Amino Acids in Human Nutrition and Health
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Amino Acids in Human Nutrition and Health

Edited by

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Preface

Rationale

Research interest in the biochemistry of amino acids continues apace, generating significant dividends for nutritional support and the elucidation of mechanisms underlying a variety of disorders in humans. The remarkable scale of recent developments has provided the impetus for publication of this first edition of *Amino Acids in Human Nutrition and Health*. It was deemed appropriate to formally acknowledge these advances within a comprehensive volume. The recruitment of authors with exceptional merit constituted an integral part of my strategy.

There appears to be a demand for a book which integrates recent advances relating to amino acids within the two disciplines of nutrition and health. Various symposia have been convened on certain aspects covered in this book, but the published proceedings are distributed in different issues of journals, thereby compromising convenience for consultation by students and research staff. It is an unfortunate reflection of our time that university libraries cannot afford to stock some of the primary journals that have, in the recent past, been judged to be essential reading for advanced students. The publication of *Amino Acids in Human Nutrition and Health* might be viewed as an attempt to rectify this deficiency. Furthermore, the symposia have focused on restricted themes, whereas this volume is designed to address a comprehensive range of issues. The reviews in published proceedings of symposia have also been restricted to a few pages per article, but there is a need for in-depth coverage to more appropriately reflect current developments.

This volume is designed for academic, research, and corporate establishments worldwide, particularly in Europe, the United States, Canada, Japan, and Australia, but generally in all countries where English is a primary medium for education and research. This book should appeal to final year undergraduate and graduate students as well as to research staff. It is anticipated that it will be recommended reading for courses in general and clinical biochemistry, medicine, nursing, human nutrition and food science. The text is also designed with the commercial sector in mind, particularly pharmaceutical companies with extensive R&D laboratories.

Overview

The chapters in *Amino Acids in Human Nutrition and Health* are arranged within a thematic structure as indicated in the sections below. The nature of the subject and the need for
interlinking chapters have meant that a limited amount of overlap was inevitable. This is not necessarily a detraction, as individual chapters are now self-contained to ensure continuity for readers, with cross-referencing kept to the minimum. This strategy has also allowed authors increased flexibility in terms of emphasis and interpretation.

**Part I Enzymes and metabolism**

This section pursues the theme of amino acid metabolism through the driving actions of the principal enzymes, emphasizing recent developments particularly with reference to localization, molecular genetics, biophysical characterization and regulation. Subsequent chapters will also demonstrate the changing facets of amino acid biochemistry. The competing actions of enzymes for critical substrates are also features of relevance in this section. A number of the enzymes under review here catalyse rate-limiting steps in important metabolic pathways, leading to synthesis of physiologically active intermediates and end products. There is scope for elaboration of the important pathways initiated by enzymes under review in this section. Part I has also been developed with the aim of underpinning subsequent chapters in this volume.

**Part II Dynamics**

This section deals with important issues relating to whole-body amino acid dynamics, with a particular objective of supporting the chapters on nutrition and health that will follow. In this chapter, authors were encouraged to adopt an integrative approach to include their own expertise and that of others in their respective fields. A basic outline of metabolic pathways appears in Part I. The theme in this section centres around kinetics and regulation in broad-spectrum reviews incorporating innovative aspects of the relevant research. In other words, the concept of metabolic networking forms an underlying theme in this series of chapters.

**Part III Nutrition**

Since the publication of *Mammalian Protein Metabolism* (Munro and Allison, 1964), there has been a steady but perceptible shift in focus towards individual or distinct groups of amino acids, and this change is most clearly seen in nutritional developments. The move away from protein to amino acid considerations is a deliberate theme in the development of the rationale for this section. However, even traditional issues, such as protein-energy malnutrition, are being investigated in the light of kinetics of specific amino acids, with reduced emphasis on whole-body protein dynamics. Against such a background, it was considered appropriate to secure reviews that would reflect a modernizing and progressive agenda in amino acid research.

The chapters cover a number of topical research investigations employing existing technologies to develop novel concepts or to underpin contemporary practices. Methods previously developed and validated with animal models are now being applied to human physiology and nutrition with significant results worthy of publication in this volume.

**Part IV Health**

The earlier sections have provided the biochemical basis of several of the conditions to be reviewed here. It is now clear that the metabolism of amino acids is associated with or
modulated by a diverse array of disorders and, in certain instances, may provide markers for risk assessment. At least four of the chapters in this section will focus on different amino acids associated with neurological issues and cognitive performance measures. The approach here is designed to reflect developments in epidemiology, monitoring, and clinical interventions in the various conditions under consideration in this section.

**Part V Conclusions**

The final section contains a plenary review designed to summarize the main findings in the foregoing chapters within an integrated account. The main theme centres around the concept of the emergence of a new momentum driving forward a progressive agenda in further elucidating the biochemical and health implications of amino acids.

**Acknowledgements**

I am indebted to my team of distinguished authors who have made publication of this volume possible despite the constraints imposed by their normal schedules. Their cooperation in submitting manuscripts promptly has ensured that the book remains up-to-date and relevant in an ever-changing scenario. Their lucid chapters have inspired me to enquire further and to challenge existing hypotheses; I trust that my readers will be similarly motivated. I am heartened by responses I have received from a number of my authors. The following words of Professor Deniz Kirik (Chapter 26, with Professor Sahin) encapsulates these sentiments: ‘It has been an interesting exercise for us to write this text as it provoked many interesting discussions in areas we thought we knew well but noticed gaps in our knowledge. We will follow on some of these points to inquire more and think that some of them could even become topics for experimentation in the next period. So it has been very valuable and pleasant for us as well.’

**Disclaimer**

This book necessarily contains references to commercial products. However, authors were asked to refrain from excessive usage of any trade names unless there were compelling reasons for doing so. No endorsement of these products is implied or should be attributed to the editor or to CAB International.

The information set out within *Amino Acids in Human Nutrition and Health* is presented in good faith and in accordance with ‘best practice’. Although every effort has been made to verify the facts and figures, neither the editor nor CAB International can accept responsibility for the data presented in individual chapters or for any consequences of their use.

At the time of preparation, I was aware of articles in the popular press extolling the virtues of citrulline and the branched-chain amino acids in the context of health and longevity. However, the publication of this book should not be interpreted as a recommendation for individuals to use these or any other amino acids for whatever purpose. *Amino Acids in Human Nutrition and Health* is intended exclusively for use as a text in education and in R&D establishments.

J.P.F. D’Mello
Editor
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Evaluation of issues underlying the role of amino acids in human nutrition and health inevitably entails an appreciation of specific nomenclature and technical descriptors. Although many of the terms and acronyms used are now in common usage outside scientific circles, it was deemed important to provide as comprehensive a list as possible to assist those readers who are new to this field. Further definitions are available in appropriate scientific dictionaries, for example in the compilations of Hodgson et al. (1998), Marcovitch (2005), Parish et al. (2006), Martin (2010) and the MedlinePlus (2010) website. Handbooks such as those by D’Mello (1997) and Longmore et al. (2010) and current textbooks in medical sciences (Bear et al., 2007; Barker et al., 2008; Baynes and Dominiczak, 2009; Naish et al., 2009) are also recommended as sources of relevant information.

### Definition of Terms and Acronyms

The important terms and acronyms are defined in Table 1. This compilation includes standard conventions as well as unique chapter-specific terms. Cross-referencing to individual chapters in this volume is provided in order to permit a greater appreciation of the context of usage of selected terms.

**Table 1.** Explanation of relevant terms and acronyms used in *Amino Acids in Human Nutrition and Health.*

<table>
<thead>
<tr>
<th>Abbreviation or Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>AAA</td>
<td>aromatic amino acid(s)</td>
</tr>
<tr>
<td>AAAH</td>
<td>aromatic amino acid hydroxylase (Chapter 9)</td>
</tr>
<tr>
<td>AADC</td>
<td>aromatic amino acid decarboxylase (Chapters 9 and 26)</td>
</tr>
<tr>
<td>AARE</td>
<td>amino acid regulatory element (Chapter 13)</td>
</tr>
<tr>
<td>Ac-CoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>Acute toxicity</td>
<td>severe adverse effects occurring within a relatively short period of exposure to a potentially harmful substance</td>
</tr>
</tbody>
</table>

Continued
**Table 1. Continued.**

<table>
<thead>
<tr>
<th>Abbreviation or Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease (Chapters 21, 22, 25, and 28)</td>
</tr>
<tr>
<td>Adduct</td>
<td>covalent product of a compound or metabolite to large biomolecules such as proteins and DNA (Chapter 28)</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder (Chapter 9)</td>
</tr>
<tr>
<td>ADI</td>
<td>acceptable daily intake(s)</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetrical dimethylarginine (Chapter 4)</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate (Chapter 1)</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product(s) (Chapters 19, 22, 28)</td>
</tr>
<tr>
<td>Agonist</td>
<td>a compound eliciting a biological response by interacting with specific cell receptors, enzymes or metabolites</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B (Chapter 17)</td>
</tr>
<tr>
<td>ALE</td>
<td>advanced lipoxidation end-product(s) (Chapters 22 and 28)</td>
</tr>
<tr>
<td>Allosteric</td>
<td>multi-site enzyme modulation of structure and activity (Chapter 10)</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase (Chapter 2)</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis (Chapter 25)</td>
</tr>
<tr>
<td>ALS/PDC</td>
<td>amyotrophic lateral sclerosis/Parkinsonism dementia complex (Chapter 19)</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase (Chapter 2)</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration (Chapter 22)</td>
</tr>
<tr>
<td>Aminoacidergic</td>
<td>relating to amino acids as neurotransmitters (Chapter 28)</td>
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<td>6-hydroxydopamine (Chapter 26)</td>
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<td>3-O-methyl-DOPA (Chapter 26)</td>
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<th>Abbreviation or Term</th>
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<td>recommended daily intake(s) (Chapter 23)</td>
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<td>RE</td>
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<td>S-methylcysteine sulfoxide (Chapter 19)</td>
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<td>streptozotocin (Chapter 3)</td>
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<td>T1R1</td>
<td>one of two class C G protein coupled receptors (Chapter 20)</td>
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<td>Teratogenic</td>
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<td>WHO</td>
<td>World Health Organization (United Nations)</td>
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**References**


Part I

Enzymes and Metabolism
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1 Glutamate Dehydrogenase

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1.1 Abstract

Glutamate dehydrogenase (GDH) is one of the most extensively studied enzymes, described by hundreds of articles spanning more than five decades of research. The enzyme catalyses the reversible oxidative deamination reaction of L-glutamate to 2-oxoglutarate. All living organisms express this enzyme and key catalytic residues have remained unchanged through the epochs. However, animal GDH exhibits complex allosteric regulation by a wide array of metabolites compared to GDH from the other kingdoms. Recent studies have demonstrated that the loss of some of this allosteric regulation causes hypersecretion of insulin, suggesting that animal GDH is not just important for amino acid oxidation. Discussed here are the atomic details of animal GDH regulation and why these features may have evolved. What is also emerging from these studies is that GDH is a highly dynamic enzyme and regulators act by controlling this movement at key junctions. These details have led to the development of a number of novel inhibitors that may find use in treating a number of GDH-related disorders. It is very clear that we are only beginning to understand the ingenious versatility of this very old enzyme.

1.2 Introduction

GDH is found in all organisms and catalyses the reversible oxidative deamination of L-glutamate to 2-oxoglutarate using NAD+ and/or NADP+ as coenzyme (Hudson and Daniel, 1993). In nearly all organisms, GDH is a homohexameric enzyme composed of subunits comprised of ~500 residues in animals and ~450 residues in the other kingdoms. While the chemical details of the enzymatic reaction have been tightly conserved through the epochs, the metabolic role of the enzyme has not. Most striking is the fact that GDH from animal sources is allosterically regulated by a wide array of metabolites, while it is mainly regulated at the transcriptional level in the other kingdoms.

1.3 GDH in Animals

In stark contrast to the other kingdoms, animal GDH is regulated by a wide array of metabolites. The two major opposing allosteric regulators, ADP and GTP, appear to exert their effects via abortive complexes. Abortive complexes are where the product is replaced by substrate before the reacted coenzyme has

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a chance to dissociate; GDH\textbullet glutamate \bullet NAD(P)\textbullet H in the oxidative deamination reaction and GDH\textbullet 2-oxoglutarate\bullet NAD(P)\textbullet in the reductive amination reaction. Once these complexes form, coenzyme binds very tightly and there is slow enzymatic turnover. ADP is an activator believed to act, at least in part, by destabilizing the abortive complex (Frieden, 1965; George and Bell, 1980). In contrast, GTP is a potent inhibitor and is thought to act by stabilizing abortive complexes (Iwatsubo and Pantaloni, 1967). GTP binding is antagonized by phosphate (Koberstein and Sund, 1973) and ADP (Dieter et al., 1981), but is proposed to be synergistic with NADH bound in the non-catalytic site (Koberstein and Sund, 1973). Finally, ADP and GTP bind in an antagonistic manner (Dieter et al., 1981) due either to steric competition or to competing effects on abortive complex formation. As discussed below, it is likely that these regulators act by modulating the enzyme dynamics (Smith et al., 2002, Banerjee et al., 2003).

Mammalian GDH is also regulated by several other types of metabolites. Leucine, as well as some other monocarboxylic acids, has been shown to activate mammalian GDH (Yielding and Tomkins, 1961) by increasing the rate-limiting step of coenzyme release in a manner similar to ADP (Prough et al., 1973). However, since ADP and leucine activation were shown to be synergistic, these activators apparently do not bind to the same site (Prough et al., 1973). Since leucine is a weak substrate for GDH, clearly one binding site is the active site. The major question is whether there is a second, allosteric, leucine-binding site. Palmitoyl-CoA (Fahien and Kmiotek, 1981) and diethylstilbestrol (Tomkins et al., 1962) are inhibitors of mammalian GDH, but nothing is known about their binding location.

1.3.1 Structure of animal GDH

The crystal structures of the bacterial (Baker et al., 1992; Stillman et al., 1993; Yip et al., 1995) and animal forms (Peterson and Smith, 1999; Smith et al., 2002) of GDH have shown that the general architecture and the locations of the catalytically important residues have remained unchanged throughout evolution. The structure of GDH (Fig. 1.1) is essentially two trimers of subunits stacked directly on top of each other with each subunit being composed of at least three domains (Peterson and Smith, 1999; Smith et al., 2002; Banerjee et al., 2003). The bottom domain makes extensive contacts with a subunit from the other trimer. Resting on top of this domain is the ‘NAD binding domain’ that has the conserved nucleotide-binding motif. Animal GDH has a long protrusion, an ‘antenna’ rising above the NAD binding domain, that is not found in bacteria, plants, fungi, and the vast majority of protists. The antenna from each subunit lies immediately behind the adjacent, counter-clockwise neighbour within the trimer. Since these intertwined antennae are only found in the forms of GDH that are
Glutamate Dehydrogenase

allosterically regulated by numerous ligands, it is reasonable to speculate that they play an important role in regulation.

1.4 Active Site

The first crystal structure of bovine GDH (bGDH) included glutamate, NADH and GTP (Peterson et al., 1998). Initial crystals of the apo form of GDH had been obtained previously, but had an extremely large unit cell and diffracted to modest resolution. Therefore, a substrate-saturated form of the enzyme was crystallized to stabilize the crystal lattice. NADH and glutamate form a tightly bound abortive complex in the active site. In addition, NADH binds to a second site and acts synergistically with the inhibitor GTP (Koberstein and Sund, 1973) to further increase the affinity of glutamate and NADH to the active site (Frieden, 1963a; Iwatsubo and Pantaloni, 1967; Koberstein and Sund, 1973). Therefore, together, it was hoped that high concentrations of these ligands would be sufficient to overcome the negative cooperativity exhibited by GDH with respect to coenzyme binding (Dalziel and Egan, 1972; Bell, 1974; Dalziel, 1975; Bell et al., 1985) so that homogenous and saturated GDH could be crystallized.

From this structure, both the bound NADH and glutamate were clearly visible in the active site (Peterson and Smith, 1999; Smith et al., 2001) (Fig. 1.1). It is important to note that at the time of these structures,
only the chemical sequence had been determined for bovine GDH (Smith et al., 1970), and had a number of errors compared to the recently determined DNA sequence (GI:32880220). Figure 1.2 shows the structural details of both NADH and glutamate bound to the active site using the updated and refined bovine GDH structure (Li and Smith, unpublished results). The residues noted by parentheses denote the equivalent amino acids in Clostridium symbiosum GDH (csGDH) (Stillman et al., 1993; Maniscalco et al., 1996).

The chemical mechanism of GDH has been extensively studied and well detailed (for a review see Brunhuber and Blanchard, 1994) and the structure of the GDH•NADH•glutamate complex is entirely consistent with this model (Fig. 1.2). In the early 1990s the chemical mechanism was being refined (Singh et al., 1993; Srinivasan and Fisher, 1985; Maniscalco et al., 1996) at the same time that the structure of bacterial glutamate dehydrogenase was being determined (Baker et al., 1992; Dean et al., 1994; Smith et al., 1996;...

![Fig. 1.2. The active site of animal GDH. At the top is a schematic diagram showing some of the important protein/ligand interactions in the active site in the GDH•NADH•glutamate complex. Shown below is a stereo diagram of the substrate and coenzyme bound to the active site.](image)
Stillman et al., 1993). In the proposed mechanism for the reductive amination reaction, the first step is the binding of the $\gamma$-carboxyl of 2-oxoglutarate to a protonated lysine. This interaction has been observed in csGDH (Stillman et al., 1993), verified by mutagenesis in csGDH (Wang et al., 1995; Baker et al., 1997), and observed in bGDH (Fig. 1.2). In bGDH, K90, S380, and R211 form hydrogen bonds with the $\gamma$-carboxyl of the substrate. These interactions help determine the substrate specificity as has been demonstrated with csGDH, where a mutation of the equivalent to K90 to a leucine decreased the activity with L-glutamate by ~2000 fold, while increasing the activity with monocarboxylic amino acids (norvaline and $\alpha$-aminobutyrate) by two- to threefold. In the next step of the proposed reaction, ammonium enters the active site and reacts with the $\alpha$-keto group. A carbinolamine intermediate is then formed when the $\alpha$-carbonyl oxygen accepts a proton from an active site lysine (K126 in bGDH and K125 in csGDH). The $\alpha$-carbonyl oxygen then accepts a second proton from an active site carboxyl group (D168 in bGDH and D165 in csGDH) that at the same time accepts a proton from the substrate amine. Water is released as the carbinolamine forms an imine that is, in turn, reduced by NAD(P)H. The product is then protonated by the active site carboxyl group and released by the enzyme. Additional amine groups (K114 and N349) hold the carboxylic acid in place with a network of hydrogen bonds. It was also proposed that a necessary step in this chemical reaction is the closure of the catalytic cleft upon substrate and coenzyme binding (Singh et al., 1993) that expels the bulk water and brings the C-4 atom of the nicotinamide ring into very close contact with the C-$\alpha$ atom of the glutamate. This is clearly the case with only a couple of water molecules found in the closed bGDH•NADH•glutamate complex and the C-4 atom is brought to within ~3.1Å from the C-$\alpha$ atom of glutamate.

### 1.4.1 GDH dynamics

From the structures of bGDH with and without active site ligands, it is possible to observe the closure of the active site cleft, along with some of the large conformational changes that occur throughout the hexamer during each catalytic cycle (Peterson and Smith, 1999; Smith et al., 2001; Smith et al., 2002; Banerjee et al., 2003). Details of these conformational changes are summarized in Fig. 1.1. Substrate binds to the deep recesses of the cleft between the coenzyme binding domain and the lower domain. Coenzyme binds along the coenzyme binding domain surface of the cleft. Upon binding, the coenzyme binding domain rotates by ~18° to close down firmly upon the substrate and coenzyme (Fig. 1.1; arrow 1). As the catalytic cleft closes, the base of each of the long ascending helices in the antenna appears to rotate out in a counterclockwise manner to push against the ‘pivot’ helix of the adjacent subunit (Fig. 1.1; arrow 2). There is a short helix in the descending loop of the antenna that becomes distended and shorter as the mouth closes in a manner akin to an extending spring (Fig. 1.1; arrow 3). The ‘pivot helix’ rotates in a counterclockwise manner along the helical axes as well as rotating counterclockwise around the trimer threefold axis. Finally, the entire hexamer seems to compress as the mouth closes (Fig. 1.1; arrow 4). The three pairs of subunits that sit on top of each other move as rigid units towards each other, compressing the cavity at the core of the hexamer. Therefore, it is quite clear that the bGDH active site cleft does not open and close in isolation, but rather that this motion involves the entire hexamer. As is further detailed in subsequent sections, it is apparent that animal GDH is controlled allosterically via ligand interactions at a number of these flex points.

### 1.4.2 GTP inhibition site

GTP is a potent inhibitor for the reaction and binds at the base of the antenna, wedged in between the NAD binding domain and the pivot helix (Peterson and Smith, 1999; Smith et al., 2001) (Fig. 1.3). It is important to note that this binding site is only available for GTP binding when the catalytic cleft is closed. Therefore, it is likely that after GTP binds to the ‘closed’ conformation, it is more difficult for the ‘mouth’ to open and release
the product. This is entirely consistent with the finding that GTP inhibits the reaction by slowing down product release and concomitantly increasing the binding affinity of substrate and coenzyme (Frieden, 1963a; Iwatsubo and Pantaloni, 1967; Koberstein and Sund, 1973). The vast majority of the interactions between GTP and bGDH involve the triphosphate moiety with the majority of the salt bridges being made with the γ-phosphate. This explains why, in terms of inhibition, GTP >> GDP > GMP (Frieden, 1965). This site is essentially an energy sensor in that if the mitochondrial energy level is high, then the GTP (and ATP) levels will be elevated and GDH will be inhibited. The environment of the GTP binding site is so favourable for phosphate binding that phosphate buffer was observed to bind in the absence of GTP (Smith et al., 2001). Interestingly, this is consistent with the fact that high phosphate concentrations compete with GTP for binding (Koberstein and Sund, 1973). Further, it is also well known that phosphate buffer stabilizes the enzyme compared to other buffers such as Tris (Frieden, 1963b). Therefore, phosphates binding to this apparently sensitive 'hinge' area somehow protect the enzyme against thermal denaturation.

1.4.3 ADP/second NADH site paradox

Perhaps one of the most confusing regulator sites on animal GDH is the allosteric activator,
ADP, binding site (Fig. 1.4). However, as will be detailed below, NADH also binds to this allosteric site and causes inhibition. In spite of having atomic details as to the interaction of these ligands with the enzyme, it is not at all clear how these regulators can cause opposite effects upon binding to the same site.

The existence of a second NADH binding site per subunit was demonstrated both kinetically and by binding analysis (Frieden, 1959a; Frieden, 1959b; Shafer et al., 1972). It was observed that NADH alone binds with a stoichiometry of 7–8 molecules per hexamer. In the presence of glutamate, NADH binds more tightly and the stoichiometry increases to 12 per hexamer (Shafer et al., 1972). Similarly, GTP also increases the affinity and binding stoichiometry (Koberstein and Sund, 1973). This second coenzyme site strongly favours NADH over NADPH with Kds of 57μM and 700μM, respectively. In the case of oxidized coenzyme, NAD', two binding sites were also observed. While the recent structures of the various complexes have demonstrated that ADP and NAD(H) bind to the same site (Smith et al., 2001; Banerjee et al., 2003), this was first suggested by ADP binding competition with NAD' (Limuti, 1983) and NADH (Dieter et al., 1981). Further, these binding studies provided direct evidence that GTP and glutamate enhance binding of NADH to a second site, and ADP blocks binding of both NAD' and NADH to a second site. A number of chemical reagents affect NADH inhibition by binding to disparate sites of the enzyme: TNBS (Goldin and Frieden, 1971) and FSBA (Pal et al., 1975; Schmidt and Colman, 1984) bind to the antenna; FSBAzA (Dombrowski et al., 1992) and FSBA (Pal et al., 1975; Schmidt and Colman, 1984) modify the core of the hexamer; and 6-BDB-TADP (Batra and Colman, 1986) modifies the outer portion of the NAD binding domain. These results demonstrate that a number of regions distal to the NADH binding site are involved in NADH inhibition. Since, in general terms, NADPH is involved in anabolic reactions in the cell while NADH is important for catabolic processes, it is possible that this regulation offers a feedback mechanism to curtail glutamate oxidation when catabolic reductive potentials (NADH) are high.

In nearly every way, ADP acts in a manner opposite to NADH binding to this site. In the oxidative deamination reaction, ADP activates at high pH, but inhibits at low pH with

![Fig. 1.4. The binding site of the activator, ADP (left), and the second, inhibitory site for NADH (right). This site is behind the NAD binding domain and immediately under the pivot helix. The highlighted residue, R459, was mutated to alanine and caused a loss in ADP activation. Shown on the right is the structure of NADH bound to this site where the adenosine/ribose moiety closely matches that of ADP and the nicotinamide/ribose portion binds into the subunit interface.](attachment:image)
either NAD\(^+\) or NADP\(^+\) as coenzyme. In the reductive amination reaction, ADP is a potent activator at low pH and low substrate concentration. At pH 6.0, high concentrations of \(\alpha\)-KG and NADH, but not NADPH, inhibit the reaction. This substrate inhibition is alleviated by ADP (Bailey et al., 1982). Therefore, while GTP and glutamate bind synergistically with NADH to inhibit GDH, ADP activates the reaction by decreasing the affinity of the enzyme for coenzyme at the active site. Under conditions where substrate inhibition occurs, this activates the enzyme. However, under conditions where the enzyme is not saturated (e.g., low substrate concentrations), this loss in binding affinity causes inhibition. Put another way, under conditions where product release is the rate-limiting step, ADP greatly facilitates the catalytic turnover. It should be noted that the fact that substrate (2-oxoglutarate) inhibition in the reductive amination reaction is only observed using NADH as coenzyme was suggested to be due to NADH (but not NADPH) binding to the second coenzyme site. Further, it was suggested that ADP activation under these conditions was due to ADP displacement of NADH from the second allosteric site (Frieden, 1965).

ADP binds behind the NAD binding domain and immediately under the pivot helix (Banerjee et al., 2003). This location is exactly consistent with chemical modification studies. The ADP analogue, AMPSBDB, reacts with R459 that lies on the pivot helix and is adjacent to the ADP binding site (Wrzeszczynski and Colman, 1994). This modification permanently activates the enzyme by essentially covalently locking an ADP molecule in the activation site. As shown in Fig. 1.4, R459 lies on the pivot helix and interacts with the phosphates of the bound ADP. It was proposed that this interaction might facilitate the rotation of the NAD binding domain and the release of product (Banerjee et al., 2003). To test this, R459 (R463 in human GDH) was mutated to an alanine and this led to a loss in ADP activation. This essentially suggests that ADP activates the reaction by ‘pulling’ on the back of the NAD binding domain to help open the active site cleft and facilitating product release.

The structures of GDH complexed with NADH, NADPH, and NAD have all been determined (Smith et al., 2001). Because NADH (but not NADPH) has been suggested to be an inhibitor of the reaction, it is somewhat surprising that it binds to the ADP activation site (Banerjee et al., 2003). The adenosine-ribose moiety location exactly matched that of ADP. The electron density of the ribose-nicotinamide moiety was much weaker and was initially built in two alternative conformations. However, the stronger density for this portion of NADH suggests that it points down into the interface between adjacent subunits as shown in Fig. 1.4. As predicted from the binding studies reviewed above, NADPH was found bound to the active site but not the second, allosteric site. From the preferred orientation shown in Fig. 1.4, this is likely due to the fact that there is not enough room to accommodate the additional phosphate on the ribose ring that is buried at the subunit interface.

NAD\(^+\) was found to bind in a manner essentially identical to NADH (Smith et al., 2001). From steady state kinetic analysis, it was initially thought that NAD\(^+\) binding to this second site causes activation of the enzyme (Frieden, 1959a), even though NADH causes apparent inhibition. However, subsequent studies demonstrated that this apparent activation was due to negatively cooperative binding with respect to coenzyme (Dalziel and Engel, 1968). Therefore, it is not clear what difference there might be, if any, between NAD\(^+\) and NADH binding to GDH at this location. It is interesting to note that modification of the ADP site with an ADP analogue did not eliminate NADH inhibition (Wrzeszczynski and Colman, 1994). Perhaps this is due to the nicotinamide moiety still binding to the pocket between the subunits in spite of AMPSBDB being bound to R459. As will be detailed below, recent studies on new GDH inhibitors have shown that compounds binding to subunit interfaces can be potent inhibitors of the enzyme. Perhaps the ribose-nicotinamide moiety is acting in a similar manner.

The physiological role of ADP activation is easily understood: when the energy level of the mitochondria is low and ADP levels are high, the catabolism of glutamate is facilitated for energy production. However, the possible in vivo role of NADH inhibition is less clear.
In mammalian mitochondria, assuming a matrix volume of 1 µl mg⁻¹ of protein, the concentrations of NAD(H) and NADP(H) are approximate 0.5–2.0 mM (Lenartowicz, 1990). However, activity of the transhydrogenase transfers much of the reductive power of NADH to NADPH. Using metabolite indicators, the mitochondrial NADH/NAD⁺ ratio was estimated to be ~0.2 and the NADPH/NADP⁺ ratio was ~200 (Hoek and Rydstrom, 1988). In experiments on submitochondrial particles, the energy-linked transhydrogenase was found to maintain NADP up to 500 times more reduced than NAD (Rydstrom et al., 1970). These results suggest that the range of NADH concentration is ~0.083–0.33 mM. NADH inhibition is observed at concentrations above 0.2 mM (e.g., see Batra and Colman, 1986), but only reaches ~50% inhibition at 1mM NADH. Therefore, if NADH inhibition is physiologically relevant, it seems more likely that its purpose is to synergistically enhance GTP inhibition; under conditions of high reductive potential, NADH acts with GTP to keep GDH in a tonic state.

At an atomic level, there is a very clear delineation between ligands binding to the open and closed conformations. NADH alone only binds to the active site. When glutamate is added, the catalytic cleft closes and NADH is able to bind to the second, allosteric site. Further, the GTP binding site collapses when the catalytic cleft opens and therefore GTP also favours the closed conformation. Therefore, the synergism between NADH and GTP is likely due to both ligands binding to, and stabilizing the closed conformation. Again, this supports the contention that NADH inhibition alone may not have a significant physiological role, but rather its main function is the enhancement of GTP inhibition.

### 1.5 Role of GDH in Insulin Homeostasis

The difference between the allosteric regulation of GDH from animals and the other animal kingdoms has been known for decades, but possible roles for allosteric regulation in animal GDH is only starting to emerge. Of growing interest is the fact that loss of inhibition of GDH causes inappropriate stimulation of insulin secretion. There have been three GDH-mediated forms of hyperinsulinism identified thus far: hyperinsulinism/hyperammonaemia (HHS) due to mutations that abrogate GTP inhibition, mutations in NAD-dependent deacetylase (SIRT4) and knockout mutations of short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD).

#### 1.5.1 HHS

HHS was one of the first diseases that clearly linked GDH regulation to insulin and ammonia homeostasis (Stanley et al., 1998). In brief, the mitochondrion of the pancreatic β-cells plays an integrative role in the fuel stimulation of insulin secretion. The current concept is that mitochondrial oxidation of substrates increases the cellular phosphate potential that is manifested by a rise in the ATP⁴ MgADP² ratio. The elevated ATP concentration closes the plasma membrane K₅AT channels, resulting in the depolarization of the membrane potential. This voltage change across the membrane opens voltage gated Ca²⁺ channels. The rise of free cytoplasmic Ca²⁺ then leads to insulin granule exocytosis (Fig. 1.5).

The connection between GDH and insulin regulation was initially established using a nonmetabolizable analogue of leucine (Sener and Malaisse, 1980; Sener et al., 1981), BCH (β-2-aminobicyclic(2.2.1)-heptane-2-carboxylic acid). These studies demonstrated that activation of GDH was tightly correlated with increased glutaminolysis and release of insulin. In addition, it has also been noted that factors that regulate GDH also affect insulin secretion (Fahien et al., 1988). Subsequently, it was postulated that glutamine could also play a secondary messenger role and that GDH plays a role in its regulation (Stanley, 2000; Li et al., 2003; Li et al., 2004). The in vivo importance of GDH in glucose homeostasis was demonstrated by the discovery that a genetic hypoglycaemic disorder, the HHS syndrome, is caused by loss of GTP regulation of GDH (Stanley et al., 1998; Stanley et al., 2000; MacMullen et al., 2001). Children with HHS have increased
β-cell responsiveness to leucine and susceptibility to hypoglycaemia following high-protein meals (Hsu et al., 2001). This is likely due to uncontrolled catabolism of amino acids yielding high ATP levels that stimulate insulin secretion as well as high serum ammonium levels. The elevation of serum ammonia levels reflects the consequence of altered regulation of GDH, leading to increased ammonia production from
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glutamate oxidation, and possibly also impaired urea synthesis by carbamoylphosphate synthetase (CPS) due to reduced formation of its activator, N-acetyl-glutamate, from glutamate (Fig. 1.5). Essentially, this genetic lesion disrupts the regulator linkage between glycolysis and amino acid catabolism. During glucose-stimulated insulin secretion in normal individuals, it has been proposed that the generation of high energy phosphates inhibits GDH and promotes conversion of glutamate to glutamine, which, alone or combined, might amplify the release of insulin (Li et al., 2003, 2004).

While the glucose and ammonium levels in patients with HHS are alone sufficient to potentially cause damage to the CNS, recent studies have suggested a high correlation between HHS and childhood-onset epilepsy, learning disabilities, and seizures (Bahi-Buisson et al., 2008). Some of these pathologies have been shown to be unrelated to serum glucose and ammonium levels. This is not entirely surprising considering the importance of glutamate and its derivative, γ-aminobutyric acid, as neurotransmitters. The current treatment for HHS is to pharmacologically control insulin secretion (e.g. diazoxide, a potassium channel activator) but this does not address the serum ammonium and CNS pathologies.

1.5.2 SIRT4 mutations

Sirt2 or sirtuins (silent information regulator two proteins) are found in all organisms and most are NAD-dependent protein deacetylases. The sirtuins have been shown to be implicated in ageing and regulate transcription, involved in stress response, and apoptosis. Recent studies have shown that SIRT4, a mitochondrial enzyme, uses NAD to ADP-ribosylate GDH and inhibit its activity (Herrero-Yraola et al., 2001; Haigis et al., 2006). When SIRT4 knockout mice were generated, the loss of SIRT4 activity led to the activation of GDH and, much like HHS due to the loss of GTP inhibition, up-regulated amino acid-stimulated insulin secretion (Haigis et al., 2006). In addition, they found that, with regard to that ADP-ribosylation, GDH activity in SIRT4 -/- mice was similar to mice on a calorie restriction diet regime. This suggests that normally SIRT4 in the β cell mitochondria represses GDH activity by ADP-ribosylation and this regulation is removed during times of low caloric intake.

1.5.3 SCHAD mutations

A form of recessively inherited hyperinsulinism has been recently identified to be associated with a deficiency of a mitochondrial fatty acid β-oxidation enzyme, the short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) encoded by the HADH gene on 4q (Jackson et al., 1991; Molven et al., 2004; Hussain et al., 2005). Children with this defect have recurrent episodes of hypoglycemia that can be controlled with the K_ATP channel agonist diazoxide. These patients also have serum accumulation of fatty acid metabolites such as 3-hydroxybutyryl-carnitine and urinary 3-hydroxyglutaric acid (Molven et al., 2004, Hussain et al., 2005). Similar to HHS, these patients also have severe dietary protein sensitivity (Hussain et al., 2005). However, the loss of SCHAD activity is at odds with increased insulin secretion observed in HHS since it is expected to decrease ATP production. In addition, other genetic disorders of mitochondrial fatty acid oxidation do not lead to hyperinsulinism (Stanley et al., 2006).

Recent studies have now explained this seeming paradox by suggesting that SCHAD regulates GDH activity via protein-protein interactions (Li et al., 2010). From immunoprecipitation and pull-down analysis, it is apparent that GDH and SCHAD interact as had been previously suggested (Filling et al., 2008). This interaction inhibits GDH activity by decreasing GDH affinity for substrate. This effect was found to be limited to the pancreas presumably because of the relatively high levels of SCHAD found in this tissue. Hussain et al. (2005) recently demonstrated that SCHAD-deficient children have protein sensitive hypoglycaemia, consistent with an activation of GDH. This would also explain why SCHAD-deficient patients do not have a concomitant increase in serum ammonium levels as observed in HHS. These results are
particularly interesting since it strongly suggests that part of GDH regulation comes from associating as a large multienzyme complex in the mitochondria. In addition, this fits in well with the linkage between fatty acid and amino acid oxidation as suggested by the evolution of allosterity that came from analysis of ciliate GDH (Allen et al., 2004; Smith and Stanley, 2008) as discussed in the next section.

1.6 Evolution of GDH Allostery

It has been known for some time that animal GDH contain an internal 48-residue insert compared to GDH from the other kingdoms. From the structure of animal GDH, we now know this insert forms the antenna region (Fig. 1.1). Further, kinetic analysis has demonstrated that animal GDH is allosterically regulated by a wide array of metabolites. What was not at all clear was when and why the antenna evolved and whether it was linked to the complex pattern of allosterity in animal GDH. With the release of the Tetrahymena Genome Project, it became evident that tetrahymena GDH (tGDH) also has this ~48-residue insert. This possible evolutionary ‘missing link’ was then further analysed via kinetic and mutagenesis analyses (Allen et al., 2004). Like mammalian GDH, tGDH is activated by ADP and inhibited by palmitoyl CoA. However, like bacterial GDH, tGDH is coenzyme specific (NAD(H)) and is not regulated by GTP or leucine. Therefore, with regard to allosteric regulation, tGDH is indeed an evolutionary ‘missing link’ between bacterial and animal GDH.

A major question was how the antenna was linked to allosteric regulation. From structural studies, it was clear that the antenna was not involved in ADP or GTP binding (Peterson and Smith, 1999; Smith et al., 2001; Banerjee et al., 2003). However, when the antenna on human GDH was replaced by the short loop found in bacterial GDH, the enzyme lost GTP, ADP, and palmitoyl-CoA regulation (Allen et al., 2004). When the ciliate antenna was spliced onto human GDH, this hybrid enzyme had a fully functional repertoire of mammalian allosterity. This demonstrated that the antenna is essential for exacting allosteric regulation by GTP and ADP while not being directly involved in their binding. Evolutionarily, this also demonstrated that the antenna in ciliate GDH is capable of transmitting the GTP inhibitory signal, but GTP regulation was apparently not needed in the ciliates.

This suggests that GDH allosteroy evolved in at least a two-step process (Fig. 1.6). The first evolutionary step may have been due to the changing functions of

![Fig. 1.6. Evolution of glutamate dehydrogenase. The ciliates represent an evolutionary missing link between animals and all of the other kingdoms. In the ciliates, fatty acid oxidation started to move from the peroxisomes to the mitochondria, the location of GDH. Perhaps in response to this, the antenna was created to facilitate allosteric regulation and coordinated control of amino acid and fatty acid oxidation. Using this new architecture, animal GDH added further complexity to allosteric regulation since glutamate has such an important role in insulin homeostasis, neurotransmission and ureagenesis.](image-url)
the cellular organelles as the ciliates branched off from the other animal kingdoms. In the other eukaryotic organisms, all fatty acid oxidation occurs in the peroxisomes (Gerhardt, 1992; Erdmann et al., 1997). In the ciliates, fatty acid oxidation is shared between the peroxisomes and the mitochondria (Müller et al., 1968; Blum, 1973). Eventually, all medium and long chain fatty oxidation moved into the mitochondria in animals (Reddy and Mannaerts, 1994; Hashimoto, 1999). Therefore, it appears that GDH regulation evolved in response to fatty acid and amino acid oxidation being brought into the same subcellular compartment. Specifically, the pattern of regulation suggests that the catabolism of amino acids is down-regulated when there are sufficient levels of fatty acids. When the mitochondria have run low on fatty acids and their energy state is low (i.e. high ADP levels), only then will amino acids be catabolized. This closely mirrors what was found in the insulin disorders described above; GDH activity is tightly repressed until other energy sources are depleted. Therefore, allosteroy was necessary to coordinate the growing complexity of mitochondrial metabolism and the antenna feature is needed to exact this regulation.

The second step of evolution was to add leucine and GTP allosteroy as animals needed even more sophisticated and rapid-response regulation of GDH activity. This is an example of exaptation, where a feature evolves for one reason and is then further refined and used for other functions in subsequent evolution. In animals, GDH is found in high levels in the central nervous system (CNS), pancreas, liver and kidneys. In each organ, the levels of glutamate need to be controlled by very different physiological signals. In the pancreas, GDH needs to coordinate amino acid oxidation with fatty acid and carbohydrate catabolism for an appropriate insulin response. In the liver, glutamate is crucial for ureagenesis. In the CNS, glutamate and its derivative, GABA, are major neurotransmission ligands. These disparate roles for GDH are more than likely why GDH in animals has such sophisticated allosteric regulation compared to all other organisms.

As a likely response to the growing complexity of multi-organ animals, two very important regulators, leucine and GTP, were added to the allosteric regulatory repertoire. In the pancreas, GDH needs to be activated when amino acids (protein) are ingested to promote insulin secretion and appropriate anabolic effects on peripheral tissues. In the glucose-fed state, triphosphate levels are high and GDH needs to be inhibited to redirect amino acids into glutamine synthesis in order to amplify insulin release. In the liver, GDH needs to be suppressed when other fuels, such as fatty acids, are available, but to be increased when surplus amino acids need to be oxidized. To this end, mammals added layers of regulation onto ciliate GDH to include leucine activation and GTP inhibition. The choice of leucine as a regulator is likely not an accident, because leucine is the most abundant amino acid in protein (10%) and provides a good measure of protein abundance. Similarly, the marked sensitivity of GDH for GTP over ATP is also not likely to be accidental. Most of the ATP in the mitochondria is produced from oxidative phosphorylation that is driven by the potential across the mitochondrial membrane created by NADH oxidation. Therefore, the number of ATP molecules generated from one turn of the TCA cycle can vary between 1 and 29. In contrast, one GTP is generated per turn of the TCA cycle and there is a slow mitochondria/cytoplasm exchange rate. Therefore, the GTP/GDP ratio is a much better metric of TCA cycle activity than the ATP/ADP ratio. Indeed, recent results have demonstrated that mitochondrial GTP, but not ATP, regulates glucose-stimulated insulin secretion (Kibbey et al., 2007). This is also consistent with the HHS disorder in that, without GTP inhibition of GDH, glutamate will be catabolized in an uncontrolled manner; the TCA cycle will generate more GTP; and more insulin will be released. Therefore, the addition of GTP and leucine regulation to GDH makes it acutely sensitive to glucose and amino acid catabolism, with obvious implications for insulin homeostasis. It is also likely that this complex network of allosteroy was also needed to accommodate the differing regulation needed by the CNS and ureagenesis.
1.6.1 Possible therapeutics for GDH-mediated insulin disorders

The current treatment for HHS is to pharmaceutically control insulin secretion (e.g. diazoxide, a potassium channel activator) but this does not address the liver and CNS pathology. One approach to circumvent the extremely high costs of developing therapeutics for diseases with a small patient base is to try to find bioactive compounds commonly found in the food chain. To this end, a broad search led to two bioactive compounds found in green tea.

According to legend, green tea was discovered by the Chinese Emperor Shen-Nung in 2737 BC and for centuries has been used as a folk remedy to treat a number of ailments. Green tea is a significant source of a type of flavonoids called catechins, including epigallocatechin gallate (EGCG); epigallocatechin (EGC); epicatechin gallate (ECG); and epicatechin (EC). One 200 ml cup of green tea supplies 140, 65, 28 and 17 mg of these polyphenols, respectively (Yang and Wang, 1993). Over the past few decades, there has been growing interest in EGCG since it has been suggested to decrease cholesterol levels (Maron et al., 2003), act as an antibiotic (Hamilton-Miller, 1995) and anticarcinogen (Katiyar and Mukhtar, 1996), repress hepatic glucose production (Waltner-Law et al., 2002), and enhance insulin action (Anderson and Polansky, 2002). The exact mechanism of action of EGCG with regard to these various effects is largely unknown and in many cases is assumed to due to its antioxidant activity.

Of the four major catechins found in green tea, only two showed inhibitory activity against GDH: ECG and EGCG (Fig. 1.7). Essentially, activity is dependent upon the presence of the third ring structure, the gallate,
on the flavonoid moiety. EGCG and ECG allo-
sterically inhibit purified animal GDH in vitro
with a nanomolar ED₅₀. Since EC or EGC were
not active against GDH, but have the same
antioxidant activity as ECG and EGCG, the
antioxidant property of these catechins can-
not be relevant to GDH inhibition. EGCG inhibi-
tion is non-competitive and, similar to GTP
inhibition, is abrogated by leucine, BCH, and
ADP. As noted above, the antenna is necessary
for GTP inhibition and ADP activation (Allen
et al., 2004). Similarly, EGCG does not inhibit
the ‘antenna-less’ form of GDH, thus is further
evidence that ECG is an allosteric inhibitor.
Most importantly, EGCG inhibits HHS GDH
mutants as effectively as wild type (Li et al.,
2006), making it a possible therapeutic lead
compound.

The next step was to ascertain whether
EGCG was active in tissue. Studies have
demonstrated that GDH plays a major role
in leucine stimulated insulin secretion (LSIS)
by controlling glutaminolysis (Li et al., 2003,
2004). Therefore, EGCG was tested on pan-
creatic β-cells using the perfusion assay (Li
et al., 2006). Importantly, EGCG, but not
ECG, blocked the GDH-mediated stimula-
tion of insulin secretion by the β-cells.
However, it did not have any effect on insu-
lin secretion, glucose oxidation, or cellular
respiration during glucose stimulation
where GDH is known to not play a major
role in the regulation of insulin secretion.
Therefore, EGCG is indeed a specific inhibi-
tor of GDH both in vitro and in situ, and
ongoing studies are evaluating whether it
will be similarly active in vivo.

More recent studies on glioblastoma cells
have demonstrated that EGCG inhibition of
GDH might have even broader utility than just
with HHS. Increased glucose and glutamine
utilization are hallmarks of tumour metabo-
lism (Kim and Dang, 2006; DeBerardinis et al.,
2008). The phosphatidylglycerol 3'-kinase/Akt
pathway is enhanced in many human tumours
and up-regulates glucose uptake and utiliza-
tion (Eliot et al., 2004; Bauer et al., 2005).
C-Myc, on the other hand, up-regulates
B-glutamine utilization by increasing cell surface
transporters and enzymes (Wise et al., 2008;
Gao et al., 2009). At least in vitro, the enhanced
utilization of one of these carbon sources also
makes the cells sensitive to its withdrawal
(Bazzai et al., 2005; Wise et al., 2008). Extending
upon all of these results, the DeBerardinis lab-
oratory demonstrated that EGCG sensitizes
glioblastoma cells to glucose withdrawal and
to inhibitors of Akt signalling and glycolysis
(Yang et al., 2009). Indeed, the addition of
EGCG mirrored the effects of knocking out
GDH in the tissue. Therefore, these results sug-
gest that anti-cancer therapy that combines
GDH inhibitors with those that inhibit glucose
utilization could be very effective in treating
tumours.

1.6.2 Other novel inhibitors of GDH

The results with EGCG/ECG offer proof of
concept that it is possible to control GDH
activity pharmaceutically. However, it is
always important to have more than one lead
compound for drug development. Therefore,
high throughput screening was used to find
additional compounds that might have better
efficacy and/or pharmacokinetics (Li et al.,
2007; Li et al., 2009).

A search of more than 30,000 compounds
yielded a number of interesting active com-
ounds (Fig. 1.7) (Li et al., 2007; Li et al., 2009).
First, in agreement with previous studies, the
screen identified EGCG as an active com-
pound but EC was found not to be active.
With physical properties similar to EGCG,
aurintricarboxylic acid (ATA) and 3,3'-
[(2-bromo-1,4-phenylene)di(E)ethene-2,1-
diylyl]bis(6-hydroxybenzoic acid) (BSB) were
also found to be efficacious inhibitors of GDH.
ATA can interfere with protein-nucleotide
interactions such as those found in kinases
and phosphatase (Myskiw et al., 2007) and
can inhibit influenza virus neuraminidases
(Hashem et al., 2009). BSB interacts with amy-
lloid polymers (Skovronsky et al., 2000). The
effects of all three of these compounds were
strongly abrogated by ADP. Therefore, while
not proof evident, it is possible that all three
compounds bind to the ADP site. This could
be akin to NADH binding to the ADP site and
causing inhibition rather than the ADP-
mediated activation, and would be consistent
with the inhibitory effects that ATA has on
kinases/phosphatases. The other group of
compounds that were identified in this screen are small, hydrophobic compounds: bithionol, hexachlorophene, GW5074, and diethylstilbestrol. Compared to ATA and EGCG, the inhibition caused by these compounds is not as easily abrogated by ADP. It is likely that these compounds are binding to a site(s) distinct from where the more soluble ATA, EGCG, and BSB compounds are binding.

Subsequent structural studies found that the small, hydrophobic compounds (GW5074, hexachlorophene (HCP), bithionol) bind to subunit interfaces in the enzyme that are critical for conformational transitions (Li et al., 2009). As shown in Fig. 1.8, six molecules of HCP form a ring in the inner cavity of the hexamer, not with sixfold symmetry but rather alternate between two different conformations around the ring. One conformer is relatively flat and tucks into a pocket at the interface between diagonal subunits while the other conformer is more vertically oriented but also interacts with diagonal subunits. Essentially, the symmetrical HCP binds at the interface between twofold related subunits with one ring interacting with one subunit and the other ring interacting with the other. The majority of the interactions between HCP and GDH are hydrophobic, but there is also an almost 'chain link' of aromatic stacking interactions.

Bithionol and GW5074 do not bind to the same site as HCP. While HCP binds to the inner core, these two drugs bind halfway between the core and the exterior of the hexamer (Fig. 1.8). This is in contrast to the binding geometry of HCP where the internal cavity is blocked off from the exterior solvent mainly by the antenna structure. Also unlike HCP, each of the six drug molecules is associated with separate subunits rather than one molecule contacting two symmetrical sites simultaneously. Instead, two drug molecules form pairs that are related by the hexameric twofold axes. The binding environments of the two drugs are nearly identical. Residues 138–155 of the glutamate-binding domain form an α-helix that makes most of the contact between diagonal subunits and draw closer together when the catalytic cleft is closed. These two drugs stack against each other and interact with hydrophobic residues and the aliphatic portions of the polar and charged side chains of

Fig. 1.8. Locations of the binding sites for the new GDH inhibitors. The small, hydrophobic compounds bind to inter-subunit flex points. The left figure is a side view of the entire hexamer showing the location of the bithionol and GW5074. Note that this is the same region that compresses during the closure of the catalytic cleft (Fig. 1.1). On the right is a cutaway view from the top of the hexamer showing that these hydrophobic compounds bind to the subunit interfaces and that HCP forms a ring of aromatic interactions in the core of the enzyme. For clarity, GW5074 is not shown since it binds to the same location as bithionol.
residues K143, R146, R147, and M150. These drugs, therefore, appear to bind directly to the area that compresses during mouth closure.

1.7 Conclusions

It has been suggested that the complex motions within GDH may initially have evolved to improve catalytic efficiency (Smith and Stanley, 2008). The negative cooperativity and extensive subunit communication may have evolved to conserve and transfer the energy involved in substrate binding to one subunit to facilitate product release from adjacent subunits (Smith and Bell, 1982). As is clear in the case of these drugs and the naturally occurring allosteric regulators, this complex ballet of motion creates numerous sites by which ligands can bind and modulate catalytic activity. This has allowed allosterity in GDH gradually to evolve in complexity to create new functions and roles, rather than creating entirely new gene products to accommodate the changing needs of the cell (e.g. linkage between amino acid catabolism and insulin secretion). While it seems that GDH regulation is overly complex for an enzyme involved in such a mundane chemical reaction, it is in fact remarkable that this allostery can adequately control a single enzyme that is apparently crucial to regulate central nervous system levels of glutamate, ureagenesis in the liver, and insulin secretion in the pancreas.

1.8 Acknowledgements

This work was supported by National Institutes of Health (NIH) Grant DK072171 (to T.J.S.), NIH Grant DK53012 and American Diabetes Association Research Award 1-05-RA-128 (to C.A.S.) and NIH Grant DK19525 for islet biology and radioimmunoassay cores.

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2 Aminotransferases

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2.1 Abstract

The aminotransferases are PLP-dependent proteins which catalyse the transfer of an amino group from the donor amino acid to α-ketoglutarate, forming glutamate and the respective keto acids. Several key aminotransferase proteins have been identified as playing central roles in whole-body nitrogen metabolism, where they share common functions as nitrogen donors. These pathways play integrated roles within cells and between tissues, shuttling metabolites alluding to distinct pockets of compartmented metabolic activity. These anaplerotic shuttles interface with key metabolic pathways, for example the glutamate/glutamine cycle and TCA cycle, facilitating the regeneration of key metabolites such as the primary neurotransmitter glutamate. Contributions to glutamate levels in the brain from these anaplerotic pathways exceed 30%, illustrating their importance in maintaining the neurotransmitter pool of glutamate in neuronal cells. Knowledge of these pathways is not only important to our understanding of normal physiological mechanisms, but even more to the ways in which they alter and contribute to the pathogenesis of disease. The pathological implications of impaired aminotransferase metabolism is discussed, in particular their potential role in glutamate toxicity, which has been implicated in the pathogenesis of neurodegenerative disease. Finally, because of their tissue distribution these proteins have additional roles as biomarkers of disease, and can be used in the differential diagnosis of acute and chronic hepatic injury.

2.2 Introduction

The compartmentation of metabolic substrates and the subcellular localization of enzymes contribute to a fascinating interplay of highly regulated pathways. Several metabolic pathways, which play central roles in protein metabolism, are governed by the aminotransferases. Although numerous aminotransferase proteins exist, this chapter details the whole-body distribution of several specific aminotransferases, with particular focus on their role in anaplerotic pathways to generate and maintain the pool of brain glutamate through their involvement in metabolic shuttles. Furthermore, their role in disease is discussed with particular reference to the clinical application of serum aminotransferases.

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Transamination reactions are facilitated by pyridoxal phosphate-dependent (PLP) transaminase enzymes (Christen and Metzler, 1985). The key aminotransferase proteins discussed here include the branched chain aminotransferases (BCAT) [E.C. 2.6.1.42], the alanine aminotransferases (ALT) [glutamate pyruvate transaminase or alanine 2-oxo-glutarate E.C. 2.6.1.2], and the aspartate aminotransferase proteins (AST) [glutamic oxaloacetic transaminase or L-aspartate:2-oxoglutarate aminotransferase, E.C. 2.6.1.1]. These proteins have a mitochondrial and cytosolic isoform with tissue-specific locations. The crystal structures of BCAT and AST have been elucidated, where the catalytically competent structure of these aminotransferases is a homodimer (Jansonius et al., 1984a,b; Yennawar et al., 2002, 2006; Goto et al., 2005). These enzymes belong to a large family of homologous proteins which operate by the same basic mechanism (Jansonius, 1998). PLP-dependent enzymes have been classified into four families with different fold types based on their three-dimensional structures (Jansonius, 1998; Mehta and Christen, 2000; Salzmann et al., 2000; Schneider et al., 2000). With the exception of the BCAT proteins which fall into the fold-type IV class of proteins, most of the PLP aminotransferases have been placed in the fold type I or L-aspartate aminotransferase family. A unique feature of the fold type IV family is that the proton is abstracted from the C4' atom of the coenzyme-imine or external aldimine on the re face instead of the si face of the PLP cofactor (Yoshimura et al., 1996).

2.2.1 Transamination

The mechanism of transamination consists of the coupled half-reaction in which the PLP cofactor transfers between its PLP and pyridoxamine (PMP) form (Fig. 2.1) (Karpeisky and Ivanov, 1966; Ivanov and Karpeisky, 1969; Kirsch et al., 1984). The substrates of the forward reaction are dictated by the specificity of the particular transaminase, whereas L-glutamate features in the reverse reaction for most transaminases. The cofactor PLP is covalently attached to the enzyme via a Schiff base linkage, as a result of the condensation of its aldehyde group with the ε-amino group of a lysine residue. Transamination occurs via a Ping-Pong Bi-Bi mechanism, where each half reaction is divided into three stages. Interaction of the transaminases with their respective substrates involves the nucleophilic attack of the α-amino group of amino acid 1 (e.g. isoleucine) with the enzyme–PLP Schiff base carbon atom to form an amino acid–PLP Schiff base (external aldimine) with release of the enzyme (e.g. BCAT) (Fig. 2.1a). After transamination the second step is keto-enol tautomerism which involves the interconversion between the keto-enol form resulting in the formation of an α-keto acid–PMP Schiff base. This is subsequently hydrolysed to PMP and an α-keto acid, the final step in the first half-reaction (Fig. 2.1b). The second half reaction involves the same three steps but in reverse. In this stage the substrate is a second α-keto acid which reacts with PMP forming a Schiff base. Tautomerization facilitates the conversion from α-keto acid PMP to amino acid 2–PLP Schiff base. Subsequently, an internal aldimine is formed through attack from the ε-amino group of the active site lysine, leading to the release of a new amino acid and the regeneration of the aminotransferase.

The BCAT enzymes catalyse the transfer of the α-amino group from the hydrophobic branched chain amino acids (BCAA) leucine, isoleucine, and valine to α-keto glutarate, releasing their respective keto acids: ketoisocaproate, keto methyl valerate and ketoisovaline, and glutamate, regenerating the enzyme (Ichihara and Koyama, 1966; Taylor and Jenkins, 1966a,b,c). Both ALT and AST operate by the same basic mechanism generating pyruvate and glutamate, and oxaloacetate and glutamate, respectively (Glinghammar et al., 2009). The transamination reaction of each aminotransferase is summarized in Box 2.1.

2.2.2 Cellular distribution of aminotransferases

Although aminotransferase activity is found in most tissues, each isoform shows tissue specificity with distinct subcellular location, which points to distinct functional roles
Fig. 2.1. Transamination of the branched chain amino acid isoleucine (first half reaction). (a) The enzyme-PLP Schiff base carbon undergoes a nucleophilic attack by the α-amino group of isoleucine resulting in the formation of an amino acid-PLP Schiff base and the release of BCAT. (b) Following keto-enol tautomerization the resulting ketamine undergoes hydrolysis generating the α-keto acid, α-ketoisocaproate and the PMP form of BCAT.

Box 2.1. Transamination reactions of the aminotransferase proteins.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucine + α-ketoglutarate</td>
<td>BCAT</td>
<td>α-Ketoisocaproate + L-glutamate</td>
</tr>
<tr>
<td>L-Valine + α-ketoglutarate</td>
<td>BCAT</td>
<td>α-Ketoisovalerate + L-glutamate</td>
</tr>
<tr>
<td>L-Isoleucine + α-ketoglutarate</td>
<td>BCAT</td>
<td>α-Keto-β-methylvalerate + L-glutamate</td>
</tr>
<tr>
<td>L-Aspartate + α-ketoglutarate</td>
<td>AST</td>
<td>oxaloacetate + L-glutamate</td>
</tr>
<tr>
<td>L-Alanine + α-ketoglutarate</td>
<td>ALT</td>
<td>pyruvate + L-glutamate</td>
</tr>
</tbody>
</table>
of these proteins in tissues. The cellular distribution of these enzymes was first characterized in murine and rat models and has since progressed to human and cell systems. These models have offered enumerable insights into the compartmentation of many metabolic pathways, and indeed the flow of metabolites between these compartments. Understanding the differential distribution of these proteins and the factors which contribute to their regulation is pivotal to our understanding of the pathogenesis of disease, as many specific enzymes or pathways of metabolism are altered in adverse conditions.

2.2.2.1 Cellular distribution of the BCAT proteins

In mammals there are predominantly two BCAT isoforms, encoded by two different genes, which show both tissue and cellular-specific locations. Although the BCAT isoforms share 58% sequence homology and are structurally very similar, they possess distinct differences in catalytic efficiency and regulation (Davoodi et al., 1998). These enzymes play significant roles in amino acid metabolism and whole-body nitrogen shuttling. The most ubiquitous isoform, BCAT_m, is found in mitochondria and is thought to be responsible for the majority of transamination outside the central nervous system with the highest levels of BCAT_m recorded in the pancreas, kidney, stomach, and brain (Suryawan et al., 1998). To date, the cytosolic isoform (BCAT) has only been isolated from brain, placenta, ovary, and the peripheral nervous system (Hutson, 1988; Hutson et al., 1992, 1998; Hall et al., 1993; Sweatt et al., 2004a,b; Garcia-Espinosa et al., 2007). Although these two isoforms predominate, two other spliced variants have been identified, namely, a novel alternatively spliced PP18b variant found in placental tissue, and a novel co-repressor for thyroid hormone nuclear receptors (P3) (Lin et al., 2001; Than et al., 2001). Both spliced variants are homologous to BCAT_m. Although the function of the PP18b variant is unknown, P3 acts as a co-repressor for thyroid hormone nuclear receptors. The biological significance of these variants remains to be determined.

Due to the ease with which the BCAA pass the blood–brain barrier and their role in glutamate metabolism, the subcellular localization of the BCAT proteins in brain has been extensively investigated (Oldendorf, 1973; Cremer et al., 1976, 1979; Bixel et al., 1997, 2001; Bixel and Hamprecht, 2000; Sweatt et al., 2004a,b; Garcia-Espinosa et al., 2007). In brief, BCAT_m was only found in neuronal cells, localized to axons and nerve terminals in glutamatergic neurons, and concentrated in cell bodies in GABAergic neurons (Bixel et al., 2001; Sweatt et al., 2004a,b). Conversely, BCAT_m was the predominant isoform in astrocytic cells. However, low levels of BCAT_m were detected in certain astrocyte populations using a cell culture model, but this was not reported in rat tissue (Bixel et al., 1997, 2001; Bixel and Hamprecht, 2000). To date the BCAT proteins have not been mapped at the subcellular level in the human brain.

2.2.2.2 Cellular distribution of the ALT proteins

Early studies investigating the cellular distribution of ALT in rats suggested that there was only one cytosolic ALT isoform, with the mitochondrial form dismissed as cytosolic contamination of mitochondrial preparations. Subsequent reports have validated that there are two ALT isoforms (ALT1, cytosolic; and ALT2, mitochondrial), encoded by separate genes (ALT1-GTP gene and ALT2-GTP2 gene) (De Rosa and Swick, 1975; Sohocki et al., 1997; Lindblom et al., 2007; Glinghammar et al., 2009). An alternatively spliced isoform of ALT2 (ALT2-2) has been suggested with a predicted sequence of 100 amino acids shorter than ALT2; however this isoform has not been characterized at the protein level and its function is unknown (Lindblom et al., 2007). The ALT proteins play a pivotal role in mediating the passage of intermediates between gluconeogenesis and amino acid metabolism from the muscle to the liver, and more recently have been described as playing a role in the hypothetical alanine–lactate shuttle between neuronal and astrocytic cells (Waagepetersen et al., 2000). Clinically, due to the high expression levels of ALT in liver it is considered to be one of the major biomarkers of liver dysfunction (see below).

Unlike BCAT, both ALT isoforms are widely expressed in rat and murine tissues.
Here high levels of ALT mRNA expression were reported in liver, muscle, and brown/white adipose tissue, with relatively lower ALT expression observed in rat colon, heart, and brain (Jadhao et al., 2004; Yang et al., 2009). With the exception of the kidney, these patterns of mRNA expression largely mapped to those observed in humans (Yang et al., 2002a, 2009). While both ALT isoforms are highly expressed in rat adipose tissue, their expression in human adipose tissue has not been described. Although ALT brain activities have been reported in several rat models there are limited data on the pattern of ALT distribution in the human brain. A study described by Lindblom et al demonstrated that expression of ALT1 was below the detection limit for all brain samples analysed, whereas ALT2 showed low to moderate levels of expression in the cerebral cortex with intense staining in the cerebellum, highlighting the importance for further studies to validate ALT distribution in the brain (Lindblom et al., 2007). These studies would extend our knowledge on the contribution of ALT proteins to glutamate metabolism (see below).

2.2.2.3 Cellular distribution of the AST proteins

Like ALT, the AST proteins are widely distributed with the highest expression found in striated muscle, myocardium and liver tissues. With the exception of the red blood cells which only contain ASTc, all tissues have both isoforms, albeit at varying levels in different cell types. Because of their role in neuronal metabolism the distribution of these isoforms has been extensively studied in various brain preparations with particular focus on the mitochondrial isoform (ASTm). ASTm is not only targeted to mitochondria but also on the cell surface, confirming the role of ASTm as both a mitochondrial and plasma membrane protein (Cechetto et al., 2002). Interestingly ASTm was found to be identical to a fatty acid-binding protein (FABPpm), which has a role in permitting the uptake of long chain free fatty acid in cells (Stremlel et al., 1990; Stump et al., 1993; Bradbury and Berk, 2000). For full activity AST must first associate with binding proteins which transfer it to lipids on the inner mitochondrial membrane (Teller et al., 1990). Functionally, AST proteins play a central role in glutamate metabolism and in the malate/aspartate shuttle which transfers reducing equivalents from the mitochondria to the cytosol in the brain. Glutamate dehydrogenase (GDH), like AST, also catalyses the conversion of glutamate to α-ketoglutarate, albeit by a different mechanism (Leong and Clark, 1984). Whereas AST catalyses transamination (Box 2.1), GDH either adds an amino group to α-ketoglutarate utilizing NAD(P)H or removes an amino group from glutamate producing α-ketoglutarate with the reduction of NAD(P). Numerous studies have reported that both these enzymes are colocalized in cells and work in concert with each other to either drive the synthesis or degradation of amino acids (Lai et al., 1977, 1986; Palaiologos et al., 1988).

Immunohistochemical studies on rat brain showed differential staining between glutamatergic or GABAergic neurons. High expression of AST was reported in periglomerular cells of the olfactory bulb and basket cells, and in stellate cells of the cerebellum and second layer cells of the neocortex, whereas ASTm was found in mitral cells and glomerular regions of the olfactory bulb and golgi cells of the cerebellum (Kamisaki et al., 1984). Similar reports of isoenzyme compartmentation were also described in the rat retina (Inagaki et al., 1985, 1987). Although high levels of AST activity have been reported in neuronal cells, controversy surrounding the actual activity and indeed its contribution to glutamate metabolism in astrocytes exists, which is in part due to reports of the absence of the malate/aspartate carrier (AGC) (see below). Further studies are required to determine the activity of AST relative to GDH in these brain preparations, their localization, and their specific roles in astrocytes.

2.3 The Role of Aminotransferases in Brain Metabolism

The compartmentalization of the aminotransferase isoforms in neuronal or astroglial cells and the observed differences in their catalytic
Aminotransferases

and regulatory mechanisms points to two functionally distinct proteins despite catalysing the same reaction. The aminotransferase proteins and their substrates play a significant role in normal brain function, driving several key metabolic pathways central to energy metabolism and neurotransmitter synthesis (Figs 2.2–2.6). A dysfunction of these pathways can potentially contribute to the pathogenesis of a number of neurodegenerative conditions such as Alzheimer’s and Parkinson’s disease (Choi, 1988; Esclaire et al., 1997). A key metabolite linking these metabolic pathways is glutamate, the major excitatory neurotransmitter in the mammalian brain as well as the immediate precursor to GABA and glutathione (Attwell and Laughlin, 2001; Danbolt, 2001). Under normal physiological conditions glutamate plays a role in dendrite and synapse formation, but also plays a dominant role in glutamatergic transmission essential for memory and learning (Danbolt, 2001). 

Fig. 2.2. The glutamate-glutamine cycle. Excess glutamate released from pre-synaptic neurons into the synaptic cleft is rapidly taken up by astrocytes through the specific glutamate receptors GLAST/EAAT1 and GLT1/EAAT2. Within the astrocyte the majority of glutamate undergoes amidation to glutamine catalysed by glutamine synthetase. Non-neuroactive glutamine is released into the ECF for uptake by pre-synaptic neurons for regeneration of the neuronal glutamate pool. Glutamate may also be oxidized or utilized for the synthesis of glutathione or purines.
amidation to the non-neuroactive amino acid glutamine, by the microsomal enzyme glutamine synthetase (GS) that is restricted to astrocytes. Glutamine synthetase utilizes the steady supply of ammonia from blood (or brain metabolism) for glutamate synthesis which is then released into the ECF allowing for uptake by the pre-synaptic neuron to recycle the store of glutamate through deamidation of glutamine by mitochondrial phosphate-dependent glutaminase (Fig. 2.2) (Yudkoff et al. 1993, 1994). Thus, astrocytes and neurons play complementary roles in the glutamate-glutamine cycle, maintaining the neuronal glutamate pool at high concentrations, and preventing toxic elevations in the synaptic space. In astrocytes there are several fates for glutamate other than glutamine synthesis, dependent on substrate availability, the differential distribution and regulation of key metabolic enzymes (e.g. glutaminase, GDH, Malate dehydrogenase (MDH), AST, BCAT, ALT, Malic enzyme (ME), and GS), or whether the source of glutamate is exogenous or endogenous (Fig. 2.6). For example, when levels of external glutamate are low, the glial glutamate synthetase pathway is favoured, whereas in glutamate excess, considerable oxidation occurs (McKenna et al., 1996a,b).

Both glutamate and glutamine can be oxidized for energy in astrocytes and neuronal cells (McKenna et al., 1996a,b; Daikhin and Yudkoff, 2000). An estimated 30% of glutamate taken up by astrocytes is metabolized to
Aminotransferases

Fig. 2.4. The branched chain aminotransferase-branched chain keto acid shuttle. Leucine is actively taken up by astrocytes where it undergoes transamination via BCATₐ, forming glutamate and 2-ketoisocaproate (KIC). The glutamate formed can enter the glutamate-glutamine cycle whereas KIC, which is poorly metabolised by astrocytes, is further metabolized in neuron cells. The keto acid is subsequently transaminated with glutamate to regenerate leucine, which can be subsequently returned to the astrocyte to complete the cycle. These cycles also operate with the TCA cycle and GDH metabolism (Yudkoff et al., 1990; Hutson et al., 2001).

lactate involving TCA cycle intermediates and the pyruvate/malate cycle (Sonnewald et al., 1993; Gamberino et al., 1997). Glutamate in astrocytes is also used in the production of purines and key metabolic amino acids such as glutathione (Fig. 2.2) (Shank and Aprison, 1981; Yudkoff et al., 1988). Therefore, anaerobic pathways must interface with the glutamate-glutamine cycle to regenerate this ‘lost’ glutamate necessary to sustain efficient neurotransmission. Pyruvate carboxylase, an enzyme found solely in astrocytes, utilizes brain CO₂ to replenish the carbon required for the TCA cycle, which as a result contributes to the overall concentration of glutamine produced (Oz et al., 2004). A limiting factor in this reaction is the source of nitrogen, where the BCAA, aspartate and more recently alanine serve as potential nitrogen donors (Shank et al., 1985; Bixel and Hamprecht, 1995; Yudkoff et al., 1996a,b; Yudkoff, 1997; Hutson et al., 1998, 2001; Kanamori et al., 1998; Lieth et al., 2001; Magistretti, 2009). The respective roles of the aminotransferase proteins are discussed in the following sections.

2.3.1 The role of BCAT in brain metabolism

The BCAA easily traverse the blood–brain barrier, with leucine more readily accepted than other amino acids (Oldendorf, 1973; Smith et al., 1987). Conversely, glutamate and glutamine are poorly taken up, highlighting the importance of glutamate synthesis in the brain. It has long been established that in peripheral tissues the role of the BCAA, in particular leucine, is to act as a major nitrogen donor for glutamate and glutamine synthesis (Fig. 2.3) (Goldberg and Chang, 1978). Skeletal muscle harbours high concentrations of BCATₐ, mediating the formation of glutamate.
Fig. 2.5. The alanine-lactate shuttle operates with the glutamate-glutamine cycle. Lactate produced in astrocytes can be exchanged in part with alanine from neuronal cells. Lactate is subsequently metabolized to pyruvate which has two fates: i) The TCA cycle, or ii) transamination with glutamate via ALT. The alanine formed through transamination is thought to carry the amino group through GDH metabolism from glutamine deamination to glutamate. The alanine produced acts as a nitrogen donor in astrocytes, ultimately used to regenerate glutamate (adapted from Waagepetersen et al., 2000).

and α-ketoglutarate from BCAA exported from the liver (Fig. 2.2). The role of leucine as a nitrogen donor in brain metabolism has since been extensively studied, where the BCAT proteins are considered to play a key role in the oxidation and synthesis of glutamate owing to both their high expression and subcellular localization.

In rat brain slices the BCAA are metabolized faster than they are incorporated into proteins, supporting the theory that the BCAA serve functions other than just an energy source (Chaplin et al., 1976). In particular, leucine is readily metabolized in astrocytes, where uptake of leucine by glial cells is mediated by a sodium-independent process (Brookes, 1992, 1993). A combination of kinetic and metabolic studies in astrocytes, measuring the incorporation of [15N]leucine into glutamate and glutamine, suggested that the rate of transamination of leucine is greatly favoured over complete oxidation with approximately 30% of the nitrogen of glutamate-glutamine derived from leucine alone (Brand, 1981; Brand and Hauschildt, 1984; Harper and Benjamin, 1984; Yudkoff et al., 1990). These studies, among others, support the hypothesis that although transamination is completely reversible it seems that BCAT transamination in the direction of glutamate and α-keto acid formation is favoured in astrocytes, whereas the reverse holds true for neuronal cells. These produce leucine and α-ketoglutarate, thought to complement the glutamate-glutamine cycle (Yudkoff et al., 1996b; Yudkoff 1997; Daikhin and Yudkoff, 2000). Both in vivo rat brain and ex vivo rat retina models (accepted models of glutamatergic
neurons) using gabapentin as an inhibitor of BCAT, have also supported the de novo synthesis of glutamate (LaNoue et al., 2001; Lieth et al., 2001). These studies confirmed that gabapentin inhibited the de novo synthesis of glutamate by 30% in retina cells and up to 50–60% of added leucine transamination.

Not only is synthesis affected but so too is oxidation. Both retinal Muller cell (retinal astroglia) and cultured astrocyte models confirmed that transamination was a necessary prerequisite step to glutamate oxidation, where oxidation was blocked by the transaminase inhibitor aminooxyacetic acid (AOAA) (McKenna et al., 1996a,b; Lieth et al., 2001). To fit with the proposed model these results would imply that the supply of branched chain keto-acids (BCKA) would affect the extent of glutamate oxidation. In cultured rat astrocytes, oxidation of [14U]glutamate was stimulated by addition of BCKA (Hutson et al., 1998). In retinal cells the addition of BCKA, gabapentin, and BCKA and gabapentin together, resulted in the stimulation of glutamate oxidation with the latter showing the greatest degree of stimulation. These findings, together with the subcellular localization of BCAT to astrocytes and BCAT, to neuronal cells, led to the development of the BCAA–BCKA shuttle hypothesis between the astrocyte and neuron, which works together with the glutamate–glutamine cycle (Fig. 2.4) (Yudkoff et al., 1996a; Yudkoff, 1997; Hutson et al., 1998, 2001). Here, mitochondrial BCAT catalyses the transamination of the BCAA in astrocytes. The resulting BCKA, which are poorly metabolized by astrocytes, are shuttled to neuronal cells for further metabolism.
while glutamate enters the glutamate–glutamine cycle. BCAT, which is neuronal specific, catalyses transamination of the BCKA with glutamate back to the BCAA, which exit the neuron and return to the astrocyte. \( \alpha \)-Ketoglutarate may also undergo reductive amination to glutamate via neuronal GDH but is thought not to be a serious contender, as the flux in this direction is low (Yudkoff et al., 1990; Hutson et al., 2001). Therefore, BCAT proteins in brain metabolism do not only facilitate energy metabolism, but also provide essential nitrogen for the anaplerotic regeneration of glutamate.

### 2.4 Alanine Aminotransferases and Glutamate

In muscle, BCAA and alanine metabolism are intrinsically linked with glutamate metabolism. During gluconeogenesis both alanine and glutamine are the predominant amino acids which leave the muscle, with alanine preferentially taken up by the liver (Fig. 2.3). Evidently ALT plays a more dominant role in protein metabolism of liver and muscle relative to brain, as the levels of ALT are significantly higher. Alanine released from muscle is derived from pyruvate and glutamate, releasing \( \alpha \)-ketoglutarate thought to drive the TCA cycle particularly during exercise; this is known as the glucose–alanine cycle (Fig. 2.3) (Rutten et al., 2005). This is closely linked to the glucose–lactate cycle (Cori cycle), which operates in parallel during gluconeogenesis. Thus, carbohydrate and protein metabolism operate cooperatively, where the direction of metabolism is substrate driven. The role of ALT in brain metabolism has been described in the hypothetical lactate–alanine shuttle between brain cells, with its metabolites potentially linked with glutamate metabolism (Fig. 2.5) (Pellerin and Magistretti, 1994; Peng et al., 1994; Waagepetersen et al., 2000; Schousboe and Waagepetersen, 2005; Bak et al., 2006).

Glucose is considered the main energy source of the brain, where both neuronal and astrocytic cells are capable of its metabolism. Relative to other metabolites glucose is readily taken up by the brain and is available in high concentrations in the blood. Conversely, lactate cannot readily pass the blood–brain barrier (Cremer et al., 1976, 1979) and its levels in the blood fall short in maintaining the energy requirements of the brain, even though neuronal cells express both lactate and glucose transporters (Bergersen et al., 2002). It has been demonstrated that astrocytes have greater glycolytic activity than neuronal cells, with the lactate produced transported to neuronal cells for further oxidation (Schousboe et al., 1997; Waagepetersen et al., 2000; Pellerin, 2003). The lack of metabolic machinery (i.e. the malate–aspartate shuttle) in astrocytes, which would otherwise drive glycolysis through to complete oxidation in the mitochondria, support these findings. In fact, with the proposed lactate-alanine shuttle it was suggested that neuronal cells may actually metabolize lactate as efficiently as glucose, a subject actively debated in the literature (Schurr et al., 1997 a,b,c; Cater et al., 2001; Waagepetersen et al., 2000; Bak et al., 2006, 2007, 2009). Metabolic studies utilizing [U\(^13\)C]lactate incubated with glutamatergic cerebellar granule cells, astrocytes and co-cultures demonstrated that alanine was preferentially formed or released into the medium in neuronal cells. Incubation of these cells with [U\(^13\)C]alanine resulted in the preferential enrichment of alanine in astrocytes relative to neuronal cells, suggesting that neurons preferentially synthesize and release alanine, and astrocytes favour uptake supporting a different functional role of alanine metabolism in brain cells. Thus, as described for BCAA in the BCAA–BCKA shuttle, the role of alanine as a carrier for nitrogen between neurons and astrocytes in exchange for lactate was suggested (Fig. 2.5). In this role it was proposed that alanine would serve to transport excess ammonia from neuronal cells to astrocytes, generated from the metabolism of glutamine to glutamate by mitochondrial glutaminase, where it could be utilized by GS for glutamine synthesis from glutamate (Waagepetersen et al., 2000). To establish a link between the alanine–lactate cycle and the glutamate–glutamine cycle, neuronal and astrocyte cells were incubated with [5\(^15\)N]glutamine and [\(^15\)N]alanine, respectively. A minor 3.3% enrichment of alanine in neuronal cells and 22% monolabelling in glutamate and glutamine in astrocytes was reported, offering
some evidence in support of the shuttle; however, the actual extent to which it contributes as an anaplerotic pathway is still in dispute.

Production of lactate in astrocytes is thought to correlate with an increase in glycolysis stimulated through glutamate uptake (Pellerin et al., 1998). In brief, it was proposed that lactate generated from astrocytes or through uptake from the peripheral blood is converted to pyruvate by lactate dehydrogenase (LDH), which is further utilized for energy production through the TCA cycle or as a substrate for ALT (Fig. 2.5). In neuronal cells ALT catalyses the transamination of pyruvate to alanine which results in the consumption of glutamate and the generation of \( \alpha \)-ketoglutarate. Alanine that is produced is released and taken up by astrocytes to undergo further transamination to pyruvate and \( \alpha \)-ketoglutarate, completing the cycle (Fig. 2.5) (Waagepetersen et al., 2000). Here, the role of alanine was proposed as a nitrogen carrier from neuronal cells to astrocytes. It was suggested that alanine formed as a result of GDH activity utilizes the excess ammonia formed from the glutaminase reaction, and channels the much-needed source of nitrogen into astrocytes. In retinal Muller cells 60% of glutamate synthesis occurred via AOAA-sensitive transamination (LaNoe et al., 2001). Furthermore, using the inhibitor L-cycloserine, \textit{de novo} synthesis of glutamate was inhibited to a greater degree relative to the specific inhibition of BCAT using gabapentin. This suggests that this alanine shuttle is considered to be particularly important in neurons and the most important in the brain (Fig. 2.6) (Cheeseman and Clark, 1988). The role of the glycerol 3-phosphate shuttle is disputed, due to conflicting reports of the subcellular localization of this shuttle in various brain cells (Nguyen et al., 2003).

The AST isoforms are central to the operation of this shuttle as they are the rate-limiting enzymes; evidence suggests that this shuttle is more active in neuronal cells relative to astrocytes (McKenna et al., 2006). The AST enzymes work with several other proteins including the aspartate/glutamate carrier (AGC – aralar 1 isoform), the \( \alpha \)-ketoglutarate carrier, and GDH and MDH, which have been shown to form metabolomic complexes through physical associations with each other (Ramos et al., 2003; McKenna et al., 2006). These physical interactions between enzymes are thought to facilitate the transfer of substrates between enzymes thus maximizing catalysis. The \textit{in vivo} importance of these interactions is currently unknown. The mitochondrial enzyme AST\textsubscript{m} operates in the direction of aspartate synthesis, and the cytosolic in the direction of aspartate conversion to oxaloacetate (OAA). Briefly, the mitochondrial membrane is impermeable to NADH formed during the glycolytic cycle. To facilitate the transfer of reducing equivalents, OAA is reduced to malate in the cytosol, which is exchanged for}

### 2.5 Aspartate Aminotransferases and their Role in the Malate–Aspartate Shuttle and Glutamate Metabolism

Functionally, AST proteins play a central role in the malate–aspartate shuttle and in glutamate metabolism. In neuronal cells, the synaptic terminals which are rich in mitochondria have high malate–aspartate shuttle activity (Cheeseman and Clark, 1988) and high levels of AST and GDH activity (McKenna et al., 2000a,b), whereas the activity of AST in astrocytes is reported to be considerably lower. Oxidation of glucose to pyruvate yields NADH, a major reducing equivalent which drives the TCA cycle to yield the maximum energy from glucose, which is essential for brain function. The function of the malate-aspartate shuttle is to avoid the build-up of the ratio of NADH/NAD\(^+\), which serves to favour metabolism moving in the direction of lactate production, rather than oxidation of pyruvate in the TCA cycle, which would decrease the energy output from glycolysis. Although other shuttle mechanisms may operate, the two most studied shuttles include the malate–aspartate shuttle and the glyceraldehyde 3-phosphate shuttle, with the malate–aspartate shuttle considered to be particularly important in neurons and the most important in the brain (Fig. 2.6) (Cheeseman and Clark, 1988). The role of the glycerol 3-phosphate shuttle is disputed, due to conflicting reports of the subcellular localization of this shuttle in various brain cells (Nguyen et al., 2003).

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\(\alpha\)-ketoglutarate across the mitochondrial membrane. Malate dehydrogenase catalyses the release of the reducing equivalents from malate with the formation of OAA. Here, mitochondrial AST catalyses the transamination of OAA and glutamate to aspartate and \(\alpha\)-ketoglutarate, respectively. Both aspartate and \(\alpha\)-ketoglutarate are subsequently transferred to the cytosol where its cytosolic counterpart, AST, regenerates glutamate and OAA from cytosolic aspartate and \(\alpha\)-ketoglutarate (Fig. 2.6). Over the last few years several groups using immunohistochemistry and in situ hybridisation have demonstrated that astrocytes have either no expression or very low levels of AGC, one of the key components of the malate–aspartate shuttle (Ramos et al., 2003; Xu et al., 2007), which suggests that the direction of metabolism results in the production of lactate that must be exported to neuronal cells for further oxidation (Fig. 2.6) (McKenna, 2007; Xu et al., 2007).

In addition to their role in the transfer of reducing equivalents, the AST proteins have been shown to play an important part in glutamate metabolism (Safer and Williamson, 1972; Scholz et al., 1998; Chatziioannou et al., 2003). The rate of oxidation of glutamate differs in neuronal and astrocytic cells. In astrocytes glutamate is oxidized at a rate almost twice that of glutamine, which supports the role of astrocytes in disposing excess glutamate. It has been reported that the role of AST\(_m\) in glutamate metabolism is dependent on the origin of glutamate. Using \(^{13}\text{C}\) NMR tracer experiments and inhibitors of aminotransferase proteins such as AOA, it was proposed that oxidation of exogenous glutamate is primarily through the action of mitochondrial GDH, whereas glutamate synthesized endogenously from glutamine (oxidized for energy by glutaminase) is metabolized by AST (Fig. 2.6) (McKenna et al., 1993, 1996b). In fact, a congenital deficiency or mutation of the BCKDH complex resulting in the accumulation of the BCAA and \(\alpha\)-keto acids leading to neuronal dysfunction (see below). Although not discussed in detail in

2.6 Pathological Conditions Resulting from Impaired Aminotransferase Metabolism

To date there are no known mutations of the BCAT, AST, or ALT aminotransferase proteins contributing to a pathological condition. However, a build-up of their substrates or products can lead to disorders leading to neurodegeneration (e.g. glutamate toxicity in Alzheimer’s disease). The most accepted consequence of altered BCAA metabolism is a mutation of the BCKDH complex resulting in the accumulation of the BCAA and \(\alpha\)-keto acids leading to neuronal dysfunction (see below). Although not discussed in detail in
this chapter, the only aminotransferase protein with a known mutation is the alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44), a PLP-dependent metabolic enzyme which catalyses the transamination of alanine and glyoxylate to pyruvate and glycine. This enzyme is characteristically found in the peroxisomes distributed largely in hepatocytes. In humans, AGT is encoded by the AGXT gene, mutations of which give rise to dysfunctional proteins resulting in the overproduction of oxalate (Coulter-Mackie and Rumsby, 2004; Danpure, 2006). Excess oxalate leads to the progressive accumulation of insoluble calcium oxalate in the kidney and urinary tract leading to urolithiasis, often accompanied by systemic oxalosis, which ultimately results in renal failure.

2.6.1 Maple syrup urine disease

Maple syrup urine disease is a congenital disease characterized by a build-up of both BCAA and their respective keto-acid derivatives. These metabolites are neurotoxic to cells in the cerebrospinal fluid, blood, and tissues resulting in the patient presenting with symptoms such as neurological dysfunction, seizures and infant death (Silberman et al., 1961; Chuang, 1998, 2006). MSUD is an autosomal recessive disorder caused by a deficiency of the multienzyme complex, the branched-chain α-keto acid dehydrogenase complex (BCKDH), the rate-limiting step of transamination (Dancis et al., 1959, 1960; Menkes, 1959, 1962; Dankis 1964). This complex is composed of three catalytic subunits, the E1, E2 and E3 subunits (Harris et al., 2004), where all three units are essential for enzymatic activity (Danner and Doering, 1998). At least 150 mutations in the BCKDH complex genes have been reported, with the most disease-causing mutations seen in E2 (Chuang, 1998; Danner and Doering, 1998, Chuang and Chuang, 2000; Chuang et al., 2006). Five classifications of MSUD have been identified (varying from severe classic forms to mild variant types, and also with a thiamine-responsive form), based on the residual BCKDH activity, the age of onset and the concentration of leucine in serum (Duran and Wadman, 1985; Chuang and Chuang, 2000; Chuang et al., 2006). Although treatment through restriction of the BCAA in the diet has most value in milder forms of the condition, patients that are not compliant with their diet or those with more severe forms of the disease are still subject to many side effects (Snyderman, 1986, 1988). Investigation into alternative therapies using animal models has been described (Klivenyi et al., 2004; Wu et al., 2004; Homanics et al., 2006). Briefly, the usefulness of these models varies widely, from creating homozygous lethal knockouts, to knockouts that phenotypically resemble BCKDH but on further investigation revealed a BCATm mutation (Wu et al., 2004). More useful models include a classical MSUD and intermediate MSUD design (Homanics et al., 2006; Zinnanti et al., 2009). In the homozygous mouse, relative to wild-type (WT), levels of BCAA to alanine were 22-fold higher and 16-fold higher for the cMSUD and iMSUD, respectively. In contrast to the increase in BCAA levels, alanine, glutamate, and glutamine in the blood were all significantly reduced in iMSUD mice compared with WT, with low levels of aspartate and gamma-aminobutyric acid also uniformly reduced. The reduced levels of neurotransmitters are likely to cause encephalopathies such as coma. These models will provide a wealth of knowledge for pathological analysis and metabolic profiling of blood, brain and other tissues (Zinnanti et al., 2009).

2.6.2 Glutamate toxicity and neurodegeneration

Under normal physiological conditions glutamate plays a role in dendrite and synapse formation, and also a dominant role in glutamatergic transmission, essential for memory and learning (Danbolt, 2001). However, high levels of glutamate result in this amino acid becoming a potent neurotoxin (Chapter 25). This has been reported to lead to increased expression of tau protein, neuronal degeneration and cell death (Choi, 1988, 1990; Esclaire et al., 1997). The effect of the excessive synaptic release of glutamate is largely mediated by an increase in the entry of calcium into neurons (Kaplan and Miller, 1997; Sattler and Tymianski, 2000; Mattson, 2003, 2007, 2008), which is the predominant secondary messenger
for neurotransmitters and neurotrophins (Choi, 1988; Kaplan and Miller, 1997). An overload of calcium, observed in the brain tissue of patients with AD has been shown to evoke acute degenerative conditions (Mattson and Chan, 2003; Mattson, 2007). Thus, a strong relationship exists between excessive calcium influx and glutamate-triggered neuronal injury. Recently published studies using targeted proteomics in neuronal cells have shown that BCAT proteins have redox-mediated associations with several neuronal proteins involved in G-protein cell signalling, indicating a novel role for BCAT in cellular redox control (Conway et al., 2008). Interestingly, the brain-derived neurotrophin factor, which mediates its action through calcium cell signalling, causes up-regulation of BCAT (Numakawa et al., 2002, 2009; Madeddu et al., 2004). These findings indicate that the BCAT proteins may have fundamental links with calcium-mediated signalling, and because of its primary role in producing glutamate, understanding this mechanism may enhance our knowledge of how glutamate can reach toxic levels in neurodegenerative diseases such as AD, offering potential sites for targeted therapy.

### 2.6.3 Redox sensitivity of BCAT

Generation of reactive nitrogen and oxygen species (RNS and ROS, respectively) can occur through calcium overload, which as previously mentioned can be generated through glutamate neurotoxicity (Kaplan and Miller, 1997; Sattler and Tymianski, 2000; Mattson, 2003, 2007, 2008). Mitochondrial dysfunction can also generate reactive species, in particular peroxynitrite. Targets of these harmful species include reactive thiols of receptive proteins, resulting in changes to the structure and/or function of a protein, ultimately leading to metabolic imbalances resulting in cell death. The BCAT proteins are unique among the mammalian aminotransferases in that they contain a redox-active CXXC motif subject to reversible modification by both ROS and RNS, potentially serving as a biological control point (Conway et al., 2002, 2003, 2004, 2008; Coles et al., 2009; Hutson et al., 2009). Response to cellular stress varies between isoforms, with BCAT$_m$ being completely inactivated by both ROS and RNS, whereas BCAT$_c$ is only partially sensitive to air oxidation and the nitric oxide donor, S-nitrosoglutathione (GSNO). Low concentrations of GSNO caused a reversible time-dependent loss in 50% of BCAT$_c$ activity, characterized predominantly through S-nitrosation (a reaction transferring a NO group to the reactive cysteine of this protein) (Coles et al., 2009). However, increased exposure to GSNO resulted in a shift towards S-glutathionylation (addition of GSH to the reactive thiol), a marker of oxidative stress (Coles et al., 2009). Recent studies have demonstrated that both S-nitrosation and S-glutathionylation of proteins occur in the brains from patients suffering with AD, which is directly correlated to the misfolding of proteins (Yao et al., 2004; Benhar et al., 2006; Uehara et al., 2006; Fang et al., 2007; Lipton et al., 2007; Nakamura and Lipton, 2007, 2008, 2009; Cho et al., 2009). Investigation as to how these mechanisms of S-nitrosation or S-glutathionylation regulate the hBCAT proteins in vivo, relative to glutamate toxicity and protein misfolding, may contribute to the understanding of these fundamental pathways involved in the pathogenesis of AD.

### 2.7 Aminotransferase Proteins as Biomarkers of Disease

Not only do the aminotransferase (AST and ALT) proteins have significant roles in whole-body nitrogen metabolism: they have also been used for decades as biomarkers of disease, most notably liver disease (Panteghini, 1990). The chapter will therefore digress to accommodate their role in clinical biochemistry, detailing how their measurements in serum can assist in the differential diagnosis of hepatic conditions. Biomarkers are used in screening, diagnostics, prognostics, or monitoring of patient outcome. The role of AST and ALT as biomarkers is primarily diagnostic with some prognostic applications. Ideally a diagnostic biomarker should be differentially specific, released in a timely fashion, with rapid robust validated methods of analysis. As both AST and ALT are found in several tissues they fall short as ‘ideal’ markers of disease. For example, erythrocyte levels of
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AST are 10–15 times greater than that measured in serum, so mild elevations could suggest haemolysis rather than hepatic injury. However, to increase their clinical utility and diagnostic relevance, AST and ALT are grouped with other tests of liver function traditionally known as ‘liver function tests’ (LFT) (Table 2.1). It is relevant to note that although these tests are generically associated as LFT, they do not reflect liver function but rather the structural integrity of liver cells. True liver function tests would include the measurement of albumin, total protein, bilirubin, and/or prothrombin time. As ALT is localized only in the cytosol of liver, and AST is found in both mitochondria and cytosol, the ratio of AST/ALT can be used as an index of the severity of hepatic damage and indeed as a good prognostic indicator. Should the level of AST exceed ALT, damage to the cell is extensive and prognosis is poor.

Abnormal levels of the aminotransferase proteins may reflect both acute and chronic conditions and are interpreted in conjunction with the clinical and biochemical presentation of the patient. Patient history is of utmost importance in this differential diagnosis, as medications (including herbal remedies and over-the-counter preparations), co-morbid conditions, risk factors for viral hepatitis, and age can considerably influence the diagnosis. The reference limits for the aminotransferase proteins can vary among laboratories; however, examples of reference ranges used are summarized in Table 2.1. The use of AST and ALT as biomarkers is normally expressed with respect to the level of magnitude above the reference range, and more so the pattern of alteration often with respect to other LFT markers (Table 2.1). Levels can be defined as mild (≤ 2x), moderate (3–5x), or a marked increase (≥ 10–100x) above the upper reference limit (URL), where the rate and nature of change also illustrates the extent of cellular damage. It is important to note that these classifications are broad and may differ among clinicians. Therefore, patient history, the relative degree or elevation, in addition to the pattern of increase of the aminotransferase proteins relative to the other LFT, can differentiate between the causes of both acute and chronic liver disease.

2.7.1 Mild elevation of ALT and AST

The most common causes for a mild elevation of ALT and AST include acute alcoholic-induced or non-alcoholic fatty liver disease (NAFLD), where the activities of AST/ALT are reported as ≤ 2x the URL (Bayard et al., 2006). Generally, levels of ALT exceed that of AST in acute liver damage, with the exception of toxin-induced or alcoholic hepatitis and Reyes syndrome (Dufour et al., 2000a,b). One explanation for this change in the ratio is due to a deficiency in PLP, common in alcoholics. This has an impact in two ways: ALT is more sensitive to this loss than AST, and alcohol induces the release of ASTm, thus increasing the total amount of serum AST (Dufour et al., 2000a,b). Mildly elevated levels of AST and ALT are also reported for chronic conditions associated with hepatitis B and C, and cirrhosis. A ratio of AST/ALT greater than 1.0 is suggestive of advanced liver disease with a greater risk of advanced fibrosis indicative of a poor prognosis (Williams and Hoofnagle, 1988; Giannini et al., 1999, 2003). An AST/ALT ratio ≥ 1 can be found in 4% of patients with chronic hepatitis C and in 79% of patients who have cirrhosis (Williams and Hoofnagle, 1988; Giannini et al., 1999, 2003). If these conditions are excluded, consideration for more rare causes of mildly elevated AST and ALT must be evaluated, such as haemochromatosis, Wilson’s disease (in younger patients), autoimmune liver disease, and α-1-antitrypsin deficiency (Krawitt, 1996; Morrison and Kowdley, 2000; Ferenci et al., 2005). Patients with autoimmune disease, based on elevated levels of hypergammaglobulinaemia, also present with low levels of aminotransferase activity.

Pathologically the first stage of alcoholic liver disease is the appearance of large fatty deposits, without evidence of clinical or biochemical abnormalities. Currently, no biomarker can detect early stage hepatic injury. In stage two, alcoholic hepatitis, increased levels of AST and ALT may be mild or moderate. Because other conditions can also give rise to mild elevations of AST and ALT, γ-glutamyl transferase, a toxin-inducible enzyme often elevated in both alcoholic and drug induced injury and alkaline phosphatase (ALP), may be assessed and taken together with the
Table 2.1. Serum levels of AST and ALT relative to other markers of hepatic injury.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cellular distribution</th>
<th>Predominant tissue localization</th>
<th>Reference range</th>
<th>Cholestasis</th>
<th>Hepatic ischaemia or toxic injury</th>
<th>Acute viral hepatitis</th>
<th>Acute alcoholic liver disease</th>
<th>Chronic liver disease</th>
<th>Hepatic cirrhosis</th>
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<tbody>
<tr>
<td><strong>Biomarkers of structural integrity</strong></td>
<td></td>
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<td></td>
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<tr>
<td>ALT</td>
<td>Cytoplasm*</td>
<td>Liver, muscle and kidney</td>
<td>5-50 IU l⁻¹</td>
<td>Mild or moderate</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>Mild or moderate</td>
<td>Normal or ↑</td>
<td>Normal or ↑</td>
</tr>
<tr>
<td>AST</td>
<td>Cytoplasm and</td>
<td>Liver, heart, skeletal muscle, brain, and kidney</td>
<td>Age range 1-5 wks (6-122 IU l⁻¹), 6 wks-1 yr (6-71 IU l⁻¹) 1-4 yrs (6-51 IU l⁻¹) ≥5 yrs (6-35 IU l⁻¹)</td>
<td>Mild or moderate</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>Mild or moderate</td>
<td>Normal or ↑</td>
<td>Normal or ↑</td>
</tr>
<tr>
<td>γ-Glutamyl</td>
<td>Cell membrane</td>
<td>Liver, biliary canals, kidney</td>
<td>≤ 55 IU l⁻¹</td>
<td>Mild or moderate</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
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<td>transferase</td>
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<tr>
<td>ALP</td>
<td>Cell membrane</td>
<td>Liver, bone, and kidney</td>
<td>≤ 150 IU l⁻¹</td>
<td>Moderate or marked</td>
<td>↑</td>
<td>↑↑</td>
<td>↑↑</td>
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<td><strong>Functional biomarkers</strong></td>
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<tr>
<td>Bilirubin</td>
<td></td>
<td>Liver (but also extra hepatic)</td>
<td>≤ 20 μmol l⁻¹</td>
<td>Mild or moderate</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>Dependent on the type of infectionb</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>Albumin*</td>
<td>Liver</td>
<td>35-45 g l⁻¹</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal or ↑</td>
<td>↑↑</td>
<td>Normal or ↑</td>
</tr>
<tr>
<td>Prothrombin time*</td>
<td>Liver</td>
<td>≤ 150 s</td>
<td>Normal</td>
<td>Mild or moderate</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>Normal or ↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

*Only cytosolic form present in the liver; other tissues have both a cytosolic and mitochondrial isofrom.

b Levels of bilirubin will be elevated relative to infection with hepatitis A, B, C, etc., and the extent of damage.

*Albumin levels may also be reduced in patients who are malnourished, have cancer, malabsorption, or immune disorders (therefore it lacks specificity).

*The increase in time it takes for the blood to clot will depend on the extent of functional damage, and this can vary greatly within conditions.

↑ mild increase; ↑↑ moderate increase; ↑↑↑ marked increase.
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2.7.2 Moderate/marked elevation of ALT and AST

Acute damage to the liver can also result in a moderate/marked increase in AST and ALT. When levels of AST/ALT reach 100× the URL, it is almost always indicative of ischaemic injury or injury due to toxic ingestion (Dufour et al., 2000a,b). Ischaemic and hypoxic acute liver damage are frequently associated with patients who are clinically challenged, such as those with sepsis (Seeto et al., 2000). Toxin ingestion can captivate prescribed and herbal medications and intended overdose use. Within several hours of an overdose of acetaminophen levels of AST can reach in excess of 7000 IU l⁻¹, reflecting the ultimate destruction of liver cells leading to an immediate release of enzymes into the blood stream (Singer et al., 1995). As also seen in ischaemic hepatic injury, the ratio of AST/ALT will be increased and reflect an increase in lactate dehydrogenase, a marker of ischaemic injury. Post insult the levels of AST and ALT can drop dramatically to within the reference range; however, this is not necessarily a good prognosis as it may reflect extensive hepatic necrosis rather than recovery, due to the short circulatory half-life of AST (17 h) and ALT (47 h) (Giannini et al., 2005). In this case the monitoring of true liver function tests such as bilirubin levels or prothrombin time can assess if the patient is at risk from hepatic failure.

It is estimated that over 80% of individuals with acute viral hepatitis are never clinically diagnosed (Giannini et al., 2005; Knight, 2005). This is mostly due to the asymptomatic presentation of those infected and is often passed off as a flu-like illness with non-specific indicators such as fatigue and fever. Although hepatitis A and hepatitis B are on the decrease worldwide due to the introduction of vaccines, hepatitis C is increasing. For those with hepatitis C, 85% develop chronic hepatitis and have a 30% increased risk of developing hepatocellular cancer. Although jaundice is evident in almost 70% of those with acute hepatitis A, it occurs in less than 50% and 33% of cases with acute hepatitis B and C, respectively, and is therefore an insensitive diagnostic indicator (Dufour et al., 2000a,b; Giannini et al., 2005). Levels of the aminotransferases can also be varied dependent on the extent of cellular damage, and can show a moderate or marked increase with or without jaundice. As a result differential diagnosis of acute viral hepatitis can be more challenging for viral hepatitis, in particular hepatitis C. Although biochemically the clinical picture may not be clear, patient history and evidence of high-risk factors such as travel to endemic areas (hepatitis A), intravenous drug use, or transfusions will play a pivotal role in diagnosis. Confirmation of acute viral hepatitis, however, is only obtained when the patient is tested for viral-specific antibodies. Although toxin or viral insult represent the vast majority of acute liver diseases with moderate or marked increases in AST and ALT, other causes such as Epstein–Barr virus, autoimmune, extrahepatic, or congenital disorders must be considered (Giannini et al., 2005).

2.8 Conclusions and Future Directions

Although the intriguing compartmentation of metabolic pathways and the subcellular localization of the aminotransferase isoforms to different brain cells offer numerous insights into whole-brain metabolism, they also highlight the enormity of what is not understood. Evidently, the cellular distribution of the aminotransferases in brain tissue requires further characterization to substantiate the biochemical findings. Confirmation of the expression and activity of the specific isoforms in brain...
cells will further assist understanding of the actual extent to which these proteins participate in anaplerotic pathways. The current studies unequivocally support the role that these aminotransferase proteins play in the supply of nitrogen for glutamate synthesis. In particular, studies detailing the role of leucine in glutamate oxidation and synthesis show that it fulfills the criteria as an external nitrogen source:

1. It freely passes the blood–brain barrier.
2. The BCAT proteins are highly expressed in brain cells showing neuronal and astrocytic specificity.
3. Their role in muscle metabolism as a nitrogen donor is substantially characterized.
4. Overwhelming evidence in several model systems support the BCAA–BCKA shuttle with direct evidence of its contribution to the glutamate–glutamine cycle.

However, as with the other aminotransferase proteins, much is still unknown. More sensitive models using inhibitors with greater specificity would further enhance knowledge of these pathways. As these aminotransferase proteins play a key role in facilitating the anaplerotic generation of glutamate it is highly likely that their metabolism will be altered in neurodegenerative disease conditions. Therefore, understanding of how these suggested shuttles are altered in disease will offer possible targets for novel therapeutic treatment to either delay onset or prevent further neuronal destruction. Finally, to date ALT and AST have for decades sustained their role as key biochemical markers of disease, despite their lack of specificity. With the advent of proteomics and the search for the ideal marker of early stage liver disease, new markers are emerging. However, ALT and AST will sustain their prominent role for the foreseeable future and their value may extend if modifications of these proteins are observed at the early stages of disease.

References


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with mitochondria from primary cultures of cortical neurons or cerebellar granule cells. *Neurochemistry International* 36, 451–459.


M. E. Conway


3 Arginase

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3.1 Abstract

Arginase is one of the several enzymes that metabolize the semi-essential amino acid, L-arginine. The other enzymes which metabolize L-arginine include:

1. Nitric oxide synthase (NO production).
2. Arginyl-tRNA synthetase (protein production).
3. Arginine:glycine aminotransferase (creatine production).
4. Arginine decarboxylase (agmatine production).

Of special note is the competition between arginase and nitric oxide synthase (NOS) for L-arginine. This has been a growing focal point for biomedical research in recent years, because a change in the balance can vastly affect cellular function.

Arginase is distributed in tissue throughout the body in two isoforms and has functions in both health and disease. Arginase converts L-arginine into urea and ornithine – in many tissues ornithine can be further metabolized to polyamines, proline and glutamate. These products have important biological functions. Arginase function in the liver is extremely important. It is part of a cycle which releases urea and produces ornithine, which then accepts hepatic metabolites of NH3 in the production of citrulline, the precursor of L-arginine. This hepatic urea cycle is essential for ridding the body of toxic NH3 via the release of urea. Arginase also is beneficial in wound healing and neuroprotection/regeneration. Arginase also has key functions in a number of disease states. These prominently include vascular and endothelial dysfunctions associated with diabetes, hypertension, sickle cell disease, ischaemia/reperfusion injury, atherosclerosis and erectile dysfunction. Other diseases involving elevated arginase activity are asthma, nephropathy, cancer, and parasitic infections.

Stimuli that increase arginase activity/expression include reactive oxygen species, inflammatory cytokines and humoral factors, including angiotensin II and thrombin. Studies of signal transduction mechanisms and development of inhibitors of the activation processes are under way with the goal of limiting arginase function to physiological levels.

3.2 Introduction

Arginase is the hydrolytic enzyme that converts L-arginine to urea and ornithine.

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(Fig. 3.1). In the liver, this enzyme is a key element of the urea cycle. This cycle removes toxic ammonia, formed through protein catabolism, by processing it along with ornithine via carbamoyl phosphate synthase-1 (CPS-1) and ornithine carbamoyltransferase (OCT) into L-citrulline. L-citrulline can be recycled back to L-arginine by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) to complete the urea cycle (Osowska et al., 2004). However, most non-hepatic tissues do not have the complete urea cycle, because they lack OCT or CPS-1 (Fig. 3.2).

![Fig. 3.1. Scheme for synthesis of L-arginine from L-glutamine. Also shown are catabolism of L-arginine to L-ornithine/urea or L-citrulline/NO, production of polyamines, and anabolism and catabolism of proline. ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; Asp, aspartate; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; OCT, ornithine carbamoyltransferase; P5CS, pyrroline-5-carboxylate synthase.](image)

![Fig. 3.2. Scheme shows the urea cycle, which is partially included in Fig. 3.1. Note the entry of ammonia (NH₃) into the cycle through the synthesis of carbamoyl-PO₄. Abbreviations in addition to those given for Fig. 3.1: CPS-1, carbamoylphosphate synthase-1; N-AG, N-acetylglutamate.](image)
When activated, arginase can compete with NOS for their common substrate, the semi-essential amino acid L-arginine. Decreases in L-arginine availability to NOS can lead to decreased production of NO, and possible NOS uncoupling and increased superoxide formation (White et al., 2006; Romero et al., 2008). These effects and characteristics of arginase can be of pathological importance when its activity and expression are elevated as in disease states such as hypertension and vascular complications of diabetes. In these situations, there is impaired endothelial-dependent vasorelaxation, proliferation of vascular smooth muscle cells, and vascular and perivascular fibrosis (Yang and Ming, 2006). Arginase activity and expression can be enhanced by inflammatory processes and reactive oxygen species associated with disease states.

Ornithine synthesis by arginase in the cytosol leads primarily to the formation of polyamines (putrescine, spermidine, and spermine) via ornithine decarboxylase (ODC) and to the formation of proline via ornithine aminotransferase (OAT). While the actions of polyamines are not completely understood, they are known to be germinally involved in cell cycling and proliferation, but are also implicated in cell death under some conditions (Durante et al., 1998; Li et al., 2001). Proline is essential in collagen synthesis and its organization into fibrous protein structure (Li et al., 2001).

Polyamines are important for processes such as neural development, wound healing and tissue regeneration (Lange et al., 2004). Proline formation and the synthesis of collagen are necessary for wound repair and structural integrity of some tissues (Witte and Barbul, 2003). However, excessive enhancement of these pathways can lead to thickened and stiff blood vessels and airways, hypertrophied and fibrotic hearts and kidneys, growth of cancers and toxicity in some neural cells.

### 3.3 Isoforms and Distribution

Arginase exists as two isoforms, arginase I and arginase II. Arginase I is a cytosolic enzyme that constitutes a majority of total body arginase activity. It is strongly expressed in the liver and is central to the urea cycle (Morris, 2002). Arginase II is a mitochondrial enzyme expressed primarily in extra-hepatic tissues, especially in the kidney (Miyanaka et al., 1998), but is also found in other tissues. Both arginase I and II have been found in endothelial cells (Romero et al., 2006; Marinova et al., 2008; Romero et al., 2008) while only arginase I has been described in vascular smooth muscle cells (Wei et al., 2000; Topal et al., 2006; White et al., 2006).

Arginase activation provides substrate ornithine for the ornithine decarboxylase (ODC) pathway, producing polyamines which are important in cellular growth and migration and can contribute to cellular hypertrophy and hyperplasia (Liang et al., 2004). In some cases, it is arginase rather than ODC which limits polyamine synthesis (Li et al., 2001; Wei et al., 2001). Arginase II plays a role in the production of proline, a critical component of collagen, through the ornithine aminotransferase (OAT)/pyrroline-5-carboxylate reductase (P5CR) pathway (Durante et al., 2001; Li et al., 2001). Therefore, elevated arginase activity is associated with excess cell growth and collagen accumulation – fibrosis.

### 3.4 Structure and Location of Arginase

Arginase I and II share 58% homology (Munder, 2009). Human arginase I consists of 322 amino acids and was cloned more than 20 years ago (Dizikes et al., 1986) whereas arginase II was cloned in 1996 and has 354 amino acids (Gotoh et al., 1996). The gene for human arginase I is mapped on chromosome 6q23 (Sparkes et al., 1986) and for arginase II on chromosome 14q24.1–24.3 (Gotoh et al., 1997). High-resolution crystal structures have been determined for arginase I and for arginase II. Each identical subunit of the trimeric enzyme contains an active site at the bottom of a 15 Å deep cleft. Mn(II) ions are located at the bottom of this cleft, separated by approximately 3.3 Å and bridged by oxygen derived from two aspartic acid residues and a solvent-derived hydroxide. This metal bridging is
proposed to be the nucleophile that attacks the guanidinium carbon of substrate arginine. Liver arginase is a 105 kDa homotrimer, and each subunit (35 kDa) contains a binuclear Mn(II) centre that is critical for catalytic activity. The overall fold of each subunit belongs to the $\alpha/\beta$ family, consisting of a parallel, eight-stranded $\beta$-sheet flanked on both sides by numerous $\alpha$-helices (Ash, 2004).

### 3.5 Involvement of Arginase in Health and Disease

#### 3.5.1 Arginase in health

##### 3.5.1.1 Ammonia detoxification

The urea cycle in liver removes excess ammonia from the body accumulated through dietary sources or due to breakdown of endogenous protein. Various enzymes involved in the urea cycle convert ammonia nitrogen to urea which is non-toxic, more water-soluble, and easily excreted through the kidney in the form of urine. Hyperammonaemia results from deregulation of urea cycle enzymes including N-acetylglutamate synthase (N-AGS), carbamyl phosphate synthetase I (CPS-I), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS), argininosuccinate lyase (AL) and arginase (Deignan et al., 2008) (see urea cycle in Fig. 3.2). Patients with urea cycle disorder are healthy at birth but develop pathological symptoms progressively. Patients with complete CPS-I or OTC deficiency show symptoms of hyperammonaemia during the first few days of life; patients with AS or AL deficiency develop symptoms during the first month; and patients with ARG deficiency usually present later in childhood (Grody et al., 1993). Deficiency in arginase I, the last enzyme in the urea cycle, causes more severe form of hyperammonaemia. If left untreated, ammonia toxicity manifests as seizures, mental disorders, and early morbidity (Deignan et al., 2008). Arginase deficiency is diagnosed by elevated plasma levels of arginine compared to the lack of any of the other enzymes in the urea cycle, which reduce plasma arginine levels.

Of the two isoforms, arginase I deletion in the mouse model has been associated with hyperammonaemia, decerebrate posture, encephalopathy, tremors in the extremities and death within 10–12 days after birth (Iyer et al., 2002). Arginase II knockout mice do not present any pathological abnormalities. Recently a study showed that arginase II knockout mice displayed hypertension though it was correlated to an up-regulation of sympathetic tone (Huynh et al., 2009a). Treatment of patients with arginase I deficiency involves reduction of protein intake, dietary supplementation with all essential amino acids other than arginine, haemodialysis and in severe cases orthotropic liver transplant and gene replacement therapy (Deignan et al., 2008).

##### 3.5.1.2 Wound healing

The first/acute phase in tissue injury involves oxidative insult through generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from the resident macrophages through up-regulation of inducible nitric oxide synthase (iNOS). This helps in eradication of pathogens (Satriano, 2004). Next is the repair phase wherein arginase converts L-arginine to L-ornithine and urea. L-ornithine is further metabolized to form proline via ornithine aminotransferase (OAT) and polyamines via ornithine decarboxylase (ODC). Both of these pathways are essential for wound healing owing to their potential effects on collagen synthesis and cell proliferation. It is reported that arginase activity/ expression is strongly induced three to five days post injury (Kamper et al., 2003). Moreover, supplementation of L-arginine has been shown to enhance wound healing in arginine-depleted animals, as well as in rats with normal dietary intake of arginine (Witte and Barbul, 2003). This response is associated with an accumulation of hydroxyproline, a key constituent of collagen synthesis. Polyamines (putrescine, spermidine and spermine) are required for cell proliferation and homeostasis. Induction or overexpression of arginase I has been shown to cause proliferation of rat aortic smooth muscle cells through increased production of polyamines (Wei et al., 2001). A critical balance between rate of consumption of L-arginine by iNOS
Arginase (NO production) and arginase (polyamine and collagen synthesis) determines the course of wound repair.

3.5.1.3 Neuroprotection/regeneration

Arginase has been reported to play important roles in neuroprotection and neural regeneration (Esch et al., 1998; Lange et al., 2004). In a model of enhanced oxidant stress (glutathione depletion) and neuronal cell death, arginase I application was found to be protective, seemingly via depletion of L-arginine and inhibition of protein synthesis. Further, it was protective against several other stimuli which induce apoptosis. Also, a number of studies have shown that up-regulation of arginase I expression can promote axonal regeneration. In one study, acidic fibroblast growth factor along with nerve grafts improved locomotor function after spinal transaction (Kuo et al., 2007). This treatment resulted in a large rise in levels of spermine and arginase I protein in area motor neurons and macrophages. Similar results have been seen by making a small lesion in neuron branches or elevating cAMP levels. This effect seems to be related to elevated levels of cAMP with resultant enhancement of arginase I expression and subsequent blockade of myelin-associated glycol-protein (MAG), a major factor in prohibiting neural regeneration. In fact, synthesis of spermidine, not putrescine, appears to be necessary for this effect. Polyamines are known to be important in neural growth, development, and regeneration (Filbin, 1996).

However, enhanced arginase activity and polyamine synthesis are not always beneficial to neuronal growth and regeneration. Enhanced arginase activity and polyamine production in the eye can cause retinal ganglion cell death. This appears to be due to excessive activation of the excitotoxic NMDA receptors. Inhibition of arginase and polyamine synthesis is neuroprotective (Pernet et al., 2007). Thus, polyamines appear to promote either cell growth or death, depending on the environment (Wallace et al., 2003).

3.5.2 Arginase in disease

3.5.2.1 Diabetes

Vascular and hepatic arginase activity are increased in diabetic rats, and arginase expression and activity are also increased in aortic and coronary endothelial cells exposed to high glucose (Romero et al., 2006; Romero et al., 2008). Our recent studies show that arginase activity is increased in aortas of diabetic rats and aortic endothelial cells treated with high glucose, and that coronary endothelial-dependent vasorelaxation is reduced in diabetic rats and restored by inhibition of arginase activity (Romero et al., 2006; Tawfik et al., 2006; Romero et al., 2008).

Decreased plasma levels of L-arginine have been reported in diabetic animals and patients (Hagenfeldt et al., 1989; Pieper and Dondlinger, 1997) and in vascular tissue of STZ-diabetic rats (Pieper and Dondlinger, 1997). Increased arginase activity seems to be involved in these conditions. Diabetic rat liver has higher arginase activity than control rats, as well as elevated manganese content, which may stimulate the enzyme (Bond et al., 1983; Spolarics and Bond, 1989). In diabetic patients, arginase activity is also increased in red blood cells (Jiang et al., 2003), and in penile vessels, associated with erectile dysfunction (Bivalacqua et al., 2001). Studies in STZ-diabetic rats show elevated arginase activity and expression in aorta and carotid arteries (Romero et al., 2008).

Diabetes and hyperglycaemic conditions cause production of ROS. These ROS have been shown to increase arginase expression and activity. Several mechanisms are involved in the high glucose-induced elevation of O₂⁻ and other reactive oxygen species. These are summarized in the Regulation of Activity (see below).

3.5.2.2 Hypertension

Elevated arginase activity in aorta, heart and lung has been reported in adult spontaneously hypertensive rats (SHR) (10 weeks and older), but arginase activity was not altered in kidney, liver and brain (Bagnost et al., 2009). Furthermore, treatment of 25-week-old SHR
with the arginase inhibitor N\textsuperscript{\alpha}-hydroxy-nor-L-arginine reduced systemic blood pressure, improved vascular function, and reduced cardiac fibrosis (Bagnost et al., 2010). However, arginase II knockout mice display a hypertensive phenotype in adulthood rather than the expected reduction in blood pressure with improved vascular endothelial function. This hypertension appears to be related to an enhanced sympathetic nervous function (Huynh et al., 2009a).

Pulmonary hypertension (PH) is also associated with increased arginase activity. Hypoxia-induced pulmonary hypertension is reported to involve elevated arginase II protein expression and activity (Chen et al., 2009; Jin et al., 2010). This elevation in arginase expression/activity is associated with lower NO production in pulmonary artery endothelial cells and proliferation of these cells (Xu et al., 2004). The enhancement of endothelial cell arginase and cell proliferation with hypoxia can be prevented by blockade of EGF receptor tyrosine kinase (Toby et al., 2010). In addition, lack of MAP kinase phosphatase-1 in mice subjected to hypoxia caused them to suffer a more severe PH and greater lung protein levels of arginase (I and II) (Jin et al., 2010). These data support the involvement of MAP kinase in the increased expression and activity of arginase (Shatanawi et al., 2010).

In secondary pulmonary hypertension (PH) resulting from haemolytic anaemias, such as in sickle cell disease, plasma arginase activity is greatly increased by its release from red cells (see sickle cell disease below). Treatment with arginase inhibitors is projected to be successful in reducing the PH (Morris et al., 2008).

### 3.5.2.3 Sickle cell disease

In sickle cell disease, haemoglobin S-containing erythrocytes become entrapped in the microcirculation, which leads to repetitive cycles of ischaemia-reperfusion tissue injury and infarction (Bunn, 1997). Primary and secondary inflammation, endothelial activation, oxidant stress and adhesion molecule expression contribute to this process. This disease state is characterized by low tissue levels of NO, sludging of red blood cells (RBC), vaso-occlusive disorders, haemolysis and pulmonary hypertension. Arginase activity and expression are substantially elevated in the plasma, RBCs, and platelets from these patients (Morris et al., 2008). Lysis of the RBCs releases large amounts of both free haemoglobin and arginase. Haemoglobin consumes endothelial NO, while arginase limits L-arginine availability to NOS, further reducing NO levels. Treatment of sickle cell disease patients with supplemental L-arginine and/or L-citrulline has been shown to reduce levels of RBC sludging, vaso-occlusion and pulmonary hypertension (Waugh et al., 2001; Fasipe et al., 2004; Morris et al., 2005). Treatment of sickle cell disease by arginase inhibitors is also a promising therapeutic strategy (Morris, 2006).

### 3.5.2.4 Erectile dysfunction

Smooth muscle relaxation in the corpus cavernosum is essential for erectile function. The NO/cyclic GMP pathway has been demonstrated to be the principal mediator of cavernous smooth relaxation and penile erection (Masuda et al., 2004). NO is produced from L-arginine by NOS, and both neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms serve as sources to produce relevant levels of NO in the corpus cavernosum. Recently, both arginase I and II isoforms have been shown to exist in human corpus cavernosum, and the inhibition of arginase resulted in the facilitation of corporal smooth muscle relaxation (Cox et al., 1999; Bivalacqua et al., 2001). It has been reported that basal NO production from the endothelium regulates intrinsic cavernous tone and that endogenous arginase activity in the endothelium modulates tone by inhibiting NO production, presumably through competition with eNOS for the common substrate L-arginine. Both increased arginase activity and reduced NO bioavailability have been strongly associated with erectile dysfunction (ED) in many conditions including obesity (Carneiro et al., 2008), hypercholesterolemia (Xie et al., 2007), ageing (Bivalacqua et al., 2007) and diabetes (Bivalacqua et al., 2001). Angiotensin II-induced elevation of arginase activity and erectile dysfunction can be prevented by...
Arginase inhibition of p38 MAP kinase (Toque et al., 2010a). It also has been recently reported that diabetic mice lacking the gene for ARG II do not exhibit the impaired nitricergic nerve and endothelial dependent relaxations of the corpus cavernosum observed in wild type diabetic mice (Toque et al., 2010b). Thus, arginase is strongly implicated as a regulator of erectile function, and penile arginase is a potential target for the treatment of male sexual dysfunction.

3.5.2.5 Asthma

Allergic asthma is a chronic inflammatory disorder of the airways characterized by allergen-induced bronchoconstriction and inflammation of airways. Lack of available L-arginine for NOS and reduced NO production appears to be centrally involved with the asthmatic state (Maarsingh et al., 2008a). Supplemental L-arginine or treatment with the arginase inhibitor nor-NOHA reduces bronchoconstriction to allergens or other stimulants (de Boer et al., 2001) and enhances nitricergic nerve-mediated airway smooth muscle relaxation (Maarsingh et al., 2006). Both arginase I and II are constitutively expressed in airways tissues, including epithelial and endothelial cells, myofibroblasts, and macrophages (Lindemann and Racke, 2003; Maarsingh et al., 2008b). Arginase activity and expression are elevated in humans with asthma and animal models of asthma (Zimmermann et al., 2003; Kenyon et al., 2008). Asthmatic subjects who are smokers exhibit an even greater elevation of arginase I in airway epithelial cells and myofibroblasts (Bergeron et al., 2007). They also display enhanced levels of ornithine decarboxylase.

Chronic inflammation with asthma also leads to airway remodelling including thickening of basement membranes, fibrosis, and enhanced smooth muscle mass. Structurally, these changes can reduce lung function. Current evidence indicates that this remodelling involves elevated production of polyamines and proline — downstream products of arginase (Meurs et al., 2008). Since several airway functions involve arginase, a key role for arginase in the pathophysiology of asthma is emerging.

3.5.2.6 Ischaemia/reperfusion injury

Reperfusion is aimed to minimize damage in an ischaemic tissue. However, ischaemia/reperfusion (I/R) injury commonly occurs as a result of induction of ROS and pro-inflammatory factors/markers. Endothelial and microvascular dysfunction result from decreased nitric oxide production. As was explained above, arginase competes with NOS for their common substrate L-arginine, thereby reducing NO production. Arginase has been shown to be up-regulated in I/R injury in cardiac tissue (arginase I), and kidney (arginase II), as well as in liver (arginase I) (Hein et al., 2003; Reid et al., 2007; Jeyabalan et al., 2008; Jung et al., 2010). Liver I/R injury releases arginase I from injured hepatocytes leading to hepatic depletion of arginine (Reid et al., 2007; Jeyabalan et al., 2008). The levels of inflammatory cytokines such as tumour necrosis factor-α (TNFa) are shown to be increased in I/R injury and contribute to increased arginase expression and generation of oxidative radicals in endothelial cells leading to endothelial dysfunction (Gao et al., 2007; Zhang et al., 2010). Treatment with the arginase inhibitor, Nω hydroxy-nor-L-arginine (nor-NOHA) has been reported to protect myocardium from I/R injury (Jung et al., 2010). Similarly, infusion with nor-NOHA prevents liver necrosis and increases hepatic arginine and citrulline levels in I/R associated with liver transplant (Reid et al., 2007; Jeyabalan et al., 2008).

3.5.2.7 Atherosclerosis

Pathogenesis of atherosclerosis involves vasoconstriction, inflammation and thrombus formation. Hyperglycaemia contributes significantly to atherosclerosis by increasing pro-inflammatory and pro-thrombotic factors that lead to plaque formation and an increased risk for thromboembolism (Retnakaran and Zinman, 2008). The central mechanism for endothelial dysfunction in atherosclerosis is considered to be due to uncoupling of NOS, reducing NO production, and increasing ROS production (Ozaki et al., 2002; Yang and Ming, 2006). Vascular arginase activity has been shown to be increased with high cholesterol
diet in atherogenic-prone mice (ApoE−/−) and wild-type mice (Ming et al., 2004). This has been attributed to an increase in the expression of the arginase II isoform as deletion of this gene prevents vascular dysfunction and oxidative stress (Ming et al., 2004; Ryoo et al., 2008). Both isoforms of arginase were expressed in atherosclerotic lesions of hyperlipidaemic rabbits (Hayashi et al., 2006). Arginase II has been reported to increase in endothelial cells of atherosclerotic mice (Zhang et al., 2001) and upon exposure to oxidized LDL in human aortic endothelial cells (Ryoo et al., 2006). Moreover, oxidized LDL induces arginase I expression through PPAR-γ and -α activation in macrophages contributing to atherosclerosis (Gallardo-Soler et al., 2008). Increasing NO bioavailability by citruline therapy or arginase inhibition improved the prognosis of atherosclerosis in diabetic patients (Hayashi and Iguchi, 2010).

3.5.2.8 Nephropathy

Nitric oxide levels are significantly reduced in chronic kidney disease and end-stage renal disease patients (Zharikov et al., 2008). One of the mechanisms is due to reduced substrate availability, i.e. L-arginine. Although, the liver is the major site of de novo L-arginine synthesis, this pool of L-arginine never enters the circulation because of quick consumption by arginase I in the liver to produce urea and ornithine. Moreover, hepatic ornithine is recycled to form citrulline and then arginine. Thus hepatic arginase I activity does not cause significant depletion of the overall arginine supply (Baylis, 2008). Arginase II is the predominant isoform in the kidney, and elevated levels of arginase II can compete with NOS to reduce arginine supply. Indeed there is evidence that inhibition of arginase protects the kidney from structural damage in the renal mass ablation/infarction model of chronic kidney disease (Sabbatini et al., 2003).

In models of nephrotic nephritis, it has been shown that both iNOS and arginase are elevated (Waddington et al., 1998a,b). An up-regulation of proline and polyamine production through the arginase II pathway has been considered in the pathology of glomerulonephritis (Waddington et al., 1998b). NO bioavailability is reported to be decreased in renal medulla of diabetic rats due to increased uptake of L-arginine by the CAT-1 transporter in the liver, manifesting in reduced plasma arginine levels (Palm et al., 2008). This is also associated with enhanced arginase activity in the renal cortex of these rats. Early stages in diabetes are characterized by hyperfiltration, postulated to be due to increased glomerular L-arginine uptake and polyamine synthesis (Schwartz et al., 2004). Both arginase I and arginase II were elevated in this 2-week diabetic model, which could affect NO levels as well as increase polyamine levels through the formation of ornithine.

3.5.2.9 Cancer

The level of NO in the tumour milieu determines the rate of cancer progression. Induction of iNOS greatly increases NO generation which can restrict the growth and metastasis of cancer. High expression of iNOS has been reported in the early stages of breast, colon, brain, lung and prostate cancers (Thomsen et al., 1994; Cobbs et al., 1995; Thomsen et al., 1995; Xu et al., 2002). In contrast, moderate levels of NO promote tumour growth. Arginase plays a major role in tumour survival, growth and metastasis. It depletes the substrate, arginine, thereby limiting NO production and increasing ornithine levels which enhance cell replication through polyamine synthesis. Arginase activity and arginase II expression have been shown to be increased in breast, colon and prostate cancers (Buga et al., 1998; Singh et al., 2000; Mumenthaler et al., 2008). A reduction of iNOS and up-regulation of arginase in macrophages causes a phenotypic shift from a tumour-repressive to tumour-supportive microenvironment in which factors that help the tumour grow are released (Mills et al., 1992; Weigert and Brune, 2008). Indeed, elevated arginase I expression has been reported in macrophages isolated from tumours of wild-type mice (Davel et al., 2002; Kusmartsev and Gabrilovich, 2005; Sinha et al., 2005) as well as in tumour cells (Lechner et al., 2005). A recent study showed that knockdown of arginase II causes apoptosis of thyroid cancer cells (de Sousa et al.,
Statin therapy in a breast cancer cell line was found to cause cell death which was attributed to a decrease in arginase II (Kotamraju et al., 2007).

### 3.5.2.10 Parasitic infection

Several parasites including *Leishmania*, *Trypanosoma*, and *Plasmodium* have been shown to express arginase (Walther et al., 2006; Cuervo et al., 2008; Stempin et al., 2008; Rogers et al., 2009; Dowling et al., 2010). Arginase activity causes ornithine production which promotes proliferation of the pathogen through the polyamine pathway. Macrophages are recruited to the site of infection by factors such as lipopolysaccharide, tumour necrosis factor-α (TNF-α), interleukin-12 (IL-12) and interferon-γ (IFN-γ). During the acute phase of infection, these classically activated macrophages (CAM) induce iNOS to produce NO and peroxynitrite (ONOO⁻) to control parasitaemia. This has been observed in infections with *Leishmania*, *Trypanosoma*, and *Plasmodium* (Plebanski and Hill, 2000; Peluffo et al., 2004; Walther et al., 2006). Depending on the host genotype, parasite virulence, and stage of the disease (progressed), hosts can produce Type 2 cytokines such as IL-4 and IL-13 which antagonize CAMs and instead up-regulate arginase I to cause collagen synthesis and cell proliferation through the polyamine pathway (Stempin et al., 2010). T helper 1 (Th1) cells generate IFN-γ and induce iNOS, whereas Th2 cells generate IL-4 and IL-10 with resultant induction of arginase I and suppression of iNOS (Munder et al., 1999). Although the polyamine pathway is essential for wound healing, it has been shown to exacerbate parasitic infections by preventing clearance by CAM and promoting parasitic proliferation (Stempin et al., 2010). Further, it has been reported that limiting arginine or inhibiting arginase activation can reduce parasite survival (Wanasen et al., 2007; Rogers et al., 2009).

### 3.5.2.11 Hyperargininaemia

As was explained above, a deficiency in arginase I leads to accumulation of L-arginine or hyperargininaemia (Dizikes et al., 1986; Vockley et al., 1994; Uchino et al., 1995). It is inherited as an autosomal recessive disease. Patients with this disorder appear normal at birth but start showing symptoms of diminished growth and mental retardation when they are 2–4 years old. They become progressively spastic and develop neurological diseases (Crombez and Cederbaum, 2005). There are sporadic instances of hyperammonaemia and hyperuraemia even though there is an increase in arginine levels by two- to fivefold (Cederbaum et al., 1979; Marescau et al., 1992). This is likely due to compensation by the arginase II isoform in the kidney. Indeed, augmented levels of kidney arginase activity have been reported in arginase-deficient patients whose liver arginase activity is reduced (Spector et al., 1983). Treatment of these patients involves restriction of dietary protein intake, supplementation of essential amino acids, and diversion of ammonia to salvage pathways (Crombez and Cederbaum, 2005).

### 3.5.2.12 Ageing

Age-related vascular changes have been investigated in humans and in a number of animal species. Arginase plays an important role in regulating vasomotor tone, and its relative contribution varies depending on the vascular bed and the vessel size and type (Santhanam et al., 2008). There is increasing evidence that up-regulation of arginase contributes to impaired endothelial function in ageing (Berkowitz et al., 2003). It has been shown that there is higher arginase activity, lower NO and higher $O_2^-$ production in old rats compared with young rats (Kim et al., 2009). Furthermore, these authors also found that acute inhibition of NOS (with N-nitro-l-arginine methyl ester) and arginase (2S-amino-6-boronohexanoic acid, ABH), can reduce $O_2^-$ production in old rats and prevent uncoupling of the eNOS dimer. Ageing is also associated with endothelial senescence which can contribute to atherosclerosis as well as impairing endothelial function. Indeed, knocking down arginase I (White et al., 2006) or increasing NO bioavailability by citrulline therapy (Hayashi and Iguchi, 2010) restores ageing-related endothelial senescence and
improves vascular function. Erectile dysfunction associated with ageing has been found to be improved by inhibiting arginase using ABH or knocking down arginase I (Bivalacqua et al., 2007). Inhibition of arginase also decreases blood pressure and improves vascular function of resistance vessels in hypertensive rats. (Demougeot et al., 2005; Durante et al., 2007; Bagnost et al., 2008). Increased arginase activity can also cause vessel stiffness with age due to collagen deposition and fibrosis through the proline and polyamine pathways.

3.5.2.13 Retinopathy

Ischaemic retinopathies are characterized by a progression of vascular damage, beginning with inflammatory reactions, endothelial cell dysfunction, and reduced blood flow, which can lead to pathological neovascularization and fibrovascular scarring, culminating in retinal detachment and blindness (Friedlander et al., 2007). During the inflammatory stage, excessive arginase activity could enhance cytokine formation by limiting NO production by iNOS. In addition, excessive arginase activity at the level of the retinal endothelial and smooth muscle cells could exacerbate retinal inflammation by reducing NO production by endothelial and neuronal NOS which would decrease blood flow and promote platelet aggregation and leukocyte adhesion to the vessel wall. The ensuing retinal ischemia would further increase the retinal injury and could contribute to pathological neovascular growth due to the induction of VEGF expression. Excessive arginase activity could also contribute to pathological vascular growth and fibrovascular scarring, by increasing the formation of polyamines and proline, which promote cell growth and collagen formation, respectively.

Arginase I was reported to be increased and localized mainly to Muller cells in rats with endotoxin-induced uveitis (EIU) (Koga et al., 2002). Arginase I is also involved in excitotoxic cell death due to polyamines (Pernet et al., 2007). Studies have shown that arginase activity and arginase I expression are increased in models of EIU and diabetic retinopathy and in cultured retinal endothelial cells, Muller cells, and microglial cells exposed to high glucose or cytokines (Zhang et al., 2009; Caldwell et al., 2010). This was correlated to a concomitant increase in cytokine expression and oxidative stress, reduced NO, vascular dysfunction and pathological angiogenesis. Furthermore, deletion of one copy of the arginase I gene and both copies of arginase II decreases cytokine production in both diabetic retinopathy and EIU (Zhang et al., 2009).

3.6 Regulation of Activity

3.6.1 Humoral factors

3.6.1.1 Reactive oxygen species

Reactive oxygen species (ROS) formation is increased in diabetes. Numerous sources of ROS in diabetes have been described and include advanced glycation end products, flux through the aldose reductase pathway, and activation of PKC (Nishikawa et al., 2000a). ROS play a role in vascular dysfunction in diabetes. Overproduction of O$_2^-$ can lead to scavenging NO and reducing its bioavailability (Yung et al., 2006). Reactive species have been implicated in increased arginase activity and expression. Studies have shown the H$_2$O$_2$ causes arginase activation in endothelial cells, leading to decreased NO production which was restored by antioxidant treatment (Thengchaisri et al., 2006). In a model of wound healing, arginase effects in promoting excessive scar formation were mitigated by inhibitors of peroxynitrite actions (Kapoor et al., 2004). Furthermore, treatment with peroxynitrite has been shown to cause increased arginase activity and mRNA and protein expression in coronary and aortic endothelial cells (Romero et al., 2006; Chandra et al., 2010).

High glucose enhances the expression of eNOS and production of O$_2^-$ (Cosentino et al., 1997). The passage of glucose through the polyol pathway generates O$_2^-$ and the toxic reaction product of NO and O$_2^-$, peroxynitrite (ONOO$^-$) (Giugliano et al., 1996). Aldose reductase, the first and rate-limiting enzyme in the polyol pathway, is activated by high
glucose and begins the production of sorbitol. In addition to production of ROS, this active pathway consumes NADPH, reducing its supply for many endothelial enzymes, including eNOS. Moreover, large amounts of ATP are consumed, which may compromise the EC energy supply and cell membrane potential. NADH and ATP are also depleted by activation of poly (ADP-ribose) polymerase by ROS (Garcia Soriano et al., 2001). Inhibitors of aldose reductase have been reported to reduce vascular dysfunction in experimental models of diabetes (Cameron and Cotter, 1992). High glucose also stimulates ROS production by mitochondria via actions through the electron transport chain complex II and oxidative phosphorylation (Nishikawa et al., 2000b). The ROS from all sources described above can activate the janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (Marrero et al., 1997; Schieffer et al., 2000), and promote vascular smooth muscle proliferation, collagen deposition and fibrosis. The JAK/STAT pathway has been shown to be involved in up-regulation of arginase (Wei et al., 2000).

3.6.1.2 Angiotensin II

This has been implicated in oxidative stress-induced endothelial dysfunction and vascular remodelling and fibrosis in diabetes. A mechanism of Ang II stimulation of ROS is through stimulation of endothelial NADPH oxidase (Touyz, 2004). In a model of hypertension, inhibitors of the AT1 receptor have been reported to inhibit increased arginase activity and to normalize endothelial function (Kotsiuruba et al., 2002). Studies in our lab have shown that treatment of endothelial cells with Ang II increases both arginase activity and arginase I protein expression (Shatanawi et al., 2011). Cytokines such as IL-4, IL-13, and TNF-α are also activators of arginase activity (Wei et al., 2000; Nelin et al., 2005). Ang II has been shown to play a critical role in inflammatory vascular disease such as atherosclerosis, and is known to be associated with endothelial nitric oxide synthase (eNOS) dysfunction/uncoupling (Satoh et al., 2008). Inhibitors of the renin angiotensin-aldosterone system (RAAS) have been effective in moderating increased vascular wall thickness clinically and Ang II receptor blockers have inhibited development of atherosclerosis in animal models (Kim and Iwao, 2000).

3.6.2 Elevation of arginase activity and signal transduction mechanisms

Enhanced arginase function can result from increased specific activity/enzyme efficiency or elevated protein levels. The activators of arginase have not been extensively investigated. S-nitrosylation of arginase I through iNOS has been reported to increase its activity by reducing the Km of arginase (Santhanam et al., 2008). Uric acid also has been reported to activate arginase by reducing its Km, seemingly for both isoforms (Zharikov et al., 2008).

There is more evidence for mechanisms that increase levels of arginase protein. Protein expression of arginase I is increased in rat smooth muscle in response to interleukins and is apparently mediated through cyclic AMP/protein kinase A and JAK/STAT6 pathways (Wei et al., 2000). In macrophages, it is reported that CAMP mediates activation of arginase I involving protein kinase A type I and histone deacetylase (Haffner et al., 2008).

We and others have shown that RhoA and Rho kinase are involved with upregulation of arginase activity/expression in atherosclerosis, inflammatory bowel disease, and diabetes (Ming et al., 2004; Horowitz et al., 2007; Romero et al., 2008). RhoA-GDP is activated by Rho guanosine nucleotide exchange factor (Rho GEF), which promotes exchange of GDP for GTP. The active RhoA-GTP stimulates ROCK to trigger downstream signalling (Loirand et al., 2008). ROS have been shown in several studies to be involved in the enhancement of arginase activity (Ming et al., 2004; Boor et al., 2007; Horowitz et al., 2007; Romero et al., 2008; Zhang et al., 2009). We recently reported that oxidative radicals can increase arginase activity/expression in endothelial cells through a pathway involving protein kinase C-activated p115-Rho GEF and subsequent activation of RhoA/Rho kinase (Chandra et al., 2011). Further, phosphorylation and activation of p115-Rho GEF through
protein kinase C alpha (PKCa) is reported to increase endothelial permeability in response to thrombin (Holinstat et al., 2003), a factor which also increases arginase activity (Horowitz et al., 2007).

Active RhoA has also been indicated as an upstream activator of mitogen-activated protein kinase (MAPK) family members such as p38 mitogen-activated protein kinase (MAPK) (Lovett et al., 2006; Guo et al., 2009). Both angiotensin II (Ang II) and high glucose (HG) are reported to activate MAPKs, among which p38 MAPK has been shown to have a central role in cardiovascular dysfunction (Wen et al., 2006; Santhanam et al., 2007). Also, p38 MAPK seems to be involved in increasing arginase I activity and expression in macrophages (Chang et al., 2000; Stempin et al., 2004). Studies also indicate a role for p38 MAPK in increasing arginase activity/expression in endothelial cells upon exposure to Ang II and ROS, which involves intermediate activation of RhoA/ROCK (Chandra et al., 2010; Shatanawi et al., 2010). Activation of p38 MAPK is known to result in activation of transcription factors.

These signal transduction intermediates may be considered targets for the therapeutic limitation of arginase activity. Those intermediate enzymes or processes closest to the enhancement of arginase activity would be expected to be the most specific.

### 3.7 Arginase Inhibitors

Arginase competes with NOS for their common substrate, L-arginine, thus inhibition of arginase can increase the pool of L-arginine and thereby enhance NO production. Several inhibitors of arginase are available commercially. The first class of inhibitors had non-specific actions and many side effects owing to the high concentrations required (Morris, 2009). For example, norvaline used as an arginase inhibitor is a substrate for amidotransferases (Davoodi et al., 1998). Similarly, N^\text{\textmu}-hydroxy-L-arginine (NOHA) is a potent inhibitor for arginase but is also a precursor for NO synthesis from NOS. α-Difluoromethylornithine (DFMO) is a non-specific weak inhibitor of arginase though it is a potent inhibitor for ornithine decarboxylase (Morris, 2009). Thus the effects of DFMO in increasing NO production are probably due to increased accumulation of ornithine which is known to inhibit arginase. Besides producing urea, arginase is also involved in synthesis of polyamines and amino acids such as ornithine, proline and glutamate. In fact, ornithine, leucine, valine, lysine, isoleucine and nor-valine inhibit arginase with ornithine being the most potent (Huynh et al., 2009b). Also, L-citrulline is an allosteric inhibitor of arginase (Shearer et al., 1997). Recently, competitive inhibitors of arginase with greater specificity for the enzyme have been synthesized. Boronic acid analogues of L-arginine, i.e. S-(2-boronoethy1)-L-cysteine (BEC) and 2(S)-amino-6-boronohexanoic acid (ABH), are highly selective arginase inhibitors that bear N-hydroxyguanidinium or boronic acid heads which bind the manganese cluster in arginase (Berkowitz et al., 2003). Their transition state structures differ significantly from those occurring in NO biosynthesis. Nor-NOHA is also a potent arginase inhibitor and it neither inhibits NOS nor is it an intermediate in NO synthesis. It has a much longer half-life than NOHA. A recent study shows that BEC and NOHA reverse the vascular tolerance to acetylcholine in rat aorta and mesenteric arteries, suggesting an increase in NO bioavailability (Huynh et al., 2009b). It has even been reported that BEC and DFMO could suppress arginase activity and restore NOS activity in the aortic rings from old rats (Berkowitz et al., 2003). L-valine, nor-valine and DFMO have been used to study the relationship of arginase and NOS function by several investigators (Ming et al., 2004; Santhanam et al., 2007; Lewis et al., 2008).

Although several specific arginase inhibitors have been developed and investigated, they are not isoform specific. RNA interference has been used to inhibit expression of specific isoforms. Administration of short hairpin RNA (shRNA) against arginase I greatly reduced IL-13 induced airway hyper-responsiveness, with a concomitant decrease in arginase I mRNA and protein levels in the lungs of mice (Yang et al., 2006). Direct delivery of anti-sense arginase I using adenovirus vectors in the corpus cavernosum improved
Fig. 3.3. Elevated arginase activity can reduce availability of L-arginine for nitric oxide synthase (NOS) and disrupt its function. Increased production of ornithine can lead to excess collagen formation and cell proliferation.

erectile function in aged mice (Bivalacqua et al., 2007). Anti-sense arginase I also increases NO production in endothelial cells exposed to high glucose (Romero et al., 2008).

### 3.8 Conclusions

Arginase function/activity is essential for the production of urea in the hepatic urea cycle and the elimination of toxic excess ammonia produced by protein catabolism (Fig. 3.1). Also, wound healing involves enhanced local tissue arginase activity through which ornithine is metabolized to proline (collagen production) and polyamines (cell proliferation). Additionally, arginase activity can be neuroprotective and support regeneration in spinal nerves.

However, elevated arginase activity/expression also occurs and is involved in a variety of disease or pathological states such as diabetes, hypertension, sickle cell disease, asthma, ischaemia/reperfusion injury and atherosclerosis. Activation and excessive function of arginase is closely associated with inflammation and elevated ROS. In these conditions, arginase can compete with NOS and reduce available L-arginine, leading to depressed NO production, and possible NOS uncoupling and increased superoxide (O$_2^-$) production (Fig. 3.3). Additionally, there is enhanced production of proline and polyamines. In these circumstances, there is impaired endothelial-dependent vasorelaxation (vasoconstriction), proliferation of vascular and other cells, and vascular and tissue fibrosis.

Thus, it is being recognized that arginase plays very important roles in physiological and pathophysiological processes in many cell types and tissues. Its activity and expression are highly regulated. Understanding these signal transduction processes is essential in any efforts to maintain normal arginase function.

### References


Arginase


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4 Bypassing the Endothelial L-Arginine–Nitric Oxide Pathway: Effects of Dietary Nitrite and Nitrate on Cardiovascular Function

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4.1 Abstract

L-Arginine (L-Arg) is a semi-essential amino acid that is a natural constituent of most dietary proteins in our daily diet. A fundamental function of this amino acid is its role as the substrate for the synthesis of the signalling molecule nitric oxide (NO). As NO regulates a variety of physiological processes in nearly every organ system of the human organism, L-Arg is a crucial element in mammalian physiology. The family of NO-generating enzymes is called NO synthases (NOS). NOS provide the availability of NO under physiological conditions and depend on an adequate supply of dioxygen (O₂) and several substrates. Under conditions of ischaemia, where O₂ is lacking, the NO production by the NOS shuts down and the generation of the important signalling molecule from L-Arg is disturbed. Until recently it was not known how the organism maintains NO availability under ischaemic conditions. In recent years, biomedical research has demonstrated that along the physiological O₂ gradient NO can be formed independently of its enzymatic synthesis from L-Arg, by reduction of inorganic nitrate and nitrite. Although for a long time both anions have been considered as inert end products of NO metabolism, it has been shown that reduction of nitrate and nitrite could maintain NO homeostasis under hypoxia and ischaemia. It has been demonstrated that low levels of supplemental nitrite and nitrate can influence blood pressure, and that dietary sources of NO metabolites may protect against various cardiovascular disease states. Thus the enzymatic generation of NO from L-Arg under normoxia and the non-enzymatic formation of NO via nitrate and nitrite can be considered as a balance that maintains NO availability along the circulating and metabolic O₂ gradient. Dietary intake of nitrate–nitrite-rich foods such as leafy vegetables may represent an opportunity for disease prevention and health modulation of human physiological functions.

4.2 Introduction

Since the 1998 Nobel Prize in Medicine or Physiology was awarded to Ferid Murad, Louis J. Ignarro, and Robert F. Furchgott for their groundbreaking investigations on the...
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role of NO in human physiology, it is inevitable that one mentions NO when talking about the role of L-arginine (L-Arg) in human nutrition and health (Howlett, 1998). Herein we summarize the role of the amino acid L-Arg as the substrate of human NO synthesis under physiological conditions. Furthermore, we will consider alternative sources of NO under physiological and pathophysiological conditions, with focus on the effects of nitrate and nitrite on cardiovascular function.

4.3 L-Arginine: a Semi-Essential Amino Acid in Human Physiology

L-arginine (2-amino-5-guanidino-pentanoic acid) is a dibasic amino acid that was first discovered over 100 years ago and that is a natural constituent of most dietary proteins (Silk et al., 1985). L-Arg serves as the substrate for the synthesis of the signalling molecule NO and takes part in protein production, endocrine function, wound healing and erectile function (Palmer et al., 1988; Rajfer et al., 1992). L-Arg serves as a precursor for creatine, which plays an essential role in the energy metabolism of muscle, nerve, and testis. As the adult human is able to synthesize L-Arg de novo from the urea cycle, L-Arg is regarded as a non-essential amino acid. However, in infants the amount of synthesized L-Arg is too low to cover the demands of the growing organism, and it becomes essential in this developmental stage. Thus the classification of L-Arg as a semi-essential or conditionally essential amino acid is given. The biosynthesis of L-Arg results from L-citrulline, a by-product of the glutamine metabolism in the gut and in the liver (Hecker et al., 1990). When L-citrulline is excreted into the circulation, it is reabsorbed in the proximal tubule of the kidney where it is converted into L-Arg (Fig. 4.1). Although endogenous synthesis is possible, the dietary intake of L-Arg is the basic determinant of L-Arg plasma levels because the biosynthesis is not able to balance inadequate intake or deficiency. Because of the fact that L-Arg is crucial for ammonia detoxification, dietary deficiency of L-Arg (hypoguargininaemia) is a significant nutritional problem in preterm infants. The resulting hyperammonaemia leads to cardiovascular, pulmonary, neurological and intestinal dysfunction, demonstrating the impact of L-Arg on endocrine homeostasis (Wu et al., 2004). The relative amounts of L-Arg in various dietary proteins range from 3% in cereals up to 15% in L-Arg rich protein sources like fish or walnuts. Typical dietary intake of L-Arg is 3.5–5.0 g daily and mainly derives from plant or animal foods (Oomen et al., 2000). Against this background, the different dietary habits between populations and regions may be the underlying reason for differences in L-Arg plasma levels in various parts of the world in recent studies. Ranges from 45–100 μmol l⁻¹ in healthy humans as well as in the plasma of patients with vascular disorders (Böger and Bode-Böger, 2001) have been reported. Women show lower levels compared to men, ranging from 72.4 ± 6.7 μmol l⁻¹ in the young to 88.0 ± 7.8 μmol l⁻¹ in the elderly. This phenomenon is due to the lower muscle mass in women (Moller et al., 1983).

Among the variety of physiological effects of L-Arg, its role as the substrate for the enzyme endothelial NO synthase (eNOS) is one of the most investigated ones and it is supposed to be one of the most important functions of this amino acid. NO exerts various physiological functions in most if not all organ systems throughout the body. To understand the relation between the effects of L-Arg and NO, it is inevitable that one must consider NO synthesis under physiological as well as under pathophysiological conditions.

4.4 L-Arginine is the Substrate of the Nitric Oxide Synthases: The L-Arginine–Nitric Oxide Pathway

The small and relatively unstable free radical NO has become one of the most studied and fascinating molecules in biological chemistry and medicine. It is an important messenger molecule involved in many physiological and
Fig. 4.1. Biosynthesis of L-arginine from citrulline in the urea cycle. The primary function of the urea cycle is the detoxification of ammonia (NH₃). The generation of L-arginine is a byproduct of this adenosine triphosphate-dependent (ATP), energy consuming process. Catalysed by cytosolic argininosuccinate synthetase, citrulline and aspartate are condensed to form argininosuccinate. The reaction involves the addition of AMP (from ATP) to the amido carbonyl of citrulline, forming an activated intermediate on the enzyme surface (AMP-citrulline), and the subsequent addition of L-aspartate to form argininosuccinate. L-arginine and fumarate are produced from argininosuccinate by the cytosolic enzyme argininosuccinase lyase (also called argininosuccinase). In the final step of the cycle arginase cleaves urea from arginine, regenerating cytosolic L-ornithine, which can be transported to the mitochondrial matrix for another round of urea synthesis. The fumarate, generated via the action of argininosuccinate lyase, is reconverted to L-aspartate for use in the argininosuccinate synthetase reaction. AMP, adenosine monophosphate; ADP, adenosine diphosphate; P, phosphate.

pathophysiological processes within the mammalian body, with both beneficial and detrimental properties (Moncada and Higgs, 1993). NO plays a key role in nearly every organ system within our body and it is primarily known for maintaining normal blood pressure by regulating blood vessel tone, being the major mediator of endothelium-dependent vasodilatation (Furchgott and Zawadzki, 1980, Palmer et al., 1987). NO furthermore regulates the vascular structure by affecting smooth muscle cell proliferation and inhibition of platelet and monocyte adhesion, by maintaining a nonthrombotic endothelial surface (Busse et al., 1987; Ignarro et al., 2001). Risk factors like hypercholesterolaemia, hypertension, diabetes mellitus, or cigarette smoking lead to endothelial dysfunction and thus to the inability of the endothelium to produce NO (Galle et al., 1991; Makimattila et al., 1996; Busse and Fleming, 1999). A decrease of endothelial NO formation is a hallmark of endothelial dysfunction which is a key element in the development of atherosclerosis. This leads to diseases such as hypertension, myocardial infarction, or apoplectic stroke. One of the main consequences of endothelial dysfunction is that the diminishing NO synthesis influences a variety of physiological NO-dependent processes. Besides its impact on cardiovascular function, NO plays a key role in immunomodulatory processes and in the central nervous system (Knowles et al., 1989).

The 'Molecule of the Year' in 1992 is biosynthesized endogenously from the amino acid L-Arg and oxygen by various NOS (Culotta and Koshland, 1992). So far three isoforms of NOS are known, named either according to their distribution within the body
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Table 4.1. Isoforms of nitric oxide synthases.

<table>
<thead>
<tr>
<th>Isoforms</th>
<th>Synonym</th>
<th>K_m(a) μmol L⁻¹</th>
<th>Molecular mass, kDa(b)</th>
<th>Ca²⁺ – calmodulin dependence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS I</td>
<td>neuronal NOS (nNOS)</td>
<td>1.4–2.2</td>
<td>160</td>
<td>+</td>
</tr>
<tr>
<td>NOS II</td>
<td>inducible NOS (iNOS)</td>
<td>2.8–32.3</td>
<td>131</td>
<td>–</td>
</tr>
<tr>
<td>NOS III</td>
<td>endothelial NOS (eNOS)</td>
<td>2.9</td>
<td>133</td>
<td>+</td>
</tr>
</tbody>
</table>

aK_m, Michaelis–Menten constant, half-saturating L-Arg concentration; b kDa, Kilo Dalton.

or in the order in which they were first purified and cloned (see Table 4.1) (Forstermann et al., 1994).

All three isoforms function as a homodimer consisting of two identical monomers, which can be functionally divided into a C-terminal reductase-carboxy domain and an N-terminal oxygenase-amino domain (Culotta and Koshland, 1992). Two types are constitutively expressed. They are calcium-calmodulin dependent and release NO for short periods in response to receptor or physical stimulation during signalling processes. The third isoform (iNOS) is inducible upon activation of macrophages, endothelial cells, and a number of other cells by endotoxin and pro-inflammatory cytokines. Once expressed, iNOS synthesizes NO for a prolonged period of time.

NOS produce NO by catalysing a five electron oxidation of guanidino nitrogen of L-Arg. The oxidation of L-Arg into L-citrulline via formation of the intermediate N³-hydroxy-L-Arg consumes two moles of dioxygen (O₂) and 1.5 moles of nicotinamide adenine dinucleotide (NADPH) per mole of produced NO (Liu and Gross, 1996). Beside O₂ and NADPH, the NOS enzymes require the binding of five cofactors to function properly. These cofactors are: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem iron, tetrahydrobiopterin (BH₄), and calcium-calmodulin (Bredt and Snyder, 1990) (Fig. 4.2). If any of these cofactors becomes limiting, NO production from NOS shuts down, and in many cases NOS then produce superoxide (O₂⁻) instead. This mechanism has been termed NOS uncoupling (Landmesser et al., 2003). Consequently a physiological oxygen concentration as well as sufficient substrate supply is necessary for a proper NOS function.

One possible pathway for NO metabolism is the stepwise oxidation to nitrite (NO₂⁻) which is rapidly oxidized in whole blood to nitrate (NO₃⁻) (Kelm, 1999). For years, both nitrite and nitrate have been considered as inert end products of the NO metabolism. However, we will later illustrate the evolving evidence that the formation of NO from the anions nitrite and nitrate may represent an alternative to the classical L-Arg–NO pathway.

NO derived from the constitutive eNOS mediates vascular smooth muscle relaxation which is the underlying mechanism for the blood pressure regulating effect of this molecule (Ignarro et al., 1986). After entering the target cell, eNOS-derived NO binds to the guanylyl cyclase and activates this enzyme by inducing a conformational change (Ignarro, 1991). Guanylyl cyclase catalyses the formation of cyclic guanylyl monophosphate (cGMP) from guanylyl triphosphate (GTP). cGMP then triggers a cascade of intracellular events that culminate in a reduction of the calcium-dependent vascular smooth muscle tone and vasodilatation (Twort and van Breemen, 1988) (Fig. 4.2).

Under ischaemic conditions the oxidation of L-Arg by NOS is inefficient and NO availability decreases. This is due to the limited O₂ as well as to the lack of substrates and cofactors. If the partial O₂ pressure (pO₂) decreases below 7.6 mmHg, eNOS activity slows down progressively (Abu-Soud et al., 2000). In comparison, a pO₂ of 15 mmHg is considered as normal O₂ level in nonexercised muscle (Marcinek et al., 2003). This limitation of NOS-derived NO is not only of interest under pathological conditions but also under physiological conditions where the O₂ concentration in blood or tissue decreases and the essential NOS cofactor O₂ is lacking, i.e. during physical exercise. Taken together the production of NO from L-Arg can be considered as a critical cellular function in most if not all organ systems throughout the body.
Fig. 4.2. Endothelial NO synthase (eNOS). (a) Upon stimuli such as shear stress via increased blood flow, nitric oxide (NO) is generated in the endothelial cells of blood vessels by the eNOS. (b) The eNOS is a homodimer consisting of two identical monomers, which can be functionally divided into a C-terminal reductase site and an N-terminal oxygenase site. (c) Once released, NO can mediate relaxation of the smooth muscles by activating the soluble guanylate cyclase (sGC). This leads to vasodilatation of the vessel and decreased blood pressure. This reaction requires five bound cofactors beside the substrate L-Arginine (see a). These are: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem iron (Fe2+), tetrahydrobiopterin (BH4), and calcium-calmodulin. Nicotinamide adenine dinucleotide (NADPH) and oxygen (O2) are also required.
4.5 L-Arginine in Cardiovascular Disease: Perspectives and Limitations

NO bioavailability is controlled by its formation through NOS and by its degradation via increased oxidative inactivation by reactive oxygen species (ROS). Considering NO as one of the most important signalling molecules in human physiology, it has been suggested that supplementation of L-Arg might increase NO synthesis in individuals with endothelial dysfunction. The first clinical application of L-Arg, an attempt to improve vascular function in patients with cardiovascular disease, was published by Drexler et al. (1991). It was shown that L-Arg enhanced the blood flow response to acetylcholine in the coronary artery of patients with coronary artery disease (CAD), but not in healthy controls. Since then, there have been numerous studies that demonstrated beneficial effects of L-Arg supplementation in both animals and humans under pathophysiological conditions such as hypercholesterolaemia, atherosclerosis and hypertension (Drexler et al., 1991; Rossitch et al., 1991; Hirooka et al., 1992; Böger et al., 1997; Brandes et al., 1997). However, the precise molecular mechanism by which L-Arg improves endothelial function remains unclear.

Increasing the circulating L-Arg concentration by supplementation could not explain the beneficial effects mentioned above, since the intracellular levels of this amino acid are in the mM range (Gold et al., 1989), whereas the enzyme's Michaelis–Menten constant ($K_m$) for substrate is in the μM (2.9 μmol l$^{-1}$) range (Bredt and Snyder, 1990). Likewise, circulating L-Arg plasma levels in healthy humans as well as in patients with vascular disorders are up to 15–30-fold higher than the concentration required to saturate the NOS (Böger and Bode-Böger, 2001). Hence, considering enzyme kinetics, eNOS should always be saturated and not depend on extracellular L-Arg supply. This discrepancy is termed the 'arginine paradox' (Tsikas et al., 2000). For this reason, in recent years other possible explanations have been proposed to explain the beneficial effects of supplemental L-Arg:

- McDonald et al. demonstrated in 1997 that the eNOS and the L-Arg transporter (CAT1) are co-localized within plasma membrane caveolae. Others proposed that the L-Arg concentration within these caveolae may be lower than in the ambient cytoplasm. It was further hypothesized that eNOS preferentially converts extracellular over intracellular L-Arg. Extracellular L-Arg is taken up into the caveolae via the CAT1 transporter. The intracellular L-Arg concentration could be increased by increasing the extracellular L-Arg concentration. Thus, the co-localization of both eNOS and CAT1 transporter in membrane caveolae may account for the 'arginine paradox', since the high intracellular level of L-Arg may not be utilized for NO synthesis (McDonald et al., 1997).

- Other authors consider the upregulation of the enzyme arginase, which converts L-Arg to ornithine and urea, which may decrease cellular levels of L-Arg and thereby impair NO production (Wei et al., 2000). It has been shown in animal models that upregulation of arginase may contribute to smooth muscle proliferation, resulting in endothelial dysfunction. In vessels of older animals, endothelial arginase activity is elevated and can thus contribute to endothelial dysfunction by reducing the capacity of NO synthesis (Berkowitz et al., 2003). Increasing L-Arg concentrations may override converting activity of arginase and thus contribute to the beneficial effects of L-Arg supplementation.

- Another proposed mechanism by which the 'arginine paradox' could be explained is that oxidized low density lipoproteins (LDL) and lysophosphatidylcholine decrease L-Arg transport into endothelial cells by competing or interfering with transporter systems (Kikuta et al., 1998, Jay et al., 1997). This could be an explanation for the beneficial effects of L-Arg observed in hypercholesterolaemic patients.

- The impact of asymmetrical dimethylarginine (ADMA) on NOS activity and its impact on endothelial dysfunction might serve as another explanation of the way in which L-Arg may affect vascular function.
In 1992, Vallance et al. were the first to describe the presence of endogenous analogues of L-Arg. ADMA and L-\(N^2\)-monomethyl-arginine-citrate (L-NMMA) exert biological activity by competing with L-Arg for the active substrate binding site on NO synthase (Vallance et al., 1992). Böger et al. showed that elevation in plasma ADMA occurs in hypercholesterolaemia and correlates with endothelial dysfunction (Böger et al., 1998). Free ADMA as a competitive inhibitor of the NOS is present in the cytoplasm and in the blood plasma. ADMA binds to NOS but could not be used as a substrate through the enzyme. ADMA is eliminated 20% renally, whereas the main excretion route is via metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline. DDAH activity is impaired by a variety of factors such as oxidized LDL, hyperglycaemia, or infectious diseases. The resulting increase in plasma ADMA concentrations may contribute to endothelial dysfunction (Stuhlunger et al., 2002). Inhibition of NOS activity might be exceeded by increased extracellular L-Arg/ADMA ratio through excessive substrate and could explain how L-Arg improves endothelial function in patients with vascular disease. The investigators conclude that nutritional supplementation with L-Arg may be able to restore physiological status via this mechanism.

Taken together it has been shown that intravenous or dietary (oral) administration of relatively large doses of L-Arg results in enhanced NO formation in subjects with impaired endothelial function under baseline conditions. According to these findings, long-term administration of L-Arg has been shown to improve the symptoms of cardiovascular disease in several controlled clinical trials. In other trials L-Arg was not beneficial, and in a single study a higher mortality of subjects receiving the amino acid has even been reported (Rector et al., 1996; Bednarz et al., 2005; Schulman et al., 2006). In conclusion more placebo-controlled clinical trials and mechanistic investigations are necessary to resolve the issue of dietary L-Arg as adjunctive therapy for different stages of cardiovascular disease. Furthermore, it will be necessary to assess if individual cofactors such as hypercholesterolaemia or elevated ADMA plasma concentration in patients selectively influence the efficiency of L-Arg supplementation, in comparison to the average population of cardiovascular disease patients.

4.6 Nitric Oxide Generation without NO-Synthase? Bypassing the L-Arginine Pathway

The ability of humans to produce NO from the classical L-Arg pathway is complex and requires undisturbed blood supply, oxygen, and substrate delivery. Under conditions where blood flow is impaired by occlusion or narrowing of vessels, the classical L-Arg pathway is no longer fully functional. A common disease that impairs blood flow by narrowing of the blood vessels and thereby mediating substrate deficiency in tissues is arteriosclerotic vascular disease. Of all diseases, CAD still remains the leading cause of death in the industrialized countries and is the fatal end-stage of endothelial dysfunction. Plaque rupture and the subsequent occlusion of a large epicardial artery cause an acute myocardial infarction. The therapeutic gold standard is the early reperfusion of the ischaemic heart muscle to protect the heart from ischaemia and from the absence of essential substrates. However, when flow through coronary vessels is restored the reperfusion may be an additional source of harm to the myocardium, a phenomenon called ischaemia and reperfusion (I/R) injury (Jennings et al., 1960). This phenomenon is not only limited to the heart but occurs also under all conditions where blood supply is disrupted or deficient over a period and restored afterwards, such as cerebral ischaemia, transplantation surgery, or haemorrhagic shock. In recent years, major advances have been made towards understanding the role of NO in the ischaemic biology of the heart. Numerous studies demonstrate that NO represents one of the most effective
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Protective mechanisms against myocardial I/R injury (Bo lli, 2001; Bell et al., 2003; Jones and Bo lli, 2006). Although NO offers protective properties, the classical L-Arg–NO pathway is not functional during ischaemia due to insufficient O2, limited substrate delivery and reduced cofactors. Thus, NOS independent mechanisms must exist to maintain NO homeostasis under hypoxic conditions. The reduction of nitrite, the oxidation product of NO by several ‘nitrite-reductases’ under hypoxia was identified to be such an alternative pathway (Millar et al., 1998; Cosby et al., 2003; Huang et al., 2005; Rassaf et al., 2007; Shiva et al., 2007). These nitrite reductases operate along the physiological and pathological O2 gradient and allow a graded nitrite reduction to NO according to the circulating and metabolic need. The reduction of nitrite to NO reflects a major mechanism by which the NO homeostasis is maintained independent of NOS. L-Arg and nitrite maintain the balance of NO sources ensuring NO availability during normoxic as well as hypoxic and ischaemic conditions.

4.7 The Nitrate–Nitrite–Nitric Oxide Pathway

Until recently the inorganic anions nitrate and nitrite were considered inert end products of NO metabolism and unfavourable dietary constituents (Mirvish, 1995). However, a new view is evolving with the accumulating evidence that nitrate and nitrite metabolism occurs in blood and tissues to recycle NO and other bioactive nitrogen oxides. This may represent an alternative to the ‘classical’ L-Arg–NOS–NO signalling. For the first time in 1994 both Lundberg et al. and Benjamin et al. independently presented evidence for the generation of NO in the stomach resulting from the acidic reduction of inorganic nitrite (Benjamin et al., 1994; Lundberg et al., 1994) (Fig. 4.3). These were the first reports of NO synthase-independent formation of NO in vivo. Further studies identified that commensal bacteria in the crypts of the tongue possess a nitrate reductase enzyme that they utilize for energy metabolism in the absence of oxygen (Duncan et al., 1995; Lundberg et al., 2004). It was known that nitrate is taken up by the salivary glands and concentrated in the saliva. However the reason for this active process could not be explained until the discovery that nitrate serves as substrate for the nitrate reductase enzyme of bacteria in the mouth. These bacteria reduce both plasma-extracted nitrate as well as dietary nitrate, to form nitrite, resulting in salivary nitrite levels that are 1000-fold higher than those found in human plasma (Lundberg and Govoni, 2004). When nitrite-rich saliva meets the acidic gastric juice after swallowing, nitrite is protonated to form nitrous acid (HNO2) which then decomposes to NO, a process termed acidic disproportionation. Further investigation showed that this gastric NO formation takes part in the human defence against pathogens entering via the alimentary tract. Furthermore it could provide protection against ulcers from drugs or stress (Dykhuizen et al., 1996; Miyoshi et al., 2003; Jansson et al., 2007). Beside the intraluminal formation of NO it has been demonstrated that some of the ingested nitrite survives and reaches the systemic circulation. Thus, ingestion of nitrate increases plasma nitrite by entero-salivary reduction of nitrate and makes it systemically available (Lundberg and Govoni, 2004). It was in 1995, a year after the discovery of gastric-derived NO when Zweier et al. demonstrated that the reduction of nitrite to NO may also occur systemically (Zweier et al., 1995). They discovered that NO was generated in the ischaemic heart from nitrite. In the following years it became clear that nitrite can be reduced in vivo via numerous pathways to form bioactive NO. These include the reduction via deoxygenated myoglobin within the heart muscle, deoxyhaemoglobin, intracellular xanthin oxidoreductase, enzymes of the mitochondrial respiratory chain, cytochrome P-450, and even via the NOS (Millar et al., 1998; Cosby et al., 2003; Kozlov et al., 2003; Castello et al., 2006; Rassaf et al., 2007; Vanin et al., 2007; Hendgen-Cotta et al., 2008). Since then there has been a growing interest in the physiological effects of dietary nitrates and nitrites in human health and disease.
4.8 Effects of Nitrite and Nitrate in Human Physiology

To understand the variety of physiological implications of nitrite and nitrate in the human organism it is essential to take a look at the biochemical background of nitrite and nitrate formation. As already outlined above, there are several mechanisms by which NO is generated in the body, including the NO synthase enzymes or the non-enzymatically acidic reduction of nitrite. Once NO is formed, it can be oxidized to nitrite or nitrate through its relatively slow reaction with oxygen. However in most in vivo environments like blood or tissue, this autoxidation reaction is not favoured since NO reacts more rapidly with other targets, particularly haem-based proteins. Joshi et al. (2002), for example, showed that NO reacts rapidly with oxygenated haemoglobin within the erythrocyte to form nitrate and methaemoglobin. Although it is kinetically unexpected that any NO would be oxidized to nitrite in the blood, it has been shown that the addition of NO to blood does generate significant concentrations of plasma nitrite (Shiva et al., 2006). This nitrite formation was due to the NO-oxidase function of the multi-copper oxidase caeruloplasmin. This was consistent with the finding that caeruloplasmin-deficient mice have lower nitrite levels when NO is added than controls (Shiva et al., 2006). Other sources of nitrite are dietary intake of both nitrite and nitrate, which maintain the plasma nitrite concentration between 300 and 500 nM, whereas the concentration in the tissue is about 1–10 μM (Bryan et al., 2004). The one-electron reduction of nitrite to NO by ferrous haem proteins such as deoxygenated haemoglobin and myoglobin or xanthin oxidoreductase can occur in a much simpler
mechanism than the five-electron oxidation that was demonstrated for the synthesis from L-arginine (see Eqn 4.1):

$$\text{NO}_2^- + \text{Fe}^{(II)} + 2\text{H}^+ \leftrightarrow \text{NO} + \text{Fe}^{(III)} + \text{H}_2\text{O} \quad (4.1)$$

The outlined reaction occurs in the presence of Fe^{(II)} in haem proteins, a redox state found in deoxygenated haemoglobin in the blood or deoxygenated myoglobin within muscle tissue. Confirming studies for these observations were made by several groups demonstrating that under conditions of low O_2 the nitrite reductase activity of these proteins contributes to NOS independent NO formation (Fig. 4.4). Experiments carried out by our group using wild-type and myoglobin deficient mice (Mb-/-) under moderate hypoxia due to acute coronary artery occlusion established a novel homeostatic mechanism mediated by myoglobin during O_2 deprivation (Rassaf et al., 2007). The imbalance of O_2 supply and demand in the working heart muscle, a consequence of acute hypoxia, results in increased levels of deoxygenated myoglobin, which is able to reduce nitrite to NO. Thus, the decrease in tissue O_2 tension switches the activity of myoglobin from being an NO scavenger under normoxic conditions to an NO producer in hypoxia. Because myoglobin must be at least partially deoxygenated to act as nitrite reductase, the latter reaction pathway can become significant only when the oxygen level falls below the half-loading point (P_50) of Mb. The P_50 of myoglobin is reached when the pO_2 falls below 3.0 mmHg. In comparison the pO_2 of working muscle in humans is 8.0 mmHg (Bylund-Fellenius et al., 1981). During ischaemia the tissue pO_2 can be certainly even lower (Heusch et al., 2005), thereby further augmenting the ability of deoxyMb to form NO from nitrite. This reflects an important O_2 sensing by deoxygenated myoglobin through which NO can regulate muscle function and energetics. The mechanism strongly resembles the characteristics described for acute hibernation (Rassaf et al., 2004). Myocardial short-term hibernation implies an adaptive reduction of energy expenditure through reduced contractile function in response to acute coronary artery inflow reduction. This restores myocardial energy balance over time and maintains myocardial integrity and viability (Heusch, 1998). The protective effects of NO during myocardial I/R are generally accepted (Bolli, 2001, Jones and Bolli, 2006). Our group could provide experimental evidence that the reduction of exogenous nitrite to NO during myocardial I/R leads to a marked decrease of myocardial infarct size which is critically dependent on the presence of myoglobin (Hendgen-Cotta et al., 2008). Concomitantly, ROS formation was attenuated accompanied by lower protein oxidation damage in wild-type mice. These data point out the impact of nitrite reductases under hypoxic conditions. Hence, the observed nitrite mediated cardio-protection was absent in mice lacking myoglobin, supporting the hypothesis that myoglobin serves as an intrinsic nitrite reduction that regulates cellular responses to hypoxia and reoxygenation (Hendgen-Cotta et al., 2008). Other authors report about a nitrite mediated blood flow regulation under conditions of hypoxia (Jia et al., 1996, Cosby et al., 2003). An observation was already made in the “pre-Nitric-Oxide-era” in 1953 from the later Nobel Prize winner Robert Francis Furchgott, who demonstrated that high concentrations of nitrite could mediate vasodilatation when applied to isolated aortic rings of rats (Furchgott and Bhadrakom, 1953). Half a century later it was Gladwin et al., who tested
whether nitrite could mediate vasodilatation in vivo by infusing physiological concentrations of nitrite into the human brachial artery and simultaneously measuring forearm blood flow. They observed that under exercise, at rest and especially in the presence of the NO synthase inhibitor L-NG\(^{-}\)monomethyl Arginine citrate (L-NMMA), nitrite could increase blood flow and hence hypothesized that the observed nitrite reductase activity of haemoglobin can mediate hypoxic vasodilatation and may contribute to blood pressure regulation (Gladwin et al., 2000; Cosby et al., 2003; Gladwin, 2005). One major challenge facing the idea that NO generated from red blood cell deoxyhaemoglobin-nitrite-reaction can mediate vasodilatation is that haemoglobin is a potent scavenger of NO. Hence any NO generated would be scavenged by surrounding haemoglobin before mediating vasodilatation. The oxygen-bound form of nitrite-methaemoglobin shows a degree of ferrous nitrogen dioxide (Fe(II)-NO\(_2^\cdot\)) character, so it may rapidly react with NO to form dinitrogen trioxide (N\(_2\)O\(_3\)), a unique species that is not scavenged by haemoglobin and can diffuse out of the red blood cell and homolyse into NO (see Eqn 4.2) (Basu et al., 2007). Briefly, one molecule of nitrite reacts with deoxyhaemoglobin and generates NO and methaemoglobin by the classical nitrite reductase reaction described. Another molecule of nitrite binds to methaemoglobin, and this nitrite bound methaemoglobin has an unusual electron configuration which appears to possess Fe(III)-NO\(_2^\cdot\) character. The NO\(_2^\cdot\) species can react rapidly with a molecule of NO to form N\(_2\)O\(_3\). This reaction may allow the NO generated from nitrite to reach distal targets such as the smooth muscle cells, without being scavenged by haemoglobin:

\[
\begin{align*}
& \text{MetHb} + \text{NO}_2^- \leftrightarrow \text{MetHb-NO}_2^- \\
& \text{MetHb-NO}_2^- \leftrightarrow \text{Hb-NO}_2^- \\
& \text{Hb-NO}_2^- + \text{NO} \leftrightarrow \text{Hb} + \text{N}_2\text{O}_3
\end{align*}
\]

Other nitrite reductases, some much more efficient in generating NO from nitrite, become active at much lower oxygen tensions than haemoglobin (Shiva et al., 2007). The distribution of these proteins in various biological compartments suggests that mechanisms of nitrite reduction may vary by tissue type, and further studies will be necessary to evaluate the physiological function of these proteins. It would go beyond the scope of this chapter to give a complete overview over all nitrite-dependent cytoprotective effects and influences on cellular functions. A comprehensive overview on the role of nitrite as regulator of hypoxic signalling in mammalian physiology is given by van Faassen et al. (2009). Furthermore, it is not only the anion nitrite that influences human physiology and serves as a source of NO, but also nitrate. An impressive example was demonstrated by Larsen et al. (2006), showing that after three days of oral treatment with inorganic nitrate a reduction of blood pressure occurred in healthy normotonic subjects. These observations could be reproduced with a natural nitrate source. Ingestion of beetroot juice could also decrease blood pressure in healthy volunteers via reduction of nitrate to nitrite (Webb et al., 2008).

However, it becomes clear that nitrate and nitrite are not only the inert end products of NO metabolism, but can be considered as a circulating NO pool that is utilized under conditions where NO synthesis via L-Arg is insufficient.

### 4.9 Dietary Nitrate and Nitrite

Considering the evidence that nitrite and nitrate mediate cytoprotective effects in human physiology and especially under pathophysiological conditions, it is not unlikely that dietary nitrate and nitrite may positively affect human health and disease. Recognizing that NO is the most important molecule in regulating blood pressure and maintaining vascular homeostasis, food sources rich in NO compounds may provide beneficial effects primarily to the heart and blood vessels. Although there are clear reports on certain foods and diets that have shown a benefit in terms of preventing cancer and cardiovascular disease, the specific nature of the active constituents responsible for the cardioprotective effects of certain foods is still unknown. Viable candidates, promoted not least by TV and food industry campaigns are fibres, minerals, or antioxidants. In order to bring light to this issue several epidemiological studies were carried...
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out. Joshipura et al. (1999) found that a high intake of fruits and vegetables was indeed associated with a reduced risk for coronary artery disease and apoplectic stroke. The large study population also allowed for analysis of the protection afforded by specific types of food, and the strongest protection against coronary heart disease was seen with a high intake of green leafy vegetables. According to these findings, Appel et al. showed that a dietary supplementation with vegetables lowers blood pressure in subjects with borderline hypertension to the same extent as monotherapy with a standard anti-hypertensive drug (Appel et al., 1997; Sacks et al., 2001). These observations are concordant with the findings of Larsen and Webb et al. who demonstrated the blood pressure-lowering effects of dietary nitrate, and ingestion of beetroot juice, respectively (Larsen et al., 2006; Webb et al., 2008). The investigators of the so-called 'Dietary Approaches to Stop Hypertension study' (DASH) attributed the observed blood pressure-lowering effect to the high calcium, potassium, polyphenols and fibre content, and to the low content of sodium and animal protein (Most, 2004). Bearing in mind that our major dietary sources of nitrate and nitrite are vegetables, these and other findings from the DASH study also point to a less widely acknowledged but highly plausible hypothesis: the observed blood pressure-lowering effect is due to the reduction of nitrate to nitrite and nitric oxide. This hypothesis is encouraged by considering the constituents of the Mediterranean diet, which has been consistently associated with lower incidence of cardiovascular disease and cancer in several studies (Fidanza et al., 1970; Willett et al., 1995; Trichopoulou and Critselis, 2004; Visioli et al., 2004). The main constituent of the Mediterranean diet pyramid is composed of fruits and vegetables, both rich in nitrate and nitrite. The nitrate/nitrite contents of the diet consumed within the DASH study illustrates this coherence. The beneficial, blood pressure-lowering diet results in a consumption of 1222 mg nitrate whereas the unfavourable diet only yields 174 mg nitrate. The latter is comparable with the estimated intake from food in Northern Europe (50-140 mg d⁻¹) (Mensinga et al., 2003). In contrast, the World Health Organization’s acceptable daily intake (ADI) for the nitrate ion is set to an exposure limit of 222 mg nitrate and 3.6 mg nitrite for a 60 kg adult.

The hypothesis that dietary nitrate might provide cardiovascular benefit is further encouraged by recent animal models of myocardial infarction, where dietary supplementation of these anions provided beneficial effects on I/R injury (Bryan et al., 2007). But even nowadays it is not possible to talk about the exciting discoveries relating to NO, nitrate, and nitrite without acknowledging the public perception of these anions; for discoveries in the 1960s suggested an increase of cancer incidence from the formation of N-nitrosamines. Despite the early findings of Tannenbaum et al. that nitrites and nitrates are formed endogenously in the human intestine, the public perception is still that these anions are harmful food additives that should be avoided (Tannenbaum et al., 1978). Direct evidence for the participation of nitrate and nitrite in human carcinogenesis is lacking, despite extensive epidemiological and animal studies (Powlson et al., 2008). Interestingly, a diet rich in vegetables, such as the Mediterranean and the traditional Japanese diets, contains more nitrate than the recommended acceptable daily intake by the World Health Organization (Katan, 2009). Even a portion of spinach commonly consumed in one serving of salad can exceed the acceptable daily intake for nitrate (Lundberg and Govoni, 2004). Taken together, the current evidence supports the conclusion of the European Food Safety Authority that the benefits of vegetable and fruit consumption outweigh any perceived risk of developing cancer from the consumption of nitrate and nitrite in these foods. The data outlined above, from observational epidemiologic and human clinical studies, support the hypothesis that nitrates and nitrites of plant origin play essential physiologic roles in supporting cardiovascular health.

4.10 Conclusions

Including food rich in nitrite, nitrate and antioxidants combined with L-arginine may
provide the optimal combination of substrates to improve NO production and homeostasis. L-arginine alone is not able to restore NO bioavailability under conditions of ischaemia or oxidative stress. The growing field of evidence for the benefit of dietary nitrate and nitrite may provide a rescue or protective pathway for people at risk of cardiovascular disease.

4.11 Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) and the German Cardiac Society (DGK). Tienush Rassaf is a Heisenberg scholar of the DFG (RA 969/5-1). Malte Kelm is supported by the DFG (Ke405/5-1). Peter Luedike is a scholar of the DGK.

References


5 Histidine Decarboxylase

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5.1 Abstract

The mammalian histidine decarboxylase cDNA encodes for a protein with a molecular mass of 74 kDa that is converted into a 53-55 kDa form during post-translational proteolytic processing through the ubiquitin-proteasome system. The resulting enzyme, histidine decarboxylase, converts L-histidine to histamine. In the brain, histamine is an important neurotransmitter and modulator which has been implicated in a wide range of physiological functions and behaviours, such as immune function, gastric acid secretion, nutrition, sleep, anxiety, reward and memory. Furthermore, histamine plays a role in human diseases such as allergy, cancer, epilepsy and autoimmune disorders. The first part of this chapter focuses on the histamine synthesizing enzyme histidine decarboxylase, and summarizes what is known about its molecular structure and catalytic function, its distribution in peripheral and central tissues, its cellular localization, the cell types in which it is expressed, and its gene structure, as well as the regulation of its transcription and translation. The second part of this chapter reviews the literature on neuro-physiological and behavioural effects of pharmacological histidine decarboxylase inhibition in the rat, and histidine decarboxylase gene knockout in the mouse. Finally, evidence implicating the brain’s histamine system in Alzheimer’s disease is discussed.

5.2 Introduction

The imidazolamine histamine is synthesized from its precursor L-histidine by the enzyme histidine decarboxylase and its cofactor pyridoxal phosphate. In the central nervous system, histamine is metabolized to tele-methylhistamine by histamine methyltransferase. Tele-methylhistamine is further degraded to t-methyl-imidazoleacetic acid by monoamine oxidase (Haas et al., 2008; Fig. 5.1a). Histamine plays a role in various physiological functions including nutrition, immune function, cell growth, gastric acid secretion, body temperature, respiration, neurotransmission, sexual behaviour (Rangachari, 1992; Falus, 2003; Ohtsu and Watanabe, 2003), sleep, arousal, anxiety, reward and memory (Dere et al., 2010). It is also involved in several pathological conditions and diseases such as allergic reactions, inflammation, atherosclerosis (Tanimoto et al., 2006), cancer, epilepsy, and autoimmune encephalomyelitis (Watanabe...
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and Yanai, 2001; Falus, 2003). The central and peripheral effects of histamine are mediated by the stimulation of four metabotropic G-protein coupled histamine receptors H1-, H2-, H3- and the recently discovered H4-receptors. Histamine receptors differ in terms of pharmacology, central and peripheral localization, and cellular transduction processes (Parson and Ganellin, 2006; Haas et al., 2008).

Although histamine is produced by mast cells in nearly all tissues it is also detected in a variety of other cell and tissue types. The concentration of tissue histamine depends on the status of histidine decarboxylase activity. Expression of histidine decarboxylase in different cell populations and tissues can be investigated, for instance by using quantitative real-time reverse transcription polymerase chain reaction to measure histidine decarboxylase mRNA levels, or by means of immunohistochemical labelling with antibodies raised against histidine decarboxylase. Histidine decarboxylase-positive cells include mast cells, enterochromaffin-like cells in the stomach, basophils, platelets, macrophages, endothelial cells and neurons (Li et al., 2008). In rats, histidine decarboxylase immunoreactive structures were demonstrated in fetal liver and peritoneal mast cells, as well as in skin, stomach and brain tissue (Taguchi et al., 1984). The highest amounts of histidine decarboxylase mRNA expression are measured in the stomach followed by the brain, skin, jejunum, spleen and liver (Kondo et al., 1995).

In the mammalian brain, histidine decarboxylase-positive neurons are clustered in the nucleus tuberomamillaris of the posterior hypothalamus. The tuberomamillary nucleus consists of five subregions known as E1–E5 which are differentiated in terms of cytoarchitecture and projections (Wada et al., 1991). Histaminergic projections are diffused widely within the central nervous system, including the septum, hippocampus, amygdala, olfactory bulb, basal ganglia, diagonal band of Broca, thalamus, forebrain, brain stem and spinal cord (Brown et al., 2001; Fig. 5.1b). In the rat brain, the majority of neurons containing histidine decarboxylase mRNA in the nucleus tuberomamillaris are also immunoreactive for glutamate decarboxylase, suggesting that histamine is co-localized with GABA.

![Fig. 5.1. Neuronal histamine. (a) Synthesis and metabolism of histamine in the brain. (b) The histamine system in the rat brain: origins and projections. AH, anterior hypothalamic area; Arc, arcuate nucleus; Cc, corpus callosum; Cer, cerebellum; CG, central grey; CX, cerebral cortex; DR, dorsal raphe nucleus; F, fornix; Hip, hippocampus; LS, lateral septum; MD, mediadorsal thalamus; MMr, medial mammillary nucleus; OB, olfactory bulb; Pn, pontine nuclei; Sol, nucleus of solitary tract; Sox, supraoptic decussation; VDB, Vertical limb of the diagonal band; VMH, ventromedial hypothalamus.](image-url)
and galanin (Kohler et al., 1986). In the human brain, histidine decarboxylase mRNA only co-localizes with GABA but not with galanin (Trottier et al., 2002).

5.3 Histidine Decarboxylase Enzyme

Mammalian histidine decarboxylase belongs in the fold type I pyridoxal phosphate-dependent enzyme category. It is the only rate-limiting enzyme that catalyses the formation of histamine. Histidine decarboxylase has been purified from various tissues and species including mouse mastocytoma and fetal rat liver. The intracellular localization and post-translational processing of histidine decarboxylase had been investigated in a rat basophilic/mast cell line RBL-2H3 (Tanaka et al., 1998). Histidine decarboxylase mRNA is translated in the cytosol to an endogenous 74 kDa form of the histidine decarboxylase enzyme. This full length 74 kDa form translocates to the endoplasmic reticulum where it undergoes post-translational processing and is truncated into a shorter form by the involvement of the ubiquitin proteasome system (Joseph et al., 1990; Yamamoto et al., 1990; Tanaka et al., 1998). It has been proposed that biologically active histidine decarboxylase is a homodimer consisting of two carboxy-truncated monomers with a molecular mass of 53–55 kDa (Taguchi et al., 1984). However, there is also evidence suggesting that other processed isoforms which are greater in size than 55 kDa might also contribute to histamine biosynthesis (Furuta et al., 2006). The histidine decarboxylase enzyme has no amino terminal signal sequence or hydrophobic membrane anchor. COS-7 cell transfection studies with histidine decarboxylase cDNA revealed that the precursor 74 kDa form of L-histidine decarboxylase is tightly associated with the membrane of the endoplasmic reticulum with its carboxyl terminal region exposed on the cytosolic part of the cell (Furuta et al., 2006).

5.4 Histidine Decarboxylase Gene

By means of in situ hybridization assays using a rat histidine decarboxylase cDNA probe, the histidine decarboxylase gene has been identified in the 15q21-q22 region of human chromosome number 15 and in the E5-G region of mouse chromosome number 2 (Malzac et al., 1996). The mouse and human histidine decarboxylase genes consist of 12 exons and cover 24 kb of genomic DNA (Suzuki-Ishigaki et al., 2000). In humans, two splicing variants of the histidine decarboxylase gene of 2.4 and 3.4 kb have been detected. In contrast, the mouse homologue does not undergo alternative splicing and generates only a single transcript of 2.4 kb. The exon-intron boundaries of the mouse gene are comparable to those of the human gene and the nucleotide sequences of the exons of the mouse gene show 60–90% homology to their human counterparts (Suzuki-Ishigaki et al., 2000). Research on the regulation mechanism of the histidine decarboxylase gene indicates that in cells that express histidine decarboxylase, the CpG islands in the promoter region of the histidine decarboxylase gene are demethylated, whereas in cells that do not express the histidine decarboxylase gene they are methylated (Watanabe and Ohtsu, 2002). The transcription of the histidine decarboxylase gene is stimulated by various agents, treatments, and physiological conditions. The transcription-initiating factors include gastrin, phorbol ester phorbol 12-myristate 13-acetate, oxidative stress, thrombopointin, tetradeacanophosphol acetate, helicobacter pylori infection and cooled-induced stress (Watanabe and Ohtsu, 2002). Gastrin and phorbol ester-induced histidine decarboxylase gene transcription is mediated through protein kinase C- and mitogen-activated protein kinase-dependent pathways (Höcker et al., 1998). In rat tissue, the expression of the histidine decarboxylase protein decreases histidine decarboxylase gene promoter activity in a dose-dependent fashion. Thus, the transcription rate of the histidine decarboxylase gene is controlled by a negative feedback loop. Within the histidine decarboxylase promoter region this negative feedback inhibition is thought to be mediated by gastrin and extracellular signal-related kinase-1 response element recognition sites (Colucci et al., 2001).
5.4.1 Gene polymorphism

Several non-synonymous polymorphisms of the human histidine decarboxylase gene have been detected, including rs17740607 Met31Thr, rs16963486 Leu553Phe, and rs2073440 Asp644Glu (Garcia-Martin et al., 2009). These genetic polymorphisms might be responsible for inter-individual variation in histamine metabolism and possibly some pathological conditions (Zhang et al., 2006).

5.5 Pharmacological Inhibition

α-Fluoromethylhistidine is a specific and irreversible inhibitor of histidine decarboxylase activity (Watanabe et al., 1990). In rats, the intraperitoneal injection of alpha-fluoromethylhistidine at a dose of 200 mg kg⁻¹ induced a substantial depletion of neocortical and hippocampal histamine levels three hours after administration of the drug (Servos et al., 1994). Onodera et al. (1992) report that one and three hours after intraperitoneal injection of α-fluoromethylhistidine (200 mg kg⁻¹) to rats the histamine levels were decreased considerably in the hypothalamus, cortex, and thalamus. Histamine levels were moderately reduced in the olfactory bulb, amygdala, pons-medulla oblongata, and midbrain. In contrast to the study by Servos et al. (1994), Onodera et al. (1992) found only minor decreases in histamine levels in the striatum, hippocampus, cerebellum, and pituitary (Onodera et al., 1992). These contradictory results might be due to methodological differences in brain tissue preparation for the measurement of histamine levels. Using a CO₂-trapping enzymatic method it has been found that α-fluoromethylhistidine induced a near-complete inhibition of histidine decarboxylase in the hypothalamus both in vivo and in vitro, but it failed to do so in the frontal cortex of rats. In the latter, maximal doses of the drug were ineffective in completely inhibiting histidine decarboxylase activity (Skratt et al., 1994). Therefore, it might be possible that a second α-fluoromethylhistidine-resistant form of histidine decarboxylase exists in the mammalian brain. However, there is also evidence that α-fluoromethylhistidine potency to inhibit histidine decarboxylase activity might vary across different brain areas. In this regard it has been suggested that α-fluoromethylhistidine might not necessarily reduce histamine levels in the rat hippocampus because of its scarce innervations by histamine-containing axon terminals (Sakai et al., 1998).

The histamine H3-receptor is an autoreceptor located on histamine-containing cells and regulates histamine synthesis and release at its terminal structures. Intraperitoneal administration of the histamine H3-receptor agonist (R)α-methylhistamine to rats reduces histidine decarboxylase gene expression in the nucleus tuberomammilaris one hour after treatment, and increases histidine decarboxylase gene expression when examined after three hours post treatment (Chotard et al., 2002). The latter might reflect a compensatory or rebound effect required to counteract histamine depletion.

5.6 mRNA Antisense and Gene Knockout

Histidine decarboxylase activity can also be inhibited by molecular genetic techniques including antisense RNAs and gene targeting. The translation of histidine decarboxylase mRNA into the histidine decarboxylase protein can be prevented by a polyclonal histidine decarboxylase-specific antisense oligonucleotide. In melanoma cells, histidine decarboxylase expression is increased by tenfold as compared to normal skin, resulting in high levels of histamine. Histidine decarboxylase-specific antisense oligonucleotides substantially decrease protein levels of histidine decarboxylase and simultaneously inhibit cell proliferation, suggesting that histamine acts as an autocrine growth factor for tumour cell proliferation (Hegyesi et al., 2001).

Histidine decarboxylase knockout mice have been generated by means of homologous gene recombination in embryonic 129/Sv stem cells, and are maintained on a homogenous 129/Sv genetic background. In these mice, the genomic sequence from intron 5 to exon 9 was replaced with an inverted PGK.
promoter-driven neomycin phosphotransferase gene, resulting in a null mutation (Ohtsu et al., 2001). Exon 8 contains the coding sequence for the binding site of pyridoxal 5'-phosphate which is the essential coenzyme of histidine decarboxylase protein. Therefore it was anticipated that the histidine decarboxylase enzyme produced from this truncated histidine decarboxylase gene would be unable to catalyse the synthesis of histamine from L-histidine. However, histidine decarboxylase gene expression was abolished already at the transcriptional level in these mice. These histidine decarboxylase knockout mice are viable and fertile (although due to their genetic background strain they breed rather poorly as compared to C57BL/6J mice). As expected, the targeted disruption of mouse histidine decarboxylase gene in these mice resulted in the near complete absence of endogenous synthesis of histamine. Furthermore, a decreased number of mast cells have been observed in these mouse mutants. Moreover, the remaining mast cells of histidine decarboxylase knockout mice were characterized by morphological aberrations, reduced levels of granular proteases and generally reduced granular content (Ohtsu et al., 2001). However, it should be noted that although the histidine decarboxylase protein expression was blocked at the transcriptional level, nevertheless some residual minor histamine content was found in the brains of histidine decarboxylase knockout mice, even under low-histamine diet conditions. It therefore remains to be determined whether there is a yet unknown brain enzyme different from histidine decarboxylase which is capable of catalysing the synthesis of histamine.

5.7 Neurophysiology and Behaviour

In the following, some of the behavioural and associated neurophysiological effects of histidine decarboxylase inhibition in rats and mice will be reviewed. The behavioural effects of pharmacological blockade of single histamine receptors, as well as studies with histamine receptor knockout mice, has been reviewed recently elsewhere (Esbenshade et al., 2008; Alvarez, 2009; Dere et al., 2010) and will not be discussed here. Nevertheless the reader should be aware that pharmacological blockade of single histamine receptors can have distinct and sometimes opposed effects on behavioural and neurophysiological measures when compared to pharmacological histidine decarboxylase blockade. For a discussion of these divergent results see Alvarez (2009) and Dere et al. (2010).

5.7.1 Brain neurotransmitters

Histamine has been implicated in the control of appetite, is a mediator of the anorexigenic action of leptin, and plays an important role in the pathogenesis of age-related obesity in mice. α-Fluoromethylhistidine has been reported to increase food intake in rats via depletion of neuronal histamine (Tuomisto et al., 1994; Sakai et al., 1995). Intracerebroventricular infusion of α-fluoromethylhistidine into the third ventricle of rats induced feeding in the early light phase when the histamine synthesis usually is high (Ookuma et al., 1993).
Chronic administration of α-fluoromethylhistidine to rats via osmotic minipumps over a period of two weeks increased feeding throughout the test period and increased body weights towards the end of the test period (Orthen-Gambill and Salomon, 1992). Furthermore, intraperitoneal administration of α-fluoromethylhistidine abolished leptin-induced suppression of food intake in C57BL/6 mice (Morimoto et al., 1999).

It is well known that the hypothalamus located in the diencephalon mediates the release of nutrition-related hormones, and that the dorsomedial hypothalamic nucleus is involved in the control of feeding and drinking behaviour, as well as in the regulation of body weight (Bellinger and Bernardis, 2002). Furthermore it has been proposed that histamine stimulates the satiety centre located in the ventromedial part of the hypothalamus (Sakata et al., 2003). Given that α-fluoromethylhistidine led to histamine depletion in the hypothalamus, without affecting concentrations of catecholamines (Ookuma et al., 1993), it is likely that α-fluoromethylhistidine increases food intake via histamine depletion in the hypothalamus.

In rats, the intraperitoneal application of the histamine precursor L-histidine decreased both drinking and food ingestion. This effect was reversed in part by the co-administration of α-fluoromethylhistidine (Vaziri et al., 1997). Histidine decarboxylase knockout mice exhibited an enhanced susceptibility to high-fat diet-induced obesity (Jorgensen et al., 2006). In sum, it appears that central histamine depletion increases food and water intake, while it is decreased by the stimulation of histamine synthesis. It remains to be determined whether the brain’s histamine system might be a potential pharmacological molecular target for the treatment of eating disorders such as anorexia and obesity (Masaki and Yoshimatsu, 2007).

5.7.3 Sleep, waking and arousal

Animal research using rodents and cats has established a central role of the histaminergic system in the regulation of sleep and wakefulness. Histidine decarboxylase-positive neurons increase their firing frequency during waking and arousal but are silent during sleep (Lin, 2000). In cats, the functional inactivation of the nucleus tuberomamillaris by the microinfusion of muscimol caused hypsomnolica associated with reduced arousal and attention (Lin et al., 1989). Similar effects have been observed after microinjections of α-fluoromethylhistidine into the ventrolateral posterior hypothalamus of cats which is innervated by histaminergic fibres. Here, α-fluoromethylhistidine decreased wakefulness and increased slow-wave sleep (Lin et al., 1986). In rats, the intraperitoneal application of the histidine decarboxylase inhibitor α-fluoromethylhistidine prevented the increase in wakefulness induced by intracerebroventricular administration of the hypocretin orexin, a recently discovered wake-promoting agent, suggesting that the sleep–wake regulation by orexin neurons recruits the histaminergic system (Yasuko et al., 2010). Furthermore, histidine decarboxylase knockout mice displayed diminished wakefulness during the dark period of the light–dark cycle and in an unfamiliar environment (Parmentier et al., 2002). Furthermore, they also exhibited an abnormal electroencephalogram, sleep fragmentation and increased paradoxical sleep during the light period (Anaclet et al., 2009). The pharmacological modulation of histamine neurotransmission in the brain has been utilized as a treatment for sleep disorders. For example, histamine H1 receptor antagonists known as ‘antihistamines’ as well as various psychotropic drugs with antihistamine properties are currently used to ameliorate insomnia (Stahl, 2008).

5.7.4 Reward and drugs

The dopamine hypothesis of reward holds that activation of the mesocorticolimbic and nigrostriatal dopamine system is a common pathway through which both drugs of abuse and natural rewards can reinforce behaviour and/or initiate memory formation (Wise, 2008). There is substantial evidence indicating
that neuronal histamine counteracts reward and reinforcement processes, mediated by the dopamine system in the brain (Hasenohrl and Huston, 2004). Accordingly, it has been hypothesized that the blockade of histamine synthesis should enhance the rewarding or hedonic aspects of addictive drugs. In line with these assumptions it was found that subcutaneous injections of α-fluoromethylhistidine potentiated the conditioned place preference induced by a sub-effective low dose of morphine (Suzuki et al., 1995). Similarly, histidine decarboxylase knockout mice showed stronger morphine-induced conditioned place preference but decreased naloxone-precipitated withdrawal jumping as compared to their wild-type controls (Gong et al., 2010). They also displayed a weaker stimulatory response to acute ethanol but showed stronger ethanol-induced conditioned place preference compared to their wild-type litter mates (Nuutinen et al., 2010). Receptor binding studies suggested that these effects are not related to changes in the expression of the GABA_A receptor in the brain of histidine decarboxylase knockout mice, but rather reflect increases in the rewarding or reinforcing properties of ethanol (Nuutinen et al., 2010). In rodents, the chronic intraperitoneal administration of methamphetamine induces a progressive and long-lasting increase in locomotor and stereotyped behaviour. The behavioural effects of methamphetamine are potentiated by pre-treatment with α-fluoromethylhistidine administered intraperitoneally (Ito et al., 1996). Histidine decarboxylase knockout mice showed increased methamphetamine-induced locomotor hyperactivity and accelerated behavioural sensitization to methamphetamine as compared to the wild-type controls (Iwabuchi et al., 2004). However, the psychostimulant effects of cocaine were lower in histidine decarboxylase knockout mice relative to their wild-type littermates. Furthermore, cocaine-induced conditioned place preference of histidine decarboxylase knockout mice was not significantly different from the controls (Brabant et al., 2007). In conclusion, the majority of findings reviewed above are in accordance with the assumption that central histamine depletion has a disinhibitory effect on the dopamine system of the brain. The meso-cortico-limbic dopamine system is thought to mediate the rewarding and reinforcing effects of natural rewards and drugs of abuse.

5.7.5 Stress, fear and anxiety

Histamine has been implicated in the regulation of the stress response mediated by the hypothalamo-pituitary-adrenocortical axis. In mice, different types of stress including water bathing, cold, body restraint, foot shock, and prolonged walking increase histidine decarboxylase activity in peripheral tissues such as skeletal muscles and the stomach (Taylor and Synder, 1971; Yoshitomi et al., 1986; Ayada et al., 2000). In rats, the application of unavoidable electrical shocks or a chronic restraint of their bodies increased histamine turnover rates in the diencephalon, nucleus accumbens and striatum (Ito, 2000; Westerink et al., 2002). Incomplete lesions of the nucleus tuberomamillaris induced anxiolytic effects in rats (Frisch et al., 1998). The amygdala is a central part of the brain’s defence system, modulating fear and anxiety-related behaviours including conditioned and genetically predetermined unconditioned fear responses. Microinfusion of histamine into the amygdala of rats increased anxiety-related behaviours in the elevated plus maze test (Zarrindast et al., 2005). From these findings one would predict an anxiolytic effect of brain histamine depletion. However, the behavioural phenotyping of histidine decarboxylase knockout mice revealed an opposite relationship. These mouse mutants exhibited increased behavioural measures of anxiety in a variety of tasks measuring experimental fear and anxiety including the open field test of anxiety, light–dark box, elevated plus maze and elevated zero maze (Dere et al., 2004). Furthermore, histidine decarboxylase knockout mice showed an increase in anxiety-related behaviours in the height–fear task and the graded anxiety test, a modified elevated plus maze based on a combination of the light–dark box and elevated plus maze (Dere et al., 2004). The role of brain histamine
in fear and anxiety-related behaviour has been investigated by means of pharmacological, lesion, and genetic approaches. However, to date no clear-cut picture on the question of whether central histamine has anxiolytic or anxiogenic effects has emerged.

5.7.6 Learning and memory

It is well known that histamine plays an important role in various forms of learning and memory, both under physiological as well as pathological conditions (Dere et al., 2010). Histamine has also been implicated in synaptic long-term potentiation and depotentiation, which are assumed to represent electrophysiological correlates of learning and memory formation (Dringenberg and Kuo, 2006; Haas et al., 2008). In rats, the working and reference memory impairment that is induced by the infusion of the muscarinergic acetylcholine receptor blocker scopolamine into the dorsal hippocampus is ameliorated by the co-administration of histamine or its precursor L-histidine (Xu et al., 2009). Moreover, intracerebroventricular injection of histamine or the intraperitoneal injection of L-histidine to rats with hippocampal lesions both ameliorated active avoidance performance (Kamei et al., 1997). Intracerebroventricular injection of the histidine decarboxylase-blocker α-fluoromethylhistidine to rats impaired spatial memory in a radial maze task (Chen et al., 1999) and prolonged the response latency after intracerebroventricular and intraperitoneal injection in an active avoidance paradigm (Kamei et al., 1993). Interestingly, the senescence-accelerated mouse-prone strain, which already exhibits learning and memory deficits at the age of 12 months as compared to an age-matched normal-rate ageing strain, also exhibited lower histidine decarboxylase activity in the forebrain including the cortex, hippocampus and striatum (Meguro et al., 1995). These studies suggest that the stimulation of histamine neurotransmission can counteract amnestic manipulations such as acetylcholine receptor blockade or hippocampal lesions, and that the reduction of histamine synthesis has detrimental effects on learning and memory performance in rodents. However, there is also evidence available suggesting the opposite relationship. For example, repeated administration of α-fluoromethylhistidine decreased histamine levels in the hippocampus and cerebral cortex, but concomitantly improved spatial memory in a working-memory version of the eight-arm radial maze (Sakai et al., 1998). Furthermore, histidine decarboxylase knockout mice showed improved cued and contextual fear-conditioning (Liu et al., 2007); and electrophysiological measurements revealed that hippocampal CA1 long-term potentiation was greater in the histidine decarboxylase knockout mice immediately after its induction, but smaller one day after. Additionally, hippocampal glutamate levels were increased in histidine decarboxylase knockout mice up to four days after the induction of long-term potentiation (Liu et al., 2007). In the Morris water maze, histidine decarboxylase knockout mice showed superior performance in the hidden and cued platform versions of the task and on the retention test of the passive avoidance task (Dere et al., 2003; Acevedo et al., 2006). However, the histidine decarboxylase knockout mice were impaired in their learning of a temporal sequence whereby novel objects were successively introduced into a familiar environment (Dere et al., 2003).

These contradictory effects of various forms of brain histamine depletion might be the result of concomitant effects on emotional and reward-mediating systems and their interactions with specific experimental conditions and technical parameters of the different learning and memory tasks used. It is well known that both too low, and excess levels of stress/arousal during testing, can counteract learning and memory performance. In aver-sively motivated tasks, such as the Morris water maze or the one-trial inhibitory avoidance task, the level of stress/arousal induced to motivate learning and memory formation has to be adjusted to an optimal intermediate range that enables the detection of impaired and improved performance. Interestingly, it has been found that a slight reduction in the brain histamine concentration by α-fluoromethylhistidine enhances attention and accuracy in solving a visuospatial task
under stressful conditions (Cacabelos and Alvarez, 1991). In positively motivated learning and memory paradigms, such as the eight-arm radial maze, conditioned place preference or the novelty preference object recognition paradigm, the amount of reward given to reinforce correct responses can likewise modulate learning and memory performance. When the amount of reward given is at the lower end of the spectrum that is sufficient to reinforce correct responses and maintain responding, brain histamine depletion might increase learning motivation by enhancing the rewarding value of natural rewards and drugs (Dere et al., 2010).

5.7.6.1 Dementia

The severe memory impairments of patients suffering from Alzheimer’s disease was originally attributed to the degeneration of cholinergic neurons, especially those located in the basal forebrain, due to extracellular amyloid plaque deposits and the intracellular accumulation of neurofibrillary tangles. However, there is substantial evidence suggesting that other neurotransmitter systems, including the brain’s histamine system, also contribute to the cognitive impairments of these patients (Musial et al., 2007; Yanai and Tashiro, 2007; Dere et al., 2010). Individuals suffering from Alzheimer’s disease displayed changes in brain histamine levels (Panula et al., 1998) concomitant with a degeneration of histamine-containing neurons in the nucleus tuberomammillaris (Airaksinen et al., 1991; Nakamura et al., 1993; Ishunina et al., 2003). A coupled radioenzymatic assay has been used to determine the activity of histidine decarboxylase in the frontal cortex of ageing Down’s syndrome patients, Alzheimer’s patients and healthy control individuals. It was found that histidine decarboxylase activity was reduced in brains of both Down’s syndrome and Alzheimer’s patients and healthy control individuals. It was found that histidine decarboxylase activity was reduced in brains of both Down’s syndrome and Alzheimer’s patients (Schneider et al., 1997). Tacrine is an anti-Alzheimer’s drug (a cholinesterase inhibitor) which increases the availability of acetylcholine, and is widely prescribed during the early stages of the disease. The catabolic activity of histamine-N-methyltransferase, which normally degrades histamine to tele-methylhistamine, is blocked by tacrine. Tacrine has been reported to increase histamine levels in the hippocampus (Nishibori et al., 1991; Morisset et al., 1996). In contrast to tacrine, physostigmine, another anti-Alzheimer’s drug, is less effective in ameliorating cognitive symptoms in Alzheimer’s disease and has a lower affinity to histamine-N-methyltransferase (Nishibori et al., 1991). Thus, it is possible that the pre-cognitive effects of cholinesterase in early-stage Alzheimer’s disease are due at least in part to increases in histamine neurotransmission, e.g. in the hippocampus. Given the growing need for effective cognitive enhancers with minor adverse effects, histamine H3 receptor antagonists which stimulate histamine synthesis and release are currently being evaluated for their potential use in Alzheimer’s disease (Medhurst et al., 2009).

5.8 Summary and Conclusions

Mammalian histidine decarboxylase is the rate-limiting enzyme that catalyses the formation of the indolamine histamine from histidine in a one-step oxidative decarboxylating process. Biologically active histidine decarboxylase takes the form of a homodimer consisting of two carboxy-truncated monomers with a molecular mass of 53-55 kDa. Within the cell, histidine decarboxylase is coupled to the membrane of the endoplasmic reticulum with its carboxyl terminal region exposed on the cytosolic part of the cell. Histidine decarboxylase-positive cells include mast cells, enterochromaffin-like cells, macrophages, endothelial cells and neurons. In the brain histidine, decarboxylase-positive neurons are exclusively located in the nucleus tuberomammillaris of the posterior hypothalamus, from where projections to wide parts of the brain arise. The mouse and human histidine decarboxylase gene consists of 12 exons and covers 24 kb of genomic DNA. Histidine decarboxylase gene expression is initiated by several agents, treatments and physiological conditions.

The depletion of brain histamine by means of systemic or intracerebral application of the histidine decarboxylase blocker α-fluoromethylhistidine to rats, or the genetic
inactivation of histidine decarboxylase expression in the mouse, modulates a wide range of behavioural and physiological processes. The effects of histamine depletion in rodents include increases in nutritive behaviour; decreased waking and arousal; potentiation of the psychostimulant, rewarding, and reinforcing effects of addictive drugs; modulation of behavioural correlates of stress, fear, and anxiety; changes in brain acetylcholine and monoamine levels; and the modulation of hippocampal synaptic plasticity and learning and memory performance. In terms of learning and memory performance the findings are inconsistent, with both improvements and impairments observed after pharmacological or genetic inhibition of histidine decarboxylase activity. These effects might be explained by simultaneous effects on emotional- and reward-mediating systems, and their interactions with specific experimental conditions and task parameters (Dere et al., 2010).

Pathological changes in the histamine system have been measured in brains of Alzheimer’s disease patients. These changes involve neurodegeneration of histamine-positive neurons in the nucleus tuberomammillaris, decreased activity of histidine decarboxylase and changes in the levels of central histamine. It remains to be determined whether some of the cognitive symptoms of patients with Alzheimer’s disease might be ameliorated after treatment with histamine-related drugs.

5.9 Acknowledgements

This work was supported by the German Science Foundation through grant DE1149/5-1 to E.D.

References


Histidine Decarboxylase


Glutamate Decarboxylase

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6.1 Abstract

Glutamate decarboxylase (GAD) catalyses the synthesis of \( \gamma \)-aminobutyric acid (GABA), an inhibitory neurotransmitter, from glutamate. Glutamate is an excitatory neurotransmitter and is also known as an umami compound. GAD was known for its localization in the central nervous system, testes and pancreas; however, recent findings suggest wide distribution including skin and digestive system, i.e. stomach, intestine and tongue. This chapter updates some of the findings of GAD in the taste signalling system. It also covers the area of GAD gene structure and reports an unusual form of GAD protein expression, probably evidence for multiple splicing. The role of GAD in diabetes is another emerging issue worthy of further investigation in relation to diagnostic and therapeutic potential.

6.2 Introduction

This chapter describes recent advances relating to glutamate decarboxylase (GAD), involving two amino acids: L-glutamate as a substrate and \( \gamma \)-aminobutyrate (GABA) as a product. GABA was first recognized for its role as an inhibitory neurotransmitter in the central nervous system (CNS) of higher animals (Ueno, 2000). Since both substrate and product are neurotransmitters but with opposite roles, GAD is a unique enzyme.

Characterization of GAD has been carried out widely and described elsewhere; however, it is important to point out that there was controversy in the properties that were published prior to early 1990, before genes for GAD had been cloned. Molecular analysis made it clear that there are two distinct gene products for GAD, namely GAD 65 and GAD 67. Since then, genetic investigations including knockout mouse studies and immunological analysis for tissue-specific GAD expression have been carried out. Furthermore, GAD 65 involvement in diabetes is also now well recognized. In the following sections, each topic on GAD-related issues is briefly reviewed.

6.3 Distribution of GABA

The role of GABA as an inhibitory neurotransmitter defines its distribution in the mammalian system, mainly at the GABAergic neuron that is estimated at around 25–45% of the total neurons. GABA concentration is estimated at

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mM level, significantly high, but its distribution is uneven (Iversen and Snyder, 1968). On the other hand, there is little GABA present at the peripheral nerves. In the nerve cells, GABA is localized at the synaptic terminals and cell bodies (Ribak et al., 1979a,b) (Fig. 6.1). In vertebrates, GABA occurs at relatively high levels in Purkinje cells in the cerebellum (Obata, 1969; Saito et al., 1974; Storm-Mathisen, 1975); hippocampus (Mihailovic et al., 1965; Delgado et al., 1971; Storm-Mathisen, 1975); substantia nigra (McGeer et al., 1974; Storm-Mathisen, 1975); posterior spinal cord (Henry, 1979); hypothalamus (Storm-Mathisen, 1975; Marczyński, 1998); and at low levels in medulla oblongata (Siemers et al., 1982); cerebral cortex (Thangnipon et al., 1983); and cerebellar cortex (Ikenaga et al., 2005). Outside of the nervous system, GABA was found in pancreatic β-cells (Smismans et al., 1997), ovary (Schaeffer and Hsueh, 1982), heart (Yessaian et al., 1969), and intestine (Taniyama et al., 1982). Crustaceans show significant amount of GABA at the connective part of neurons.

6.3.1 GABA storage, release and uptake

GABA is stored in synaptic vesicles by vesicular GABA transporter (VGAT). Ligand specificity of VGAT is low: i.e., glycine can sometimes be transported (Dumoulin et al., 1999). Nerve impulses release GABA into the synaptic space, where the process depends upon Ca²⁺. Released GABA molecules are taken up to the nerve ending and/or glia cells. The process depends upon energy and is associated with Na⁺ and Cl⁻. Currently, four subtypes are known to be GABA transporters; these actions may be inhibited by β-alanine or L-2, 3-diaminobutyrate (Guimbal et al., 1995).

6.3.2 GABA receptors

GABA binds to the GABA receptor to raise Cl⁻ permeation that leads to hyperpolarization of the membrane in inhibiting nerve excitation. There are three subtypes of GABA receptor, namely GABAₐ, GABAₐ, and GABAₐ. GABAₐ and GABAₐ are Cl⁻ ion channel hetero-pentameric subunits. GABAₐ is mainly found in the central nervous system (CNS), particularly around synapses, and its subunit construction is quite diverse: five subunits, with four transmembrane domains selected from eight groups of polypeptide chains classified into 19 isoforms. The exact location of GABAₐ receptor with one specific combination of subunits defines the role of the particular GABAₐ receptor. GABAₐ receptor has two GABA binding sites and several allosteric sites; thus, it exhibits complex pharmacological characteristics.

![Fig. 6.1. Metabolic pathway of GABA.](image-url)
GABA\textsubscript{B} receptor is a typical G-protein coupled type with seven transmembrane domains and constructed with dimeric subunits. GABA\textsubscript{B} is located at the peripheral nerves, pre- and post-synaptic terminals.

GABA\textsubscript{C} receptors are ligand-gated Cl\textsuperscript{-} ion channel type receptors constructed with 5\(\alpha\) family subunits (Qian et al., 1997, 1998). GABA\textsubscript{C} receptors mediate slow and sustained responses in contrast to GABA\textsubscript{A} receptors. GABA\textsubscript{C} receptors are expressed in many brain regions, including retinal neurons. Affinity towards the GABA molecule is tenfold higher than that of GABA\textsubscript{A} receptors (Table 6.1).

### 6.3.3 Metabolism of GABA

In the metabolic network, GABA locates at the GABA shunt (Fig. 6.1), adjacent to the TCA cycle (Erecinska et al., 1996). L-glutamate can be synthesized from \(\alpha\)-ketoglutarate by aminotransferase(s), and is decarboxylated by an enzyme, glutamate decarboxylase (GAD, EC: 4.1.1.15) to produce GABA (Martin et al., 1991; Zhang et al., 1999). GABA is then converted to succinic semialdehyde by GABA transaminase, and to succinate by succinic semialdehyde dehydrogenase. Succinate is now returned to the TCA cycle (Bouche et al., 2003). GABA lowers high blood pressure (Daniels and Pettigrew, 1975), and also has diuretic (Krantis and Kerr, 1981) and anti-tumour activity (Fukushima and Toyoshima, 1975; Lin et al., 1996). These physiological activities might act upon non-neuronal systems. Recently many food products such as chocolate, having GABA as a main ingredient, have been developed commercially for their relaxation effect. However, it is still unclear how GABA acts on non-neuronal systems. GABA is not transported into the brain since it is unable to cross the blood–brain barrier (Purpura and Carmichael, 1960).

### 6.3.4 Decarboxylation reaction by GAD

Decarboxylation of L-glutamate is a one-step and irreversible reaction catalysed by GAD. GAD requires a coenzyme, pyridoxal 5'-phosphate (PLP) that binds to the active site lysine residue to form an internal Schiff base (Choi and Churchich, 1986), where PLP is an active form of vitamin B\(\textsubscript{6}\). GAD takes L-glutamate as a substrate and cleaves the \(\alpha\)-carboxyl group to form CO\(_2\) and GABA. During the catalytic cycle, GAD also utilizes one H\(^+\).

GAD belongs to a family of PLP-dependent amino acid decarboxylases that includes histidine decarboxylase (HDC) and aromatic amino acid decarboxylase (AroDC), also called DOPA decarboxylase (DDC). The three decarboxylases have a common function that cleaves \(\alpha\)-carboxyl group of the substrate amino acids to form CO\(_2\) and the corresponding amines. Comparison of the amino acid sequences should show that, if common amino acid residues are found, these should participate in the recognition and cleavage of the \(\alpha\)-carboxyl group. When amino acid sequences were compared, it was of interest to note that AroDC is the shortest among the three: mammalian GAD isoforms have a long extra complement of residues, about 100 amino acids, at the N-terminal side, and HDC shows about 150 extra amino acid residues at

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Further studies are needed on substrate-recognition mechanisms of these three decarboxylases. Those residues with amino acid similarity being highly heterologous should be candidates for substrate recognition.

Fig. 6.2. Amino acid sequence alignment of amino acid decarboxylases. Amino acid sequences of rat GAD 67, rat GAD 65, human HDC, and rat ADC were compared by using the homology program (DNASIS®, Hitachi). Protein weight matrix of Gonnet 250 was employed with gap open penalty of 10, and gap extension penalty of 0.1. Amino acid sequences were obtained from public database (Swiss-Prot).
Glutamate Decarboxylase

recognition sites for the distal carboxyl group, the aromatic group, and the imidazole group for GAD, AroDC, and HDC, respectively. The answer to this may emerge from the completion of X-ray crystal structure studies.

### 6.3.5 Distribution and characterization of GAD

GAD is distributed widely, from microbes to mammals (Miyashita and Good, 2008; Ueno, 2000). In higher mammals, GAD is found in the brain, particularly in Purkinje cells in the cerebellum, neuronal cells (Saito et al., 1974; Ogoshi and Weiss, 2003; Sueiro et al., 2004; Castaneda et al., 2005), pancreas (Faulkner-Jones et al., 1993), liver (Wu et al., 1978), kidney (Tursky and Bandzuchova, 1999), and sexual organs, including testis and ovary (Medina-Kauwe et al., 1994).

While attempting to clone a gene for GAD, it was found that there are two separate genes for GAD (Erlander et al., 1991; Erlander and Tobin, 1991; Kaufman et al., 1991; Bu et al., 1992; Soghomonian and Martin, 1998). Due to the difference in the expected molecular mass for the protein products, now called isoforms, those gene products were named GAD 65 and GAD 67. Localization of each isoform was extensively studied and it was found that GAD 65 is localized at the nerve ending, particularly at the vesicular membrane region, whereas GAD 67 is localized in the cytosolic space. Knockout studies showed that mice in which individual genes were depleted showed different morphological behaviour (Asada et al., 1996, 1997). Results suggested each isoform has separate biological functions. GAD 67 knockout induced developmental defects in newborn mice, resulting in immediate death after birth, whereas GAD 65 knockout caused development of seizures but allowed survival (Asada et al., 1996, 1997; Choi et al., 2002). Distribution of GAD isoforms in mammalian tissues and organs has not been totally clear. The GAD/GFP transgenic (knock-in) mouse might be a useful tool for the purpose (Tamamaki et al., 2003; Obata, 2004). The GAD 67/GFP knock-in mouse was used successfully to show that GAD 67 is expressed in the stomach, jejunum, and type III taste buds (Akamatsu et al., 2007; Nakamura et al., 2007). The specific expression of GAD in the non-neuronal system is of great interest and could be a target for future research.

Figure 6.3 shows amino acid homology between two isoforms from the same animal species; also shown is homology among various species. There is about 60% sequence homology between the isoforms from the same species; however, homology reduces to 30% if only N-terminal 100 amino acid sequences are compared. Sequence homology between the isoforms from the same species is lower than that of one of the isoforms from difference species; for example, GAD 65 from human versus GAD 65 from rat, or GAD 67 from human versus GAD 67 from rat.

Different properties for GAD isoforms were reported. PLP affinity appeared to be different: GAD 67 has high affinity to PLP, or in other words, both subunits of GAD 67 bind to PLP with the same affinity. GAD 65 may be asymmetrically constructed; one subunit is bound to PLP but another subunit has no PLP, and this is known as the half-apo form. Therefore, the specific activity of GAD 67 is obviously higher than that of GAD 65. The reason why one subunit of GAD 65 has low affinity for PLP is still unclear.

GAD 65 was reported to be a target antigen for autoantibody found in patients of type 1 diabetes (Baekkeskov et al., 1987, 1990, 2000). Similar autoantibody was found in patients of Stiff-person syndrome (Bjork et al., 1994; Daw et al., 1996). A part of GAD 65 protein, showing sequence homology with a virus coat protein commonly found in type 1 diabetes patients, may be the target site that activates B-cells for antibody production (Von Boehmer and Sarukhan, 1999; Yoon et al., 1999). In an extension of this finding, GAD 65 protein has become a target protein for developing an early diagnostic tool of type 1 diabetes patients, since autoantibody against 63-64 kDa protein circulates as early as 15-18 years prior to onset of the diabetic symptoms. For this reason, extensive attempts have been made for large-scale preparation of GAD 65 protein, stability tests on GAD 65, immunogenicity examination of GAD 65, and other studies to
develop a suitable ELISA system (Daw et al., 1996; Law et al., 1998; Papakonstantinou et al., 2000; Kono et al., 2001).

Besides PLP binding, post-translational modification has been observed with GAD. Palmitoylation of cysteine residue(s) at the N-terminal region of GAD 65 (Christgau et al., 1992; Shi et al., 1994; Solimena et al., 1994; Dirkx et al., 1995; Wei and Wu, 2008), and phosphorylation were reported (Bao et al., 1994, 1995; Namchuk et al., 1997; Hsu et al., 1999; Wei et al., 2004; Wei and Wu, 2008).

Fig. 6.3. Amino acid sequence alignment of GAD from different species. GAD protein sequences from the following species were compared for the amino acid homology: rat GAD 67, human GAD 67, mouse GAD 67, feline GAD 67, pig GAD 67, rat GAD 65, human GAD 65, mouse GAD 65, pig GAD 65, Escherichia coli GAD, E. coli GAD, Lactococcus GAD, baker’s yeast GAD1, petunia GAD, tomato GAD, Arabidopsis GAD, and fruit fly GAD. Computational condition was as described in Fig. 6.2.
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The significance of palmitoylation is not clear but it is postulated to show increased membrane affinity of GAD 65. Phosphorylation and dephosphorylation on GAD were reported. It is quite rare that both palmitoylation and phosphorylation are observed on PLP-dependent enzymes.

A typical GAD protein from mammalian sources comprises a dimer, each subunit has one PLP (Denner et al., 1987). Studies on the mechanism of action of GAD, as well as biochemical characterization were extensively carried out on the Escherichia coli enzyme; however, mammalian GAD was not fully understood, because until two separate GAD genes were identified, investigators assumed there was only one GAD gene for mammals. Ever since multiple GAD genes became evident, two separate gene products, exhibiting about 60% amino acid sequence homology, are characterized to have separate biological roles. Nevertheless, the difference or similarity between the two GAD protein properties is not fully characterized. Hence it is prudent to be cautious about references to enzymatic studies on GAD that were conducted prior to the discovery of two independent genes.

6.3.6 Purification of GAD protein

Rat (Wu et al., 1985) and mouse sources (Wu et al., 1973) have been used for GAD protein purification. Other attempts to purify brain GAD have been made on rabbit (Brandon, 1986), pig (Nathan et al., 1994), monkey (Inoue et al., 2008) and human tissues (Davis et al., 2000), for example. Soon after the identification of the GAD genes, attempts were made to clone and express GAD isoforms in E. coli (Kaufman et al., 1986; Chu and Metzler, 1994), yeast (Kanai et al., 1996), and other host cells (Mauch et al., 1993). More recently, the use of tag, i.e. His-tag and GST, attached at either end of the proteins expressed, has made the purification step easier. By using recombinant GAD proteins, X-ray crystal structures of both isoforms of GAD have been determined, although N-terminal 100 amino acid residues were missing from both preparations (Fenalti et al., 2007). N-terminal region of GAD exhibits low sequence homology and is highly susceptible to proteolysis (Chu and Metzler, 1994). It is desirable to construct the in vitro expression system for GAD proteins with full length.

6.3.7 Gene structure of GAD

Human GAD 67 gene is located on chromosome 2 constructed with 16 exons (Erlander et al., 1991; Karlsen et al., 1991; Bu and Tobin, 1994). It encodes a 594 amino acid residue protein with PLP attached at K405. Two truncated isoforms of GAD 67 have been reported: one has not been fully characterized and the other, known as GAD 25, has no enzymatic activity (Chessler and Lernmark, 2000). Rat, mouse, and chicken GAD 67 genomes contain 17 exons, where the 5' untranslated region of GAD 67 mRNA is found within an additional exon, exon 0, together with a part of exon 1. The exon 16 of GAD 67 covers the entire 3' untranslated region of GAD 67 mRNA. Human GAD 65 gene is located on chromosome 10, also constructed with 16 exons. It encodes a 585 amino acid residue protein with PLP attached at K396. Like GAD 67 genomes, gene structure of GAD 65 exhibits high resemblance among animals. Particularly interesting are the locations of exon/intron boundaries, nearly identical for all mammalian GAD genomes.

6.4 GAD 65 in Blood Leucocytes

GAD 65 was found as a target antigenic protein of autoantibody produced by type 1 diabetic patients (Baekkeskov et al., 1990; Richter et al., 1992; Kaufman and Tobin, 1993). It is noteworthy that the autoantibody appears many years before onset of the diabetic symptoms (Atkinson et al., 1994; Hou et al., 1994; Richter et al., 1994; Schloot et al., 1997; Tong et al., 2002; Kawashima et al., 2004). It is thought that a part of GAD 65 amino acid sequence is homologous with a part of the coat protein of Coxsackie virus, a virus suspected for its infection in type 1 diabetes (Fairweather and Rose, 2002). Since GAD 65
is suspected of causing diabetes by inducing antibody production, we have investigated its expression profile in peripheral blood leucocytes (Matsukawa and Ueno, 2007).

Human blood cells were collected and divided into polynuclear and mononuclear leucocyte fractions. After cellular proteins were solubilized, SDS-PAGE was performed and GAD protein was visualized by Western blot analysis. We used two antibodies specific to C-terminal region of GAD 65: one, G4913, is specific to GAD 65 and the other, G5163, recognizes a common sequence in GAD 65 and 67.

G5163 antibody was raised against peptide having a sequence of KDIDFLIEEIERLGQDL that corresponds to residues 570–585 locating at the end of C-terminal region of human GAD 65, and also to residues 579–594 of GAD 67. Those two sequences of GAD 65 and 67 are highly conserved among mammalian species; thus, G5163 should bind to both mammalian GAD 65 and GAD 67. When Western blot analysis was carried out on the leucocyte fractions, no bands appeared, although the control brain sample exhibited two bands at 67 and 65 kDa (Fig. 6.4). The results suggested that both mono- and polynuclear leucocytes may not express GAD proteins.

G4913 antibody was raised against KRTLEDNEERMSRLSKVA that corresponds to residues 514–530 of GAD 65, slightly away from the C-terminal. This sequence is common between human and rat; thus, rat brain homogenate was used as a control. Western blot analysis with G4913 showed positive bands at 80 and 30 kDa for mononuclear leucocyte but only 30 kDa for polynuclear leucocyte extracts, whereas the rat brain extract gave bands at 65 and 30 kDa (Fig. 6.4). Results were unexpected; although there was no 65 kDa band for both mono- and polynuclear leucocytes, an 80 kDa band that is larger than expected GAD 65 was observed, and polynuclear leucocyte extract

![Fig. 6.4](image_url)

*Fig. 6.4.* Detection of anti-GAD antibody reactive protein by Western blotting. Lysate from human mononuclear leucocytes or rat brain were separated on polyacrylamide gel, then blotted to PVDF membrane and probed by G5163 anti-GAD 65/67 antibody (a) or G4913 anti-GAD 65 antibody (b). Lane 1, molecular ladder marker; lanes 2–3, mononuclear leucocyte fraction; lanes 4–5, polynuclear leucocyte fraction; lanes 6–7, rat brain extract.
showed a band at 30 kDa. The results suggest, first, that both mono- and polynuclear leucocytes do not express a functional 65 kDa protein; and second, that the 80 kDa band may be the result of multi-splicing specific to mononuclear leucocyte or a fault positive. The small 30 kDa band may be a GAD isoform reported earlier that could be a result of early termination in the biosynthesis of GAD 65.

In addition to Western blot analysis, further study was carried out by using RT-PCR, targeting the antibody recognition region for G5163 and G4913. mRNA isolated from mononuclear leucocytes was converted to cDNA and used as a template for RT-PCR. Two sets of primer pair were designed: primer set 1 was designed to produce a 154 bp fragment that covers G4913 epitope and extends toward 3’ (C-terminal region) just prior to the G5163 epitope. Primer set 2 was designed to produce a 58 bp fragment that includes the G4913 epitope. As a positive control, a primer set for GAPDH was employed. When PCR products were analysed by the Agilent Bioanalyzer, a positive band was observed for the cDNA obtained from mononuclear leucocytes with the primer 2 set only (Fig. 6.5). Both GAPDH and brain samples showed positive bands. The results support the Western blot analysis that GAD 65 is expressed in mononuclear leucocytes, but the C-terminal region may be missing or truncated. In order to confirm the finding, PCR was carried out by using primer set 2 as primers and mononuclear leucocyte cDNA as a template (Fig. 6.5). After the PCR product was integrated into pGEM®-Teasy, the inserted region was confirmed after DNA sequencing as a valid sequence (data not shown).

The location of G4913 epitopes on the gene sequence of GAD 65 was further examined. The exon/intron interface at the end of exon 15 happens to be correlated with the end of epitope for G4913 antibodies. cDNA sequence at the C-terminal of human GAD 65 is shown with PCR primers and epitopes. The epitope for G4913 or G5163 with amino acid above is shown with underline or highlight, respectively. Left primer (>) and right primer (<) are represented for primer set 1 (upper) and set 2 (lower), respectively. The border of exon 15 and 16 is shown by a dotted line.
supports the idea that exon 16 may be omitted during the RNA splicing process. There are two bands with 80 and 30 kDa for mononuclear leucocyte extracts on SDS-PAGE and Western blot analysis. At the present time, there is no evidence whether or not the mRNA isolated corresponds to particular protein bands. Also unknown is the size problem for 80 kDa species: deletion would produce a smaller size protein than the native one, but 80 kDa is larger. There are two possibilities: either some duplication of exons might occur during the splicing process, or post-translational modification might occur. Both possibilities should be considered in the future.

6.5 Taste Signalling

GAD protein has long been known for its localization in few specific organs, including brain, nerves, pancreas and testes. Recently, other localization sites for GAD have been demonstrated in various tissues related to the digestion of food: the stomach, intestine (Fig. 6.7), salivary gland, skin and taste buds (Kuno et al., 2001; Iwahori et al., 2002; Watanabe et al., 2002; Wang et al., 2006; Akamatsu et al., 2007; Ito et al., 2007; Nakamura et al., 2007). Localization is specific, and conventional methods of solubilizing the tissues and by detecting the activity or Western blot analysis might not be sensitive enough to detect the minute amounts of GAD proteins expressed. Recent advancements in RT-PCR and transgenic mouse studies have made the following findings possible.

Mammals have taste buds on their tongue to sense five distinct tastes: sweet, bitter, umami, salt, and sour, where umami is recognized most recently as sodium monoglutamate (MSG) isolated from kelp in 1908 by Kikunae Ikeda (Ajinomoto, 2009; Kurihara, 2009). Taste buds are mainly distributed in papillae, and these are classified into four groups: circumvallate, fungiform, foliate, and filiform. The latter lack taste buds (Fig. 6.8). The first three types of papillae contain about 100 or more taste buds. Taste buds are divided into four types, I–IV, where type I is the connecting cell, type II serves to express taste receptors, type III is for linkage to the nerve, and type IV is the stem cell (Fig. 6.9). There are dedicated receptor proteins for each of the five tastes. For example, sweet materials are considered to bind to sweet-specific receptors, sour materials to sour-specific receptors, and so on to convey the respective taste signals to the brain. It is of interest to understand whether or not each of the type II taste buds express all three receptor types for sweet, bitter, and umami; and whether or not each of the type III taste buds expresses both receptor types for salt and sour tastes. In addition,
the nerve from the brain ends up at the tongue, where the nerve connects only with type III taste buds; thus, a question has been raised as to how the signal from type II travels to the nerve.

Expression of GAD protein was examined on the mouse tongue. Thin sections of mouse tongue were subjected to immunohistochemical study with antibodies specific to GAD, as explained in the previous section. There was positive staining at the area where papillae are located (data not shown). More precise evidence was obtained when GAD 67/GFP transgenic mouse was used; under fluorescent light, there was a single spot having green fluorescence on the tongue where the circumvallate papilla locates (Fig. 6.10). Thin sections with the fluorescent spot showed GAD 67 and GABA positive for antibody reactions, and positive
results for RT-PCR probing for GAD 67. The finding supports the notion that GAD 67 is expressed in mouse taste buds, particularly at circumvallate papilla. RT-PCR studies on the sample taken from the fluorescent spot supports the immunohistochemical results (Fig. 6.11). In the next step, taste cell type that may express GAD 67 was determined. Antibodies specific to cell markers for type II and type III were used. The number of cells reacting with antibodies and those having fluorescence were counted. Results suggested that PGP9.5 and serotonin-positive cell populations overlap the most: it indicates that type III cells express GAD 67 protein (Fig. 6.12) (Nakamura et al., 2005; Nakamura et al., 2007). The results suggest a scheme (Fig. 6.13) as to how the salt (or acid) signal is transmitted via GABA in type III cells.

6.6 Suggestions for Future Research

Studies on the chemistry of glutamate have been relatively subdued recently, partly because of
the lack of high-sensitivity analytical tools for amino acids. Recent advancements in chromatography have made it possible to analyse just above femtomol amounts of amino acids within 8 min. This is the most significant breakthrough for amino acid analysis since Moore-Stein’s original instrument and the Pico-tag® system developed later by Waters. Enzymological studies on GAD should benefit from this analytical technique as more precise assays should now be possible. Researchers are now more optimistic about the ability to conduct single-cell enzymology for GAD expression and characterization.

6.7 Conclusions

The glutamate-metabolizing enzyme GAD produces GABA, an amino acid credited with the role of inhibitory neurotransmitter in the CNS of higher animals. Mammals have two isoforms of GAD, with a slightly different molecular mass encoded in two independent genes. Differences in subcellular localization and characteristics probably reside in the heterogeneity of their N-terminal amino acid residues. Blood cell analyses have shown that mononuclear leucocytes exhibit a GAD-like protein apparently having a truncated C-terminal region that corresponds to exon 16. The finding implicates a possible multi-sPLICing phenomenon that may explain the diverse expression pattern of GAD. Recent studies also show that GAD67 is expressed at the taste bud cells, specifically in the type III cells that contain salt and acid receptors, and also have a physical connection with taste nerve terminals. Since GABA acts as a ligand for GABA₆ receptor, also known as a chloride-ion channel, the possibility is strongly suggested that GABA is a mediator for taste signal transduction. In other words, if GAD activity could be altered by external factors, an altered taste signal might result that may lead, for example, to the salt-enhancement effect. The role of GAD in diabetes is another emerging field for potential exploitation in the development of novel diagnostics and therapeutics for this disease.
6.8 Acknowledgements

We appreciate the long-term collaboration with Dr Kenichi Ito of Ichimaru Pharcos Co.; with Professors Masahito Watanabe and Yoshinori Otsuki of Osaka Medical College; with Professor Kunihiko Obata of Riken; and with Professor Yuchio Yanagawa of Gunma University Graduate School of Medicine. We are also grateful for funds provided by Urakami Food and Food Culture Foundation, Nakano Research Foundation, and the Society for Research on Umami Taste.

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Glutamate Decarboxylase


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Glutamate Decarboxylase


7 Glutaminase

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7.1 Abstract
A key role for phosphate-activated glutaminase has been proposed for the synthesis of neurotransmitter glutamate and γ-aminobutyric acid (GABA) in the brain. This synthesis must be exquisitely regulated because of its potential harmful effects giving rise to excitotoxic damage. It is noteworthy that two glutaminase isozymes coded by different genes are expressed in the brains of mammals. The need for two genes and two isozymes to support the single process of glutamate synthesis is unexplained, and identifying the role of each glutaminase is an important factor in understanding glutamate-mediated neurotransmission. Furthermore, simultaneous expression of glutaminase isoforms has been reported in other mammalian tissues and cells. Validation of glutaminase expression and regulation as therapeutic tools for brain lesions will be considered here.

7.2 Introduction
Phosphate-activated glutaminase (EC 3.5.1.2; GA) is a glutamine (Gln) amidohydrolase, a true hydrolytic enzyme because the acyl chain and ammonium acceptor is water and not another molecule. Some of the main physiological functions of GA include renal ammoniagenesis, nitrogen supply for urea biosynthesis in the liver, synthesis of the excitatory neurotransmitter glutamate (Glu) in the brain and energy supply for the bioenergetics of many normal and transformed cell types (Kovacevic and McGivan, 1983; Curthoys and Watford, 1995). In brain, although several different precursors have been proposed for the synthesis of transmitter Glu, Gln is considered the most important source through GA reaction (Kvamme, 1984). Besides being the major excitatory neurotransmitter in the central nervous system (CNS) (Fonnum, 1984), Glu fulfils many other crucial roles in synaptogenesis, synaptic plasticity, pathogenesis of neuropsychiatric diseases (Conti and Weinberg, 1999), synthesis of GABA and brain energy metabolism (Erecińska and Silver, 1990).

On the other hand, the role of two key genes of Gln metabolism, glutamine synthetase (GS, EC 6.3.1.2) and GA, has also attracted considerable attention in tumour biology, because Gln behaves as a central metabolite for growth and proliferation (Matés et al., 2002). The high rate of glutaminolysis observed in a wide variety of tumours may be essential to maintain their proliferative capacity (Souba, 1993).

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7.3 Mammalian Glutaminase Genes and Transcripts

In humans, the GA family consists of two main members which are encoded by separate genes in different chromosomes (Table 7.1): the Gls gene, located in chromosome 2, encodes isoforms known as kidney (K-type) glutaminases; and the Gls2 gene, located on chromosome 12, codes for liver (L-type) isozymes (Aledo et al., 2000). Orthologous genes have been described in other mammalian species, such as mouse and rat, for Gls (Mock et al., 1989) and Gls2 (Chung-Bok et al., 1997).

7.3.1 Gls gene and transcripts

The human Gls gene spans 82 kb. By comparison with available human cDNAs, the gene was split into 19 exons (Porter et al., 2002). At least two different transcripts arise from this gene: the KGA mRNA formed by joining exons 1-14 and 16-19, and the alternative spliced transcript named glutaminase C (GAC) mRNA which uses only the first 15 exons, omitting exons 16-19 (Porter et al., 2002). The K-type cDNA named GAC was originally isolated from an HT-29 human colon cDNA library (Elgadi et al., 1999) but it has also been reported to be present in rat kidney and porcine kidney cell line (Porter et al., 2002). The K-type cDNA named GAC was originally isolated from an HT-29 human colon cDNA library (Elgadi et al., 1999) but it has also been reported to be present in rat kidney and porcine kidney cell line (Porter et al., 2002). In human tissues, GAC mRNA is expressed predominantly in cardiac muscle and pancreas, appreciably in placenta, kidney and lung, but not in liver and brain (Elgadi et al., 1999).

Table 7.1. Mammalian glutaminase genes and transcripts. Data are for human glutaminases except for the LGA isoform (rat). The expression data have been collected from different mammalian glutaminases.

<table>
<thead>
<tr>
<th>Gene</th>
<th>KGA</th>
<th>GAC</th>
<th>LGA</th>
<th>GAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Gls</td>
<td>Gls</td>
<td>Gls2</td>
<td>Gls2</td>
</tr>
<tr>
<td>Chromosome</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Transcript (nt)</td>
<td>4348</td>
<td>3183</td>
<td>2257</td>
<td>2408</td>
</tr>
<tr>
<td>Number of exons</td>
<td>18</td>
<td>15</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Expression</td>
<td>Ubiquitous in most non-hepatic mammalian tissues</td>
<td>Breast cancer cells, colon, heart, kidney, lung, pancreas, placenta</td>
<td>Liver</td>
<td>Brain, breast cancer cells, pancreas</td>
</tr>
</tbody>
</table>

KGA mRNA was found to be ubiquitous in most non-hepatic human tissues (Aledo et al., 2000). K-type cDNAs have been cloned from different mammalian tissues including kidney (Shapiro et al., 1991), brain (Nagase et al., 1998) and colon cancer cells (Elgadi et al., 1999). Differential expression of multiple K-type mRNAs have been detected in LLC-PK1, FBPase-1 porcine proximal tubule-like cells (Porter et al., 1995), rat lymphocytes (Sarantos et al., 1993), mouse splenocytes (Aledo et al., 1998), Ehrlich ascites tumour cells (Aledo et al., 1994) and human kidney (Elgadi et al., 1999). The length of these transcripts is in the range of 3.0–6.0 kb and can be produced by use of alternative polyadenylation sites (Porter et al., 2002) or by alternative splicing (Elgadi et al., 1999).

In kidney, the KGA enzyme is strongly induced by metabolic acidosis. The presence of a pH-response element (AU-rich sequences) in the 3'-UTR region of the KGA mRNA is responsible for its selective stabilization through binding of the protein α-crystallin/NADPH-quinone reductase (Tang and Curthoys, 2001). However, in tissues other than kidney, expression of the Gls gene is likely to be regulated at the level of transcription. For example, in small intestinal mucosal cells, dexamethasone increased KGA mRNA and specific activity. The increase in message preceded the increase in activity, consistent with de novo RNA synthesis followed by protein synthesis. Glucocorticoids may accelerate intestinal Gln utilization by increasing glutaminase expression, an adaptive response that could provide more energy for mucosal cells in stress states (Sarantos et al., 1992). The primary role of glutaminase in the intestine is...
to regulate enterocyte metabolism. This is because Gln is the main respiratory fuel of enterocytes and GA catalyses the rate-limiting step of Gln degradation (Kong et al., 2000).

In proliferative cells and tumours transcriptional regulation of the Gls gene seems to be operative and associated with cell growth and proliferation. Glutamine is the principal fuel used by lymphocytes, and a fast rise of KGA mRNA levels was observed after mitogenic challenge with endotoxin (Sarantos et al. 1993). Cytokines such as interleukin-1, interleukin-6, tumour necrosis factor-α, and γ-interferon decreased KGA activity, protein content and mRNA levels in cultured human fibroblast (Sarantos et al., 1994). In Ehrlich ascites tumour cells, a long-term regulation for tumour KGA expression during tumour development was deduced, in such a way that maximum activity and mRNA levels were found in mitochondria isolated from cells in the exponential phase of growth, when compared with the stationary phase of growth (Aledo et al., 1994).

With regard to mammalian brain regulation, it is noteworthy that both rat KGA and human KGA and GAC cDNAs contain variable CAG trinucleotide repeats in their 5' ends (Shapiro et al., 1991). However, the CAG repeats are located in the 5'-nontranslated region of the human K-type mRNAs, while in the rat they are located within the coding region. CAG repeat-length polymorphisms are shown to be the cause of various neurodegenerative diseases, including Huntington’s disease, spinocerebellar ataxias and myotonic dystrophy (Singer, 1998).

7.3.2 Gls2 gene and transcripts

The human Gls2 gene has a length greater than 18 kb and is split into 18 exons (Aledo et al., 2000). Exon 1 shares 62.5% similarity, but it codes for 129 amino acids in KGA and only for 61 amino acids in human glutaminase L, accounting for the 67 extra amino acids of KGA protein at the N-terminal. The sequences encoded by exon 1 contain the signals involved in mitochondrial targeting and translocation processes (Shapiro et al., 1991). Interestingly, exon 1 also contains an LXXLL signature motif for human glutaminase L, which might explain the nuclear localization recently demonstrated for glutaminase L in mammalian brain (Olalla et al., 2002). Likewise, exon 18, which codes for the C-terminal region of both proteins, shows the lowest sequence similarity (29.4%). This region of the human L-type GA protein has been demonstrated recently to be involved in the recognition of PDZ (PSD95/Dlg/ZO1 domains)-interaction modules (Márquez et al., 2009). Therefore, the most significant differences between human Gls and Gls2 exons are located in regions involved with organelle targeting and protein–protein interactions, which may help to explain their differential function and regulation. L-type transcripts derived from the Gls2 gene were originally thought to be present in adult liver tissue and absent in extra-hepatic tissues (Smith and Watford, 1990; Curthoys and Watford, 1995). This restricted pattern of expression was generally accepted until quite recently, when results showed clear evidence that expression also occurs in extra-hepatic tissues like brain, pancreas and breast cancer cells (Gomez-Fabre et al., 2000).

Two different L-type GA transcripts of the mammalian Gls2 gene have been characterized so far: a long transcript, named GAB, was isolated as a cDNA clone from ZR75 breast cancer cells, having an ORF of 1806 nucleotides encoding a protein of 602 amino acids (Gomez-Fabre et al., 2000; de la Rosa et al., 2009). A short transcript termed LGA was isolated from rat liver (Smith and Watford, 1990; Chung-Bok et al., 1997). Sequence comparisons showed that human GAB cDNA was very similar (89% identity) to the rat liver LGA cDNA. With regard to the deduced amino acid sequence, human L-type GAB shares a considerable degree of identity (94%) with the rat liver LGA enzyme, but the human enzyme extends over 67 residues at the N-terminal end (Gomez-Fabre et al., 2000). In contrast, the human L-type GAB showed only 68.5% identity with the rat kidney KGA cDNA, a percentage similar to that found between rat liver and kidney GA cDNA species (Chung-Bok et al., 1997).

Glutamine is a major substrate for hepatic gluconeogenesis and urea synthesis (Watford,
Glutaminase

In contrast to KGA, hepatic GA is not affected by changes in acid-base status. Analysis of the rat hepatic GA promoter revealed a cAMP-responsive element (CRE) that may explain the gene’s responsiveness to low insulin and/or high glucagon levels (Chung-Bok et al., 1997). The proximal promoter of the rat Gls2 gene lacks a functional TATA box, but contains hepatocyte nuclear factor recognition elements (HNF-1 and HNF-5), and CAAT-enhancer binding protein (C/EBP) that may be important for its basal expression in liver (Chung-Bok et al., 1997).

The core promoter regions of both the human and rat Gls2 genes do not contain canonical TATA boxes, but do have G+C-rich domains (Pérez-Gómez et al., 2003). This characteristic is also found in the human KGA promoter (Porter et al., 2002). Unlike rat LGA, human Gls2 has a canonical CAAT box and Ras-responsive element-binding transcription factors (RREBs), though it lacks the HNF-5 site present in rat Gls2. Mutagenesis and transient transfections clearly demonstrated that two CAAT boxes play crucial roles in the transcriptional regulation of the human Gls2 gene, both in cells of liver origin (HepG2), and in MCF-7 breast cancer cells (Pérez-Gómez et al., 2003). C/EBPs control cell growth and differentiation, causing growth arrest and inducing cellular differentiation in several adipocyte, granulocyte, and keratinocyte lineages (Darlington et al., 1998). Furthermore, HepG2 hepatoma cells express significantly lower levels of C/EBPα and C/EBPβ than those found in normal terminally differentiated hepatocytes (Friedman et al., 1989). Thus conversion of hepatocytes into proliferating hepatoma cells might require strong down-regulation of C/EBPα and C/EBPβ expression.

The GAB enzyme from ZR75 human breast cancer cells also showed a long-term regulation depending on the cell proliferation state: maximal activities were found at the beginning of the exponential growth phase, with a remarkable decrease at the stationary phase of growth when cell confluence was achieved. These results agree with those previously reported for Ehrlich ascites tumour cells, and indicate a long-term regulation of GA in tumours by differential gene expression (de la Rosa et al., 2009).

7.4 Mammalian Glutaminase Enzymes

Besides differences in molecular structures, the distinct kinetic behaviour has been a hallmark frequently used to distinguish between GA isoforms (Table 7.2). These enzymes are intricately regulated by a number of low-molecular mass effectors. The main kinetic differences have been observed in the dependence of the activator inorganic phosphate (Pi) (low for L-type, high for K-type); the relative affinity for the substrate Gln (higher in K- than in L-types); and the inhibitory effect of Glu, a unique characteristic reported only for K-type isozymes (Kovacevic and McGivan, 1983; Curthoys and Watford, 1995).

Table 7.2. Mammalian glutaminase proteins. The kinetic data have been collected from different mammalian glutaminases.

<table>
<thead>
<tr>
<th>Purification source</th>
<th>KGA</th>
<th>LGA</th>
<th>GAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissues/cells</td>
<td>Cow, mouse, pig, rat</td>
<td>Rat</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>Brain, EATC, kidney</td>
<td>Liver</td>
<td>Recombinant protein expressed in Sf9 insect cells</td>
</tr>
<tr>
<td>Length (aas)</td>
<td>669</td>
<td>535</td>
<td>602</td>
</tr>
<tr>
<td>Native molecular mass (kDa)</td>
<td>90–137</td>
<td>162–170</td>
<td>Not determined</td>
</tr>
<tr>
<td>Glutamine affinity</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Pi dependence</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Glutamate inhibition</td>
<td>Yes</td>
<td>No</td>
<td>Moderate</td>
</tr>
<tr>
<td>Ammonia activation</td>
<td>No</td>
<td>Strong</td>
<td>Very low</td>
</tr>
</tbody>
</table>

4EATC, Ehrlich ascites tumour cells.
7.4.1 Molecular structures and kinetics properties

KGA protein has been purified from pig kidney (Kvamme et al., 1991); pig brain (Svenneby et al., 1973; Nimmo and Tipton, 1980), rat kidney (Curthoys et al., 1976a), rat brain (Haser et al., 1985; Kaneko et al., 1987), cow brain (Chiu and Boeker, 1979) and from Ehrlich ascites tumour cells (Quesada et al., 1988; Segura et al., 1995). These enzymes are not activated by ammonia but inhibited by Glu, have a relatively low $K_{0.5}$ for Gln, a high $K_{0.5}$ for Pi, and their antibodies showed crossed reactions, but not with the LGA isoenzyme (Curthoys et al., 1976b). Purified native KGA in the absence of polyvalent anions is an inactive protomer with an apparent relative molecular mass ranging from 90,000 to 137,000.

Rat renal and brain KGA contain two different peptides of Mr 66,000 and 68,000 that are present in the ratio 3:1 and are produced in vivo from a common precursor (Haser et al., 1985; Perera et al., 1990). In rat kidney, the two mature KGA subunits forming the tetramer are produced by mitochondrial processing of a common 74 kDa precursor (Srinivasan et al., 1995). In contrast, KGA from pig brain has been reported to contain a single polypeptide of Mr 64,000 (Svenneby et al., 1973) or 73,000 (Nimmo and Tipton, 1980). These discrepancies can be ascribed to species-specific differences or, alternatively, they could be explained by proteolytic degradation and the use of different molecular mass standards for calibration of the SDS gels (Quesada et al., 1988; Segura et al., 1995).

In contrast to KGA, the liver LGA enzyme is not inhibited by Glu, has a higher $K_{0.5}$ for Gln, is fully activated at lower concentrations of Pi, and shows activation by ammonia. Rat liver LGA possesses a unique subunit with an Mr of 56,000 and 68,000 that are present in the ratio 3:1 and are produced in vivo from a common precursor (Haser et al., 1985; Perera et al., 1990). These discrepancies can be ascribed to species-specific differences or, alternatively, they could be explained by proteolytic degradation and the use of different molecular mass standards for calibration of the SDS gels (Quesada et al., 1988; Segura et al., 1995).

In contrast to KGA, the liver LGA enzyme is not inhibited by Glu, has a higher $K_{0.5}$ for Gln, is fully activated at lower concentrations of Pi, and shows activation by ammonia. Rat liver LGA possesses a unique subunit with an Mr of 56,000 and 68,000 that are present in the ratio 3:1 and are produced in vivo from a common precursor (Haser et al., 1985; Perera et al., 1990). These discrepancies can be ascribed to species-specific differences or, alternatively, they could be explained by proteolytic degradation and the use of different molecular mass standards for calibration of the SDS gels (Quesada et al., 1988; Segura et al., 1995).

Glutamate is a competitive inhibitor and the relative concentrations of Gln and Glu in glutamatergic terminals (Kvamme et al., 2000) suggest that GA can be strongly inhibited in nerve cells. GAB and LGA enzymes differ markedly in their molecular mass, kinetic characteristics, regulatory properties, tissue distribution and subcellular localization (de la Rosa et al., 2009). Human GAB is 67 amino acids longer than rat liver LGA protein. Nuclear GA exhibited a kinetic behaviour that resembles that of the LGA enzyme with regard to the low Pi concentration.
requirement; however, nuclear GA showed a strong and unexpected inhibition by Glu, a property that is absent in the LGA enzyme (Olalla et al., 2002). Purified human GAB is an allosteric enzyme: measurements of the Gln binding kinetics yielded a sigmoidal curve with a Hill index of 2.7, and \( S_{0.5} \) values of 32 and 64 mM for high and low Pi concentrations, respectively (de la Rosa et al., 2009). Whereas the protein showed a low Pi dependence typical for L-type GA, the enzyme was unexpectedly inhibited by Glu, a kinetic characteristic exclusive of K-type isozymes. At low Pi concentration (5 mM) and suboptimal Gln concentration (20 mM) the IC value for Glu was 50 mM. These data are in the range of Ki values reported for Glu competitive inhibition of K-type enzymes at high Pi concentrations (de la Rosa et al., 2009).

### 7.4.2 Subcellular locations

It is now accepted that at least two GA isoforms, with different kinetic and regulatory properties, are expressed in mammalian brain (Olalla et al., 2002). The regional distribution of both GA transcripts in human brain showed a similar pattern of expression: they were ubiquitously expressed in all regions of the brain examined, with the strongest signal in the cerebral cortex. Furthermore, expression of K- and L-type transcripts in brain was also demonstrated in other mammalian species such as cow, mouse, rabbit and rat. Simultaneous expression of both K- and L-type GA isoforms in the same cell type is more frequent than previously thought: apart from neurons, it has been found in human colorectal tumour cells (Turner and McGivan, 2003), human hepatoma HepG2 cells, medullar blood mononuclear cells from patients suffering from leukaemia, KU812F human myeloid cells and human breast cancer cells MCF7 and ZR-75-1 (Pérez-Gómez et al., 2005).

A crucial difference between GAB and the rest of the GA isoforms so far described is that GAB has been found in extramitochondrial localizations, while KGA and LGA have always been exclusively confined to mitochondria (Erecińska and Silver, 1990; Curthoys and Watford, 1995). In the cerebral cortex, hippocampus, cerebellum and striatum the immunolocalization revealed an L-type GA immunostaining concentrated in the neuronal nuclei (Olalla et al., 2002). The staining was seen in rat and monkey brains. The nuclear role of this L-type GA has yet to be determined, but this novel location has been linked to a potential function as a transcriptional coregulator (Márquez et al., 2006; Szeliga et al., 2009).

Whereas GAB seems to have structural determinants needed for mitochondrial targeting (Gómez-Fabre et al., 2000), it does not possess a discernible classical nuclear localization signal. However, human GAB has other sequence motifs and conserved modules that may be essential for its nuclear import (Márquez et al., 2006). For example, a PDZ-recognition motif would be implicated in GAB specific targeting to selective cellular locations (Olalla et al., 2008; Márquez et al., 2009). A second extra-mitochondrial localization of GAB, apart from cell nuclei, has been found in human polymorphonuclear neutrophils (PMN) (Castell et al., 2004). The presence of L-type GA in these leukocytes was related to their bactericidal action through a Gln-dependent mechanism of superoxide production. These results point towards a cell-specific subcellular location of GAB, increasing the number of potential roles this protein may fulfil (de la Rosa et al., 2009).

### 7.5 Glutaminase Expression in Mammalian Brain

Glutamate is the principal excitatory neurotransmitter in the CNS (Fonnum, 1984; Chapter 25). Glutamate homeostasis is achieved by multiple interactions in the tripartite synapsis where this amino acid is utilized as a releasable transmitter or for general metabolism (Erecińska and Silver, 1990). Considerable attention has been dedicated to study the regulation of Glu release and inactivation, leading to an exhaustive characterization of Glu receptors, which are
among the most studied and best-understood molecules of the nervous system (Watkins and Jane, 2006). However, understanding the mechanisms by which Glu plays its role in diverse processes requires not only the detailed knowledge of the implicated postsynaptic receptors but also of the enzymes involved in the production of Glu in the presynaptic neurons (Márquez et al., 2009).

Despite extensive investigations, the source of neurotransmitter Glu has not been completely resolved. Although there is more than one pathway for Glu synthesis in cells, the vesicular Glu pool in neurons is derived primarily from glutamine through GA. Furthermore, neurotransmitter Glu synthesis has also been shown to be dependent on transamination of α-ketoglutarate involving tricarboxylic acid cycle reactions (Palaiologos et al., 1988; Waagepetersen et al., 2005). Most of the released Glu is taken up by the glial compartment and converted to Gln. A Glu–Gln shuttle between neurons and glial cells has been postulated for neurotransmitter recycling (Hertz, 2004). This cycle assumes that Gln, in turn, is released from the glial cells, taken up by neurons, and converted back to Glu. The model nicely explains the neuronal input of a suitable precursor for Glu synthesis, taking into account the lack of quantitatively important anaplerotic enzymes in neurons (Fig. 7.1). The Glu–Gln cycle was also based on the exclusive localization of glutamine synthetase in astrocytes and GA in neurons (Norenberg and Martinez-Hernández, 1979; Aoki et al., 1991; Laake et al., 1995).

Key issues will be to answer the main question of why two GA isoenzymes are needed in mammalian brain, and also to elucidate the functions that glutaminase L fulfills in nuclei. Potential nuclear functions for glutaminase L include the regulation of Gln/Glu levels or its role as a transcriptional coregulator. In this regard, a recent study has revealed that over-expression of human L-type GA gene in the T98 glioblastoma cell line induced a marked change in the cells' transcriptome correlated with a reversion of their transformed phenotype (Szeliga et al., 2009). Human malignant gliomas have been

![Fig. 7.1. Glutaminase in the glutamate–glutamine cycle between astrocytes and neurons, including pathways followed in energy metabolism. Astrocytes transform Glc to Glc-6P, which is further metabolized to substrates for subsequent transformation into the TCA cycle. The main routes of carbon are from Glc to pyruvate through glycolysis, the anaplerotic pathway at astrocytic oxaloacetate, astrocytic, and neuronal TCA cycles, and the Glu–Gln cycle. Glu is converted to Gln by GS in the astrocytes. Glu can also be degraded in astrocytes, via formation of α-KG. Ac-CoA can also be formed from acetate, but only in astrocytes. Gln is released by the astrocytes, transported into the neurons, and converted to Glu by GA. Ac-CoA, Acetyl coenzyme A; α-KG, alpha-ketoglutarate; GA, glutaminase; GABA, γ-aminobutyric acid; Glc, glucose; Glc-6P, glucose-6Phosphate; GS, Gln synthetase; OAA, oxaloacetate; Pyr, pyruvate; TCA, Tri-carboxylic acid cycle.]

**Glc** → **Ala** → **Glu** → **GS** → **Gln** → **TCA** → **GA** → **Glu** → **NAD+** → **NADH**

Astrocyte

Neuron
shown to express K-type GAs (KGA and GAC isoforms) but with a negligible expression of L-type GA (Szeliga et al., 2005). Taking into account its presence in the nuclei of the cells, it has been speculated that glutaminase L over-expression may contribute to alter the transcriptional programme of glioma cells yielding a less-malignant and more differentiated phenotype, but the concrete molecular mechanisms underpinning this phenotypical change are unknown (Marquez et al., 2009).

The other main neurotransmitter in the CNS – GABA – is produced by L-glutamic acid decarboxylase (EC 4.1.1.15; GAD (Chapter 6)). Interestingly, two GAD isoforms, encoded by different independently regulated genes, are also expressed in brain (Bu et al., 1992). Both forms can synthesize transmitter GABA, but have different roles in the coding of information by GABA-containing neurones and different subcellular localizations (Soghomonian and Martin, 1998). Although it is not fully clear why two GAD enzymes are needed in the brain, recent structural studies are revealing the molecular basis for selective isoform activation and its implication in the regulation of GABA homeostasis (Fenalti et al., 2007). Thus, each of the two main neurotransmitters in the CNS (Glu and GABA) can be synthesized by two isozymes coded by distinct genes, a unique situation to the other neurotransmitters. Production of glutamate and GABA must be a process exquisitely regulated to ensure a proper Glu function (Marquez et al., 2009).

### 7.5.1 Expression of glutaminase L in astrocytes

The presence of functional GA in astrocytes is a phenomenon which is hard to reconcile with the current model of Glu recycling. This does not consider that any GA activity takes place in astrocytes; rather, astrocytes take up most of the neurotransmitter Glu by specific carriers and convert it into Gln through the Gln synthetase enzyme, exclusively expressed in these cells. We have found L-type GA label in rat brain astrocytes from the cerebral cortex by immunocytochemical analyses (Olalla et al., 2008). Although primary cultures of astrocytes displayed GA activity (Yudkoff et al., 1988), immunohistochemical studies have shown KGA immunoreactivity in rat brain astrocytes (Aoki et al., 1991). Recently, KGA, GAC, and LGA mRNAs have been detected in cultured rat astrocytes (Szeliga et al., 2008). However, no GA signal was found in astrocytes from rat cerebellum via post-embedding immunocytochemistry with colloidal gold (Laake et al., 1999). While an in-depth study of GA isoform expression in glial cells is still lacking (further work is in progress), these findings complement early studies describing GA expression in glial cells (Márquez et al., 2009).

The concept of GAs as exclusive neuron-specific enzymes has been challenged by recent findings reporting expression of GA in astrocytes. These novel findings open a new avenue of research on how Glu and Gln may affect the synopsis. As the interactome of brain glutaminases is being investigated, they can be envisioned as multifunctional proteins having additional tasks that seem to be operative in different processes relating to Glu homeostasis, besides their roles in glutamatergic transmission. Some of these novel roles for brain glutaminases may include transcriptional control, neuronal growth and differentiation, and cerebrovascular regulation (Márquez et al., 2009).

On the other hand, the Glu–Gln cycle proposes that astrocytes transform Glu to Gln, which fuels the neuron (Fig. 7.1). The carbon skeletons of oxaloacetate and other intermediates of the tri-carboxylic acid cycle (TCA) can be derived from Gln and Glu as well as glucose (Chambers et al., 2010). The conversion of Gln to Glu occurs in both the extracellular and intracellular compartments in many cell types and organs (Welbourne et al., 2001). Fluxes of nitrogen to and from alanine have been reported to be high in both cultured astrocytes and neurons (Fig. 7.1).
In addition to a role in neuronal Glu synthesis, transamination reactions are critical for Glu utilization by neurons (Erecińska et al., 1994). The expansion of the available pool of oxaloacetate can reflect an increased oxidation of Glu and Gln through a portion of the TCA cycle and enhanced production of oxaloacetate from Glu and Gln carbon (Yudkoff et al., 2000). Interestingly, cerebral Glu and Gln are predominantly localized in glutamatergic neurons and astroglia, respectively (Shen, 2006). Independent studies using in vivo microdialysis and mass spectrometry to determine the labelling of extracellular Glu and Gln have shown that neuronal Glu (through Glu–Gln cycling) is the precursor for 80–90% of glial Gln synthesis. In fact, the reported values of the Glu–Gln neurotransmitter cycle flux in humans and the relationship between Glu–Gln cycle flux and neuronal TCA cycle flux are in relatively good agreement (Shen et al., 2009). In addition to its interaction with the predominantly neuronal Glu, the astroglial Gln is also in exchange with blood, allowing for unlabelled Gln from blood to be transported into brain, and the glial compartment, effectively diluting the astroglial Gln pool (Bröer and Brookes, 2001).

Astrocytes play a critical role in the regulation of brain metabolic responses to activity. Astrocytes prepared from different brain regions all gave rise to the same capacity to enhance glycolysis upon Glu stimulation. Thus, this property appears to be an intrinsic feature of astrocytes and is not specifically linked either to culture conditions or cell origin (Pellerin et al., 2007). Interestingly, neuronal consumption of glucose to maintain their antioxidant status may take priority over the use of glucose to fulfil their bioenergetic requirements, which can be met by other sources. Increasing evidence indicates that neurons can use lactate generated by astrocytes to produce energy and that this is not a uniform process but varies as a result of glutamatergic activation. It is likely that the increased use of lactate by neurons is coupled to an increase in their regeneration of reduced glutathione from glucose (Herrero-Méndez et al., 2009). Moreover, incubation of neurons with a plasma membrane-permeable form of glutathione (glutathione ethyl ester) prevented apoptosis. These results indicate that production of reactive oxygen species (ROS) in neurons might be consequent to the diversion of glucose-6-phosphate from the pentose–phosphate pathway to glycolysis (Bolaños et al., 2008).

### 7.6 State of Art and Perspectives

In most tested experimental models the Gln-responsive genes and the transcription factors involved correspond tightly to the specific effects of Gln in cell proliferation, differentiation and survival, and metabolic functions. Indeed, in addition to the major role played by nuclear factor-kappaB in the anti-inflammatory action of Gln, the stimulatory role of activating protein-1 and the inhibitory role of C/EBP homology binding protein in growth promotion, and the role of c-Myc in cell survival, many other transcription factors are also involved in the action of Gln to regulate apoptosis and intermediary metabolism in different cell types and tissues (Brasset-Lagnel et al., 2009). The influence of Gln on intestinal proteome expression in apoptotic conditions has been studied and evaluated in the human epithelial intestinal cell line HCT-8 (Deniel et al., 2007). By comparing the mitochondrial proteomes of tetracycline-treated and untreated cells with high Myc expression, it has been found that GA expression was increased tenfold in response to Myc. GA levels diminish with decreased Myc expression, and recover on Myc re-induction (Gao et al., 2009). Gln is converted by GA to Glu for further catabolism by the TCA cycle, and previous studies indicate that over-expression of Myc sensitizes human cells to Gln-withdrawal-induced apoptosis (Yuneva et al., 2007).

In both a murine microglia model and the microglial cell line BV-2, inhibition of GA suppresses chromatin condensation and annexin-V labelling. Gln and ammonium enhanced production of ROS. Apoptosis, induced by Gln, was inhibited either by the radical scavenger α-tocopherol or by a nitric
Glutaminase catabolizes Gln for ATP and glutathione synthesis; its reduction affects proliferation and cell death, presumably through depletion of ATP and augmentation of ROS, respectively (Gao et al., 2009). The predominant neurotoxic factor released from both activated microglia and macrophages is Glu. They synthesize Glu via GA from extracellular Gln, and release it through hemicannels of gap junctions, not through Glu transporters. Inhibiting GA and/or gap junctions with specific antagonists effectively suppresses neuronal dysfunction caused by both macrophages and activated microglia. Both the glutaminase inhibitor DON, and the gap junction inhibitor carbenoxolone (CBX), effectively suppressed Glu production and subsequent neurotoxicity. Therefore, these drugs may be clinically valuable as components of a therapeutic strategy to limit the severity or progression of neurological disorders that promote neurodegeneration (Yawata et al., 2008).

Glucose metabolism via pyruvate dehydrogenation provides energy in both neurons and astrocytes and may include gap junction-mediated lactate transport into astrocytes. The importance of glycogen reflects that it selectively supports de novo synthesis of transmitter Glu by combined pyruvate dehydrogenation and carboxylation in astrocytes (Hertz and Gibbs, 2009). Because de novo synthesis from \( \alpha \)-ketoglutarate is minimal in neurons, metabolic studies have suggested that most neurotransmitter Glu is recycled via the Gln–Glu neuron–astrocyte shuttle, involving GA (Fig. 7.1).

The critical role of GA has been supported further by pharmacological experiments in which a near-complete depletion of neuronal Glu was observed after inhibition of Glu synthesis. Mice Gls null mutants failed to feed because of deficits in goal-directed behaviour and died within the first postnatal day with a phenotype of altered respiration (Masson et al., 2006). Gls knockout perturbed glutamatergic neural networks such as brainstem respiratory circuits that depend on robust summation of synaptic inputs, and thus could account for the altered respiration phenotype. Despite the conclusions of many previous studies that GA is the principal source of neurotransmitter Glu, knocking out the Gls gene had a surprisingly subtle effect on glutamatergic synaptic transmission (Masson et al., 2006). This finding implies that other Glu-synthetic pathways are operative but, surprisingly, glutaminase L was not considered as a plausible compensatory source of transmitter (Masson et al., 2006). Besides Glu synthesis by LGA/GAB, the persistence of glutamatergic synaptic transmission in GA null mutants could be accounted for by other Glu-synthetic pathways such as transamination of \( \alpha \)-ketoglutarate with alanine, branched-chain amino acids, or lysine (Hertz, 2004), or by direct reuptake of Glu into neurons (Waagepetersen et al., 2005). In spite of alternative synthetic pathways allowing temporary postnatal survival, the mice breathe poorly and die, indicating that KGA/GAC function is essential for survival (Masson et al., 2006).

7.7 Conclusions

Glutaminase converts Gln to Glu, which is further catabolized through the TCA cycle for the production of ATP, or serves as a substrate for glutathione synthesis. Under pathological conditions, Gln and ammonium are elevated globally in the brain. The Trojan horse hypothesis of L-Gln toxicity assumes that intramitochondrial hydrolysis of L-Gln enhances ammonium locally and leads to mitochondrial dysfunction (Albrecht and Norenberg, 2006). Exposure of brain cells to L-Gln can promote apoptosis (Fig. 7.2). Glu catabolism through GA is critical for cell proliferation induced by Myc, and protection against ROS generated by enhanced mitochondrial function in response to c-Myc (Lora et al., 2004). Intriguingly, elevated levels of c-Myc protein in human cancer
Fig. 7.2. Glutaminase and ammonium are involved in glutamine-dependent apoptosis in brain cells. Gln is accumulated in mitochondria and metabolized to Glu and ammonium. Ammonium contributes to the generation of reactive oxygen species (ROS) by blocking respiratory chain in mitochondria. The Trojan horse hypothesis of Gln toxicity assumes that accumulation and hydrolysis of Gln in mitochondria lead to toxic concentrations of ammonium. Because of the small volume of mitochondria, ammonium interacts rapidly with mitochondrial enzymes involved in energy metabolism leading to production of ROS and mitochondrial membrane depolarization. The efflux of cytochrome c contributes to the induction of caspase-3 and triggers apoptosis. GA, glutaminase; Gln, glutamine; Glu, glutamate; MMP, mitochondrial membrane potential; ROS, reactive oxygen species. Correspond to levels of GA, which are not increased in the accompanying normal tissue from the same patients (Gao et al., 2009). Taken together, the data indicate that GA is a promising pharmacotherapeutic target. The fact that both GA activity and Glu levels are reduced in adulthood suggests that a reduction in GA, and the ensuing decrease in Glu levels, are plausible explanations for the phenotypes observed in heterozygous Gls gene knockout mice, and argues against the possibility that these effects are due to neurodevelopmental alterations unrelated to GA or to Glu neurotransmission (Gaisler-Salomon et al., 2009).

7.8 Acknowledgements

This work was supported by Excellence Grant CVI-1543 from the regional Andalusian government (Junta de Andalucía), Grant SAF2007-61953 from the Ministry of Education and Science of Spain, and Grant RD06/1012 of the RTA RETICS network from the Spanish Health Institute Carlos III.

References


8 D-Serine and Serine Racemase in the Retina

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8.1 Abstract

Recent data suggest that D-serine is the endogenous ligand for the ‘glycine’ binding site of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. This chapter reviews evidence that this is the case, not only in brain, but also in the retina. Information is provided documenting the location of D-serine and serine racemase, in retinal Müller glial cells. The transport of D-serine by these cells is discussed. New evidence demonstrating that D-serine and serine racemase are present in retinal neurons is provided, along with data evaluating the effects of excess D-serine on retinal neuronal viability.

8.2 Introduction

The retina is the light-sensitive tissue of the eyeball. It develops from two layers of the invaginated optic vesicle. Figure 8.1 provides a photomicrograph of the mammalian retina. The outermost lamina becomes a single layer of epithelial cells, termed the retinal pigment epithelial cell layer. It is sandwiched between a vascular bed, the choriocapillaris, and the inner retina, frequently referred to as the neural retina. The neural retina is stratified into nuclear layers, which harbour cell bodies and plexiform layers that represent synapses between cells. There are five broad classes of neuronal cells and at least three types of glial cells. The cell bodies of retinal photoreceptors (rods and cones), specialized to capture photons of light, occupy the outer nuclear layer and their processes comprise the inner and outer segments. In the outer plexiform layer, photoreceptor cells synapse with first-order neurons of the visual system, the bipolar cells whose cell bodies are found in the inner nuclear layer. Other neurons in the inner nuclear layer include horizontal cells that modulate the activity of bipolar cells and amacrine cells that modulate the synaptic transmission from bipolar cells to ganglion cells. Synaptic connections to ganglion cells are present in the inner plexiform layer. Ganglion cells are the second-order neurons of the visual pathway and their cell bodies occupy the innermost cellular layer (ganglion cell layer) of the retina. Their axons form the optic nerve and are visualized as the nerve fibre layer in retinal histological sections. The cell bodies of some amacrine cells can also be found in the ganglion cell layer. The major glial cell of the retina,
Fig. 8.1. Haematoxylin and eosin-stained JB-4 plastic embedded section of C57Bl/6 adult mouse retina. Note the well-organized, laminar appearance of the retina, distinguished by its orderly nuclear and plexiform (synaptic) layers. Light passes through the thickness of the retina to strike the outer segments of photoreceptor cells initiating a conversion of the photic stimulus into a neuronal stimulus. The impulse travels from the photoreceptor to bipolar cells of the inner nuclear layer, and then to ganglion cells whose axons form the nerve fibre layer that gives rise to the optic nerve.

The Müller cell, is radially oriented and its end-feet form the inner and outer retinal limiting membranes. Müller cells subserve many of the metabolic, ionic, and extracellular buffering requirements of adjacent neurons. Müller cells play a key role in transporting substances from the extracellular milieu into the cell and vice versa. Other glial cell types in the retina are astrocytes and microglia.

In the retina, L-glutamate serves as the major excitatory transmitter; it is used in the retinal forward transmission of visual signals by photoreceptors, bipolar, and ganglion cells (Massey and Miller, 1987; Massey and Miller, 1990). Receptors for glutamate are broadly divided into the two categories ionotropic and metabotropic, depending upon whether the channel is gated directly or indirectly. The ionotropic glutamate receptors are further subdivided into three classes, NMDA, AMPA, and kainate, based upon the synthetic agonists that activate them (N-methyl-D-aspartate, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, and kainate).

The focus of this review, the NMDA subtype of glutamate receptor, is arguably one of the most important synaptic receptors in the CNS. This chapter reviews recent evidence that D-serine is an endogenous ligand for the NMDA receptor in the retina. It provides an overview of data showing that both retinal glial cells and retinal neurons express D-serine and serine racemase, the enzyme that converts L-serine to D-serine. Mechanisms by
which cells take up D-serine are discussed, as well as the possible effects of excessive levels of D-serine on retinal neuronal viability.

### 8.3 NMDA Receptor and D-Serine as a Co-agonist

NMDA receptors are heterotetrameric protein complexes, typically comprising two NR1 and two glutamate-binding NR2 subunits. NMDA receptor variety is provided in large part by the four different NR2 subunits (NR2A-D) (Monyer et al., 1994). In addition to the binding of glutamate, NMDA receptors require the binding of a co-agonist on the NR1 subunit to exert physiological actions. The obligatory co-agonist was identified originally as glycine (Johnson and Ascher, 1987). Indeed, this 'glycine binding' site must be occupied to allow glutamate to open the ion channel of the NMDA receptor. Subsequent studies revealed that other compounds could serve as a glutamate co-agonist; indeed some were more effective than glycine. Included among these compounds was D-serine (Kemp and Leeson, 1993; Czepita et al., 1996).

The observation that D-serine could serve as a co-agonist of the NMDA receptor in vitro was intriguing. While it was well known that bacteria and many invertebrate species use both D- and L-enantiomers of amino acids for cellular functions, it was generally believed that higher organisms had a more restricted stereospecificity and were confined to the use of L-amino acids. D-Amino acids detected in vertebrates were presumed to arise from intestinal flora or ingested material (Corrigan, 1969). Hashimoto et al. (1992) asked whether D-serine was present in the brain, and found that nearly 25% of the total brain serum was the D-enantiomer. These investigators were the first to suggest that D-serine may be an endogenous, co-agonist for the NMDA receptor. Mc Bain et al., (1989) reported that D-serine was much more effective than L-serine as a co-agonist of the binding site.

An important additional indicator that D-serine might play a key role in NMDA receptor activation came in the form of discovery of the enzyme that catalyses the conversion of L-serine to D-serine (Wolosker et al., 1999a,b). This enzyme, serine racemase, is a 37 kDa protein and was isolated from rat brain. In addition, the enzyme D-amino acid oxidase (D-AAOX) is also present in the brain and represents the principal pathway of serine degradation. D-serine has been studied in other regions of the brain including spinal cord and cerebellum. Levels of D-serine are relatively high in the neonatal rat cerebellum, though levels decrease as the animals develop (Rabacchi et al., 1992).

### 8.4 D-Serine in the Retina

For almost a decade, studies related to D-serine centred on its presence and role in the brain. In 2003, however, Stevens and colleagues investigated D-serine and serine racemase in the retina for the first time. Their findings implicate D-serine as an NMDA receptor co-agonist in the mammalian and non-mammalian retina (Stevens et al., 2003). D-serine and serine racemase were analysed comprehensively in rats and larval tiger salamanders using immunohistochemistry, immunoblotting, analytical chemistry (HPLC), and electrophysiology. D-serine and serine racemase were localized to Müller glial cells and astrocytes. Exogenous D-serine enhanced NMDA receptor responses and modulated light-evoked activity in retinal ganglion cells. It is noteworthy that several years prior to the report by Stevens, Harsanyi and colleagues (1996) were experimenting with NMDA receptor activation in a fish model and used D-serine in their superfusate. They detected horizontal cell activity in the presence of D-serine, but did not explore this as an NMDA receptor co-agonist. Elegant analytical studies of D-serine levels in the tiger salamander retina complemented the findings of Stevens. O’Brien et al. (2003) used microdialysis-capillary electrophoresis with laser-induced fluorescence detection in a sheath flow detection cell to analyse D-serine levels in their model system. Levels of D-serine in retinal homogenates of larval tiger salamander retinas averaged 11.9 nmol mg$^{-1}$ protein. The investigators were also able to study the amount of D-serine released from the isolated retinal preparations (O’Brien et al., 2004). These early studies of D-serine in the retina have been
reviewed by Miller (2004). In addition to analyses in rat and salamander, D-serine has been detected in human (fetal) retina (Diaz et al., 2007) and in mouse retina (Dun et al., 2008).

### 8.5 Mechanisms of D-Serine Uptake in the Retina

The initial studies of mechanisms used by cells to transport D-serine were performed in the brain and investigated the potential role of a variety of neutral amino acid transporters (ASCT1, ASCT2, system A, system L). Studies by Hayashi et al. (1997) and by Ribeiro et al. (2002) provided functional evidence that D-serine uptake is mediated by the ASC type of transporters. No functional experiments were performed to distinguish between ASCT1 and ASCT2 transporters, nor were molecular analyses performed to determine which transporter mediated D-serine transport.

Using whole-retina preparations from the larval tiger salamander, O'Brien et al. (2005) examined whether neutral amino acid transporters mediate D-serine uptake. They argued that an amino acid exchanger that can take up and release D-serine would offer a mechanism for regulation of D-serine levels in the retina. In experiments using capillary electrophoresis, D-serine uptake was shown to be dependent upon Na⁺, thus ruling out the Na⁺-independent asc-1 transporter (SLC7A10, a transporter system for small neutral L- and D-amino acids), as a transporter for D-serine in retina. The observation that Na⁺-dependent uptake of D-serine was inhibited by L-alanine, L-serine, and L-cysteine was consistent with transport mediated by the ASC types of neutral amino acid transporters. The ASCT1 (SLC1A4, alias: SATT) and ASCT2 (SLC1A5, alias: ATB°) transport systems are Na⁺-dependent and have high affinity for alanine, serine, and cysteine. They exhibit distinct substrate selectivity: in addition to the common substrates of ASCT transporters, ASCT2 also accepts glutamine and asparagine as high affinity substrates, whereas ASCT1 does not (Kanai and Hediger, 2003).

The studies by O'Brien et al. (2005) did not distinguish between the ASCT1 and the ASCT2 transporters, nor did they rule out transport by a unique amino acid transporter ATB°, which is energized by Na⁺- and Cl⁻-gradients and membrane potential. ATB° (SLC6A14) has broad substrate specificity and concentrative ability and recognizes neutral as well as cationic amino acids. Both ASCT2 and ATB° have been shown to mediate D-serine uptake (Hatanaka et al., 2002; Thongsong et al., 2005). Immunohistochemical analysis of ocular tissues, using an antibody specific for ATB°, provided evidence that this transporter is present in the mammalian retina (Hatanaka et al., 2004). Given our familiarity with analysis of transporter systems and our interest in retinal Müller cells, we analysed the mechanism(s) of D-serine uptake in a rat Müller cell line and in primary cultures of Müller cells isolated from mouse retina (Dun et al., 2007a). We used functional methods to distinguish among the three likely transporter candidates (ASCT1, ASCT2, ATB°), and used molecular methods to establish the identity of the transporter likely to be responsible for the uptake and/or efflux of D-serine in Müller cells. We detected serine racemase mRNA and protein in rMC-1 and primary mouse Müller cells. We examined the uptake of D-serine uptake in the cell line and in the primary Müller cells. We found that D-serine uptake in rMC-1 cells is absolutely dependent on Na⁺ and that Cl⁻ is not obligatory for the process (Fig. 8.2a). The Na⁺-activation kinetics were analysed to determine the Na⁺: D-serine stoichiometry. The dependence of D-serine uptake on Na⁺ concentration displayed a hyperbolic relationship and the analysis of the data by the Hill equation yielded a value of 0.8 ± 0.03 for the Hill coefficient, indicating an Na⁺: D-serine stoichiometry of 1:1 (Fig. 8.2b). We confirmed the ion dependence of D-serine uptake in primary Müller cells. The uptake in these cells exhibited ion dependence that was similar to that observed in rMC-1 cells (Fig. 8.2c).

We examined the substrate specificity of the transport system responsible for D-serine uptake in retinal Müller cells and found that uptake of D-serine was inhibited markedly by L-alanine, L-serine, L-cysteine, L-asparagine, and L-glutamine. The anionic amino acids (L-aspartate and L-glutamate), and cationic amino acids (L-lysine and L-arginine) failed to inhibit the uptake of D-serine, showing...
Fig. 8.2. Ion dependence of D-serine uptake in retinal Müller cells. (a) Time course and ion dependence of D-serine uptake in rMC-1 cells. Uptake of [3H] D-serine (0.1 μM) in rMC-1 cells incubated at various times in NaCl-, Na-gluconate-, and NMDG-Cl-containing uptake buffers. (b) Na⁺-activation kinetics of D-serine uptake in rMC-1 cells. Uptake of [3H] D-serine (0.1 μM) was determined following a 30 min incubation in uptake medium containing increasing concentrations of Na⁺ (0–140 mM). The concentration of Cl⁻ was kept constant at 140 mM (inset, Hill plot). (c) Ion dependence of D-serine uptake in primary Müller cells; uptake of [3H] D-serine (0.1 μM) was determined following a 30 min incubation in NaCl-, Na-gluconate- and NMDG-Cl-containing uptake medium. Values are means ± SE for three determinations from two independent experiments. (*) Significantly different from NaCl value, p < 0.05.) (Adapted from Dun et al., 2007a, and used with permission.)
that the transport process that mediates the uptake of D-serine in Müller cells is specific for neutral amino acids and excludes anionic and cationic amino acids. Our studies ruled out the participation of ATB° in the uptake process, leaving two family members of the ASC transporter group as likely mediators of D-serine uptake in Müller cells. The mRNAs encoding ASCT1 and ASCT2 were both expressed in the rMC-1 and primary Müller cells. To distinguish between the two isoforms, we used a battery of known substrates for ASCT1 and ASCT2. Owing to the finding that uptake of D-serine was inhibited by not only alanine, serine, and cysteine, but also by asparagine and glutamine, substrates specific for ASCT2, ASCT2 emerged as the mediator of D-serine transport in Müller cells. Our immunodetection analyses confirmed the presence of ASCT2 in neural retinal and in primary Müller cells. Our studies with the *Xenopus laevis* oocyte heterologous expression system and the cloned human ASCT2 showed that ASCT2 was able to mediate not only the uptake, but also the efflux, of D-serine, a distinct characteristic of ASC-type transporters. Thus, these findings provided definitive evidence that ASCT2 is the transporter for D-serine in retinal Müller cells.

**8.6 D-Serine and Serine Racemase in Retinal Neurons**

The early analyses of D-serine and serine racemase were performed in the brain, and immunohistochemical studies localized D-serine exclusively to type II astrocytes in the brain (Schell et al., 1995). Shortly thereafter, serine racemase was shown to be enriched in cortical astrocytes (Wolosker et al., 1999a,b). These findings were supportive of the proposed dynamic role of glial cells in influencing neuronal activity, particularly as a third element of the chemical synapse. In the retina, the initial reports of D-serine localized it and serine racemase to Müller glial cells. This location fit well with postulated mechanisms of glial to neuron signalling. Newman speculated that release of D-serine by Müller cells could facilitate NMDA synaptic transmission by binding to the NMDA co-agonist site (Newman, 2004).

Interestingly, two years after the discovery of serine racemase, Yasuda and colleagues published a brief report that D-serine was present not only in glial cells, but also in some neurons of the rat brain (Yasuda et al., 2001). Using highly sensitive immunohistochemical methods, they detected D-serine at low levels in pyramidal neurons of the cerebral cortex and in neurons of the nucleus of trapezoid body. A few years later, other groups examined the notion that D-serine is localized exclusively to glial cells more comprehensively. Investigators from Wolosker's lab provided unambiguous evidence for the presence of D-serine and serine racemase in neurons as well as in glia (Kartvelishvily et al., 2006). Using neurons purified from the embryonic cerebral cortex, they found that virtually all of these cells were immunoreactive for serine racemase. They also detected D-serine in their neuronal cell cultures. At about the same time, studies from Hashimoto's lab were published, reporting the expression of serine racemase in primary cultures of neurons from rat brain (Yoshikawa et al., 2006). High levels of D-serine were reported also during the first three weeks of postnatal development in glial cells of rat vestibular nuclei (Puyal et al., 2006). This time period corresponds to an intense period of plasticity and synaptogenesis. Interestingly, upon maturation, D-serine levels were low and mainly localized in neuronal cell bodies and dendrites. The reports that D-serine and serine racemase are present in neurons isolated from the brain, coupled with the finding that the expression of D-serine may be developmentally regulated, led us to investigate the neuronal expression of D-serine and its synthesizing enzyme in the retina, specifically focusing on expression in retinal ganglion cells (Dun et al., 2008). It is well established that the processes of Müller cells span much of the retinal thickness and ensheath the neurons of the retina, including the ganglion cells. Our studies demonstrated robust serine racemase expression and appreciable D-serine content in the ganglion cell layer, as well as in other neurons of the intact retina and in the purified cultures of ganglion cells.

As shown in Fig. 8.3, serine racemase was detected in abundance in the ganglion cell layer of mouse retina. Serine racemase also localized to the inner segments of photoreceptor
cells and the inner limiting membrane (Fig. 8.3b). It was detected in some of the cells of the inner nuclear layer. Incubation of sections with the antibody that had been pre-incubated with the serine racemase blocking peptide (Santa Cruz Corp.) yielded minimal staining. Our data were acquired in retinas harvested from three-week-old mice. When the pattern of immunostaining for serine racemase was examined in adult mouse retina (18-week-old), the expression levels were considerably less. Figure 8.4f shows a photomicrograph of an
adult retina in which the immunodetection procedure was performed exactly as for the retinas of the young mice shown in Figs 8.3b, c, and e panels (including exposure time in capturing the image). There was minimal labelling in the ganglion cell layer of the adult retina. There was a very faint immunopositive reaction associated with some of the cells in the inner nuclear layer, but it was reduced dramatically compared with that observed in the three-week-old retinas. These data suggested that there is a developmental regulation of serine racemase expression in retina such that the protein levels are much higher in neonatal and young mice compared with mature animals. This was investigated further using a more quantitative method (Western blotting, Fig. 8.3g). For these experiments, we isolated retinal proteins from very young (1 day) and very old (1 year) mouse retinas and subjected them to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose membranes and incubation with the antibody against serine racemase, the protein was detected at robust levels in the neural retina from 1-day-old mice, but the level of detection was reduced in the 1-year-old mouse. When data were normalized using β-actin as a loading control and quantified by densitometry, the ratio of serine racemase to β-actin at 1 day was 0.7 and at 1 year was 0.4 (Fig. 8.3g). Thus our studies suggested that serine racemase levels are also developmentally regulated in the retina, with high levels detected during the early postnatal period, but diminishing considerably as the retina matures.

In performing these studies, we recognized that the close proximity of glial processes with neuronal processes complicates the interpretation of immunohistochemical data. Therefore, in addition to studying D-serine and serine racemase gene and protein expression in intact tissue, we used an immunopanning procedure to isolate ganglion cells from the neonatal mouse retina and investigated serine racemase expression and D-serine content in these neuronal cells.

Proteins were isolated from these immunopanned ganglion cells, subjected to SDS-PAGE, transferred to membranes, and incubated with commercially available monoclonal or polyclonal antibodies against serine racemase (Figs 8.4a and 8.4b, respectively.) Primary Müller cells known to express serine racemase served as a positive control. A band of ~38 kDa, consistent with the published size of the monomeric form of serine racemase, was detected in both Müller cells and RGCs using both antibodies. We confirmed the neuronal phenotype of the isolated cells immunocytochemically using NF-L (Fig. 8.4c, note the intense fluorescence of the cell body and the axon (arrowhead)). These cells were immunopositive for serine racemase using two different antibodies (Figs 8.4d, 8.4e). Figure 8.4f depicts immunocytochemical analysis of serine racemase in the neuronal processes, which can be better visualized if the exposure time of the camera is increased; the cell body labelling is saturated (extremely intense fluorescence), axon is more visible (arrowhead). These primary ganglion cells are positive for the ganglion cell marker Thy 1.2 (Fig. 8.4g), but are negative for the amacrine cell marker ChAT (choline acetyltransferase, Fig. 8.4h).

This was the first report of neuronal expression of D-serine and serine racemase in the vertebrate retina and suggested an important contribution of neuronal D-serine during retinal development. Others have confirmed our findings. Takayasu et al. (2008) used the rat retina as a model system and employed in situ hybridization techniques to detect serine racemase mRNA. They found expression in ganglion cells, amacrine cells, bipolar cells, horizontal cells, and Müller cells of the retina as well as in the astrocytes of the optic nerve head and the lamina cribrosa. They interpreted their findings to suggest that both neuron- and glia-derived D-serine could modulate neurotransmission via the glycine site of the N-methyl-D-aspartate receptors in the retina.

### 8.7 Role of D-Serine in the Retina

Recent elegant studies have provided compelling evidence that endogenous D-serine plays an essential role as a co-agonist for NMDA receptors, allowing it to contribute to light-evoked responses of retinal ganglion cells (Gustafson et al., 2007). In subsequent studies analysing the retina of the tiger salamander
Fig. 8.4. Immunodetection of serine racemase in primary mouse ganglion cells. Primary ganglion cells (1°GC) were isolated by immunopanning from 1–3-day-old mouse retinas and were used for immunoblotting (a, b) or immunocytochemistry (c–h). Proteins were isolated, subjected to SDS-PAGE, transferred to membranes, and incubated with commercially available monoclonal (a) or polyclonal (b) antibodies against serine racemase (SR). Primary Müller cells (1°MC) known to express serine racemase were used as positive controls. A band of ~38 kDa, consistent with the published size of the monomeric form of serine racemase, was detected in both Müller cells and ganglion cells using the two antibodies. (c) Immunocytochemical detection of NF-L, a marker for neurons, in the cultured ganglion cells. Note the intense fluorescence of the cell body and the axon (arrowhead). (d) Immunocytochemical detection of serine racemase in ganglion cells using the Alexa Fluor® 488 secondary antibody. The intense fluorescence reflects the positive reaction in the cell bodies. (e) Immunocytochemical detection of serine racemase in ganglion cells using the Alexa Fluor® 555 for serine racemase; labelling of processes is minimal. (f) Neuronal processes of ganglion cells can be visualized well if the exposure time of the camera is increased; the cell body labelling is saturated (extremely intense fluorescence), axon is more visible (arrowhead). (g) Primary ganglion cells are positive for Thy 1.2 as indicated by fluorescence; arrow points to the axon. (h) Incubation of the cells with an antibody to detect choline acetyltransferase (ChAT), an amacrine cell marker, revealed no positive labelling (which would fluoresce if present). 1°, primary; MC, Müller cell; GC, ganglion cell; arrow heads point to axons; NF-L, neurofilament light polypeptide; SR, serine racemase, SR-En, serine racemase immunofluorescence with increased exposure time. (Adapted from Dun et al., 2008, with permission; original figure shows fluorescent images in colour.)
(Ambystoma tigrinum), this same team of investigators blocked the synthesis of D-serine by exposing the retina to phenazine ethosulphate and validated the changes in the tissue levels of D-serine using capillary electrophoresis methods. They found that phenazine ethosulphate exposure decreased D-serine levels in the retina by about 50% and significantly reduced the NMDA receptor contribution to light responses of the inner retina. Theirs was the first report of a linkage between D-serine synthesis and NMDA receptor activity in the vertebrate retina (Stevens et al., 2010). Daniels and Baldridge (2010) have shown that D-serine enhances glutamate-induced calcium responses in immunopanned retinal ganglion cells. Endogenous D-serine degradation by treatment with D-amino acid oxidase caused ∼45% decrease in NMDA-induced responses, which were reversible by co-application of D-serine. Interestingly, in their in vitro model, D-serine and glycine were equally effective in enhancing glutamatergic calcium responses. Endogenous D-serine contributes to NMDAR activation in retinal wholemounts and some but not all retinal ganglion cells may experience saturating levels of D-serine or glycine.

8.8 Role of D-Serine and Serine Racemase in Neuronal Cell Death

It is well documented that glutamate, under certain circumstances, can contribute to disease. Excessive amounts of glutamate are highly toxic to neurons. Brief exposure to high concentrations of glutamate to cells in tissue culture can kill many neurons by an action called glutamate excitotoxicity. In many cell types, glutamate excitotoxicity is thought to result predominately from excessive inflow of Ca²⁺ through NMDA-type channels. High concentrations of intracellular Ca²⁺ may activate Ca²⁺-dependent proteases and phospholipases and may produce free radicals that are toxic to cells. Excessive glutamate has been implicated in various diseases such as stroke and Huntington’s disease, and where the retina is involved, diabetic retinopathy. The possible role of glutamate (and other excitotoxic amino acids such as homocysteine) in neuronal cell death associated with diabetic retinopathy has prompted a number of studies in our laboratory, including investigation of D-serine in the retina. Sasabe et al. (2007) published data showing that D-serine plays a key role in the glutamate toxicity associated with amyotrophic lateral sclerosis.

We were interested in determining whether D-serine would augment glutamate-induced toxicity in primary ganglion cells. To examine this, we isolated ganglion cells using our published method (Dun et al., 2007b) and exposed the cells to varying concentrations of D-serine [1, 25, or 50 µM] in the presence/absence of glutamate [10 or 20 µM]. Cells were treated for 18 h and then analysed in situ by detection of DNA Fragmentation (TUNEL assay). The TUNEL assay was performed using the ApopTAG® Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Temecula, California, USA) following our published method (Dun et al., 2007b). Cells were viewed by epifluorescence using a Zeiss Axioplan-2 microscope, equipped with the axiovision programme, and an HRM camera. Cells in five randomly chosen fields for each coverslip were counted for positive fluorescent indicating apoptosis. Three coverslips were examined for each treatment group. The data from these experiments are shown in Fig. 8.5. We found that exposure of ganglion cells to D-serine decreased their viability, especially at 25 and 50 µM concentrations. Exposing ganglion cells to higher levels of glutamate [20 µM] induced significant death of primary ganglion cells. Given that ganglion cell death is a feature of diabetic retinopathy, we investigated the levels of serine racemase in retinas of the diabetic Ins2Δm1 congenic mouse. We found that the levels of this enzyme were similar in mice 8 and 18 weeks post-onset of diabetes (unpublished observations) to those levels determined for non-diabetic control animals. Whether alterations in serine racemase are detectable in other models of retinal degeneration remains to be investigated.

Earlier studies from the Akaike laboratory examined the contribution of endogenous glycine site NMDA agonists in the cell death of retinal neurons (Hama et al., 2006) and neurons of the brain (Katsuki et al., 2004). In studies of the retina, the investigators quantified surviving retinal ganglion cells following injections into the superior colliculus of a
Fig. 8.5. Effects of D-serine on retinal ganglion cell viability. Retinal ganglion cells were isolated from neonatal mice by immunopanning. The primary neuronal cultures were maintained for several days in neurobasal media and then exposed to varying concentrations of D-serine, or D-serine and L-glutamate. Cell viability was determined using the TUNEL assay and the data were expressed as the percentage of cells that were alive compared to all cells in the field. For each experiment, at least five fields were examined and the experiments were performed three times. (*Significantly different from control, p < 0.05.)

competitive antagonist of the glycine-binding site following administration of 200 nM NMDA. They showed that blocking the glycine site rescued ganglion cells from excitotoxic death. In addition, they found that retinas exposed to 20 nM NMDA in the presence of 10 nM glycine or 10 nM D-serine increased the death of ganglion cells. They concluded that ganglion cell death under excitotoxic conditions depended upon the levels of glycine and D-serine.

8.9 Conclusions

The NDMA sub-type of glutamate receptor plays a key role in neurotransmission in the retina as well as in the brain. Though for years
it was thought that glycine was the endogenous agonist required for activation of the receptor, evidence over the past decade indicates that D-serine is at least as potent an activator of this site as glycine. Indeed, recent work on the retina suggests that D-serine is the endogenous co-agonist of the receptor. While originally thought to be limited to glial cells of the retina, it appears as if D-serine and its synthesizing enzyme serine racemase are present in retinal neurons also, including the ganglion cells. Future analyses of D-serine are likely to uncover the contributions of this NMDA receptor ligand to retinal disease.

8.10 Acknowledgements

This work was supported by NIH R01 EY014560 to S.B.S.

References


9 Tryptophan Hydroxylase

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9.1 Abstract

Tryptophan hydroxylases (TPH1 and TPH2) are the rate-limiting enzymes in the serotonin biosynthesis and have the most restricted tissue distribution of the aromatic amino acid hydroxylases. TPH1 is mainly found in the pineal gland and enterochromaffin cells of the gut, whereas TPH2 is the main TPH in the brain. Here we summarize the structure, reaction mechanism, and physiological effect of both TPHs. Since much of the research was performed before the identification of TPH2, it has been difficult to distinguish what is known for each of the different TPH enzymes. In this review we try to clarify this aspect. In addition we discuss the regulation of the enzymes, both in vitro and in vivo, and how to use this information in drug treatment. Finally we discuss the implications of TPH dysfunction in human health, based on studies both in patient groups and knockout mice.

9.2 Introduction

The basic processes involved in serotonin biosynthesis, degradation, storage, uptake and release have been well characterized and are schematically shown in Fig. 9.1. In brief, serotonergic neurotransmission depends on three major steps:

1. Synthesis and degradation of serotonin, where the main enzymes are tryptophan hydroxylase (TPH) and monoamine oxidase A (MAO A).
2. Serotonin reuptake from the synaptic cleft, mediated by the monoamine transporters.
3. Activity of serotonin receptors. In the pineal glands, serotonin is further processed to melatonin. This chapter will focus on TPH, the rate-limiting enzyme in the synthesis of serotonin, with emphasis on its role in the mammalian nervous system.

9.3 General Properties

TPH [E.C.1.14.16.4] is a member of the aromatic amino acid hydroxylase enzyme superfamily (AAAH), together with phenylalanine hydroxylase (PAH) [E.C. 1.14.16.1] and tyrosine hydroxylase (TH) [E.C.1.14.16.2], which hydroxylate their respective amino acid substrates in the presence of tetrahydrobiopterin (BH₄), molecular oxygen (O₂) and iron (Fig. 9.2). They are all closely related, i.e. having approximately 60% overall DNA sequence
Two distinct TPH genes code for their respective enzymes, TPH1 and TPH2. It was only in 2003 that the latter gene was reported (Walther et al., 2003), although many researchers had already distinguished between brain and peripheral TPH (Fig. 9.3) (Kim et al., 1991; Cash, 1998). TPH1 is found in the enterochromaffin cells in the gut, pineal gland, spleen, thymus, skin, and retina, whereas TPH2 is predominantly distributed in the serotonergic neurons of the raphe nuclei in the brain stem and in the gut (Cote et al., 2003; Hornung, 2003; Slominski et al., 2003; Liang et al., 2004; Zill et al., 2004). TPH2 appears to be the major TPH protein in the brain, although TPH1 mRNA levels
Fig. 9.2. Hydroxylation of L-tryptophan (L-Trp) using tetrahydrobiopterin (BH$_4$) as cofactor. 5-OH-Trp, 5-hydroxy tryptophan; q-BH$_2$, quinonoid dihydrobiopterin.

(around one-third of TPH2 levels) have also been detected in the anterior and posterior pituitary and in the hypothalamus in addition to the pineal gland (Sugden et al., 2009, Zill et al., 2009). When analysing total brain lysates, these findings will have to be taken into consideration. The possible role of TPH1 in brain function is still being debated since TPH1 appears to be important in the early development of the brain (Nakamura et al., 2006b; Cote et al., 2007; Abumaria et al., 2008; Halmoy et al., 2010).

9.4 Structure and Function of TPH

9.4.1 Domain organization

The two TPH enzymes have a subunit and domain organization that is similar to the
other AAAH enzymes. Limited proteolysis, deletion mutagenesis, and crystallographic studies have demonstrated that they are organized in three domains:

1. An amino (N)-terminal regulatory domain of variable length with one or more serine phosphorylation sites, which mediate 14-3-3 binding in the case of TPH and TH.

2. A catalytic core with substrate and cofactor binding sites.

3. A short carboxy (C)-terminal oligomerization domain (Mockus et al., 1998) (Fig. 9.4).

TPH has been shown to exist as homotetramers, similar to TH (Nakata and Fujisawa, 1982; Okuno and Fujisawa, 1982), whereas PAH has been shown to exist in a state of equilibrium between dimeric and tetrameric forms (Iwaki et al., 1986; Kappock et al., 1995; Kleppe et al., 1999). ‘Molecular dissection’ of TPH has shown that the final 17 C-terminal residues comprise the tetramerization domain, which forms a leucine zipper motif and is conserved in TH (Mockus et al., 1997; Carkaci-Salli et al., 2006; Tenner et al., 2007). However, it has also been suggested that some N-terminal residues may contribute to subunit assembly in TPH1 (Yohrling et al., 1999). X-ray crystal structures of N- and C-terminally truncated forms of rat TH and human PAH have shown that the tetrameric form is held together by a long helix, which forms an antiparallel coiled-coil, and that C-terminal residues contribute to oligomerization via two antiparallel β-strands that contribute to subunit contacts (Erlandsen et al., 1997; Goodwill et al., 1997; Fusetti et al., 1998). A pentapeptide (Val-Pro-Trp-Phe-Pro, 105–109 in TPH1 and 151–155 in TPH2) is conserved in all of the hydroxylase enzymes, and demarcates a border between the catalytic domain and the end of the regulatory region. Removal of the regulatory region dramatically improves the solubility while enzyme activity is retained (Mockus et al., 1998; Carkaci-Salli et al., 2006). Both TPH1 and TPH2 have a tendency to form aggregates when purified, which is attributed to residues in the N-terminus. In TPH2, the N-terminal region is 46 aa longer than in TPH1 and contains several aromatic and basic residues (theoretical pI = 8.69) as well as an important PKA/CaMKII phosphorylation site, Ser19, which modulates 14-3-3 binding in TPH2 (Winge et al., 2008). The TPH2 deletion mutants NA41 and NA44 show that it exerts a negative effect on enzyme activity in vitro and in vivo, i.e. they have a higher V_max without affecting the K_m for Trp, higher expression levels and decreased half-life (Murphy et al., 2008; Tenner et al., 2008). Recently a TPH2 isoform (TPH2b) was identified, the product of alternative mRNA splicing, which has two extra amino acids in the N-terminal region, (Gly146-Lys147) (Grohmann et al., 2010), which interrupts a

![Fig. 9.4. The domain organization of the hAAAhs (PAH, TH1, TPH1, and TPH2); an N-terminal region containing one or more serine (S) phosphorylation sites, the catalytic domain, and the oligomerization domain.](image-url)
highly acidic sequence in TPH2a, i.e. Glu144-Asp150 where 6 of 7 residues are acidic (theoretical pI = 3.3).

Both TPH1 and TPH2 contain putative ACT domains in the N-terminal regulatory region, based on the consensus sequence and homology with PAH, which correspond to amino acids 18–94 in TPH1 and 66–142 in TPH2 (Liberles et al., 2005). Proteins containing the ACT domain are often involved in amino acid metabolism, and it was originally hypothesized that binding of regulatory ligands was its common function (Chipman and Shaanan, 2001). Several proteins that contain the ACT domain motif βαββββ have been crystallized, including PAH (PDB: 1PHZ) (Grant, 2006). There is no evidence that phenylalanine binds to the ACT domain in PAH nor tryptophan in TPH, and it has been hypothesized that the ACT domain has adopted other functions in the AAAH enzymes (Siltberg-Liberles and Martinez, 2009).

### 9.4.2 Ligand binding

The catalytic core domain has been crystallized for TPH1, PAH, and TH and demonstrates that a 2-His-1-carboxylate facial triad is a conserved motif that anchors the catalytic mononuclear non-haem iron at the active site (Erlandsen et al., 1997; Goodwill et al., 1997; Hegg and Que, 1997; Wang et al., 2002; Windahl et al., 2008). They correspond to residues His272, His277 and Glu317 in TPH1; and to His318, His323 and Glu363 in TPH2. A variety of different investigations including rational site directed mutagenesis, naturally occurring missense variants, and computational studies of crystal structures in complex with ligands and ligand analogues demonstrate important ligand selectivity determinants (Daubner et al., 2006; Teigen et al., 1999; Daubner et al., 2000; Jiang et al., 2000; McKinney et al., 2001). Electrostatic interactions that are important for amino acid substrate orientation are conserved across the hydroxylase enzymes, i.e. between the carboxyl group in tryptophan and phenylalanine with the guanidium group of the Arg257 side chain, the hydroxyl group of the Ser336 side chain, and the nitrogen backbone of Thr265. They were first identified in NMR/modelling studies (Teigen et al., 1999; McKinney et al., 2001) and in crystal structures with substrate analogues in a ternary complex with PAH and BH₄ (Andersen et al., 2001). The importance of those studies was later demonstrated for a naturally occurring Arg303Trp missense variant in TPH2 (McKinney et al., 2008), which had no detectable activity, probably due to loss of the charged Arg residue (McKinney et al., 2001). In the chicken TPH1 structure, tryptophan stacks against Pro268, which is different from both the docked structure and the substrate analogue, which stacked against the imidazole group of His272 (Windahl et al., 2008).

A conformational change is inferred from a comparison of the crystal structure for human TPH1 catalytic domain in complex with cofactor analogue (Wang et al., 2002) and a recent crystal structure of chicken catalytic domain in complex with substrate (Windahl et al., 2008). It was reported that the substrate-bound chicken TPH1 structure is more similar to the PAH structure with bound substrate analogue (PDB: 1MMK) than to the TPH1 structure without substrate: rmsd 0.9 versus 1.47 Å respectively for the Ca atoms. Two loops that
are located on either side of the active site channel form a more closed active site in the substrate bound structure; that is, the Ca atoms of Leu130 and Thr368 are 7.1 Å apart with tryptophan bound, which is 10 Å less than in the structure with complex with the cofactor analogue (Windahl et al., 2008). Those loop residues are conserved in human TPH1 and TPH2: Leu124-Asp139 and Ile367-Thr369, Leu123-Asp140 and Ile366-Thr368 in TPH1 and Leu169-Asp184 and Ile412-Thr414 TPH2, respectively. Thus, the crystal structures provide an explanation for the substrate inhibition seen for L-Trp both for the catalytic domain and the full-length enzyme (Wang et al., 2002; McKinney et al., 2005).

There has been a keen interest in the regulation of BH₄-dependent enzymes, the pharmacological effects of BH₄ administration, and the development of selective inhibitors of BH₄-dependent enzymes (Werner-Felmayer et al., 2002). The first TPH crystal structure was of the catalytic domain, aa 102–402, in complex with ferric iron and 7,8-dihydrobioppterin (Wang et al., 2002), which is similar to the binding mode for all the AAAH enzymes as was shown for PAH (Teigen et al., 2004) and TH (Martinez et al., 1993). The BH₄ binding site is similar in the hydroxylase enzymes and comprises aa Tyr235-Pro238 and Phe241 in TPH1 which are also conserved in TPH2. The pterin ring pi-stacks with Phe241 and is hydrogen bonded to G1u273. The binding pocket for the BH₄ dihydroxypropyl side chain is different for each enzyme due to non-conserved residues there, i.e. Pro in TPH1 and TPH2, Ser in PAH, and Ala in TH. Thus, the pterin binding site has important differences that may be exploited for designing hydroxylase-specific inhibitors, which has been demonstrated for TPH using 8-methyl-6,7-dimethyl-5,6,7,8-tetrahydropterin (Teigen et al., 2007).

Besides its function as an essential cofactor for substrate hydroxylation in the AAAH enzymes, BH₄ has also been shown to enhance both PAH levels and activity and TH levels in the brain, especially at nerve terminals, whereas TPH does not appear to be affected (Sumi-Ichinose et al., 2001; Takazawa et al., 2008; Thony et al., 2008). Thus, an interest in the identification of ligands that might affect AAAH enzyme stability differently also led to discovery of a compound in a commercially available chemical library that dramatically increased the thermostability of the TPH2, as well as PAH and TH (Pey et al., 2008; Calvo et al., 2010).

### 9.4.3 Catalytic mechanism

Due to structural and functional similarities, TPH and the other AAAH enzymes are assumed to share a common catalytic mechanism; however, studies on the catalytic mechanism are mainly reported for TH and PAH due to their greater stability in vitro. According to enzyme nomenclature, they are categorized as oxidoreductases, i.e. they transfer electrons from a reductant to an oxidant. AAAH enzymes employ a paired electron donor (BH₄), with incorporation or reduction of molecular oxygen, i.e. into tryptophan and transiently to BH₄ (IUPAC-IUB, 1984). Catalysis requires a ferrous iron atom, one molecule of reduced pterin per catalytic site, which is oxidized to a dihydropterin. One molecule of O₂ is consumed in the reaction; one atom of oxygen is incorporated into the amino acid substrate, whereas the other is ultimately reduced to water (Fitzpatrick, 2003). Although a variety of data suggests that BH₄ is the physiological substrate (Frantom et al., 2006), many different synthetic tetrahydropterins, e.g. 6-methyltetrahydropterin (6-MPH₄) and 6,7-dimethyltetrahydropterin (DMPH₄), are sufficient for catalysis and have been used in experiments that have generated important data about the catalytic mechanism (Eser et al., 2007; Chow et al., 2009).

Upon purification, the enzymes are typically isolated with ferric iron, whereas the reaction cycle starts and stops with iron in the ferrous state (Fitzpatrick, 2003). Thus, the mechanism for iron reduction has been somewhat controversial due to the stoichiometric incompatibility between the two-electron
reductant BH$_4$ and the one-electron oxidant ferric iron (Nieter Burgmayer, 1998). The reaction mechanism is typically described as two partial reactions: formation of the hydroxylating intermediate and oxygen transfer to the aromatic amino acid (Fitzpatrick et al., 2003; Haahr et al., 2010). From kinetic data this mechanism, as shown for PAH and TH, appears to be sequential (Hosoda, 1975; Fitzpatrick et al., 2003). The Fitzpatrick laboratory has made many important findings regarding the nature of the reaction. It has shown evidence for an electrophilic aromatic substitution reaction mechanism with a cationic amino acid intermediate, where a ferryl-oxy species is responsible for generating the 4a-OH-pterin intermediate. The 4a-OH-pterin intermediate has been demonstrated for all of the hydroxylases including TPH (Haavik and Flatmark, 1987; Moran et al., 2000).

The binding order of amino acid substrate and cofactor is deemed to be somewhat random; however, kinetic data and crystal structures of enzyme–ligand complexes show that binding of the cofactor before substrate enhances enzymatic activity and that catalysis only occurs when both substrate and pterin are bound (Fitzpatrick, 2003). This hypothesis is supported by crystallographic and kinetic data for TPH1 (Windahl et al., 2009). The enzyme exhibits substrate inhibition for L-Trp, i.e. $K_i$ 385 µM for the full-length enzyme (McKinney et al., 2004) and 71.7 µM for double-truncated catalytic domain (Windahl et al., 2009) and is in a more closed conformation when in complex with substrate (Windahl et al., 2008) than with cofactor analogue (Wang et al., 2002). For TPH2, no crystal structure has been reported yet, but the kinetic data show that it is less susceptible to substrate inhibition, i.e. $K_i$ 970 µM for the full-length enzyme (McKinney et al., 2004), and no substrate inhibition up to 100 µM for the isolated catalytic domain (Windahl et al., 2009).

Many researchers have reported that TPH (both TPH1 and TPH2) easily aggregate and are difficult to purify. Kinetic values are also critically dependent on reaction conditions, such as pH and buffer compositions. Together, this can probably explain some of the diverging kinetic values shown in Table 9.1. With plasma and brain levels of L-Trp of about 30 µM (for rats) (Grahame-Smith, 1964; Fernstrom and Wurtman, 1971), and diverging $K_m$ results, as for BH$_4$, it is difficult to conclude whether the substrate availability is limiting in the cells.

### 9.5 Enzyme Regulation

#### 9.5.1 Inhibition of TPH

The regulation of TPH activity has been studied extensively. One way of regulating enzymes is by substrate or product inhibition. All AAAHs seem to be rather insensitive to product inhibition, whereas they are more sensitive towards their substrate. TPH appears to be regulated by substrate inhibition, by high concentrations of L-Trp or L-Phe, although the inhibition by L-Trp is more pronounced than by L-Phe (Friedman et al., 1972; Tong and Kaufman, 1975). For a long time it was unclear if this had an effect in vivo, but it has been shown that patients with phenylketonuria (PKU) have decreased platelet (Friedman et al., 1972; Tong and Kaufman, 1975), cerebrospinal fluid (CSF) (Schulpis et al., 2002), and brain (Burlina et al., 2000) serotonin levels. When treated with BH$_4$, these levels increased in almost all patients, although the L-Trp and L-Phe levels remained the same, suggesting that this is a direct effect of BEI, on TPH, compensating for the inhibition by high L-Phe levels (Ormazabal et al., 2005).

The sensitivity of TPH towards inhibition is also dependent on its cofactor. Substrate inhibition of brain TPH is more pronounced in the presence of BH$_4$ than DMPH$_4$. However, it is debated whether this inhibition is significant in vivo, as inhibition in the presence of BH$_4$ has only been seen at concentrations of 100 µM (Ormazabal et al., 2005), exceeding normal tissue levels of BH$_4$.

As for both PAH and TH, TPH has been shown to be very sensitive towards inhibition by catecholamines (Kaufman, 1974), although catecholamines do not seem to be synthesized in TPH1- and TPH2-containing cells. Nevertheless, dopamine and serotonin appear to have a similar distribution in the paraventricular organ, most probably due to uptake of dopamine in the serotonergic cells
### Table 9.1. Kinetic parameters (Km values) of TPH1 and TPH2.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>TPH1</th>
<th>TPH2</th>
<th>Species</th>
<th>Reference</th>
</tr>
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<tr>
<td>L-Trp (μM)</td>
<td>50</td>
<td>32</td>
<td>Native rabbit hind brain</td>
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<td></td>
<td>8</td>
<td>71.2</td>
<td>Rabbit (cloned)</td>
<td>Tong and Kaufman (1975)</td>
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<td></td>
<td>12</td>
<td>8</td>
<td>Mouse (cloned)</td>
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<td></td>
<td>26</td>
<td>20</td>
<td>Human (cloned)</td>
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<td></td>
<td>22.8</td>
<td>40.3</td>
<td>Human (cloned)</td>
<td>Kowlessur and Kaufman (1999)</td>
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<td>24.26</td>
<td>41.3</td>
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<td>28</td>
<td>33</td>
<td>Human (cloned), 92–444</td>
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<td>7.8</td>
<td>26.8</td>
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<td></td>
<td>22.8</td>
<td>15</td>
<td>Human TPH1:100–413 TPH2:</td>
<td>Windahl et al. (2009)</td>
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<td></td>
<td></td>
<td></td>
<td>146–459 (recombinant)</td>
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<td>BH4(μM)</td>
<td>31</td>
<td>39</td>
<td>Rabbit hind brain, 92–444</td>
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<td>273</td>
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Only mammalian forms of the enzyme (preferentially human) are listed. For the measurements of Km values for the amino acid substrate L-Trp only the data with the natural cofactor BH4 are selected.

As TPH may be involved in many different disorders, there is an increased interest in finding specific inhibitors of TPH1 and TPH2. High concentrations of L-Trp and L-Phe inhibit TPH1 and TPH2. In addition, derivatives of L-Trp and L-Phe such as p-chlorophenylalanine (PCPA), 6-fluorotryptophan and 6-chlorotryptophan are also potent TPH inhibitors (McGeer et al., 1968; Nicholson and Wright, 1981; Pandey et al., 1983; Nakamura et al., 2006a). PCPA was first introduced as an agent for depleting brain serotonin (McGeer et al., 1968; Nicholson and Wright, 1981; Pandey et al., 1983; Nakamura et al., 2006a), and inhibition
of TPH was reported as the main cause of serotonin depletion (Koe and Weissman, 1966). It was shown that in vivo the drug would lead to an irreversible inhibition of the enzyme. Pineal TPH (now known as TPH1) has been reported to be less sensitive to inhibition by PCPA (Jequier et al., 1967) than TPH2. The effect of TPH-inhibition has been shown to be dependent on the pterin used, i.e. the inhibition of PCPA is stronger in the presence of BH$_4$ than in the presence of DMPH$_4$ (Deguchi and Barchas, 1972). Although PCPA is a strong inhibitor, 6-fluorotryptophan appears to be more potent in vivo (Tipper et al., 1994). More recently, p-ethynylphenylalanine has been found to be an even more selective, reversible, and potent inhibitor than PCPA (Stokes et al., 2000).

Tetrahydroisoquinolines (TIQs) have been proposed to be neurotoxic in vitro and constitute another class of TPH inhibitors (Stokes et al., 2000). TIQs such as salsolinol and tetrahydropapaveroline have been identified in the urine of parkinsonian patients receiving L-DOPA therapy. They inhibit the dopamine biosynthesis by a direct binding of TH (Shin et al., 1999; Kim et al., 2001; Scholz et al., 2008). In addition to inhibiting TH, the rate-limiting enzyme in the catecholamine biosynthesis, these TIQs have also been found to inhibit TPH at a similar concentration range (Ota et al., 1992; Kim et al., 2003; Kim et al., 2004).

Recently, Cianchetta and co-workers published their work on TPH1 crystal-structures with the novel inhibitors LP533401, LP521834, and LP534193 (Ota et al., 1992; Kim et al., 2003; Kim et al., 2004). They appear to fill the tryptophan-binding pocket of TPH1 without reaching into the binding site of the cofactor and induces a major conformational change of the enzyme. Since all three inhibitors share a phenylalanine moiety, they are competitive with L-Trp and assume a compact complex that is similar to the TPH1-Trp complex. Apart from the phenylalanine moiety, LP521834 contains a 2-amino-triazine, LP521834 a 2-amino-pyrimidine, and LP-534193 contains a pyrazine as the second component of the molecule (Cianchetta et al., 2010).

LP533401 has also been tested in a group of patients with osteoporosis (Yadav et al., 2010). Gut-derived serotonin (GDS) has been described to be a powerful inhibitor of osteoblast proliferation and bone formation (Yadav et al., 2010). Oral administration of this TPH1-inhibitor decreased the GDS synthesis and may therefore be a potential treatment for osteoporosis (Yadav et al., 2010). Armed with such enzyme-specific inhibitors, it will be easier to explore the biology and pathophysiology of TPH1 and TPH2.

### 9.5.2 Regulation of TPH

In contrast to the growing numbers of genetic association and functional studies of TPH1 and TPH2, little is known about the normal regulation of TPH expression. In 1997, it was reported that glucocorticoids are involved in the tissue-specific regulation of TPH mRNA levels. Treatments of adrenalectomized rats with the synthetic glucocorticoid dexamethasone led to an increase in TPH mRNA in the pineal gland, whereas in the raphe nuclei it was decreased (Yadav et al., 2010). The identification of TPH2, and the knowledge that the promoters of TPH1 and TPH2 lack sequence homologies, suggests that the main reason for the apparently different regulation was different mRNA species, and not different tissue localization.

Both TPH1 and TPH2 have been shown to exhibit a diurnal rhythm of gene expression, although TPH2 to a lesser extent than TPH1 (Liang et al., 2004; Malek et al., 2004; Malek et al., 2007). The nocturnal increase in TPH1 appears to be dependent on a cyclic adenosine monophosphate (cAMP) increase that both increases mRNA production (Liang et al., 2004; Malek et al., 2004; Malek et al., 2007) and phosphorylation of TPH1 by the cAMP-dependent protein kinase (PKA) (Huang et al., 2008). Phosphorylation appears to stabilize the protein, which may explain the increase in TPH1 at night-time (Huang et al., 2008). The increase in TPH2 mRNA, however, has been found to be stimulated by an increase in corticosterone (Malek et al., 2004; Malek et al., 2007). In addition to being regulated by the diurnal rhythm, expression of both TPH1 and TPH2 mRNA have been shown to be regulated by various hormones and stressors, such as haemorrhaging shock, glucocorticoids, oestrogen...
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and ovarian steroids (Clark et al., 2005; Malek et al., 2005, 2007; Sanchez et al., 2005; Brown et al., 2006; Hiroi et al., 2006).

Recently, a truncated isoform without a catalytic domain (ENST00000266669, g.22879A >G) (Haghighi et al., 2008) and an alternatively spliced variant of TPH2 with six extra nucleotides between exons 3 and 4 (Haghighi et al., 2008) have been described. This supports the suggestion that alternative splicing and RNA editing may be an additional way of regulating TPH activity. It has also been shown that the N-terminal domain of TPH2 is involved in regulation of enzyme expression, and this might partially explain the different expression levels and stability of TPH1 and TPH2 in vivo (Grohmann et al., 2010).

9.5.3 Phosphorylation of TPH

Many enzymes are regulated by reversible phosphorylation at Ser, Thr, or Tyr residues. Multiprotein serine/threonine specific protein kinases such as PKA and the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CaMKII) have been reported to phosphorylate and activate the AAAHs. Particularly, regulation of TH by phosphorylation has been thoroughly studied (Kumer and Vrana, 1996; Toska et al., 2002; Dunkley et al., 2004; Fujisawa and Okuno, 2005). Compared to TH, less is known about the phosphorylation of TPH1 and TPH2, and it is sometimes difficult to know whether the findings involve TPH1 or TPH2, as most experiments were performed before the knowledge about TPH2. The pineal gland is, however, known to express mainly TPH1. The phosphorylation site on Ser58 in TPH1 was later confirmed by site-directed mutagenesis (Kuhn et al., 1997; Kumer et al., 1997), and although Ser260 has been suggested as an additional phosphorylation site (Jiang et al., 2000), this has not been verified by other groups. Recently, it has been shown that phosphorylation on Ser58 in TPH1 increases during the night, and this may play a regulatory role on the TPH1-levels in the pineal gland, as phosphorylation also appears to stabilize TPH1 (Huang et al., 2008).

Although phosphorylation of TPH2 was not explicitly studied before 2003, it was early shown that addition of ATP and Mg\textsuperscript{2+} in rat brain stem lysates, containing mainly TPH2, increases TPH activity by 40–50%, and that addition of Ca\textsuperscript{2+} increased the activity even more (70–200%). Removing Ca\textsuperscript{2+} by adding EDTA had the opposite effect (Knapp et al., 1975; Hamon et al., 1977). Similar results were also reported using total rat brain lysates (Boadle-Biber, 1978; Boadle-Biber, 1979; Boadle-Biber, 1982). In the presence of Ca\textsuperscript{2+}, calmodulin was reported to be an activator of TPH activity (Boadle-Biber, 1978; Boadle-Biber, 1979; Boadle-Biber, 1982), and it was demonstrated that TPH from rat brain stem (most likely TPH2) is phosphorylated by CaMKII (Yamauchi and Fujisawa, 1979a,b, 1981; Ehret et al., 1989). However, it was not until 2007 that the phosphorylation site for CaMKII in TPH2 was identified as Ser19, using mass spectrometry (MS) and site directed mutagenesis (Kuhn et al., 2007; Winge et al., 2008).

The evidence for activation of TPH by adding cAMP to brain stem extracts is not as clear as for addition of Ca\textsuperscript{2+}. Several investigators state that addition of cAMP activates rat brain stem TPH (TPH2) up to twofold (Kuhn et al., 2007; Winge et al., 2008), whereas others have not observed this increase in enzyme activation (Hamon et al., 1978; Kuhn et al., 1978; Yamauchi and Fujisawa, 1979b; Boadle-Biber, 1982; Johansen et al., 1995). However, these reports are based on findings using brain lysate that may already contain cAMP, so there is no extra activation effect when it is added. Some groups Johansen et al. (1995) and Makita et al. (1990) found that TPH2 was indeed phosphorylated by
exogenous PKA. In 2005 the first PKA-phosphorylation site in TPH2 was identified, i.e. Ser19 (McKinney et al., 2005), and later a second phosphorylation site, Ser104, corresponding to Ser58 in TPH1, was identified (Winge et al., 2008). Phosphorylation on Ser19 by PKA appears to increase the stability of TPH2 (Murphy et al., 2008; Winge et al., 2008); however, this may be related to the binding of 14-3-3 proteins (Winge et al., 2008).

So far, little is known about the functional effects of phosphorylation of TPH. However, there appears to be a consensus that phosphorylation activates TPH, although it is still unclear whether the activation is direct, as suggested for TPH1 (Banik et al., 1997; Kuhn et al., 1997), or due to increased stability of the protein, as proposed for TPH1 and TPH2 (Huang et al., 2008; Murphy et al., 2008; Winge et al., 2008). To make any further conclusions about the regulatory effect of phosphorylation, more studies on both enzymes are needed.

### 9.5.4 14-3-3 binding to TPH

The mammalian 14-3-3 protein family consists of seven different structurally related acidic proteins that are widely distributed and involved in many physiological processes. Activation of TPH and TH by 14-3-3 were the first biological functions associated with binding of 14-3-3 proteins (Ichimura et al., 1987). Most research has been done on 14-3-3 binding to TH, but there are also some findings for TPH. Most of the findings were reported prior to the discovery of TPH1 and there is no clear distinction between the effects on TPH1 and TPH2. Yamauchi and coworkers reported as early as in 1981 that rat brain stem TPH (mainly TPH2) phosphorylated by CaMKII was activated in the presence of an activator protein that later turned out to be a 14-3-3 (Ichimura et al., 1987). Later, a similar activation was observed for TPH in brain stem phosphorylated by endogenous or exogenous PKA in the presence of 14-3-3 (Ichimura et al., 1987). However, other studies showed no enzyme activation when incubating either rat brain stem TPH or recombinant TPH2 with 14-3-3 (Johansen et al., 1995; McKinney et al., 2005). Binding of 14-3-3 to TPH1 has also been shown to maintain the enzyme in a phosphorylated state, and thereby prolong the phosphorylation-dependent increase in activity (Johansen et al., 1995; McKinney et al., 2005) (Fig. 9.5). This stabilizing effect on the protein activity was also reported for TPH2 (Winge et al., 2008).

![Fig. 9.5.](image-url) Schematic representation of the interaction of TPH with 14-3-3 proteins. Figure adapted from Winge et al. (2008).
Phosphorylation studies and site-directed mutagenesis have revealed that phosphorylation is necessary for the binding of 14-3-3 to TPH2 (McKinney et al., 2005; Winge et al., 2008), and an interesting finding is that there appears to be a different binding site for TPH2 compared to TPH1. Specific binding sequences of 14-3-3 in TPH1 and TPH2 are yet to be confirmed, but it has been suggested that Ser58 is involved in the binding of 14-3-3 (McKinney et al., 2005; Winge et al., 2008), whereas mutation studies indicate that phosphorylation on Ser19 and not Ser104 (corresponding to Ser58 in TPH1) is essential for binding of 14-3-3 to TPH2 (Winge et al., 2008). This may indicate a different regulation of the enzymes with the 14-3-3 proteins.

9.6 TPH Knockout Studies

Recently, several reports have been published regarding TPH knockout (KO) studies in mice. The first TPH1 KO report was particularly important as it revealed the existence of a second TPH gene (TPH2). In these TPH1 KO mice serotonin was found in normal levels in the brain, but was not present in the periphery (Walther et al., 2003). The earliest reports of TPH1 -/- mice showed no morphological or behavioural changes, except for a cardiac dysfunction due to an increased heart size, compared with the wild type (WT) (Cote et al., 2003). The first TPH2 KO reports were also negative with respect to changes in the visible or behavioural features. The phenotypic features that were reported were decreased body size during the first 2 months of life, reduced body fat, and the TPH1/TPH2 double KO mice had also decreased immobility (Gutknecht et al., 2008; Savelieva et al., 2008). However, a recent report showed that TPH1 KO also led to 50% lethality during the first 4 weeks of postnatal life. In addition, the pups had altered regulation of sleep, breathing, thermoregulation, heart rate and blood pressure. As adults, the mice were more aggressive and showed maternal neglect (Alenina et al., 2009). This is also reflected in another report showing that maternal serotonin is crucial for murine embryonic development, as pups born to TPH1 KO mice showed dramatic abnormalities in the development of the brain and other organs (Alenina et al., 2009). In addition to these KO mice, a mutant form (P449R) of TPH2 has also been found in Balb/c mice. These mice had reduced serotonin levels and behaved aggressively (Zhang et al., 2004). All these results indicate that although TPH1 and TPH2 are not essential for adult life, they are involved in both behaviour and autonomic pathways. However, further studies of KO organisms are needed to establish the full effect of TPH-deletion and serotonin depletion.

9.7 Implications of TPH Dysfunction in Human Health

Serotonin is an important neurotransmitter in the CNS, and serotonin-containing diffuse projections from the raphe nuclei in the brainstem are found in many brain regions. Thus, although constituting less than 1% of the total transmitters in the human brain, serotonin may be considered to be one of the most widely distributed neurotransmitters (Hensler et al., 1994) and it has been linked to a variety of CNS functions such as temperature control, attention, pain, and memory (Mochizucki, 2004; Maurer-Spurej, 2005; Mendelsohn et al., 2009). In recent years there has been a dramatic increase in clinical evidence associating dysregulation in central serotonin activity with symptoms such as aggression, impulsivity and hyperactivity, in addition to human disorders such as depression, anxiety, obsessive-compulsive disorder, schizophrenia and attention deficit hyperactivity disorder (ADHD) (reviewed in Arango et al., 2003; Haavik et al., 2008; Matthes et al., 2010).

Although TPH2 is found mainly in neurons, it has been reported that TPH1 is predominantly expressed during the late developmental stage of the brain (Nakamura et al., 2006b). Therefore, both TPH1 and TPH2 have been regarded as potential susceptibility genes for psychiatric disorders. The coding regions of TPH1 and TPH2 are small compared to the total gene sequence, so most of the single nucleotide polymorphisms (SNPs) that have been reported have been located in the introns and promoter regions (Nakamura et al., 2006b).
TPH1 markers have been reported to be associated with suicidal behaviour (reviewed in Haavik et al., 2008), depression (Sun et al., 2004; Jokela et al., 2007), bipolar disorder (Bellivier et al., 1998; Zaboli et al., 2006; Chen et al., 2008) schizophrenia (Bellivier et al., 1998; Zaboli et al., 2006; Chen et al., 2008) and ADHD (Allen et al., 2008). Since the characterization of the neuronal TPH2 (McKinney et al., 2005), the focus has mainly been shifted towards this enzyme. Since then, variants of TPH2 have been reported to be associated with different psychiatric disorders as well, such as major depression, bipolar disorder, suicidal behaviour, personality disorders and ADHD (reviewed in Haavik et al., 2008; Matthes et al., 2010).

One of the first variants reported was in intron 7 of the TPH1-gene (Haavik et al., 2008; Matthes et al., 2010), whereas the first coding polymorphism, p.TPH1 V1771, was found in a patient exhibiting motor- and neurodevelopmental problems (Ramaekers et al., 2001). Recently, six other coding polymorphisms have been found in patients with ADHD (Ramaekers et al., 2001): p.TPH1 K54Q, R142C, R145X, L2741, A300T, and I410N. These polymorphisms have been linked to impaired maternal serotonin production that may have long-term effects on brain development and increase the risk of ADHD-related symptoms and behaviour in offspring (Halmoy et al., 2010) (Fig. 9.6 a and b). So far, for TPH1, there are hundreds of variants identified across

![Fig. 9.6. Genomic structure and location of variants in human TPH1 (a and b) and TPH2 (c and d).](image-url)

(a and c) Exons encoding the regulatory domain are shown in dark grey (p.Met1-Thr104 (TPH1) and p.Met1-Asp150 (TPH2)), the catalytic domain in lighter grey (p.Val105-Asn402 (TPH1) and Val151-Asn448 (TPH2)), and the oligomerization domain in light grey (p.Pro403-Ile444 (TPH1) and Pro449-Ile490 (TPH2)). Modified from Yadav et al., 2010. (b and d) Molecular model of a full-length subunit of TPH1 (b) and TPH2 (d) WT, illustrating the position of missense mutations and phosphorylation sites. A dark grey ball represents the active site iron atom. Arbitrarily determined domain boundaries are as shown in Fig. 9.6a. Figure adapted from Haavik et al. (2008) with permission from John Wiley and Sons.

The polymorphisms A218C (rs1800532) and A779C (rs1799913) in the introns of TPH1 are the two most studied SNPs in TPH1 and have been reported to be associated with several disorders such as schizophrenia (Allen et al., 2008), bipolar disorder, suicidality, and depression. However, other groups have not been able to replicate these findings. In a recent large multicentre study, common variants in either TPH1 or TPH2 were not significantly associated with persistent ADHD (Johansson et al., 2010).

So far, almost 2000 SNPs have been identified for TPH2 across all species, and more than 700 of these are found in the human gene (www.ncbi.nlm.nih.gov/snp?term=TPH2). Among these, eight non-synonymous variants are in the coding region, ten in humans and one in mouse: human p.TPH2 L36V, L36P, S41Y, R55C, P206S, R303W, A328V, R441H, D473N, and D479E; and mouse p.TPH2 P447R (Winge et al., 2005; Zhang et al., 2005; Cichon et al., 2008; McKinney et al., 2009; Halmoy et al., 2010) (Fig. 9.6c and d). There are also five synonymous coding SNPs (Y212Y, P312P, L327L, A375A, and C406C) and many non-coding SNPs reported (470 in humans, June 2010) (www.ncbi.nlm.nih.gov/snp?term=TPH2).

In addition to exerting an impact on psychiatric disorders, TPH2 has been suggested to be involved in sudden infant death syndrome (SIDS) by the observation of decreased protein levels in the SIDS cases compared with controls (Duncan et al., 2010), and to be involved in coronary artery lesions in children with Kawasaki disease (Park et al., 2010). TPH1, as a peripheral enzyme, has been
reported to be involved in gastrointestinal disorders such as irritable bowel syndrome (Park et al., 2010) and colonic inflammation (Ghia et al., 2009), in both of which decreased mRNA levels have been observed. In addition, the inhibition of TPH1 by synthetic inhibitors increases bone formation and could potentially be important in the treatment of osteoporosis (Yadav et al., 2010).

Further implications of the reduced serotonin levels on brain function and neuropsychological disorders are discussed in Chapter 24 of this volume.

9.8 Concluding Remarks and Future Research

Studies on TPH have been carried out for several decades, although it was only recently that TPH2 was identified. Most of the early studies were done in cell lysates, but during the last two decades the purified recombinant proteins have also been studied. The mechanism of hydroxylation by both TPH1 and TPH2 appears to be similar to that of the other two AAAHs, with a sequential order of substrate binding and formation of an activated oxygen intermediate. Knowledge of the ligand-binding and catalytic mechanism is also increasing with the aid of crystal structures, spectroscopy, and binding assays. However, the detailed mechanism of hydroxylation is still not known, and more investigation is needed.

Another question that is yet to be answered is why the TPHs are so difficult to purify, and why they aggregate rapidly in vitro. TPH2 has been reported to be somewhat less stable than TPH1, and the N-terminal has been suggested to be involved in the susceptibility of the protein to aggregation. Phosphorylation, in contrast, has been suggested to stabilize the protein, and also to label it for degradation. There are still some controversies regarding the different phosphorylation sites in TPH1 and TPH2, and which enzymes truly phosphorylate TPH1 and TPH2 in vivo. The effects of TPH phosphorylation on enzyme kinetics and turnover also need further studies.

TPH1 and TPH2 have been reported to be associated with psychiatric disorders, SIDS, and disorders in the gut. However, these aspects need to be clarified as there are contradictory results in the literature, in particular for the different psychiatric disorders. As a consequence of the growing amount of articles concerning TPH and disorders there is increased interest in finding new enzyme inhibitors that can be used as drugs. Some of the naturally occurring mutations reported in TPH1 and TPH2 have been reported to destabilize the enzyme in a way that is compatible with a role in brain disorders. The discovery of pharmacological chaperones that can stabilize these enzymes may therefore be an important finding that should be studied further.

Inhibitors of TPH1 have been suggested as a possible treatment in different diseases such as osteoporosis. However, one should keep in mind that TPH1 has been found to be important for early development of the brain, and pregnant women should probably not be treated with these inhibitors. These findings show the complexity of the serotonin pathway, how important it is to consider all aspects of the enzymes, and the importance of TPH1 and TPH2 in the normal development of the brain and other organs.

References


Tryptophan Hydroxylase


Tryptophan Hydroxylase


Tryptophan Hydroxylase


10 Methionine Metabolism

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10.1 Abstract

The steatosic and hepatocarcinogenic effect of diets deficient in methyl groups (methionine and choline) has been known for decades. Knockout mice with either a deficiency or excess in hepatic S-adenosylmethionine (SAMe, the first step in methionine metabolism and the main biological methyl donor) spontaneously develop steatosis (fatty liver) and hepatocellular carcinoma (HCC). Thus, a model has emerged in which changes in cellular pools of hepatic SAMe, fluctuating in response to a variety of conditions such as hepatocyte growth and differentiation, could provide the rheostat by which the methylation status and consequently the activity of critical proteins can be modulated. The relevance of this observation to human health is obvious since SAMe synthesis is impaired in humans with liver cirrhosis and HCC, and SAMe treatment increases survival in patients with alcoholic liver cirrhosis. Several molecular mechanisms have emerged to explain how abnormal fluctuations in hepatic SAMe content lead to the development of non-alcoholic fatty liver disease and HCC. This review analyses the methionine requirement of resting and proliferating hepatocytes as well as of liver cancer cells, in an attempt to understand why abnormal methionine metabolism can lead to liver injury and HCC. It is concluded that a less efficient methionine metabolism facilitates hepatocyte proliferation, and that manipulation of methionine metabolism holds promise for improving HCC prognosis.

10.2 Introduction

The body of a normal, adult, lean individual has an energy reserve of about 138,000 kcal, the majority of them (about 135,000 kcal) stored in the white adipose tissue as triglycerides (TGL). In addition, a small amount of TGL (about 450 kcal) playing a critical role in maintaining metabolic homeostasis for the whole body is stored in the liver (Cahill, 1976). Current estimates indicate that about 20–40% of the general adult population living in Western countries has non-alcoholic fatty liver disease (NAFLD, defined as more than 5% of fat by weight) (Adams and Lindor, 2007). In these individuals, the liver loses its characteristic reddish colour and turns a yellowish tint, due to the excessive accumulation of TGL. NAFLD is mainly associated with obesity although it is also frequent in individuals

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suffering from essential nutrient deficiency. NAFLD is a progressive disease that moves from the simple accumulation of fat in the liver (steatosis) to steatosis with inflammation, necrosis, and fibrosis (non-alcoholic steatohepatitis, NASH). In turn, NASH may progress to cirrhosis and hepatocellular carcinoma (HCC), indicating that metabolic imbalance may be at the origin of the association of cancer with NAFLD (Reid, 2001).

While our current knowledge of the existence of this synchronization of nutrient metabolism and cell growth is principally based on studies on glucose consumption (Vander Heiden et al., 2009), the hepatocarcinogenic activity of diets deficient in methyl groups (methionine and choline) (Mikol et al., 1983) indicates that the metabolism of methionine may also be adapted to meet the needs of proliferating cells. This realization has brought renewed attention to Charles Best’s observation in 1932 that choline prevented the deposition of fat in the liver, a phenomenon known as ‘lipotropism’ (Fig. 10.1) (Best, 1956). Best also realized that the rate of oxygen uptake by the slices of livers from rats on a diet low in choline was appreciably less than that of the normal liver slices, indicating a synchronization of the metabolism of choline and mitochondrial oxidative phosphorylation. In a subsequent study carried out in 1937, Tucker and Eckstein discovered the lipotropic action of methionine (Best, 1956). Then in 1940 du Vigneaud and his colleagues administered deuterium-labelled methionine to rats fed a diet low in methionine and choline, finding a massive accumulation of the isotope in choline (Best, 1956). This process, referred as ‘transmethylation’, undoubtedly established the synthesis of choline from methionine. Later work by Stetten established that ethanolamine (we know now that in the form of phosphatidylethanolamine) receives methyl groups from methionine to form choline (Best, 1956). In 1953 Cantoni showed that, in order to transfer its methyl group, methionine needs first to be converted to an active sulphonium ion by reacting with ATP and forming S-adenosylmethionine (SAMe) (Best, 1956). In 1983, Poirier observed that a severe deficiency in methyl groups causes liver cancer (Mikol et al., 1983). More recently, Lu and Mato (2008) demonstrated that knockout mice deficient in hepatic SAMe synthesis fed a normal diet spontaneously develop fatty liver (Lu et al., 2001) and HCC (Martinez-Chantar et al., 2002). An explanation for these observations has remained elusive, since the connection of methionine metabolism with carbohydrate and lipid utilization as well as with hepatocyte proliferation is, at first glance, not obvious (Fig. 10.2).

10.3 Proliferating Hepatocytes and Liver Cancer Cells Show a Less Efficient Methionine Metabolism than Normal Differentiated Hepatocytes

SAMe is synthesized by methionine adenosyltransferase (MAT), an enzyme extremely well conserved through evolution, with 59% sequence homology between the human and Escherichia coli isoenzymes (Markham and Pajares, 2009). MAT is one of the minimal set of about 300 proteins that sustain independent life (Fraser et al., 1995) because it catalyses the only reaction that generates SAMe, the main biological methyl donor (Mato and Lu, 2007; Mato et al., 2008). In mammals there are three isoforms of MAT (MATI, MATII, and MATIII).
that are encoded by two genes (\textit{MAT1A} and \textit{MAT2A}). \textit{MATI} and \textit{MATIII} are tetrameric and dimeric forms, respectively, of the same subunit (a1) encoded by \textit{MAT1A}, whereas the \textit{MATII} isomer is a tetramer of a different subunit (a2) encoded by \textit{MAT2A}. A third gene, \textit{MAT2P}, encodes for a β subunit that regulates the activity of \textit{MATII} (lowering the \(K_m\) and \(K_v\) for methionine and SAMe, respectively) but not of \textit{MATI} or \textit{MATIII} (Halim \textit{et al.}, 1999; Yang \textit{et al.}, 2008). Adult differentiated liver expresses mainly \textit{MAT1A}, whereas extrahepatic tissues, fetal liver, and HCC express \textit{MAT2A} and \textit{MAT2P} (Halim \textit{et al.}, 1999; Yang \textit{et al.}, 2008). The question why there are three different \textit{MAT} isoforms in the liver is intriguing.
The predominant liver form, MATIII, has lower affinity for its substrates, a hysteretic response to methionine (a hysteretic behaviour, defined as a slow response to changes in substrate binding, has been described for many important enzymes in metabolic regulation), and higher $V_{\text{max}}$, contrasting with the other two enzymes (del Pino et al., 2000). Based on the differential properties of hepatic MAT isoforms, it has been postulated that MATIII is the truly liver-specific enzyme.

In a quiescent differentiated liver, when the levels of methionine are low, MATI would, as MATII outside the liver, synthesize most SAMe required by the hepatic cells to maintain the methylated status of DNA, histones, etc., and the homocysteine thus generated is mainly used for methionine regeneration (Fig. 10.3). However, after an increase in methionine concentration, i.e. after a protein-rich meal, conversion to the high activity MATIII would occur and methionine excess will be eliminated (del Pino et al., 2000). This will lead to an accumulation of SAMe and to the activation of glycine N-methyltransferase (GNMT), the main enzyme involved in hepatic SAMe catabolism (Luka et al., 2009). Consequently, the excess of SAMe will be eliminated and converted to homocysteine via S-adenosylhomocysteine (SAH). Once formed, the excess of homocysteine will be used mainly for the synthesis of cysteine and $\alpha$-ketobutyrate as result of its transsulphuration (oxidative methionine metabolism) (Fig. 10.3).

**Fig. 10.3.** Methionine metabolism in hepatocytes. In quiescent differentiated hepatocytes, when the levels of methionine are low, MATI (methionine adenosyltransferase I) synthesizes most SAMe required by the hepatic cells to maintain the methylated status of DNA, histones, and numerous other proteins and small molecules. The homocysteine thus generated is mainly used for methionine regeneration and only a small part is channelled through the transsulphuration pathway to be converted into cysteine and $\alpha$-ketobutyrate (aerobic methionine metabolism). On the contrary, when the levels of methionine are high (i.e. after a protein-rich meal), the excess of this amino acid will be eliminated by the concerted action of MATIII (methionine adenosyltransferase III) and GNMT (glycine N-methyltransferase), and the resulting homocysteine is mainly channelled into the transsulphuration pathway to be converted into cysteine and $\alpha$-ketobutyrate. Cysteine is then utilized for the synthesis of glutathione, while $\alpha$-ketobutyrate penetrates the mitochondria and is decarboxylated to carbon dioxide and propionyl CoA (oxidative methionine metabolism). See text for details.
This pathway involves two enzymes: cystathionine \( \beta \)-synthase (CBS), which is activated by SAMe, and cystathionase. Cysteine is then utilized for the synthesis of glutathione as well as other sulphur-containing molecules such as taurine, while \( \alpha \)-ketobutyrate penetrates the mitochondria where it is decarboxylated to carbon dioxide and propionyl CoA. Since SAMe is an inhibitor of methylene-THF reductase (MTHFR), this will prevent the regeneration of methionine after an oral load of this amino acid. At the mRNA level, SAMe maintains MAT1A expression while inhibiting MAT2A expression (Mato et al., 2002). This modulation by SAMe of both the flux of methionine into the transsulphuration pathway and the regeneration of methionine maximizes the production of cysteine and \( \alpha \)-ketobutyrate after a methionine load and minimizes the regeneration of this amino acid (Fig. 10.3).

In contrast to normal non-proliferating hepatocytes, which rely primarily on MAT1/III to generate SAMe and maintain methionine homeostasis, embryonic and proliferating adult hepatocytes – as well as liver cancer cells – instead rely on MATII to synthesize SAMe (Cai et al., 1996; Gil et al., 1996; Huang et al., 1998; Garcia-Trevijano et al., 2002). Moreover, liver cancer cells often have very low levels of GNMT and CBS expression and increased expression of MAT2B, which as mentioned above lowers the \( K_m \) for methionine and the \( K_i \) for SAMe of MATII (Huang et al., 1998; Martinez-Chantar et al., 2003). Consequently, MAT2A/MAT2B-expressing hepatoma cells have lower SAMe levels than cells expressing MAT1A favouring the regeneration of methionine and THF. This mechanism maximizes the incorporation of methionine into biomass, which in turn facilitates hepatocyte and hepatoma growth regardless of whether methionine is present in high or low amounts. By analogy with the ‘Warburg effect’, a well-known phenomenon that maximizes the conversion of glucose into biomass (facilitating rapid cell division even in the presence of enough oxygen to support mitochondrial oxidative phosphorylation) (Vander Heiden et al., 2009), we designate this process as aerobic methionine metabolism.

The finding that MAT1A, GNMT, and CBS knockout mice spontaneously develop steatosis (Lu et al., 2001; Robert et al., 2005; Martinez-Chantar et al., 2008; Varela-Rey et al., 2009a) and, in the case of MAT1A- and GNMT-deficient animals, also HCC (Martinez-Chantar et al., 2002, 2008; Liao et al., 2009) additionally
demonstrates the synchronization of methionine metabolism with lipid metabolism and hepatocyte growth. It has been observed that genetic polymorphisms that associate with reduced MTHFR activity and increased thymidylate synthase (TYMS) activity, both of which are essential in minimizing uracyl misincorporation into DNA (Fig. 10.2), may protect against the development of HCC in humans (Yuang et al., 2007). This further supports the view that this synchronization may be an adaptive mechanism that is programmed to fit the specific needs of hepatocytes, and that alterations in the appropriate balance between methionine metabolism and proliferation may be at the origin of the association of cancer with fatty liver disease.

10.4 How Does a Less Efficient Methionine Metabolism Facilitate Hepatocyte Proliferation?

From these results it becomes evident that proliferating hepatocytes and hepatoma cells are not very tolerant of high SAMe levels or converting methionine via the transsulphuration pathway to cysteine and α-ketobutyrate. The underlying question is whether a less efficient methionine metabolism is simply an adaptation to the ‘Warburg effect’, allowing the conservation of this amino acid to optimize its utilization for protein and DNA synthesis (methionine metabolism is coupled to the synthesis of dTMP, and consequently of DNA, through the folate cycle), or if it is also a primordial factor in the hepatocyte proliferation process and hepatocarcinogenesis. There is increasing evidence that SAMe works as a rheostat that regulates hepatocyte and liver cancer cell growth. As a rule, low SAMe levels facilitate hepatocyte and hepatoma cell growth, and elevated SAMe prevents cell growth. Thus, whereas in hepatoma cells over-expression of MAT2B reduces SAMe content and increases DNA synthesis, down-regulation of MAT2B expression increases SAMe content and reduces DNA synthesis (Halim et al., 1999; Martinez-Chantar et al., 2003; Yang et al., 2008). Similarly, hepatoma cells transfected with MAT1A grow more slowly than control tumour cells (Li et al., 2010), and the addition of SAMe to hepatoma cells, but not to normal hepatocytes, induces apoptosis (Ansorena et al., 2002; Lu et al., 2009). In vivo, SAMe treatment is also effective in preventing the establishment of HCC, although in a rodent model it is ineffective as a treatment for existing liver tumours (possibly due to the adaptation of the surrounding normal liver tissue to eliminate excess exogenous SAMe) (Lu et al., 2009). The mitogenic effect of HGF (hepatocyte growth factor) in hepatocytes requires the induction of both MAT2A and MAT2B expression, and is blocked by exogenous SAMe administration (Latasa et al., 2001; Martinez-Chantar et al., 2002; Luka et al., 2009; Vázquez-Chantada et al., 2009). Moreover, the administration of SAMe preceding partial hepatectomy (PH) in mice prevents the fall in SAMe concentration and impairs liver regeneration (Vázquez-Chantada et al., 2009). Intriguingly, although in hepatoma cells the mitogenic effect of leptin (an adipose tissue-derived hormone that plays a pivotal role in the progression of liver fibrogenesis and carcinogenesis) is blocked by SAMe administration, inducing apoptosis, this adipokine increases hepatic SAMe levels by about 45% despite inducing both MAT2A and MAT2B expression (Ramani et al., 2008). Our hypothesis is that for hepatoma cells, like non-hepatocytes that express only MAT2A and have low levels of SAMe, an increase in this molecule may be beneficial in supporting polyamine synthesis and growth. However, at higher doses SAMe inhibits growth and induces apoptosis. With regards to MAT2B, this protein may have other activities independent of the modulation of MATII, including the regulation of the activity of several mitogen-activated protein kinases by mechanisms that we do not know yet (Ramani et al., 2008; Yang et al., 2008). These results demonstrate that the effect of SAMe can vary drastically in normal versus malignant hepatocytes.

A signalling pathway that senses the cellular SAMe content and that involves AMPK (adenosine 5’-monophosphate (AMP) activated protein kinase) has been recently identified in hepatocytes (Fig. 10.5) (Martinez-Chantar et al., 2006; Vázquez-Chantada et al., 2009). AMPK is a serine/threonine protein kinase that plays a key role
AMPK (AMP-activated protein kinase) is a protein kinase that plays a key role in the regulation of energy homeostasis. AMPK is activated by an increase in the AMP/ATP ratio. This activation is dependent on LKB1, a protein kinase that phosphorylates AMPK. Binding of AMP is thought to be responsible for allosteric regulation of AMPK by making the enzyme less accessible to phosphoprotein phosphatase inactivation and facilitating phosphorylation by LKB1. Once activated, AMPK shuts down anabolic pathways that mediate the synthesis of proteins, lipids, and glycogen and stimulates catabolic pathways such as lipid oxidation and glucose uptake. This coordinated action of AMPK on metabolism restores ATP levels, keeping the cellular energy balance. In hepatocytes, LKB1 and AMPK are phosphorylated in response to HGF. SAMe blocks this activation by HGF of LKB1 and AMPK phosphorylation, through a mechanism possibly involving increased dephosphorylation of the enzymes, by making them more accessible to PP2A (phosphoprotein phosphatase 2A). PP2A enzymes exist as heterotrimeric complexes consisting of catalytic (PP2Ac), structural (PP2Aα), and regulatory (PP2Aβ) subunits. Different PP2Aβ subunits have been described that determine the substrate specificity of the enzyme. PP2Aβ subunit is methylated by LCMT1 (leucine carboxyl methyltransferase-1) and demethylated by PME1, a specific phosphoprotein phosphatase methylesterase. PP2Aβ methylation has no effect on PP2A activity but has a crucial role in the recruitment of specific PP2Aβ subunits to the PP2Aα,β complex and therefore on PP2A substrate specificity. AMPK activation leads to the mobilization of HuR from the nucleus (HuRn) to the cytoplasm (HuRc). HuR is a RNA-binding protein that increases the stability and translation of numerous target mRNAs involved in cell proliferation and apoptosis under conditions of stress. SAH, S-adenosylhomocysteine.
AMPK are phosphorylated in response to HGF (Fig. 10.5) (Martínez-Chantar et al., 2006; Vázquez-Chantada et al., 2009). SAMe blocks this activation by HGF of LKB1 and AMPK phosphorylation through a mechanism that may involve increased dephosphorylation of the enzymes by making them more accessible to PP2A (phosphoprotein phosphatase 2A) (Martínez-Chantar et al., 2006). PP2A enzymes exist as heterotrimeric complexes consisting of catalytic (PP2A<sub>c</sub>), structural (PP2A<sub>s</sub>), and regulatory (PP2A<sub>r</sub>) subunits (Sontag et al., 2008). Different PP2A<sub>r</sub> subunits have been described, that determine the substrate specificity of the enzyme. PP2A<sub>c</sub> subunit is methylated by SAMe-dependent leucine carboxyl methyltransferase-1 (LCMT1), and demethylated by a specific phosphoprotein phosphatase methylsterase-1 (PME1). PP2A<sub>c</sub> methylation has no effect on PP2A activity but has a crucial role in the recruitment of specific PP2A<sub>r</sub> subunits to the PP2A<sub>c</sub> complex, and therefore on PP2A substrate specificity. In hepatocytes, AMPK interacts with PP2A through a process stimulated by SAMe (Martínez-Chantar et al., 2006) and which is associated with increased PP2A methylation (MLMC, SCL, JMM, unpublished). In the brain, alterations in methionine metabolism that lead to a decrease in the ratio of SAMe/SAH (SAH is a potent competitive inhibitor of LCMT1) associate with decreased SAMe content and its association with AMPK.

Down-regulation of LKB1 or AMPK with specific RNA interference (RNAi) inhibits the mitogenic effect of HGF in hepatocytes, through a mechanism that involves HuR mobilization from the nucleus to the cytoplasm and that is inhibited by SAMe (Fig. 10.5) (Vázquez-Chantada et al., 2009). HuR is a RNA-binding protein that increases the stability and translation of numerous target mRNAs involved in cell proliferation and apoptosis under conditions of stress (Doller et al., 2008). HuR can regulate both cell proliferation (by varying the levels of proteins that control cell-cycle progression such as cyclins D1 and A2) and apoptosis (by changing the expression of pro- and anti-apoptotic proteins such as p53 and Bcl-2) (Doller et al., 2008). As a rule, elevated cytoplasmic HuR associates with cell survival and proliferation, whereas reduced cytoplasmic HuR associates with apoptosis. In MAT1A knockout (KO) mice, a reduction in hepatic SAMe content is associated with LKB1- and AMPK-hypophosphorylation, HuR mobilization to the cytoplasm, increased expression of proliferative genes and mitogenesis (Martínez-Chantar et al., 2006; Vázquez-Chantada et al., 2009). In contrast, in GNMT-KO animals the large increase in hepatic SAMe associates with LKB1- and AMPK-hyperphosphorylation, reduced cytoplasmic HuR, increased expression of apoptotic genes and cell death (Varela-Rey et al., 2009a). From this perspective, it seems that increased SAMe content is a problem when hepatocytes have to grow. This explains, at least in part, the selective advantage provided to proliferating hepatocytes by the switch from MAT1A to MAT2A expression. Uncontrolled liver proliferation is prevented because hepatocytes do not normally express MATII unless forced to do so by growth factors. Cancer cells overcome this growth factor dependence by silencing MAT1A expression (Gil et al., 1996; Avila et al., 2000).

10.5 Regulation of Methionine Metabolism is a Crucial Step in Liver Regeneration

Hepatocyte proliferation is infrequent in normal adult liver, since these cells are arrested in the G0 phase of the cell cycle (Taub, 2004). However, after PH the majority of the hepatic cells re-enter the cell cycle within hours of surgery, achieving liver mass restoration in several days. Re-entering the cell cycle after PH is preceded by a decrease in MAT1A expression and inhibition of MATI/III activity, possibly mediated by the nitric oxide and reactive oxygen substances (ROS) generated in response to the stress imposed by the loss of liver mass (Corrales et al., 1990), an increase
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in the expression of MAT2A, and a reduction of SAMe content (Huang et al., 1998; Ramani et al., 2008). This reduction in hepatic SAMe content releases the inhibition that this molecule exerts on HGF-mediated phosphorylation of LKB1 and AMPK, HuR mobilization, and the expression of mitogenic genes such as cyclin D1 (Fig. 10.5) (Vázquez-Chantada et al., 2009). These results suggest that switching to a less efficient methionine metabolism with the concomitant reduction in SAMe content is a crucial step in liver regeneration. Accordingly, SAMe administration preceding PH to prevent the fall in hepatic SAMe content during liver regeneration inhibited LKB1- and AMPK-phosphorylation, HuR mobilization, and the activation of cyclin D1, thereby blocking progression through G1 and entry into the DNA-synthesis phase of the cell cycle (Vázquez-Chantada et al., 2009). Similarly, in vivo down-regulation of GNMT with specific RNAi to prevent the fall in SAMe content following PH also induced a marked reduction in the expression of cyclin D1 (Varela-Rey et al., 2009a); and MAT1A- and GNMT-KO mice show impaired liver regeneration after PH (Chen et al., 2004; Varela-Rey et al., 2009a).

As mentioned above, a switch accompanies cell growth from complete catabolism of glucose, using mitochondrial oxidative phosphorylation to maximize the generation of ATP required for cellular processes, to aerobic glycolysis (conversion of glucose into lactate, the Warburg effect). Hepatocytes are the exception to this rule. Unlike most growing cells, after PH regenerating hepatocytes not only need to proliferate rapidly but also continue functioning to maintain metabolic homeostasis for the whole organism (Taub, 2004). This fundamental difference between proliferating hepatocytes and other growing cells is reflected in their metabolic needs. Hepatic regeneration results in a rapid mobilization of FA from the white adipose tissue to the liver, causing a transient accumulation of fat in hepatocytes. These FA are then oxidized, mainly in the mitochondria, providing the necessary ATP to support the metabolic demands of rapid proliferation (catabolic metabolism), a process that is activated by AMPK. In parallel, liver regeneration induces the expression of gluconeogenic genes (anabolic metabolism), a critical function to maintain the serum glucose level and compensate for the hypoglycaemic effect of AMPK activation (Taub, 2004). Hepatic regeneration also induces the activation of the protein kinase complex mTOR, another key energy sensor controlling gluconeogenesis and directing amino acids into protein synthesis (Taub, 2004).

Steatosis occurs when the rate of hepatic FA uptake from blood and lipogenesis is greater than the rate of FA oxidation and secretion (as TGL forming very low density lipoproteins, VLDL). Isotope-labelling experiments have shown that adipose tissue-derived FA account for up to 60% of the liver TGL in steatosis, whereas hepatic lipogenesis accounts for about 25%, and other pathways, such as impaired lipid oxidation or secretion, are less important (Parks and Hellerstein, 2006). In MAT1A-KO mice, chronic deficiency in hepatic SAMe induces hepatocyte proliferation which possibly stimulates a constant flux of FA from the adipose tissue to the liver and steatosis (Fig. 10.6). Similarly, in GNMT-KO mice the excess of hepatic SAMe triggers activation of the Ras and JAK/STAT pathways, inducing hepatocyte proliferation and possibly leading to an increased flux of FA from the adipose tissue to the liver, and to steatosis (Fig. 10.6). Additionally, altered SAMe metabolism may impair the flux of VLDL from hepatocytes to other parts of the body, as suggested by the finding that phosphatidylethanolamine methyltransferase (PEMT) KO mice are defective in VLDL secretion and develop liver steatosis (Zhao et al., 2009). Interestingly, patients with liver cirrhosis have impaired SAMe synthesis and reduced PEMT activity (Duce et al., 1988).

Hepatic steatosis has been proposed as an essential precursor of steatohepatitis, but the simple accumulation of fat in the liver is not sufficient to induce the necroinflammatory lesions associated with steatohepatitis, as seen in models of chronically obesity such as in obese (ob/ob) mice (Koteish and Diehl, 2002; Varela-Rey et al., 2009b). One of the factors associated with the progression of steatosis to steatohepatitis is the oxidative stress originated by toxic lipid peroxidation catalysed by
cytochrome P4502E1 (CYP2E1), the main enzyme involved in NADPH-dependent reduction of oxygen leading to lipid peroxidation (Lieber, 1997). The CYPs constitute a superfamily of haem-containing microsomal mono-oxygenases that play a central role in the detoxification of xenobiotics, as well as in the metabolism of endogenous compounds including FA. CYP2E1 expression is up-regulated in MAT1A-KO mouse liver (Martinez-Chantar et al., 2002). In contrast, CYP2E1 expression in GNMT-KO mouse liver is reduced, but the expression of two alternative FA-hydroxylases (CYP4A10 and CYP4A14, the two major CYP4A genes) is markedly induced (Latasa et al., 2001). It has been demonstrated that CYP4A enzymes are key intermediates of an adaptive response to perturbation of hepatic lipid metabolism. Thus, in CYP2E1-KO mice, lipid peroxidation induced by the accumulation of hepatic FA in response to a methyl-deficient diet is mediated by the up-regulation of CYP4A10 and CYP4A14 expression (Leclerq et al., 2000).

Alterations in the appropriate balance of CYPs that deal with FA oxidation may underline the predisposition to develop steatohepatitis and HCC associated with methionine deficient metabolism. A better understanding of how hepatic SAMe levels differentially interact with CYP2E1, CYP4A10, and CYP4A14 expression, and with lipid metabolism, may help to identify points for therapeutic intervention.

10.6 How do Both a Defect and an Excess of Liver SAMe Trigger HCC?

Through its regulation of the LKB1/AMPK signalling pathway, SAMe is linked to both growth control and metabolism, and through

**Fig. 10.6.** Schematic representation of fatty acid mobilization in normal regenerative liver and in the liver of animals with altered SAMe metabolism. Liver regeneration after partial hepatectomy induces the mobilization of fatty acids (FA) from the white adipose tissue to the liver causing a transient accumulation of triglycerides (steatosis). These FA are then oxidized, mainly in the mitochondria, providing the necessary ATP to support the metabolic demands of rapid proliferation. In MAT1A-KO and GNMT-KO mice, regardless of whether the liver is regenerating or not, abnormal hepatic SAMe content induces hepatocyte proliferation, which stimulates a constant flux of FA from the adipose tissue to the liver causing chronic steatosis. The increased FA flux to the liver and decreased FA oxidation results in toxic lipid peroxidation catalysed by CYP2E1 in the case of MAT1A-KO mice, and CYP4A10 and CYP4A14 in the case of GNMT-KO animals, leading to steatohepatitis (steatosis with inflammation, necrosis and fibrosis).
the methylation of DNA and histones, SAMe regulates gene expression. In addition, there is evidence indicating that SAMe also regulates proteolysis, widening its spectrum of action (Fig. 10.7). In hepatocytes, the protein levels of both prohibitin 1 (PHB1) and the apurinic/apyrimidinic endonuclease (APEX1) are stabilized by SAMe (Santamaria et al., 2003; Tomasi et al., 2009). PHB1 is a chaperone-like protein involved in mitochondrial function and APEX1 is a key protein involved in DNA repair. Proteasomal chymotrypsin- and caspase-like activities are increased in MAT1A-KO livers and cultured hepatocytes, but were blocked by SAMe treatment (Tomasi et al., 2010). Moreover, SAMe inhibits chymotrypsin- and caspase-like activities in 20S proteasomes and causes rapid degradation of some of the 26S proteasomal subunits (Tomasi et al., 2010). Recently, methylated proteins have been identified in mammalian 20S proteasome complex (Zong et al., 2008), suggesting that this may be a key regulatory mechanism of proteasome function.

Under normal conditions, liver SAMe content is low enough not to induce aberrant DNA and histone hypermethylation, which would result in epigenetic modulation of specifics and critical carcinogenic pathways, such as the Ras and JAK/STAT signalling pathways. Nevertheless it is sufficiently elevated to avoid the spontaneous activation of the LKB1/AMPK signalling pathway resulting in uncontrolled hepatocyte growth and, possibly, undesired proteolysis. In MAT1A-KO mice, liver SAMe content is reduced chronically about threefold, resulting in the continuous activation of the LKB1/AMPK signalling pathway, abnormal proteasomal activity, and inefficient DNA repair. This leads to uncontrolled hepatocyte growth and HCC formation (Lu et al., 2001; Martínez-Chantar et al., 2002). Tumorigenic progenitor oval cells (OC), capable of forming epithelial tumours, have been isolated from MAT1A-KO mouse liver (Rontree et al., 2008; Ding et al., 2009), indicating that liver cancer stem cells contribute to carcinoma formation in this mouse model of SAMe deficiency. OC are liver stem cells found in the nonparenchymal fraction of the liver, and reside near the terminal bile ducts, at the hepatocyte–cholangiocyte interface. In normal adult liver, OC are quiescent and few in number, and proliferate only during severe, prolonged liver injury and in various models of experimental carcinogenesis (Lee et al., 2009). OC-derived carcinomas tend to have a more aggressive phenotype, with as many as 50% of HCC defined as having a progenitor cell phenotype. Related to this is a recent

![Fig. 10.7. Schematic representation of the spectrum of SAMe action in the liver. The modulation of the activity of critical genes and proteins by modification of their methylation status supports a model in which changes in cellular pools of hepatic SAMe, fluctuating in response to a variety of conditions, regulates key cellular functions such as hepatocyte growth and differentiation, oval cell proliferation, apoptosis, proteasomal activity, oxidative stress and lipid and carbohydrate metabolism.](image-url)
report finding that patients with HCC where MAT1A expression was silenced had more aggressive tumours and shorter survival (Calvisi et al., 2007).

In GNMT-KO mice, hepatic SAMe content is chronically increased about 50-fold, resulting in the inhibition of the LKB1/AMPK signalling pathway and increased apoptosis (Martinez-Chantar et al., 2008; Varela-Rey et al., 2010). These supra-physiological levels of hepatic SAMe also induce loss of expression of the Ras and JAK/STAT inhibitors RASSF1, RASSF4, SOCS1, SOCS2, SOCS3, and CIS, resulting in uncontrolled growth and HCC (Martinez-Chantar et al., 2008). These inhibitors of the Ras and JAK/STAT signalling pathways, which are frequently inactivated by promoter methylation, have been involved in carcinogenesis, including HCC formation (Calvisi et al., 2006). In GNMT-KO mouse liver the methylation of RASSF1 and SOCS2 promoters has been demonstrated, as well as the hypermethylation of H3K27 (lysine 27 in histone 3) (Martinez-Chantar et al., 2008), which is also linked to gene repression and carcinogenesis (Calvisi et al., 2006). Tumorigenic OC have also been identified in GNMT-KO mouse liver (Martinez-Chantar et al., 2010).

This effect of GNMT silencing on HCC formation is possibly related to the more common phenomenon of methionine auxotrophy in cancer cells. Methionine auxotrophy (or methionine stress) is defined as the inability of a cell to grow in a medium devoid of methionine but containing instead homocysteine (Kokkinakis et al., 2006). Most cancer cells, but not normal cells, are methionine dependent (Kokkinakis et al., 2006). The biochemical basis of methionine dependency of tumour cells is not totally clear. Normal cells grow slowly and possibly have lower needs of methionine for protein synthesis than cancer cells, being able to cover their need for this amino acid by regenerating methionine from homocysteine. In contrast, an imbalance may exist in tumour cells between the needs of methionine and the capacity of the cells to regenerate this amino acid from homocysteine. As a consequence, tumour cells cannot compensate for a relative deficiency in methionine and therefore cease growing. From this perspective, silencing MAT1A, GNMT, and CBS (a most efficient pathway to remove methionine from protein synthesis and convert it to cysteine and α-ketobutyrate) will facilitate liver tumour cell growth.

GNMT is also present in large amounts in the prostate (Mato et al., 2008; Luka et al., 2009). GNMT-KO mice, however, do not develop prostate pathology, indicating that the function of GNMT in this organ is different than that in the liver. Interestingly, down-regulation of GNMT with RNAi results in a significant reduction in cell invasion in DU145 prostate cancer cells (Sreekumar et al., 2009), demonstrating that when it comes to methionine metabolism and cancer, generalization is very difficult.

10.7 Does Changing the Metabolism of Hepatocytes through Manipulation of Methionine Metabolism Hold Promise for Improving HCC Prognosis?

An explanation of Best’s effect is that where the cellular levels of SAMe and the activity of the transsulphuration pathway are maintained low, a less efficient methionine metabolism enhances the capacity of hepatocytes and hepatoma cells to utilize nutrients for anabolic processes, and supports growth. In principle, this dependency of HCC on methionine metabolism may be utilized for cancer treatment. Understanding how MAT1A, GNMT, and CBS are silenced and how MAT2A and MAT2B are activated in hepatoma cells may identify potential points for therapeutic intervention. Recently, we have provided some mechanistic insight into how this switch between MAT1A and MAT2A occurs (Vázquez-Chantada et al., 2010). It was first observed that under a variety of conditions (embryonic liver development, hepatocyte growth, and liver regeneration), the timely switch from MAT1A to MAT2A coincides with an increase in the expression of the mRNA binding proteins HuR and AUF1. We also observed that HuR associates with MAT2A increasing its stability, while AUF1 associates with MAT1A mRNA decreasing its
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stability. Understanding how HuR and AUF1 expression is regulated during NAFLD progression and hepatocarcinogenesis may identify potential points for therapeutic intervention. Additionally, understanding how SAMe interacts with HGF-stimulated LKB1/AMPK/HuR signalling may better define how alterations in methionine metabolism lead to fatty liver, abnormal growth, and increased cancer predisposition. Furthermore, a better understanding of how liver regeneration mobilizes FA from the adipose tissue to the liver and of how SAMe interacts with CYP2E1, CYP4A10, and CYP4A14 expression and, perhaps, with VLDL secretion, may help to prevent the progression of fatty liver disease from steatosis to steatohepatitis. The finding that SAMe regulates proteolysis in the liver has widened its spectrum of action, opening new and unexpected areas of clinical interest. From this perspective, maintaining a tight control of hepatic SAMe and methionine metabolism is likely to have a major impact in the development and progression of fatty liver disease and on the predisposition to cancer in these patients. Both patients with alcoholic and non-alcoholic liver cirrhosis have low MAT1A expression and MATI/III activity (Cabrero et al., 1988; Duce et al., 1988; Corrales et al., 1990), and SAMe treatment has been shown to increase survival in patients with alcoholic liver cirrhosis (Mato et al., 1999). Additionally, patients with HCC where MAT1A expression is silenced have more aggressive tumours and shorter survival (Calvisi et al., 2007). Treatment of GNMT-KO mice with nicotinamide, a substrate of nicotinamide N-methyltransferase – an enzyme mainly expressed in the liver (Cantoni, 1951) – leads to the normalization of hepatic SAMe content and prevents liver injury. The clinical importance of this finding is obvious, since children with mutations that lead to a drastic reduction of GNMT develop liver injury (Mudd et al., 2001; Augoustides-Savvopoulou et al., 2003).

10.8 Conclusions

Mammalian cells metabolize methionine by converting it into SAMe, the main biological methyl donor. Widespread methylation of DNA and proteins indicates methylation is important in controlling cell function. Changes in the availability of methionine could directly affect the methylation status, and hence the activity of critical substrates. The issue then is how, after an increase in dietary methionine, mammals metabolize it and, at the same time, control SAMe content intracellularly. This is crucial for the liver, where methionine is mainly metabolized. Two genes, MAT1A and GNMT, play critical roles in controlling methionine metabolism and hepatic SAMe content. MAT1A- and GNMT-KO mice spontaneously develop NAFLD and HCC. SAMe-regulation of AMPK, and proteasomal activity, as well as that of DNA and histone methylation, have been causally implicated in these animal models and in human studies. Changing the metabolism of methionine in hepatocytes through manipulation of methionine metabolism provides potential for improving NAFLD and HCC prognosis.

10.9 Financial Support

This work is supported by grants from NIH AT-1576 (to S.C.L., M.L.M-C. and J.M.M.); SAF 2008-04800, HEPADIP-EULSHM-CT-205, and ETORTEK-2008 (to J.M.M. and M.L.M.-C); and from Sanidad Gobierno Vasco 2008111015 (to M.L.M.-C). CIBERehd is funded by the Instituto de Salud Carlos III.

References


Part II

Dynamics
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11 Amino Acid Transport Across Each Side of the Blood–Brain Barrier

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11.1 Abstract

Brain capillary endothelial cells form the blood–brain barrier (BBB). They are connected by extensive tight junctions, and are polarized into luminal (blood-facing) and abluminal (brain-facing) plasma membrane domains. The polar distribution of transport proteins mediates amino acid (AA) homeostasis in the brain. The existence of two facilitative transporters for neutral amino acids (NAA) on both membranes provides the brain access to essential AA. Four Na+-dependent transporters of NAA exist in the abluminal membranes of the BBB. Together these systems have the capability to actively transfer every naturally occurring NAA from the extracellular fluid (ECF) to endothelial cells and thence to the circulation. The presence of Na+-dependent carriers on the abluminal membrane provides a mechanism by which NAA concentrations in the ECF of brain are maintained at about 10% of those of the plasma. Also present on the abluminal membrane are at least three Na+-dependent systems transporting acidic AA (EAAT) and a Na+-dependent system transporting glutamine (N). Facilitative carriers for glutamine and glutamate are found only in the luminal membrane of the BBB. This organization promotes the net removal of acidic and nitrogen-rich AA from brain, and accounts for the low level of glutamate penetration into the central nervous system (CNS). The presence of a γ-glutamyl cycle at the luminal membrane and Na+-dependent AA transporters at the abluminal membrane may serve to modulate movement of AA from blood to brain. The γ-glutamyl cycle is expected to generate pyroglutamate within the endothelial cells. Pyroglutamate stimulates secondary active AA transporters at the abluminal membrane, thereby reducing net influx of AA to the brain. It is now clear the BBB participates in the active regulation of the AA content of the brain.

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11.2 Introduction

The brain is sheltered from the changing metabolite concentrations in the blood by a blood-brain barrier (BBB) that surrounds the entire central nervous system (CNS) including the spinal cord (Fig. 11.1). The BBB is necessary to provide an optimal chemical environment for cerebral function. Several layers exist between blood and brain: capillary endothelial cells; a basement membrane comprising type IV collagen, fibronectin, and laminin that completely covers the capillaries; pericytes that are embedded in the basement membrane; and astrocyte processes that surround the basement membrane. Each of these layers could, potentially, restrict the movement of solutes (Fig. 11.2).

Endothelial cells were demonstrated to be the site of the BBB when it was observed that horseradish peroxidase could not pass the endothelial layer from either direction (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Brightman et al., 1971). Pappenheimer (1970a) challenged this concept, arguing that the astrocytes were a more likely site of the barrier. Crone decided the issue by demonstrating that brain capillaries from amphibians, which have no surrounding layer of astrocytes, have high electrical impedance, =2000 Ω x cm² indicative of a restriction to the movement of ions. The cerebral endothelium is now accepted as the site of the BBB in higher animals (Crone and Olesen, 1982).

Cerebral capillary endothelial cells differ from other mammalian capillary endothelial cells; they have few cytoplasmic vesicles, more mitochondria (Oldendorf and Brown, 1975), and a larger number of tight junctions between overlapping cells. The tight junctions inhibit paracellular movement, prevent membrane molecules from moving from one cell to another (van Meer et al., 1986), and divide the membranes of the endothelial cells into two distinct sides: luminal (blood side), and abluminal (brain side) (van Meer and Simons, 1986). Different populations of both lipids and intrinsic proteins (e.g. transporters) exist on the luminal and abluminal side (Betz and Goldstein, 1978; Betz et al., 1980; Lewes and Galla, 2001). Therefore, hydrophilic nutrients must pass two sheaths of membrane, the combined characteristics of which determine which particles traverse the barrier and how quickly. Pappenheimer and Setchell (1973) recognized the implication of molecules having to pass two membranes in series to gain entry to the CNS, and Oyler et al. (1992) demonstrated the effect of two membranes in series by computer simulation.

Various methods are used to study the transport of solutes across the BBB in vivo and in vitro including single-pass indicator diffusion (Crone, 1963, 1965); the brain uptake index (Oldendorf, 1970); in situ brain perfusion (Takasato et al., 1984); isolated brain microvessels (Brendal and Meezan, 1974; Goldstein et al., 1975); and cultured endothelial cells (DeBault and Cancilla, 1980; Vinters et al., 1985; Dehouck et al., 1992). These techniques give valuable information about transport, but they did not distinguish between the different functions of the luminal and abluminal membranes.

Fig. 11.1. The blood–brain barrier extends throughout the central nervous system. This sagittal section through a mouse shows the distribution pattern of 1131 labelled Renografin®, an hydrophilic dye that does not pass the blood–brain barrier, 15 min after injection. All tissues take up the dye except the entire central nervous system including the spinal cord (Nair and Roth, 1961). Photo courtesy of Professor V. Nair, Rosalind Franklin University of Medicine and Science. (A similar figure was previously published in IUBMB Life.)
The studies in vivo led to the concept that the BBB, at least with regard to metabolites, was a passive system. The various facilitative transporters were considered to play a role in the regulation of brain metabolism through their ability to limit access (Pardridge, 1983). On the other hand it was known that active transport of ions exists. Bicarbonate and other...
ions are actively secreted across the BBB (Pappenheimer, 1970b; Bradbury, 1979). (Na⁺/K⁺)-ATPase is present in the abluminal membrane (Vorbrodt, 1988). One of the most important functions of (Na⁺/K⁺)-ATPase is to maintain the high concentration gradient of Na⁺ (external > internal) so Na⁺-dependent transport can occur. Furthermore, cerebral endothelial cells have a high density of mitochondria compared with other endothelial cells and, therefore, the capacity for greater energy production (Oldendorf and Brown, 1975).

11.3 A New Approach to Studying the BBB

Betz et al. (1980) developed a procedure to separate the respective plasma membrane domains: they convincingly demonstrated a polarity between the two sides. Sánchez del Pino and associates recognized the potential of using these membranes to study transport under controlled conditions (Sánchez del Pino et al., 1992, 1995a,b). On isolation, luminal and abluminal membranes form sealed spherical vesicles that are predominantly right-side-out, and are suitable for the study of transport in vitro (Sánchez del Pino et al., 1992, 1995b) (Fig. 11.3). The isolated membranes maintain functional transport properties, and thus may be used to characterize the contribution of each membrane domain to BBB activity under controlled conditions in vitro. For instance the ionic composition inside and outside the vesicles permits the exploration of such conditions as the influence of the transmembrane potential and Na⁺-dependence. This advance allowed the study of the BBB in a completely different manner and resulted in a change in the concept of the BBB and the synergy between its two membranes. The following sections illustrate that the BBB is an active participant in the regulation of the brain’s amino acid (AA) content.

11.4 Facilitative Amino Acid Transporters of the BBB

Early studies of AA transport in vivo identified facilitative transporters on the luminal
membrane that were saturable and stereoselective (Oldendorf, 1971a,b, 1973). Luminal carriers of AA have no dependence on Na⁺ gradients (Battistin et al., 1971; Oldendorf, 1971; Schain and Watanabe, 1972; Sershen and Lajtha, 1976; Christensen 1979; Smith and Stoll 1998). Three broad classes of facilitative carriers exist: large neutral amino acids (LNAA), cationic AA (CAA) and acidic AA (AAA) (Oldendorf and Szabo, 1976). Currently, four facilitative carriers have been identified: L1, y⁺, x⁻, and n. L1 and y⁺ are present in both membranes (Sánchez del Pino et al., 1995a), while x⁻ and n are restricted to the luminal membrane (Lee et al., 1998).

### 11.4.1 Facilitative transport of large essential neutral amino acids: system L1

Early studies of transport in vivo revealed a distinct pattern of LNAA uptake by the brain: movement of essential NAA (neutral amino acids) from blood to brain was greater than non-essential NAA (Battistin et al., 1971; Oldendorf, 1971b); the movements of the latter were minimal (Oldendorf and Szabo, 1976). Transport was facilitative, Na⁺ independent and NAA were preferred (Oldendorf and Szabo, 1976). Therefore, the carrier seems to belong to the L-system (leucine preferring) originally described by Oxender and Christensen (Oxender and Christensen, 1963) and it is probably the high affinity form currently referred to as L1 (Smith and Stoll, 1998; Boado et al., 1999; Segawa et al., 1999; Killian and Chikhale, 2001). Measurements in membranes indicate L1 is present in both membranes in a 2:1 ratio (luminal-to-abluminal) (Sanchez del Pino et al., 1995). The substrates carried by L1 include leucine, valine, methionine, histidine, isoleucine, tyrosine, tryptophan, phenylalanine and threonine, most of which are essential. The affinity constants (Kₘ) are in the μM range and similar to the plasma concentrations (Smith and Stoll, 1998). Glutamine has also been described as a substrate of L1, but glutamine transport is not completely inhibited by BCH1 (2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid), a specific inhibitor of the L1 system. Therefore it seems likely that glutamine is also transported by system N (Lee et al., 1998).

L1 is undoubtedly the most important source by which essential NAA gain access to the brain. Fernstrom and Wurtman (1972) demonstrated the important role of the L1 system and the competition amongst LNAA by showing that brain tryptophan and serotonin contents were correlated with the ratio of tryptophan-to-LNAA existing in plasma. They concluded that competition between tryptophan and other LNAA for entry to the brain is an important factor in determining the content of serotonin in brain.

### 11.4.2 Facilitative transport of cationic amino acids: system y⁺

Smith (1991) concluded that system y⁺ is the primary CAA transporter of the BBB from experiments conducted in vivo that examined the BBB only from the luminal side. More recent study of plasma membranes isolated from bovine brain microvessels allowed characterization of the CAA transporters on both sides of the BBB to be studied in close detail (O’Kane et al., 2006).

Two families of proteins: Cat, commonly referred to as the system y⁺, and Bat, which comprise systems b⁰⁺⁺ (Van Winkle et al., 1985, 1988, 1990), B⁰⁺ (Van Winkle et al., 1985), and y⁻ (Deves et al., 1998; Deves and Boyd, 1998; Palacin et al., 1998) have been found to transport CAA in various tissues. Transporter system B⁰⁺⁺ is the only Na⁺-dependent carrier that carries CAA, as well as some NAA (although with less affinity) (Van Winkle et al., 1985). There is no evidence for system B⁰⁺ in the BBB and no evidence of Na⁺-dependence of CAA transport (O’Kane and Hawkins 2003; O’Kane et al., 2006). In this regard the CAA are unique because all other naturally occurring AAA and NAA examined to date have Na⁺-dependent transporters on the abluminal membrane that are capable of coupling the Na⁺ gradient existing between the extracellular fluid (ECF) of brain and BBB endothelial cells to transport AA out of the ECF (Hawkins
Facilitative transport seems to be the only mechanism in the BBB to allow the movement of CAA.

No evidence was found for the presence of Na⁺-independent systems b₀, or of y'L systems in the BBB (O’Kane et al., 2006). Therefore as posited by Smith (1991) only system y⁺ is available to transport CAA. In addition to transporting CAA, y⁺ exhibits weak interactions with NAA if Na⁺ is present and hence it is referred to as y⁻ (White, 1985; Mann et al., 2003). In the BBB y⁺ may transport several essential NAA (phenylalanine, threonine, histidine, valine and methionine) as well as non-essential NAA (serine, glutamine, alanine, and glycine) but the affinity constants are about tenfold greater than those of system L₁ (O’Kane et al., 2006). Thus while y⁺ may contribute to the ‘first-order’ transport component observed in studies of AA transport (Pardridge, 1983), system L₁ must be considered the principal provider of essential NAA while y⁻ is primarily a purveyor of arginine.

The ability of system y⁺ to transport several non-essential amino acids (serine, glutamine, alanine, and glycine), with affinity values similar to their plasma concentrations, may explain the small, but finite, permeability of the BBB to small NAA (Oldendorf, 1971a).

Both membranes of the BBB contain y⁺, but its activity is greater on the abluminal side and it is voltage sensitive (O’Kane et al., 2006). The affinity of y⁺ is much greater for arginine compared with the other CAA and y⁺ is probably important in the provision of arginine for nitric oxide (NO) synthesis, a diffusible gas, originally called endothelium-derived relaxing factor. NO regulates numerous physiological actions including smooth muscle contraction, blood flow and pressure (Palmer et al., 2003). The biosynthesis of NO requires L-arginine and O₂ for the NO synthase (NOS) catalysed reaction. Three isoforms of NOS have been identified: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) (Wu and Meininger, 2002). Real-time PCR and Western blotting techniques established the presence of all three known NOS in cerebral endothelial cells, suggesting that NO can be produced in brain endothelial cells (O’Kane et al., 2006).

Endothelial cells do not have the ability to synthesize arginine de novo (Wu and Meininger, 1993). They must therefore rely on an external source of arginine; it seems likely that the availability of arginine is the determining factor in NO production by endothelial cells (Wu and Meininger, 1993, 2002).

### 11.4.3 Facilitative transport of glutamine: system n

Lee et al. (1998) described facilitative transport of glutamine across the luminal membrane of the BBB that was not inhibited by BCH and did not demonstrate transstimulation. This transport system is similar to system n described in hepatic plasma membrane vesicles (Pacitti et al., 1993). The BBB system n is inhibited by asparagine and histidine (Hawkins et al., 2006a) as was found in hepatic vesicles by Pacitti et al. (1993). System n exists solely on the luminal membrane (Lee et al., 1998).

### 11.4.4 Facilitative transport of acidic amino acids: system x₅⁻

Benrabh and Lefauconnier (1996) studied glutamate uptake in vivo and found no evidence of Na⁺-dependent transport when glutamate was presented to the luminal membrane. They concluded the carrier was facilitative and probably the x₅⁻ form because no evidence for the cystine-glutamate exchanger x₅⁻ could be found: this was confirmed in isolated luminal membranes. Cystine did not compete with glutamate for uptake while aspartate did. Furthermore, cystine did not accelerate glutamate uptake in vesicles preloaded with 2 mmol L-cystine (Hawkins et al., 2006a).

Lee et al. (1998) measured facilitative glutamate transport in both luminal and abluminal membranes, and found facilitative glutamate transport only on the luminal border in
a position to allow the release of glutamate from endothelial cells to the plasma.

A compilation of the substrates carried by the various facilitative systems is presented in Table 11.1 and their kinetic characteristics in Table 11.2. The organization of the transporters is depicted in Fig. 11.4.

### Table 11.1. Amino acids transported by facilitative transport systems.

<table>
<thead>
<tr>
<th>System</th>
<th>L1</th>
<th>y*</th>
<th>n</th>
<th>x*-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-essential</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Arginine</td>
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<td></td>
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<tr>
<td>Ornithine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Essential in brain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Histidine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
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<td></td>
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<tr>
<td>Isoleucine</td>
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<td></td>
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<tr>
<td>Phenylalanine</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Tyrosine</td>
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<td></td>
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<tr>
<td>Tryptophan</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

AA transported, or shown to inhibit transport, are indicated by a +. Facilitative transport (weak) of NAA by y* in the presence of Na* is indicated by *. Systems L1 and y* exist on both membranes while systems x*- and n are restricted to the luminal membrane (Lee et al., 1998). AA in italics are essential in brain (Laterra et al., 1999). The distribution of these transporters is depicted in Fig. 11.4.

### Table 11.2. Kinetic characteristics of facilitative amino acid transporters on the blood-brain barrier.

<table>
<thead>
<tr>
<th>Transporter (substrate)</th>
<th>Apparent $K_m$ (mmol l⁻¹)</th>
<th>Apparent $V_{max}$ (pmol mg⁻¹ min⁻¹)</th>
<th>Clearance (µl mg⁻¹ min⁻¹)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 (Phe)</td>
<td>0.012 ± 0.02</td>
<td>94 ± 9</td>
<td>8</td>
<td>Luminal &amp; abluminal</td>
</tr>
<tr>
<td>y* (Lys)</td>
<td>0.8 ± 0.3</td>
<td>5800 ± 1600</td>
<td>7</td>
<td>Luminal &amp; abluminal</td>
</tr>
<tr>
<td>n (Gln)</td>
<td>1 ± 0.5</td>
<td>1100 ± 230</td>
<td>1</td>
<td>Luminal</td>
</tr>
<tr>
<td>x*- (Glu)</td>
<td>0.9 ± 0.9</td>
<td>700 ± 300</td>
<td>1</td>
<td>Luminal</td>
</tr>
</tbody>
</table>

The radiolabelled substrate used for measurements are in parentheses. Clearance was calculated to the nearest integer as $V_{max} / K_m$. Values were taken from Sánchez del Pino et al. (1992, 1995b).

### 11.5 Amino Acid Gradients between Brain and Plasma

The concentrations of all naturally occurring AA in the cerebrospinal fluid (CSF), presumably similar to the ECF, with the exception of glutamine, are about 10% or less than the plasma concentrations (Fig. 11.5) (Laterra et al., 1999). This situation cannot be explained by the consumption of AA by the brain because the arteriovenous differences of most AA across the brain are imperceptible (Felig et al., 1972; Drewes et al., 1977; Sacks et al., 1982) as are the arteriovenous differences of ammonia (NH₄⁺), a by-product of AA catabolism (Cooper and Plum, 1987). These observations indicate that AA leave the brain against a concentration gradient. From this it may be concluded that active (e.g. Na*⁻-dependent) systems on the abluminal membrane have an important role in maintaining both homeostasis of brain AA content as well as the lower concentration in the extracellular fluid. Based on similar observations Bradbury wrote ‘there is a strong indirect argument in favour of the hypothesis that most AA must be moved against a concentration gradient from interstitial fluid to blood’ (Bradbury, 1979).

### 11.6 Na*⁻-dependent Transport Systems of the BBB

Several Na*⁻-dependent systems have been identified to date in the abluminal membrane of the BBB. They include: A (alanine preferring), which was first characterized and shown to actively transport small non-essential NAA (Betz and Goldstein, 1978; Sanchez del Pino et al., 1992; O'Kane et al., 2004); ASC (alanine,
Fig. 11.4. Amino acid transporters of the BBB. The brain gains access to all essential AA through the facilitative systems L1 and $y^+$ that exist on each membrane. Facilitative transporters $x_{G^-}$ and $n$ exist only on the luminal membrane and in a position to allow glutamate, aspartate and glutamine egress. Each facilitative transporter carries several substrates (see Table 11.1). The Na⁺-dependent transport systems provide mechanisms for the elimination of non-essential AA, toxic AA, as well as maintaining the optimal concentrations of all other AA. As with the facilitative systems there is considerable overlap of substrates (please see Table 11.3). All naturally occurring AA, except basic AA, are transported by at least one system and some by as many as three, A, Na⁺-dependent system A; N, Na⁺-dependent system N; EAAT, Na⁺-dependent glutamate transporter; $x_{G^-}$, facilitative glutamate transporter; $n$, facilitative glutamine transporter. (A similar figure was previously published in the Journal of Nutrition.)

Fig. 11.5. Amino acid concentrations in plasma and brain. The plasma and CSF concentrations were grouped and the CSF-to-plasma ratio expressed as percentages of the plasma. CSF concentrations are assumed to approximate brain ECF (Davson and Welch, 1971; Laterra et al., 1999). With the exception of glutamine, the concentrations of all AA in the ECF are much lower than the concentrations of AA in plasma. (A similar figure was previously published in the Journal of Nutrition.)
serine, and cysteine preferring) (Tayarani et al., 1987; Hargreaves and Pardridge, 1988; Tovar et al., 1988; O’Kane et al., 2004); N (e.g. glutamine, asparagine, and histidine preferring) (Lee et al., 1998; O’Kane et al., 2004); the excitatory acidic AA family (EAAT, e.g. aspartate and glutamate preferring) (Hutchison et al., 1985; O’Kane et al., 1999); and a recently described system that primarily transports essential LNAA (O’Kane and Hawkins, 2003). The latter system has not been named and is referred to as Na+-LNAA.

Na+-dependent transport of AA exists only in abluminal membranes. No Na+ dependency has been detected in luminal membranes, which appear to have only facilitative carriers (Oldendorf, 1971a,b; Sershen and Lajtha, 1976; Christensen, 1979; Smith and Stoll, 1998). Therefore, the Na+-dependent transporters are in a position to remove AA from the brain, utilizing the Na+-gradient that exists between the ECF and the endothelial cells of brain capillaries comprising the BBB.

11.6.1 Na+-dependent transport of large neutral amino acids: system Na+-LNAA

Initial studies by Sánchez del Pino et al. (1995b) found Na+-dependent phenylalanine transport that was inhibited by BCH. Studies by Van Winkle et al. (1985) had demonstrated that system B°, is a Na+-dependent carrier which recognizes NAA and is inhibited by BCH. Because of this characteristic and the observed inhibition, the authors thought carrier system B°, was likely to be responsible for the transport activity. A characteristic of system B°, is the ability to transport CAA (Van Winkle et al., 1985). However, the rate of lysine transport was not inhibited by the presence of BCH of up to 10 mmol l⁻¹ concentrations, casting doubt on the presence of system B°, (O’Kane and Hawkins, 2003). Further investigation led to the discovery of Na+-LNAA as the carrier responsible for the BCH-inhibited, Na+-dependent phenylalanine transport and other LNAA (O’Kane and Hawkins, 2003).

Na+-LNAA was discovered as a distinct transporter in abluminal membrane microves- sels, and its kinetic characteristics cannot be ascribed to any other currently known systems (O’Kane and Hawkins, 2003). Na+-LNAA has a high affinity for leucine (Kₘ = 21 μM ± 7 μM) and is inhibited by other NAA including glutamine, histidine, methionine, phenylalanine, serine, threonine, tryptophan and tyrosine. Transport is Na+- dependent, voltage sensitive, and inhibited by BCH. The spectrum of AA carried by Na+-LNAA is similar to the facilitative system L1 that allows the entry of essential LNAA down their concentration gradients (compare Tables 11.1 and 11.3). The presence of a Na+-dependent carrier on the abluminal membrane, capable of removing LNAA (most of which are essential) from the brain, seems to provide a mechanism for the control of the LNAA content of brain.

11.6.2 Na+-dependent transport of small non-essential neutral amino acids: system A

The activity of system A, named for its preference for transporting alanine (Oxender and Christensen, 1963) may be distinguished from other Na+-dependent carriers by its acceptance of MeAIB (N-methylamino-isobutyric acid) as a unique substrate (Christensen et al., 1967). System A is voltage sensitive; three positive charges are translocated per MeAIB molecule (O’Kane et al., 2004). System A is inhibited by small non-essential AA such as proline, alanine, histidine, serine, asparagine and glutamine, as well as by the essential AA histidine. Some laboratories (Oxender and Christensen, 1963; Betz and Goldstein, 1978) reported a similar AA spectrum for system A but also included glycine. Glycine transport was not mediated by system A in isolated membrane vesicles but was a putative substrate of system ASC (O’Kane et al., 2004).

ASC activity was measured in abluminal membranes, after blocking system A with MeAIB, confirming the findings of others who have reported its presence (Tayarani et al., 1987; Hargreaves and Pardridge, 1988; Tovar et al., 1988). In addition to alanine, serine,
cysteine and glycine, several essential AA were putative substrates including methionine, valine, leucine, isoleucine and threonine (O’Kane et al., 2004). ASC activity is independent of the transmembrane potential (O’Kane et al., 2004).

### 11.6.4 Na+-dependent transport of nitrogen-rich amino acids: system N

System N has a preference for NAA that are nitrogen-rich, such as glutamine, histidine and asparagine, hence its designation (Kilberg et al., 1980, 1993). BBB abluminal membranes also transport serine via this system. System N was not affected by the transmembrane potential (O’Kane et al., 2004). Li+ could substitute for Na+, suggesting that system N in the BBB is similar to system N in liver cells (Kilberg et al., 1980; O’Kane et al., 2004).

### 11.6.5 Na+-dependent transport of acidic amino acids: the EAAT family

Na+-dependent glutamate transporters exist on the abluminal membrane. They are voltage dependent, and collectively have an apparent $K_m$ of 14 μM at a transmembrane potential of −61 mV (Lee et al., 1998; O’Kane et al., 1999). Analysis of mRNA demonstrated that three transporters were expressed (EAAT1, 2, and 3) in brain capillary endothelial cells. Western blot analysis confirmed the glutamate transporters to be present only on the abluminal membranes; none were detectable on luminal membranes (O’Kane et al., 1999). The activity of the three transporters was 1:3:6, EAAT1: EAAT2: EAAT3, respectively. Collectively the EAAT family is the most powerful of the Na+-dependent AA transporters; they show the greatest ability to clear AA at low concentrations (Table 11.4).

### 11.7 Organization of the Various Transport Systems

The brain gains access to all essential AA through the facilitative systems L1 and y+.

There is considerable substrate overlap within the facilitative systems as well as within the Na+-dependent systems (tables 11.1 and 11.3).

Five Na+-dependent AA transport systems are present exclusively on the abluminal membrane of the BBB (Fig. 11.4), and the capacities of these transporters are similar or greater than those of the facilitative transporters. Because the electrochemical gradient for Na+ is oriented to flow from the extracellular fluid into the endothelial cells, these Na+-dependent transport systems are in a position to export AA from the brain extracellular fluid.

Thus, AA that pass both endothelial cell membranes and enter the basement membrane space could be actively, and selectively, pumped back across the abluminal membrane. This asymmetrical distribution of Na+-dependent carriers has the potential, therefore, to reduce the content of AA in brain.

| Table 11.3. Amino acids transported by Na+-dependent systems of the abluminal membrane. |
|---------------------------------|----------|----------|--------|---|
| System                         | A  | N   | ASC  | Na+-LNAAS     | EAAT |
| Non-essential                  |    |     |      | +            |     |
| Glycine                        |    | +   | +    | +            |     |
| Alanine                        |    | +   | +    | +            |     |
| Serine                         |    | +   | +    | +            |     |
| Proline                        |    | +   |      |             |     |
| Asparagine                     |    | +   | +    | +            |     |
| Glutamine                      |    | +   | +    | +            |     |
| Aspartate                      |    | +   |      |             | +   |
| Glutamate                      |    |     |      |             | +   |
| Essential in brain             |    |     |      |             |     |
| Histidine                      |    | +   |      |             |     |
| Threonine                      |    | +   |      |             |     |
| Cysteine                       |    | +   |      |             |     |
| Methionine                     |    | +   |      |             | +   |
| Valine                         |    | +   |      |             | +   |
| Leucine                        |    | +   |      |             | +   |
| Isoleucine                     |    | +   |      |             | +   |
| Phenylalanine                  |    | +   |      |             |     |
| Tyrosine                       |    | +   |      |             |     |
| Tryptophan                     |    |     |      |             | +   |

AA that are transported, or shown to inhibit transport, are indicated by a +. Values for systems A, N, and ASC are from O’Kane et al. (2004). Values for Na+-LNAAS are from O’Kane and Hawkins (2003). Values for the EAAT1-3 family are from O’Kane et al. (1999). AA in italics are essential in brain (Laterra et al., 1999).


### Table 11.4. Kinetic characteristics of Na\(^+\)-dependent amino acid transporters in abluminal membranes.

<table>
<thead>
<tr>
<th>Transporter (substrate)</th>
<th>Apparent $K_m$ (mmol L(^{-1}))</th>
<th>Apparent $V_{max}$ (pmol mg(^{-1}) min(^{-1}))</th>
<th>Clearance (µl mg(^{-1}) min(^{-1}))</th>
<th>Voltage sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (MeAlB)</td>
<td>0.4 ± 0.16</td>
<td>500 ± 60</td>
<td>1*</td>
<td>Yes</td>
</tr>
<tr>
<td>N (Gln)</td>
<td>1.3 ± 0.4</td>
<td>4400 ± 700</td>
<td>3</td>
<td>No</td>
</tr>
<tr>
<td>ASC (Ala)</td>
<td>0.11 ± 0.06</td>
<td>660 ± 70</td>
<td>6</td>
<td>No</td>
</tr>
<tr>
<td>Na(^+)-LNAA (Leu)</td>
<td>0.021 ± 0.007</td>
<td>114 ± 6</td>
<td>5*</td>
<td>Yes</td>
</tr>
<tr>
<td>EAAT (Glu)</td>
<td>0.014 ± 0.004</td>
<td>151 ± 20</td>
<td>11*</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The radiolabelled AA used for measurements are in parentheses. Clearance was calculated as $V_{max}/K_m$. Kinetic values were from: Na\(^+\)-LNAA, (O’Kane and Hawkins, 2003); EAAT-1-3, (O’Kane et al., 1999); A, ASC and N (O’Kane et al., 2004). Values marked by an asterisk were measured at a transmembrane potential of -61 mV. MeAlB (20 mmol L\(^{-1}\)) was included in measurements of systems N and ASC to exclude transport by system A.

The Na\(^+\)-dependent transport systems provide a mechanism for the elimination of non-essential AA and toxic AA, as well as maintaining the optimal concentrations of all other AA. As with the facilitative systems, there is considerable substrate overlap. All naturally occurring AA are transported by at least one system and some by as many as three (Table 11.3). The kinetic characteristics are summarized in Table 11.4. The following sections illustrate how both membranes of the BBB may play an active role in maintaining homeostatic concentrations.

### 11.8 Branched-chain Amino Acids and Brain Function

It has been suggested that the plasma concentrations of branched-chain AA (BCAA) may influence brain function and affect appetite (Fernstrom, 1985); physical and mental fatigue (Newsholme et al., 1992; Newsholme and Blomstrand, 1996; Yamamoto and Newsholme, 2000); mental performance (Castell et al., 1999); physical endurance (Blomstrand et al., 1991; Hassmen et al., 1994); sleep (Castell et al., 1999); and hormonal function, blood pressure and affective state (Fernstrom, 2005). Presumably, BCAA influence brain function by altering the availability of aromatic AA (Fernstrom and Wurtman, 1972). As mentioned, transport of LNAA is mediated by the facilitative system L1, which is shared by several LNAA; BCAA are especially effective in competing with aromatic AA for entry. Consequently, when plasma BCAA concentrations rise – which can occur in various normal and abnormal situations – they impair the entry of aromatic AA, notably tryptophan (Fernstrom, 2005). Serotonin synthesis in brain depends directly on the availability of tryptophan. Therefore, when plasma BCAA concentrations rise, the contents of brain tryptophan and serotonin fall (Fernstrom, 2005). While the focus of LNAA transport has been on the facilitative system L1, the recent discoveries that Na\(^+\)-dependent carrier systems are present on the abluminal membrane of the BBB (O’Kane and Hawkins, 2003; O’Kane et al., 2004) adds new elements that must be considered. These Na\(^+\)-dependent carriers are capable of propelling all NAA, including BCAA and aromatic AA, back towards the plasma. Thus, future directions should consider mechanisms that affect the retention of AA by the brain once they have entered.

### 11.9 Glutamate in Plasma and Brain

Glutamate, a non-essential amino acid, is the most abundant free AA in the brain (Meldrum, 2000). In CNS, glutamate functions as a neurotransmitter (Meldrum, 2000); as a link between the redox states of the pyridine nucleotides (NAD\(^+\) and NADP\(^+\)) (Krebs and Veech, 1969); and as a fuel reserve (Miller et al., 1975). The oxidation of glutamate to oxaloacetate yields 12 ATP per molecule. Therefore, when the
brain has insufficient glucose levels, or glycolytic flux is reduced, it mobilizes glutamate as a fuel (Miller et al., 1975).

11.9.1 Compartmentation of glutamate

In CNS glutamate exists as the free AA divided between two separate metabolic compartments located in astrocytes and neurons. These compartments were first recognized in the brain on the basis of radioisotope precursor-product relationships between glutamine and glutamate (Balazs et al., 1972a,b; Martinez-Hernandez et al., 1977; Cooper et al., 1979; Cooper and Plum, 1987). Compartmentation is almost absent at birth and develops in parallel with glial cells (Balazs et al., 1972b) since glutamine synthetase is found only in astrocytes (Martinez-Hernandez et al., 1977).

Neuronal glutamate is contained in at least two pools: in neuronal perikarya and dendrites, and in nerve terminals (vesicles) (Balazs et al., 1972a,b; Meldrum, 2000). Nerve impulses trigger release of glutamate from the pre-synaptic cell, which in turn binds to the glutamate receptors on the opposing post-synaptic cell. Neurotransmission is terminated by astrocytes and neurons that take up glutamate. Very little glutamate is believed to diffuse away from the synapse.

11.9.2 Excitotoxicity hypothesis of neuronal death

Early studies that used pharmacological doses of glutamate demonstrated damage to areas of the brain that were not protected by the BBB (Olney and Sharpe, 1969; Price et al., 1981). These studies led to the concept that neuronal death could be produced by over-stimulation of excitatory AA receptors (Schwarcz et al., 1984; Kirino, 1989; Albin and Greenamyre, 1992). Subsequently, this hypothesis became a popular explanation of the pathogenesis of neuronal death in a variety of acute conditions. However, the source of glutamate arises from within the brain in most circumstances. For instance, during an ischaemic episode, release of glutamate from brain cells (Choi et al., 1987; Castillo et al., 1996) may result in an excessive concentration of glutamate in the ECF (Martin et al., 1994; Rothstein et al., 1996). The extreme excitation of neurons by glutamate may in turn result in the opening of receptor-coupled ionophores, of which calcium channels are of particular importance. A large influx of calcium associated with impaired intracellular calcium sequestration mechanisms (which activate catabolic enzymes) may ultimately result in neuronal death (Benveniste et al., 1984). However, plasma glutamate is stable and does not change much unless glutamate is raised by artificial means.

11.9.3 Glutamate in circulation

Plasma glutamate concentrations are in the range of 50–100 µM in humans and other species (Laterra et al., 1999). Even when relatively large quantities of monosodium glutamate are added to the food of mice, monkeys or humans, only small changes in the plasma concentration of glutamate were found (Stegink et al., 1982, 1983, 1985; Tsai and Huang, 2000).

11.10 Facilitative and Active Transport Systems for Glutamate in the BBB

Early studies of the BBB, using whole brain perfusions or animals in vivo, identified facilitative transporters in the BBB membrane that are saturable and stereoselective (Oldendorf, 1970, 1971b). Because the substrate was presented to the capillary lumen it may be deduced that these transporters are present at least in the luminal membrane. On the other hand, it has been shown in several studies that glutamate does not enter the brain in material quantities, except in the circumventricular organs (Drewes et al., 1977; Hawkins et al., 1995; Viña et al., 1997). Until recently this has been a puzzle. Why should there be a transport system for an AA that is synthesized within the brain in large
quantities? Examining the luminal and abluminal membranes separately provided an explanation.

11.10.1 Facilitative transport of glutamate in the luminal membrane

Lee et al. (1998) measured facilitative glutamate transport separately in luminal and abluminal membranes, and found that facilitative glutamate transport exists only in the luminal border in a position to allow transport in both directions between plasma and endothelial cells. However, the function of a transporter for AAA that had a high affinity and a low capacity (Pardridge, 1983; Benrabh and Lefauconnier, 1996; Smith, 2000) was not clear: both glutamate and aspartate are non-essential amino acids that are synthesized and accumulated in high concentrations in brain.

11.10.2 Active transport systems expel glutamate from the ECF

Ordinarily, ECF glutamate is kept very low (∼0.5–2 µM) (Meldrum, 2000). In fact the concentration of glutamate and aspartate in CSF is lower than any other AA group (Fig. 11.5). The large gradient between brain cells and ECF is maintained by a family of Na⁺-dependent glutamate transporters known as EAAT. These transporters couple the steep Na⁺ gradient that normally exists between the ECF and brain cells. Currently five members of the EAAT family have been identified (Eliasof et al., 1998; Meldrum, 2000). They reside in the plasma membranes of astrocytes (Rothstein et al., 1994, 1996; Swanson et al., 1997; Meldrum, 2000); neurons (Kanai and Hediger, 1992, 2004; Lehre et al., 1995; Velaz-Faircloth et al., 1996; Attwell, 2000; Meldrum, 2000); and the BBB (O’Kane et al., 1999). The Na⁺-dependent transporters work at the limit of their ability to maintain the glutamate gradient between the brain cells and ECF, and of course, the steep Na⁺ gradient as well (extracellular >> intracellular) that is maintained by Na⁺/K⁺-ATPase. If the oxygen supply is insufficient to maintain ATP levels, membrane Na⁺/K⁺-ATPase cease to function. Under these circumstances the Na⁺ gradient is dissipated and glutamate is released from both astrocytes and neurons by reversal of the EAAT family of transporters. If ECF glutamate rises, nerve cells may be damaged.

Of the five known Na⁺-dependent glutamate transporters (EAATs 1–5) (Shigeri et al., 2004), at least three are proven to exist in the abluminal membrane of the BBB (Hutchison et al., 1985; O’Kane et al., 1999), and the transcript of EAAT 4 has been found in the BBB (Enerson and Drewes, 2006). The EAATs are voltage dependent, and collectively have an apparent Kᵣ of 14 µM (Lee et al., 1998; O’Kane et al., 1999). Western blot analysis confirmed the glutamate transporters to be present exclusively in the abluminal membranes (O’Kane et al., 1999). Collectively the EAAT family is the most powerful of the Na⁺-dependent AA transporters found in the abluminal membrane to date (Table 11.4).

11.10.3 Current concept of glutamate transport across the BBB

The current concept is that when glutamate concentrations increase above optimal in the ECF, the abluminal membrane of the BBB pumps glutamate into the endothelial cells. The facilitative transport system in the luminal membrane allows glutamate egress to the circulation (Fig. 11.6).

The organization of the BBB explains why various investigators have found that glutamate entry to brain is almost undetectable (Drewes et al., 1977; Hawkins et al., 1995; Viña et al., 1997; Smith, 2000). Glutamate may enter the endothelial cells, but net movement of glutamate from endothelial cells to brain is nearly impossible. This is a consequence of the steep Na⁺-gradient that powers the EAAT family of glutamate transporters at the border between the ECF and the abluminal membrane of the endothelial cells. Because of this organization the BBB is virtually impermeable to the net movement of glutamate from the circulation into the brain.
Fig. 11.6. Glutamate and glutamine transport between neurons, astrocytes, and endothelial cells. The presence of Na⁺-dependent carriers capable of pumping glutamine and glutamate from brain into endothelial cells, glutaminase within endothelial cells to hydrolyze glutamine to glutamate and NH₄⁺, and facilitative carriers for glutamine and glutamate at the luminal membrane provides a mechanism for removing nitrogen and nitrogen-rich AA from brain (Lee et al., 1998). EAAT1, 2, and 3 are present in endothelial cells (O’Kane et al., 1999), and astrocytes (Miralles et al., 2001). A transcript of EAAT4 has also been found in endothelial cells (Enerson and Drewes 2006). EAAT3 is present in nerve cells (Rothstein et al., 1994). A, Na⁺-dependent system A; N, Na⁺-dependent system N; EAAT, Na⁺-dependent glutamate transporter; xG, facilitative glutamate transporter; n, facilitative glutamine transporter. (A similar figure was previously published in the Journal of Nutrition.)
11.11 Glutamine and Ammonia Balance

Glutamine is the second most abundant AA in brain, about 6.2 µmol g⁻¹ (Hawkins and Mans, 1983). As mentioned, glutamine differs from other amino acids insofar as the CSF and plasma concentrations are similar (Fig. 11.4). Glutamine is synthesized in the mitochondria of astrocytes from α-ketoglutarate and NH₄⁺ that constantly enters the brain from the circulation, as well as from recycled NH₄⁺ that arises from glutamine metabolism by neurons (Fig. 11.6). Astrocytes are the sole source of glutamine because glutamine synthetase is only found in their mitochondria: no other brain cells have the capability to synthesize glutamine (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979).

11.11.1 Facilitative transport of glutamine at the luminal membrane

Lee et al. (1998) described facilitative transport of glutamine across the luminal membrane of the BBB that was not inhibited by BCH and did not demonstrate trans-stimulation. This transport system is similar to system n described in hepatic plasma membrane vesicles (Pacitti et al., 1993). The BBB system n is inhibited by asparagine and histidine (Hawkins et al., 2006a) as was found in hepatic vesicles by Pacitti et al. (1993). System n exists solely on the luminal membrane (Lee et al., 1998). Glutamine is also transported weakly by system L1 (see below) in competition with many of the essential NAA.

11.11.2 Na⁺-dependent transport of glutamine at the abluminal membrane

There are two Na⁺-dependent systems that transport glutamine, systems A and N, both of which are located exclusively on the abluminal membrane. System N has a preference for NAA that are nitrogen-rich, such as glutamine, histidine and asparagine, hence its designation (Kilberg et al., 1980, 1993). BBB abluminal membranes also transport serine to some degree via this system. System A is named for its preference for transporting alanine (Oxender and Christensen, 1963). In addition to glutamine and alanine, system A also transports small non-essential amino acids such as proline, alanine, serine and asparagine, as well as the essential AA histidine. There are, therefore, two Na⁺-dependent transport systems capable of moving glutamine from the ECF to the endothelial cells. Glutamine is free to diffuse to the plasma, facilitated primarily by system N and to a lesser degree by system L1. Thus it may be surmised that the Na⁺-dependent systems of the abluminal membranes, together with the facilitative system n in the luminal membrane, remove glutamine from the brain (Fig. 11.5).

11.11.3 Ammonia balance

The organization of the BBB also provides an explanation for a long-standing puzzle regarding brain NH₄⁺ metabolism. Various measurements have shown that 20–50% of the NH₄⁺ circulating through the blood vessels in the brain passes the BBB and is incorporated quantitatively into the amide group of glutamine by astrocytes (Cooper et al., 1979; Cooper and Plum, 1987). It is intriguing, however, that it has not been possible to consistently measure arteriovenous differences of NH₄⁺ (Cooper and Plum, 1987). If there were no mechanism for the removal of glutamine it would accumulate in the brain, thereby raising the osmolarity and causing swelling. For instance, taking cerebral blood flow to be 1 ml min⁻¹ g⁻¹ and plasma NH₄⁺ to be 50–100 µmol l⁻¹, it may be calculated that glutamine accumulation could be 14–72 µmol g⁻¹ each day. Clearly this would be an osmotic challenge for the brain. The situation is now clearer. Glutamine may be pumped from ECF into endothelial cells and is at least partially metabolized to NH₄⁺ and glutamate. The remaining glutamine, as well as NH₄⁺ and glutamate, are free to diffuse across the luminal membrane into the blood (Lee et al., 1998; O’Kane et al., 1999). This would provide an explanation of why the rate of NH₄⁺ uptake and release are balanced.

This new knowledge also explains how the entry of glutamine (and glutamate) to the
CNS is restricted (Hawkins et al., 1995; Hawkins, 2009) even though carrier activities for both amino acids have been described (Oldendorf and Szabo, 1976; Smith et al., 1987). Glutamine and glutamate can traverse the luminal membrane on facilitative systems. However, movement into the brain, across the abluminal membrane, is small because of the lack of facilitative carriers in the abluminal membrane. Furthermore, the three Na⁺-dependent carriers in the abluminal membrane that are driven by the steep Na⁺ gradient that exists between brain ECF and the cell interior forcefully oppose glutamate entry and promote its removal from the brain.

The BBB seems to be arranged in such a manner as to not only restrict the entry of glutamine and glutamate into the brain, but also actively to export these amino acids and NH₄⁺ to the circulation (Fig. 11.5). Therefore, the BBB participates in the regulation of brain nitrogen metabolism, and protects against the development of neurotoxicity by preventing the accumulation of glutamate as well as the accumulation of NH₄⁺.

11.12 The γ-Glutamyl Cycle and the Role of Pyroglutamate on Na⁺-dependent Carriers

The γ-glutamyl cycle proposed by Meister (Orlowski and Meister, 1970; Meister, 1973) accounts for the synthesis and degradation of reduced glutathione (GSH) and has been shown to influence AA transport in various tissues. The original suggestion that the cycle is involved directly in AA translocation into cells is controversial, having received both support and criticism. However, studies using lactating mammary glands and placenta of pregnant rats showed that pyroglutamate (also known as oxoproline), an intermediate of the γ-glutamyl cycle, serves to stimulate Na⁺-dependent AA transport (Viña et al., 1985, 1989).

The first reaction of the cycle occurs extracellularly and is catalysed by γ-glutamyl transpeptidase (GGT) (Fig. 11.7) (Meister, 1973). The substrates for GGT are glutathione, which is exported across the luminal membrane of endothelial cells to the plasma side, and extracellular AA in the plasma. The γ-glutamyl-AA that results enters cells by a transport system that is not shared by free AA. Intracellularly, γ-glutamyl-AA are substrates of γ-glutamyl cyclotransferase, which converts the γ-glutamyl-AA into pyroglutamate and the corresponding free AA. Subsequently pyroglutamate is hydrolyzed to glutamate by oxoprolinase (Van Der Werf et al., 1974).

Using abluminal membrane vesicles from the BBB, it was shown that pyroglutamate stimulates the Na⁺-dependent system A by 70% in a concentration-dependent manner (Lee et al., 1996). Thus, the affinity for MeAIB was increased by 50%, with no change in Vₘₐₓ. Pyroglutamate had no effect on luminal transport of L-phenylalanine (a representative substrate of the facilitative transport system L1); the effect of pyroglutamate was restricted to the Na⁺-dependent AA transport systems of the abluminal membrane (Lee et al., 1996).

The effect of pyroglutamate on the other Na⁺-dependent transporters of the BBB was studied recently (Hawkins et al., 2006b). It was found that preloading membrane vesicles with 2 mmol L-pyroglutamate stimulated all Na⁺-dependent AA transport systems with the exception of system N, which transports glutamine. The latter is interesting because glutamine is the only AA present in similar concentrations in plasma and ECF, and is synthesized from NH₄⁺ that enters brain continuously (Cooper and Plum, 1987). Also of interest was the finding that pyroglutamate stimulated γ⁺, a transporter that transports cationic AA, but also transports a range of NAA in the presence of Na⁺ (White, 1985; Mann et al., 2003).

The presence of GGT in the BBB has been an enigma. GGT activity is high in tissues that actively transport AA, such as the brush border of the proximal convoluted tubules of the kidney (Curto et al., 1988), the lactating mammary gland (Curto et al., 1988), and the apical portion of the intestinal epithelium. The BBB differs from these tissues in that it is not associated with active AA uptake from plasma. While brain requires essential AA for its function and growth, their supply is not much greater than the demand, and it is difficult to detect arteriovenous differences of AA across
the brain (Drewes et al., 1977; Sacks et al., 1982). It has, therefore, been puzzling why brain capillaries have such high GGT activity.

The data support the hypothesis that the γ-glutamyl cycle influences AA transport systems indirectly through pyroglutamate, produced intracellularly as an intermediary metabolite of the γ-glutamyl cycle. Pyroglutamate, in turn, acts to stimulate Na⁺-dependent AA transport systems. The γ-glutamyl cycle and GGT may serve to monitor the availability of AA to the brain, and constitute the first step in a control mechanism that influences the accessibility and content of brain AA (Fig. 11.7). The question arises whether the pyroglutamate concentrations that exist in vivo are sufficient to stimulate Na⁺-dependent transport. While data on the concentrations of pyroglutamate in cerebral microvessels seem to be unavailable, the concentrations in normal human plasma and various tissue extracts are between 20 and 50 μM (van der Werf et al., 1974; van der Werf and Meister, 1975), and as high as 6 mmol l⁻¹ in plasma and CSF in pathological conditions (Meister and Anderson, 1983). Stimulation of the Na⁺-dependent transport of system A was a linear function of the pyroglutamate concentration up to 2 mmol l⁻¹, a range that does not seem unreasonable.

The transpeptidation activity of GGT is a function of the plasma concentration and spectrum of AA (Allison and Meister, 1981), both of which may vary considerably depending on nutritional status. This provides a feedback mechanism in which the γ-glutamyl-AA produced by GGT enter cerebral capillary endothelial cells and are converted to pyroglutamate, which in turn activates four of the five Na⁺-dependent systems at the abluminal...
membrane. Since these systems are oriented to remove AA from the brain in an energy-dependent fashion, their up-regulation could provide at least a part of a control mechanism to guard against elevations of AA in the brain when their availability is excessive. This is of particular interest with regard to smaller non-essential AA for which systems A and ASC have a relatively high affinity. Thus, this process may serve to modulate the entry of AA that serve as neurotransmitters, or their precursors.

11.13 Concluding Comments

The present-day view of the BBB is that cerebral endothelial cells participate actively in regulating the composition of brain ECF and the AA content of the brain. The luminal and abluminal membranes work in a complementary fashion, with Na⁺-dependent transport of AA occurring at the abluminal membrane and facilitative transport at the luminal, or in the case of LNAA, at both membranes (Hawkins et al., 2002).

While the BBB determines the availability and therefore the brain content of essential AA, astrocytes and neurons participate in maintaining the extracellular concentrations. Astrocytes and neurons have Na⁺-dependent transport systems capable of transporting NAA and acidic AA (Broer and Brookes, 2001; Miralles et al., 2001; Schousboe, 2003). These systems are actively involved in regulating AA concentrations in ECF and are especially important in the maintenance of low concentrations of neurotransmitter AA such as glutamate, aspartate and glycine. On the other hand, it now seems clear that BBB also participates in the active regulation of brain ECF composition, and the abluminal membrane is especially important in this role.

11.14 Acknowledgements

Some sections of this article, and Figures 11.1, 11.2a, 11.5 and 11.6 are from an article published in IUBMB Life (Hawkins et al., 2002). Figures 11.2b, 11.4, 11.5, 11.6 and 11.7 were from an article published in the Journal of Nutrition (Hawkins et al., 2006a). The authors express their gratitude to Mary Regina DeJoseph for her skilled technical assistance. Financial support: portions of these works were supported by: National Institutes of Health Grants NS 31017 (R.A.H); NS 041405 (I.A.S); the Ministerio de Educacion y Ciencia, Plan Nacional I+D+i(BFU2007-62036) (J.R.V); the International Glutamate Technical Committee (R.A.H).

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12 Inter-organ Fluxes of Amino Acids

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12.1 Abstract

Plasma amino acid pools are regulated by production and disposal. Whereas essential amino acids are dependent on protein breakdown for their plasma flux, non-essential amino acids are produced de novo within the body. In many instances these production processes are encased in metabolic cycles, such as the urea cycle, that do not interact with the plasma pool. However when cells or organs lack the enzymes to complete a full cycle they must take up their substrate from the circulation and release the products of their metabolism back to it. In this way these organs regulate plasma pools of certain amino acids. In addition an intricate flux of amino acids between different organs exists by virtue of this phenomenon.

Glutamine is generated within the muscle and taken up by the kidney and the gut, where it is converted to ammonia. Intestinal-derived ammonia is taken up by the liver and detoxified to glutamine, and probably to a lesser extent to urea, which is generated from amino nitrogen and molecular ammonia generated within the liver itself. Since the liver extracts all ammonia generated by the intestines from the circulation, the kidneys are the only ammonia-producing organs that release ammonia to the systemic circulation.

Another product of intestinal glutamine metabolism is citrulline, which is released into the portal vein and largely passes the liver unchanged. Subsequently citrulline provides substrate for the kidneys that convert it to arginine. However, this process contributes only partly to systemic arginine flux. Moreover, many cells metabolize and generate arginine in an NO-cycle that is independent of plasma arginine.

Enteral administration of glutamine is accompanied by increased production of citrulline by the gut, and urea by the liver. Since hepatic arginase activity is compartmentalized within the urea cycle, hepatic extraction of enterally administered arginine is limited, and enteral arginine supplementation is feasible.

Since the intestinal conversion of glutamine to citrulline is crucial for total plasma citrulline flux, reduction of intestinal functional mass leads to low citrulline levels. Although the role of citrulline as a marker of enterocyte mass is becoming more and more established, the potential benefit of its supplementation in short-bowel patients is still unclear.

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12.2 Introduction

Apart from being mere building blocks of protein, virtually all free amino acids fulfil specific functions, for example in neurotransmission, signal transduction, or cell proliferation (van de Poll et al., 2005). Consequently, maintenance of the free amino acid pool within the body is of vital importance. The free amino acid pool is separated in extracellular (plasma) and intracellular compartments which are connected and equilibrated by several transporters situated on the cell membrane. These regulate amino acid uptake from the plasma into the cell or secretion from the cell into the plasma, thereby maintaining transmembrane gradients. All free amino acids are metabolized continuously, and therefore their different pools must be replenished at a similar rate to keep them at a constant level. The plasma concentration of an amino acid is a poor reflector of its flux. For example, increased plasma levels of glutamine are found during exogenous supplementation (higher influx) but also during liver failure (lower clearance); conversely, lower glutamine concentrations during experimental sepsis are initially accompanied by an increased flux of glutamine from the muscles to the liver (Bruins et al., 2003).

The appearance of essential amino acids in the free amino acid pool depends completely on protein breakdown, whereas non-essential amino acids can be formed from other amino acids by specific enzymatic processes or by transamination. Such enzymatic processes may be encased in metabolic cycles within a single cell, such as the urea cycle which continuously turns over citrulline, arginine and ornithine within the hepatocyte. These cycles are highly compartmentalized and therefore do not contribute to the appearance rate of amino acids in the plasma (Cheung et al., 1989). When there is no full enzymatic cycle present within a single cell, it must take up its substrate from the plasma. In line with this, non-essential amino acids that cannot be further metabolized are excreted.

These non-cyclic amino acid conversions are particularly important in the regulation of the extracellular free amino acid pool. Due to the tissue-specific distribution of most enzymes, non-essential amino acids are produced and released by specific organs. This leads to an intricate flux of amino acids between various organs resulting in the exchange of substrate through the bloodstream, thus forming inter-organ pathways. In some instances even cells within a single organ are distributed along the bloodstream in a way that allows them to exchange substrate efficiently. This is most strikingly exemplified by the differential distribution of glutaminase and glutamine synthetase within parenchymal cells lining the hepatic sinusoid. Appreciation of enzymatic distribution is crucial to the understanding of these intra- and inter-organ pathways of amino acid metabolism.

In this chapter some of the most important of these pathways will be outlined. In past years many data specifically concerning inter-organ nitrogen exchange in humans have emerged. Although similarities between different species are remarkable, so are some differences. In this chapter recent data that have elucidated and quantified inter-organ pathways of amino acid and nitrogen flux between the liver, intestines, kidney and skeletal muscle in health and disease will be reviewed.

12.3 Glutamine and Ammonia

12.3.1 Metabolism

Glutamine is the most abundant free amino acid in plasma and is central in amino acid metabolism as a nitrogen donor and transporter (Fig. 12.1). It is used as an energy source for enterocytes and immune cells, and amongst others plays a role in the regulation of cell size. In postabsorptive conditions it is released into the plasma at a rate of approximately 250 μmol kg⁻¹ h⁻¹ in healthy adults, which is amply sufficient to fulfil physiological demand (van de Poll et al., 2007a). Therefore glutamine is a non-essential amino acid under normal physiological conditions. It is derived from protein breakdown and synthesized from glutamate and ammonia by the enzyme glutamine synthetase, particularly found in skeletal muscle and the liver. It is broken down again in a reverse reaction to glutamate and ammonia by the
Fig. 12.1. Physiology of inter-organ amino acid exchange. Central is glutamine which is produced by skeletal muscle from glutamate. Glutamine is taken up by the gut, the kidneys, and the intestines. Ammonia is formed by glutaminase activity in these organs. Renal-derived ammonia is released into the systemic circulation, intestinal-derived ammonia is transported to the liver and eventually converted to glutamine again by perivenous glutamine synthetase. Hepatic glutaminase activity, which is confined to the periportal hepatocytes, serves to provide molecular ammonia to the urea cycle. The glutamate yielded in this process is used for glutamine synthesis by perivenous glutamine synthetase activity. Alternatively, the liver takes up glutamine as a nitrogen donor for the urea cycle and to serve gluconeogenesis after deamination. Ammonia released during gluconeogenesis is partly bound to glutamate and excreted to prevent loss of nitrogen via urea synthesis. Urea is released to the systemic circulation and excreted in the urine. Glucose is released into the systemic circulation and utilized ubiquitously. The carbon skeleton of glucose can be recycled to alanine within the muscle and subsequently serve hepatic gluconeogenesis again, thus creating an alanine-glucose cycle. Thirteen per cent of glutamine metabolized by the intestines is converted to citrulline. This is transported to the kidney where it is converted to arginine which is released into the systemic circulation. UC, urea cycle; TA, transamination.
enzyme glutaminase, which is predominantly present in the kidney, gut, and again in the liver.

Most endogenously synthesized plasma glutamine is derived from skeletal muscle glutamine synthetase activity. The ammonia needed for this process is mainly derived from amino acid breakdown within the muscle itself, since under normal circumstances skeletal muscle glutamate uptake by far exceeds ammonia uptake from the bloodstream (Olde Damink et al., 2002a). Although in the postabsorptive situation skeletal muscle is the most important glutamine producer, the liver is in fact the most active site of glutamine metabolism. However as mentioned above, the liver expresses both glutamine synthetase and glutaminase, and since their activity is approximately similar, net hepatic glutamine uptake or release is negligible (van de Poll et al., 2007a). Within the hepatic lobule glutaminase and glutamine synthetase are differentially expressed, with glutaminase being located in hepatocytes around the branches of the portal vein (upstream) and glutamine synthetase being located in hepatocytes that line the perivenous (downstream) part of the hepatic sinusoid. This distribution leads to an intercellular, but intrahepatic pathway of glutamine breakdown and synthesis that facilitates urea synthesis and ammonia clearance (Haussinger, 1986). This mechanism will be discussed in detail later in this chapter.

Net glutamine uptake is found within the intestines and the kidneys. In both organ systems, glutamine is deamidated by glutaminase to glutamate and ammonia immediately after uptake in the cell, and consequently both organ systems release ammonia to their venous effluent, the portal and renal veins, respectively (van de Poll et al., 2008b). Glutamate is further metabolized intracellularly and can either serve as a nitrogen donor for transamination processes or be metabolized further to form other non-essential amino acids.

Intestinal ammonia production is stoichiometrically related to intestinal net glutamine uptake, which both approximate 30 µmol kg⁻¹ h⁻¹ in the postabsorptive state. Intestinal ammonia production exceeds renal ammonia production threefold. However, ammonia – which is released from the intestines into the portal vein – must pass the liver before it can reach the systemic circulation. In the absence of portosystemic shunting, all ammonia that is released from the intestines is easily taken up by the liver which has two potential ‘ammonia detoxification mechanisms’. The first and most important of these is the urea cycle which turns over at a rate of approximately 200 µmol kg⁻¹ h⁻¹ and that binds one molecule of ammonia per turn. The other is glutamine synthesis which occurs at a rate of approximately 75 µmol kg⁻¹ h⁻¹ (van de Poll et al., 2007a,c).

Considering the activity of hepatic glutamine synthetase and the activity of the urea cycle, it can be calculated that the liver can bind at least 275 µmol kg⁻¹ h⁻¹ of molecular ammonia in the postabsorptive state. From this it can easily be conceived that the 30 µmol kg⁻¹ h⁻¹ of ammonia that is generated by the intestines is only a minor substrate for hepatic nitrogen disposal. Urea synthesis is quantitatively the most important and also the only definitive way of disposing of nitrogen. Urea can readily be excreted in the urine by the kidney, whereas glutamine, like any other amino acid, does not get excreted from the body in significant amounts in subjects with a normal renal function. The urea cycle metabolizes one molecule of free ammonia per turn (Fig. 12.2). As indicated, regarding the low portal ammonia flux when compared to the total ammonia flux in the liver, the portal venous ammonia load is no driving force behind hepatic ammonia metabolism. Instead, the urea synthesis rate is closely regulated by the supply of amino nitrogen (Vilstrup, 1980) which is, next to molecular ammonia, the other source of nitrogen for urea synthesis (Fig. 12.2). In line with this, the primary function of the urea cycle appears to be the detoxification and disposal of amino nitrogen derived from degraded amino acids rather than to scavenge molecular ammonia derived from intestinal glutamine metabolism. Additionally, since net hepatic ammonia uptake is only a fraction of urea production, most of the molecular ammonia that is used in urea synthesis must be generated within the liver itself. This occurs within the
periportal hepatocytes, amongst other sites, by glutaminase activity (Fig. 12.3).

Hepatic ammonia production and disposal is a tightly regulated process, and in fact the hepatic venous ammonia concentration equals the arterial ammonia concentration, meaning that the net ammonia balance across the entire splanchnic region (intestines plus liver) equals zero. Since intestinal-derived ammonia does not gain access to the systemic circulation owing to its hepatic detoxification, glutaminase activity in the kidneys is the only process in the human body that substantially adds ammonia to the systemic circulation.
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Fig. 12.3. Zonation of glutamine metabolism in the liver. In the periportal hepatocytes amino nitrogen (α-AN) is taken up and converted to urea, together with molecular ammonia, partly derived from periportal glutaminase activity. Portal venous ammonia is only a minor contributor to hepatic ureagenesis and may be the primary substrate for perivenous glutamine synthesis from glutamate that is produced by periportal glutaminase activity or transamination processes. The amount of glutamine being released in the hepatic vein is identical to the amount of glutamine taken up by the liver. The amount of ammonia taken up by the liver is equal to the amount of ammonia produced by the intestines.

Critical illness and trauma result in an acute catabolic state. Under such conditions increased skeletal muscle breakdown leads to a higher plasma appearance of most amino acids. In addition, an increment of skeletal muscular endogenous glutamine production by increased glutamine synthase activity has been found in a pig model of early sepsis (Bruins et al., 2003). In the same experiment an increased de novo synthesis of alanine by skeletal muscle was found. The enhanced glutamine and alanine fluxes were accompanied by an increase of hepatic glucose production. Increased gluconeogenesis from amino acids is accompanied by the production of amino nitrogen. A simultaneous rise of the transamination rate of α-ketoglutarate to glutamate and a higher efflux of glutamate from the liver can be regarded as an attempt to prohibit nitrogen wasting and to preserve nitrogen balance. None the less, ureagenesis and hence nitrogen loss is higher during acute illness. The increased amino acid fluxes during catabolic states are seemingly contradictory, accompanied by a decline in plasma concentrations of most amino acids, particularly of glutamine. The lowered plasma concentrations may indicate that increased substrate demands are insufficiently compensated.
for by the increased supply from endogenous supplies. Consequently, low glutamine levels are seen by many as a sign of (impending) glutamine deficiency. Therefore glutamine is generally considered a conditionally (or semi-) essential amino acid, and indeed, in some selected patient categories such as surgical ICU patients and those suffering from severe burns, the positive effects of glutamine supplementation on clinical outcome parameters have been shown (Singer et al., 2009).

Interestingly, the catabolic response to trauma or illness and the increased flux of amino acids from peripheral to central organs is tightly orchestrated by a series of transcription factors and signalling molecules, and cannot be simply reversed by nutritional interventions (Tisdale, 2005). Moreover, attempts to reduce catabolism and improve the nitrogen balance in septic patients by growth hormone administration not only resulted in an attenuation of muscle wasting, but also in increased mortality (Jakala et al., 1999).

12.3.2.2 Hyperammonaemia

In patients with liver disease, increased amounts of amino nitrogen and ammonia are found in the blood. This is associated with the development of hepatic encephalopathy. The increased amount of amino nitrogen can be ascribed to hepatocellular dysfunction and a decreased nitrogen clearance rate, since a higher load of amino nitrogen is needed to maintain urea synthesis (Vilstrup, 1980). In contrast, the hyperammonaemia which is characteristic for liver disease, and which is commonly used as a clinical parameter for liver function, must in most cases not be ascribed to metabolic liver dysfunction but rather to increased portosystemic shunting consequent to portal hypertension. Owing to the large reserve capacity of the liver for ammonia detoxification, only in severe cases of (acute) liver failure does hepatocellular dysfunction directly influence hepatic ammonia clearance and systemic ammonia levels. The first important cause of hyperammonaemia in chronic liver disease is portosystemic shunting. Due to portosystemic shunting, portal venous blood and hence ammonia produced in the intestines can access the systemic circulation without passing through the liver, thus escaping hepatic ammonia detoxification mechanisms (Olde Damink et al., 2002b).

Apart from portosystemic shunting, renal ammonia production is an important source of hyperammonaemia. As outlined above, under normal conditions the kidney is the only source of ammonia in the systemic circulation, owing to its high glutaminase activity. The hyperaminoacidemia which accompanies liver disease leads to an increase of renal ammonia production. It has been shown that in patients with chronic liver disease and a portosystemic shunt, renal ammonia production is as important as total splanchnic ammonia production (i.e. intestinal ammonia production minus hepatic ammonia uptake) for the total systemic ammonia flux (Olde Damink et al., 2002b). On the other hand, it has been shown in an experimental model of liver failure that urinary ammonia excretion can be increased while systemic ammonia release is reduced (Dejong et al., 1993). As such, the kidney appears to change from an ammonia-producing organ to one that causes a net removal of ammonia from the body, providing an alternative to urea synthesis and urinary urea excretion. An acute reduction of systemic ammonia release from the kidneys, observed in patients with chronic liver disease who received a transjugular intrahepatic portosystemic shunt, seems to confirm these experimental data (Olde Damink et al., 2006). As outlined above, skeletal muscle takes up only a limited amount of ammonia from the blood under normal conditions. In patients with hyperammonaemia due to chronic liver disease and portocaval shunting, however, skeletal muscle converts plasma ammonia to glutamine. In fact skeletal muscle consumes as much ammonia as the liver in such patients (Olde Damink et al., 2002b). However, since the body is not capable of excreting glutamine as a whole, this is only a temporary means of ammonia detoxification. Renal glutaminase activity and urinary ammonia excretion is mandatory to complete this alternative pathway of nitrogen disposal.
12.4 Glutamine, Citrulline and Arginine

12.4.1 Physiology

12.4.1.1 Glutamine and citrulline

After its deamidation to glutamate and ammonia in the gut, the carbon skeleton becomes metabolized further. Intestinal glutamine uptake is correlated with glutamine influx and therefore intestinal glutamine metabolism appears to be a substrate-driven process that can be stimulated by increasing glutamine plasma concentration. In the postabsorptive state, 13% of the glutamine taken up by the gut is eventually converted to ornithine and subsequently to citrulline. This comes down to a rate of approximately 4 μmol kg⁻¹ h⁻¹. The enzymes that catalyse this process (carbamoylphosphate synthase and ornithine transcarbamylase) are identical to those that catalyse the conversion of ornithine to citrulline in the liver as part of the urea cycle. The human gut does express the arginine-synthesizing enzymes argininosuccinate synthase and argininosuccinate lyase, and therefore it does possess the intrinsic capacity to generate arginine from citrulline (and glutamine). However, after infancy the activities of these enzymes rapidly disappear and the adult human intestines produce only limited amounts of arginine (Kohler et al., 2008). Since the gut cannot further metabolize citrulline, most of the citrulline is released into the portal vein after its generation.

On a whole-body level, plasma citrulline flux depends for 60–90% on intestinal citrulline production (van de Poll et al., 2007b). Citrulline is also synthesized within the liver by the urea cycle, at a rate that exceeds its plasma flux by a factor of 50. However, given the cyclic and compartmentalized nature of this process, it does not lead to net citrulline release. In contrast to prior assumptions, recent findings indicate that the liver takes up citrulline from the portal circulation (van de Poll et al., 2007a,b) although the aim of hepatic citrulline metabolism outside the urea cycle remains unclear.

In addition to its focal intestinal production, citrulline is generated throughout the body during nitric oxide (NO) synthesis, which occurs by the conversion of arginine to citrulline in a reaction that is catalysed by one of three isoforms of the enzyme nitric oxide synthase (NOS) (Fig. 12.2). Nitric oxide is a pluripotent signalling molecule that is generated, amongst other sites, by the vascular endothelium and macrophages. It plays a role in the regulation of vascular tone, organ perfusion and in regulation and mediation of the inflammatory response. Insufficient NO production has been implicated in a wide array of clinical conditions including sepsis and ischaemia-reperfusion injury. Since arginine is the immediate precursor for NO synthesis, arginine supplementation is widely studied as a method to modulate NO synthesis.

Human studies with isotopically labelled tracers show that 1 μmol kg⁻¹ h⁻¹ of citrulline generated during NO synthesis is released into the circulation (Castillo et al., 1996). This thus accounts for approximately 20% of total citrulline plasma flux. The total rate of the conversion of citrulline being produced during NO synthesis within the entire body, however, is difficult to determine since in many cells NOS is co-localized with argininosuccinate lyase and synthase. Because of this, citrulline can be resynthesized to arginine within the single cell where it is generated. As such an NO cycle is formed that does not depend on the exchange of citrulline and arginine across the cell membrane to maintain intracellular substrate supply (Mori, 2007). In fact it has been shown that activated macrophages are able to produce nitric oxide in an arginine-depleted medium, using glutamine as a nitrogen donor (Murphy and Newsholme, 1998). Due to the limited exchange between the citrulline plasma pool and the citrulline carbon skeletons that are confined to the NO cycle (Fig. 12.4), the total amount of citrulline that is generated during nitric oxide synthesis cannot accurately be assessed by classical intravenous tracer methods (Castillo et al., 1996).

Since plasma citrulline flux relies so heavily on intestinal glutamine metabolism, impairment of glutamine availability or reduction of intestinal function or mass are immediately reflected by changes in plasma
Fig. 12.4. Enzymes and substrates of the nitric oxide cycle. (1) Glutaminase. (2) Aminotransferase. (3) Argininosuccinate synthetase. (4) Argininosuccinate lyase. (5) Nitric oxide synthetase. Nitric oxide arises by the conversion of arginine to citrulline, catalysed by nitric oxide synthase. In many cells that express argininosuccinate synthase and argininosuccinate lyase, they are co-localized and also induced simultaneously, facilitating a rapid production of nitric oxide. Note that during this process only the input of a single amino nitrogen derived from amino acid transamination (bold italics) is required as input for the cycle, while the carbon skeleton and amino group (bold) of citrulline and arginine remains unaffected and is not transported across the cell membrane. Glutamine is probably the most important nitrogen donor for this cycle.

12.4.1.2 Citrulline and arginine

Citrulline is taken up from the systemic circulation by the kidneys at a rate that approximately equals the intestinal release of citrulline. The kidneys convert citrulline to arginine by the consecutive actions of argininosuccinate synthase and argininosuccinate lyase, and release arginine to the circulation. This forms an inter-organ pathway between the intestines (which take up glutamine and release citrulline) and the kidneys (which take up citrulline and release arginine). Through this pathway glutamine becomes the ultimate precursor for endogenous arginine production. The enzymatic steps of the pathway are almost identical to those of the urea cycle, but owing to the distribution throughout different organs its intermediates enter the circulation and may

flux and concentration of citrulline. It has been found that experimental glutamine depletion leads to diminished citrulline levels in humans (Rouge et al., 2007). In addition, diminished citrulline fluxes have been found in association with low glutamine levels in septic patients (Luiking et al., 2009). Even more striking is the close correlation between citrulline and functional enterocyte mass that is firmly established in patients who had undergone extensive small bowel resection (Crenn et al., 2000); patients with coeliac disease (Crenn et al., 2003); and the treatment of oncological patients suffering from radiation-induced enteritis (Lutgens et al., 2005). Although citrulline flux and levels decrease markedly in these patients, no evidence of the occurrence of clinically relevant citrulline depletion has been found to date.
thereby become available for other metabolic processes. Almost 70% of endogenously produced plasma arginine is derived from this inter-organ pathway (van de Poll et al., 2007a,b; Ligthart-Melis et al., 2008). The importance of intestinal citrulline production for renal arginine synthesis is illustrated by the fact that a reduction in intestinal citrulline production leads to diminished renal arginine synthesis (Luiking et al., 2009). On the other hand it must be pointed out that endogenous renal arginine synthesis (9 μmol kg⁻¹ h⁻¹) accounts for only 10% of total plasma arginine flux (Castillo et al., 1993), which depends for the largest part on protein breakdown. Moreover, only 1% of plasma arginine flux serves NO synthesis. Considering this low number and the putatively high rate of intracellular NO cycling, the importance of renal arginine synthesis for NO synthesis remains to be elucidated.

12.4.2 Metabolism after enteral administration

12.4.2.1 Glutamine

Since enterocytes express such high glutaminase activity, enterally administered glutamine is rapidly metabolized during absorption. In fact, the proportion of glutamine that is metabolized by the intestines during absorption from the intestinal lumen (50%) is threefold higher than the proportion of glutamine that is metabolized by the intestines during a single pass, while being supplied through the bloodstream (Ligthart-Melis et al., 2007). Enteral glutamine supplementation therefore results in a lower rate of glutamine appearance in the plasma and in a lower systemic glutamine concentration than parenteral glutamine supplementation. On the other hand, enteral glutamine administration results in increased intestinal citrulline production and to increased plasma levels of citrulline and glutamate, compared to parenteral glutamine supplementation (Melis et al., 2005). In contrast, no differences in arginine production or plasma level have been found between subjects receiving either enteral or parenteral glutamine supplementation. This can be explained by the small proportion of intestinal glutamine metabolism (13%) that is involved in the glutamine–citrulline–arginine pathway and by the limited influence renal arginine synthesis exerts on total plasma arginine flux (10%).

Simultaneous with the increased amount of glutamine-derived amino acids that is released into the systemic circulation after enteral glutamine administration, an increased rate of glutamine oxidation and urea synthesis can be found. This indicates that a substantial part of the glutamine undergoes transamination and serves gluconeogenesis after enteral administration. In conclusion it must be assumed that enteral and parenteral administration of glutamine are two completely different entities, which also explains the diverse clinical effects that are exerted by both interventions.

12.4.2.2 Arginine

Like glutamine, arginine is considered by many to be an essential amino acid, although the indications for supplementation of arginine are much less defined than those of glutamine. Positive effects of arginine supplementation in experimental studies are most frequently ascribed to its role as the precursor for NO synthesis. Concerns have been raised regarding the systemic availability of enterally administered arginine. Although the intestines only show limited arginine metabolism and almost all enterally administered arginine becomes available in the portal vein, it is believed by many that systemic availability of enterally administered arginine is limited by hepatic arginase activity. However, as underlined earlier, hepatic arginase activity is part of the urea cycle which is highly compartmentalized. This means that there is little or no exchange between the arginine that is being formed and broken down within the urea cycle, and the arginine that reaches the liver through the portal vein. Indeed there is some hepatic arginine uptake, but the fractional extraction of arginine approximates a mere 10%, which is within the same range as the fractional extraction of citrulline and other amino acids. Therefore hepatic arginine uptake appears to be a physiological process that is not substrate driven.
Most arginine extracted by the liver is probably used for protein synthesis. In addition it is pragmatically shown that enteral arginine supplementation does effectively increase systemic arginine levels.

Hepatic arginase can only affect systemic arginine availability when it is released from injured hepatocytes into the circulation. However the specific activity and plasma half-life of arginase is very low, and in fact the only clinical situation where a relation between increased plasma arginase activity and diminished arginine levels was convincingly shown is the immediate reperfusion phase after liver transplantation. Increased plasma arginase levels due to other causes of hepatocellular injury or to red blood cell injury does not result in arginase activity of any clinical importance (van de Poll et al., 2008a).

12.4.2.3 Citrulline

The assumptions that arginine deficiency may occur in certain conditions and that arginine levels cannot effectively be raised by enteral arginine supplementation have led to the hypothesis that enteral citrulline supplementation, by stimulation of renal arginine synthesis, may be a more effective way of increasing systemic arginine levels than enteral arginine supplementation itself. Indeed, it has been shown that arginine plasma levels do increase after citrulline supplementation. However, at a citrulline dosage that exceeds endogenous citrulline flux five times, the plasma arginine concentration increased twofold while plasma citrulline concentration increased fivefold (Rouge et al., 2007). Moreover renal citrulline uptake and arginine production seem to be limited at high citrulline doses (Moinard et al., 2008). In contrast, in rats with short bowel syndrome, citrulline supplementation restored citrulline and arginine levels as well as nitrogen balance more effectively than did arginine supplementation (Osowska et al., 2004). These experimental data suggest that citrulline may become a conditionally essential amino acid under certain conditions, although it is far from clear that the effects of citrulline on nitrogen homeostasis are mediated by increased renal arginine synthesis.

12.5 Recommendations for Future Research

The physiological aspects of inter-organ flux of amino acids in humans seems to be firmly established. Data on the metabolism of glutamine and arginine after enteral administration and absorption from the lumen, however, are not that clear yet. Some very recent studies in a murine stable isotope model indicate that the carbon skeleton of enterally administered glutamine does not get converted to citrulline. The same experiments suggested that arginine, rather than glutamine, is the major dietary source for citrulline synthesis (Marini et al., 2010). Obviously these intriguing findings contrast sharply with existing views and warrant careful pursuit, since (if confirmed in other species and using intravenous tracers), they may ultimately lead to a rewriting of the textbooks.

Moreover, the physiological and clinical importance of renal arginine synthesis in the light of the much larger arginine flux from protein breakdown should be addressed. The question remains whether enhancement of renal arginine production (for example by glutamine or citrulline supplementation) is a fruitful way to increase arginine availability. In line with this question, and the relative inaccessibility of the intracellular NO cycle for plasma arginine, it remains to be seen if enhancement of plasma arginine levels is useful at all.

In past decades many studies have been performed that attempted to modify substrate metabolism by increasing substrate supply. The results of most of these studies have been disappointing. Given the fact that many processes (including NO synthesis in most cells) are enzyme rather than substrate driven, the rationale for substrate supplementation may be flawed. Pharmacological induction of enzymatic pathways (with or without additional supplementation of substrate) may prove to become an interesting alternative to increase metabolic conversions, especially when these metabolic conversions are captured in intracellular cycles that are not freely accessible for plasma amino acids.
12.6 Conclusions

Intercellular and inter-organ amino acid exchange occurs via an intricate network that serves to maintain plasma amino acid pools and nitrogen homeostasis. Glutamine produced by the muscles is taken up by the liver to provide substrate for ureagenesis, which is driven by amino nitrogen supply and not by intestinal ammonia production. Glutamate released from the liver preserves nitrogen, and again is the precursor for muscular glutamine synthesis. Sepsis leads to an increased flux of substrate from the muscle to the liver, partly due to increased muscle protein catabolism and partly to increased amino acid synthesis. Reversal of this catabolic response by anabolic steroids does not improve survival. During liver insufficiency, muscular ammonia uptake and urinary ammonia excretion increase, to detoxify the ammonia that escapes liver metabolism due to portosystemic shunting.

The intestines convert glutamine to citrulline, which subsequently gets converted to arginine by the kidney. This inter-organ pathway is particularly important for whole body citrulline plasma flux and for endogenous renal arginine synthesis. However, it comprises only 13% of intestinal glutamine metabolism and only 10% of whole-body arginine plasma flux, which is much more dependent on protein breakdown. The true synthesis rates of citrulline and arginine, however, remain elusive since the largest turnover of these amino acids occurs within intracellular cycles such as the urea cycle and the NO cycle.

Enteral supplementation of glutamine is followed by significant first-pass extraction, largely serving hepatic oxidation. It is accompanied by an increased production of citrulline, but not of arginine. The bioavailability of enterally supplied arginine is not limited by hepatic arginase activity, although the indications for arginine supplementation remain to be established. Citrulline supplementation leads to increased arginine levels at very high doses. It remains to be seen, however, if the putatively beneficial effects of citrulline supplementation in experimental settings are mediated via its conversion to arginine.

Future research should be aimed at elucidating the fate of enterally administered glutamine and at the modulation of metabolic pathways by enzyme induction, rather than by enhancing substrate supply.

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13 Cellular Adaptation to Amino Acid Availability: Mechanisms Involved in the Regulation of Gene Expression

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13.1 Abstract

In mammals, the impact of nutrients on gene expression has become an important area of research. Nevertheless, the current understanding of amino acid-dependent control of gene expression is limited. Amino acids have multiple and important roles, so their homeostasis has to be finely maintained. However, the blood amino acid content can be affected by certain nutritional conditions or various forms of pathology. It follows that mammals have to adjust several of their physiological functions involved in the adaptation to amino acid availability by regulating expression of numerous genes. The aim of this review is to examine the role of amino acids in regulating mammalian gene expression and physiological functions.

A limitation for several individual amino acids strongly increases the expression of target genes such as insulin-like growth factor-binding protein1 (IGFBP-1), C/EBP homologous protein (CHOP) and asparagine synthetase (ASNS) genes. The molecular mechanisms involved in the regulation of CHOP and ASNS gene transcription in response to amino acid starvation have been partly identified. In particular, a signalling pathway requiring the protein kinase general control non-derepressive 2 (GCN2) and the activating transcription factor 4 (ATF4) has been described as sensing the amino acid limitation. In the case of an amino acid-imbalanced food source, this pathway has been shown to decrease food intake by activating a neuronal circuit. Taken together, the results discussed in this review demonstrate that amino acids by themselves can act as ‘signal’ molecules, with important roles in the control of gene expression and physiological functions.

13.2 Introduction

Regulation of metabolism is achieved by coordinated actions between cells and tissues, and also by mechanisms operating at the cellular level. These mechanisms involve the conditional regulation of specific genes in the presence or absence of appropriate nutrients. In multicellular organisms, the control of gene expression involves complex interactions of hormonal, neuronal, and nutritional factors.
Although not as widely appreciated, nutritional signals play an important role in controlling gene expression in mammals. It has been shown that major (carbohydrates, fatty acids, sterols) and minor (minerals, vitamins) dietary constituents participate in the regulation of gene expression (Towle, 1995; Foufelle et al., 1998; Pégorier, 1998; Duplus et al., 2000; Vaulont et al., 2000; Grimaldi, 2001). In the last decade, significant progress has been achieved in the understanding of molecular mechanisms involved in the control of mammalian gene expression in response to amino acid availability (Jousse et al., 2004; Kimball and Jefferson, 2004; Kilberg et al., 2005). This review summarizes recent work on the effect of amino acid availability in the regulation of biological functions. On the basis of the physiological concepts of amino acids homeostasis, we will discuss specific examples of the role of amino acids in the regulation of physiological functions, particularly focusing on the mechanisms involved in the amino acid regulation of gene expression.

13.3 Regulation of Amino Acid Metabolism and Homeostasis in the Whole Animal

Mammals are composed of a series of organs and tissues with different functions and, consequently, different metabolic demands. As a consequence, the regulation of protein and amino acid metabolism in the whole animal is made up of the sum of the regulatory responses in all individual parts of the body, and is achieved through a series of reactions that are both integrated and cooperative. Enzymes regulating the reactions of amino acid metabolism are distributed differently in various tissues. Consequently, there is a continuous exchange of amino acids between tissues, and metabolic regulation responds to the flow of compounds arriving at each cell. For example, arginine is mainly synthesized by liver and kidney but can be degraded by nitric oxide synthase in most of the tissues. Arginine is also catabolized by arginase, which is primarily found in the liver (Cynober, 2007).

In healthy adult humans, nine amino acids (valine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, histidine and tryptophan) cannot be synthesized de novo and are designated as indispensable (or essential) amino acids and have to be supplied by the food (Munro, 1970; Young et al., 1994). In addition, under a particular set of conditions certain non-indispensable amino acids may become indispensable. These amino acids are called 'conditionally indispensable'. For example, enough arginine is synthesized by the liver (urea cycle) and by the kidney (from citrulline) to meet the needs of an adult but not those of a growing child. Second, there are no large and specific stores of amino acids. Consequently, when necessary, an organism has to hydrolyse muscle protein to produce free amino acids. This loss of protein will be at the expense of essential elements. Therefore, complex mechanisms that take these amino acid characteristics into account are needed for maintaining the free amino acid pools.

13.3.1 Free amino acid pool

The size of the cellular pool of each amino acid is the result of a balance between input and removal (Fig. 13.1). The metabolic outlets for amino acids are protein synthesis and amino acid degradation, whereas the inputs are de novo synthesis (for non-indispensable amino acids), protein breakdown, and dietary supply. Changes in the rates of these systems lead to an adjustment in nitrogen balance. For example, the plasma concentration of amino acids has been reported to rise following the administration of a protein-containing meal to animals or humans. The concentration of leucine and some other amino acids approximately doubles in peripheral blood following a protein-rich meal (Aoki et al., 1976) and reaches much higher concentrations within the portal vein (Fafournoux et al., 1990). It has been well demonstrated that the increased concentrations of circulating amino acids resulting from a protein-rich meal ingestion is involved in the regulation of protein turn-over (Yoshizawa et al., 1995; Svanberg et al., 1997).

Due to the properties of amino acids (indispensable AA cannot be synthesized and there is no AA storage), adjustment of the amino acid metabolism is even more critical.
9 essential amino acids (Val, Ile, Leu, Lys, Met, Phe, Thr, His, Trp) 
No amino acid storage

Fig. 13.1. Biochemical systems involved in the homeostasis of proteins and amino acids.

in case of protein malnutrition. A dramatic drop in the plasma concentrations of certain indispensable amino acids has been shown to occur following insufficient amino acid or protein intake.

13.3.2 Specific examples of the role of amino acids in the adaptation to protein deficiency

13.3.2.1 Protein undernutrition

Prolonged feeding on a low-protein diet causes a fall in the plasma level of most indispensable amino acids. For example, leucine and methionine concentrations can be reduced from about 100–150 μM and 18–30 μM to 20 μM and 5 μM, respectively, in plasma of children affected by kwashiorkor (Grimble and Whitehead, 1970; Baertl et al., 1974). It follows that individuals have to adjust several physiological functions in order to adapt to this amino acid deficiency. Protein undernutrition has its most devastating consequences during growth. One of the main consequences of feeding a low-protein diet is the dramatic inhibition of growth. Growth is controlled by a complex interaction of genetic, hormonal, and nutritional factors. A large part of this control is due to growth hormone (GH) and insulin-like growth factors (IGF). The biological activities of IGF are modulated by the IGF-binding proteins (IGFBP) that specifically bind IGF-I and IGF-II (Lee et al., 1993; Straus, 1994). Straus et al. (1993) demonstrated that a dramatic overexpression of IGFBP-1 was responsible for growth inhibition in response to prolonged feeding on a low-protein diet. Known regulators of IGFBP-1 expression are GH, insulin, or glucose. However, the high IGFBP-1 levels found in response to a protein-deficient diet cannot be explained by these factors. It has been demonstrated that a fall in the amino acid concentration was directly responsible for IGFBP-1 induction (Straus et al., 1993; Jousse et al., 1998). Therefore, amino acid limitation, as occurring during dietary protein deficiency, participates in the down-regulation of growth through the induction of IGFBP-1.

13.3.2.2 Imbalanced diet

The ability to synthesize protein is essential for survival, and protein synthesis is dependent on the simultaneous supply of the 20 precursor amino acids. Because mammals cannot synthesize all of the amino acids, the diet must provide the remainder. Thus, in the
event of a deficiency in one of the indispensable amino acids, body proteins are broken down to provide the limiting amino acid (Munro, 1976). It follows that mammals need mechanisms that provide for selection of a balanced diet. Amino acid-imbalanced diets can be a frequent nutritional situation for wild omnivorous animals. For example, rodents are often confronted with poor food availability with a single plant protein source, which is most likely partially deficient for one indispensable amino acid.

After eating an amino acid-imbalanced diet, animals first recognize the amino acid deficiency and then respond by reducing their food intake (see Chapter 19). Recognition and anorexia resulting from an amino acid-imbalanced diet take place very rapidly (Harper et al., 1970; Rogers and Leung, 1977; Gietzen, 1993). The mechanisms that underlie the recognition of protein quality must act by the way of the free amino acids resulting from intestinal digestion of proteins. The decrease in the blood concentration of the limiting amino acid become apparent as early as few minutes after feeding an imbalanced diet, and depends on the extent of deficiency. The anorectic response is correlated with a decreased concentration of the limiting amino acid in the plasma. Several lines of evidence have suggested that the fall in the limiting amino acid concentration is detected in the brain. Gietzen (1993, 2000) reviews the evidence that a specific brain area, the anterior pyriform cortex (APC), can sense the variations of the amino acid concentrations. This recognition phase is associated with a localized decrease in the concentration of the limiting amino acid and changes in protein synthesis rate and gene expression. Subsequent to recognition of the deficiency the second step, development of anorexia, involves another part of the brain.

These two examples suggest that a variation in blood amino acid concentration can regulate several physiological functions including growth and appetite, and the expression of target genes (IGFBP-1). Recent progress has been made in understanding the mechanisms by which amino acid limitation controls the expression of several genes. The present review focuses on the regulatory role of low amino acid availability. The effects of an excess of amino acids are not considered here.

13.4 Molecular Mechanisms Involved in the Regulation of Gene Expression by Amino Acid Limitation

In mammalian cells, specific mRNAs that are induced following amino acid deprivation have been reported (Marten et al., 1994). Most of the molecular mechanisms involved in the amino acid regulation of gene expression have been obtained by studying the up-regulation of C/EBP homologous protein (CHOP), asparagine synthetase (ASNS), and the cationic amino acid transporter (Cat-1) genes.

Three distinct mechanisms of regulation of gene expression have been identified so far:

1. A post-transcriptional component involving stabilization of mRNA under starved conditions has been shown for CHOP and ASNS, but the molecular mechanisms involved in this process have not been fully identified (Gong et al., 1991; Bruhat et al., 1997; Aulak et al., 1999).

2. A translational control. It was shown that translation of the arginine/lysine transporter Cat-1 mRNA increases during AA starvation via the presence of internal ribosome entry site (IRES) located in the 5'UTR (Fernandez et al., 2001). It has also been shown that translation of the Cat-1 transcript can be regulated by miRNA (Bhattacharyya et al., 2006).

3. A transcriptional control. Most of the information we have concerning the regulation of gene transcription by AA availability has been obtained studying CHOP and asparagine synthetases genes. In this article we will focus on the transcriptional control of gene expression in response to amino acid deficiency.

13.4.1 Transcriptional activation of mammalian genes by amino acid starvation

It was established that the increase in CHOP or ASNS mRNA following amino acid starvation is due mainly to an increased transcription
By first identifying the genomic cis-elements and then the corresponding transcription factors responsible for regulation of these specific target genes, it is anticipated that one can progress backwards up the signal transduction pathway to understand the individual steps required.

13.4.1.1 Regulation of the human CHOP gene by amino acid starvation

CHOP encodes a ubiquitous transcription factor that heterodimerizes avidly with the other members of the C/EBP (Fawcett et al., 1996) and jun/fos (Ubeda et al., 1999) families. The CHOP gene is tightly regulated by a wide variety of stresses in mammalian cells (Luethy and Holbrook, 1992; Sylvester et al., 1994; Wang et al., 1996). Leucine limitation in human cell lines leads to induction of CHOP mRNA and protein in a dose-dependent manner (Bruhat et al., 1997).

We have identified a cis-positive element located between -313 and -295 that is essential for amino acid regulation of its transcription in the CHOP promoter (Bruhat et al., 2000) (Fig. 13.2). This short sequence can regulate a basal promoter in response to starvation of several individual amino acids and so can be called an amino acid regulatory element (AARE). The sequence of the CHOP AARE region shows some homology with the specific binding sites of the C/EBP and ATF/CREB transcription factor families. Using gel shift experiments and chromatin immunoprecipitation, we have shown that several transcription factors belonging to the ATF or C/EBP family and regulatory proteins have the ability to bind to the CHOP AARE (ATF2, ATF3, ATF4, and CCAAT/enhancer-binding proteinβ (C/EBPβ)). Among these factors, ATF2 and ATF4 are indispensable for the amino acid control of CHOP expression: in cell knockout for these two proteins, the amino acid regulation of CHOP expression is abolished (Bruhat et al., 2000; Averous et al., 2004).

13.4.1.2 Regulation of the asparagine synthetase gene by amino acid starvation

At the same time, the Kilberg laboratory identified a sequence in the ASNS promoter (between -70 to -62) responsible for its regulation in response to amino acid starvation. This sequence was referred to as the nutrient-sensing response element-1 (NSRE-1) (Barbosa-Tessmann et al., 1999, 2000). Further promoter analysis indicated that a second sequence, 11 nucleotides downstream from NSRE-1, was also required for activation of the ASNS promoter by both amino acid starvation and endoplasmic reticulum (ER) stress. This sequence was referred to as NSRE-2. The genomic unit encompassing NSRE-1 and NSRE-2 was named NSRU, for nutrient-sensing response unit. NSRU is also responsible for the induction of ASNS by glucose starvation or ER stress. The same group demonstrated that several transcription factors and regulatory proteins (including ATF3, ATF4, C/EBPβ, TBP, and TFII) bind ASNS-NSRE-1. Using a chromatin immunoprecipitation approach they analysed precisely the time course of interaction between these transcription factors and the ASNS promoter (Chen et al., 2004).

Comparison between CHOP and ASNS transcriptional control elements shows that ASNS NSRE-1 and CHOP AARE share a similar nucleotide sequence (Brurat et al., 2002) (Fig. 13.2). The core sequences of CHOP AARE and ASNS NSRE-1 differ only by two nucleotides (Fig. 13.3). It is thus expected that a common set of transcription factors bind to both ASNS-NSRE-1 and CHOP-AARE.
Fig. 13.3. The mammalian GCN2/ATF4 signalling pathway. The signal transduction pathway triggered in response to amino acid starvation is referred to as the amino acid response (AAR). The initial step in AAR is activation by uncharged tRNAs of GCN2 kinase which phosphorylates the α subunit of translation initiation factor eIF2α (eIF2α) on serine 51. This phosphorylation decreases protein synthesis by inhibiting the formation of the pre-initiation complex. However, eIF2α phosphorylation also triggers the translation of specific mRNAs including ATF4. Once induced, ATF4 induces transcription of specific target genes such as CHOP, ASNS, TRB3 or SNAT2. In mammals, three other eIF2α kinases leading to ATF4 expression have been identified: PKR (double-stranded RNA dependent protein kinase) which is activated by double-stranded RNA during viral infection, HRI (haem-regulated translational inhibitor) which is activated by heme deficiency, and PERK (PKR-like endoplasmic reticulum kinase) which is activated by protein load in the endoplasmic reticulum.

13.4.1.3 Transcription factors binding the AARE

Several transcription factors have been described for their ability to bind the CHOP or the NSRE1-AARE. However, only ATF4 is indispensable for the amino acid regulation of ASNS expression, and both ATF2 and ATF4 are indispensable for the regulation of the CHOP-AARE.

13.4.1.3.1 ATF4. ATF4 belongs to the basic region/leucine zipper (bZIP) family of transcription factors that also includes members of the Jun/Fos (AP-1) family (Karpinski et al., 1992; Ameri and Harris, 2008). Its key role in amino acid regulated transcription has been clearly established in the past few years (Chen et al., 2004; Averous et al., 2004; Kilberg et al., 2005; Pan et al., 2007). In the case of CHOP, it has been shown that:

1. the expression of ATF4 and its binding to CHOP AARE sequences are increased following amino acid starvation;

2. in cells devoid of ATF4 expression, the induction of CHOP upon amino acid starvation is completely lost; and

3. when over-expressed, ATF4 by itself is able to activate the CHOP AARE-dependent transcription.

13.4.1.3.2 ATF2. ATF2 belongs to the bZIP family of transcription factors and is an important member of activating protein 1 (AP-1) (Wagner, 2001). The transactivation capacity of the N terminal domain of this transcription factor can be enhanced through phosphorylation of two threonine residues, Thr-69 and Thr-71 (Gupta et al., 1995; Livingstone et al., 1995; Ouwens et al., 2002). The role of ATF2 has been studied in the context of CHOP regulation by amino acid starvation (Bruhat et al., 2000; Averous et al., 2004; Bruhat et al., 2007). It was shown that:

1. in cells devoid of ATF2 expression, the induction of CHOP transcription upon amino acid starvation is lost;
2. ATF2 binds to the CHOP AARE in both starved and unstarved conditions; and
3. ATF2 phosphorylation is necessary for the activation of the CHOP AARE-dependent transcription.

In a further study, it has been reported using a chromatin immunoprecipitation approach that in vivo binding of phospho-ATF2 to CHOP AARE is associated with acetylation of histones H4 and H2B in response to amino acid starvation (Bruhat et al., 2007). A time course analysis revealed that ATF2 phosphorylation precedes histone acetylation, ATF4 binding, and the increase in CHOP mRNA. Using cells devoid of ATF2 expression, it has also been demonstrated that ATF2 is essential for the acetylation of histones H4 and H2B within AARE sequences in response to amino acid starvation. Phosphorylation of this transcription factor may have a key role in stimulating an unidentified histone acetyl transferase (HAT) activity. Thus, ATF2 appears to be involved in promoting the modification of the chromatin structure to enhance CHOP transcription in response to amino acid starvation.

13.4.1.3.3 Role of ATF4 and ATF2 in the Control of the AARE-Dependent Transcription. Although the CHOP AARE and NSRE1 are very similar, some differences prompt us to think that CHOP and ASNS induction following amino acid starvation do not entirely occur through a unique and common mechanism:

1. no cis-DNA sequence equivalent of NSRE2 has been identified in the CHOP gene;
2. it has been shown (Bruhat et al., 2002) that ATF2 does not bind the ASNS NSRE; and
3. the amino acid specificity in relation to the degree of induction of these two genes is different (Jousse et al., 2000).

Hence, CHOP AARE and ASNS NSRE-1 are structurally related but functionally distinct.

Identification and studies of AARE sequences located in other genes allowed the identification of two sets of AARE-regulated genes (Bruhat et al., 2002; Chaveroux et al., 2009). Both are dependent on ATF4 but only one depends on ATF2 for its regulation. For example, regulation of CHOP and ATF3 transcription by leucine starvation depends on the expression of both ATF4 and ATF2, whereas regulation of ASNS and SNAT2 is dependent on ATF4 but not on ATF2 (Averous et al., 2004; Chen et al., 2004; Palii et al., 2004, 2006; Bruhat et al., 2007). These last genes contain an AARE that is identical or very homologous to CHOP or ATF3 AARE in sequence, and binds ATF4 but not ATF2. Taken together, these results demonstrate that the flanking sequences of the AARE are important for precise control of AARE-dependent transcription. These differences in mechanism would permit flexibility among amino acid-regulated genes in terms of the rapidity, magnitude, and cell specificity of the transcriptional response for the same initial signal.

13.4.2 Signalling pathways regulated by amino acid limitation

In mammals, the signalling pathways that mediate regulation of gene expression in response to amino acid starvation are partially understood. A pathway leading to the up-regulation of ATF4 protein has been described at molecular level, and a second pathway leading to ATF2 phosphorylation has recently been identified.

13.4.2.1 The GCN2/ATF4 pathway (the AAR pathway)

In mammals, limiting the extracellular supply of an indispensable amino acid or blocking the synthesis of an otherwise non-indispensable one results in activation of a signal transduction pathway that is referred to as the amino acid response (AAR). Ron’s group identified this signalling pathway for regulating gene expression in mammals (Harding et al., 2003). The AAR is homologous to the well-characterized yeast general control response to amino acid deprivation (Dever et al., 1992). Its components include the mammalian homologue of GCN2 kinase, the initiation factor of translation eIF2α, and ATF4 (Fig. 13.3). The initial step in AAR is activation by uncharged tRNA of mammalian GCN2 protein kinase, which phosphorylates eIF2α on serine 51 (Dever et al., 1992). This phosphorylation decreases the cap-dependent translation of most mRNAs. Like the GCN4
transcript, ATF4 mRNA contains uORFs in its 5'UTR that allow translation when cap-dependent translation is inhibited (see Lu et al., 2004 and Vattem and Wek, 2004 for the molecular mechanisms). Therefore, under circumstances of amino acid starvation, transcription factor ATF4 is translationally up-regulated. Once induced, ATF4 directly or indirectly induces transcription of specific target genes.

13.4.2.2 Signalling pathway leading to ATF2 phosphorylation

Several data (described above) established that ATF2 is phosphorylated in response to amino acid starvation (Bruhat et al., 2000; Averous et al., 2004). Collectively, these data suggested the existence of a specific amino acid-regulated pathway leading to phosphorylation of ATF2. This pathway was partially identified recently (Chaveroux et al., 2009). We first showed that the MAPK module MEKK1/MKK7/JNK2 is responsible for ATF2 phosphorylation in response to leucine starvation. Then we progressed backwards up the signal transduction pathway and showed that GTPase Rac1/Cdc42 and protein Ga12 control the MAPK module, ATF2 phosphorylation, and AARE-dependent transcription.

13.4.2.3 Other signalling pathways

In yeast, the mechanisms involved in amino acid detection have been well documented (Hinnebusch, 1988) and several signalling pathways identified. By homology to yeast we can imagine that pathways other than mGCN2 are involved in sensing amino acid availability in mammals. Notably, mTOR is well known to be involved in the regulation of translation by amino acid availability. However, several studies have shown that mTOR is not widely involved in regulation of gene expression (Peng et al., 2002; Deval et al., 2009). Recent studies suggest that other signalling mechanisms could be involved in regulation of gene expression in response to amino acid limitation. These conclusion were obtained either by measuring the response of gene expression to deprivation of different amino acids (Palii et al., 2008) or by using transcription profiling from cells devoid of GCN2 expression (Deval et al., 2009). In addition, it has been shown that amino acids can interfere directly or indirectly with signalling pathways and/or various transcription factors (distinct from the AAR). In particular, it was shown that glutamine, the most abundant amino acid, modulated activity of various transcription factors through mechanisms that remain to be identified (see Brasse-Lagnel et al., 2009 for review). Hence we can conclude here that the response to amino acid starvation in mammals is a complex process that involves different regulatory processes.

13.5 Control of Physiological Function by the GCN2/ATF4 Pathway

Although several signalling mechanisms modulated by amino acid starvation have been described, the GCN2/ATF4 pathway is the most studied and is likely to be the most important (Deval et al., 2009). In this chapter we focus on the role the GCN2/ATF4 pathway plays in the control of several physiological functions.

13.5.1 Amino acid deficiency sensing by GCN2 triggers food aversion

Food intake results from a complex behavioural pattern in which innate factors play an important role, particularly in the case of omnivores. A remarkable example of an innate mechanism governing food choice is presented by the fact that omnivorous animals will consume substantially less of an otherwise identical experimental meal lacking a single indispensable amino acid (see Chapter 19) (Leung et al., 1968; Harper et al., 1970). Although it seems likely that the signalling pathway leading to this response comprises the sensing of amino acid variations, the basis for this innate aversive response is poorly understood. Blood concentration of an indispensable amino acid decreases rapidly when it is missing in the diet. As a consequence, the protein kinase GCN2, which is ubiquitously expressed, could be activated in most tissues. Therefore GCN2 could be an important sensor of amino
acid homeostasis inside cells and could activate downstream-rectifying responses mediated by phosphorylated eIF2α (Hinnebusch, 1994; Harding et al., 2000; this review).

Recent results (Hao et al., 2005; Maurin et al., 2005) establish that the aversive response of wild-type mice to a diet deficient in one indispensable amino acid is likewise blunted in GCN2-/- mice (Fig. 13.4a), whereas serum amino acid levels are decreased to similar levels by the imbalanced diet in both genotypes (Fig. 13.4b). These results indicate an altered response to amino acid deficiency in mice lacking GCN2 activity. Using conditional GCN2 knockout mice, we further demonstrated that GCN2 ablation specifically in the brain also impairs the aversive response to an imbalanced diet. Thus, even if the consumption of an imbalanced meal also activates GCN2 and promotes eIF2α in peripheral tissues, particularly in the liver, our observation implicates brain GCN2 signalling in initiating the aversive response.

13.5.2 Role of GCN2 in the regulation of neuronal plasticity

Several studies have shown a link between messenger RNA translation, learning, and memory (Kandel, 2001). Costa-Mattioli et al. (2005, 2007) provided evidence that GCN2 regulate synaptic plasticity, as well as learning and memory. The molecular basis for enhanced memory in GCN2-/- mice with reduced eIF2α phosphorylation is explained by the fact that GCN2-mediated eIF2α phosphorylation causes an increase in translation of ATF4 mRNA in the brain. ATF4 suppresses memory because it inhibits transcription factor c-AMP response element binding (CREB) protein-mediated gene expression. It is likely that the basal activity of GCN2 is required for its function. Modulation of GCN2 activity in the brain by various factors has not been addressed in these studies.

13.5.3 Role of GCN2 in the regulation of fatty-acid homeostasis during leucine deprivation

Guo and Cavener (2007) brought to light the role of GCN2 in the regulation of lipid metabolism in the liver during deprivation of an indispensable amino acid. These authors showed that expression of lipogenic genes in the liver were repressed and lipid stores in adipose tissue were mobilized upon leucine deprivation. In contrast, GCN2-deficient mice developed
liver steatosis and exhibited reduced lipid mobilization. Liver steatosis in GCN2−/− mice was found to be caused by unrepressed expression of lipogenic genes, including Srebp-1c. The signalling pathway linking GCN2 activity to the regulation of Srebp-1c expression is not identified at present.

In the same way, two recent articles demonstrated that phosphorylation of eIF2α was a key regulator of several aspects of intermediary metabolism. Oyadomari et al. (2008) demonstrated that eIF2α phosphorylation signalling in the liver was regulating transcription factors involved in carbohydrate and lipid metabolism. The Kaufman group showed that translation attenuation through eIF2α phosphorylation prevented oxidative stress and optimized ER protein folding to support insulin production in pancreatic β cells (Back et al., 2009).

### 13.5.4 Role of GCN2 in the immune system

It was shown recently that the AAR pathway was involved in several aspects of regulation of the immune response (Mellor and Munn, 2004, 2008; Pierre, 2009). In particular, associated with the induction of indoleamine 2,3-dioxygenase (IDO initiates the degradation of tryptophan), AAR is involved in communication between cells. For example, after stimulation, IDO-expressing antigen presenting cells (APC) cells reduce extracellular tryptophan concentrations so that adjacent T-cells, which depend on tryptophan from the extracellular environment, are unable to activate and proliferate upon encountering antigens. Therefore IDO might play a role in preventing the initiation of autoimmune disease by enforcing T-cell tolerance by suppressing their proliferation (Moffett and Namboodiri, 2003). Hence, high local expression of IDO by IDO-expressing APC may represent an anti-inflammatory and immunosuppressive mechanism attempting to counterbalance tissue damage (Wolf et al., 2004). In addition, IDO/GCN2 activities are implicated in several other contexts, such as the inhibition of maternal T-cell immunity to fetal tissues during mammalian gestation (Munn et al., 1998) and T-cell tolerance to tumours (Friberg et al., 2002; Uyttenhove et al., 2003).

Taken together, these examples highlight the need for relying on physiological observations to molecular events described in vitro to improve our knowledge on physiological consequences of nutritional status.

### 13.6 Conclusions

The idea that amino acids can regulate gene expression is now well established. In concert with hormones, amino acids can by themselves play an important role in the control of physiological function; however, the underlying processes have only begun to be discovered. Defining the precise cascade of molecular events by which the cellular concentration of an individual amino acid regulates gene expression will be an important contribution to our understanding of metabolic control in mammalian cells.

The molecular basis of gene regulation by dietary protein intake is an important field of research for study of the regulation of physiological functions of individuals living under conditions of restricted, imbalanced, or excessive food intake. Beyond gaining a basic understanding of the amino acid control of biological mechanisms, the characterization of how these processes contribute to the pathology of various diseases represents an important field of investigation in molecular nutrition.

### References


Part III

Nutrition
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14 Endogenous Amino Acids at the Terminal Ileum of the Adult Human

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14.1 Abstract
During the digestion of food very considerable quantities of proteins of body origin (endogenous protein) are voided into the digestive tract of the adult human. Much of this material is recycled, with the protein being digested and the amino acids reabsorbed. Nevertheless large quantities of endogenous protein, peptides and amino acids remain unabsorbed at the end of the small intestine, and these along with endogenous protein originating from the colon, are largely catabolized by the colonic microflora, and represent a loss of amino acids from the body. In the adult human around 2 g d⁻¹ of endogenous nitrogen (13 g protein) flow from the terminal ileum into the colon, and such a loss of amino acids is of importance metabolically.

This chapter reviews methods which may be applied in humans for collecting ileal digesta and measuring the endogenous protein component. Literature estimates of endogenous ileal nitrogen flow in humans are reviewed and factors influencing the endogenous flows discussed. The practical relevance of the estimates of endogenous N flow, both physiologically and nutritionally, is discussed in the context of gut and body metabolic rate, the daily amino acid requirement and the determination of estimates of ileal amino acid digestibility.

14.2 Introduction
During the digestion of food copious quantities of nitrogen-containing materials originating from the body are voided into the lumen of the digestive tract. These are referred to as the endogenous nitrogen and are significant metabolically.

Sources of endogenous nitrogen include digestive enzymes, bile salts, phospholipids, desquamated cells, mucoproteins, urea, proteins and amino acids originating from lysed cells, plasma proteins (especially serum albumen), and other nitrogenous compounds found in the gastrointestinal secretions. Although strictly 'non-dietary' as opposed to 'endogenous', microbial nitrogen is often included as part of the endogenous measure. The overall amounts of material entering the digestive tract from different sources are not known precisely, with estimates varying widely. Table 14.1 provides some approximate values based on the wider literature. In the adult human, it is considered that endogenous

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nitrogen flowing through the digestive tract to the terminal ileum amounts to some 11-16 g d\(^{-1}\) (FAO et al., 2007), values consistent with the estimates for the total tract given in Table 14.1.

During the digestive process, endogenous sources of nitrogen become mixed with proteins of dietary origin. Nasset (1965) suggested that a considerable amount of nitrogen in the duodenal digesta is of endogenous origin, and a study with humans (Nixon and Mawer, 1970) showed that after a protein meal as much as 53% of intraluminal protein in the small intestine was of endogenous origin. Johansson (1975) found endogenous protein to be about one third of the amount of that from the diet.

Much of the endogenous protein entering the gut lumen is digested and the amino acids reabsorbed, with the absorbed amino acids representing a significant supply of amino acids for metabolism. Up to 80% of endogenous nitrogen has been reported to be reabsorbed by the end of the small intestine (Krawielitzki et al., 1990; Souffrant et al., 1993). Based on such a rate of reabsorption and an endogenous nitrogen inflow (mouth to terminal ileum) of 13.5 g d\(^{-1}\) (mean of 11 and 16 g N d\(^{-1}\) (FAO et al., 2007)), this would equate to a loss of endogenous nitrogen from the small bowel of 2700 mg N d\(^{-1}\). Such a value is higher than an estimate given by Chacko and Cummings (1988) of 1000 mg nitrogen d\(^{-1}\), but is in line with more recent estimates obtained in subjects receiving protein-containing test diets (see below). A terminal ileal loss of 2700 mg N d\(^{-1}\) equates to a loss of endogenous protein of around 17 g d\(^{-1}\), which is quantitatively significant.

The nitrogen-containing material remaining undigested at the end of the ileum, of both endogenous and dietary origin, passes into the colon where it is catabolized extensively by the resident microflora. Amino acids are not considered to be absorbed as such from the mammalian large bowel in quantitatively significant amounts (Wrong et al., 1981; McNeil, 1988), and thus amino acid absorption studies and studies of endogenous secretion and excretion have centered on the upper digestive tract (Moughan, 2003). The objectives of this contribution are to outline recent developments in the methodology for measuring endogenous ileal nitrogen loss in humans and the practical importance of such measures, and to give an overview of current estimates of endogenous ileal nitrogen and amino acid losses, and factors influencing endogenous nitrogen flow.

**14.3 Endogenous Ileal Amino Acid Losses – How Should They be Determined?**

The determination of endogenous ileal amino acid losses from the terminal ileum into the colon necessitates the collection of samples of fresh ileal digesta. The total collection or sampling of digesta flowing at the terminal ileum (end of small intestine) is more easily undertaken with animal models (laboratory rat or growing pig) than with human subjects. With animal models digesta may be collected via surgically implanted cannulas, or directly from the deeply anesthetized or euthanized animal, the latter being a common method with birds and smaller mammals. With humans, a number of workers have enlisted the cooperation of ileostomates, who have undergone an ileostomy operation usually due to ulcerative colitis. After colorectomy, the distal small bowel is attached to the abdominal wall as a fistula, and this allows a total collection of

### Table 14.1. Approximate amounts of endogenous nitrogen from different sources entering the digestive tract of the adult human.

| Endogenous nitrogen (g d\(^{-1}\))
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Source</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Saliva</td>
</tr>
<tr>
<td>Gastric juice</td>
</tr>
<tr>
<td>Bile</td>
</tr>
<tr>
<td>Pancreatic secretions</td>
</tr>
<tr>
<td>Urea</td>
</tr>
<tr>
<td>Mucus</td>
</tr>
<tr>
<td>Epithelial enzymes/sloughed cells</td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

*Based on literature data for growing pig and adult human.*
ileal digesta. A strength of this method is that no dietary markers are required to relate the sample of digesta collected to the food intake. A major drawback, however, is the largely unknown effects that the surgery or underlying disease states may have on endogenous protein loss. Colorectomy leads to physiological and metabolic adaptations (Christl and Scheppach, 1997), and it is well known that there is a very considerable microbial colonization of at least the lower small intestine in ileostomates (Gorbach et al., 1967). In this case, observations obtained using ileostomates as a model must be interpreted with some caution.

An alternative approach is naso-intestinal intubation (Schedl and Clifton, 1961; Modigliani et al., 1973) which allows the sampling of ileal digesta from conscious human volunteers with an intact digestive tract. The method consists of inserting a triple-lumen polyvinyl chloride (PVC) tube via the nose to the stomach, progressing down to the terminal ileum aided by peristalsis and by a terminal inflatable balloon containing mercury. Digesta are collected continuously by siphoning or by slight aspiration through the distal opening of the tube. The method relies on the use of a non-absorbable marker of the liquid phase, and may thus be subject to greater error than total collection methods. Further potential concerns with the method are whether the presence of a tube in the gut alters food transit rate and whether the method allows representative sampling of digesta. The second concern, at least, has been addressed experimentally (Deglaire, 2008), where it was concluded that the method allows accurate estimation of intestinal fluid flows and representative samples of digesta. The method, however, remains time-consuming, expensive and certainly not routine.

Given the difficulties with collecting ileal digesta samples from humans, it is not surprising that animal models have been evaluated for application in studying digestion in man. The growing pig, a meal-eating omnivore, has been shown to be a particularly useful model (Moughan et al., 1992, 1994; Rowan et al., 1994; Deglaire et al., 2009) and can be used routinely to provide samples of ileal digesta.

14.3.2 Quantification of the endogenous component

Having obtained a representative sample of ileal digesta, it is then necessary to distinguish endogenous sources of nitrogen from materials of undigested dietary origin. To this end a number of methods have been developed. Such methods have been the subject of comprehensive review (Moughan, et al., 1998). Those that would seem most applicable to humans are:

1. feeding the subject a protein-free diet;
2. the enzyme hydrolysed protein/ultrafiltration method; and
3. the isotope dilution method (15N labelled diet).

All three methods have inherent strengths and weaknesses.

14.3.2.1 Protein-free diet

When a diet formulated to be devoid of protein is fed to a human subject for several days, all of the nitrogen and amino acids recovered in the terminal ileal digesta must, by definition, be of endogenous origin. Questions have been raised however about the physiological normality of the protein-depleted state and it is generally considered that the use of protein-free diets leads to lowered ileal endogenous nitrogen flows compared with protein-containing diets (Moughan et al., 2005). Also, the absence of protein in the diet can induce enhanced gut-endogenous losses of the amino acid proline.

14.3.2.2 Enzyme hydrolysed protein/ultrafiltration method

The enzyme-hydrolysed protein method, sometimes referred to as the peptide alimentation method, was proposed by Moughan et al. (1990). It allows endogenous ileal nitrogen and amino acid flows to be determined in subjects fed a diet containing dietary peptides and free amino acids as the sole source of nitrogen. Usually, the subject or animal is fed an enzymatically hydrolysed casein (EHC)-based diet, containing a mixture of free amino acids and oligopeptides with no peptides
being larger than 5000 Da, and thus simulating the products of gastric digestion. Digesta are collected from the terminal ileum and first centrifuged and then physically ultrafiltered, thus removing any material with a molecular weight lower than the filtration cut-off of 10,000 Da. This removes any undigested dietary peptides and free amino acids. The high molecular weight fraction (>10,000 Da) contains the endogenous material. An advantage of the peptide alimentation method over several other methods used to determine endogenous flows is that it allows the endogenous flows of total nitrogen and all amino acids to be determined directly.

With the peptide alimentation method, however, there may be some loss of small peptides and free amino acids of endogenous origin when the ultrafiltrate is discarded, leading to an underestimation of the total endogenous amino acid flow. Such underestimation is considered to be of a relatively small magnitude. The method has been criticized by some workers as leading potentially to inflated estimates of endogenous loss, as the hydrolysed casein may present a higher concentration of bioactive peptides to the gut lumen than would occur with the natural digestion of the parent casein. Further, the extent of hydrolysis of the casein hydrolysate may affect the outcome. A critical assumption with the enzymatically hydrolysed protein method is that the hydrolysed protein source, as fed, simulates the products of gastric digestion. Recent experimental findings with the laboratory rat and adult human (Deglaire et al., 2008) suggest that the latter concerns are unfounded, with the administration of an extensively hydrolysed casein leading to similar endogenous ileal amino acid and nitrogen flows as found when intact casein was given. The enzymatically hydrolysed protein method allows the determination of endogenous nitrogen and amino acid flows associated with the ingestion of peptide-containing dry matter, under seemingly physiologically normal conditions. Dietary factors such as antinutritional factors (ANF) and fibre will lead to enhanced endogenous amino acid excretions above the basal level determined with this method. The effects of these factors cannot be readily determined using the enzymatically hydrolysed protein method, other than using purified diets and isolated ANF and fibre sources.

14.3.2.3 Isotope dilution

The use of stable isotopes allows determination of the endogenous nitrogen at the terminal ileum while the subject is fed a diet containing protein or peptides. Either the subject’s body nitrogen pool or the food protein may be labelled, but in human studies it has been more common to label the food source. Use of the stable isotope of nitrogen (15N) has been particularly popular. The technique has been used in animal studies and with humans (Mahe et al., 1994) and over the years the spectrum of 15N-labelled proteins has increased greatly.

When the food proteins are labelled, it is assumed that the labelled and unlabelled food amino acids are absorbed equally, and that endogenous nitrogen secreted into the gut during digestion does not become labelled to a significant extent during the course of the experiment. Leterme et al. (1996) have reported, however, that a proportion of the absorbed labelled dietary amino acids is rapidly synthesized into body protein and resecreted as gut-endogenous proteins. This will lead to an underestimation of the endogenous loss value. In more recent work with the growing pig and adult human (Deglaire, 2008), the extent of recycling of the dietary 15N label has been determined. Some 10–20% of ileal endogenous protein was 15N-labelled due to tracer recycling, contributing to a likely underestimation of endogenous ileal nitrogen flow of around 6–13%.

The 15N isotope dilution method is a valuable method for determining gut-endogenous protein flows and in particular for allowing study of the relative in situ effects of dietary factors such as plant fibre and ANF that influence the recovery of endogenous protein in ileal digesta.

14.4 Determined Estimates of Endogenous Ileal Nitrogen and Amino Acid Losses in Humans

A number of studies using either the isotope dilution method (15N-labelled diet), the enzyme hydrolysed protein ultrafiltration method, or
Table 14.2. Mean estimates* of endogenous ileal nitrogen flow in the adult human.

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Digesta collection</th>
<th>Nitrogen flow (mg d⁻¹)</th>
<th>Study reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-free</td>
<td>Ileostomate</td>
<td>836</td>
<td>Rowan et al. (1993)</td>
</tr>
<tr>
<td>Protein-free</td>
<td>Ileostomate</td>
<td>719</td>
<td>Fuller et al. (1994)</td>
</tr>
<tr>
<td>Protein-free</td>
<td>Ileostomate</td>
<td>845</td>
<td>Moughan et al. (2005)</td>
</tr>
<tr>
<td>Hydrolysed protein/ ultrafiltration</td>
<td>Ileostomate</td>
<td>1736</td>
<td>Moughan et al. (2005)</td>
</tr>
<tr>
<td>Isotope dilution (¹⁵N milk protein)</td>
<td>Naso-ileal intubation</td>
<td>1638</td>
<td>Gaudichon et al. (2002)</td>
</tr>
<tr>
<td>Isotope dilution (¹⁵N casein)</td>
<td>Naso-ileal intubation</td>
<td>2184</td>
<td>Deglaire (2008)</td>
</tr>
<tr>
<td>Isotope dilution (¹⁵N hydrolysed casein)</td>
<td>Naso-ileal intubation</td>
<td>1851</td>
<td>Deglaire (2008)</td>
</tr>
</tbody>
</table>

Overall mean (protein-free) = 800 mg d⁻¹.
Overall mean (protein or peptides) = 1852 mg d⁻¹.

*Some data were initially reported in units of mg 8h⁻¹. The experimental meal was then assumed to represent one third of the daily food intake, and gut-endogenous flows were assumed to be constant over 24 h.

Factors Influencing Endogenous Ileal Amino Acid Losses

The secretion of endogenous protein into the digestive tract and its subsequent digestion and reabsorption are influenced by a number of factors both related to the subject (e.g. body weight) but especially to the diet. Most studies investigating the influence of dietary factors on endogenous ileal nitrogen and amino acid flow conducted to date have used animals, and such studies have been the subject of review (Boisen and Moughan, 1996; Nyachoti et al., 1997). It seems that dietary dry matter intake, protein content, dietary fibre (amount and type), and the presence of ANF (e.g. lectins, tannins and enzyme inhibitors) can significantly influence the endogenous amino acid flow at the terminal ileum. This has particular implications for developing countries where foods such as cereals, starchy roots, legumes, and pulses make up a disproportionate contribution to the total daily protein supply. The latter types of food contain plant fibre, and may contain ANF, both of which may increase metabolic losses of amino acids from the body and lead either directly or indirectly to lowered digestibility and bioavailability of the dietary amino acids.

Practical Relevance of Measures of Endogenous Ileal Nitrogen

Metabolic cost

The gut is a highly active organ metabolically, and the synthesis and excretion of endogenous protein makes a significant contribution to overall gut and therefore body metabolism. Gut-endogenous amino acid losses contribute
to maintenance amino acid and energy requirements, and also to the efficiency of utilization of dietary amino acids for body protein retention. A higher production of endogenous protein in the digestive tract caused by dietary factors (e.g. fibre, ANF), will be accompanied by a higher rate of gut protein turnover, an increased transport of nutrients and rate of blood flow through the intestinal tissue, and therefore increased energy expenditure. It is known that higher intakes of dietary protein are associated with higher flows of endogenous protein at the terminal ileum (Hodgkinson et al., 2000; Hodgkinson and Moughan, 2007), which in turn implies higher rates of gut metabolic activity and energy expenditure. This is one of several reasons why high protein diets may support body weight loss.

Zebrowska and Kowalczyk (2000) have calculated that for a 30 kg body weight pig, gut protein synthesis accounts for some 8% of the total maintenance energy requirement. Nyachoti et al. (1997) calculated that the energy cost of synthesizing endogenous gut proteins in a 13 kg body weight pig ranged from 6% to 13% of the maintenance energy requirement, dependent upon dietary composition. The gastrointestinal tract is estimated to account for around 25% of total body oxygen consumption in the 3.5 month old pig (Yen et al., 1989). There is no doubt, therefore, that gut-endogenous amino acid losses, which are highly variable and dependent upon a number of dietary factors, can have a significant bearing on the maintenance energy requirement. This often goes unrecognized.

It has also been reported that gut-secreted proteins may account for as much as 25% of total daily body protein synthesis (Simon et al., 1983) and that gut-endogenous amino acid losses are the single most important factor contributing to the maintenance amino acid requirement (de Lange et al., 1995).

### 14.6.2 Contribution to amino acid requirement

How important are the gut-endogenous amino acid losses as contributors to the daily amino acid requirement of the adult human? In a paper presented at the 18th International Congress of Nutrition, Moughan (2005) attempted to calculate the contribution of the gut losses for the two dietary essential amino acids, lysine and threonine. Current best estimates for ileal endogenous lysine and threonine losses for the adult human consuming a normal protein-containing diet were taken to be 657 and 960 mg kg⁻¹ food dry matter intake, respectively. These values were increased by around 10% to allow for hindgut-endogenous losses (estimates in the literature suggest that hindgut protein loss is 10% of that in the foregut) to give values of 723 and 1056 mg kg⁻¹ food dry matter intake, respectively, for the endogenous losses over the total digestive tract. It was assumed that the amino acid compositions of the hindgut and foregut losses are similar. Data presented by Schulze (1994) were used to allow calculation of the effect of dietary fibre. It was assumed that a 1 g kg⁻¹ increase in dietary neutral detergent fibre (NDF) would lead to a 0.22% increase in endogenous ileal protein excretion (Tammenga et al., 1995). The latter authors assuming calculated endogenous N losses of 0.04 and 15g per g diet fibre or tannin, respectively.

The calculated total gut-endogenous lysine and threonine flows are given in Table 14.3 and highlight how factors such as daily food dry matter intake and dietary composition (in this case plant fibre) can greatly influence gut amino acid losses and thus the daily amino acid requirement. The daily amino acid requirement is not a constant. The highest predicted gut lysine loss presented in Table 14.3 accounts for some 73% of the stated daily requirement value for lysine in the adult (FAO et al., 1985) and some 29% of the more recent MIT estimated requirement value (see Waterlow, 1996). The highest predicted threonine loss exceeds the corresponding requirement value (FAO et al., 1985) and, in line with the high concentration of threonine in digesta, is 86% of the MIT estimate. The gut-endogenous amino acid losses clearly make a significant contribution to the daily amino acid requirement.

A similar analysis has been conducted by Gaudichon et al. (2002) who concluded that amino acid losses at the terminal ileum of the
adult human are substantial and depend on the type of protein ingested. Their estimates of endogenous amino acid loss made up a substantial proportion of current estimates of the daily amino acid requirement for adult humans.

### 14.6.3 True ileal amino acid digestibility

It is now widely accepted that for simple stomached animals including humans, the digestibility of dietary protein and amino acids should be determined based on the collection of ileal digesta (terminal ileum) rather than faeces (Moughan, 2003). Faecal amino acid digestibility values may be quite misleading, either overestimating (usually the case) or underestimating digestibility. When digestibility is determined at the end of the small intestine, however, it needs to be recognized that the digesta contain large quantities of endogenous amino acids and nitrogen, and ideally this should be corrected for when determining digestibility.

If total amino acids in ileal digesta are simply deducted from the dietary amino acids ingested, ‘apparent’ estimates of digestibility are obtained. Apparent estimates of digestibility are strongly influenced by the protein content of the test diet, and can thus be misleading. ‘True’ estimates of amino acid digestibility (i.e. corrected for the endogenous amino acid component), however, are independent of the dietary protein concentration and are a fundamental property of the food itself. True digestibility is a superior measure for determining the dietary amino acids that are absorbed from the gut.

It is particularly important when determining the protein digestibility-corrected amino acid score (PDCAAS) for human foods that standardized true digestibility coefficients be used, otherwise significant unintended biases may occur.

A range of true ileal protein digestibility values for humans is given in Table 14.4. Protein and amino acid digestibility can vary widely. Most ileal protein digestibility values in humans to date have been determined on

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### Table 14.3. Influence of food dry matter intake and high and low dietary fibre contents on the predicted endogenous lysine and threonine losses (mg kg⁻¹ bodyweight d⁻¹) from the total digestive tract in adult 70 kg body weight humans.

<table>
<thead>
<tr>
<th></th>
<th>Low DMI (350 g d⁻¹)</th>
<th>High DMI (700 g d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low fibre (0% NDF)</td>
<td>High fibre (10% NDF)</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*aDMI, dry matter intake.

*bNDF, neutral detergent fibre.

### Table 14.4. Selected values for the true ileal digestibility of dietary nitrogen in humans.

<table>
<thead>
<tr>
<th>Protein source</th>
<th>True ileal digestibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw egg</td>
<td>51.3</td>
</tr>
<tr>
<td>Rapeseed isolate</td>
<td>87.1</td>
</tr>
<tr>
<td>Peas</td>
<td>89.4</td>
</tr>
<tr>
<td>Lupins</td>
<td>91.0</td>
</tr>
<tr>
<td>Soya-protein isolate</td>
<td>91.0</td>
</tr>
<tr>
<td>Cooked egg</td>
<td>90.9</td>
</tr>
<tr>
<td>Cow’s milk</td>
<td>95.5</td>
</tr>
<tr>
<td>Casein</td>
<td>97.6</td>
</tr>
</tbody>
</table>

*aEvenepoel et al. (1998).

*bBos et al. (2007).

*cGausserès et al. (1997); Bos et al. (1999); Mariotti et al. (1999, 2002).

*dDeglaire et al. (2009).
Table 14.5. Gross lysine and true ileal digestible lysine contents (g kg\(^{-1}\)) of four types of breakfast cereal\(^a\).

<table>
<thead>
<tr>
<th>Cereal product</th>
<th>Gross</th>
<th>Digestible(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat-based (shredded)</td>
<td>1.74</td>
<td>0.8</td>
</tr>
<tr>
<td>Corn-based (flaked)</td>
<td>0.45</td>
<td>0.2</td>
</tr>
<tr>
<td>Rice-based (puffed)</td>
<td>1.22</td>
<td>0.6</td>
</tr>
<tr>
<td>Mixed-cereal (rolled)</td>
<td>3.65</td>
<td>1.9</td>
</tr>
</tbody>
</table>

\(^a\)Adapted from Rutherfurd et al. (2006).
\(^b\)Based on a digestibility value (laboratory rat) determined using reactive lysine values in diet, ileal digesta, and ileal endogenous protein.

relatively well digested foods. In developing countries, where less-refined cereals and grain legumes are used as major sources of protein, much lower values for true digestibility can be expected (Sarwar Gilani et al., 2005).

Many foods consumed by humans have been processed, including thermal processing, and during processing amino acids can undergo structural changes rendering them less digestible and also lowering their availability. Lysine, often the first-limiting amino acid, is particularly susceptible to damage. By way of example, the data given in Table 14.5 for cereal products show that lysine availability can be greatly affected by processing. These results imply a major extent of damage to lysine in what are staple foods commonly consumed by both adults and children. In some cases more than half of the lysine in the food, usually considered to be nutritionally available, is unavailable, largely due to the formation of Maillard and Maillard-like complexes. The latter data highlight the crucial need to have better information on the digestibility of amino acids in human foods. Such decreases in nutritional value have important consequences for the assessment of diets in the developing world where dietary protein and lysine intakes may be marginal.

### 14.7 Conclusions

The amounts of endogenous nitrogen entering the human digestive tract are substantial, and gut protein turnover makes a significant contribution to total body metabolism. In particular the energetic costs associated with the synthesis and secretion of the various proteins makes up a significant proportion of the maintenance energy requirement.

Whereas much of the material entering the gut is digested with the amino acids being absorbed, relatively large amounts of undigested endogenous amino acids are found at the terminal ileum along with undigested dietary amino acids. The loss of these endogenous amino acids into the colon, with subsequent catabolism by the colonic microbes, represents a loss of amino acids from the body which needs to be replaced. The endogenous amino acid flow at the terminal ileum, therefore, is an important measure in terms of the daily amino acid requirement.

Moreover, as the terminal ileal amino acid flow is highly variable, dependent upon a number of dietary influences, this implies that the daily amino acid requirement is also variable and strongly influenced by dietary habit.

It is also important to have accurate estimates of endogenous ileal amino acids to allow the correction of 'apparent' amino acid digestibility to 'true' digestibility. True amino acid digestibility varies among food sources, with vegetable proteins generally having a lower digestibility than animal proteins. Vegetable proteins, containing fibre and sometimes containing active ANF, also induce higher losses of endogenous amino acids at the terminal ileum. Amino acid digestibility, and particularly that of lysine, may be especially impaired in proteins that have been heat-treated during processing.

Traditionally protein digestibility in human foods has been based on a faecal digestibility assay. This approach, however, is known to be flawed, and true ileal amino acid digestibility represents a considerable step forward in dietary protein quality evaluation.

There is a general lack of information on ileal endogenous amino acid flow in humans and on the true ileal digestibility of amino acids in diverse foods. Particularly lacking is sound information on true ileal amino acid digestibility in proteinaceous foods commonly consumed in developing countries.
For the gut-endogenous amino acid losses, studies with humans are required to determine the relationship between ileal endogenous amino acid loss and food dry-matter intake, and to elucidate how these losses are affected by variables such as body weight, age, and physiological state. There is virtually no information available from studies in humans on the effects of plant fibre and various ANF on ileal amino acid flow. Also, the contribution that the hindgut tissue itself makes to endogenous amino acid losses in humans is unknown.

Over recent years, it has become evident that gut microbes can synthesize essential amino acids, which are absorbed and available for metabolism. It is unclear, however, as to what source of material the microbes utilize for such synthesis, and whether the microbial synthesis represents a meaningful net contribution to the host’s amino acid supply. This is an area that requires further elucidation.

Finally, it is doubtful, at least in the foreseeable future, that true ileal amino acid digestibility measures will be made on a routine basis with human subjects. This necessitates adoption of an animal model for humans, and in this context the growing pig has a number of advantages. Predictive equations, relating amino acid digestibility for a wide range of foods in humans with that in the pig, would be particularly valuable. At the moment there is only a limited set of published observations of digestibility in the two species.

References


Endogenous Amino Acids


15 Metabolic Availability of Amino Acids in Food Proteins: New Methodology

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15.1 Abstract

Protein quality is a term used to describe the capacity of food proteins to meet the amino acid requirement estimates for humans. It is a measure of the nutritional value of food protein sources which depends on the concentration and balance of amino acids and on the digestibility and availability of the food protein for metabolic processes. However, not all foods are equal in their capacity to supply protein and amino acids. The protein digestibility-corrected amino acid score (PDCAAS) was proposed as the method of choice for assessing protein quality in humans. Although it is an improvement over previous methods, the digestibility correction factor used to calculate PDCAAS is based on a rat fecal digestibility model and thus does not account for losses associated with incomplete digestion and absorption, gut endogenous amino acid losses, or absorbed amino acids which are unavailable due to the effect of heat processing and anti-nutritional factors on protein quality. Metabolic availability is a new method for protein quality evaluation utilizing the indicator amino acid oxidation technique. Oxidation of the indicator amino acid is inversely proportional to whole body protein synthesis, and responds rapidly to changes in the bioavailability of amino acids for metabolic processes, and therefore reflects the true metabolic availability (MA) of amino acids. MA is minimally invasive, determined directly in humans, and is based on minimal assumptions which have been validated in animals. It can be used to assess all protein sources, measures the bioavailability of individual amino acids and can be routinely applied in humans. Studies in animals and humans have demonstrated that MA provides an accurate estimate of the protein quality from various protein sources. Practical application of the MA method has the potential to significantly advance protein quality evaluation in humans.

15.2 Introduction

Practical application of amino acid requirement estimates for humans depends on the capacity of food proteins to meet the requirements...
(Elango et al., 2009). The protein quality, or capacity of food proteins to meet the amino acid requirements, is a measure of their nutritional value, and this depends on the concentration and balance of amino acids (AA) and on their digestibility and availability for metabolic processes (i.e. growth or maintenance) (Humayun et al., 2007). The measurements of protein quality of food proteins demonstrate that protein sources are not equal in their ability to supply protein and AA (Schaaifsma, 2005).

The quality of a protein is primarily determined by the relationship between the pattern of the AA composition of the protein source and the nitrogen and amino acid requirement of the subject (Schaaifsma, 2005). Table 15.1 shows the differences in lysine, sulphur AA (methionine and cysteine) and threonine concentration in common foods. Cereals tend to be very low in lysine and threonine content, whereas legumes and animal foods are rich sources of lysine and threonine. Animal foods tend to have greater concentrations of essential AA compared to plant proteins. Peas have a similar lysine and threonine content as found in cereal grain, but are a very poor source of methionine and cysteine. Therefore, a combination of protein sources is necessary to meet the daily nitrogen and amino acid requirements.

### 15.3 Methods to Estimate Protein Quality

Numerous methods have been used to estimate protein quality of food sources (Elango et al., 2009); however, the PDCAAS was proposed as the method of choice for assessing protein quality in humans (FAO, 1991). The PDCAAS is calculated as the proportion of limiting AA from a test protein expressed as a percentage of the content of the same AA in a reference pattern of essential AA, corrected for the digestibility of the test protein as measured in a rat fecal digestibility study (Schaaifsma, 2005).

There are a number of limitations with PDCAAS: it utilizes fecal rather than ileal digestibility; it does not account for conditionally indispensable AA, for variations in digestibility between entire protein and individual AA, or for the effect of heat processing and antinutritional factors on digestibility (Elango et al., 2009). Due to these disadvantages of the PDCAAS method, new techniques need to be developed (Elango et al., 2009).

Stable isotope-based methods have the potential to more accurately estimate the nutritional value of food protein sources than PDCAAS. Within the last decade, three new technologies using stable isotope techniques have been developed: net postprandial protein utilization (NPPU) (Gausseres et al., 1997; Bos et al., 1999); postprandial protein utilization (PPU) (Millward et al., 2000, 2002); and MA (Moehn et al., 2005, 2007). Net postprandial protein utilization is calculated as the ileal digestibility of \(^{15}N\)-labelled dietary protein corrected for \(^{15}N\)-labelled AA deaminated in the body nitrogen pool, measured by plasma urea and urinary nitrogen (Gausseres et al., 1997). Although NPPU is a major advancement in protein quality evaluation,

<table>
<thead>
<tr>
<th>Food</th>
<th>Lysine</th>
<th>Methionine</th>
<th>Cystine</th>
<th>Threonine</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice, white</td>
<td>0.258</td>
<td>36</td>
<td>0.168</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Wheat flour</td>
<td>0.378</td>
<td>28</td>
<td>0.212</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Peas</td>
<td>0.317</td>
<td>59</td>
<td>0.082</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Chickpeas</td>
<td>1.291</td>
<td>67</td>
<td>0.253</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Soybean, cooked</td>
<td>1.108</td>
<td>67</td>
<td>0.224</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Egg, raw</td>
<td>0.914</td>
<td>72</td>
<td>0.380</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Chicken, raw</td>
<td>1.818</td>
<td>85</td>
<td>0.592</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Beef, raw</td>
<td>1.785</td>
<td>83</td>
<td>0.565</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

\(^{1}\)Values are per 100 g of food; \(^{2}\)values are mg g\(^{-1}\) protein.
it is limited to foods which can be intrinsically labelled with $^{15}$N, requires collection of ileal digesta via naso-intestinal intubation, and does not estimate digestibility of individual AA. These limitations suggest that routine or widespread application of this technique is unlikely. Postprandial protein utilization is expressed in terms of the efficiency of nitrogen utilization, i.e. nitrogen utilization/nitrogen intake (Millward et al., 2002). Nitrogen utilization is determined from leucine utilization (leucine intake minus cumulative leucine oxidation) assuming a leucine:nitrogen ratio of 625 mg g$^{-1}$ nitrogen in body tissue protein. Leucine and nitrogen balances are estimated based on the oxidation of intravenously infused L-[1-$^{13}$C]leucine, using the cumulative difference between the postabsorptive and fed-state leucine oxidation rates. This method involves several assumptions which have not been validated. Since it is not possible to estimate the proportion of leucine oxidation in the 6 h postprandial period arising from exogenous (dietary) or endogenous sources, the PPU for nitrogen present in the test meal cannot be accurately determined (Kurpad and Young, 2003). Furthermore, the administration route of isotope labelled AA or proteins to estimate digestion and absorption of food proteins should be the same as that of the test protein (i.e. orally) (Ball et al., 1995).

Metabolic availability is determined by measuring the slope of the response (tracer oxidation) when a test protein is consumed, and calculating the ratio of the test response to the slope of the response of a reference protein or free amino acid (Elango et al., 2009). Metabolic availability utilizes the indicator amino acid oxidation (IAAO) technique in a slope ratio assay, which is considered the optimum method or comparison (Levesque et al., 2010). In addition, because the method measures the change in whole-body protein synthesis, it should account for all losses of AA during digestion, absorption, and metabolic utilization (Moehn et al., 2005). Therefore, this method has been called true metabolic bioavailability of AA in food protein. It is based on minimal assumptions which have been validated in animals, can be used to assess all protein sources, and measures bioavailability of individual AA. Metabolic availability is readily adaptable to routine use for evaluation of proteins in human nutrition because it is the least invasive of the new methods, and thus will be the focus of the remainder of this chapter.

### 15.4 Metabolic Availability of Amino Acids in Food Protein Sources

#### 15.4.1 Concepts of indicator amino acid oxidation

The IAAO method is based on the principle that excess AA cannot be stored, so AA are either utilized for protein synthesis or must be oxidized. Therefore, the change in oxidation of the indicator AA is inversely proportional to the change in protein synthesis (Fig. 15.1) (Ball and Bayley, 1986). When one indispensable AA is deficient for protein synthesis, all other AA are therefore in excess and must be oxidized (Elango et al., 2008b). As the intake of the limiting AA increases, the oxidation of an indicator AA (another indispensable AA, typically [L-$^{13}$C]phenylalanine) decreases linearly until the requirement for the limiting AA is reached (Pencharz and Ball, 2003). Increases in intake of the test AA beyond the requirement do not increase protein synthesis further, and therefore there is no further change in oxidation of the indicator AA (Fig. 15.1); IAAO reaches a plateau. The inflection point between the linear decrease and plateau in oxidation represents the estimated average requirement (EAR) for the test amino acid and is determined with the use of bi-phase linear regression analysis (Elango et al., 2008a,b).

The IAAO technique has been extensively developed to determine AA requirements and was chosen as the gold standard by the World Health Organization for determination of AA requirements of humans (WHO, 2007; Pencharz and Ball, 2003). Initial development of the IAAO technique was in young pigs using $^{13}$C phenylalanine to determine the requirement for histidine (Kim et al., 1983) and tryptophan (Ball and Bayley, 1984a) and was validated against the traditional method of nitrogen balance (Kim et al., 1983). Extensive work followed to adapt the IAAO technique to humans, including the use of stable rather
Metabolic Availability of Amino Acids

Fig. 15.1. Inverse relationship between protein synthesis and oxidation of an indicator amino acid with increasing intake of limiting amino acid. EAR, estimated average requirement. Adapted from Ball and Bayley (1986).

than radioactive isotope (Zello et al., 1993); the route of isotope administration (intravenous versus oral delivery) (Bross et al., 1998; Kriengsinyos et al., 2002); the time of adaptation required for the level of test AA (Zello et al., 1990, Elango et al., 2009); and the time of adaptation necessary for the level of dietary protein (Thorpe et al., 1999). Thus the current protocol involved a 2 d adaptation to a fixed daily protein intake followed by at least 8 h adaptation to the test AA intake on the day of study (Elango et al., 2008a, 2009). The IAAO technique has been used in the pig model to assess the effect of enteral versus parenteral nutrition on AA requirements (e.g. Bertolo et al., 1998), the development of advanced parenteral nutrition solutions (Brunton et al., 2007), the effect of gut AA utilization on whole body AA requirements (Law et al., 2007, Shoveller et al., 2003), the availability of amino acid supplements (Shoveller et al., 2010) and the inter-animal variability of AA requirements (Bertolo et al., 2005; Moehn et al., 2008).

Indicator AA oxidation relies on selection of an appropriate indicator AA. There are three key criteria when selecting an AA as indicator:

1. a dietary indispensable AA;
2. the labelled carbon is irreversibly lost as CO₂ during oxidation, preferably during the first steps of AA catabolism; and
3. the AA should undergo no other significant reactions apart from oxidation to CO₂ and incorporation into protein (Zello et al., 1995).

Since accurate measurement of label in the free AA pool and the end products of degradation are essential to the oxidation technique, ubiquitously labelled carbon atoms would not be appropriate (Zello et al., 1995). Although lysine (Ball and Bayley, 1984a), threonine (Soliman and King, 1969), [¹⁴C-methyl]methionine (Brookes et al., 1972), and leucine (Kurpad et al., 1998; Hsu et al., 2006) have all been used as indicator AA, in comparison to phenylalanine these have all been found to be less responsive or less accurate in determining the requirement of the test amino acid. Therefore, L-[¹³C]- or L-[¹⁴C]-phenylalanine (Kim et al., 1983; Ball and Bayley, 1984a,b), in the presence of excess tyrosine, has been used most often because it satisfies all three key criteria above. It also has a number of additional advantages: the free pool size is comparatively small, with a rapid turnover rate compared to other indispensable amino acids (Neale and Waterlow, 1974); and the size of the intracellular free phenylalanine pool is tightly regulated (Flaim et al., 1982). Phenylalanine oxidation was more responsive to dietary changes than lysine or leucine (Neale and Waterlow, 1974; Hsu et al., 2006). The advantage of the rapid
turnover and small, tightly regulated size of the free phenylalanine pool is that it responds rapidly to changes in test AA intake. Ball and Bayley (1984b) showed in baby pigs that AA requirement could be determined with as little as 4 h of adaptation to the diet. In sows and growing pigs, phenylalanine oxidation responded within 1–2 d of a change in test AA intake, and remained constant for up to 10 d after a change in diet (Moehn et al., 2004). Therefore, 2 d of adaptation to a new dietary test AA level are deemed sufficient. Recently, Elango et al. (2009) showed in humans that the adaptation period to the test AA can be even shorter (~8 h), provided the subjects are adapted to a standard protein intake prior to receiving the test diet.

15.4.2 Application of indicator amino acid oxidation to determine metabolic availability of amino acids in food protein sources

The change in appearance of $^{13}$CO$_2$ or $^{14}$CO$_2$ in breath from oxidation of the indicator amino acid reflects the change in whole-body protein synthesis; therefore, the IAAO can be used to determine the MA of AA in foodstuffs. At a given AA intake below requirement, the change in indicator oxidation reflects the response of whole-body protein synthesis to graded levels (or intakes) of the limiting AA (Moehn et al., 2005). This slope indicates the change in IAAO per unit change in limiting AA. A shallower slope indicates that less AA per unit intake is available to support protein synthesis (Fig. 15.2). Therefore, the relative difference in the rate of change (slope) of IAAO between test and reference proteins will be proportional to the whole-body MA of the test AA for protein synthesis. Metabolic availability, therefore, takes into account all losses associated with incomplete digestion and absorption, gut endogenous AA losses, amino acid oxidation and absorbed AA which are unavailable due to anti-nutritional factors, damage caused during heat processing (Maillard reaction compounds), D-amino acids and cross-linked proteins such as lysinoalanine.

The MA method utilizes incremental increases in crystalline test AA to determine the reference slope. The true digestibility of crystalline (free) AA is essentially 100% (Baker, 1992). Thus, the slope of the indicator AA oxidation obtained with the crystalline form of the test AA represents the maximal unit increase in protein synthesis and is equivalent to 100% MA of the test AA. Oxidation of the indicator AA is regressed against the AA intake above that provided by the reference diet, supplied by crystalline test AA, and the protein source (Moehn et al., 2005). The ratio of the slope of the indicator

![Fig. 15.2. Metabolic availability of lysine in peas and heated peas. Metabolic availability is calculated from the ratio of the slope from the test AA (i.e. peas or heated peas) to the slope of the free AA. Adapted from Moehn et al., 2005.](image-url)
oxidation due to the test protein source compared to the indicator oxidation slope due to the free AA gives the MA of the food protein.

A number of key criteria must be met when determining MA:

1. the test AA must be first limiting to ensure that the intake of the test AA drives the change in indicator oxidation;
2. the change in indicator oxidation to incremental changes in test AA must be linear to allow calculation of availability; and
3. the observed response must not be influenced by other dietary nutrients in the test feed ingredient (Batterham, 1992; Littell et al., 1995). In addition, when applying the IAAO in a slope-ratio assay, an isotopic steady state must be achieved.

The determination of MA using the slope-ratio assay assumes a linear response to increasing test AA (Batterham, 1992). To ensure a linear response, the upper limit of test AA intake should be below the lower confidence interval of the requirement in every individual (Elango et al., 2009) or -2 SD below the requirement (Moehn et al., 2005). Limiting the intake of the test amino acid to 2 SD below the animal’s requirement ensures that each animal will respond to increasing intake in a linear fashion. If intake were to exceed the requirement, the slope would not be linear because oxidation of the indicator amino acid would be in the plateau phase. Figure 15.3 shows change in indicator oxidation with incremental changes in test AA intake above and below daily intake requirement. At AA intakes at least 2 SD below the daily intake requirement, there is a linear change in indicator oxidation.

One of the advantages of using the indicator amino acid oxidation technique to determine MA of AA in food proteins is the use of repeated measurements within each subject. Each study subjects receives each of a series of four reference diets supplying free test AA (crystalline form) at incremental increases to a maximum of 80% of the EAR of the test AA (Moehn et al., 2005). The reference diets supply all AA, except the test AA, in excess and identical in content to that supplied by the test proteins. Free AA is then replaced by protein-bound AA from the protein source being measured. Again, each subject receives the diet containing the protein-bound AA. The use of repeated measurements within the same subject reduces the effect of individual variability, and increases the sensitivity of the estimate accounting for between-subject variation which can be up to 30% in humans (Zello et al., 1993).

15.4.3 Validation of the metabolic availability method

The initial validation of MA methodology using the IAAO technique was conducted by Moehn et al. (2005) in growing pigs. The MA of lysine from peas and heat-treated peas in growing pigs (15–18 kg) was assessed by comparing the oxidation of the indicator AA to that when pigs were fed crystalline lysine. Replacing the free lysine with equal amounts of protein-bound lysine from peas and heated peas led to a lesser decrease in oxidation than addition of free lysine, as represented by a lower slope (Table 15.2; Fig. 15.2). This indicated an availability of lysine in peas (88.8%) and heated peas (54.8%) for protein synthesis. The MA of lysine in peas was similar to previously published estimates of the true ileal digestibility of lysine in peas (NRC, 1998). Ileal digestibility does not measure the reduction in bioavailability due to heat-damaged AA (Moughan and Rutherford, 2008); however, the MA of lysine in heated peas was comparable to the MA of lysine in similarly heated peas (48%), determined using slope ratio growth assay (van Barneveld et al., 1994). Similarly, Ball et al. (1995) reported impaired lysine availability in heated cottonseed meal, but found similar oxidation responses for lysine in soybean meal and for free lysine. However, in this experiment, the intake of lysine was in excess of the requirement and thus MA could not be calculated.

The MA method has been further validated with other protein sources in growing pigs (Moehn et al., 2007) and pregnant sows (Levesque, 2010) (Table 15.2). Using the method described by Moehn et al. (2005), the MA of
threonine fed to pregnant sows was determined to be 88% in corn and 89.3% in barley. As with that observed for lysine (Moehn et al., 2005), addition of protein-bound threonine from corn or barley resulted in a slower rate of decrease in indicator oxidation than from crystalline threonine. The quality of a protein increases as the AA composition of the protein more closely matches the individual’s requirement for different AA (Reeds and Beckett, 1996).
Table 15.2. Metabolic availability of amino acids in common protein sources fed to swine.

<table>
<thead>
<tr>
<th>Amino acid intake</th>
<th>IAAO response (% of dose g⁻¹ amino acid intake)</th>
<th>Metabolic availability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moehn et al., 2005&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystalline lysine</td>
<td>-3.16 ± 0.39</td>
<td>100.0</td>
</tr>
<tr>
<td>Raw peas</td>
<td>-2.81 ± 0.44</td>
<td>88.8</td>
</tr>
<tr>
<td>Heated peas</td>
<td>-1.73 ± 0.41</td>
<td>54.8</td>
</tr>
<tr>
<td>Moehn et al., 2007&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystalline lysine</td>
<td>-3.63 ± 0.43</td>
<td>100.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>-3.18 ± 0.32</td>
<td>87.5</td>
</tr>
<tr>
<td>Canola meal</td>
<td>-2.59 ± 0.31</td>
<td>71.4</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>-2.73 ± 0.38</td>
<td>75.1</td>
</tr>
<tr>
<td>Raw peas</td>
<td>-2.75 ± 0.29</td>
<td>75.8</td>
</tr>
<tr>
<td>Heated peas</td>
<td>-2.48 ± 0.30</td>
<td>68.3</td>
</tr>
<tr>
<td>Heated peas plus free lysine</td>
<td>-2.78 ± 0.27</td>
<td>76.5</td>
</tr>
<tr>
<td>Levesque, 2010&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystalline threonine</td>
<td>-1.59 ± 0.53</td>
<td>100.0</td>
</tr>
<tr>
<td>Corn</td>
<td>-1.40 ± 0.62</td>
<td>88.0</td>
</tr>
<tr>
<td>Barley</td>
<td>-1.42 ± 0.81</td>
<td>89.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are mean ± SEM, n = 4 growing pigs.
<sup>b</sup>Values are mean ± SEM, n = 8 growing pigs.
<sup>c</sup>Values are mean ± SEM, n = 6 pregnant sows.

However, the relative requirements for different AA changes with developmental and physiological state, thus the quality of a protein source may not be constant if the physiological state of the animal changes (Reeds and Beckett, 1996). The MA of threonine in corn fed to growing pigs was 82.3%, similar to published estimates of the true ileal digestibility of threonine in corn (NRC, 1998), but lower than the MA of threonine from the same sample of corn when fed to adult sows (Levesque, 2010). Therefore, age appears to have an effect on the availability of AA from food sources in pigs.

The MA method has also been adapted for use in humans and was used to determine the MA of sulphur AA from casein and soy protein isolate (Humayun et al., 2007). Casein and soy protein isolates were tested at 60% of the total sulphur AA requirement. Oxidation of indicator AA when healthy young men were fed casein or soy protein isolate diets supplying 60% of the total sulphur AA requirement was compared to the IAAO response when the same subjects consumed diets supplemented with crystalline methionine. The study was repeated in the same subjects and the MA, unsupplemented and supplemented, respectively, for both casein (90.5 and 90.6%) and soy protein isolate (70.6 and 69.3%) were obtained (Humayun et al., 2007). The methods commonly used in animals to determine AA availability are not ethical or applicable to humans and thus there are very few data in humans. The ability to routinely measure AA availability in humans in a non-invasive manner is important because AA availability is not currently used to adjust recommendations for amino acid intake in humans.

Using the IAAO in a slope-ratio assay allows determination of the availability of AA in vivo, and is capable, contrary to digestibility studies, of detecting reduced protein quality of heat-treated food sources. This method can also be used to measure the availability of amino acid supplements (Shoveller et al., 2006, 2010) and to detect differences in availability due to age.

15.5 Conclusions

Nutritional application of amino acid requirement estimates must be combined with an understanding of the capacity of food proteins to meet the amino acid
requirements of humans. Although numerous methods for assessing protein quality have been developed, the MA method can be applied for rapid and routine use in humans. The MA method is minimally invasive, can be determined in the subject of interest (i.e. human) and accounts for all losses of AA during digestion, absorption, and metabolic utilization. It is based on minimal assumptions validated in animals, can be used to assess all protein sources, and measures the bioavailability of individual AA. Studies in animals and humans have demonstrated that the MA methodology provides an accurate estimate of the protein quality from various food protein sources.

References


Shoveller, A.K., Moehn, S., Rademacher, M., Htoo, J.K. and Ball, R.O. (2010) Methionine-hydroxy analogue was found to be significantly less bioavailable compared to DL-methionine for protein deposition in growing pigs. *Animal* 4, 61–66.


16 Amino Acid Requirements: Quantitative Estimates

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16.1 Abstract

There are now several direct experimental methods that are available to measure indispensable amino acid (IAA) requirements. In all these, increasing intakes of amino acids are given until a change is observed in the response curve. The response could be nitrogen (N) balance or growth, although current methods measure amino acid oxidation or its surrogate by the use of stable isotope tracers. N balance or growth progressively increase until the requirement level of intake of the IAA is reached, after which it plateaus. Oxidation of the test IAA is measured as the primary route of loss of that amino acid, such that the IAA balance (intake-oxidation) can be measured. For oxidation, there is no change in response as graded levels of the IAA are fed below the requirement level. However, once the requirement level is reached there is a linear increase in oxidation. The IAA balance follows the same pattern as the N balance. However, both these methods have several requirements and assumptions, and are technically demanding. A third method has also been developed, related to measuring the oxidation of a selected amino acid whose kinetics are well described, in response to graded intakes of the test IAA. The selected amino acid (other than the test IAA) is called an indicator amino acid, and its oxidation (or balance) response is measured. The oxidation of the indicator, particularly in the fed state, is an index of protein synthesis, and will fall as increasing levels of the test IAA are fed until the requirement level is reached, after which there is no further change. The advantage of this method is that it is relatively non-invasive, and effectively is a breath test, since the oxidation of the tracer-labelled amino acid itself acts as a surrogate for whole-body amino acid oxidation. A modification of the indicator amino acid oxidation method relates to using the measured IAA balance as a surrogate for N balance, in 24-h indicator amino acid balance (IAAB) studies. Here, the balance response follows the same pattern as that of N balance and growth. The indicator amino acid oxidation (and balance) technique is now used primarily in both developed and developing countries, to derive estimates of amino acid requirements in humans. Values of IAA requirements based on the direct and indicator amino acid oxidation and balance (IAAO and IAAB) techniques are considerably higher than those derived from N balance studies, and these tracer-based estimates are now the basis of the recent WHO et al. (2007) recommendations for amino acid requirements.

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16.2 Introduction

Determinations of human IAA requirements have been based on several strategies, and these offer insights into actual N balances, or surrogates of balance. The strategy of these determinations is to expose an individual or a group of individuals to different intakes of the experimental amino acid, ranging from below to above the putative requirement, and to look for quantifiable responses. An inspection of the intake-response curve should allow for the determination of a change in the response pattern, which could be interpreted within a plausible framework, to determine the requirement. Thus far, the methods that have been used include measurements of the N balance method, inspection of plasma amino acid responses to different intakes of amino acids, and a number of methods related to the use of stable isotopes to accurately quantify amino acid oxidation or flux. While the N balance method has been criticized and is difficult to perform, the amino acid response method is imprecise and no longer in general use, since better tracer techniques are now available. The more recent and precise stable isotopic techniques that have been used include direct measurements of amino acid oxidation (DAAO), and measurements of the oxidation of an indicator amino acid, which is presumed to indicate the status of other indispensable amino acids at different intakes (IAAO), as well as methods that measure the actual daily balance of indispensable amino acids. As with oxidation measurements, the measurements of balance could be associated with measurements related to the experimental amino acid (direct amino acid balance, DAAB) or measurements related to the balance of an indicator amino acid (indicator amino acid balance, IAAB). The latter measurements were usually made over a period of 1 d. In addition to all these methods, one can calculate the daily requirement of amino acids by a factorial method, based on the obligatory amino acid oxidative losses (OAAL, which can be predicted from the obligatory N loss from the body). Finally, more recently, short-term feeding and fasting studies of leucine oxidation and balance have also been applied to measurements of utilization of milk and wheat proteins in terms of the relative efficiency of postprandial protein utilization, from which the requirements for wheat protein and lysine have been derived. The different methods are described in more detail below.

16.3 Nitrogen Balance

Nitrogen balance is a deceptively simple concept, simply stated as the difference between the total nitrogen intake and total nitrogen excretion over a day. This technique has been used for many years in all aspects of protein and amino acid nutrition research, and remains the principal method used to estimate adult human protein requirements. However, there are several technical problems associated with measuring all the N intake and loss accurately (Manatt and Garcia, 1992). Measuring the N intake would appear to be easier, since it can be achieved by analysing duplicate portions of food and by careful attention to the collection of all food not consumed, such as spillage and residue on plates. If this error was present, it would lead to an overestimation of the N balance. On the other hand, exact N excretion is even more difficult to measure. While complete collections of urine and faeces are difficult but still possible, there are serious difficulties in determining the skin-based integumental and other minor or unmeasured routes of N loss such as hair, nails, and various bodily secretions. Attempts have been made to quantify these losses, and in a comprehensive and detailed study, Calloway and Margen (1971) concluded that dermal and miscellaneous losses amounted to about 0.5 g N daily (or about 7.1 mg N kg\(^{-1}\) d\(^{-1}\)) in sedentary, healthy young men. In older men these losses were found to be lower, at about 3 mg N kg\(^{-1}\) d\(^{-1}\) (Zanni et al., 1979), and it is possible that the miscellaneous losses decline with advancing age. The precise magnitude of these miscellaneous losses cannot be stated with any confidence. The FAO et al. (1985) expert consultation proposed a value of 8 mg N kg\(^{-1}\) d\(^{-1}\) for adults as a reasonable estimate of miscellaneous losses, but in the more recent WHO et al.
consultation (2007), a value of 5 mg N kg\(^{-1}\) d\(^{-1}\) was selected. The value assumed for the miscellaneous N loss could vary downward or upward (depending on environmental conditions or the nature of work the individual was engaged in).

Nevertheless, assuming different amounts of these miscellaneous N losses to add to the urinary and faecal N loss will affect the quantitative interpretation of N balance data in any individual study. For example, in a reanalysis of published N balance data in adult experiments designed to measure the amino acid requirement, Hegsted (1963) assumed an additional miscellaneous loss of 0.5 g N daily (amounting to about 8 mg N kg\(^{-1}\) d\(^{-1}\) in an individual weighing 60 kg). This meant that the N balances were made more negative by 0.5 g N daily. Based on this apparently small adjustment, the amino acid requirement values were significantly increased above those proposed in the original N balance experiments. The range of increase was in many cases quite dramatic; for example, the requirement for threonine and methionine increased by about 500%. If an even smaller value was used for the miscellaneous N loss, for example 0.3 g N daily or about 5 mg N kg\(^{-1}\) d\(^{-1}\), Millward (1999) still found that there was an increase in the estimated amino acid requirement values, although the increases, as expected, were lower than when a daily miscellaneous loss of 0.5 g N d\(^{-1}\) was assumed. Nevertheless, these recalculations underline the importance of even small, unmeasured routes of N loss in N balance experiments. Adding N losses to the N balance estimate leads to different effects relating to the degree of slope of the N balances to the N intakes response, as the balances approach zero when the intake of the test amino acid is near adequate. If this is a relatively low slope, the effect of adjusting for a given value for miscellaneous N losses on the amino acid requirement estimate is even more dramatic. It is even likely that the response line is not linear and that the slope varies depending on the range of intakes being studied. For example, the gradient is higher at very low intakes, and declines appreciably as the balance point is approached (Young et al., 1973; Inoue et al., 1974). In essence, with a linear approach, it is likely that results from an experiment that included very low intakes are likely to lead to an underestimate of the requirement. It is therefore critical to study a range of intakes that encompass the putative requirement level. This is illustrated in a simulation (Rand et al., 1977; Rand and Young, 1999), in which different curve-fitting techniques were used on data acquired in an earlier study on the lysine requirement. Four different shaped curves: linear (A), logarithmic (B), square root (C), and exponential asymptotic (D) were fitted (Fig. 16.1). While all curves intersected at the same point, the intake value at which they reach the zero balance point was different. It is clear that curves B, C, and D had gradients that declined sharply as the zero balance point was approached, underlining the problem of selecting appropriate intakes to study. In addition, the median lysine requirements obtained by these three procedures were about 28–29 mg kg\(^{-1}\) d\(^{-1}\) when the miscellaneous N losses were assumed to be 8 mg kg\(^{-1}\) d\(^{-1}\), and 20.4–22.7 mg kg\(^{-1}\) d\(^{-1}\) when miscellaneous losses of 5 mg N kg\(^{-1}\) d\(^{-1}\) were assumed. This illustrates the relatively high sensitivity of the nitrogen balance method to the assumed value of miscellaneous N losses.

The quantification of N losses may also be lower than expected because of unexplained losses. For example, it has often been found that there is a progressively better or more positive N balance with a continued increase in N intake, even above that known to be required to maintain body N equilibrium, including in adults who have ceased to grow and accrete protein (Hegsted, 1976; Kurpad and Vaz, 2000). A careful recovery study of N in piglets given alanine N infusions showed that even after accounting for expanded urea and α-amino nitrogen pools, the excretion of N was significantly lower than 100% (Davis et al., 2000). In addition, it has been thought that the IAA can be made available to the body by the gut microflora (Fuller and Garlick, 1994). It is known that there is some recycling of urea by intestinal bacteria (Jackson, 1993), and in humans, this is thought to occur to the extent of about 20% in subjects in whom the bacterial flora were reduced by antibiotics (Raj et al., 2008). There is also the question about the source of N needed for bacterial amino acid synthesis. If it
Fig. 16.1. Non-linear regression of N balance against lysine intake, using four different functions. The horizontal line at the balance of +5 mg N kg\(^{-1}\) d\(^{-1}\) represents the assumed additional miscellaneous N losses that were not measured. The dashed line at +8 mg N kg\(^{-1}\) d\(^{-1}\) represents another assumption of the miscellaneous N loss; the true value could lie between 5 and 8 mg N kg\(^{-1}\) d\(^{-1}\) depending on environmental conditions or nature of work. (From Rand and Young, 1999; actual data points removed for clarity.)

comes from the ingested dietary N in the form of amino acids in dietary protein, then the input of amino acids from microbial synthesis will not provide a net benefit to the host. The importance of the microbial source of amino acids is when nitrogenous end-products of no use to the host – such as urea or ammonia – are utilized by the microbes, or when the microbial IAA comes from surplus non-IAA N in the diet. In a recent study to assess the source of N used in microbial synthesis in the upper gut of normally nourished pigs fed their normal diets with additional pectin, it was found that preformed amino acids from dietary protein – as well as endogenous protein (from mucus glycoproteins and epithelial cell turnover) – contributed much more to microbial amino acids than de novo synthesis (Libao-Mercado et al., 2009). Further, when human subjects are fed resistant starch along with adequate dietary N, it appears that there is no net extra IAA input into the body (Kurpad et al., unpublished). Therefore, the nutritional significance of this finding, and in particular the extent to which this bacterial IAA can spare dietary IAA, is not yet clear. From a purely technical viewpoint, N balance approaches used in amino acid requirement experiments are beset by problems that may lead to an underestimate of those requirements (Young, 1987; Young et al., 1989; Young and Marchini, 1990).

Experimental design also matters. The effect of dietary energy and energy balance on the N balance is well known, and the N balance experiments should typically be performed when subjects are in a near-zero energy balance. In the N balance-based estimates of amino acid requirements (Rose, 1957), one criticism was that the energy intakes in those studies were too high. This could account, at least in significant part, for an apparent underestimation of the minimum amino acid intakes actually needed to maintain body N equilibrium. Maintaining a perfect zero energy balance over many days is impractical and perhaps impossible. For example, Roberts et al. (1990) attempted to match precisely energy
intake with estimated energy expenditure in one study, and found that they had overestimated the maintenance intake by about 3 kcal kg\(^{-1}\)d\(^{-1}\). Although this overestimation might seem negligible, over short-term N balance experiments it would be sufficient to change N retention by about 6 mg N kg\(^{-1}\)d\(^{-1}\) (Young et al., 1992). In addition, adaptation to the new level of protein intake is important, as not only does metabolism take time to adapt to the new intake, but the body urea pool must also adjust to the change. Therefore, the length of the dietary adjustment period, and the degree to which the prior dietary intake differed from the experimental protocol intake, are also counted as important factors in the estimation of the N balance. This applies as a general principle to all studies in which a marker of equilibrium is sought on a particular amino acid intake, and will also be discussed under the other methods below. In N balance studies in experienced hands, where protein intake levels have been changed, a new and relatively steady state of N excretion is achieved, usually within about 4–6 d (Rand et al., 1976), and in general, this does not change by much with longer exposure to the changed protein intake (Rand et al., 1979, 1985). Other studies have been interpreted to indicate that a period of about 2 weeks or longer is necessary to achieve a new steady state N output with a change in total N intake (Quevedo et al., 1994). As detailed above, it is critical to ensure that confounders such as energy intake are adequately addressed in the design and interpretation of studies that seek to define N equilibrium at different N intakes.

In general, it would appear that the N balance approach is technically difficult and potentially confounded by many variables. For these various reasons the amino acid requirement values that were proposed by earlier N balance studies (Table 16.1) must be viewed with some caution, and more recent research with better techniques have indicated that the amino acid requirements are higher. Nevertheless, the N balance, when obtained via appropriately designed experiments, measured carefully, and the results analysed appropriately, can be a useful marker of the relative adequacy of dietary nitrogen or even of a specific, indispensable amino acid intake in comparison with an acceptable reference point.

### 16.4 Isotopic Tracer Methods

With advances in the measurement of stable isotope enrichment in biological matrices, and the expanded use of tracers enriched with these isotopes in human metabolic research, a second paradigm of measuring IAA requirements based on a stable isotope tracer technique to measure body amino acid balance was developed as the criterion of adequacy. A series of tracer studies was begun at MIT by Vernon Young and his colleagues in the early 1980s, to revisit the determination and estimation of the amino acid requirements in adults, using stable isotopic techniques to measure amino acid oxidation (Young and Bier, 1981; Young et al., 1984, 1987; Meguid et al., 1986a,b; Meredith et al., 1986). Amino acid oxidation studies are based on the principle that any amino acid, provided in excess of the needs of protein synthesis, is preferentially oxidized (Zello et al., 1995). This means that amino acid oxidation, measured in multiple amino acid intake experiments, will yield a pattern of a low or maintenance level of oxidation, which would increase as the intake of the test amino acid increased above the requirement level. The tracer used is an indispensable amino acid labelled at its carboxyl carbon (typically with \(^{13}\)C for humans and \(^{14}\)C for animals). This method relies on the assumption that the requirement of specific IAA in adults is the

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Requirement (mg kg(^{-1})d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>10</td>
</tr>
<tr>
<td>Leucine</td>
<td>14</td>
</tr>
<tr>
<td>Lysine</td>
<td>12</td>
</tr>
<tr>
<td>Methionine</td>
<td>13</td>
</tr>
<tr>
<td>Phenylalanine + tyrosine</td>
<td>14</td>
</tr>
<tr>
<td>Threonine</td>
<td>7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.5</td>
</tr>
<tr>
<td>Valine</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^{a}\)Values from WHO et al. (2007).
dietary intake of that amino acid which balances all routes of loss in which oxidation is primary.

Since that time, the paradigm has evolved into a number of different methods, all of which have used tracer-based studies of human amino acid requirements. These methods are both short and long term, and measure either the oxidation of the amino acid, or its daily balance. Studies that have used a tracer of the test dietary amino acid, with a measure of its rate of oxidation at various test intake levels are called the direct amino acid oxidation (DAAO) method, which can be extended to the measurement of the body \(^{13}\text{C}\)-amino acid balance, or the direct amino acid balance (DAAB) method. These techniques have been used to assess the requirements for leucine, valine, lysine, and threonine (Young et al., 1989). Another key advance in the field is the use of an ‘indicator’ tracer to assess the status of whole-body amino acid oxidation (IAAO) or indicator amino acid balance (IAAB) at varying levels of a test dietary amino acid. An example of the IAAO approach is seen in the study by Zello et al. (1993) which included a determination of the rate of \(^{13}\text{C}\)-phenylalanine oxidation at varying levels of lysine intake. Finally, and more recently, there have been studies designed to assess the retention of protein during the post-prandial phase of amino acid metabolism, using \(^{13}\text{C}\)-leucine as a tracer (Millward, 2000; Millward et al., 2000). This is called the post-prandial protein utilization (PPU) approach. These tracer-based methods are described in sequence below.

### 16.4.1 Direct amino acid oxidation and balance

The potential advantage of the direct amino acid oxidation and balance (DAAO/DAAB) technique is that the rate of oxidation of the dietary amino acid of interest is directly estimated. This technique is based on the measurement of the irreversible oxidation of tracer-labelled test amino acids (Motil et al., 1981; Meguid et al., 1986a,b; Meredith et al., 1986; Zhao et al., 1986; Marchini et al., 1993; Hiramatsu et al., 1994), and involves correlating the measured oxidation rate with controlled diet studies at different levels of intake of the test amino acid. It is then possible to evaluate both the pattern of change in the oxidation rate of the test amino acid and the body balance (24 h intake–24 h predicted oxidation) of the test amino acid under study. The requirement level of the test amino acid is the minimum intake at which the estimate of daily balance was close to or equal to zero. Over a run-in period of up to a week in duration, the procedure involves giving subjects diets based on amino acid mixtures. These supply limiting to generous levels of the amino acid being tested, and the test amino acid balance at each level of test amino acid intake is measured. This balance could be measured by the intravenous infusion (or oral administration) of a stable isotope \((^{13}\text{C})\) labelled amino acid, such that the flux and the irreversible oxidation of the test amino acid could be measured. The measurement of this irreversible oxidation is based on the highly accurate quantification of the respiratory loss of oxidized tracer as carbon dioxide (by indirect calorimetry and isotope ratio mass spectrometry), as well as the measurement of the isotopic enrichment (by gas chromatography–mass spectrometry) of the precursor pool of the amino acid in the body.

This tracer approach was first applied in radio-tracer studies to determine the requirement for lysine in young rats (Brookes et al., 1972) and then later to estimate the requirement for leucine (Harper and Benjamin, 1984), threonine (Kang-Lee and Harper, 1978), and histidine (Kang-Lee and Harper, 1977) in growing rats, and lysine in young rats (Bergner et al., 1978) and pigs (Chavez and Bayley, 1976). These animal models revealed that oxidation of the amino acid remained low and relatively constant at sub-maintenance amino acid intakes and then began to increase as the intake approximated and then further exceeded the requirement for maximum growth. The amino acid oxidation rate continued to increase linearly with further increases in intake.

In humans, Young and co-workers used this approach in the 1980s, using stable isotope rather than radiolabelled tracers, for
ethical and safety reasons. Until 1986, all of the published estimates of IAA requirements in adult humans had been obtained using either nitrogen balance or, for a few, using changes in plasma amino acid levels (Tontisirin et al., 1973; Young et al., 1971). In 1986, a series of DAAO studies were reported (Meguid et al., 1986a; Meredith et al., 1986; Zhao et al., 1986). Thereafter, a number of studies were performed with DAAO/DAAB methods (Table 16.2) to assess the selected IAA requirements in adults.

However, there are some limitations and disadvantages to this specific tracer approach (Table 16.3):

1. A precise determination of the rate of trace oxidation is difficult to achieve, since for most amino acids, the isotopic enrichment of the pool directly supplying the substrate for oxidation is not actually known. For practical reasons, the isotopic enrichment of the venous plasma-free amino acid pool is routinely sampled and analysed for purposes of determining the oxidation rate. Except for leucine (El-Khoury et al., 1994a) and possibly methionine where the plasma enrichments of α-ketoisocaprate and homocysteine (MacCoss et al., 1999), respectively, can be used as an index of labeling in the intracellular pool of the parent amino acid, it is likely that the DAAO and DAAB approaches with other amino acids have underestimated oxidation and overestimated balance. However, it is likely that this is a systematic error, which would change the absolute value of the amino acid oxidation rate, but not the pattern of change in amino acid oxidation in response to altered intakes. Since the identification of the requirement level of the test amino acid is considered to be where the pattern of amino acid oxidation changes in response to altered amino acid intake, this may not necessarily be a major problem. However, for the accurate measurement of amino acid balance, it remains to be a problem.

2. Initial human studies of DAAO were of relatively short duration (about 3h) and were conducted in subjects who were in the fed state (Meguid et al., 1986a,b; Meredith et al., 1986; Zhao et al., 1986). To estimate the DAAB, assumptions had to be made about the rates of amino acid oxidation during the remaining

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Method</th>
<th>Value obtained (range) mg kg⁻¹ d⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>Short-term (3 h &amp; 8 h)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Meguid et al. (1986a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cortiella et al. (1988)</td>
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<tr>
<td></td>
<td>Krempf et al. (1993)</td>
<td></td>
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<tr>
<td></td>
<td>Young et al. (1991)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Meguid et al. (1986b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>El-Khoury et al. (1994a)</td>
<td></td>
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<tr>
<td></td>
<td>El-Khoury et al. (1994b)</td>
<td></td>
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<td>El-Khoury et al. (1995)</td>
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<td></td>
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<td></td>
<td>El-Khoury et al. (1997)</td>
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<tr>
<td></td>
<td>Kurpad et al. (2001c)</td>
<td></td>
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<td></td>
<td>Kurpad et al. (2002)</td>
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<td></td>
<td>Kurpad et al. (2003)</td>
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<td></td>
<td>El-Khoury et al. (1998)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;20–30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6–13</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>Meredith et al. (1986)</td>
<td></td>
</tr>
<tr>
<td>Methionine + cysteine</td>
<td>Fukagawa et al. (1998)</td>
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</tr>
<tr>
<td>Phenylalanine + tyrosine</td>
<td>Raguso et al. (1997)</td>
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<tr>
<td></td>
<td>Raguso et al. (2000)</td>
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<td>Storch et al. (1988)</td>
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<td>Hiramatsu et al. (1994)</td>
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<tr>
<td></td>
<td>Cortiella et al. (1992)</td>
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<tr>
<td>Threonine</td>
<td>Zhao et al. (1986)</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>Meguid et al. (1986b)</td>
<td></td>
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<tr>
<td></td>
<td>Pelletier et al. (1991)</td>
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<tr>
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<td></td>
<td>10–20</td>
<td></td>
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<tr>
<td></td>
<td>&gt;16–20</td>
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</tbody>
</table>
Table 16.3. DAAO/DAAB methods limitations and disadvantages.

- Prior adaptation is required
- Can only be used to determine the requirements of amino acids whose carboxyl group is directly released to the bicarbonate pool and so will appear in breath
- In short-term studies, measurements are made only after a few hours of feeding making it difficult to extrapolate findings to a normal day with no / any level of confidence; therefore whether fed state studies represent the daily (24 h) requirement is unclear
- The precursor pool from which oxidation takes place expands as the level of the test amino acid intake increases
- Infusion of nutritionally significant amounts of labelled amino acid is a potential problem
- 24-h studies are complex and difficult method to carry out, and limits the number of studies that can be conducted in any single subject

9 h of the fed period and then also during the 12-h post-absorptive period. These assumptions have been described (Meguid et al., 1986a) but a precise estimate of daily balance would require ideally use of a 24-h tracer protocol. A further reason is that the rate of amino acid oxidation during the fed period does not necessarily remain constant but can vary throughout the 12-h phase of the fed-fast cycle with the rhythm changing according to the adequacy of intake of the test amino acid (El-Khoury et al., 1994b; Sánchez et al., 1995).

To measure the fasted state oxidation rate, a short, 8 h infusion protocol, in which no food is given for the first 3 h and small meals are given during the remaining 5 h, has been employed. This circumvents many of the problems associated with the fed-only procedure, as separate measurements are made for the fed and fasting states.

3. The stable isotopic label requires a higher amount of enrichment in the body amino acid or carbon dioxide pool for precise detection, because of the high background, or natural tracer enrichment that exists with these isotopes. This is in contrast to radioactive isotopes, in which this problem does not exist. Therefore, the stable isotope-labelled amino acid is given in larger or ‘non-tracer’ amounts, which effectively contribute to the test amino acid intake, with possible modification of the status of endogenous amino acid metabolism, particularly in the fasted state. Such effects may require relatively high tracer intakes, possibly equivalent to more than 10% of the plasma amino acid flux. In all recent 13C tracer studies that have sought to measure balance, the tracer input has been included in the estimation of total test amino acid intake for purposes of estimating amino acid balance, but it is difficult to know whether the tracer, especially when given during a post-absorptive phase of amino acid metabolism, actually affects the rate of test amino acid oxidation. Later, more extensive DAAB studies with leucine (El-Khoury et al., 1994a) and lysine (El-Khoury et al., 2000) and additional metabolic studies on leucine and methionine (Raguso et al., 1999) suggest that the doses of labelled tracers usually given in these experiments do not significantly affect the oxidation rates of the trace. A more recent study has suggested that this error might not be important, as infusions of three different levels of leucine during the fasting phase were shown to have no significant effect on leucine oxidation (Kurpad et al., 2002a), suggesting that additional leucine would therefore be available for protein synthesis in the fasting state.

4. Although the short duration of study problem was solved through the deployment of 24-h protocols of measurement of amino acid balance (El-Khoury et al., 1994a,b, 1995, 2000; Kurpad et al., 2001c), such studies are complex and difficult to carry out, often limiting the number of repeat 24-h studies that can be conducted in any one single subject or the total number of 24-h studies that are feasible in any one investigation. It has been shown that the daily rate of protein oxidation, calculated from the leucine oxidation measured during this protocol, is closely similar to that derived from the measurement of nitrogen excretion in urine and faeces (El-Khoury et al., 1994a). The intake point at
which a zero balance is achieved is taken to be the requirement. Although this longer protocol answers questions about the shorter durations of the fasted-fed experiments, there is one controversy relating to the protocol, which involves the feeding of small meals for 12 h during the fed state in order to achieve a steady state of feeding. The question is whether this is representative of a ‘normal’ day, involving larger and fewer meals. This was tested in a study that compared the protocol described above with an otherwise similar protocol in which three discrete meals were given (El-Khoury et al., 1995). Despite receiving the same total dietary leucine intake with the two protocols, the daily leucine balances were quite different. Whereas the half-hourly small meal protocol gave an approximately zero balance, the discrete meal protocol gave a balance that was significantly positive. It is possible that the half-hourly small meals protocol is less conducive to protein retention and therefore might give rise to higher estimates of amino acid requirements. Nevertheless, in most 24-h studies, the small meal protocol has been used successfully, and was internally valid in that it demonstrated that an adequate intake of the test amino acid gave a zero leucine balance in adult men.

6. There is some uncertainty over the measured rates of amino acid oxidation, because labelled CO₂ is sequestered in the body bicarbonate pool, leading to an incomplete recovery of the oxidized label in the breath. Then, the rate of labelled CO₂ output in the breath will be lower (by up to 30%), than the true cellular production rate. Typically, this incomplete recovery of label can be measured and corrected for by performing infusions of ¹³C-labelled bicarbonate in prior experiments using the same fasting and fed protocol, preferably in the same subjects on the same diet. However, it is not certain how well intravenously infused bicarbonate mimics the endogenous production of bicarbonate in the cells where the metabolite of the labelled amino acid is oxidized, and this introduces a degree of imprecision in the method. The ¹³C-enrichment of the natural CO₂ in the breath will also vary during the day, depending on the substrate being oxidized. This occurs because different dietary sources of carbon have different natural enrichments (for example, cane sugar and maize products have a much higher enrichment than potato or wheat products). Standardizing all diets with low enrichment ingredients minimizes this problem; in case a correction is required to improve precision, prior experiments can also be done with the same diet and subjects, in the same protocol, without any tracer, in which the natural variation in ¹³C enrichment in breath CO₂ is characterized. The enrichments so obtained are then subtracted from those obtained during infusions of the labelled amino acid. This procedure is necessary to avoid serious systematic errors in the determination of oxidation rates, but as with the bicarbonate correction, it adds substantially to the overall variability of the method.

Despite these limitations, the 24-h tracer balance approach (DAAB) might be regarded currently as the most rigorous tracer-based paradigm for directly determining amino acid balance when its conditions are satisfied. Typically, this is for leucine only at present, and for this amino acid, is the method of choice for determining its requirement in adults.
16.4.2 Indicator amino acid oxidation and balance

The indicator amino acid oxidation (IAAO) method was applied initially in studies of amino acid requirements in young growing pigs and validated against traditional approaches based on criteria of growth, N balance, and body composition (Kim and Bayley, 1983; Kim et al., 1983a,b; Ball and Bayley, 1984). The requirement for a test IAA is determined from the pattern or rate of change in oxidation of another (indicator) amino acid (e.g. 13C-phenylalanine). Therefore, unlike the carbon balance method, the test amino acid and the tracer are separate, and there is now no problem linked to the intake of nutritionally significant quantities of the tracer. It is also critical that the intake of the indicator amino acid is kept constant, since changes in amino acid concentrations in plasma or extracellular fluid can alter the rates of its transport into cells, such that the intracellular to extracellular or plasma enrichment ratio is changed (Mortimore et al., 1972). For amino acids such as phenylalanine and lysine, for which there are no surrogate measures of the intracellular enrichment – such as α-ketoisocaproate for leucine – the change in this ratio might confound the results. Thus, this method is not a direct measure of the maintenance requirement in the same way that the 24-h carbon balance method is, but is mainly a measure of the intake of the test amino acid as a proportion of its content in the amino acid mixture required for post-prandial protein deposition.

Typically, when the intake of the test IAA is limiting, it is expected that there would be an increased oxidation pattern of all the other amino acids owing to inefficiency in protein synthesis. As the requirement level of the test IAA is approached, the excess oxidation of the indicator amino acid will decline, until it is at its lowest when the test IAA intake is at requirement. Thus, an inflection point occurs, at which the oxidation of the indicator amino acid reaches its nadir, and this is considered to be the requirement level of the test amino acid. Then, even if the intake of the test IAA goes above the requirement, the low rate of oxidation of the indicator amino acid will remain at its nadir. This of course assumes that the indicator amino acid, and indeed all other IAA, are being supplied at a constant requirement or above requirement level in the diet. The approach was first applied in adult humans in a study designed to determine the dietary requirement for lysine (Zello et al., 1993). It was extended to estimate the requirements of healthy adults for tryptophan (Lazaris-Brunner et al., 1998), threonine (Wilson et al., 2000), methionine with no cysteine (Di Buono et al., 2001a), minimum methionine with cysteine (Di Buono et al., 2001b; Ball et al., 2006), total branched-chain amino acids (Riazi et al., 2003), and total aromatic amino acids (phenylalanine, with no tyrosine) (Hsu et al., 2006; Pencharz et al., 2007), as well as a follow-up study on the lysine requirements of adults (Duncan et al., 1996), and a recent study on tyrosine-phenylalanine relationships (Roberts et al., 2001).

Two experimental approaches are followed in the IAAO method. The first is a short-term protocol, in which subjects are given an adequate, constant diet for a few days followed by 13C-phenylalanine (or 13C-lysine) tracer study at a test intake level of the amino acid whose requirement is being estimated, while the second is a longer term 24-h protocol (described below). The tracer protocol involves giving subjects small hourly meals for 7 h, beginning 3 h before the infusion of labelled indicator tracer. The enrichment of the breath 13CO2 for the last 2 h of the 4-h tracer period is used to estimate the indicator amino acid oxidation rate. This is normalized for the amount of tracer given, and expressed as a proportion of the administered dose. Therefore, the actual oxidation of the tracee is not measured, since one is interested only in the oxidation of the tracer. Individual subjects are studied at multiple test amino acid intake levels, with as many as six or more levels in some of the investigations. If measurements of tracee oxidation are required, an invasive nature is added to the experiment, since enrichments of the precursor pool, normally estimated in the plasma, need blood samples. There have been attempts to use urinary enrichments to approximate the enrichment of the tracer amino acid in arterialized blood (Bross et al., 1998). Reasonably
good correlations between plasma and urinary amino acid enrichments have been obtained with \(^{13}\text{C}\)-leucine, \(^{15}\text{N}\)-glycine, and \(^{13}\text{C}\)-phenylalanine (De Benoist et al., 1984, Wykes et al., 1990; Zello et al., 1994). Furthermore, Bross et al. (1998) developed an IAAO model which used oral dosing of tracer along with breath sampling alone in order to make the whole method completely non-invasive. Nevertheless, given the similar breakpoints obtained with the tracer or tracee oxidation response, it might seem reasonable to continue with the tracer oxidation method until further validations are performed for the tracee oxidation method.

There are a number of advantages to this short-term IAAO approach. In comparison to longer protocols which do not permit more than two or three studies at different test IAA intakes in a single subject, this method offers the possibility of carrying out a relatively large number of short-term tracer studies within the same subject, allowing for within-subject estimates to be made of the inflection point on the response curve. Given the assumptions of this method, problems arising from changes in pool sizes and kinetics that might affect the behaviour of a direct tracer and interpretation of the isotopic data obtained are largely avoided; there is no a priori reason to determine the actual rate of indicator amino acid oxidation, since the pattern of release of the \(^{13}\text{C}\) label in expired air can provide the basis for requirement estimate derived from the inflection on the intake-oxidation response curve. A further advantage accrues from the fact that there is no need to measure the \(\text{CO}_2\) recovery, in which some imprecision occurs.

Since the basis for the measurement of leucine kinetics is well validated, it might seem that only leucine is ideally suited as an indicator, as its carbon balance can be accurately measured. However, in the short-term IAAO method, actual balance of the indicator amino acid is not measured, allowing for the choice of other indicator amino acids, such as phenylalanine. This is also because of the low concentration of phenylalanine in blood and tissues, which allows its metabolism to be rapidly sensitive to changes in protein balance or other amino acid intake (Zello et al., 1993). It is important to understand that since \(^{13}\text{CO}_2\) release occurs by tyrosine oxidation, which in turn is formed from phenylalanine, it is important to minimize the loss of \(^{13}\text{C}\) into the protein-bound tyrosine pool by giving a high tyrosine diet prior to the study (Zello et al., 1990, 1993). According to the concept on which this approach is based, this pattern of \(^{13}\text{C}\) appearance as a result of oxidation of the tracer should, in theory, parallel that for the absolute oxidation rate of the indicator (tracee). In one study concerned with the lysine requirement of adult males (Duncan et al., 1996) this was not found to be the case; while the absolute rate of phenylalanine oxidation showed a generally similar pattern to that of \(^{13}\text{CO}_2\) release, the variation precluded use of this parameter to estimate the requirement for lysine. In other studies using leucine as an indicator for the phenylalanine requirement, however, the pattern of \(^{13}\text{CO}_2\) release did mimic the pattern of leucine (indicator) oxidation.

The disadvantages of the short-term IAAO method are that it has been based essentially on a short-term fed-state model, even though it gives results similar to more complex models in many other studies. There is therefore uncertainty as to whether the same pattern of change or at least 'breakpoint' in IAAO response would apply similarly to a later (or even earlier) period within the 12 h fed phase as compared with the specific 2 h period used to elaborate the relationship between amino acid intake, oxidation, and requirements. From 24-h tracer studies (below) it is now clear that the rate of amino acid oxidation changes throughout a constant-fed period in a complex way depending upon the adequacy of amino acid intake (El-Khoury et al., 1994b; Sánchez et al., 1995). Second, Zello et al. (1995) state that the IAAO technique has the advantage of permitting oxidation measurements to be taken with no prior adaptation to the level of the test amino acid, in contrast to the DAAO and DAAB studies where adaptation periods of about 6–7 d have been included in the study design. This may not be a particular advantage of the IAAO technique, since the lack of a period of adaptation to a test amino acid intake level is potentially a serious design limitation.
El Khoury et al. (1994a,b) showed an adaptation of leucine oxidation in the fasting state over a twofold range after 6 d of leucine intakes at 89, 38, and 14 mg kg\(^{-1}\)d\(^{-1}\) at a constant nitrogen intake. These are similar to changes in fasting and fed nitrogen losses or leucine oxidation shown to occur in response to 2-week periods of widely varying protein intakes (Millward, 1999). Thus, adaptation should influence the overall need for amino acids, even when measurements are limited to the fed state. However, two separate studies of the lysine requirement at total protein intakes of 0.8 (Zello et al., 1993) or 1.0 g kg\(^{-1}\)d\(^{-1}\) (Duncan et al., 1996) gave similar inflection points on the oxidation intake response curve. More recent studies on the pattern of \(^{13}\)CO\(_2\) production after different periods of adaptation have shown that there really is no adaptive effect of prior feeding in the short-term protocol (Moehn et al., 2004). Furthermore, \(^{13}\)C-phenylalanine oxidation, measured as F\(^{13}\)CO\(_2\), was not significantly affected by 8 h, 3 d or 7 d of adaptation to a wide range of lysine intakes in healthy young men (Elango et al., 2009).

However, even if the potential drawback does exist, it must be recognized that at present several studies using this method give fairly similar answers about IAA requirements, in comparison to more rigorous and theoretically well-defined protocols of IAAO and IAAB (below). Millward (1998) has argued that, without a suitable adaptation period to a specific and lower test lysine intake, the IAAO approach would effectively give a higher value than the minimum physiological requirements for lysine. On the other hand, Young (1999) has argued the opposite, namely that the minimum requirement might, in theory, be underestimated when applying the IAAO approach under conditions where there is no adaptation to a lower intake than usual. Recent studies by Millward et al. (2000) on the post-prandial utilization of milk and wheat proteins would support this view; since their estimate of the nutritional quality of wheat protein was higher than the authors had predicted, presumably due to the ‘buffering’ effect of a significant and replete free tissue (perhaps muscle) lysine pool over the course of their short-term tracer study. Nevertheless, there is a need to establish whether or not, and for how long, an adaptation period should be included in studies involving the fed state and the IAAO technique. The strengths and limitations of the IAAO/IAAB methods have been summarized in Table 16.4.

In general, it is potentially useful to develop the short-term IAAO method for

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**Table 16.4. Strengths and limitations of IAAO/IAAB methods.**

- Short-term IAAO measurements are made on only fed state method and is a short-term study with minimal complexity
- Short-term, minimally invasive IAAO can be applied on vulnerable groups such as malnourished children, pregnant or lactating women, and the elderly
- The requirement of any amino acid can be determined with a single indicator
- The pool from which oxidation takes place does not change in size as the level of the test amino acid is altered
- Prior adaptation to the level of the test amino acid is not needed in the short-term IAAO protocol, but this still needs to be rigorously evaluated
- A full range of intakes of the test amino acid can be used
- This method is not conditional on high levels of precision or accuracy in measurement of amino acid oxidation
- The demonstration of breakpoint is much less convincing since the net protein synthesis may be less than in a growing animal
- Choice of best indicator and oxidation is limited, at present phenylalanine (in the presence of an excess of tyrosine), lysine and leucine have been used; the only other possible choices are valine and isoleucine
- Fasted as well as in the fed state measurements can be performed in 24-h experiments
- 24-h studies require adaptation to the level of the test amino acid for 5–7 d; more invasive than the short-term IAAO method
further studies in low-resource, developing countries as well as in vulnerable groups such as children, pregnant or lactating women, and the elderly (Elango et al., 2008; Pillai et al., 2010). These estimates form the first direct experimental evidence of amino acid requirements in school-age children, where it was earlier estimated by a factorial method (Table 16.5). It has also been used in innovative ways to determine other metabolic indices. Recently, the IAAO method has adapted to determine protein requirements in adults, although this still needs to be validated (Humayun et al., 2007a). This method has also been applied recently to determine the metabolic availability of sulphur amino acid (SAA) from casein versus soy protein isolate using 13C phenylalanine as an indicator amino acid (Humayun et al., 2007b).

To circumvent some of the limitations of the short-term IAAO technique, a 24-hour indicator amino acid oxidation balance (24-hour IAAO) and 24-hour IAAB approach was developed (Kurpad et al., 1998), the latter having an approach similar in concept to that of the IAAO technique but based on an indicator amino acid oxidation balance protocol conducted over an entire day, providing a true 24-h experimental observation. The 24-h IAAB could be regarded as a functional criterion of dietary amino acid adequacy in contrast to a measure of the short-term, fed state IAAO, which might be taken to be a biomarker of adequacy. This method has been applied in 13C-leucine (indicator) tracer studies of the lysine requirement of adult Indian subjects (Kurpad et al., 1998; 2001a,b) and recently in studies of the threonine requirement in US adults (Borgonha et al., 2002) and Indian subjects (Kurpad et al., 2001b). The disadvantage of the 24-h IAAO and 24-h IAAB approaches relates to the complexity of the 24-h tracer study and possibly the rather stringent demands and restraints that it places on the experimental subject. Furthermore, the 24-h paradigm has been most often conducted using a 12-h fed-state period that involves giving small, frequent, isocaloric, isonitrogenous meals. Current meal patterns in most parts of the world tend toward a larger single, evening meal, and it is not known how this pattern of meal ingestion might impact on the minimum physiological requirement for amino acids. As stated above, leucine oxidation is lower when three discrete meals are used versus a multiple frequent meal schedule (El-Khoury, et al., 1995; Raguso et al., 1999). Hence, it raises the question of what might be the appropriate or best meal pattern for estimating amino acid requirements in adults when using the IAAO and IAAB techniques. Even with these caveats, it appears that the IAAO/IAAB method works best when the indicator (particularly in the case of leucine) is given at or just above the daily requirement level (Pencharz and Ball, 2003), since at higher intakes which approach the normal intake values, the IAAB balances response tends to be far more positive than can be explained (Kurpad et al., 2001b). Current data do not permit a sufficient resolution of this matter, but the best practice might be that the multiple, frequent meal paradigm is the most appropriate for the quantitative interpretation of current amino acid tracer data. Another issue of importance with respect to estimating the minimum physiological requirements of amino acids is the molecular form of the amino acid intake. The earlier amino acid requirement studies of Rose (1957) and of many of the other investigators (Irwin and Hegsted, 1971) used D- and L-amino acid mixtures as a principal source of amino acids. The more recent 13C-tracer studies have generally used a mixture of L-amino acids. Studies of the comparative utilization of protein-bound versus free

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Author's reference</th>
<th>Requirement (mg kg⁻¹ d⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Lysine</td>
<td>Pillai et al. (2010)</td>
<td>33.5</td>
</tr>
<tr>
<td>Methionine (no cysteine, total SAA)</td>
<td>Elango et al. (2007)</td>
<td>35</td>
</tr>
<tr>
<td>Methionine (with cysteine)</td>
<td>Turner et al. (2006)</td>
<td>12.9</td>
</tr>
<tr>
<td>Total BCAA (isoleucine + leucine + valine)</td>
<td>Humayun et al. (2006)</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>Mager et al. (2003)</td>
<td>147</td>
</tr>
</tbody>
</table>
leucine indicated better retention of the leucine when given in protein-bound form.

The amino acid requirement estimates generated from the 13C-tracer 24-h DAAB, 24-h IAAB, and 24-h IAAO collectively provide the best primary estimates of the minimum physiological requirements for the indispensable amino acids. Table 16.6 gives the estimates for different amino acids that have been measured thus far. Nevertheless, the 24-h approach is open to the criticism that it may not be an entirely physiological construct. In addition, the short-term IAAO method also gives estimates of IAA requirements that are consonant with the more rigorous 24-h methods. However, given the theoretical framework underpinning the 24-h IAAB technique (with its specific requirements), this method might be regarded as the reference method by which to validate other and possibly less complex tracer paradigms.

16.4.3 Post-prandial protein utilization

Short-term fast/fed tracer [1-13C] leucine balance experiments have been used in another innovative way, to evaluate the utilization of wheat compared with milk protein, with a further calculation of the average requirement for lysine (Millward et al., 2000). In theory, this approach could be adapted to estimate the requirements for other indispensable amino acids, especially if L-amino acid mixtures, or combinations of proteins and L-amino acids, were used in place of intact proteins. The tracer protocol lasts for 9 h with three consecutive 3-h phases: a post-absorptive phase, then a low protein meal phase, followed and terminated by a higher protein meal phase. The lysine requirement is derived from an estimate of the relative efficiency of wheat nitrogen retention compared with milk, assuming that lysine limits wheat utilization. This technique has the advantage of being based on an indicator tracer amino acid, in this case leucine. Thus, it has the potential for providing a reliable means of estimating the change in protein balance with protein intake in order to calculate the efficiency of post-prandial protein utilization (PPU) with one protein source and compare this with another.

There are areas where this protocol could be optimized for purposes of estimating the minimum daily requirement for an amino acid. Some of these are technical issues, which have been reviewed (Kurpad and Young, 2003a). For example Millward et al. (2000) found that at limiting intakes of an indispensable amino acid the estimate of PPU can differ with the passage of time within the meal-feeding phase. Also, it seems likely that the low-protein meal phase would influence the leucine balance that occurs during the succeeding high protein meal phase, so affecting the value of the PPU obtained.

Table 16.6. Estimation of different IAA requirements by the IAAO/IAAB method.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>IAAO</th>
<th>IAAB</th>
<th>Value obtained (range) mg kg⁻¹ d⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>Zello et al. (1993)</td>
<td>Kurpad et al. (2001b)</td>
<td>29–45</td>
</tr>
<tr>
<td></td>
<td>Duncan et al. (1996)</td>
<td>Kurpad et al. (2002a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kriengsinyos et al. (2002)</td>
<td>Kurpad et al. (2003a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kriengsinyos et al. (2004)</td>
<td>Kurpad et al. (2003c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kurpad et al. (2002b)</td>
<td></td>
</tr>
<tr>
<td>tyrosine</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Lazaris-Brunner et al. (1998)</td>
<td>Kurpad et al. (2003b)</td>
<td>13–16</td>
</tr>
<tr>
<td>Methionine</td>
<td>Di-Buono et al. (2001b)</td>
<td>Kurpad et al. (2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td></td>
<td>Kurpad et al. (2005)</td>
<td>17–20</td>
</tr>
<tr>
<td>BCAA</td>
<td>Riazi et al. (2003)</td>
<td></td>
<td>144</td>
</tr>
</tbody>
</table>
It might be critical to include changes in the size of the free leucine pool in estimating the change in protein-bound leucine balance; if not, the relative PPU may be overestimated, such that the apparent lysine requirement would be underestimated. Finally, when conducted in nutritionally replete individuals, the PPU of wheat protein would be expected to be higher than for individuals whose body, especially muscle, free lysine pool is lower as a consequence of an habitually lower intake of lysine. This underscores the fact that it is necessary to evaluate the question of an adaptation period, particularly with this method.

In summary, a number of different tracer techniques and protocols have been applied with the purpose of determining the requirement for specific indispensable amino acids in healthy adults. None are without important limitations. The 24-h IAAO and/or IAAB technique would appear to be the best current tracer-based approaches to date for estimation of adult amino acid requirements. Fundamentally, all of the methods used are surrogates for measuring protein synthesis, which is hard to measure directly. All the three different patterns of metabolic responses to graded intakes of an essential amino acid are summarized in Fig. 16.2.

16.5 Factorial Prediction of Amino Acid Requirements

One of the earlier paradigms for assessing IAA requirements was suggested by Young et al. (1989), based on estimates of the intakes of amino acids necessary to balance the minimum obligatory losses of amino acids (OAAL) as predicted from the composition of mixed body proteins (Table 16.7). In addition, when intakes of energy and other nutrients are adequate but the diet is essentially protein-free, the rate of body N loss, principally via urine and faeces, reaches a new, relatively steady-state level within about 1 week (Rand et al., 1976). This new level is called the obligatory N loss (ONL) (FAO and WHO, 1973), and amounts to 54 mg N kg⁻¹ d⁻¹ in healthy adults (FAO et al., 1985). Moreover, various studies on obligatory nitrogen loss in different parts of the world revealed that they are remarkably uniform (Bodwell et al., 1979). Thus, it is assumed that the amounts of the different IAA contributing to these N losses occur in proportion to their concentrations in body mixed proteins, providing that those proteins contributing to the major proportion of the total N loss do not have amino acid patterns (concentrations) that differ markedly from the average of the body mixed proteins.

Fig. 16.2. The three different patterns of metabolic responses to graded intakes of an indispensable amino acid. Line 1 represents daily N balance, direct amino acid balance (DAAB) or indicator amino acid balance (IAAB). Line 2 represents the oxidation rate of the test amino acid (DAAO), in both short and 24-h measurements. Line 3 indicates the oxidation of an indicator amino acid (IAAO) in both short and 24-h measurements. The point of inflection of each line represents the requirement for the test amino acid. (Adapted from Pencharz and Ball, 2003.)
Table 16.7. Estimates of IAA requirements by the factorial prediction method.

| Amino acid             | Requirement (mg kg\(^{-1}\) d\(^{-1}\))
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>23</td>
</tr>
<tr>
<td>Leucine</td>
<td>40</td>
</tr>
<tr>
<td>Lysine</td>
<td>30</td>
</tr>
<tr>
<td>Methionine + cystine</td>
<td>13</td>
</tr>
<tr>
<td>Phenylalanine + tyrosine</td>
<td>39</td>
</tr>
<tr>
<td>Threonine</td>
<td>15</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6</td>
</tr>
<tr>
<td>Valine</td>
<td>20</td>
</tr>
</tbody>
</table>

\(a\)Values from Young et al. (1989).

The assumption that the amino acid requirement pattern for maintenance of protein nutritional status in adults is similar to that for mixed proteins in the whole body has been criticized (Millward et al., 1990; Fuller and Garlick, 1994; Waterlow, 1996). In addition, it was assumed that the efficiency of absorption of the IAA (at requirement level), in humans, was about 70%. This method is still used for those amino acids that have not yet been examined in tracer studies. The pattern of OAAL is assumed to be proportional to the relative concentrations of amino acids in whole-body mixed proteins. The question is whether the 70% value that has been assumed is valid for all the IAA. In general, however, there is a reasonable agreement between the body mixed protein–amino acid pattern and the estimations of the amino acid requirements in adults by tracer methods.

16.6 Estimates of the Amino Acid Requirement in Potentially Adapted States

From a homeostatic viewpoint, it might be anticipated that there would be a reduction in the body amino acid oxidation rate as a consequence of adaptation to the acute or chronic ingestion of decreased amounts of amino acid. That is indeed what is observed in the relatively acute conditions of the graded test intakes of amino acids described in the tracer section above, such that the oxidation of an amino acid reduces to a minimum when intakes of amino acid are below requirement. However, it might also be that under chronic conditions of under-nutrition, there is a constitutional decline in amino acid oxidation, as a conservation mechanism with lower than normal intakes. If this was the case, one might expect to observe an even greater reduction in oxidation at very low intakes, such that the degree of negativity of the amino acid balance at those low intakes is less than expected in the adapted individual. This was indeed the case in at least one set of comparisons of leucine balance at low intakes of leucine, between normal and chronically undernourished men (Kurpad et al., 2001c, 2003a). The chronically undernourished men were those with a low body mass index, and who came from a low socioeconomic stratum, living in slums in Bangalore, India. When comparing the response to graded intakes of leucine in these individuals, it becomes evident that the breakpoint for leucine requirement on the DAAB response line is about the same in both groups (Fig. 16.3). In this case, an adaptive response for conserving leucine only results in a shallower slope at leucine intakes that are very low and well below normal, since at subnormal intakes that are closer to the requirement level of 40 mg kg\(^{-1}\) d\(^{-1}\), both groups have similar balances.

In effect, this does argue for an adaptive response, but one that is more evident or operative at very low intakes, in a manner that would improve survival, but probably not affect the actual requirement level. Given the issues with detecting a breakpoint in shallow response lines (discussed in the N balance section above, and Fig. 16.1), it might be that such a response could obtain a breakpoint in the response curve at a higher, rather than a lower intake. This emphasizes the need for measuring the amino acid oxidation or balance response at many intakes that cluster around the putative requirement, in order to define the breakpoint with more confidence. In any event, using the 24-h IAAB method in chronically undernourished men has yielded a higher breakpoint-based value for lysine intake, at about 45 mg kg\(^{-1}\) d\(^{-1}\), in comparison with the normal value of 30 mg kg\(^{-1}\) d\(^{-1}\) (Kurpad et al., 2003b). In that case, however, it was found that intestinal parasites contributed to about half the increase in the
Fig. 16.3. Comparison of the 24-h leucine balance response to graded intakes of leucine in normal and chronically undernourished men. Filled circles, well-nourished subjects; open circles, undernourished subjects.

requirement, since de-worming the subjects reduced the requirement level (Kurpad et al., 2003d), and it might be that under the physiologically ‘perfect’ experimental conditions during the 7 d adaptation period, there was some amount of tissue accretion, since the muscle mass in these subjects was also low. The same reasoning applies to children. In measurements of the lysine requirements in similar aged and otherwise healthy children in India (Pillai et al., 2010) and Canada (Elango et al., 2007), the daily requirements were the same, when measured by the relatively unadapted short-term IAAO method (Table 16.5), when ingesting their habitual levels of protein and energy intake. However, the Indian children were shorter and lighter than their Canadian counterparts, and this raises the question of how much the catch-up requirement of the children could be, under adapted and perfect conditions.

It is tempting to think that exposure to a chronically low and subnormal intake of protein and amino acids may result in adaptations that limit the oxidation of these amino acids, and therefore reduce the daily requirement. However, from the perspective of a requirement for optimal health in normal conditions, it is very likely that the requirement in such chronically undernourished individuals is the same, or even higher, than in normally nourished individuals, for the reasons stated above.

16.7 Conclusions

Depending on the method (N balance or tracer based), there appear to be greatly differing estimates of the IAA requirements. While the N balance method suffers from many technical difficulties and unresolved theoretical issues, the direct tracer-based methods potentially offer more accuracy and precision of measurement. The carbon balance method using $^{13}$C-labelled amino acids is theoretically sound, but is subject to considerable uncertainty, with reasonably reliable estimates only available for amino acids that lose the label quantitatively and irreversibly when oxidized, and for which reliable estimates of precursor pool enrichment can be obtained. Another theoretically sound method is the adapted 24-h IAA or carbon balance approach, in which most of the potential theoretical and practical problems for measuring the minimum physiological requirements have been addressed; in that sense, this is probably the closest one can get to a reference method. However, this method is difficult and cumbersome to use, particularly on vulnerable populations in whom IAA estimates are still based on factorial estimates. The short-term IAAO approach is easy to use and is effectively a breath test that can define the minimum requirements of an IAA, and has obtained estimates of IAA requirement that are close to those obtained by the 24-h adapted IAAO method (or the reference method). In that sense,
it is probably the best method to deploy in resource poor environments, or in vulnerable populations. There is now a sufficient body of evidence based on the tracer methods that has led to a change in the estimates of human daily IAA requirements, and the currently accepted IAA requirements (WHO et al., 2007) are shown in Table 16.8. As always, additional studies on the quantitative aspects of the whole-body metabolism of indispensable and dispensable amino acids by these tracer approaches, particularly in subjects under different dietary conditions, are needed.

### Table 16.8. Currently accepted IAA requirements (WHO et al., 2007).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Value (mg kg⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>39</td>
</tr>
<tr>
<td>Lysine</td>
<td>30</td>
</tr>
<tr>
<td>Threonine</td>
<td>15</td>
</tr>
<tr>
<td>Phenylalanine + tyrosine</td>
<td>25</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>4</td>
</tr>
<tr>
<td>Methionine + cysteine</td>
<td>14</td>
</tr>
<tr>
<td>Valine</td>
<td>26</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>20</td>
</tr>
</tbody>
</table>

**References**


Amino Acid Requirements


R.R. Pillai and A.V. Kurpad


Amino Acid Supplements and Muscular Performance

T.A. Churchward-Venne, D.W.D. West and S.M. Phillips*
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17.1 Abstract

Free form and protein source amino acid supplements are widely available, and are popular within the athletic community. Amino acids (AA) independently stimulate muscle protein synthesis (MPS) independently of hormones such as insulin, and suppress muscle protein breakdown (MPB) to a small degree. Concomitant with AA ingestion is an activation of cell-signalling pathways involved in the formation of translation-competent ribosomes, which occurs through the mammalian target of rapamycin (mTOR). As such, AA and protein (PRT) supplements represent a potentially effective strategy to increase skeletal muscle mass and strength, particularly when combined with anabolic modes of training such as resistance exercise (RE) training. The potential of these supplements is of great interest not only among athletes and their coaches, but also among researchers and clinicians as their application also represents a potentially effective strategy to counteract the loss of skeletal muscle mass that occurs with ageing (sarcopenia). Additionally, PRT and AA supplements have been reported to enhance endurance exercise (EE) performance, although this is a topic of considerable debate. In young subjects, PRT/AA supplements are capable of enhancing RE training-induced increases in skeletal muscle mass and strength; however, their role in facilitating similar adaptations in the elderly is less clear. Other factors such as timing (relative to exercise) of PRT/AA ingestion, PRT source or type and the AA composition may be particularly important in determining the efficacy of these supplements in the elderly. For example, the AA leucine is a key regulator of MPS that has been shown, in higher concentrations, to ameliorate the diminished anabolic sensitivity to AA that is characteristic of aged muscle. In addition, bovine milk and whey PRT, in particular, appear to be superior supplemental PRT sources capable of increasing MPS and enhancing RE-induced gains in lean mass. Overall PRT/AA supplements represent a convenient and effective means to stimulate MPS and enhance RE-induced gains in skeletal muscle mass; however, there is no evidence that they are more efficacious than consumption of a mixed meal containing high quality protein.

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17.2 Introduction

There is great interest within the athletic community about the potential of nutritional interventions to enhance exercise training-induced increases in skeletal muscle mass and strength and/or improve endurance performance. In addition, both nutrition and exercise-based interventions to enhance muscle mass are of great interest clinically, as their application holds potential as an effective strategy to counteract the loss of muscle mass that occurs with ageing (sarcopenia) and other chronic diseases. Dietary supplements that purportedly increase skeletal muscle mass and strength, decrease fat mass, reduce fatigue or enhance athletic performance are widely available. Athletes in particular appear to be the greatest consumers of these supplements (Burke et al., 2006; Erdman et al., 2006; Huang et al., 2006), although individuals engaged in regular physical activity also report the use of such supplements (Morrison et al., 2004; Gaston and Correia, 2009), of which those containing PRT or AA appear to be the most popular (Gaston et al., 2009). But what is the scientific basis for the use of such supplements? The aim of this chapter is to examine the potential of AA/PRT supplements to support increases in skeletal muscle mass and strength, particularly in conjunction with RE, as well as to examine the potential for these supplements to augment EE performance and recovery. In addition, the potential of PRT/AA supplements in attenuating the loss in skeletal muscle mass that is characteristic of biological ageing will be examined.

Important considerations such as timing of ingestion relative to exercise and the food source of dietary PRT supplements will be discussed, as these factors appear to modulate the anabolic response. This chapter will not specifically address the issue of protein requirements in resistance- and endurance-trained athletes and whether they are different from an average individual. Instead, we will simply highlight evidence supporting a role for PRT/AA supplements in augmenting adaptations to both RE and EE, and attempt to provide some insight into potential mechanisms.

17.3 Amino Acids and Protein Turnover

In addition to the well-recognized role of AA as substrate for protein synthesis, AA also stimulate the phosphorylation of various cell-signalling proteins and are capable of regulating protein synthesis (Fig. 17.1). Traditionally, AA have been classified as being either essential (indispensable) or non-essential (dispensable), based on requirements from the diet for attaining nitrogen balance. Thus, while non-essential AA may be synthesized de novo, essential AA (EAA) cannot be synthesized by the body and must be consumed through dietary intake. On average, the musculoskeletal system comprises 75% of the lean body mass of a healthy individual (Forbes, 1987), and so represents the largest reservoir for AA within the body. Although muscle is capable of oxidizing several AA, particularly the branched-chain amino acids (BCAA) (leucine, isoleucine, and valine) (Goldberg and Chang, 1978), AA contribute only ~2–5% of the energy contribution during prolonged dynamic exercise (Tarnopolsky, 2004; Rennie et al., 2006). Despite this relatively low energy contribution (as compared to carbohydrates and lipids), AA have a profound influence on skeletal muscle, particularly when combined with exercise. Body proteins are in a continuous state of turnover; that is, they are continuously being synthesized and degraded. The continual synthesis of new, and degradation of old proteins is an energetically expensive process that allows for damaged proteins to release their constituent AA into the local and systemic free pools where they may be used for the synthesis of new functional proteins, provide energy via gluconeogenesis, and/or direct oxidation.

The balance between MPS and MPB is the basis for determining the net protein balance (NPB) of a given tissue. When MPS > MPB, the result is a positive NPB and protein accretion. On the other hand, when MPB > MPS, NPB becomes negative and net tissue catabolism occurs. However, of the NPB equation, MPS is the variable that undergoes the greatest fold change in response to anabolic stimuli such as AA and RE (Chesley et al., 1992; Biolo et al., 1995; Biolo et al., 1997; Phillips et al., 1997; Phillips et al., 1999; Rasmussen and Phillips, 2003). Specifically, MPS undergoes changes
that are three- to fourfold greater than any change in MPB during the typical fasted- to fed-state transitions. This is not to say that MPB can be overlooked; however it plays a comparatively lesser role in determining muscle protein mass. As such, factors capable of augmenting both the magnitude and duration of MPS are likely to represent a relevant means to increase skeletal muscle mass.

### 17.4 Muscle Protein Synthesis

Protein synthesis involves the translation of ribonucleic acid (RNA) into a polypeptide before undergoing post-translational processing to produce the functional protein target. The translation of mRNA into a polypeptide involves the action of ribosomes and is conventionally described as consisting of three stages: initiation, elongation, and termination. Initiation brings the mRNA to the ribosome and involves a multitude of eukaryotic initiation factors that are involved in the assembly of the translation-ready ribosome. The elongation phase involves the addition of AA to the growing polypeptide chain and requires the presence of eukaryotic elongation factors. At the end of elongation, the newly synthesized polypeptide is released from the translational machinery when the termination codon is reached, thus completing the process of protein translation. Newly synthesized proteins can undergo post-translational modifications such as phosphorylation, carboxylation, or glycosylation that can modify the function of a given protein. Current research suggests that AA and exercise increase MPS primarily through increasing translation initiation, although alterations in the elongation stage of protein translation may also occur (Fujita et al., 2007). The cell signalling pathways and the various proteins that respond to AA and exercise are discussed in further detail in the section ‘Cell signalling response to amino acids and exercise’ (below).

### 17.5 Enhancing Adaptations to Resistance Exercise with Amino Acid and Protein Supplements

High protein diets and the use of dietary protein supplements are largely grounded in the...
belief that such practices will increase skeletal muscle mass and strength and aid in recovery from exercise. A detailed review of protein requirements in strength and endurance trained athletes is beyond the scope of this chapter, however several reviews have been written on this topic elsewhere (Phillips, 2004; Phillips et al., 2007). Briefly, strength athletes tend to habitually consume PRT in amounts that are far in excess of the current American and Canadian recommended dietary allowance (0.8 g kg\(^{-1}\)d\(^{-1}\)). Previously, it was thought that while exorbitant from a physiology standpoint, high PRT intakes were still contributing to a positive energy balance that was thought to be crucial to muscle anabolism. Furthermore, there is currently no tolerable upper limit for PRT, and since the conversion pathway of AA to lipid stores is biochemically more energetically expensive and inefficient compared to either excess carbohydrate or fat calories, energy from high PRO consumption may offer some advantage to athletes.

Interestingly however, it has recently been observed that gains in lean mass can be achieved despite being in a negative energy balance (S.M. Phillips and A.R. Josse, unpublished observations). In addition, PRT consumed immediately after RE is able to augment adaptations to RE even when dietary PRT intake is as high as 1.4-1.5 g kg\(^{-1}\) d\(^{-1}\) (Hulmi et al., 2009). Thus protein ‘requirements’ are likely to be dependent upon important outcomes such as lean mass accrual, fat mass loss, and strength, rather than attainment of nitrogen balance.

The remainder of this section will discuss the potential of PRT/AA supplements to enhance adaptations to RE training. It is important to state, however, that consumption of a mixed meal containing adequate amounts of high quality PRT is thought to be as efficacious as consumption of AA/PRT supplements in terms of facilitating adaptations to RE training (Rodriguez et al., 2009). Indeed, provision of AA in close temporal proximity to a bout of RE increases MPS (Biolo et al., 1997; Tipton et al., 1999a, 2001; Bohe et al., 2001, 2003; Katsanos et al., 2006; Symons et al., 2007; Wilkinson et al., 2007; Tang et al., 2009) and appears to enhance training-induced gains in skeletal muscle mass (Burke et al., 2001; Candow et al., 2006; Kerksick et al., 2006; Cribb et al., 2007; Hartman et al., 2007; Willoughby et al., 2007; Hulmi et al., 2009; Josse et al., 2010).

### 17.5.1 Acute studies

The provision of a complete mixture of AA, intravenously (Biolo et al., 1997; Bohe et al., 2001, 2003) or via oral ingestion (Tipton et al., 1999a), has been shown to increase MPS. Likewise, ingestion of whole proteins is also capable of increasing MPS (Tipton et al., 2004; Katsanos et al., 2006; Symons et al., 2007; Wilkinson et al., 2007; Tang et al., 2009). The shift from the fasted- (postabsorptive) to fed-state following AA provision results in a positive NPB and tissue protein accretion (Biolo et al., 1997). The ability of AA to stimulate an increase in MPS forms the rationale for their use as a dietary supplement, particularly when combined with RE. For example, an acute bout of RE also stimulates an increase in MPS within as little as 2-4 h, but also increases the rate of MPB (Phillips et al., 1997). This increase in MPS can be 40-150% above basal levels (Chesley et al., 1992; Biolo et al., 1995; Phillips et al., 1997, 1999), and remain elevated for up to 48 h in the untrained state (Phillips et al., 1997). However, when RE is performed in the fasted state, NPB remains negative because MPB is greater than MPS, despite MPS being stimulated to a greater extent (Biolo et al., 1995; Phillips et al., 1997). However, when PRT/AA are consumed following RE, NPB becomes positive due to large increases in MPS and a suppression of the normal exercise-induced rise in MPB (Biolo et al., 1997). Biolo and colleagues reported that infusion of mixed AA after RE increased MPS >200% relative to basal rates, whereas AA infusion in the absence of prior exercise was associated with increases closer to 100% (Biolo et al., 1997). Thus, the response to combined feeding and exercise is additive such that the increase in MPS is greater than that produced by either intervention alone (Biolo et al., 1997). It is interesting, however, that the increase in MPS following AA provision can be attributed to the EAA, as non-essential AA appear to offer no further benefit (Tipton et al., 1999b; Borsheim et al., 2002; Miller et al., 2003). When performed
chronically, RE and ingestion of quality PRT/AA is thought to result in small net gains in muscle protein content that summate with each exercise/feeding session to produce gains in skeletal muscle mass (for review see Phillips, 2004).

17.5.2 Chronic studies

A valid question regarding the acute changes observed in MPS after RE and feeding is whether such findings, at least qualitatively, are predictive of long term changes in skeletal muscle mass following chronic training. While not definitively shown, there is evidence that the acute response of MPS to exercise and feeding is predictive of long-term changes in skeletal muscle mass following chronic exercise (Hartman et al., 2007; Wilkinson et al., 2007). For instance, consumption of fluid milk after acute RE increases MPS to a greater extent than consumption of soy PRT (Wilkinson et al., 2007). When examined chronically following 12 weeks of RE training, milk consumption was shown to translate into greater gains in fat and bone-free mass (i.e. muscle) than consumption of soy PRT (Hartman et al., 2007) (Fig. 17.2).

In the absence of any planned dietary intervention, RE is able to increase skeletal muscle mass and strength in both young and old individuals (Frontera et al., 1988; Fiatarone et al., 1990; Kalapotharakos et al., 2004; Kosek et al., 2006). However, several studies have shown that PRT/AA supplements consumed in close temporal proximity RE increase skeletal muscle fibre cross-sectional area (CSA) following several weeks of training to a significantly greater degree than a carbohydrate- or energy-void placebo supplement (Anderson et al., 2005; Bird et al., 2006; Cribb et al., 2007; Hartman et al., 2007). At the whole-body level, chronic consumption of PRT/AA supplements in the time period surrounding exercise also appears to increase lean tissue mass and muscle CSA following chronic RE training to a significantly greater extent than either carbohydrate or a non-energetic supplement (Burke et al., 2001; Candow et al., 2006; Kerkisick et al., 2006; Cribb et al., 2007; Hartman et al., 2007; Josse et al., 2009; Willoughby et al., 2007; Hulmi et al., 2009). For example, Hartman and colleagues (2007) reported that consumption of fat-free fluid milk immediately and 1 h after

Fig. 17.2. The acute (left) and chronic (right) effects of different post-exercise nutrition sources.
RE resulted in significantly greater increases in type I and type II fibre CSA and lean body mass following 12 weeks of RE than consumption of isonitrogenous and isoenergetic soy beverages (Hartman et al., 2007). However, other studies have failed to report any benefit from supplemental PRT/AA on skeletal muscle fibre CSA or lean body mass following chronic RE (Godard et al., 2002; Candow et al., 2006a; Verdijk et al., 2009). Verdijk and colleagues (2009) reported no benefit in elderly men from 10 g of PRT both immediately before and after RE on lean body or limb mass or skeletal muscle fibre CSA, following 12 weeks of training. However, 10 g PRT post-workout is insufficient to maximize MPS (Moore et al., 2009a), particularly in the elderly who show a diminished sensitivity to the anabolic effects of AA (Cuthbertson et al., 2005) and pre-exercise protein is of questionable benefit (Tipton et al. 2007). Thus, the collective evidence suggests that when PRT/AA supplements are consumed in conjunction with chronic RE training, skeletal muscle mass (whole-body lean mass, and both limb muscle and fibre CSA) is increased to a greater degree than consumption of an energy-matched placebo/carbohydrate, at least in young subjects.

The roles that PRT/AA play in promoting gains in skeletal muscle mass with RE in the elderly are, however, less clear than in the young. Given that muscle in the elderly appears to be resistant to the anabolic effects of both RE (Kumar et al., 2009) and AA (Cuthbertson et al., 2005), factors such as timing of intake (Esmarck et al., 2001), PRT source (Dangin et al., 2003), leucine content (Katsanos et al., 2006), and PRT/AA dose (Katsanos et al., 2005) may be particularly important in the elderly population to maximize post-exercise MPS and promote training-induced gains in skeletal muscle mass.

17.5.3 Dose and distribution considerations to maximize MPS

Oral ingestion of ~20 g of complete high quality PRT appears sufficient to maximize the response of mixed MPS following acute RE in young men (Moore et al., 2009a). Consumption of twice as much PRT saw no further increase in MPS but increased leucine oxidation rate (Moore et al., 2009a). These findings are consistent with the dose-response relationship between EAA ingestion and the increase in myofibrillar protein synthesis reported during resting conditions (Cuthbertson et al., 2005). Cuthbertson and colleagues (2005) reported that 10 g of EAA were sufficient to maximize rates of myofibrillar protein synthesis in young and old men, an amount comparable to that found in 20 g of high quality PRT (~8.6 g). The amount of EAA required to maximize MPS after RE may actually be slightly less than requirements at rest. This is due to the elevation in MPB following RE that presumably allows for liberated AA to be re-utilized for the synthesis of new muscle proteins. In addition, meal distribution of PRT intake over the course of a day is also an important consideration from the perspective of maximizing gains in muscle protein content and minimizing AA oxidation and irreversible loss. However, current guidelines for dietary PRT do not address distribution of intake over the course of a day. Bohe and colleagues (2001) have shown that MPS becomes refractory despite continued elevations in aminoacidaemia. In addition, it is well known that increasing dietary PRT intake increases the activity of enzymes involved in AA catabolism (Das and Waterlow, 1974). Thus, 20 g PRT servings (or ~8–10 g EAA) spread out in even temporal intervals during the day may serve to maximize MPS and gains in RE-induced skeletal muscle protein gains in young subjects (Moore et al., 2009a). Intakes beyond this are unlikely to offer further benefit as far as maximizing the feeding-induced stimulation of MPS. However, given their reduced ability to elevate MPS in response to EAA (Cuthbertson et al., 2005), it appears that elderly muscle may require a higher dose of PRT to maximize MPS post-exercise.

17.6 Enhancing Endurance Exercise Performance and Recovery with Amino Acid and Protein Supplements

The oxidation of AA during EE contributes ~1–4% to the total energy provided, although sex and substrate availability from
carbohydrate appear to influence the energy contribution from AA (Tarnopolsky, 2004; Rennie et al., 2006). Despite the relatively minor contribution to energy provision from AA, several investigations have suggested that the addition of PRT to a carbohydrate beverage can improve EE performance (Ivy et al., 2003; Saunders et al., 2004; Saunders et al., 2007). Other studies, however, have found no benefit to exercise performance from the addition of PRT to a carbohydrate beverage (van Essen and Gibala, 2006; Romano-Ely et al., 2006; Osterberg et al., 2008; Valentine et al., 2008; Cermak et al., 2009). However, as with RE, PRT consumption after EE has been shown to increase post-exercise MPS and NPB (Howarth et al., 2009) and may thus represent an effective strategy to enhance the recovery process following EE (Fig. 17.3).

### 17.6.1 Amino acids and recovery from endurance exercise

Similar to RE, an acute bout of EE is associated with an increase in post-exercise MPS (Carraro et al., 1990; Sheffield-Moore et al., 2004). However unlike RE, EE training is not generally associated with increases in skeletal muscle mass. Wilkinson and colleagues (2008) examined the response of individual protein fractions (myofibrillar and mitochondrial) following 45 min of unilateral cycling exercise at 75% VO2 max performed over several weeks. Regardless of the training status of the individuals, cycling exercise was associated with a pronounced increase in mitochondrial protein synthesis, yet no change in the synthesis of myofibrillar proteins (Wilkinson et al., 2008). Thus the increase in mitochondrial (and to some extent sarcoplasmic) but not myofibrillar protein following EE is likely to play a vital role in facilitating the increases in aerobic capacity that are associated with this type of training.

Consumption of AA/PRT following EE may enhance the recovery process by increasing the synthesis of proteins involved in force production and energy metabolism (Rodriguez et al., 2007), as well as by helping to restore muscle glycogen (van Loon et al., 2000; Ivy et al., 2002). When PRT is consumed immediately after EE, both skeletal muscle (Howarth et al., 2009) and whole body (Levenhagen et al., 2002)

**Fig. 17.3.** Schematic showing the benefits of amino acids to endurance exercise. ‘Insulinotropic’ indicates activation of the insulin/IGF-1-derived pathway. ‘Subsequent performance’ refers to exercise or athletic event performed in close proximity (<24 h) to a previous bout. Dashed arrow pathways remain to be demonstrated convincingly in humans.
protein synthesis are increased. For example, Howarth and colleagues (2009) reported that co-ingestion of PRT with carbohydrate after cycling exercise increased mixed MPS and resulted in a more positive NPB than ingestion of a drink matched for carbohydrate or total energy content. Additionally, Koopman and colleagues (2004) reported that addition of PRT to a carbohydrate beverage improved whole-body NPB both during and after 6 h of ultra-endurance exercise, via increased protein synthesis and reduced protein breakdown. Thus, consumption of a source of AA with carbohydrate appears to increase MPS and improve whole-body NPB, but whether this practice enhances the adaptive response to chronic EE remains to be shown definitively. However, given that the mitochondrial protein fraction is sensitive to exogenous AA provision (Bohe et al., 2003), it may be inferred that PRT/AA immediately after EE is likely to represent an effective strategy to facilitate the adaptive response to EE by increasing the synthesis of mitochondrial proteins (Fig. 17.3).

In addition to promoting post-exercise protein synthesis, the addition of a source of AA to a carbohydrate beverage has been reported to modulate the synthesis of muscle glycogen (van Loon et al., 2000; Ivy et al., 2002). The increased rates of glycogen re-synthesis may be attributed to the insulino-tropic effects of AA, which appears to enhance GLUT-4 translocation (the main insulin-sensitive glucose transporter) and stimulate glycogen synthase activity when carbohydrate is limiting (van Loon et al., 2000). Indeed, when carbohydrate content and rate of delivery are increased to 1.2 g kg\(^{-1}\)h\(^{-1}\), glycogen synthesis was increased such that it was no different from the AA-containing solution (van Loon et al., 2000). A separate study from the same group (Jentjens et al., 2001) showed that when carbohydrate was increased and provided at a rate of 1.2 g kg\(^{-1}\)h\(^{-1}\), addition of a source of AA (0.4 g kg\(^{-1}\)h\(^{-1}\)) did not increase rates of glycogen synthesis above that of carbohydrate. Thus, for those on a carbohydrate-restricted diet, provision of AA after exercise may represent an effective strategy to increase muscle glycogen while reducing the amount of carbohydrate intake.

17.6.2 Role of protein and amino acids in endurance exercise performance

Several studies have reported that PRT ingestion during exercise improves EE performance (Ivy et al., 2003; Saunders et al., 2004, 2007). Ivy and colleagues (2003) reported that adding PRT to a carbohydrate drink improved cycling endurance capacity (time to exhaustion) by ~30% compared to carbohydrate alone. However, other studies have failed to find any performance benefit from the addition of PRT to a carbohydrate beverage (van Essen and Gibala, 2006; Romano-Ely et al., 2006; Osterberg et al., 2008; Valentine et al., 2008; Cermak et al., 2009). For example, van Essen and Gibala (2006) found that addition of whey PRT to a carbohydrate solution did not improve cycling time trial performance relative to carbohydrate alone. Differences between these studies may relate to carbohydrate intake during exercise, and thus the rate of delivery. For instance, the studies demonstrating a benefit from added PRT (Ivy et al., 2003; Saunders et al., 2004, 2007) have typically provided carbohydrate at rates between 37 and 47 g carbohydrate h\(^{-1}\). These rates are below peak exogenous oxidation rates for carbohydrate, which have been reported to be 60–72 g h\(^{-1}\) (Jentjens et al., 2004a,b,c). Thus, when carbohydrate is provided at a rate of 60 g carbohydrate h\(^{-1}\), a rate considered optimal (Gibala, 2007), added PRT does not appear to improve cycling time-trial performance (van Essen and Gibala, 2006; Cermak et al., 2009) or alter markers of metabolic control such as muscle glycogen use, phosphocreatine hydrolysis, or tricarboxylic acid cycle intermediates (Cermak et al., 2009). In light of these recent data, use of AA or PRT with EE may best be directed to the post-exercise recovery period to enhance MPS, NPB, and glycogen re-synthesis.

17.7 Cell Signalling Responses to Amino Acids and Resistance Exercise

The molecular regulation of MPS in response to feeding and exercise is thought to involve multiple converging cell-signalling pathways.
Current understanding of the molecular regulation of MPS precludes the ability to predict quantitative changes in MPS based on acute cell-signalling responses to feeding and exercise. The cellular signalling response(s) to anabolic stimuli in the form of AA and/or RE remains to be fully elucidated, although this is currently an area of immense research interest. This section will provide an overview of current understanding of the ability of AA and RE to activate anabolic signalling pathways, with particular emphasis on mTOR and its downstream targets.

17.7.1 Cell signalling pathways involved in translation initiation and elongation

EAA stimulate MPS, an effect that is due to an increase in the concentration of extracellular EAA (Bohe et al., 2003). The extracellular AA concentration (or changes in it) is somehow 'sensed', and a signal activates translation initiation. Insulin and EAA activate cell-signalling pathways that converge on mTOR, activate its downstream targets, and augment both translation initiation and elongation of protein synthesis (Fig. 17.1) (for review see Drummond et al., 2009). That is, while insulin activates the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt) pathways, EAA can promote translation initiation in a manner that is independent of Akt (Wang et al., 1998). Anthony and colleagues (2000) have shown that the mTOR inhibitor rapamycin is able to inhibit the leucine-induced stimulation of MPS and subsequent phosphorylation of p70S6k, supporting the notion that EAA such as leucine increase MPS through mTOR. Recently it has been proposed that hVps34, a class III PI3K, may play an important role in the AA-induced activation of mTOR and its downstream targets (Byfield et al., 2005). Another study has shown a similar role for MAP4K3, a MAP kinase that may detect AA and signal mTOR independently of insulin (Findlay et al., 2007). A role for either hVps34 or MAP4K3 in mediating the AA-induced activation of mTOR in human muscle remains to be shown.

17.7.2 Cell signalling response to amino acids

In human muscle, increases in MPS following ingestion of EAA and carbohydrate have been shown to be associated with activation of mTORC1 (complex 1) (Cuthbertson et al., 2005; Fujita et al., 2007). Fujita and colleagues (2007) have shown that a leucine-enriched mixture of EAA and carbohydrate increases MPS, concomitant with an increase in the phosphorylation of mTOR and its downstream targets p70S6k and 4E-BP1, and a decrease in eEF2. Leucine independently stimulates MPS in rats, and results in phosphorylation of factors associated with initiating mRNA translation primarily through the mTOR signalling pathway such as p70S6k and 4E-BP1 (Anthony et al., 2002; Crozier et al., 2005). Together, these findings suggest that provision of EAA and leucine are capable of increasing activation of components of the mTOR pathway including 4E-BP1 and p70S6k.

17.7.3 Cell signalling response to amino acids and exercise

It has been firmly established that RE stimulates MPS (Chesley et al., 1992; Biolo et al., 1995; Phillips et al., 1997, 1999), and that this effect is synergistic with the provision of a source of AA (Biolo et al., 1997; Dreyer et al., 2008). Karlsson and colleagues (2004) demonstrated that p70S6k and S6 phosphorylation were increased post-exercise following the ingestion of EAA, as compared to exercise alone. In addition, PRT after acute RE results in greater phosphorylation of p70S6k than exercise performed without PRT (Koopman et al., 2007). Leucine-enriched EAA and carbohydrate provided after acute RE have been shown to increase MPS and the phosphorylation status of Akt and components of the mTOR pathway including mTOR, p70S6k, and 4E-BP1, suggesting increased translation initiation (Dreyer et al., 2008). Collectively, these findings support the notion that activation of the mTOR signalling pathway, and in particular phosphorylation of p70S6k, is associated with increases in MPS following RE.
17.8 Timing Considerations

Timing of nutrient intake involves the strategically planned consumption of either whole foods or dietary supplements, usually in the period surrounding an exercise stimulus in an attempt to enhance the adaptive response to exercise (Kerksick et al., 2008). Indeed, AA/PRT ingestion in close temporal proximity to a bout of RE appears to result in greater increases in lean body mass than nutrients provided at later times (Burk et al., 2009; Cribb and Hayes, 2006; Esmarck et al., 2001; Hartman et al., 2007). In this section we will discuss the relevance of timing of AA provision around an exercise stimulus and the potential of this strategy to augment adaptations to exercise.

17.8.1 Nutrient timing and acute exercise

An acute bout of RE stimulates an increase in MPS for up to 48 h (Phillips et al., 1997). Feeding any time within this 48-h window should simply add to this elevated rate of MPS to a greater extent than feeding during rested conditions in the absence of a previous bout of resistance exercise (Burd et al., 2009b). Indeed, Burd and co-workers (2009a) reported that acute RE results in enhanced anabolic sensitivity to AA provision that persists for 24 h post-exercise. However, early provision of AA after RE may confer greater benefit than delayed consumption, as MPS rates are elevated to a greater magnitude early after exercise (Phillips et al., 1997). In addition, cell-signalling proteins involved in increasing translation initiation after RE, such as mTOR and p70S6k are activated early and appear to undergo peak activation within 30–60 min after exercise (Camera et al., 2010). The exact duration of this ‘anabolic window’ is, however, not known. However, other reports suggest that this window may be at least 3 h long. For example, when 6 g of EAA and carbohydrate (35 g) were consumed at 1 or 3 h after RE, there was no difference in protein synthesis rates in young men (Rasmussen et al., 2000). In an early investigation, Tipton and colleagues (2001) reported greater increases in AA uptake across the leg when EAA were consumed before as opposed to after RE, and attributed the effect to a greater delivery of AA to the muscle as a result of exercise-induced hyperaemia. However, Fujita and colleagues (2009) reported no difference in the increase in post-exercise MPS relative to exercise without nutrients when EAA and carbohydrate were consumed before RE. Thus, the role of EAA ingestion prior to a bout of RE on post-exercise MPS appears to be less than the additive response that is observed with post-exercise feeding (see Drummond et al., 2009). The physiological relevance of providing a source of AA during acute exercise has also been investigated (Beelen et al., 2008). It appears that rates of MPS are reduced during the contractile activity associated with exercise (Dreyer et al., 2006). As both MPS and muscle contraction are energy-consuming processes, it has been suggested that the muscle contractile activity associated with physical exercise is of a higher metabolic priority than the synthesis of new proteins (Atherton and Rennie, 2009). However, co-ingestion of carbohydrate and PRT during exercise appears to increase MPS, although this increase was not associated with increased muscle protein accretion during subsequent overnight recovery (Beelen et al., 2008). Thus, AA/PRT consumption immediately after acute exercise appears to be more efficacious in promoting muscle protein accretion than consuming these nutrients immediately before or during exercise.

17.8.2 Nutrient timing and chronic exercise

In terms of long-term adaptations to chronic RE, Hartman and colleagues (2007) have shown that PRT consumption within 2 h following RE results in greater increases in muscle fibre hypertrophy and lean mass gains following 12 weeks of training, than consuming carbohydrate during this 2-h window (Hartman et al. 2007). Similarly, Cribb and Hayes (2006) examined changes in body composition and muscle strength following consumption of a supplement containing PRT, creatine and glucose immediately before and after RE relative to the same supplement consumed in the morning
and evening. Following 10 weeks of training, the group consuming the supplement immediately before and after exercise experienced greater increases in lean body mass and strength, in conjunction with greater increases in type-II fibre CSA (Cribb and Hayes, 2006). Interestingly, Esmarck and colleagues (2001) reported that delaying PRT consumption after RE by only 2 h prevented training-induced gains in muscle mass and strength in older men, as compared to subjects who consumed a beverage containing PRT, fat, and carbohydrate immediately after workout. Overall, consuming a source of EAA immediately after RE (within 2 h) appears to result in greater gains in lean mass and strength than delayed consumption following several weeks of training; however, skeletal muscle appears to display enhanced sensitivity to the anabolic effects of AA for at least 24 h after exercise (Burd et al., 2009a).

17.9 Amino Acid Source

Sources of dietary PRT differentially affect the rate of whole-body (Boirie et al., 1997; Dangin et al., 2001) and skeletal MPS (Tang and Phillips 2009; Tang et al., 2009), as well as support RE-induced gains in lean mass and strength than delayed consumption following several weeks of training; however, skeletal muscle appears to display enhanced sensitivity to the anabolic effects of AA for at least 24 h after exercise (Burd et al., 2009a).

17.9.1 Acute studies

Whey comprises ~20% of the proteins found in bovine milk (Ha and Zemel, 2003), with casein making up the other 80%. Whereas whey is acid soluble and digested quickly, casein coagulates in the stomach, a process delaying exit from the stomach and greatly slowing its absorption and subsequently the rate at which its constituent AA enter peripheral circulation. Thus whey is considered a ‘fast’ PRT that induces large but transient levels of hyperaminoacidaemia, whereas casein is a ‘slow’ PRT associated with a moderate but sustained rise in plasma AA (Boirie et al., 1997). Boirie and colleagues (1997) demonstrated that whey and casein differ in their ability to stimulate whole-body protein synthesis and attenuate whole body protein breakdown at rest. For example, ingestion of casein promoted a greater NPB than whey, due to a greater suppression of whole-body proteolysis (Boirie et al., 1997). Later work by Dangin and colleagues (2001) pointed to differences in the rate of PRT digestion (and subsequent hyperaminoacidaemia) between whey and casein as the key variable influencing the observed differences in whole-body protein kinetics. Interestingly, whole-body protein retention appears to be increased to a greater degree in the elderly following consumption of whey (Dangin et al., 2003). Thus, at the whole-body level, slowly digested proteins that induce a moderate but sustained rise in plasma AA appear to increase protein retention in young individuals. The elderly, however, respond differently, as wholebody-protein retention is increased in response to a large but transient increase in plasma AA concentration (Dangin et al., 2003). Skeletal muscle accounts for ~25–30% of whole-body protein synthesis (Nair et al., 1988), with a turnover rate that is ~20-fold lower than both splanchnic (Nakshabendi et al., 1995, 1999) and plasma (Carraro et al., 1990a) proteins. Thus, it is not entirely clear if the whole-body protein data demonstrating differential effects of PRT source on whole-body synthesis and breakdown (Boirie et al., 1997) is indeed reflective of the anabolism of skeletal muscle proteins. To address this question, Tang and colleagues (2009) examined the effects of whey PRT hydrolysate, soy PRT isolate, and micellar casein on rates of mixed MPS at rest and after acute RE. Whey PRT stimulated mixed MPS to a greater degree at rest than casein, and to a greater degree than both soy and casein after exercise; however, soy was more effective than casein after RE (Tang et al., 2009). Interestingly,
fat-free fluid milk increases MPS and net AA uptake across the leg to a greater degree than soy (Wilkinson et al., 2007). This finding is consistent with reports that milk and soy proteins are preferentially partitioned to different body pools for use following their ingestion (Fouillet et al., 2003). For example, soy appears to be preferentially directed towards the splanchnic tissues to support protein synthesis, while milk proteins are directed toward peripheral tissues (i.e. skeletal muscle) to support protein anabolism (Bos et al., 2003; Fouillet et al., 2003; Morens et al., 2003). Thus, differences between PRT sources may in part be explained by differences in digestion/absorption kinetics, and thus the rates at which AA enter circulation (Boirie et al., 1997; Dangin et al., 2001, 2003). However, whey PRT contains a greater amount of the BCAA leucine, a known regulator of the mTOR/p70S6k signalling cascade of translation initiation (Anthony et al., 2002). Thus, differences in leucine concentration may also affect the response of MPS following ingestion of different sources of high quality PRT. It is interesting to note that the anabolic effects of whey PRT are not simply due to its EAA content, as 15 g of whey PRT acutely increased muscle protein accrual in the elderly more than its constituent EAA content (6.72 g EAA) (Katsanos et al., 2008). The mechanism behind this finding is not clear, but may relate to the greater insulin response observed with whey as compared to EAA (Katsanos et al., 2008).

We conclude that in terms of increasing the anabolic effects of RE, consumption of whey or milk appears to be superior to consumption of micellar casein or soy (Fig. 17.2). Indeed, chronic RE training/feeding studies support this thesis (Cribb et al., 2006; Hartman et al., 2007).

17.10 Role of Leucine and Amino Acid Supplements in the Sarcopenia of Ageing

Nutrition and exercise-based interventions to counteract the loss of muscle mass that occurs with ageing (sarcopenia) are of great interest clinically. Concurrent use of nutrition and RE-based interventions to promote MPS and muscle hypertrophy represent potentially effective therapeutic strategies to minimize, and potentially reverse, the loss of muscle mass and strength that occurs with ageing. Also important, however, is that a relatively large quantity of skeletal muscle may help to offset the morbidities associated with the sarcopenia of ageing such as type II diabetes, declines in aerobic capacity, and reductions in metabolic rate that can ultimately lead to fat-mass accumulation (Wolfe et al., 2006). Here we will discuss the potential role of leucine and AA/PRT supplements to help combat the loss of muscle mass that occurs in concert with biological ageing.

Current research supports a unique role for the AA leucine as a nutrient regulator of translation initiation of protein synthesis.
Amino Acid Supplements

Leucine, isoleucine, and valine represent the BCAA that account for ~14% of the total AA found in skeletal muscle tissue (Riazi et al., 2003). In humans, infusion of a large dose of leucine is able to stimulate MPS (Smith et al., 1992). In rats, leucine alone stimulates MPS and phosphorylates factors associated with initiating mRNA translation, primarily through the mTOR signalling pathway such as p70S6k and 4E-BP1 (Anthony et al., 2002; Crozier et al., 2005). Plasma leucine concentration directs the peak activation of MPS in the post-prandial state. However, the length of time that MPS remains elevated following feeding is affected by other factors, which remain to be elucidated (Norton et al., 2009). Given the role of leucine as a primary regulator of MPS, an obvious avenue of research has been to investigate its potential as a nutritional intervention to attenuate age-related muscle wasting. Aged muscle has been shown to be resistant to the anabolic effects of AA (Cuthbertson et al., 2005) and the antiproteolytic effects of insulin (Wilkes et al., 2009). However, studies in both rats (Rieu et al., 2003) and humans (Katsanos et al., 2006; Rieu et al., 2006) have shown that a high concentration of leucine is able to restore rates of protein synthesis in aged muscle. For example, Katsanos and colleagues (2006) showed that the ingestion of 6.7 g of EAA (26% leucine) was unable to increase MPS above basal rates in the elderly, but effectively increased MPS in the young. However, when the leucine content was increased (41%), MPS was elevated above basal levels to the same extent as observed in young subjects. However, when larger doses of PRT are consumed, increased leucine does not appear to offer additional benefit (Tipton et al., 2009), suggesting that when leucine concentration is already high, additional leucine is not beneficial. In a long-term investigation, Verhoeven and colleagues (2009) reported that chronic (3 months) free-form leucine supplementation at 7.5 g d\(^{-1}\) (2.5 g at breakfast, lunch, and dinner) in the elderly did not increase lean muscle mass or strength as compared to an energy-matched placebo. In this context, it is interesting that other studies have reported increases in lean muscle mass in elderly subjects following chronic (3–16 months) mixed EAA supplementation (Borsheim et al., 2008; Solerte et al., 2008; Dillon et al., 2009). When leucine is provided in supplemental form, plasma levels of the other BCAA may become depleted (Dardevet et al., 2000); thus it has been suggested that a balanced mixture of BCAA may be more efficacious than free-form leucine supplementation (Balage and Dardevet, 2010). In addition, it may be that leucine supplementation is more effective when consumed in close proximity to acute RE. Moore and co-workers (2009b) have shown that while PRT ingestion stimulates the synthesis of both the myofibrillar and sarcoplasmic protein fraction at rest, PRT consumption after RE is associated with an increase in the synthesis predominantly of the myofibrillar fraction of muscle protein. Thus, leucine supplementation is likely to be most efficacious when combined with RE, as it may potentiate the synthesis of myofibrillar proteins. In conclusion, both nutritional and exercise-based interventions represent potentially effective therapeutic strategies to enhance skeletal muscle mass in aged individuals. A combination of RE and AA/PRT feeding is likely to be more efficacious than either stimulus alone, but more research is needed to fully elucidate the conditions under which supplemental leucine may be most effective. However, aged muscle appears to be resistant to the anabolic effects of leucine at doses that stimulate MPS in the young (Katsanos et al., 2006), suggesting that the elderly may have a higher leucine 'threshold' to stimulate MPS. Thus, the elderly should focus on obtaining adequate protein at each meal to ensure an adequate leucine concentration to maximally stimulate MPS.

17.11 Conclusions and Future Directions

Exercise and AA, particularly in combination, have profound effects on muscle protein turnover and NPB. Athletes in particular report the use of AA/PRT supplements, although use of these supplements is also popular among physically active individuals in commercial exercise settings. Both feeding and RE are known to activate anabolic cell-signalling
pathways, including the mTOR/p70S6k signalling cascade important in translation initiation. More research is required to fully elucidate these pathways and their respective proteins. Understanding how AA and RE are ‘sensed’ such that these anabolic signalling pathways are activated would greatly advance current understanding of the molecular basis of how muscle mass is regulated. It appears that in young subjects, PRT/AA supplements are more capable of enhancing RE-induced increases in skeletal muscle mass, and possibly strength, than are carbohydrate supplements. In particular, use of whey PRT and/or milk appears to result in greater gains in lean muscle mass than soy or carbohydrate when used in conjunction with RE. It is likely, however, that high-quality food-source animal proteins such as egg, chicken, or beef are also efficacious in this respect, although chronic studies incorporating these PRT sources are scarce (Campbell et al., 1999; Haub et al., 2002). The role of AA/PRT supplements in enhancing adaptations to RE in the elderly is a contentious issue and one that requires further investigation. Given that aged muscle is resistant to the anabolic effects of both RE and AA, factors such as PRT dose, leucine concentration, and timing of intake, may be of greater relevance to the elderly. Indeed, timed intake of a source of AA immediately post-exercise appears to result in greater muscle protein anabolism than intake at other times.

Although the addition of PRT to a carbohydrate beverage has been reported by some to increase EE performance, this is a very debatable issue. Indeed, there is currently no established mechanism to show how AA might be expected to acutely enhance EE performance. Future research needs to examine the role of PRT/AA supplements in enhancing EE-induced increases in oxidative capacity following a period of chronic training. In summary, AA/PRT supplements are efficacious in many respects. They clearly increase rates of MPS, and appear to enhance RE-induced gains in skeletal muscle mass in certain populations. Any benefits derived from these supplements, however, need to be weighed against the benefits of consuming PRT from a nutritionally balanced mixed meal. While these supplements may offer greater convenience under certain circumstances, they can be quite expensive. In addition, there is currently no evidence that these supplements are any more efficacious than high-quality food-source proteins such as milk, eggs, beef or chicken. Indeed, consumption of a complete meal is likely to provide other important macronutrients in addition to providing a complement of vitamins and minerals.

References


Amino Acid Supplements


Amino Acid Supplements


Nutritional support improves outcome in several clinical conditions. Glutamine (Gln), a non-essential amino acid, has distinct properties, apart from providing nitrogen for protein synthesis. This chapter aims to provide an in-depth review on the efficacy of oral Gln on protein metabolism and clinical outcomes in children with Duchenne muscular dystrophy (DMD), a severe muscle-wasting disease. Because muscle (the main source of endogenous Gln production) is severely reduced, DMD could be proposed as a ‘model’ for the role of muscle in whole-body Gln metabolism. Although short-term studies of supplemental Gln in DMD children suggest an acute ‘protein-sparing’ effect, resulting from a decrease in whole-body protein breakdown, the beneficial effects on protein metabolism have not been translated into prolonged benefits on clinical outcomes, such as muscle mass, muscle strength and muscle function. Better targeting of specific subgroups is necessary fully to evaluate the presence or absence of benefits of exogenous Gln in DMD children, particularly during acute ‘stress’, when demands of Gln might outweigh its endogenous synthesis.

Glutamine (Gln) is the most abundant free amino acid in the muscle and plasma of humans (Bergstrom et al., 1974). Although Gln is a non-essential neutral amino acid, it is necessary for optimal growth of mammalian cells in tissue culture (Eagle et al., 1956) and has important physiological functions. Apart from providing nitrogen (N) for protein synthesis, Gln is a precursor for nucleic acids, nucleotides (Newsholme et al., 1985), hexosamines (Neu, 2001), the nitric oxide precursor-arginine (Arg) (Ligthart-Melis et al., 2008) and the major antioxidant - glutathione (Neu, 2001; Duggan et al., 2002). Gln is also an important oxidative fuel for rapidly proliferating cells such as those of the gastrointestinal tract (Windmueller and Spaeth, 1980) and immune system (Newsholme et al., 1985), reticulocytes (Rapoport et al., 1971), fibroblasts (Darmaun et al., 1988) and so on. It plays a central role in N transport between tissues (Lacey and Wilmore, 1990), specifically from muscle to gut, kidney, and liver. In addition to its role as a gluconeogenic substrate (Nurjhan et al., 1995; Hankard et al., 1997; Mithieux, 2001),
Gln is involved in the renal handling of ammonia, serving as a regulator of acid-base homeostasis (Welbourne et al., 1986) and might play a role in the regulation of protein synthesis (Hankard et al., 1996b). Present data also indicate that Gln functions as a signalling molecule (Curi et al., 2005), particularly under catabolic conditions.

Traditionally Gln is considered a non-essential amino acid, because it is synthesized from carbon (C) and N precursors in most tissue (skeletal muscle being the main producer and storage site) (Darmaun and Dechelotte, 1991, Neu et al., 1996). Gln synthetase catalyses the terminal step in Gln de novo synthesis and is a key enzyme in Gln metabolism (Labow et al., 1998, 1999) (Fig. 18.1). In mammals, Gln synthetase expression is regulated by transcriptional and post-transcriptional mechanisms, and increases Gln synthetase mRNA in response to stress (e.g. glucorticoids) and regulation of Gln synthetase protein turnover in response to its product (plasma Gln concentrations) (Labow et al., 2001).

Under normal conditions Gln is released into circulation for consumption by other tissues, whereas in serious diseases associated with acute catabolic stress the production of Gln may be insufficient to meet the increased requirements by other tissue (gut, immune system/inflammatory cells, liver, and kidneys). Demands are partly met by skeletal muscle proteolysis and the release of large amounts of Gln to maintain normal concentrations in the plasma, resulting in depletion of Gln stores. In those situations associated with acute ‘stress’ (Ziegler et al., 1992), Gln might become ‘conditionally essential’ (Lacey and Wilmore, 1990). Recent meta-analyses, however, failed to show any improvement in morbidity or mortality in children with gastrointestinal disease (Grover et al., 2007) or in premature babies (Tubman et al., 2008).

Whereas abundant literature has been published on Gln administration in critical care (e.g. premature infants of low birth weight), fewer data are available for other childhood diseases such as DMD. The purpose of this chapter is to provide an in-depth review on Gln administration in children with DMD, a serious muscle-wasting disease, and the most common muscular dystrophy of childhood. The effects of this muscle-wasting disease on Gln metabolism are discussed first, followed by a detailed discussion of the clinical studies that have examined the efficacy of oral Gln supplementation on protein metabolism, and clinical outcomes in DMD.

18.3 Duchenne Muscular Dystrophy: the Role of Muscle in Glutamine Metabolism

Duchenne muscular dystrophy is a serious X-linked disease caused by a defect in the gene encoding for the cytoskeletal protein dystrophin (Hoffman et al., 1988). The absence of dystrophin expression is associated with a progressive and severe loss of muscle mass and function. By the age of 10 years, muscle mass is reduced by 75% in DMD boys (Griffiths and Edwards, 1988, Hankard et al., 1996a, 1999).

Because of the dramatic muscle mass loss observed in this paediatric condition,
we studied DMD as a 'model' to examine the role of muscle in both Gln and protein metabolism. Protein and Gln kinetics in vivo were assessed in DMD children and controls using stable non-radioactive isotope tracers (Hankard et al., 1999). Whole-body protein kinetics can be determined from a primed continuous intravenous infusion of L-[1-13C] labelled leucine (Leu) by measuring at isotopic steady state, plasma [1-13C]Leu enrichment, expired 13CO2 enrichment, and CO2 production rate (Matthews et al., 1980) (Fig. 18.2). After infusion [1-13C]Leu is either oxidized and appears in the breath as 13CO2 or is incorporated into tissue protein through protein synthesis. The isotope enrichment in expired air and plasma are determined by isotopic ratio mass spectrometry and gas chromatography mass spectrometry, and CO2 production rate by indirect calorimetry. In the post-absorptive state, Leu appearance rate (Ra,Leu), an index of whole-body protein degradation, is determined from plasma [1-13C]Leu enrichment. Similarly, Leu oxidation rate (OxLeu) is determined from 13CO2 enrichment in expired air and CO2 production rate. Estimates of whole-body protein synthesis are then calculated as Ra,Leu - OxLeu. For simultaneous determination of Leu and Gln kinetics, we infused L-[1-13C] Leu with l-15N labelled Gln (i.e. L-[2-15N]Gln) (Darmaun et al., 1985, 1986). We observed that compared to control boys of the same age, the 75% muscle mass loss in DMD boys was associated with a 25% decrease in the rate of plasma Gln appearance in the postabsorptive state (resulting from a decrease in estimates of Gln de novo synthesis), and a more negative whole-body Leu balance (Hankard et al., 1999), indicating that these children are in a state of hypercatabolism. Thus in DMD when muscle (the main Gln-producing organ) is severely reduced, Gln turnover is also reduced (Fig. 18.3).

Because skeletal muscle is the major source of endogenous Gln, Gln released into plasma from skeletal muscle must meet the Gln needs of other tissue. It is possible that in DMD, the lower Gln production might be the primary event (a direct consequence of the reduced muscle mass). With the progressive decline in muscle mass, the Gln needs of other tissue might outstrip the Gln synthetic capacity of skeletal muscle, particularly during increased 'stress' (Fig. 18.4). Similar observations were reported in DMD for another non-essential amino acid, alanine (Haymond et al., 1978). Because the rate of Gln released from protein degradation was unaffected in DMD, decreased Gln turnover results from a decrease in Gln de novo synthesis. This supports the role of Gln synthetase in regulating Gln metabolism (Smith et al., 1984). Likewise, decreased Gln de novo synthesis accounts for the decreased Gln appearance rate observed in healthy adults receiving exogenous Gln infusion (Hankard et al., 1995), as well as in adults with short bowel syndrome (SBS) (Darmaun et al., 1991). The latter condition might, however, represent an opposite 'model' to DMD, because the mass of the small intestine (a prominent Gln-consuming tissue) is severely reduced.

We studied SBS infants, and similar to DMD, we observed reduced Gln turnover (Gln appearance rate in the plasma) compared to control infants, whereas whole-body protein turnover (Leu appearance rate) was unaltered by intestinal resection (Hankard et al., 1994). This suggests that for infants with SBS, Gln production is scaled down to meet a lower Gln demand by the gut (i.e. the lower Gln production rate is secondary to an initial decline in Gln needs for other tissue). Because Gln uptake by the gut can modulate muscle Gln production, the infant small intestine might also play a prominent role in Gln metabolism, but as a preferential user of Gln. This inter-organ adaptive process also supports the role of Gln
(a) Normal conditions

Muscle

Gln production

Plasma

Gln appearance

Other tissue

Gln utilization

(b) Duchenne muscular dystrophy

Muscle

Gln production

↓

Plasma

Gln appearance

↓

Other tissue

Gln utilization

Fig. 18.3. Duchenne muscular dystrophy: a ‘model’ for the role of muscle in glutamine metabolism. (a) Under normal conditions, glutamine is released into circulation for consumption by other tissue (gut, immune system/inflammatory cells, liver, and kidneys). (b) In Duchenne muscular dystrophy, the severe reduction in muscle mass (the main source of endogenous glutamine production) is associated with a decrease in the rate of plasma glutamine appearance, resulting from a decrease in glutamine de novo synthesis. The lower glutamine production may be a direct consequence of reduced muscle mass.

Fig. 18.4. Proposed model for the ‘stressed’ Duchenne muscular dystrophy patient. The progressive muscle mass depletion combined with increased ‘stress’ (e.g. prolonged use of corticosteroids) results in a persistent imbalance between glutamine utilization and glutamine production (i.e. during acute ‘stress’, demands of glutamine by other tissue outweigh glutamine de novo synthesis by muscle). This may ultimately result in a depletion of glutamine tissue stores, requiring exogenous support.

Gln synthetase in regulating Gln metabolism, since plasma Gln concentration is known to exert feedback inhibition on Gln synthetase.

Because skeletal muscle is the body’s main producer and exporter of Gln and muscle mass is drastically reduced in DMD, the need for Gln may be increased in persons who have this disease. Whereas the body has evolved normally effective mechanisms to maintain Gln homeostasis, it may fail to accomplish this
homeostasis when muscle mass is severely depleted, limiting its ability to convert protein stores to free Gln (via Gln synthetase) (Labow et al., 2001). Furthermore in DMD, as in other protein-wasting conditions, the intramuscular Gln concentration is low (Rennie et al., 1989; Sharma et al., 2003). Sharma et al. (2003) suggested that this may be an underlying reason for muscle wasting in DMD. Thus in DMD, as in other acute ‘stress’ conditions, Gln might be considered a ‘conditionally essential’ amino acid.

18.4 Glutamine Supplementation in Children with Duchenne Muscular Dystrophy

While there is no cure for this fatal disease, current therapies aim at targeting downstream events in the pathologic progression of DMD muscle wasting. Nutritional support with amino acids (e.g. Gln, Arg, Leu) in conjunction with drug, cellular or gene therapy might have potential for slowing disease progression in these children (Archer et al., 2006; Payne et al., 2006). However, much of the data showing benefits are based on experimental evidence in the mdx mouse model (Granchelli et al., 2000; Barton et al., 2005; Chazalette et al., 2005; Voisin et al., 2005; Archer et al., 2006; Payne et al., 2006; Mok et al., 2008) and few randomized controlled trials have been conducted in DMD children (Mendell et al., 1984; Escolar et al., 2005; Mok et al., 2006, 2009).

18.4.1 Acute glutamine on protein metabolism

Studies on protein metabolism suggest that muscle wasting in DMD could result from a reduction in muscle protein synthesis, an increase in protein degradation, or from both (Rennie et al., 1982; Griggs and Rennie, 1983; Goldstein et al., 1989). We conducted two separate studies using stable isotope methodology (Hankard et al., 1998; Mok et al., 2006), to test the effect of oral Gln on whole-body protein and Gln metabolism in DMD children during the postabsorptive state. In the initial study (Hankard et al., 1998), Leu and Gln kinetics were measured in six DMD boys aged 8–13 years on 2 consecutive days. Children received a 5-h oral administration of flavoured water (Kool-Aid) on the first study day, followed by Gln (0.6 g kg⁻¹) dissolved in the same flavoured water on study day 2. During Gln administration, Leu release from protein breakdown and Leu oxidation rate both decreased by 8% and 35% respectively (P<0.01), resulting in no change in non-oxidative Leu disposal (an index of protein synthesis). Whole-body Gln exchange in the plasma doubled (P<0.01), Gln from protein degradation and Gln de novo synthesis both decreasing during oral Gln administration. These preliminary data suggested that acute oral Gln might have an acute ‘protein-sparing’ effect in children with DMD, resulting from a decrease in whole-body protein degradation and Gln de novo synthesis. However, results should be viewed with caution, due to the small sample size. Additionally, the order of treatment allocation was not randomized and participants and assessors were not blinded to treatment. Moreover, the specificity of the effect of Gln on protein metabolism could not be tested, as measurements were not performed using an isonitrogenous control group as well.

We addressed these shortcomings more recently by conducting a double-blind randomized controlled trial (Mok et al., 2006) in 26 DMD boys (aged 7–15 y) to test whether the acute ‘protein-sparing’ effect of Gln persisted when oral Gln (0.5 g kg⁻¹ d⁻¹) was given for 10 d, and whether the effect was specific to Gln, by comparing the Gln supplemented group to an isonitrogenous control (amino acid mixture). Whereas plasma Gln concentrations were not altered this time (since kinetic studies were performed 24 h after Gln or amino acid administration), the decrease in Leu release from protein breakdown persisted after 10 days of Gln supplementation (−9%, P<0.05), and endogenous Gln from protein degradation also decreased. Similar effects were observed after 10 days of amino acid supplementation; however, the magnitude of the decrease in whole-body proteolysis was less (−4%, P<0.05). There were no significant effects on other estimates of Leu or Gln turnover or on body composition.
(fat-free mass, % fat mass, muscle mass and weight) after 10 days of supplementation in either group. The lack of significant difference between Gln and isonitrogenous control could be explained by the variability in disease progression among the study population, since Gln treatment in DMD may have different effects depending on the stage of the disease process (Escolar et al., 2005). It is also possible that a higher dose or longer treatment duration may be necessary to demonstrate the specific effect of Gln, separate from its role of providing N. This highlights the need for dose and time-course data on Gln administration in DMD. Alternatively, the route of administration could partly explain the lack of significant difference, since the 'protein-sparing' effect of Gln may be less dramatic when it is given enterally (Darmaun et al., 1997; Parimi et al., 2004) as opposed to parenterally, as demonstrated in studies on protein metabolism in premature infants of low birth weight (des Robert et al., 2002; Kalhan et al., 2005).

### 18.4.2 Long-term glutamine on clinical outcomes

Based on experimental data showing that Gln improved performance in the *mdx* mouse model of DMD (Granchelli et al., 2000), Escolar et al. (2005) conducted a 6-month randomized double-blind placebo-controlled multi-centre study to test the efficacy and safety of oral Gln (0.6 g kg⁻¹d⁻¹) in 35 ambulant steroid naïve boys with DMD aged 4–10 years. While there were no significant differences in the primary outcome (manual muscle-testing score) or on quantitative measurements of muscle strength, subgroup analysis showed that in younger boys (<7 years) the Gln group had significantly less deterioration over 6 months in timed functional tests versus placebo. Although there was a trend toward less deterioration in quantitative and functional measures of muscle strength with Gln treatment over 6 months, the effect did not reach significance for the cohort as a whole. The inability to detect a significant difference in the primary outcome could be explained by the unexpected lack of strength deterioration in the placebo group (measured by manual muscle testing) over the 6-month trial. Thus the study may have been underpowered, since power calculations were based on previous natural history data of DMD (Brooke et al., 1983). The significant age-related results must be interpreted with caution as they were based on an unplanned subgroup analysis in a small group of patients. Larger trials incorporating *a priori* age stratification are required to test the disease-modifying effect of long-term Gln supplementation in DMD.

More recently, our group completed a multi-centre double-blind randomized crossover trial in 30 ambulatory DMD boys aged 2–10 years, to test whether 4 months’ administration of oral Gln can slow the progressive loss in muscle mass and therefore provide functional benefit for these children (Mok et al., 2009). Subjects received 4 months’ administration of Gln separated by a 1-month washout, followed by 4 months of placebo (malodextrin) or vice versa. The order of treatment allocation was randomized. Selection of the Gln dose (0.5 g kg⁻¹d⁻¹) was based on our previous studies in DMD boys (Hankard et al., 1998) and in healthy humans (Hankard et al., 1995, 1996b) that showed a twofold increase in plasma Gln concentrations during Gln administration. This same dose decreased whole-body protein degradation in DMD boys during Gln administration (Hankard et al., 1998), which persisted 24 h after supplementation ceased, while plasma Gln concentrations had returned to normal (Mok et al., 2006).

Overall, there was no apparent functional benefit as tested by comparing Gln versus placebo on change in walking speed at 4 months (primary outcome) or in secondary measures of muscle function (2-min walk test, work, power). We observed no differences in muscle mass (urinary creatinine), markers of protein breakdown (urinary 3-methylhistidine/creatinine) or serum creatine phosphokinase in the Gln group compared with placebo, except for a blunted increase in fat-free mass in the Gln group which led to a greater increase in fat mass percentage. Our findings that functional measures did not deteriorate during the 4-month placebo phase or over the course of the 9-month trial were not as expected. Based on natural history data...
(Brooke et al., 1983), the trial was powered to detect a 10% difference in walking speed after 4 months of Gln compared to placebo. However, we did not consider the greater placebo effect reported in children (Rheims et al., 2008) which could have narrowed the expected effect size of Gln treatment.

Interestingly, subgroup analysis revealed a differential decline in functional measures in DMD boys taking corticosteroids (versus those not on corticosteroids) (Mok et al., 2009). For example, in the subgroup of boys taking corticosteroids (n=5), there was a 30% decrease in walking speed from baseline to 9 months (P<0.05), whereas those not on corticosteroids (n=25) showed no deterioration in function over time, similar to the cohort as a whole. There was also a significant effect of Gln treatment on functional measures in boys taking corticosteroids (P<0.05). Specifically, boys taking corticosteroids showed a significant decline in walking speed during the placebo phase (~40%, P<0.05), whereas walking speed remained stable when corticosteroid-treated boys received Gln treatment for 4 months. Although the findings must be interpreted with caution, because they derive from an unplanned analysis in a small subgroup of boys, they might suggest a rationale for Gln supplementation in conjunction with corticosteroid therapy (Hickson et al., 1995; Salehian et al., 2006), which needs to be investigated.

18.5 Conclusions and Future Research

DMD could be considered a ‘model’ for the role of muscle in Gln metabolism (Fig. 18.3). In DMD, the severe decrease in muscle mass (the main source of endogenous Gln production) is associated with a reduction in the rate of plasma Gln appearance (resulting from a decrease in Gln de novo synthesis).

Studies on protein metabolism in DMD children suggest a ‘protein-sparing’ effect of supplemental Gln, resulting from a decrease in whole-body proteolysis (Hankard et al., 1998; Mok et al., 2006). Although the acute benefits on protein metabolism (Hankard et al., 1998; Mok et al., 2006) and experimental data in mdx mice (Granchelli et al., 2000; Mok et al., 2008) indicate a role for Gln in the treatment of DMD, long-term supplemental Gln does not appear to improve muscle strength in DMD children (Escolar et al., 2005). However a disease-modifying effect of long-term Gln in younger DMD children cannot be ruled out. Our recent randomized crossover trial did not show added benefit on muscle mass or function, when oral Gln administered for 4 months was compared to placebo in ambulatory DMD boys (Mok et al., 2009). Hence, current data cannot support its routine use in this population as a whole. Better targeting of specific subgroups is necessary fully to evaluate the presence or absence of benefits, particularly in DMD children under ‘stress’.

Over the long run, increased ‘stress’ (e.g. prolonged corticosteroid therapy) combined with progressive muscle mass depletion could result in a persistent imbalance between Gln utilization and Gln production, depleting Gln tissue stores (Fig. 18.4). A decrease in plasma Gln concentrations has also been observed in other diseases associated with acute ‘stress’ (e.g. neonates suffering from necrotizing enterocolitis (Becker et al., 2000)). In the ‘stressed’ DMD patient, Gln may be used in amounts exceeding the endogenous synthetic capacity of the remaining muscle, requiring exogenous support. Gln could therefore be considered a ‘conditionally essential’ amino acid in specific diseases such as DMD during acute ‘stress’.

References


Adverse Effects

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19.1 Abstract

The adverse effects of amino acids in human nutrition and health have been reviewed in the context of the classical categories of imbalance, antagonism, and toxicity, first established in studies with animal models. This system has long existed as a conceptual framework primarily confined within the academic domain. However, there is now enhanced awareness that these categories may be relevant to a greater or lesser extent in human nutrition and health. A number of conclusions emerge in the light of this analysis, as detailed below.

It is suggested that two subdivisions, namely ‘clinical amino acid imbalance’ and ‘excitotoxicity’, are required within this classification to more accurately reflect observations in humans.

Clinical amino acid imbalance appears to be a regular feature of specific disease conditions. In patients with septic encephalopathy or with chronic liver disease, plasma ratios of branched-chain to aromatic amino acids are consistently reduced. Abnormal plasma amino acid patterns may also be seen in patients with cancer, cystinuria and Huntington’s disease.

It is proposed that the category of antagonisms should include the effects of a wide range of plant non-protein amino acids. Canavanine, homoarginine and indospicine share structural similarity and compete with arginine for cellular transport and in the modulation of nitric oxide synthesis. Selenium analogues of the sulphur amino acids act by competing with methionine and cysteine at the levels of transport and protein synthesis. β-N-Oxalylamino-L-alanine and β-N-methylamino-L-alanine are potent glutamate receptor agonists implicated, respectively, in neurolathyrism and in amyotrophic lateral sclerosis and Parkinsonism-dementia (or Guam dementia). However, the aetiology of Guam dementia remains under review. On the other hand, it is widely acknowledged that hypoglycin A is associated with the seasonal incidence of a specific condition known as ‘vomiting sickness’.

The category of toxicity is of particular significance in human health as it includes the adverse effects of glutamate, homocysteine, modified lysine residues and phenylalanine. The human health implications of these amino acids are reviewed later in this volume. Glutamate is the principal excitatory amino

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acid implicated in neurodegenerative disorders. Thus, the status of excitotoxicity as the second significant subdivision in the classification of adverse effects of amino acids is amply justified.

Despite the adverse health implications cited above, there is increasing evidence that imbalance may be adapted for clinical purposes. In several studies, acute imbalance has been induced by the administration of drinks or mixtures rich in the indispensable amino acids but devoid of tryptophan or tyrosine and phenylalanine. This technique thus conforms with the classical method of precipitating amino acid imbalance. The resulting reductions in concentrations of the respective neurotransmitters provide a metabolic basis for psychiatric investigations. The potential of amino acid imbalance in cancer therapy has been briefly reviewed. The emerging consensus relates to the efficacy of branched-chain amino acid depletion in the inhibition of tumours in experimental models. However, the potential scope for specific non-protein amino acids in cancer therapy appears to be relatively more promising. Varying degrees of success have been reported with canavanine, sulphur amino acid analogues and mimosine. In experimental models, certain synthetic congeners of canavanine exhibit enhanced potential, but Se-methylselenocysteine is highly active, particularly in optimizing the therapeutic efficacy of anti-cancer drugs.

The wide interpretation of ‘adverse effects’ of amino acids adopted in this chapter thus allows a perspective for further consideration of clinical implications relating to cardiovascular, genetic and neurological disorders. Appropriate cross-referencing to subsequent chapters is provided for detailed review of these conditions.

19.2 Introduction

The distinctive and critical features of amino acids as multifunctional molecules have long been recognized and continue to be the subject of much research and innovative developments (see Chapters 1–13). As a corporate group, amino acids provide the building blocks for the biosynthesis of tissue proteins, but individual members play equally vital roles as signalling molecules and as precursors of specific neurotransmitters, hormones, purines, pyrimidines, creatine, haem and polyamines. Furthermore, arginine yields nitric oxide, while tryptophan is used to generate nicotinamide. Individual amino acids cannot be retained indefinitely as free molecules and must proceed along these synthetic pathways or follow catabolic routes involving deamination and synthesis of urea. The residual carbon skeletons are metabolized further in the processes of gluconeogenesis and/or ketogenesis. The metabolic fate of amino acids is often encapsulated in these terms in standard texts on biochemistry, and for most intents and purposes such a synopsis is eminently acceptable. Within such a benign scenario it is tacitly assumed that any surplus of amino acids is disposed of without harmful effects. However, this simplistic view is no longer tenable as increasingly more evidence accumulates to demonstrate the adverse effects of amino acids in several mammalian species, including humans.

The adverse effects of amino acids are well-established in studies with animals and the results extensively documented and reviewed (Harper, 1959; Harper et al., 1970; D'Mello, 2003). The literature relating to this field is in a reasonably advanced state. There are now compelling reasons to review the expanding evidence that these adverse effects may be replicated, to different degrees, in human subjects. The terminology developed with animal models is finding common usage in clinical sciences and it is opportune to examine the relevant data within the context of existing evidence.

19.3 Classification

In any scientific enquiry, classification of findings invariably facilitates a more orderly presentation and discussion of relevant data and this approach also applies to the question of adverse effects of amino acids. However, there are often notable exceptions to established
schemes of rationalization which, equally, may deserve further exploration.

Using animal models, the diverse manifestations have been classified within three categories: imbalances, antagonisms and toxicity (Harper, 1959; Harper et al., 1970). As D'Mello (2003) observed, this system has long existed as a conceptual model primarily confined to the academic domain by virtue of its origin in contrived experiments with laboratory animals. However, there is now enhanced awareness that these categories of deleterious effects may be relevant to a greater or lesser extent in human nutrition and health. Accounts of adverse effects generally commence with the classification advanced by Harper (1959) and more fully expressed in the review of Harper (1964). The system emerged as a result of extensive research with a rat model (Table 19.1). His three-way framework has been retained in this chapter as many of the terms presented have become part of the terminology used in human nutrition and health.

It is immediately obvious that the classification was designed primarily to accommodate data obtained with the indispensable and dispensable amino acids (IAA and DAA, respectively). It is also apparent that in the development of this system, emphasis was placed on the adverse effects brought about solely by dietary manipulation of amino acids. Furthermore, no recognition was given to observations available at that time pertaining to those non-protein amino acids occurring in plants with the propensity to induce deleterious reactions in mammals (see D'Mello, 1991). Moreover, the system now requires updating to include particular amino acids that are considered to be risk factors in a number of human disorders, and efforts will be directed at integrating the associated effects into the classical three-way scheme presented in Table 19.1.

19.4 Amino Acid Imbalance

19.4.1 Concept

The term imbalance is relatively simple in concept, based on the premise that all amino acids must be available in balanced proportions at tissue level, with any deviation being accompanied by adverse effects. However, amino acid imbalance is often used out of context. There is, therefore, a need to begin with first principles to enable a more comprehensive discussion of the various issues that have appeared in the literature.

Amino acid imbalance is firmly embedded within the nutritional domain, where a precise definition has been applied for several decades. However, the term is also used in clinical investigations, where the terminology is less formal and has emerged independently of the nutritional work with animal models. It is intended to integrate observations from these parallel lines of investigation into a unifying framework. For the purposes of this review, it is intended to consider two forms of amino acid imbalance: dietary or nutritional and clinical.

19.4.2 Dietary or nutritional amino acid imbalance

Nutritional amino acid imbalance has been defined as a change in the dietary pattern of amino acids which results in the precipitation

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Table 19.1. Classification of adverse effects of amino acids: a three-way model proposed by Harper (1964). (Based on nutritional studies with rats.)

<table>
<thead>
<tr>
<th>Category</th>
<th>Dietary conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imbalance</td>
<td>Addition of second-limiting amino acid; or addition of an incomplete amino acid mixture. Amelioration of gross effects by supplementation with first-limiting amino acid</td>
</tr>
<tr>
<td>Antagonism</td>
<td>Specific, conditionally reversible interactions induced by structurally-related indispensable amino acids, e.g. lysine and arginine; or by leucine, isoleucine and valine. Lysine antagonism reversed by arginine supplementation; leucine antagonism reversed by combinations of isoleucine and valine</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Diverse effects of disproportionate levels of indispensable or dispensable amino acids, resulting in organ damage, e.g. renal or ocular lesions. Effects normally irreversible</td>
</tr>
</tbody>
</table>
of ill-effects that are completely reversed by supplementation with the first-limiting amino acid (Harper, 1964). The prerequisite for a limiting amino acid may be satisfied by the use of suitably deficient proteins such as casein, gelatin or zein, but more generally this condition is fulfilled by employing low-protein diets. The definition of imbalance was conceived and developed in studies with laboratory animals, but is now widely applied to the nutrition and well-being of human subjects even when the strict conditions of this phenomenon are not fulfilled. Thus, the precipitation of imbalance in animals has generally focused on IAA, whereas in the application to human health all amino acids are considered, irrespective of nutritional classification.

It is therefore, instructive to recall some of the fundamental tenets embodied within the original development of the concept of dietary amino acid imbalance. Two types of such imbalances are generally recognized (Table 19.2):

1. that induced by the addition of a relatively small quantity of IAA to a low-protein diet; and
2. that precipitated by an incomplete mixture of amino acids.

In the first type, there is normally a specific requirement that the agent inducing the imbalance should be the second-limiting amino acid (Winje et al., 1954). A more reliable procedure involves the addition of an amino acid mixture devoid of one IAA to a low-protein diet limiting in that amino acid (Pant et al., 1972). In both types, the effects of imbalances are rectified by supplementation with the first-limiting amino acid, which therefore acts as the antidote (Table 19.2). Other studies show that imbalances may also be created by the use of mixtures of DAA (Tews et al., 1980). In such cases, the most reliable method involves the use of amino acids, individually or in mixtures, that compete with the dietary limiting amino acid for transport into the brain. Thus, the ratio of imbalancing amino acids to the limiting one is critical in this type of adverse effects. Therefore, the minimal requirements for unequivocal demonstration of an amino acid imbalance must involve the use of a suitable control, an imbalanced diet, and a corrected diet (Table 19.2).

Several studies with animal models indicate that amino acid imbalances may occur at the tissue level even though the diet may appear to be in ideal balance. These types of imbalances may, for example, occur with the use of crystalline amino acids in diets based on cereals. Such considerations may well apply in human nutrition if the intention is to provide a perfect balance at tissue level. It has long been recognized that free amino acids used as supplements are absorbed more rapidly than protein-bound amino acids, resulting in an imbalanced supply at the sites of protein synthesis (see D’Mello, 2003). For example, Leibholz et al. (1986) observed that the concentration of free lysine in plasma of pigs increased 1–2 h after feeding a diet containing pure lysine, declining thereafter, whereas the circulating concentrations of

<table>
<thead>
<tr>
<th>Protein source and amino acid supplements</th>
<th>First-limiting amino acid</th>
<th>Method of precipitating imbalance</th>
<th>Dietary regime</th>
<th>Growth response (proportion of control)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg albumen + Thr + Val</td>
<td>His</td>
<td>Addition of second-limiting amino acid (Lys)</td>
<td>Control</td>
<td>1.00</td>
<td>Winje et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Imbalanced</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Corrected</td>
<td>0.95</td>
<td>(1954)</td>
</tr>
<tr>
<td>Casein + Met</td>
<td>Trp</td>
<td>Addition of amino acid mixture devoid of Trp</td>
<td>Control</td>
<td>1.00</td>
<td>Pant et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Imbalanced</td>
<td>0.71</td>
<td>(1972)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Corrected</td>
<td>1.10</td>
<td></td>
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other amino acids originating from the protein-bound fraction of the diet peaked 2–6 h post-prandial. In pigs fed once daily, this lack of synchrony in absorption would precipitate an imbalance at the cellular level. Under these circumstances growth and efficiency of dietary nitrogen utilization would be impaired, but the deleterious effects could be offset by more frequent feeding. This expectation was confirmed by Batterham (1974) who showed that the efficiency of utilization of free lysine supplements for growth of pigs fed once daily was only 0.43–0.67 of values recorded for pigs fed the same quantity of food in six equal portions at 3-hourly intervals. In contrast, no such benefit occurred on feeding the unsupplemented diet more frequently. Subsequent investigations by Partridge et al. (1985) extended the benefits of increased feeding frequency and lysine supplementation to improvements in nitrogen utilization.

19.4.2.1 Anorexia

Reports of amino acid imbalances conventionally focus on the growth-depressing effects in animal models (Harper, 1964; Tews et al. 1979). However, it has been consistently recorded that a predisposing factor is a rapid and marked reduction in food intake of affected animals. Thus, Harper and Rogers (1965) reported that rats fed an imbalanced diet reduced their food intake within 3–6 h, implying that appetite depression was the primary event responsible for the ensuing retardation of growth. A considerable body of evidence supports this hypothesis. If food intake in animals is increased by force-feeding, by insulin injections, by adjusting dietary protein to energy ratios, or by exposing animals to cold environmental temperatures, then commensurate improvements in growth also occur (D’Mello, 1994).

19.4.2.2 Dietary preferences

Alteration in dietary preference is another feature of nutritional amino acid imbalance, at least in the rat, with possible wider extrapolation to other mammals, including humans. When offered a choice, rats consume a balanced diet in preference to an imbalanced one, but more remarkably, select a protein-free diet incapable of supporting growth instead of an imbalanced diet which would allow growth, albeit at a low level (Sanahuja and Harper, 1962; Leung and Rogers, 1987).

19.4.2.3 Mechanisms

The biochemical mechanisms underlying the anorectic effects of amino acid-imbalanced diets have been described by Harper and Rogers (1965), following extensive studies with the rat. It was proposed that surplus amino acids arriving in the portal circulation following ingestion of the imbalanced diet stimulates synthesis or suppresses protein degradation in the liver, leading to enhanced retention of the limiting amino acid relative to that in control groups (Table 19.3). The supply of the limiting amino acid for utilization by peripheral tissues such as muscle is thereby reduced, but protein synthesis proceeds without interruption in these tissues. Ultimately, however, the free amino acid patterns of both muscle and blood plasma become so deranged as to invoke the intervention of the appetite-regulating system to reduce food intake. The growth-depressing effects are a direct consequence of reduced appetite and intake of nutrients. As D’Mello (2003) stated, this hypothesis is still accepted as a satisfactory explanation of the effects of amino acid imbalance in the rat. A central tenet within the hypothesis advanced by Harper and Rogers (1965) is the association between appetite depression and changes in tissue patterns of amino acids. In both blood plasma and muscle, concentrations of the limiting amino acid decline, while there is an accumulation of those amino acids added to create the imbalance. Since these events occur within a few hours of ingestion of imbalanced diets, it has been suggested that changes in the plasma amino acid pattern provide the metabolic signal that ultimately results in anorexia and abnormal feeding behaviour. In subsequent attempts to validate this hypothesis, the role of the first-limiting amino acid was placed in sharper focus. For example, preliminary work by Leung and Rogers (1969) indicated that the
Table 19.3. Nutritional amino acid imbalance: biochemical cascade leading to depressed appetite and growth in animal models. (Based on the hypothesis of Harper and Rogers, 1965.)

<table>
<thead>
<tr>
<th>Site</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Surplus amino acids stimulate synthesis or suppress breakdown of proteins; efficient utilization of limiting amino acid</td>
</tr>
<tr>
<td>Muscle</td>
<td>Protein synthesis continues normally; greater retention of limiting amino acid; deranged free amino acid pattern</td>
</tr>
<tr>
<td>Plasma</td>
<td>Deranged free amino acid pattern</td>
</tr>
<tr>
<td>Brain</td>
<td>Abnormal pattern in blood monitored by appetite-regulating regions; GCN2 signalling*</td>
</tr>
<tr>
<td>Whole-body</td>
<td>Depressed appetite</td>
</tr>
<tr>
<td>responses</td>
<td>Reduced nutrient intake</td>
</tr>
<tr>
<td></td>
<td>Reduced growth</td>
</tr>
</tbody>
</table>

*See Chapter 13.

depression in appetite may be prevented by the infusion of a small quantity of the first-limiting amino acid via the carotid artery, whereas administration through the jugular vein was ineffective. These studies also provided the basis of the proposition that food intake and feeding behaviour may be linked with changes in brain uptake and metabolic disposition of critical amino acids. It was soon established that the concentration of the first-limiting amino acid declined more rapidly in cerebral tissues than in blood plasma (Peng et al., 1972). This observation led to the proposal that the fall in brain concentrations of the limiting amino acid provides the signal that initiates the changes in food intake and dietary choice, although the definitive mechanism remains obscure (Leung and Rogers, 1987). However, the regions of the central nervous system sensitive to dietary amino acid imbalance have been delineated in the rat. These include the anterior prepyriform cortex, the medial amygdala, and certain sites of the hippocampus and septum. In particular, the sensitivity of the prepyriform cortex to amino acid imbalance has been extensively investigated by Gietzen et al. (1986) and Beverly et al. (1990a,b, 1991a). Thus the selection of a protein-free diet in preference to an imbalanced one is reversed when the limiting amino acid is directly injected into the prepyriform cortex. Beverly et al. (1991b) showed that injected dose levels are important, exerting separate effects on dietary selection and on intake of imbalanced diets. Gietzen et al. (1998) developed this concept further by demonstrating that different neural circuits mediate the initial recognition and secondary conditioned responses to amino acid imbalanced diets.

Amino acid imbalance may affect food intake and dietary selection by modulating the synthesis and metabolic disposition of neurotransmitters in the brain. In one study, feeding imbalanced diets reduced production of noradrenaline in the anterior prepyriform cortex of rats (Leung et al., 1985). However, Harrison and D'Mello (1987) showed that an imbalance induced by the addition of a mixture devoid of tyrosine and phenylalanine to a diet deficient in these two amino acids reduced food intake of chicks, without affecting noradrenaline or dopamine levels in brain homogenates. This discrepancy may have more to do with neurotransmitter synthesis and metabolism at specific sites in the brain than with any genuine differences between species or type of imbalance used in the two studies.

19.4.2.4 Effects on nutrient utilization

The effect of amino acid imbalance on nutrient utilization continues to be the subject of some debate. An imbalance might be expected to impair overall efficiency of utilization of
dietary protein. Studies with a rat model confirm this expectation, with N retention efficiency declining from 0.60 to 0.44 on addition of an imbalancing amino acid mixture to a control diet. However, further examination reveals that in rats pair-fed the control diet to match food intakes of the imbalanced group, efficiency of N retention decreased to 0.33, indicating that the effects of imbalance are mediated via reductions in appetite (Kunta et al., 1958). Despite these observations, the accepted consensus is that amino acid imbalances reduce the overall efficiency of protein utilization in other animals. Thus, Moughan (1991) attributed the low efficiency of protein utilization in pigs partly to dietary amino acid imbalance. In addition, Partridge et al. (1985) demonstrated that imbalances at the tissue level, induced by the differential absorption of amino acids from crystalline and protein-bound sources, can reduce overall efficiency of protein utilization in pigs fed once daily. Furthermore, Wang and Fuller (1989) maintained that manipulation of the composition of a mixture of amino acids to simulate the pattern in casein enhanced N retention in pigs by reducing imbalances. However, Langer and Fuller (1994) demonstrated that the addition of an imbalancing mixture containing leucine, isoleucine, and valine to a diet limiting in methionine enhanced N efficiency in growing pigs. This effect was attributed to increased metabolic availability of methionine due to inhibition of enzymes associated with methionine degradation (Langer et al., 2000). The concept of enhanced utilization of the limiting amino acid under such imbalances is not new. Thus Harper and Rogers (1965) reported that rats fed a threonine-imbalanced diet reduced oxidation of this amino acid. In subsequent studies, Yoshida et al. (1966) and Benevenga et al. (1968) demonstrated increased incorporation of the first-limiting amino acid into hepatic proteins of rats fed imbalanced diets. Thus both whole-animal and biochemical studies with rats have demonstrated enhanced utilization and retention of the limiting amino acid following feeding of amino acid imbalanced diets. Despite this evidence, other investigators continue to invoke such imbalances to explain reductions in utilization of limiting amino acids in high-protein (Abebe and Morris, 1990a,b) or imbalanced dietary regimes (Yuan et al., 2000). In the light of recent evidence, there may be some merit in reviewing the results of Abebe and Morris (1990a,b). A more plausible explanation for these observations may reside in recent reports indicating the formation of advanced glycation end-products (AGE) in high-protein dietary regimes (Uribarri and Tuttle, 2006). The synthesis of AGE via Maillard-type reactions, occurring under normal physiological conditions, would be accompanied by a depletion of metabolic pools of lysine and other amino acids, leading to the reported reductions in utilization of these amino acids. Furthermore, AGE might then induce nephrotoxicity and other physiological aberrations such as intestinal inflammation and endothelial dysfunction. Thus, reduced biochemical availability of critical amino acids and AGE-induced metabolic stress may well combine to produce the discrepant responses reported by Abebe and Morris, (1990a,b). The diverse effects of AGE are of sufficient biomedical significance to justify a full review in this volume (see Chapter 22).

19.5 Clinical Amino Acid Imbalance

Although the classification of adverse effects is firmly embedded in the nutritional literature, the term imbalance, in particular, is widely used in the context of human health. However, the concept of imbalance in human health has evolved along parallel lines with little or no overlap between studies on nutritional and clinical amino acid imbalance. There is no formal definition of the latter term, and a definitive review of this subject is therefore justified here. For the purposes of this chapter, clinical amino acid imbalance is used to represent abnormal patterns and ratios of amino acids in human subjects enduring a variety of disorders. Clinical amino acid imbalance is used here primarily to denote adverse ratios between two or more specific groups of amino acids in physiological fluids of patients. However, this term is also used to describe abnormal plasma variations in amino
acid profiles of diseased patients, relative to those of control healthy individuals. In both sets of cases, it should be noted that the literature rarely contains any reports in which an explicit attempt has been made to identify the physiologically limiting amino acids associated with these abnormalities. Furthermore, there are only limited data as to whether restoration of normal ratios might improve outcomes in patients with manifestations of clinical amino acid imbalance. This contrasts with the complete efficacy of the limiting amino acid in the dietary form of imbalance (Table 19.2).

A summary of research on clinical amino acid imbalance in human subjects is shown in Table 19.4. The table is not designed to be comprehensive, but rather illustrative of the incidence of such imbalances in diverse syndromes and diseases ranging from septic encephalopathy to cancer.

### 19.5.1 Septic encephalopathy

Basler et al. (2002) reported that within 12h of the onset of septic encephalopathy, plasma amino acids were altered, with a decrease in the ratio of branched-chain to aromatic amino acids, although no severe liver dysfunction was seen in any of the patients under observation. Nakamura et al. (2003) commented that metabolic alterations including amino acid imbalance are involved in the pathogenesis of septic encephalopathy and further showed that within 12 h of the development of the syndrome, plasma ratios of branched-chain to aromatic amino acids in septic patients with encephalopathy declined relative to ratios in septic patients without encephalopathy or those in healthy controls. It was concluded that such imbalances in patients with septic encephalopathy might serve as a marker for the severity of the septic syndrome.

<table>
<thead>
<tr>
<th>Research titles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid imbalance early in septic encephalopathy</td>
<td>Basler et al. (2002)</td>
</tr>
<tr>
<td>Effects of polymyxin B-immobilized fibre haemoperfusion on amino acid imbalance in septic encephalopathy</td>
<td>Nakamura et al. (2003)</td>
</tr>
<tr>
<td>Amino acid imbalance and hepatic encephalopathy</td>
<td>Bernardini and Fischer (1982)</td>
</tr>
<tr>
<td>Albumin dialysis has a favourable effect on amino acid profile in hepatic encephalopathy</td>
<td>Kolvusalo et al. (2008)</td>
</tr>
<tr>
<td>Albumin dialysis improves hepatic encephalopathy and decreases circulating phenolic aromatic amino acids in patients with alcoholic hepatitis and severe liver failure</td>
<td>Pares et al. (2009)</td>
</tr>
<tr>
<td>Plasma amino acid imbalance in patients with liver disease</td>
<td>Cascino et al. (1978)</td>
</tr>
<tr>
<td>Evaluating responses to nutritional therapy using the branched-chain amino acid/tyrosine ratio in patients with chronic liver disease</td>
<td>Kawamura-Yasui et al. (1999)</td>
</tr>
<tr>
<td>Measurement of serum branched-chain amino acids to tyrosine ratio level is useful in prediction of a change of serum albumin level in chronic liver disease</td>
<td>Suzuki et al. (2007)</td>
</tr>
<tr>
<td>Plasma amino acid imbalance in patients with chronic renal failure on intermittent dialysis</td>
<td>Young and Parsons (1970)</td>
</tr>
<tr>
<td>Plasma amino acid imbalance in patients with lung and breast cancer</td>
<td>Cascino et al. (1995)</td>
</tr>
<tr>
<td>Amino acid imbalance in cystinuria</td>
<td>Asatoor et al. (1974)</td>
</tr>
<tr>
<td>Imbalance of the amino acid pattern in patients with AIDS: special treatment with adapted amino acid solution</td>
<td>Althoif et al. (1989)</td>
</tr>
<tr>
<td>Huntington's disease: imbalance of free amino acids in the cerebrospinal fluid of patients and offspring at risk</td>
<td>Oepen et al. (1982)</td>
</tr>
<tr>
<td>Sporadic ulcerative mutilating acropathy with imbalance of free amino acids in the cerebrospinal fluid</td>
<td>Monaco et al. (1975)</td>
</tr>
</tbody>
</table>
In view of the foregoing, it is instructive to consider the effects of one study designed to evaluate the effects of remedial procedures. Garcia-de-Lorenzo et al. (1997) examined the clinical and metabolic responses of parenteral administration of different amounts of branched-chain amino acids in septic patients. Overall, the results indicated that formulas rich in these amino acids induced a beneficial effect in septic patients.

19.5.2 Liver disorders

According to Kolvusalo et al. (2008), hepatic encephalopathy is generally regarded to be at least partly caused by an imbalance in plasma amino acid levels. The ratio between the branched-chain and aromatic amino acids correlates with the severity of hepatic encephalopathy. The lower the ratio, the greater is the manifestation of this disorder. Pares et al. (2009) confirmed this correlation and also showed the benefits of albumin dialysis in reducing circulating levels of aromatic amino acids in patients with alcoholic hepatitis and severe liver failure. These studies reflect earlier studies showing high plasma levels of aromatic and sulphur amino acids, and low levels of branched-chain amino acids, in cirrhotic patients (Cascino et al., 1978). Kawamura-Yasui et al. (1999) suggested that the branched-chain to tyrosine ratio may serve as a useful marker for monitoring the response to nutritional therapy in patients with chronic liver disease. A similar theme appeared in the investigation of Suzuki et al. (2007) who measured serum branched-chain amino acid to tyrosine ratios in patients with chronic liver disease. They concluded that the incidence of amino acid imbalance was significantly higher in subjects with liver cirrhosis, and that the ratio of these amino acids is useful for diagnostic purposes. The expression of the relationship between the branched-chain and aromatic amino acids is sometimes referred to as the Fischer ratio (Bernardini and Fischer, 1982; Kolvusalo et al., 2008). Thus, from the foregoing accounts, it may be concluded that an abnormal Fischer ratio is a primary manifestation of amino acid imbalance in patients with liver failure.

19.5.3 Cancer and other conditions

Plasma amino acid imbalance may occur during carcinogenesis. In patients with lung cancer, a significant reduction of glucogenic amino acids (threonine, serine and glycine) and a significant increase of free tryptophan and glutamate were found in plasma. In patients with breast cancer, plasma concentrations of ornithine, glutamate and tryptophan were significantly elevated (Cascino et al., 1995). Amino acid imbalance can be demonstrated in cystinuric patients. After consumption of a free amino acid mixture, plasma increments of lysine and arginine were reduced and those of many other amino acids were significantly higher than those found in control subjects (Asatoor et al., 1974). Cerebrospinal fluid has also been used to investigate amino acid imbalance. In Huntington's disease, significant reductions were recorded for asparagine, isoleucine, leucine, phenylalanine, histidine and arginine in patients with the condition, compared to non-choreic control subjects (Oepen et al., 1982). It was further suggested that amino acid imbalance is an early metabolic disturbance in Huntington's disease. On the other hand, the levels of most amino acids in cerebrospinal fluid were increased in a case of sporadic ulcerative mutilating acropathy (Monaco et al., 1975).

19.5.4 Appetite

In laboratory models, imposition of dietary amino acid imbalance is consistently accompanied by profound reductions in food intake, as described above. Dietary choice is also markedly affected. Loss of appetite is seen in patients with hepatic encephalopathy (Takeda et al., 1993) and with sepsis (Vary and Lynch, 2004). It is pertinent to enquire whether clinical amino acid imbalance is the cause or the effect of anorexia in these and other disorders reviewed above. Furthermore, it would be of considerable academic and practical interest to ascertain whether correction of clinical imbalance by parenteral administration of formulas rich in branched-chain amino acids would enhance appetite in septic patients.
Adverse Effects

(Garcia-de-Lorenzo et al., 1997) or, indeed, in any of the hepatic disorders cited above.

19.6 Amino Acid Antagonisms

An amino acid antagonism has been defined as a deleterious nutritional interaction between structurally related amino acids. This category of adverse effects was devised to accommodate the unique and separate effects of lysine and leucine in the rat and in other animal models (D’Mello and Lewis 1970a,b,c). The effects are most pronounced when excesses of these antagonists are employed in diets that are deficient in their respective ‘target’ amino acids. The target for lysine is its structural analogue arginine, while valine and isoleucine are antagonized by leucine. Demonstrations of antagonisms have now been extended to a wide range of experimental models including avian species (D’Mello and Lewis, 1970a,b). In addition, it is now recognized that antagonisms may be precipitated by particular analogues occurring naturally in plants as non-protein amino acids. In most instances, the action of these analogues is targeted at the metabolism and utilization of specific structurally related IAA and DAA.

19.6.1 Branched-chain amino acid antagonisms

Demonstrations of the antagonisms involving the branched-chain amino acids (BCAA) in animal models have been sustained by the knowledge that food staples such as maize and sorghum grains contain disproportionate quantities of leucine. Other reports that leucine may be linked with the deficiency condition pellagra in humans has provided impetus for further research.

Following initial observations with leucine-induced antagonisms in the rat, much evidence has emerged to confirm the specificity and complexity of interactions among BCAA in other animal models, including avian species. In one study, D’Mello and Lewis (1970b) showed that excess dietary leucine permitted the growth response to the first-limiting amino acid, methionine, only in the presence of supplementary isoleucine. The specificity of the leucine–isoleucine antagonism was thus established for the first time. However, other results led D’Mello and Lewis (1970b) to conclude that the leucine–valine interaction was relatively more potent. That conclusion was based on growth and plasma amino acid levels. Circulating concentrations of valine were markedly more sensitive than isoleucine to excess leucine in the diet. Nevertheless, despite the primacy of the leucine–valine interaction, it is possible to devise dietary conditions to enhance the sensitivity of isoleucine in BCAA antagonisms (see D’Mello, 2003).

Based on studies principally with the rat, Harper et al. (1984) attributed the leucine-induced antagonism to increased oxidation of valine and isoleucine, having previously discounted any effects associated with competition for intestinal or renal transport. The catabolism of BCAA is initiated by a reversible aminotransferase reaction (Chapter 2). The branched-chain keto acids (BCKA) so formed then undergo irreversible oxidative decarboxylation to yield acyl-CoA compounds, which are degraded further in a series of reactions analogous to those involved in fatty acid oxidation. Harper et al. (1984) proposed that enhanced BCKA oxidation might account for the depletion of plasma pools of valine and isoleucine in animals fed excess leucine. Studies with preruminant lambs support this view, in that marked reductions in plasma concentrations of keto acids derived from valine and isoleucine were observed in response to excess leucine intake (Papet et al., 1988a). Furthermore, Papet et al. (1988b) showed enhanced activities of aminotransferases in the liver and jejunum in response to excess leucine, which also caused activation of BCKA dehydrogenase in the jejunum. BCAA-induced antagonisms may, additionally, deplete brain pools of other amino acids, particularly those that are the precursors of the neurotransmitters (Harrison and D’Mello, 1986). It is generally conceded that changes in brain metabolism of amino acids and their neurotransmitter derivatives may be associated with alterations in food intake and
feeding behaviour (Leung and Rogers, 1987; Gietzen et al., 1998). Consistent with this concept has been the observation that a substantial element of the adverse effects of excess leucine resides in food intake depression (Calvert et al., 1982; Papet et al., 1988a), which subjugates effects arising from oxidative catabolism of isoleucine and valine.

19.6.1 Leucine and pellagra
Excess leucine intake from maize and sorghum grains has been implicated in pellagra, a condition associated with nicotinic acid deficiency. In a general review, Dickerson and Wiryanti (1978) imply that leucine excess may increase requirements for this vitamin, although there is no suggestion of a structural antagonism in the classical sense. It is of interest, however, that isoleucine is effective in improving electroencephalograms of pellagrins with mental disturbances (Krishnaswamy and Gopalan, 1971). It is relevant to enquire here whether valine might have further enhanced the efficacy of isoleucine, judging by the complexity of interactions among the BCAA in other species (D’Mello, 2003).

19.6.2 The lysine–arginine antagonism
The deleterious effects of lysine, particularly with respect to its target, arginine, is the classical example of an amino acid antagonism. The emergence of other analogues of arginine (see below) and continuing research on nitric oxide (NO) biosynthesis have re-focused interest in the lysine–arginine antagonism. Although this antagonism has been demonstrated in rats fed casein diets (Jones et al., 1966), its most potent form occurs in avian models (D’Mello and Lewis (1970a,c) since arginine is an indispensable amino acid for this species. The unique specificity of the lysine–arginine antagonism was examined in several experiments by D’Mello and Lewis (1970a), who designed basal diets that were first-limiting in methionine, tryptophan, histidine or threonine, with arginine marginally deficient. Addition of excess lysine to each of these diets precipitated a severe growth depression which, in every case, was reversed by arginine supplementation and not by the amino acid shown to be deficient in the basal diets. Plasma arginine was also specifically and consistently depressed on addition of excess lysine to these diets. However, a number of factors can affect the severity of this antagonism. For example, excess chloride augments the adverse effects, whereas alkaline salts of monovalent mineral cations reduce or eliminate the potency of this antagonism. As will be apparent later in this chapter, another analogue of arginine, namely canavanine, enhances the potency of the lysine–arginine antagonism.

By virtue of their uricotelism, avian species are unable to synthesize arginine and are, therefore, particularly sensitive to the lysine–arginine antagonism. The most significant factor in the avian manifestation of this antagonism is the enhanced activity of kidney arginase which results in increased catabolism of arginine. If arginase activity is suppressed by the use of a specific inhibitor, then the severity of the antagonism is also attenuated. A second factor is the depression in appetite, presumably caused by lysine-induced disruption of brain uptake and metabolism of other amino acids and their neurotransmitter derivatives. Secondary mechanisms include enhanced urinary excretion of arginine and inhibition of hepatic transamidinase activity, with consequent reduction of creatine biosynthesis (D’Mello, 2003). In addition, lysine may affect nitric oxide (NO) production from arginine.

It has been suggested that the arginine to lysine ratio might influence cholesterol concentrations in plasma and liver of humans. However, recent results with rats failed to support this contention (Spielmann et al., 2008).

19.6.2.1 Hyperlysinaemia
Although not a manifestation or a result of an antagonism, hyperlysinaemia is relevant here since there is potential for adverse effects (Woody, 1964). Despite some evidence to the contrary (Jones et al., 1966), there are reliable data to show that mammals are significantly less sensitive to the adverse effects of lysine than avian species (Edmonds and Baker, 1987; Tsubuku et al., 2004). This notion is corroborated by studies on patients with hyperlysinaemia. Dancis et al. (1983) examined patients
with familial hyperlysinaemia caused by lysine-ketoglutarate reductase deficiency. No adverse physical or mental effects could be attributed to this condition. Similarly, Saudubray and Rabier (2007) reported that severe hyperlysinaemia in patients deficient in aminoacidic semialdehyde dehydrogenase was accompanied by minimal health consequences. However, biochemical studies are required to investigate any effects on transport of arginine and on NO production in these subjects. Luiking and Deutz (2007) maintain that lysine competes with arginine for transport into the cell, and it is logical to enquire whether arginine uptake and metabolism are affected in patients with hyperlysinaemia.

19.6.3 Antagonisms induced by non-protein amino acids

A wide array of amino acids, occurring naturally in unconjugated forms in plants (Table 19.5), may provoke adverse reactions in both animals and humans. These non-protein amino acids are ubiquitous in the plant, but the seed is normally the most concentrated source. Legumes contain higher concentrations and a more diverse range of non-protein amino acids than any other plant species (D’Mello, 1991). In many instances these compounds bear structural analogy with the physiologically important amino acids or their neurotransmitter derivatives. Consequently, manifestations of deleterious effects range from loss of appetite and reduced nutrient utilization to profound neurological disorders and even death (Table 19.6). Although the data are derived primarily from studies with rodent models, it is appropriate and instructive to consider the implications for human health as outlined in Table 19.7.

It will be apparent that the classification shown in Table 19.6 contains examples that are not consistent with the stringent definition of antagonisms. However, this scheme has been designed in order to integrate a wider range of the non-protein amino acids into a framework that would allow a comprehensive exposition of the diverse distribution, toxicology, and mechanisms of action of these amino acids. Furthermore, at least two of these amino acids may be regarded as potent glutamate receptor agonists and their activities are, therefore, of relevance here.

19.6.3.1 Analogues of arginine

19.6.3.1.1 Canavanine. Of the three analogues of arginine, canavanine is more ubiquitous (Table 19.5) and present in higher concentrations in leguminous seeds (D’Mello, 2003).

| Table 19.5. Distribution of non-protein amino acids in plants. (Adapted from D’Mello, 1991.) |
|---------------------------------------------------|---------------------------------------------------|
| **Category** |
| **Plant species** | **Non-protein amino acid** |
| **Legume** |
| Canavalia ensiformis, | Canavanine |
| Medicago sativa (lucerne; alfalfa) | |
| Gliricidia sepium, | |
| Dioclea megacarpa, | |
| Robinia pseudoacacia | |
| Lathyrus cicera | |
| Indigofera spicata | |
| Astragalus spp. | |
| Leucaena leucocephala (ipil ipil) | |
| Lathyrus sativus | |
| Vicia sativa | |
| Lathyrus sylvestris | |
| **Brassica** |
| Brassica oleracea (cabbage, Brussels sprouts) | |
| Brassica oleracea (broccoli) | |
| **Other** |
| Cycas circinalis (false sago palm) | |
| Blighia sapida (ackee) | |
Table 19.6. Adverse effects of some non-protein amino acids in animals. (Adapted from D’Mello, 1991.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-protein amino acid</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine analogues</td>
<td>Canavanine</td>
<td>Inhibition of growth and nitrogen retention; suppression of reduced glutathione levels</td>
</tr>
<tr>
<td></td>
<td>Homoarginine</td>
<td>Inhibition of growth and nitrogen retention; hypersensitivity; modulation of NO production; death</td>
</tr>
<tr>
<td></td>
<td>Indospicine</td>
<td>Teratogenic effects; liver damage; arginase inhibition</td>
</tr>
<tr>
<td>Analogues of sulphur amino acids</td>
<td>Selenocystine</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td></td>
<td>Selenomethionine</td>
<td>Pancreatic acinar cell necrosis; anorexia; dermatitis; hypothermia</td>
</tr>
<tr>
<td></td>
<td>S-Methylcysteine sulphoxide</td>
<td>Haemolytic anaemia; organ damage; death (in ruminants)</td>
</tr>
<tr>
<td>Aromatic</td>
<td>Mimosine</td>
<td>Inhibition of hair growth; loss of facial hair; irregular or termination of oestrous cycling; teratogenic effects; reduced collagen synthesis; uterine perforations; deformities of cranium, thorax and pelvis in fetus</td>
</tr>
<tr>
<td>Neurotoxins</td>
<td>β-N-Oxalylamino-L-alanine</td>
<td>Tremors; convulsive seizures; death</td>
</tr>
<tr>
<td></td>
<td>β-Cyanoalanine</td>
<td>Hyperactivity; tremors; convulsions; prostration; death</td>
</tr>
<tr>
<td></td>
<td>α,γ-Diaminobutyric acid</td>
<td>Hyperirritability; tremors; convulsions; death</td>
</tr>
<tr>
<td></td>
<td>β-N-Methylamino-L-alanine</td>
<td>Damage of motor neurons; excitotoxicity</td>
</tr>
<tr>
<td>Other</td>
<td>Hypoglycin A</td>
<td>Fetal malformations; inhibition of gluconeogenesis</td>
</tr>
</tbody>
</table>

Table 19.7. Plant non-protein amino acids implicated in human disorders or affecting metabolic processes. (See text for references.)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Adverse or metabolic effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canavanine</td>
<td>Reactivation of systemic lupus erythaematosus (presumption); inhibition of iNOS</td>
</tr>
<tr>
<td>Homoarginine</td>
<td>Inhibition of lysine and arginine transport</td>
</tr>
<tr>
<td>Indospicine</td>
<td>Inhibition of cNOS and iNOS</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>Cautious approach to use as a Se supplement to avoid cytotoxic effects</td>
</tr>
<tr>
<td>Mimosine</td>
<td>Hair loss; inhibition of DNA replication</td>
</tr>
<tr>
<td>β-N-Oxalylamino-L-alanine</td>
<td>Neuroathyriasis: muscular rigidity, weakness and paralysis of leg muscles (epidemiological evidence); potent glutamate receptor agonist</td>
</tr>
<tr>
<td>β-N-Methylamino-L-alanine</td>
<td>Amyotrophic lateral sclerosis and Parkinsonism-dementia in Guam (inconclusive evidence); potent glutamate receptor agonist</td>
</tr>
<tr>
<td>Hypoglycin A</td>
<td>Vomiting, convulsions, coma, death; hepatic encephalopathy; hypoglycaemia</td>
</tr>
</tbody>
</table>

Canavanine is now recognized as an inhibitor of constitutive and inducible forms of NOS (Pass et al., 1996). Canavanine contributes significantly to the toxicity of *Canavalia ensiformis* in avian species. Adverse effects may also arise after synthesis of canaline, a structural analogue of ornithine, by the action of arginase on canavanine. The mammalian metabolism of canavanine is analogous with that of arginine in the urea cycle (Fig. 19.1; D’Mello, 1991). Since this cycle is non-functional in avian species, they are unable to synthesize arginine and, consequently, readily succumb to the adverse effects of canavanine (Table 19.6). Arginine is an effective antidote and on the basis of this evidence,
D'Mello (2003) proposed the existence of a canavanine–arginine antagonism analogous to that between lysine and arginine.

Canavanine also occurs in Medicago sativa (lucerne or alfalfa) seeds, regularly recommended as a herbal supplement. In monkeys, feeding lucerne sprouts induced haematological and serological abnormalities similar to those observed in human systemic lupus erythematous (SLE). Subsequent feeding of canavanine reactivated this condition in these monkeys (see Spencer and Berman, 2003). Notwithstanding the absence of direct definitive data, it is assumed here that canavanine might precipitate similar effects in humans consuming lucerne supplements (Table 19.7).

19.6.3.1.2 HOMOARGININE. The primary source of homoarginine is the seed of Lathyrus cicer (Table 19.5). In rodent models, homoarginine has been shown to induce hypersensitivity and mortality as well as a depression in brain concentrations of lysine (O’Kane et al., 2006; Zinnanti et al., 2007), ornithine, and arginine (Table 19.6; see also D'Mello, 1991). Kakoki et al. (2006) showed that homoarginine can influence NO production in endothelial cells by altering cellular arginine transport mechanisms. Homoarginine exacerbates the canavanine–arginine interaction in avian species (D'Mello, 2003), serving to illustrate the complexity of interactions among the analogues and antagonists of arginine. Since L. cicer (Table 19.5) is not a normal constituent of
human diets, the toxicity of homoarginine is of minor relevance as regards health issues. Nevertheless, there is continuing research interest in the activities of the pure form of this amino acid as a substrate for modulation of NO production in infection and immunity (Degnan et al., 1998), in human pregnancy (Valtonen et al., 2008), and in the functional metabolism of the renal medulla (Kakoki et al., 2004). Furthermore, Henningsson and Lundquist (1998) reported that homoarginine was less potent than arginine as an insulin secretagogue but equally effective for inducing glucagon release.

19.6.3.1.3 INDOSPICINE. It is widely acknowledged that indospicine is a powerful teratogen, inducing cleavage of the secondary palate and general somatic dwarfism in rats given a single oral dose of an extract of Indigofera spicata seeds (Tables 19.5 and 19.6). Furthermore, subcutaneous injections of indospicine precipitate fat deposition and cytological abnormalities in the liver of mice. The fat accretion can be prevented by simultaneous injections of arginine but not of canavanine (see D'Mello, 1991). In experimental models, indospicine has been shown to inhibit both constitutive and inducible forms of nitric oxide synthase (NOS) (Pass et al., 1996) as well as arginase (Hey et al., 1997). As I. spicata is not a normal component of human diets, it appears that interest in indospicine primarily resides in its ability to alter the partition of arginine metabolism between urea and NO production (Hey et al., 1997).

19.6.3.2 Analogues of sulphur-containing amino acids

Arguably, the most striking structural analogues are those of methionine, cystine and cystathionine. When selenium replaces the sulphur atom of these amino acids in certain leguminous and brassica plants (Table 19.5), a number of congeners are formed. Those of primary interest here are selenomethionine, Se-methylselenocysteine, and selenocystine. Other analogues of the sulphur-containing amino acids include S-methylcysteine sulphoxide, also found in brassica plants (Table 19.5), and djenkolic acid, occurring in the djenkol bean (D'Mello, 1991).

19.6.3.2.1 SELENOAMINO ACIDS. The selenoamino acids are undoubtedly toxic to animals (Cukierski et al., 1989). For example, selenomethionine is as harmful as selenite or selenate in acute tests (Schrauzer, 2000), precipitating pancreatic acinar cell necrosis in rats (Hietaranta et al., 1990) and histopathological aberrations in liver and kidneys of juvenile white sturgeon (Tashjian et al., 2006). Nakamuro et al. (2000) maintain that disturbances in the methylation reactions of the detoxification pathway (Fig. 19.2) may precede manifestations of hepatotoxicity. Selenomethionine is readily incorporated into tissue proteins of

![Fig. 19.2. Metabolism of selenomethionine, S-methylcysteine sulphoxide (in the plant) and hypoglycin A. (See text for further details and references.)](image)
animals (Schrauzer, 2000), but the balance between synthetic and detoxification pathways is dependent upon methionine status. At sub-optimal intakes of methionine, selenomethionine is directed towards tissue protein incorporation. With adequate intakes of methionine, the Se analogue follows the pathway shown in Fig. 19.2. According to Schrauzer (2000), release of selenomethionine from body proteins by catabolic processes during illness should not result in toxicity, as a steady state is established which prevents the uncontrolled accumulation of Se.

19.6.3.2.2 S-Methylcysteine sulphoxide. The deleterious effects of S-methylcysteine sulphoxide (SMCO) in ruminant animals fed mainly or solely on brassica forage are well recognized (see D'Mello, 1991). The condition haemolytic anaemia is precipitated by the synthesis of a reactive derivative during rumen fermentation by bacteria. In the plant, SMCO co-occurs with its metabolite, methyl methane thiosulphinate (Fig. 19.2). At doses of 0.5 and 1.0 mmol kg⁻¹, this metabolite was observed to induce severe toxicity in mice (Marks et al., 1993). There is no evidence that SMCO or methyl methane thiosulphinate exerts any adverse effects in humans consuming high levels of brassica vegetables.

19.6.3.3 Mimosine

Mimosine, present in Leucaena leucocephala (Table 19.5) is widely regarded as a structural analogue of tyrosine and its neurotransmitter derivatives, dopamine and norepinephrine. The biochemical or physiological evidence for such an assumption has yet to emerge. The extensive deleterious consequences of mimosine intake appear to be unrelated to any structural properties and include disruption of reproductive processes, teratogenic effects, and loss of hair. Similar reactions may be observed on feeding the seeds or foliage of L. leucocephala to laboratory animals (Table 19.6). The leaves, pods, and seeds of this legume are widely used in Central America, Indonesia, and Thailand to prepare a soup. Anecdotal evidence (Table 19.7) indicates that loss of hair is a frequent outcome among those individuals who regularly consume this broth. As will be elaborated later, current interest in mimosine has shifted to its role as an inhibitor of DNA replication and cell cycle progression (Dong and Zhang, 2003; Perry et al., 2005).

19.6.3.4 Neurotoxic amino acids

A number of neurotoxic amino acids occur in plants of economic importance (Table 19.5). β-N-Oxalylamino-L-alanine (BOAA) is a common constituent of Lathyrus sativus, while β-cyanoalanine and α,γ-diaminobutyric acid are components of Vicia sativa and Lathyrus sylvestris, respectively. The three amino acids are often referred to collectively as the neurolathrogens. The structural homology of the lathyrergic amino acids has been emphasized by D'Mello (1991). Another neurotoxic amino acid, β-N-methylamino-L-alanine (BMAA), occurs in the seed of Cycas cirinalis.

19.6.3.4.1 β-N-Oxalylamino-L-Alanine. There is overwhelming evidence that BOAA is one of the most potent neurotoxic amino acids found in plants. Neurological and biochemical lesions in animal models include tremors, convulsive seizures, and increased brain concentrations of ammonia and glutamine (Table 19.6; see also D'Mello, 1991). The precipitation of adverse effects is influenced by age and by physiological factors such as the induction of an acidotic state, but not by animal species. Other studies confirm that BOAA is a powerful glutamate receptor agonist (Andersson et al., 1997). Intake of BOAA via the grain of L. sativus has been positively associated with the incidence of neurolathyrism in humans living in India and in parts of China. This condition (Table 19.7) is characterized by muscular rigidity, weakness and paralysis of leg muscles. Neurolathyrism affects all ages and several members of the same family may be similarly affected (Spencer and Berman, 2003).

19.6.3.4.2 β-Cyanoalanine. The neurotoxicity of β-cyanoalanine is well established (Table 19.6) following systematic work with animal models (see D'Mello, 1991). Single doses of this amino acid are sufficient to provoke severe metabolic and neurological manifestations of toxicity.
Effects include cystathioninuria, hyperactivity, tremors, convulsions, rigidity, and death. Pyridoxal hydrochloride administration delays the onset of the neurological lesions and increases the dose required to induce adverse reactions. The role of β-cyanoalanine in human neurolathyrism has been discounted as V. sativa generally only occurs as a contaminant of other legume grains.

19.6.3.4.3 α,γ-DIAMINOBUTYRIC ACID. Toxicological investigations with α,γ-diaminobutyric acid reveal increased susceptibility of mammals relative to avian species (D’Mello, 1991). Effects in the rat include upper extremity tremors, convulsions, and death. Biochemical changes may also occur, including increased ammonia concentrations in blood and brain, elevated glutamine levels in brain, and inhibition of ornithine carbamoyltransferase with reduced urea production. Human exposure to α,γ-diaminobutyric acid is limited as L. sylvestris is not a normal dietary component.

19.6.3.4.4 P-N-METHYLAMINO-L-ALANINE. Following extensive research, BMAA has been designated as a potent glutamate receptor agonist (Table 19.6; Andersson et al., 1997) and, controversially, has been implicated in a specific neurodegenerative disorder in humans (Table 19.7). In a mouse model, BMAA induced neuronal cell death in vivo (Santucci et al., 2009) while in dissociated rat brain cells, the amino acid caused an elevation in intracellular Ca levels (Brownson et al., 2002). Using dissociated mixed spinal cord cultures, Rao et al. (2006) observed that pure BMAA selectively induced motor neuron loss via AMPA/kainate receptor activation, which was replicated with the use of cycad seed extracts.

Spencer and Berman (2003) discounted any link between cycad-derived BMAA and the incidence of amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS/PDC) (Duncan et al., 1990). However, contemporary views are that BMAA may well be the causative agent. Murch et al. (2004) found the amino acid in free form in 83% of ALS/PDC patients and in protein-bound form in 100% of these individuals. Both forms were also found in two Canadians who had died of progressive neurodegenerative disease. BMAA is now thought to be more ubiquitous, being a product of all cyanobacteria which are universally distributed in terrestrial, freshwater, and marine environments (see Santucci et al., 2009). Thus, the amino acid can be biomagnified within the ecosystem and accumulate in the food chain. Furthermore, BMAA concentrations in the protein fraction of cycad seeds have hitherto been underestimated. In addition, evidence demonstrating similar efficacy of cycad seed extracts compared with the pure form of BMAA in precipitating motor neuron injury is consistent with the hypothesis that BMAA exerts a major role in the etiology of ALS/PDC (Rao et al., 2006). Nevertheless, it is still possible that BMAA and the aglycone of cycasin may act additively, synergistically or in a potentiating mode to precipitate ALS/PDC in Chamorro communities.

19.6.3.5 Hypoglycin A

The fruit of the ackee tree (Blighia sapida) is the source of hypoglycin A (Table 19.5) and its glutamyl derivative, hypoglycin B. In animal models, hypoglycin A induces fetal malformations and inhibits gluconeogenesis (Table 19.6). Research continues on the toxicology of hypoglycin A. For example, Blake et al. (2006) suggested that the form in which the amino acid is administered to rats could affect outcome in dose-response investigations, and concluded that for the purpose of risk assessment, hypoglycin A should be incorporated within the matrix of the fruit.

In the West Indies, consumption of the arils and seed of unripe fruits is associated with violent vomiting, convulsions, coma and mortality (Table 19.7; see also Spencer and Berman, 2003). Incidence of this ‘vomiting sickness’ tends to be familial, affecting undernourished children during periods of scarcity of mature fruits.
19.6.3.6 Mechanisms

There is overwhelming evidence to indicate that the adverse effects of non-protein amino acids are mediated via diverse mechanisms (Table 19.8). This polymorphism is observed individually and collectively for most of the non-protein amino acids present in plants. In addition, a number of structural analogues share common mechanisms. The following account represents an updated version of comprehensive reviews by D’Mello (1991), D’Mello (2003) and Spencer and Berman (2003).

19.6.3.6.1 Arginine Analogues. There are compelling arguments to support the hypothesis that the adverse effects of arginine

Table 19.8. Diverse mechanisms and reactions underlying the adverse effects of non-protein amino acids. Biochemical changes observed in animal models and/or humans. (See text for further details and references.)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Biochemical changes</th>
<th>Outcome/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canavanine</td>
<td>Enhanced arginase activity</td>
<td>Increased arginine catabolism</td>
</tr>
<tr>
<td></td>
<td>Reduced activity of ornithine decarboxylase following synthesis of canaline</td>
<td>Depressed polyamine synthesis</td>
</tr>
<tr>
<td></td>
<td>Competition with lysine and arginine for transport</td>
<td>Reduced cellular levels of lysine and arginine</td>
</tr>
<tr>
<td></td>
<td>Inhibition of transaminase activity</td>
<td>Suppression of creatine synthesis</td>
</tr>
<tr>
<td></td>
<td>Synthesis of aberrant proteins</td>
<td>Enhanced protein turnover†</td>
</tr>
<tr>
<td></td>
<td>Inhibition of NO synthesis</td>
<td>Impaired immunocompetence†</td>
</tr>
<tr>
<td></td>
<td>Depression of intracellular reduced glutathione</td>
<td>Effect unrelated to inhibition of NO synthesis</td>
</tr>
<tr>
<td>Homoarginine</td>
<td>Competition with lysine and arginine for transport</td>
<td>Reduced brain lysine levels; modulation of NO synthesis</td>
</tr>
<tr>
<td>Indospicine</td>
<td>Competitive inhibition of arginase</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td></td>
<td>Inhibition of aminoacylation of arginine</td>
<td>Protein synthesis impaired</td>
</tr>
<tr>
<td></td>
<td>Disruption of DNA synthesis (in vitro data)</td>
<td>Substantial reversal by increasing arginine supply</td>
</tr>
<tr>
<td></td>
<td>NOS inhibitor</td>
<td>Diverse physiological effects</td>
</tr>
<tr>
<td>Selenoamino acids</td>
<td>Replacement of sulphur amino acids in protein synthesis</td>
<td>Reduced functional properties†</td>
</tr>
<tr>
<td></td>
<td>Competition with sulphur amino acids for transport</td>
<td>Impaired intestinal absorption of sulphur amino acids</td>
</tr>
<tr>
<td></td>
<td>Suppression of selenium methylation</td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>S-Methylcysteine sulphoxide</td>
<td>Synthesis of reactive metabolites</td>
<td>Inactivation of key proteins; endocrine stimulation</td>
</tr>
<tr>
<td>Mimosine</td>
<td>Complex formation with pyridoxal phosphate</td>
<td>Cystathioninuria</td>
</tr>
<tr>
<td>β-N-Oxalylamino-L-alanine</td>
<td>Inhibition of DNA replication</td>
<td>Reduced keratin synthesis†</td>
</tr>
<tr>
<td>β-Cyanoalanine</td>
<td>Potent glutamate receptor agonist</td>
<td>Neurotoxicity</td>
</tr>
<tr>
<td>α,γ-Diaminobutyric acid</td>
<td>Complex formation with pyridoxal phosphate</td>
<td>Cystathioninuria</td>
</tr>
<tr>
<td></td>
<td>Inhibition of ornithine carbamoyltransferase</td>
<td>Liver dysfunction; brain lesions</td>
</tr>
<tr>
<td>β-N-Methylamino-L-alanine</td>
<td>Potent glutamate receptor agonist</td>
<td>Neurotoxicity</td>
</tr>
<tr>
<td>Hypoglycin A</td>
<td>Synthesis of methylene cyclopropylacetyl-coenzyme A</td>
<td>Hypoglycaemia; organic acidemia</td>
</tr>
</tbody>
</table>

*Speculative.
anallogues are mediated via diverse and complex mechanisms (Table 19.8). The major route of canavanine metabolism in mammals follows the urea cycle, where the amino acid undergoes arginase-induced hydrolysis to canaline and urea (Fig. 19.1). Canavanine administration elicits striking increases in serum and urinary concentrations of ornithine. This pathway should, therefore, provide a mechanism for at least partial detoxification of canavanine. However, canaline forms a covalent complex with pyridoxal phosphate, thereby inhibiting the activities of enzymes dependent upon this cofactor. One such enzyme is ornithine decarboxylase, and its inhibition by canaline would account for the substantial accumulation of ornithine in body fluids. Ornithine decarboxylase is a key enzyme in the synthesis of specific polyamines required for regulation of cell growth and differentiation. Inhibition of polyamine synthesis by canaline thus provides an important focal point for the action of canavanine (Bence et al., 2002). Canavanine may also act by competing with lysine and arginine for transport across cell membranes. Other studies suggest that canavanine may, like lysine, act by inhibiting transamidinase activity, thus reducing creatine synthesis. It is widely recognized that canavanine can replace arginine during protein synthesis, resulting in the formation of aberrant macromolecules with modified functional properties. Thus in rats, canavanine is incorporated into liver enzymes and into brain components, yielding proteins of reduced activity. However, other studies with rats show negligible rates of canavanine incorporation into tissue proteins. D’Mello (1991) suggested that canavanyl proteins may be degraded as rapidly as they are formed. Any cycle of synthesis and degradation of these proteins might well result in increased protein turnover rates. Arguably, the most interesting biochemical feature to emerge is the inhibition of constitutive and inducible forms of NOS by canavanine (Pass et al., 1996; Rios-Santos et al., 2007). The consequences of this effect on essential physiological processes have yet to be elucidated, but will probably impact adversely on signalling, immunocompetence, and cardiovascular function. Finally, canavanine may act in glial cells by decreasing levels of reduced glutathione, an effect not related to inhibition of NOS (Riganti et al., 2003).

Current indications are that homoarginine competes with lysine for transport across the blood–brain barrier (O’Kane et al., 2006). In the work of Zinnanti et al. (2007), for example, brain lysine concentrations were reduced by 50%. In the perfused rat kidney, it has been shown that homoarginine can modulate NO production by altering cellular arginine transport via y’ and y’ L mechanisms (Kakoki et al., 2006).

Indospicine operates via at least four mechanisms. It acts as a competitive inhibitor of arginase (Hey et al., 1997), a property which accounts for the precipitation of hepatotoxicity in mammals owing to the critical role of this enzyme in the elimination of nitrogen in the urea cycle. Furthermore, indospicine strongly inhibits aminoacylation of arginine, whereas charging of leucine remains unaffected. Incorporation of arginine into proteins is thereby impaired. Other evidence suggests that DNA synthesis is inhibited by indospicine and that this effect may be substantially reversed by increasing arginine supply. Indospicine is another inhibitor of constitutive and inducible forms of NOS, a property it shares with canavanine (Pass et al., 1996). Thus indospicine has the capacity to alter the pathway of arginine utilization depending upon the cellular status of inducible NOS (Hey et al., 1997).

19.6.3.6.2 ANALOGUES OF THE SULPHUR-CONTAINING AMINO ACIDS. Three types of reactions have been associated with the selenoamino acids (Table 19.8):

1. replacement of sulphur amino acids in protein synthesis;
2. competition with sulphur amino acids for transport; and
3. suppression of selenium methylation.

Schrauzer (2000) suggested that substitution of methionine by its Se analogue does not significantly alter protein structure, but the functional properties may be affected if the replacement occurs near an active site. The release of Se stored in body proteins during illness is not considered to result in adverse
effects, as no mechanism exists for the selective release of Se-methionine during degradative processes (Schrauzer, 2000). However, disturbances in the methylation pathway (Fig. 19.2) may lead to manifestation of selenium (Nakamuro et al., 2000).

In ruminant animals, toxicity of SMCO arises after its metabolism to a reactive metabolite (Table 19.8). In brassica vegetables, SMCO co-exists with its metabolite, methyl methane thiosulphinate (Fig. 19.2). At relatively high doses (0.5 and 1.0 mmol kg\(^{-1}\) bodyweight), this metabolite precipitated severe acute toxicity in mice (Marks et al., 1993).

SMCO itself is considered to be safe for humans; indeed, it may confer beneficial effects, as reported below.

19.6.3.6.3 MIMOSINE. The two features of mimosine commonly associated with its adverse effects are complex formation with pyridoxal phosphate, and inhibition of DNA replication (Table 19.8). Lin et al. (1996) noted that mimosine is an extremely effective inhibitor of DNA replication, and specifically indicated that it targets serine hydroxymethyltransferase, involved in the penultimate step of thymidylate biosynthesis. Perry et al. (2005) concluded that mimosine attenuates serine hydroxymethyltransferase transcription by chelating Zn, and considered the implications for inhibition of DNA replication. Other relevant studies include the effect of mimosine in cell cycle progression (Kulp and Vulliet, 1996; Dong and Zhang, 2003) and its role in the induction of apoptosis (Hallak et al., 2008).

19.6.3.6.4 NEUROTOXIC AMINO ACIDS. Of the four neurotoxic amino acids listed in Table 19.8, two (BOAA and BMAA) act as potent glutamate receptor agonists (Andersson et al., 1997). Brownson et al. (2002) suggest the involvement of a product of BMAA and CO\(_2\) which mimics the structure of other excitatory amino acids, such as glutamate, in its mechanism of toxicity. Rao et al. (2006) demonstrated the capacity of BMAA to selectively damage motor neurons via AMPA/kainate receptor activation.

In the case of \(\beta\)-cyanoalanine, complex formation with pyridoxal phosphate appears to be the major mechanism of action. With \(\alpha, \gamma\)-diaminobutyric acid, inhibition of ornithine carbamoyltransferase induces primary liver dysfunction accompanied by secondary brain lesions in mammals, while avian models are unaffected by this neurotoxin (D’Mello, 1991).

19.6.3.6.5 HYPOGLYCIN A. According to Spencer and Berman (2003), hypoglycin A metabolism (Fig. 19.2) results in inhibition of fatty acid transport, acyl-CoA dehydrogenases, and neoglucogenesis. Tanaka (2005) showed that glucose-6-phosphatase activity is also impaired. The resulting hypoglycaemia is a characteristic feature of hypoglycin A toxicity (Table 19.8).

19.6.3.6.6 UNDERLYING THEMES. A number of general rules may be formulated in the light of the evidence presented above. Urea cycle enzymes appear to be an important focal point for the action of at least three amino acids (Table 19.8). Canavanine enhances and indospicine inhibits arginase activity. In mammals, the effect of canavanine is observed after synthesis of canaline, which then precipitates the adverse effect, but the action of indospicine is more direct due to competitive inhibition of arginase, required for the proper functioning of the urea cycle. The inhibition of ornithine carbamoyltransferase by \(\alpha, \gamma\)-diaminobutyric acid is also of significance in mammals due to disruption of the urea cycle.

Another common theme relates to the modulation of NO production by canavanine, homoarginine, and indospicine (Table 19.8), while competition during cellular amino acid transport is a feature of the individual actions of canavanine, homoarginine, and the selenoamino acids.

Despite their diverse nature, a number of non-protein amino acids, including canaline, mimosine, and \(\beta\)-cyanoalanine form complexes with pyridoxal phosphate and are thus associated with inhibition of certain aminotransferases and decarboxylases. The three amino acids also inhibit cystathionase, a feature which accounts for the cystathioninuria observed in rats administered with mimosine or with \(\beta\)-cyanoalanine (Table 19.8).
19.7 Amino Acid Toxicity

In the conventional classification of adverse effects, ‘amino acid toxicity’ has always represented a somewhat heterogeneous cluster, based on nutritional assessments of the most toxic individual amino acid. Disproportionate levels have been employed with the additional aim of discerning unique effects (see Benevenga and Steele, 1984 and D'Mello, 2003). Results have remained largely within the academic domain with methionine emerging as the most toxic IAA. A defining issue for this category, however, is that there is no simple antidote for reversing the ensuing adverse effects (Table 19.1). However, in human nutrition and health, the study of amino acid toxicity is, arguably, as important as that of imbalances or antagonisms. A number of examples are relevant in this context, including glutamate, homocysteine, modified lysine residues and phenylalanine. These topics are summarized below but considered more comprehensively in specified chapters of this volume.

19.7.1 Glutamate

Glutamate provides a prime example of investigations in amino acid toxicity that impact on human nutrition and health. Interest has arisen from its use as an additive and its role as an important excitatory neurotransmitter. The disquiet over the use of monosodium glutamate (MSG) in foods continues despite the conclusions of a consensus meeting indicating that the amino acid is ‘harmless for the whole population’ (Beyreuther et al., 2007). Indeed, it was suggested that glutamate salts may improve appetite in particular circumstances where such an effect is desirable, e.g. geriatric nutrition. However, others have questioned their use on the basis that hypothalamic regulation of appetite may be damaged (Hermanussen et al., 2006). Earlier evidence linking MSG to ‘Chinese restaurant syndrome’ has been adequately reviewed by Simon and Ishiwata (2003). Nevertheless, the ‘umami’ flavour of this additive continues to be the subject of research. McCabe and Rolls (2007) presented some elegant results based on functional brain imaging, and a comprehensive review appears in Chapter 20 of this volume.

Glutamate is also the principal excitatory neurotransmitter in the mammalian central nervous system. Lipton and Rosenberg (1994) stated that in several neurodegenerative disorders, injury to neurons may be caused, at least in part, by excessive activity of receptors for excitatory amino acids. The title of their paper, ‘Excitatory amino acids as a final common pathway for neurologic disorders’ provides a succinct analysis of emerging views concerning glutamate in particular (Pitt et al., 2000; Sarchielli et al., 2003). Thus, the concept of ‘excitotoxicity’ as a significant subset in the classification of adverse effects of amino acids is now firmly established. Further consideration of the pivotal role of excitatory amino acids in neurodegenerative disorders appears in Chapter 25.

19.7.2 Homocysteine

Homocysteine is considered to be an independent risk factor for cardiovascular disease, and recently Wagner and Koury (2007) questioned whether S-adenosylhomocysteine might be a better indicator for this disorder. Although there is no generally accepted mechanism for the pathophysiology involved, Wagner and Koury (2007) proceeded to consider various factors for the ‘toxic action’ of homocysteine. Hyperhomocysteinaemia has also been associated as an independent risk factor for cognitive impairment (Kim et al., 2007). A full review of factors affecting homocysteine status and health risks is presented in Chapter 21.

19.7.3 Modified lysine residues

As stated earlier in this chapter, amino acids may participate in the post-translational modification of proteins in vivo yielding AGE. Much information is available on the carboxymethyl and carboxyethyl residues of lysine in AGE and on the implications of these
proteins in cardiovascular disease (Hartog et al., 2007), acute lung injury (Calfee, 2008), renal dysfunction in diabetic patients (Lieuw-A-Fa et al., 2004) and intestinal inflammation (Andrassy et al., 2006). In addition, high dietary protein regimes may precipitate adverse reactions by increasing the AGE burden (Uribarri and Tuttle, 2006). These and related issues are of sufficient importance to merit detailed consideration (Chapter 22).

19.7.4 Phenylalanine

Inborn errors of amino acid metabolism may be regarded as manifestations of toxic reactions. Phenylketonuria (PKU) represents the classical and most significant example of such a genetic disorder, with detrimental effects arising from accumulation of phenylalanine and other catabolic intermediates in affected subjects. Issues such as the molecular basis of PKU, maternal influence, diet and compliance, and screening are under regular review. Clinical presentation and novel strategies relevant to this condition are discussed in Chapter 23.

19.8 Potential Applications

19.8.1 Neuropsychological investigations

The imposition of dietary amino acid imbalance is now increasingly and deliberately employed as a technique to investigate psychiatric disorders in humans. For example, Stadler et al. (2007) reported the results of a study on the effects of rapid tryptophan depletion on laboratory-provoked aggression in children with attention deficit/hyperactivity disorder (ADHD). Porter et al. (2007) used a similar technique to examine the effects of tryptophan depletion in patients recovered from depression, while Norra et al. (2008) investigated effects on acoustic startle response in females. In all cases, acute tryptophan depletion was induced by the administration of a drink or a mixture rich in IAA but devoid of tryptophan. This technique thus conforms with the classical method of precipitating amino acid imbalance as described earlier (Tables 19.1 and 19.2). Peripheral plasma tryptophan levels may fall by up to 80% within 5–7 hours (Porter et al., 2007). Under these conditions, tissue and circulating levels of serotonin also decline and provide a metabolic basis for psychiatric investigations. Stadler et al. (2007) observed that there is an inverse relationship between serotonin and aggression in children with ADHD. Tryptophan depletion is reviewed in greater depth in Chapter 24. Acute tyrosine depletion has also been used in a similar context, with the aim of reducing metabolic pools of dopamine. In this instance, amino acid mixtures devoid of tyrosine and phenylalanine are provided in a drink. As before, this is another example of the imposition of an imbalance in the classical sense. In examining the effects of tyrosine depletion in normal healthy volunteers, McLean et al. (2004) considered the implications for unipolar depression, while Ellis et al. (2005) used a similar technique to investigate effects on working memory performance. The procedure and effects are short term, but it is salutary to recall that in laboratory models, imposition of an imbalance is attended by unexpected and profound alterations in diet selection (see above). If similar effects occur in humans during amino acid depletion, then it is reasonable to enquire whether the responses observed in psychiatric investigations can be isolated from other underlying aspects of behaviour associated with imbalance.

19.8.2 Therapeutic aspects

Despite the negative attributes presented in this chapter, there is increasing evidence that imbalances and non-protein amino acids may be used for clinical benefit. The therapeutic potential of amino acid imbalance is presented in Table 19.9, which represents a selection of anti-cancer studies with animal or in vitro models and one clinical investigation with septic patients. An unmistakable feature is the use of BCAA deprivation in the quest
for adjuncts to cancer therapy. It is clear that research has proceeded with the opposing objectives of restricting tumour growth but providing sufficient BCAA to replenish host tissue losses. Nishihira et al. (1988) reported that valine depletion elicited the most significant inhibition of hepatoma and mammary tumours in a rat model, and concluded that this treatment is a promising tool in cancer therapy. He et al. (2003) further showed that simultaneous omission of valine and methionine was more effective than either amino acid alone in reducing growth of tumours in rats. Sun et al. (2003) confirmed the efficacy of BCAA imbalance in selectively suppressing growth of gastric carcinoma cells in vitro. The role of amino acid imbalance in cancer therapy awaits further elucidation and evaluation in clinical trials.

Depressed BCAA to aromatic amino acid ratios appears to be a characteristic feature of septic encephalopathy and chronic liver disease (Table 19.4). It is logical to expect that BCAA supplementation might be beneficial in these conditions. The data of Garcia-de-Lorenzo et al. (1997) suggest that formulas rich in BCAA elicit ameliorative effects in septic patients.

The potential scope for specific non-protein amino acids in cancer therapy (Table 19.10) appears to be more promising than that for imbalance. Canavanine, Se-methylselenocysteine, Se-allylselenocysteine, selenomethionine, SMCO, mimosine and selected congeners have been tested in animal and in vitro models with varying degrees of efficacy. For example, NaPhuket et al. (1998) reported that several ester derivatives of canavanine were markedly more effective than the parent compound in suppressing the growth of cultured pancreatic carcinoma cells, whereas analogues based on modification of carbon chain length or of the terminal functional group were less successful. More substantive evidence is available for the selenoamino acids. Thus, Whanger (2002) concluded that Se-methylselenocysteine was most effective against mammary tumours, while Rustum et al. (2004) reported that this amino acid was highly effective in potentiating the efficiency of anti-cancer drugs and in protecting against drug-induced toxicity. Indeed, Azrak et al. (2007) suggested that the increased therapeutic efficacy of the anticancer drug, irinotecan, when in combination with methylselenocysteine, was dependent upon the dose of the amino acid. While there is good evidence of the therapeutic potential of methylselenocysteine (see also El-Bayoumy and Sinha, 2004; and Rayman, 2005), it should be recalled that in the studies of Ip et al. (1999), Se-allylselenocysteine was more effective than a number of other selenoamino acids for chemoprevention of mammary cancer in a rat methylnitrosourea model. Limited work points to the potential therapeutic use of selenomethionine (Kumar et al., 2005), but Wu et al. (2009) recommended a cautious approach to minimize cytotoxic effects of the amino acid as a supplement. Marks et al.
Table 19.10. Therapeutic potential of non-protein amino acids. (Selected studies with animal and in vitro models and supporting reviews.)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Examples of investigations/conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canavanine</td>
<td>Synthesis and structure-activity studies of some antitumor congeners of L-canavanine</td>
<td>NaPhuket et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Effects of NO donors and NO synthase substrates for possible use in myopia prevention</td>
<td>Beauregard et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>The anti-proliferative and immunotoxic effects of L-canavanine and L-canaline</td>
<td>Bence et al. (2002)</td>
</tr>
<tr>
<td>Se-Methylselenocysteine</td>
<td>Effective against mammary tumorigenesis Highly effective in potentiating efficacy of anti-cancer drugs and protecting against drug-induced toxicity</td>
<td>Whanger (2002)</td>
</tr>
<tr>
<td></td>
<td>Appraisal of evidence relating to selenocysteine and methyl selenol</td>
<td>Rustum et al. (2004) and Azrak et al. (2007)</td>
</tr>
<tr>
<td>Se-Allylselenocysteine</td>
<td>More effective than other selenoamino acids for chemoprevention of mammary cancer</td>
<td>Ip et al. (1999)</td>
</tr>
<tr>
<td>Selenomethionine</td>
<td>Prevents degeneration induced by overexpression of wild-type human α-synuclein during differentiation of neuroblastoma cells</td>
<td>Kumar et al. (2005)</td>
</tr>
<tr>
<td>S-Methylcysteine sulphoxide</td>
<td>Efficacy of S-methylcysteine sulphoxide and its metabolite, methyl methane thiosulphinate, on mouse genotoxicity</td>
<td>Wu et al. (2009)</td>
</tr>
<tr>
<td>Mimosine</td>
<td>Mimosine blocks cell cycle progression in asynchronous human breast cancer cells</td>
<td>Kulp and Vulliet (1996)</td>
</tr>
<tr>
<td></td>
<td>Mimosine attenuates serine hydroxymethyltransferase transcription</td>
<td>Perry et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>Mimosine-induced apoptosis: molecular mechanism</td>
<td>Hallak et al. (2008)</td>
</tr>
</tbody>
</table>

(1993) examined the efficacy of SMCO and its metabolite, methyl methane thiosulphinate, on mouse genotoxicity, and concluded that these two organosulphur compounds may contribute to the anti-carcinogenic properties of brassica vegetables. However, epidemiological evidence only supports the case for prevention of gastric and lung cancers through consumption of these vegetables (Kim and Park, 2009). Finally, interest in mimosine as a potential anti-cancer agent is typified by three investigations cited in Table 19.10. It is well known that mimosine blocks cell cycle progression and the report of Kulp and Vulliet (1996) with asynchronous human breast cancer cells is consistent with this particular property. The work of Dong and Zhang (2003) provides data illustrating part of the mechanism for this effect. Mimosine attenuates serine hydroxymethyltransferase transcription by chelating Zn, and Perry et al. (2005) consider the implications for inhibition of DNA replication, a well-recognized effect of this non-protein amino acid (Lin et al., 1996). The third investigation relates to mimosine-induced apoptosis. A molecular mechanism has been proposed involving oxidative stress and mitochondrial activation, with both factors exerting functional roles in the induction of cell death (Hallak et al., 2008).

19.9 Conclusions

The traditional categories of imbalance, antagonism and toxicity established in nutritional studies with experimental models constitute a satisfactory basis for classifying the
adverse effects of amino acids in human subjects. However, two subdivisions, namely ‘clinical amino acid imbalance’ and ‘excitotoxicity’, have been introduced to more accurately incorporate current interpretations of deleterious reactions observed in humans.

The concept of clinical amino acid imbalance has been advanced here to accommodate the incidence of adverse ratios of groups of amino acids in specific disease conditions. In patients with septic encephalopathy or with chronic liver disease, plasma ratios of branched-chain to aromatic amino acids are consistently reduced.

Antagonistic effects of a wide range of plant non-protein amino acids have also been reviewed. \( \beta-N \)-Oxalylamino-L-alanine occurs in the grain of \textit{L. sativus}, while \( \beta-N \)-methylamino-L-alanine is a constituent of the seed of the false sago palm. Both amino acids are potent glutamate receptor agonists implicated, respectively, in neurolathyrism and in amyotrophic lateral sclerosis and Parkinsonism-dementia. However, the aetiology of ALS/PDC remains contentious and may be associated with other underlying factors. On the other hand, there is universal agreement that hypoglycin A is the dominant agent causing vomiting sickness with hypoglycaemia in individuals consuming the unripe fruit of the ackee tree.

The issue of toxicity is currently of much greater significance in human health than it has been in nutritional investigations with animal models. A number of examples have been highlighted here, including glutamate, homocysteine, lysine adducts, and phenylalanine. The human health implications of these amino acids are considered more comprehensively in specified chapters of this volume. Interest in glutamate, in particular, has arisen due to its dual role as a food additive and as the pre-eminent excitatory amino acid implicated in neurodegenerative disorders (Chapter 25). Thus, the introduction of excitotoxicity as the second significant subdivision in the classification of adverse effects of amino acids is fully justified.

Notwithstanding the overall theme of negativity in this chapter it is, nevertheless, important not to overlook a number of potential applications for clinical research. For example, the imposition of dietary amino acid imbalance is now deliberately employed as a technique to investigate behavioural and psychiatric disorders in humans. In several studies, acute imbalance has been induced by the administration of a mixture rich in IAA but devoid of tryptophan. This technique thus conforms with the classical method of precipitating amino acid imbalance. Under these conditions, tissue and circulating levels of serotonin decline and provide a metabolic basis for psychiatric investigations. The results are considered in greater depth in Chapter 24 of this volume. The potential of amino acid imbalance in cancer therapy has also been the subject of a limited number of investigations. A consistent feature is the efficacy of BCAA-depleted amino acid mixtures in the inhibition of tumours in laboratory models. However, the potential scope for specific non-protein amino acids in cancer therapy appears to be more promising than that for imbalance. Varying degrees of efficacy have been reported with canavanine, \( Se \)-methylselenocysteine, \( Se \)-allylselenocysteine, selenomethionine, SMCO, mimosine and selected congeners and metabolites in studies involving animal and \textit{in vitro} models. Of these amino acids, \( Se \)-methylselenocysteine was most effective against mammary tumours, with the capacity of potentiating the efficacy of anti-cancer agents and protecting against drug-induced toxicity. Thus the interpretation of adverse effects of amino acids, as presented in this chapter, transcends the original conceptual model to accommodate significant food safety and clinical issues.

References


Adverse Effects


20 The Umami Taste of Glutamate

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20.1 Abstract

Umami, the savoury taste of L-glutamate, is one of the five basic taste qualities detected by humans. The umami taste receptor is a heteromeric complex of two class-C G protein-coupled receptors, T1R1 and T1R3. Breakthrough discoveries have been made in the molecular biology of the mammalian taste system since identification of the first taste receptors a decade ago. This chapter provides an overview of the molecular biology and physiology of umami taste, including the identification and characteristic of the umami receptor, the signal transduction pathway and the recent attempts to understand the neuronal representation of umami taste in the brain. Particular focus is given to umami synergy, a unique feature of umami taste.

20.2 Introduction

Protein is considered one of the basic nutrients. Amino acids serve as building blocks for proteins and can also be used as an energy source. It is essential for animals to detect and consume protein-rich food. The appetite for protein-rich food is mediated primarily by the gustatory system in humans.

Humans can detect at least five basic taste qualities, including sweet, umami, bitter, salty, and sour. Umami taste is the specific taste quality dedicated to detection of selective L-amino acids. This taste quality was discovered by Kikunae Ikeda (Ikeda, 1909), who named it ‘umami’, a Japanese word meaning ‘delicious’. The closest English words for umami are ‘savoury’ or ‘meaty’. The primary umami tastant is L-glutamate, one of the most abundant free amino acids in protein-rich food. Other umami tastants include L-aspartate and purinic ribonucleotides, such as inosine-5'-monophosphate (IMP) (Kodama, 1913) and guanosine-5'-monophosphate (GMP) (Kuninaka, 1960).

The most unique feature of umami taste is the synergy (Kuninaka, 1960) among its natural ligands: the mixture of umami-tasting amino acid with purinic ribonucleotide gives a much stronger umami taste than the sum of the either class of umami tastants alone. In fact, sub-milimolar concentrations of IMP or GMP, which elicit no umami taste on their own, can greatly potentiate the

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umami taste of glutamate or aspartate. IMP and GMP are thus considered umami taste ‘enhancers’, and probably the only known natural enhancers of any G protein-coupled receptors (GPCR).

In contrast to other four taste qualities, umami taste is a subtle sensation not so easy to describe. There has been a long debate over the validity of umami as the fifth basic taste quality. In fact, the concept was not generally accepted until the umami taste receptor was identified.

20.3 The Taste Sensory System

Taste is mediated by a group of specialized chemosensory cells known as the taste receptor cells (TRC). Clusters of 50–100 taste cells form a taste bud (Fig. 20.1), an onion-shaped assembly distributed on the surface of the tongue and soft palate. The majority of taste buds on the tongue sit on raised protrusions of the tongue surface called papillae. There are three types of taste papillae present in the human tongue:

1. Fungiform papillae: as the name suggests, these are slightly mushroom-shaped if viewed in longitudinal section. They are present mostly at the tip of the tongue, as well as at the sides, and are innervated by the chorda tympani nerve.
2. Circumvallate papillae: most people only have about 10–14 of these papillae. Located at the back of the oral part of the tongue, they are arranged in a circular row just in front of the sulcus terminalis of the tongue. They are associated with ducts of Von Ebner’s glands and are innervated by the glossopharyngeal nerve.
3. Folate papillae: these are ridges and grooves towards the posterior part of the tongue, and are found on lateral margins; they are innervated by the chorda tympani nerve (anterior papillae) and glossopharyngeal nerve (posterior papillae).

The cluster of elongated TRC project microvillae to the apical surface and form the ‘taste pore’ at the top of the taste bud. Taste receptor proteins are concentrated on the taste pore and exposed to the oral cavity. This is where the tastant molecules come into contact with the receptor proteins and taste detection is initiated. Taste stimuli activate the taste receptors, which trigger their specific signal transduction pathways and lead

Fig. 20.1. Diagram of a human tongue, highlighting the structure of a single taste bud (left), the three different types of taste papillae (middle), and their corresponding topographic distribution (right). Reproduced with permission from Chandrashekar et al. (2006).
to activation of the TRC. The signal is relayed to the brain through either the chorda tympani or the glossopharyngeal nerves.

Cells in a taste bud can be categorized into three types based on their morphology (Fig. 20.2) (Murray, 1973; Finger, 2005):

1. Type I cells, sometimes called ‘dark cells’ extend lamellate processes around other types of taste cells and express glutamate-aspartate transporter (Lawton et al., 2000). These features suggest a glial function for Type I cells, e.g. transmitter clearance and functional isolation of other taste cell types.

2. Type II taste cells have a characteristic large, round nucleus and express all of the elements of the taste transduction cascade for GPCR-mediated taste qualities (sweet, umami, and bitter) (Boughter et al., 1997; Yang et al., 2000b; Clapp et al., 2001).

3. Type III cells are characterized by morphologically identifiable synaptic contacts with the gustatory nerve fibres and expression of the synaptic membrane protein SNAP25 (Yang et al., 2000a), as well as the neural cell adhesion molecule (NCAM) (Nelson and Finger, 1993). The presence of a prominent synaptic contact implicates these cells in transmission of information to the nervous system.

The TRC can also be divided based on the taste quality they mediate. Each TRC expresses only a single type of taste receptor and can therefore only respond to the single specific taste quality, consequently being categorized as umami-, sweet-, bitter-, salty-, or sour-responding cells. There is no overlap among the five types of TRC (Chandrashekar et al., 2006, 2010). This functional segregation of TRC is believed to be important for the brain to differentiate the taste qualities, as the cells are believed to be hard-wired to the brain (the labelled-line model) (Yarmolinsky et al., 2009). The umami, sweet and bitter TRC belong to type II, while sour cells belong to type III.

### 20.4 The T1R Family of Taste Receptor

Multiple candidate receptors have been proposed for umami taste over the years. Only recently have molecular biology and mouse genetics studies demonstrated that the mammalian umami taste receptor is a heteromeric complex of T1R1/T1R3. There are three genes in the T1R family. T1R1 and T1R2 were identified in 1999 by sequencing a subtracted cDNA library derived from rat taste tissue (Hoon et al., 1999), and T1R3 was identified in 2001 by sequencing a subtracted cDNA library derived from rat taste tissue (Hoon et al., 1999), and T1R3 was identified in 2001 (Bachmanov et al., 2001; Kitagawa et al., 2001; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2001; Sainz et al., 2001).

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**Fig. 20.2.** Schematic diagram showing the major features of the different taste cell types. Reproduced with permission from Finger (2005).
In situ hybridization revealed selective expression of T1R in mouse taste tissue, with T1R1 more enriched in the fungiform taste buds and T1R2 in the circumvallate taste buds. T1R3 was also found to be selectively expressed in a subset of TRC. Although T1R1 and T1R2 are expressed in different cells, they are each coexpressed with T1R3 (Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2001). Besides the T1R1/T1R3 and T1R2/T1R3 cells, a fraction of TRC expresses T1R3 only.

An important clue about the function of T1R came from the genetic locus of T1R3. Importantly, mouse T1R3 was mapped to a genomic interval containing Sac (Fuller, 1974), a locus that influences sweet-taste sensitivity in mice. Different inbred strains of mice are known to have different sweet-taste sensitivities. The difference was found to be dependent solely on the Sac locus. The dominant Sac allele (taster) is associated with higher taste sensitivity than the recessive allele (nontaster). To prove that the Sac locus does encode T1R3, transgenic mice were generated to introduce the T1R3 gene from a taster into a nontaster strain. As a result, the taste deficiency of nontaster mice was fully rescued, indicating that T1R3 was indeed the Sac gene (Nelson et al., 2001).

The T1R belong to class-C GPCR (Fig. 20.3). Other renowned members of this class of GPCR include the metabotropic glutamate receptors (mGluR), γ-aminobutyric acid receptor B (GABA<sub>B</sub>R), and calcium-sensing receptor (CaSR). The defining motif of this class of GPCR is the extracellular Venus flytrap (VFT) domain, which is their ligand-binding domain. The VFT domain is so named because of its structural resemblance to the leaves of the Venus flytrap plant, a carnivorous plant that catches animal prey. The crystal structures of mGluR VFT domains have been solved (Kunishima et al., 2000; Tsuchiya et al., 2002; Muto et al., 2007). The domain is composed of two globular subdomains connected by a threestranded flexible hinge. The bi-lobed architecture can form an ‘open’ or ‘closed’ conformation (Fig. 20.4). The closed conformation of the VFT domain is stabilized by glutamate, analogous to the closure of the Venus flytrap leaves with a trapped prey.

### 20.5 Functional Expression of T1R

Functional assays were developed for T1R in mammalian cell lines (Li et al., 2002; Nelson et al., 2001, 2002). According to their in vivo expression pattern, T1R2 and T1R1 were each
coexpressed with T1R3 in a HEK cell line that expresses Ga15, a promiscuous G protein. Binding of T1R agonists would activate Ga15, which leads to release of Ca\(^{2+}\) from intracellular stores. The elevated Ca\(^{2+}\) concentration can be monitored with a calcium-sensitive fluorescent dye. The cells were stimulated with different taste stimuli and the T1R receptor activities were monitored. The heteromeric human T1R2/T1R3 receptor selectively responded to all of the ~20 known sweeteners tested at physiologically relevant concentrations, and the responses were inhibited by lactisole (Li et al., 2002), a human sweet-taste inhibitor. Similarly, rat T1R2/T1R3 also responded to all of the dozen molecules that generate similar behavioural responses to sucrose (Li et al., 2002).

In contrast, human T1R1/T1R3 receptor selectively responded to L-glutamate (Li et al., 2002). The activities of human T1R1/T1R3 in the functional assay correlated well with umami taste (Fig. 20.5). The heteromeric receptor recognized glutamate with an EC\(_{50}\) closely matching the umami detection threshold. More importantly, the hallmark umami synergy is reconstituted in the assay: IMP or GMP can strongly potentiate the response of the receptor to glutamate or aspartate. The human receptor was highly selective for umami stimuli (Li et al., 2002), responding only to glutamate, aspartate, and L-AP4. Interestingly, mouse T1R1/T1R3 was found to be far more promiscuous (Nelson et al.,

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**Fig. 20.4.** Stereoview of mGluR1 VFT domain in a closed conformation. Reproduced with permission from Kanishima et al. (2000).

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**Fig. 20.5.** Human T1R1/T1R3 activity correlated with umami taste. (a) Calcium imaging of T1R1/T1R3-expressing cells in response to glutamate with or without IMP. (b) T1R1/T1R3 dose response curves in the presence and absence of 0.2 mM IMP. The X axis circles represent average psychophysical detection threshold values for L-glutamate in the presence and absence of 0.2 mM IMP. Reproduced with permission from Li et al. (2002).
2002), responding to virtually all L-amino acids in heterologous cells.

The same functional assays for human T1R1/T1R3 and T1R2/T1R3 were adopted for high-throughput screening of synthetic compound libraries. Multiple novel chemical classes of umami-tasting compounds, sweeteners, and sweet-taste enhancers have been identified through this effort (Zhang et al., 2008, 2010; Servant et al., 2010). These results validated the functional assay data. The human umami and sweet taste are mediated by related heteromeric receptors sharing one common subunit (Fig. 20.6).

20.6 T1R Knockout Mice

Behavioural and physiological studies using knockout mice demonstrated that T1R were required for rodent sweet and umami taste. Knockout mice were generated for each T1R gene (Zhao et al., 2003), and analysed using brief access taste tests and chorda tympani nerve recording. As expected, T1R1 null mice exhibited complete loss in preference for umami tastants; T1R2 null mice exhibited complete loss in preference for artificial sweeteners and greatly diminished preference for sugars; and T1R3 null mice lost preferences for both umami stimuli and artificial sweeteners completely, displaying greatly diminished responses to sugars. The CT nerve responses were consistent with the behavioural data.

Independently, a T1R3 knockout mouse model was generated and analysed using the two-bottle preference test (Damak et al., 2003). The results were somewhat different: the preference for monosodium glutamate (MSG) in T1R3-null mice was reduced but not abolished. The animals showed no preference for artificial sweeteners, and a diminished preference for sucrose, but essentially the same preferences for glucose and maltose as the wild-type mice.

The discrepancies between the behavioural results from these two reports could be due to the different taste test protocols. The brief access taste tests were carried out within 30 min and stimuli were presented in 5-s trials, while in two-bottle tests, the animals were exposed to the tastants for 48 h. It is now known that post-ingestive effects can greatly distort the outcome of two-bottle preference tests. Genetically engineered mice lacking the cellular machinery required for sweet-taste transduction can still develop a robust preference for sucrose solutions based solely on caloric content (de Araujo et al., 2006). The post-ingestive effect of MSG is also documented in the literature (Bachmanov et al., 2000; Zhao et al., 2003). Therefore, data from the brief access tests are more relevant to taste physiology.

20.7 The Molecular Mechanism of Umami Synergy

The unique feature of synergy in umami taste is scientifically intriguing. The molecular mechanism for the synergy has been revealed recently using a combination of chimeric receptors, mutagenesis and molecular modeling approaches (Zhang et al., 2008). The first step of this study was to discover the umami ligand binding sites on the receptor. Since the umami and sweet-taste receptors recognize different taste stimuli, the primary ligand binding sites should reside on the unique subunits T1R1 and T1R2, not the shared

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Fig. 20.6. Schematic drawing of umami and sweet receptors. T1R1 is used as an example to illustrate the overall domain structure of this class of GPCR. CRD, cys-rich domain; TMD, transmembrane domain; VFT, Venus flytrap domain.
subunit T1R3. A chimeric receptor (Fig. 20.7) with the T1R1 N-terminal domain and the T1R2 transmembrane domain (T1R1-2) displayed essentially the same ligand specificity as the umami taste receptor, and the activity was enhanced by IMP. Conversely, chimera with T1R2 N-terminal domain and T1R1 transmembrane domain (T1R2-1) displayed the same ligand specificity as the sweet-taste receptor. These data indicate that the T1R1 N-terminal domain is critical for binding IMP as well as glutamate.

Mutagenesis analysis further defined the binding site of glutamate and IMP to the VFT domain of T1R1. In the VFT domain of T1R1, 38 residues were mutated individually, and the mutants were tested for their response to glutamate and IMP. Among the 38 residues, four were found to be essential for glutamate recognition, and another set of four residues was found to be critical for IMP activity, suggesting that glutamate and IMP occupy different parts of the space within the VFT domain.

Molecular modelling of T1R1 VFT based on the crystal structure of metabotropic glutamate receptors revealed the relative positions of the eight critical residues. The four residues important for glutamate binding are located near the hinge region, while the four for IMP are located near the opening or ‘lips’ of the bi-lobed structure. It is known that the negatively charged phosphate group of IMP is important for its umami-enhancement activity. A cluster of positively charged residues was found near the lips of the T1R1 VFT. In fact, three of the four residues important for IMP activity are positively charged, and they could interact with the phosphate group of IMP through a salt bridge. A molecular model was constructed (Fig. 20.8), where both glutamate and IMP were positioned in the cleft of the T1R1 VFT domain. A mechanism for the synergy is proposed: glutamate binds close to the hinge region of the VFT domain, and induces the closure of the lobes and activates the receptor. IMP binds close to the opening of the VFT domain and coordinates the positively charged residues from both sides of the bi-lobed structure, thereby stabilizing the closed conformation and enhancing the activity of the receptor.

GPCR modulators can be categorized as orthosteric or allosteric, depending on their binding sites. The orthosteric modulators work on the same binding site as the natural ligand of the receptor, while the allosteric modulators work on different sites. In recent years, researchers have become more and more interested in allosteric modulators of GPCR as novel therapeutic agents. In many cases, allosteric modulators have advantages over orthosteric ones. For instance, the family of mGluR shares the same ligand and highly conserved ligand binding domain, making it...
very difficult to develop a selective orthosteric modulator for an individual receptor. Allosteric modulators, on the other hand, can target the less conserved portion of the receptors and achieve subtype selectivity. Many synthetic allosteric modulators for class-C GPCR have been developed over the years (Brauner-Osborne et al., 2007); however, all of them target the transmembrane domains. IMP is a naturally occurring allosteric modulator of the umami taste receptor, as it occupies a different part of the VFT binding pocket from glutamate. The cooperative binding of glutamate and IMP to the T1R1 VFT domain represents a novel mechanism, and could stimulate new ideas to allow the development of allosteric modulators of other class-C GPCR.

This mechanism of enhancement also applies to the sweet-taste receptor. Recently, synthetic enhancers have been identified for the human sweet-taste receptor through high-throughput screening (Servant et al., 2010). Similar to the umami enhancers, the sweet-taste enhancers elicit no sweet taste on their own, but can strongly potentiate the sweet taste of selective sweeteners in human taste test. A molecular mechanism of these molecules was proposed (Zhang et al., 2010) based on data from the same chimeric receptor, mutagenesis, and molecular modelling approaches, and turned out to be very similar to that of umami synergy. Using the sucralose enhancer SE-2 as an example (Fig. 20.9), sucralose binds deep inside the VFT domain of T1R2 and induces the closure of the two lobes by interacting with the hinge region, while the enhancer molecule binds near the lips of the Venus flytrap and stabilizes the closed conformation. SE-2 is not a charged molecule like IMP. Based on the model, it interacts with the T1R2 VFT domain mostly through hydrogen bonding and hydrophobic interactions.

Besides the VFT domain, other ligand interaction sites (Fig. 20.10) have also been identified on both umami and sweet-taste receptors in studies using similar chimeric

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**Fig. 20.8.** A molecular model for the umami synergy. Glutamate and a potent analogue of IMP molecules are fitted inside the binding pocket of T1R1 VFT domain. LB1 is the upper lobe of T1R1 VFT domain and LB2 the lower lobe. The cartoon of a clam shell in the upper right corner shows the overall orientation of the VFT domain.

**Fig. 20.9.** A molecular model of the T1R2 VFT domain in closed conformation with bound sucralose and its enhancer molecule SE-2. Reproduced with permission from Zhang et al. (2010).
receptor and mutagenesis analysis (Jiang et al., 2004, 2005a,b; Xu et al., 2004; Winnig et al., 2007; Zhang et al., 2008, 2010).

### 20.8 Umami Signal Transduction

A number of different signalling pathways have been proposed over the years for umami, sweet, and bitter taste (Gilbertson et al., 2000). It became clear only recently that the three groups of taste receptors share the same signal transduction cascade (Chandrashekar et al., 2006). Studies using mouse knockout models proved that gustducin (Wong et al., 1996), phospholipase Cβ2 (PLC-β2) (Zhang et al., 2003), inositol 1,4,5-trisphosphate receptor type 3 (IP3R3) (Hisatsune et al., 2007), and a transient receptor potential protein (TRPM5) (Zhang et al., 2003; Damak et al., 2006) are required for the pathway. Knockout of each component results in severe electrophysiological and behavioural defects in umami, sweet, and bitter taste.

The transduction cascade (Fig. 20.11) emerges after identification of the critical components: activation of umami receptor triggers conformation changes in the heterotrimeric G protein gustducin, leading to release of the Gβγ subunits (Huang et al., 1999; Zhang et al., 2003), which in turn activate PLC-β2. Two messenger molecules are generated by PLC-β2: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 binds to IP3R3 and leads to Ca²⁺ release from intracellular stores. The elevated Ca²⁺ level results in gating of the TRPM5 channel and depolarization of the taste-receptor cell. The depolarized TRC release neurotransmitter and the signal is transmitted to the chorda tympani or glosopharyngeal nerves.

Adenosine 5'-triphosphate (ATP) is believed to be the key neurotransmitter linking taste buds to the taste nerves (Finger et al., 2005). Stimulation of taste buds in vitro evokes release of ATP. The ionotropic purinergic receptors P2X₂ and P2X₅, which respond to ATP, are present on the taste nerve fibres (Bo et al., 1999; Finger et al., 2005). Knockout of the two receptors specifically eliminates the taste responses without affecting other responses to touch or temperature in the nerves (Finger et al., 2005; Eddy et al., 2009).

The umami, sweet and bitter TRC all belong to type II, which do not form morphologically identifiable synaptic contact with the taste nerves. It is not clear how neurotransmissions occur between type II cells and the nerves. One hypothesis is that type II cells use type III
cells as intermediates for neurotransmission (Roper, 2006). However, data from transneuronal tracing experiments (Damak et al., 2008; Ohmoto et al., 2008) argue against the intermediate cell hypothesis. Transgenic mouse lines were engineered to express the transneuronal tracer wheat germ agglutinin (WGA), in sweet and umami TRC under the control of the mouse T1R3 gene promoter. WGA protein was transferred not laterally to other TRC, but directly to a subset of neurons in the geniculate and nodose/petrosal ganglia, and further conveyed to a subpopulation of neurons in the rostrocentral region of the nucleus of the solitary tract. Therefore, direct neurotransmissions exist between type II TRC and the taste nerves. Recently, it was proposed that ATP was released from type II taste cells via a non-exocytotic mechanism, most likely through the connexin or pannexin hemichannels (Fig. 20.11) (Huang et al., 2007; Romanov et al., 2007).

### 20.9 Functional Neuroimaging of Umami Taste

Umami tastants increase the palatability of a variety of foods. However, umami alone is
not a pleasant taste. In fact, many find the taste of glutamate solution very unpleasant. It is clear that the pleasant taste perception associated with umami results from the integration of multiple sensory cues in the central nervous system. Recently, the mechanisms for the pleasantness associated with umami taste were investigated in humans using functional magnetic resonance imaging (fMRI) (McCabe and Rolls, 2007).

This study was based on findings from neuronal recordings in macaque monkeys. In primates, taste signals generated at the taste buds are transmitted to the primary taste cortex through relays at the nucleus of the solitary tract and taste thalamus (Rolls, 2009). A region of secondary taste cortex is found in the primate orbitofrontal cortex. More importantly, neurons in the orbitofrontal taste cortex appear to be related to food reward, as their activities are potentiated by hunger and inhibited by satiety (Rolls et al., 1989; Critchley and Rolls, 1996).

Neurons that are best tuned to umami were identified in the primary taste cortex as well as the orbitofrontal cortex of macaques (Baylis and Rolls, 1991; Rolls et al., 1996). These neurons responded to gustatory stimuli by glutamate or IMP. The neuronal representation of umami taste is separate from those of other taste qualities (sweet, sour, salty and bitter). Interestingly, the responses of some of these neurons decreased when the monkeys were fed to satiety with monosodium glutamate solution (Rolls et al., 1996). Based on this inverse correlation between the sensory-specific neuronal activity and satiety, it was proposed that the pleasantness of umami is represented in the orbitofrontal cortex.

Human studies were carried out to address the fundamental questions of what makes umami pleasant and how the pleasantness correlates with the neuronal activities. fMRI revealed umami-responding neurons (de Araujo et al., 2003) in the insular-opercular taste cortex, the putative human primary taste cortex, and the orbitofrontal cortex. The combination of glutamate and a consonant vegetable odour was found to result in a much more pleasurable response (McCabe and Rolls, 2007). More importantly, the combination of umami taste and the olfactory stimulus elicited synergistic effect on the activity of certain brain regions, i.e., the activity induced by the combination is significantly larger than the sum of either stimulus alone. This supralinear additivity was observed in the medial orbitofrontal cortex, and in a part of the ventral striatum which receives inputs from the orbitofrontal cortex. Activation of these brain regions correlated with the pleasantness (McCabe and Rolls, 2007). In contrast, the supralinear additivity was significantly less between salty taste and the vegetable odour.

There are likely to be many other ways to make umami taste pleasant. Sometimes it is just as simple as changing the label of the taste sample. The same sample was rated significantly more pleasant when labelled ‘rich and delicious taste’ than ‘MSG’ (Grabenhorst et al., 2008), while the umami intensity rating was not significantly changed. Interestingly, this cognitive modulation also correlated with the neuronal activities in the medial orbitofrontal cortex: samples with the ‘rich and delicious taste’ label elicited much higher response in this brain region.

20.10 Conclusions

The last decade has witnessed many breakthrough discoveries in taste biology. Identification of the taste receptors led to revolutions in our understanding of the taste mechanism. Many important questions are being addressed by different research groups, including the mechanisms of signal transduction between TRC to the taste nerves, and the brain representation of the different taste qualities. As the labelled-line model becomes generally accepted, an interesting question arises: how is the specific wiring between the taste nerves and the TRC maintained, given the rapid turnover of TRC? Many other interesting questions still remain: is there any other more subtle taste quality besides the basic five, such as a fat taste? How do experience and cognition modulate people’s taste sensations and food preferences?

The presence of taste receptors is not limited to taste buds. Specialized chemosensory cells expressing taste receptors and
their downstream signalling elements have been found in many tissues, including the gastrointestinal tract (Wu et al., 2002; Kaske et al., 2007) and the respiratory tree (Finger et al., 2003; Kaske et al., 2007; Merigo et al., 2007; Lin et al., 2008; Sbarbati et al., 2009; Shah et al., 2009). Important functions of these chemosensory cells are being revealed (Kokrashvili et al., 2009; Tizzano et al., 2010).

References

The Umami Taste of Glutamate


Part IV

Health
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21 Homocysteine Status: Factors Affecting and Health Risks

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21.1 Abstract

A significant finding in recent times is the classification of high homocysteine levels as a potential risk factor in a number of chronic vascular conditions, particularly cardiovascular disease – though neural tube defects and decreased cognitive performance have also been demonstrated. Several factors influence the level of homocysteine concentrations, including age, gender, smoking and diet. Interactions with folate and vitamin B_{12} may also be important in the risks imposed by high levels of homocysteine. Genetic factors also influence homocysteine metabolism, and therefore risk of vascular disease. Observational studies, both prospective and case-control, have shown a moderate risk between homocysteine and coronary heart disease, stroke and venous thromboembolism. The majority of randomized clinical trials testing the effectiveness of vitamin B on reducing homocysteine levels, and putatively decreasing the risk of vascular disease, are null. The epidemiological evidence is reviewed in this chapter.

21.2 Introduction and Objectives

Homocysteine is a sulphur-containing amino acid first identified for its role in methionine metabolism in the 1930s by DuVigneaud (1952) as well as others. But only recently have elevated homocysteine levels been associated with putative deleterious consequences, including cases of mental retardation, fatty liver, vascular lesions, thrombosis (McCully, 2005) and arterial stiffness (Nestel et al., 2003).

In 1969, McCully identified the index case of methionine synthase deficiency associated with arteriosclerosis, which led to the theory of homocysteine-related arteriosclerosis. Since then, the influence of homocysteine on risk of cardiovascular disease, stroke, venous thromboembolism and cognitive function has been examined in observational studies and randomized clinical trials. Neural tube defects have also been associated with elevated homocysteine concentrations along with reduced levels of folate, vitamin B_{12} and/or vitamin B_{12}. The objective for this chapter is to review the epidemiology of homocysteine
and its relation to chronic disease. Specifically, the topics reviewed include:

1. the metabolism of homocysteine;
2. the distribution of homocysteine in the US population;
3. the determinants of total serum homocysteine concentrations;
4. homocysteine as a risk factor for selected chronic diseases and conditions;
5. randomized clinical trials (RCT) testing the effectiveness of vitamin B supplementation to reduce the risk of chronic disease, including coronary heart disease, stroke and venous thromboembolism through the putative reduction in elevated homocysteine levels; and
6. a discussion of differences in study designs and methods that may influence results.

21.3 The Metabolism of Homocysteine

Homocysteine is a sulphur-containing amino acid formed during the metabolism of methionine (Chapter 10) and is either metabolized into cysteine through transsulphuration or into methionine through methylation, depending on methionine levels. When excess methionine is present, homocysteine is conjugated to cysteine by the enzymatic action of cystathionine β-synthase, requiring vitamin B₆ as a cofactor. When methionine levels are low, homocysteine is methylated by the enzyme methionine synthase that requires vitamin B₁₂ as a cofactor, methyltetrahydrofolate as a co-substrate, adequate folate intake and the enzyme methylene tetrahydrofolate reductase (MTHFR) to form methionine (Selhub, 1999). Accumulation of homocysteine may occur through defects in either transsulphuration or remethylation (Herrmann, 2001).

21.4 Distribution of Homocysteine Concentrations in the US Population

Serum total homocysteine concentrations were measured in adolescents and adults (ages 12 to more than 80 years) attending the Third National and Health and Nutrition Examination Survey (NHANES III), phase 2. Generally, serum total homocysteine levels increased with age and were higher among males than females (Jacques et al., 1999a). Other investigators observed that higher oestrogen levels were associated with lower homocysteine levels, which may explain the male–female difference in levels (Morris et al., 2000). Mexican-American females across all ages had lower levels than non-Hispanic white or African-American females (7.4 μmol l⁻¹ versus 7.9 μmol l⁻¹ versus 8.2 μmol l⁻¹, respectively), whereas there were no race/ethnicity differences reported in men (ranging from 9.4 to 9.8 μmol l⁻¹) (Jacques et al., 1999a). Average serum homocysteine concentrations ranged from 6.1 μmol l⁻¹ in those 12–15 years to 15.0 μmol l⁻¹ in adults aged 80+ years. Plasma homocysteine ranged from 4.54 μmol l⁻¹ in children less than 4 years to 6.89 for those aged 16–18 years (Ganji and Kafai, 2005). Clinically, normal values of homocysteine range from 5 μmol l⁻¹ to less than 16 μmol l⁻¹, mild to moderately elevated levels range from 16 to 100 μmol l⁻¹, and severely elevated levels are above 100 μmol l⁻¹ (Eikelboom et al., 1999).

21.5 The Determinants of Serum Total Homocysteine Concentrations

Serum homocysteine levels are influenced by genetics and environmental factors, including demographic characteristics, diet, smoking, chronic medical conditions and medication use.

21.5.1 Demographic characteristics

As previously discussed, total serum homocysteine concentrations have been shown to increase with age, in the male sex, and with menopause in women (Jacques et al., 1999a; Morris et al., 2000).

21.5.2 Diet

An important environmental factor influencing homocysteine levels is dietary intake of nutrients required for homocysteine
Homocysteine metabolism, including folate (folic acid), vitamin B₆ (pyridoxine), and vitamin B₁₂ (cyanocobalamin) (Malinow et al., 1999). Observational and clinical studies have demonstrated inverse associations of plasma or dietary folate and vitamins B₆ and B₁₂ with total serum homocysteine concentrations (Homocysteine Lowering Trialists’ Collaboration, 1998). Unsurprisingly, the majority of elderly adults and patients with elevated homocysteine levels are deficient in folate, vitamin B₆ and/or vitamin B₁₂ (Selhub et al., 1993).

In a randomized, double-blind trial, 189 middle-aged and elderly, relatively healthy adults who did not consume dietary supplements were randomly assigned to a treatment group (daily consumption of one cup breakfast cereal fortified with 440 μg folic acid, 1.8 mg vitamin B₆, and 4.8 μg vitamin B₁₂) or a control group (no fortified cereal) for 12 weeks. Compared to the control group, the treatment group showed an increase in plasma levels of B vitamins – that is, plasma folate and vitamins B₆ and B₁₂ – and a concomitant decrease in homocysteine levels overall, representing a 4.8% decrease in the prevalence of hyperhomocysteinaemia (Tucker et al., 2004).

Apart from folic acid, vitamin B deficiency has also been associated with elevated homocysteine concentrations. For example, vegetarian diets are low in vitamin B₁₂, and hyperhomocysteinaemia has frequently been observed in vegetarians (Mezzano, 2000). Supplementation with vitamin B₁₂ corrects the hyperhomocysteinaemia. Whether this supplementation further improves the already reduced cardiovascular morbidity and mortality associated with a vegetarian diet has yet to be demonstrated. Expectedly, individuals consuming greater quantities of animal protein, i.e. red and processed meat and poultry, have higher levels of homocysteine (Gao et al., 2003).

In 1998, the US Food and Drug Administration mandated folate fortification of grain products in the United States to reduce the incidence of neural tube defects, a common birth defect in newborn infants. The effect of folate fortification on homocysteine levels was determined using pre- and post-fortification blood samples of adults enrolled in the Framingham Heart Study who did not consume vitamin supplements. The prevalence of mild hyperhomocysteinaemia was reduced by about 50% as a result of fortification (Jacques et al., 1999b). Similarly, in a study conducted among 2695 adolescents 5 years after the start of folate fortification, lower serum homocysteine levels were observed among adolescents consuming greater intakes of whole and refined grains and dairy products (Lutsey et al., 2006). These studies provide evidence that homocysteine levels are lowered by supplementation of folate, vitamin B₆ and B₁₂.

21.5.3 Smoking

In a population-based surveillance study of adults aged 40–67 years, current smokers had higher plasma homocysteine levels than non-smokers, with a dose response between the number of cigarettes smoked per day and level of homocysteine (Nygard et al., 1995). There was no difference in homocysteine levels between former smokers and non-smokers. Though the mechanism accounting for increased homocysteine levels in smokers remains unclear, a case control study of individuals less than 60 years old demonstrated that current smokers had lower levels of plasma folate, vitamin B₆ and vitamin B₁₂ than non-smokers (O’Callaghan et al., 2002).

21.5.4 Medical conditions and medication use

Chronic disease may also alter homocysteine levels. Total serum homocysteine was found to be positively correlated with glomerular filtration rate, and higher levels have been observed in patients with kidney disease (van Guldener, 2006). Furthermore, elevated levels of homocysteine have been seen frequently in patients with coronary heart disease, stroke, venous thromboembolism, or other vascular disease (Makris, 2000), as well as in individuals with high blood pressure (Nygard et al., 1995), and retinal artery and vein occlusions (Wright et al., 2008).
Anticoagulated patients may be at risk of hyperhomocysteinaemia, since patients receiving oral anticoagulant therapy are normally asked to restrict vitamin K-rich foods, including green vegetables that are good sources of folate. Homocysteine levels significantly increased while folate levels decreased in individuals on anti-coagulation therapy with restricted vitamin K intake, thus raising the risk of thrombosis (Murua et al., 2001). Apart from anti-coagulation therapies, other medications including antiepileptic drugs, methotrexate, fibric acid derivatives, and metformin have been hypothesized to influence the absorption of vitamin B or interfere with the pathways involved in the metabolism of homocysteine, resulting in elevated homocysteine levels (Desouza et al., 2002).

21.5.5 Genetic factors

Some individuals are genetically predisposed to elevated homocysteine levels. A common genetic variant in methylenetetrahydrofolate reductase (MTHFR), an enzyme that regulates the conversion of homocysteine to methionine, is the 677C → T polymorphism, which has been shown to result in increased levels of homocysteine. Notably, the 677 C → T polymorphism is involved in two major functions of one-carbon metabolism, DNA synthesis and DNA methylation, which play key roles as cofactor substrates in one carbon metabolism. Additional genetic mutations may also influence the metabolism of homocysteine and folate, including defects in vitamin B₁₂ metabolism or homozygous deficiency of folate, vitamin B₁₂ cystathionine β-synthase, or methionine synthase (Eikelboom et al., 1999). Current treatment for these mutations/conditions is ensuring adequate folate and vitamin B intakes.

21.6 Homocysteinaemia is a Risk Factor

21.6.1 Coronary heart disease, stroke and venous thromboembolism

It is well known that homocysteinuria, an inherited disorder of methionine metabolism, increases the risk for venous and arterial thrombosis. Individual observational studies have shown that moderately elevated homocysteine levels increase the risk of coronary heart disease (CHD), stroke, venous thromboembolism (VTE), and peripheral arterial disease, though reports are inconsistent. To increase the power and precision of these studies, meta-analyses of observational studies have been conducted to determine the predictive value of elevated homocysteine levels for incident CHD, stroke, and VTE. A recent meta-analysis of 18 nested case-control and 8 prospective observational studies showed a positive association between homocysteine and incident CHD (Humphrey et al., 2008). More specifically, the relative risk (RR) for incident CHD associated with each 5 μmol l⁻¹ increase in homocysteine was 1.18 (95% C.I. 1.10–1.26). The RR was stronger for studies with follow-up less than 5 years (RR 1.39; 95% C.I. 1.20–1.62), while the RR for studies with follow-up time of 10 or more years was 1.13 (95% C.I. 1.00–1.28). Importantly, statistical models were adjusted for traditional risk factors for CHD, including age, gender, smoking, physical activity, blood pressure, and renal dysfunction. The major strength of this study was the inclusion of data from population-based prospective studies of incident CHD events. Other meta-analyses that included persons with known CVD reported similar findings (Boushey et al., 1995; Homocysteine Studies Collaboration, 2002; Wald et al., 2002). One such meta-analysis by Wald et al. (2002) included 72 observational studies that examined the prevalence of MTHFR genetic mutation in 16,849 cases and controls and 20 prospective studies (n = 3820) that examined homocysteine and risk of heart disease, stroke, and VTE. The odds for a 5 μmol l⁻¹ increase in serum homocysteine for these vascular diseases ranged from 1.42 to 1.65 in the genetic studies and 1.32 to 1.59 in the prospective studies. An updated meta-analysis showed similar findings of prospective and genetic studies for VTE among first and recurrent events: a 20% increased risk for a first VTE event for those with the TT genotype compared to those with the CC genotype (den Heijer et al., 2005).
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21.6.2 Cognitive function, dementia and Alzheimer’s disease

Age is a major risk factor for the decline in cognitive function and incident dementia. According to estimations from the Aging, Demographics, and Memory Study in 2002, the prevalence of dementia and Alzheimer’s disease (AD) was 13.9% and 9.7%, respectively, among US adults aged 70+ years (Plassman et al., 2007). Among adults aged 71–79 years, the prevalence of dementia was 5% and increased to 37.4% among those aged 90+ years. Apart from age, other risk factors for cognitive decline and dementia include cardiovascular risk factors such as obesity, hypertension, dyslipidaemia, diabetes, and hyperhomocysteinaemia (Lighart et al., 2010). Of these risk factors, hyperhomocysteinaemia was associated with AD and dementia as reported in several case-control and prospective studies (Van Dam and Van Gool, 2009). Thus, homocysteine may be an important modifiable risk factor that may delay the onset of cognitive decline and dementia, except in multimorbid elderly patients (Hengstermann et al., 2009).

The prevalence of hyperhomocysteinaemia among US adults aged 60+ years participating in NHANES III (1988–1994) was 43.2% in men and 46.5% in women (Jacques et al., 1999a). Janson et al. (2002) reported a higher prevalence of hyper-homocysteine, ranging from 66% to 75% in elderly men and women, respectively, who participated in a cross-sectional study. In a recent systematic review, higher homocysteine levels were reported in AD cases compared to controls, while the relative risk of hyperhomocysteinaemia with AD was 2.5 (95% C.I. 1.38–4.56, p < 0.01) (Van Dam and Van Gool, 2009).

21.7 Clinical Efficacy of Folate and Vitamins B₆ and B₁₂

21.7.1 Homocysteine, folate, vitamin B₆, vitamin B₁₂, and vascular disease

Dietary folate and vitamins B₆ and B₁₂ are known to lower homocysteine concentrations. However, it is not clear whether homocysteine reduced by supplementation or dietary intake of the B vitamins influences the risk of a vascular event.

21.7.2 Coronary heart disease, stroke and venous thromboembolism

21.7.2.1 Observational studies

Briefly, the epidemiologic evidence has shown a moderate effect of dietary or supplemental vitamin B intakes on lowering the risk of CHD, stroke, and VTE (Rimm et al., 1998; Steffen et al., 2003, 2007).

21.7.2.2 Randomized clinical trials

Over the past 10 years, many large randomized clinical trials (RCT) have been conducted to evaluate the effectiveness of vitamin B supplementation in reducing homocysteine concentrations and putatively to lower the risk of recurrent CVD, including myocardial infarction (MI), stroke and CHD death (see Table 21.1). Generally, homocysteine-lowering treatment (folic acid, vitamin B₆ and vitamin B₁₂ supplementation) versus placebo has not lowered the risk of vascular disease in study participants with pre-existing disease. A Cochrane review and meta-analysis reported the risk ratio and 95% confidence intervals for 8 RCT as 1.03 (0.94, 1.13) (Marti-Carvajal et al., 2009). Conversely, homocysteine-lowering treatment may lower the incidence of strokes. Lee et al. (2010) recently conducted a meta-analysis of 13 RCT that enrolled over 13,000 participants with or without a history of stroke. They reported an 11% lower risk of the incidence of stroke with vitamin B supplementation, while secondary prevention of stroke was not reduced with these supplements. Further, den Heijer et al. reported no effect of vitamin B supplements folic acid, vitamin B₆ and vitamin B₁₂ in the secondary prevention of VTE (den Heijer et al., 2007).

21.7.3 Cognitive function, dementia and Alzheimer’s disease

Haan et al. (2007) provide evidence from a prospective study that the association between hyperhomocysteinaemia and dementia over
Table 21.1. Summary of the major randomized clinical trials testing the effectiveness of vitamin B supplementation in reducing homocysteine concentrations to lower the risk of recurrent CVD.

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Data collection</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOLARDA</td>
<td>Hx MI</td>
<td>N=140 Usual care 5 mg d⁻¹ FA</td>
<td>1 year Recurrent MI, revascularization CHD death</td>
</tr>
<tr>
<td>Liem et al., 2004</td>
<td>283 multicentre</td>
<td>N=143 Usual care</td>
<td></td>
</tr>
<tr>
<td>GOES</td>
<td>Hx MI; unstable angina, revascular.</td>
<td>N=300 Usual care 0.5 mg d⁻¹ FA</td>
<td>42 months Recurrent MI CVD death</td>
</tr>
<tr>
<td>Liem et al., 2005</td>
<td>593 Single centre</td>
<td>N=293 Usual care</td>
<td></td>
</tr>
<tr>
<td>HOPE2</td>
<td>Hx CVD; age &gt; 55</td>
<td>N=2758 2.5 mg d⁻¹ FA 50 mg d⁻¹ B₆ 1 mg d⁻¹ B₁₂</td>
<td>5 years Recurrent MI, stroke, sudden death (CVD)</td>
</tr>
<tr>
<td>Investigators (2006)</td>
<td>5522 multicentre</td>
<td>N=2764 Placebo</td>
<td></td>
</tr>
<tr>
<td>NORVIT Bonaa et al., 2006</td>
<td>Hx MI</td>
<td>N=943 Placebo</td>
<td>3.5 years Recurrent MI, stroke, CVD death</td>
</tr>
<tr>
<td>VISP</td>
<td>Hx stroke; age ≥ 42</td>
<td>N=1827 2.5 mg d⁻¹ FA 0.4 mg d⁻¹ B₁₂ 25 mg d⁻¹ B₆</td>
<td>2 years Recurrent stroke</td>
</tr>
<tr>
<td>Toole et al., 2004</td>
<td>3680 multicentre</td>
<td>N=1853 20 mcg d⁻¹ FA 6 mcg d⁻¹ B₁₂ 200 mcg d⁻¹ B₆</td>
<td></td>
</tr>
<tr>
<td>WAFACS Albert et al., 2008</td>
<td>With or w/o CVD</td>
<td>N=2721 Placebo</td>
<td>Incident/recurrent CVD</td>
</tr>
<tr>
<td>5442 women; multicentre</td>
<td>N=2721 Placebo</td>
<td>12.1–12.5 μmol l⁻¹ 7.3 years</td>
<td></td>
</tr>
<tr>
<td>WENBIT</td>
<td>Hx CVD; age ≥ 18</td>
<td>N=772 Gp1 N=772 Gp2 N=780 Placebo</td>
<td>Recurrent CVD</td>
</tr>
<tr>
<td>Ebbing et al., 2008</td>
<td>3096 multicentre</td>
<td>N=772 Gp1</td>
<td>10.8 μmol l⁻¹ Median 38 months</td>
</tr>
<tr>
<td>Study</td>
<td>Code</td>
<td>Hx/RF Description</td>
<td>Age (yrs)</td>
</tr>
<tr>
<td>---------------------</td>
<td>------</td>
<td>-------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SEARCH Collaborative Multicentre Group (2000)</td>
<td>VITATOPS Trial Study Group (2010)</td>
<td>VITRO den Heijer et al., 2007</td>
<td>SU.FOL.OM3 Galan et al., France</td>
</tr>
<tr>
<td>2 mg d⁻¹ Fol, 1 mg d⁻¹ B₁₂</td>
<td>Placebo</td>
<td>20 simvastatin</td>
<td>N=6031</td>
</tr>
</tbody>
</table>

B₁₂, vitamin B₁₂; B₆, vitamin B₆; CVD, cardiovascular disease; Fol, folic acid; Hcyst, homocysteine concentration; MI, myocardial infarction; HR, hazard ratio; Hx, history of; NR, not reported; RF, risk factors; RR, relative risk; TIA, transient ischaemic attack; 95% C.I., 95% confidence intervals.

* NORVIT: Gpl = 0.8 mg d⁻¹ folic acid, 0.4 mg d⁻¹ B₁₂, 40 mg d⁻¹ B₆; Gp2 = 0.8 mg d⁻¹ folic acid, 0.8 mg B₁₂, d⁻¹; Gp3 = 40 mg d⁻¹ B₆.
** WENBIT Gpl = 0.8 mg d⁻¹ folic acid, 0.4 mg d⁻¹ B₁₂, 40 mg d⁻¹ B₆; Gp2 = 0.8 mg d⁻¹ folic acid, 0.4 mg d⁻¹ B₁₂; Gp3 = 40 mg d⁻¹ B₆.
4.5 years of follow-up may be explained by lower levels of vitamin B₁₂ but not low folate. However, in a randomized, double-blind, placebo-controlled trial, results showed that among 406 adults aged 50–70 years taking folic acid supplements for 3 years versus 413 adults assigned to the placebo group, homocysteine levels were lowered by 26% and cognitive function improved (Durga et al., 2007). Results from a meta-analysis of nine placebo-controlled randomized trials with a median duration of 6 months showed that folic acid with or without other B vitamins did not prevent cognitive decline in over 2835 participants (Wald et al., 2010). Similarly, two randomized controlled trials demonstrated that daily intake of high-dose B vitamin supplements for 6–18 months did not slow cognitive decline among elderly adults with mild to moderate AD (Sun et al., 2007; Aisen et al., 2008). Meanwhile, several randomized clinical trials are under way, testing whether folate, vitamin B₆, and vitamin B₁₂ reduce levels of homocysteine and whether these will slow the progression of cognitive decline and dementia.

21.8 Neural Tube Defects

Neural tube defects (NTD) are severe congenital malformations that occur in early pregnancy. The development of NTD is multifactorial. Both genetic and environmental factors are involved in the metabolism of homocysteine in NTD, one potential pathway. Besides dietary intake, a number of environmental factors are associated with the development of NTD including socioeconomic status, geographic differences, and maternal characteristics, such as maternal medication use during pregnancy as well as status of maternal diabetes and obesity (Blom, 2009). One genetic risk factor associated with the development of NTD is ethnicity. After folate fortification of grain products in the United States in 1998, the prevalence of NTD was reduced by 50% to 70%, especially among non-Hispanic whites and Hispanics, but not among non-Hispanic blacks (Williams et al., 2005). Molloy et al. (2009) observed that low (i.e. deficient or inadequate intake) maternal vitamin B₁₂ status was associated with increased risk for neural tube defects. Genetic factors may influence both homocysteine and folate metabolism. Despite the MTHFR 677T allele, adequate folate intake may reduce the risk of NTD, while inadequate intake of folate may have the opposite effect (Gueant et al., 2003). Further, the pathogenesis of NTD may be related to one-carbon metabolism that is influenced by intakes of folate and vitamin B₁₂ and by several other genetic factors. Mechanisms that may play a role in the aetiology of NTD include folate receptor autoantibodies and methylation patterns. This literature has been reviewed by Blom (2009).

21.9 Methodological Issues

21.9.1 Differences among studies of homocysteine, B vitamins and vascular disease

Generally, the results from the majority of single RCT or meta-analyses that tested the effectiveness of vitamin B supplementation (including folate, vitamin B₆ and B₁₂) in reducing homocysteine levels did not influence the risk of recurrent CHD (Bazzano et al., 2006) and thus far, in most but not all trials, of cognitive dysfunction and dementia (Wald et al., 2010). For stroke, some benefit was demonstrated in some subgroups (Bazzano et al., 2006; Lee et al., 2010). In contrast, results from a meta-analysis of observational studies examining the relation between homocysteine levels and risk of vascular disease showed lower risk of CHD and stroke (Homocysteine Studies Collaboration, 2002).

Several factors that might explain these study results include differences in study design, study population, duration of follow-up, data collected (i.e., confounding factors), and study outcome. Cohort studies reported weaker relative risks of CVD outcomes than case-control studies, while the majority of RCT did not demonstrate an impact of supplements on the risk of a recurrent event. Another factor that influences the outcome of the study is the study population. Many of the reported studies on CHD included adults
Homocysteine Status

with a prior history of CHD or advanced vascular disease. For studies of dementia, the populations were those who had mild to moderate dementia or Alzheimer’s disease, but did not include low-risk or intermediate-risk individuals. Even though homocysteine can be reduced with vitamin B supplementation in high risk patients, it may still not be possible to lower the risk of vascular disease in these individuals who are depleted of thioctic acid in cellular membranes (McCully, 1993). Sufficient sample size and power are needed to detect a significant difference between (treatment) groups. In some RCT, the sample size may have been inadequate or the observational studies inadequately powered considering the fortification of the US food supply with folic acid since 1998. Duration of follow-up is an important factor that affects the power of the study. Length of follow-up in published studies or RCT has varied from a few months to several years. Wang et al. (2007) found that greater benefit was shown with duration of vitamin B treatment greater than 36 months. Further, in the analysis of observational studies, the statistical models may not have been adjusted for the same confounding factors, such as the cardiovascular risk factors. Finally, in many of the studies, a variety of study outcomes was reported, instead of a single, defined outcome-of-interest. The primary endpoint for most of the trials testing the B vitamins to reduce risk was recurrent vascular disease, including CHD and stroke, while cognitive function and dementia were secondary endpoints for several of the studies. Similar methods are necessary for comparison of studies and to appropriately interpret the study results. Several meta-analyses have been conducted to synthesize the findings of the individual trials. One such study was a Cochrane review of the homocysteine-lowering interventions (Marti-Carvajal et al., 2009). Results from this meta-analysis provide no evidence to support homocysteine lowering to prevent CVD.

An important point to consider, however, is that folate may not be the most effective therapy or intervention to reduce risk of a recurrent event. As observed by Haan et al. (2007) in a prospective observational study, vitamin B₁₂ appeared to provide the most benefit in reducing the risk of vascular events. Vitamin B₁₂ status has also associated with lower risk of NTD in nested case-control studies (Molloy et al., 2009). Another potential therapy is the combination of omega-3 fatty acid and folic acid or vitamin B supplementation that may improve cognitive function or prevent dementia and Alzheimer’s disease, as well as beneficially influencing CHD and stroke outcomes (Das, 2008). The SU.FOL.OM3 trial is evaluating the effect of supplementation of the B vitamins, folate, vitamin B₆ and vitamin B₁₂, with and without omega-3 fatty acids versus placebo on reducing the risk of recurrent ischaemic disease (Galan et al., 2003). However, more than one RCT is needed to test these hypotheses. Finally, the RCT have tested the effect of supplementation of the B vitamins on preventing recurrent disease, but not primary prevention. Epidemiologic evidence has shown, however, the cardiovascular benefits of a dietary pattern rich in fruit, vegetables, grain products and fish/seafood – foods that are rich in folate, vitamin B₆ and omega-3 fatty acids (Appel et al., 1997; Steffen et al., 2007).

21.10 Conclusions

Homocysteine is a risk factor for vascular disease and neural tube defects. Meta-analysis of the observational studies on homocysteine concentrations and risk of vascular events showed that a 5 μmol l⁻¹ increase in homocysteine level conferred a 9–18% increase in risk for CHD, independent of CHD risk factors (Ganjii and Kafai et al., 2006; Humphrey et al., 2008). Further, intakes of dietary folate or folic acid supplementation have been shown to lower homocysteine levels in adolescents and adults, both young and old. However, results have been null in the majority of RCT testing the effectiveness of folate, vitamin B₆ and vitamin B₁₂ supplementation to reduce the risk of recurrent CHD, stroke, and VTE, as well as cognitive function or dementia. However, vitamin B supplementation may prevent the incidence of stroke events. Several RCT with large sample sizes are currently under way, and these may soon provide a definitive answer to these significant public health problems.
Although national folic acid fortification in the United States has been associated with over 50% lower prevalence of NTD, there are potentially other modifiable risk factors that may be targeted, including maternal status of obesity, diabetes and vitamin B\textsubscript{12} status, to further reduce the risk of NTD. Large epidemiologic studies are warranted to further elucidate its multiple causes.

References


Industrial processing of food has not only improved the management and safety of foods, but also its taste. Unfortunately however, most of these processes – including plant breeding, gene manipulation, fractionation, separation, condensation, drying, freezing, heating, irradiation, roasting, microwave, toasting, smoking, emulsification and homogenization – appear to be negative, as they reduce the nutritional quality of the food and also contribute significantly to increased vulnerability to development of diseases, especially those referred to as endemic and chronic.

This chapter deals especially with the negative consequences of heating and mainly with the impact of heat-produced glycated and lipoxidated molecules, often referred to as Maillard products. These products are more specifically referred to as advanced glycation end-products (AGE) and advanced lipoxidation end-products (ALE). The negative effects on health of other heat-produced compounds, such as heterocyclic aromatic amines, are outside the scope of this review.

Modern molecular biology has made it possible to explore the impact of these and other process-induced molecules on the body and its functions. The detection in 1992 of a specific receptor in the body for such products provided the opportunity for a better understanding of their effects in health and disease. This receptor for advanced glycation end products (RAGE) is recognized as a key member of the immunoglobulin superfamily of cell surface molecules. It functions as a master switch, induces sustained activation of NF-κB, suppresses a series of endogenous autoregulatory functions, and converts long-lasting pro-inflammatory signals into sustained cellular dysfunction and disease. Its activation is associated with much increased levels of dysfunctioning proteins in body fluids and tissues, and is strongly associated with a series of diseases from allergy and Alzheimer’s disease to rheumatoid arthritis and urogenital disorders. It is important to observe that heat treatment and other forms of processing of foods will dramatically increase the content of these dysfunctional molecules, and thereby, with time, significantly contribute to the epidemic of chronic diseases seen around the world. An increased
consumption of raw foods, fruits and vegetables; foods rich in polyphenols and other antioxidants; as well as live bacteria, probiotics and plant fibre seems appropriate in order to counteract these undesirable developments.

22.2 Introduction

Modern food is often extensively processed prior to distribution and sale. Drying, freezing, irradiation, roasting, microwaving, toasting, emulsifying, homogenizing and the addition of numerous compounds are all aimed at enhancing the appeal of the food, its palatability and its shelf-life. The effects of each of these manipulations on human health are not fully explored, and even when documented, often not considered to the extent they should be, either by industry or the consumer.

Heating food to higher temperatures is generally regarded to improve both the taste and smell of the foods we eat. High temperature makes food proteins change structure: coagulate, aggregate and produce crusts. Modern food chemists, chefs and cooks use this information every day to produce delicious new foods.

The French biochemist Louis-Camille Maillard described the chemical process which occurs non-enzymatically in foods (Maillard, 1912), and which is much accelerated by heating. This process is now referred to as the Maillard reaction and its products collectively named Maillard products. Reducing sugars (fructose, glucose, glyceraldehyde, lactose, arabinose and maltose) will during the process bind to amino acids, nucleic acids, including DNA, RNA, peptides and proteins, to produce transitional compounds, most often referred to as Amadori products. In time these undergo complex changes: cyclization, dehydration, oxidation, condensation, cross-linking and polymerization, to form irreversible chemical products. In particular, reactive carbonyls such as glyoxal and methylglyoxal have been found to rapidly modify reactive side chains of proteins. Important amino acids such as lysine (essential amino acid) and histidine (essential for children) are often involved.

During the process significant amounts of pigments (melanoids) but also thousands of often good-tasting and good-smelling so-called volatile compounds will be released. These pigments often make the food or parts of the food look brown or black, which is why the process is sometimes referred to as 'browning'. Common browning products are bread crusts and roasted surfaces of fried meat and fish, all sorts of broths, irrespective of vegetable or animal origin, and all smoked food, as well as Asian sauces, balsamic products, Chinese soy, and cola products, all rich in brown/black Maillard products. But not all Maillard products are dark in colour; there are also white Maillard products, especially dairy products such as cheese and powdered milk. Maillard himself suggested that the Maillard process might be negative to health, as these products will accumulate in the body, as we now know, for many years and sometimes for the rest of life. The process might also reduce the availability in the body of important and essential amino acids.

22.3 Effects of Heating on Food Quality

Heat-induced alterations of foods are considered to commence at around 28°C – the highest processing temperature allowed for olive oil to be called virgin. Most enzymes in foods become deactivated after approximately 42°C. Some antioxidants are resistant to heat but a majority will disappear in the interval 30°-100°C, and almost all during microwaving. The heat-enhanced production of Maillard products – glycated and lipoxidated molecules – is said to start and accelerate from around 80°C. Similarly the heat-induced production of carcinogens such as heterocyclic amines is said to start from the interval 100°-130°C, after which the production accelerates dramatically.

Mailard products based on association of carbonyl groups in sugars and proteins are collectively referred to as AGE. Similar products, formed between reactive fatty acids and proteins, are referred to as ALE. Numerous such synthetic products are now identified, but two or three previously unknown compounds are each year added to the list. The most commonly studied AGE
are pentosidine, $N^\epsilon$-carboxymethyl-lysine (CML) and $N^\epsilon$-(carboxyethyl)lysine (CEL).

It is important to note that the production of both AGE and ALE are not dependent on enzymes. The intensity in their production increases, not only with increase in temperature, but also with length of storage at elevated temperatures, and even at room temperature. The content of the AGE furosine increases dramatically with heat treatment such as pasteurization and especially with the production and storage of powdered milk (Baptista and Carvalho, 2004). Other industrial practices commonly used in food processing such as irradiation, ionization, microwaving and smoking also contribute significantly to increased production of AGE/ALE. Vegetable-based foods are no exception - industrial treatment of plant products, such as roasting, drying and ‘curing’ will lead to amounts of AGE/ALE that are as great as those found in animal products. One such example is roasted peanuts.

Fresh tobacco leaves, fresh coffee beans, and fresh peanuts are rich in powerful antioxidants, most of which will disappear during the industrial process (‘curing’, roasting) and be replaced by often large amounts of AGE/ALE. As the temperature increases above 130°C, carcinogens, especially heterocyclic amines, will also be produced, and their production increases dramatically as the temperature increases. AGE/ALE will not exclusively reach the body through the food we eat, or the smoke we inhale – they are also produced spontaneously in the body, especially in the presence of increased levels of sugars and fatty acids in body fluids and tissues, and in those suffering chronic diseases such as diabetes and chronic renal diseases.

### 22.4 AGE/ALE Accumulation in the Body

The accumulation of late/matured Maillard products – AGE/ALE – in the body is in principle irreversible; what is accumulated in the tissues persists for a very long time and most often forever. Hitherto, the finding of larger amounts of AGE/ALE in the tissues of elderly individuals has simply been regarded as a normal effect of ageing. However, it might not be so. Instead it might mainly be a result of the lifestyle chosen, smoking, and eating habits, and thus in theory preventable. Large to extreme increases in AGE/ALE are regularly observed in body fluids and tissues of patients with chronic diseases, particularly those with diabetes and chronic renal diseases, and in patients suffering complications of these diseases. It is commonly observed in diabetic patients, who suffer from reduced wound healing (Peppa et al., 2003), retinopathy, nephropathy (Zheng et al., 2002), and angiopathy (Vlassara et al., 2002; Lin et al., 2003). Accumulation of AGE/ALE in tissues is seen as intracellular or extracellular deposits referred to as tau proteins, amyloid $\beta$ proteins (Smith et al., 1994) and in neurofibrillary tangles (Smith et al., 1994; Vitek et al., 1994). Such depositions in various body tissues were long regarded as degenerative but biologically inert structures. However, increasing evidence supports the conclusion that these structures are foci with very strong pro-inflammatory potential, which maintain the systemic chronic inflammation at a high level in the tissues, and thereby accelerate further production of AGE/ALE and exacerbation of disease.

### 22.5 Modern Molecular Biology: Essential for Understanding the Effects of AGE/ALE

Almost 100 years ago, Maillard suggested that accumulation in the body of AGE/ALE would significantly contribute to the progression of diseases, especially of chronic urinogenital diseases, and in particular uraemia. He created what he called an ‘index of urinogenital imperfection’, which he used to document the association between the degree of accumulation in the body of Maillard products and the severity of disease, especially chronic renal disease.

The time was, however, not opportune for such radical thinking, and the concept was largely ignored by scientists and clinicians of the time, remaining so for several decades to come. It was the introduction of modern molecular biology and particularly the identification of specific receptors in the body for these
substances that would dramatically change the attitude and interest in these substances by both biologists and physicians. The turning point seems to be the identification in 1992 by the American Ann Marie Schmidt of a specific receptor for AGE/ALE named RAGE (Schmidt et al., 1992, 1993, 1994a,b). Since then increasing numbers of publications have appeared in the literature. From the year 2000, several international scientific organizations have become involved in the concept, arranging special symposia on RAGE and AGE/ALE, and publishing special issues relating to these topics. New societies have also been founded with the aim of specifically investigating the effects of food-derived AGE/ALE on health and well-being. The New York Academy of Science seems to have taken the lead and many scientific contributions on AGE/ALE are published each year in its Annals. Searches on PubMed relating to AGE and ALE reveal more than 5000 publications, of which more than 25% appeared in 2009. In addition almost 20,000 titles on PubMed are about glycated haemoglobin, HbA1c.

Several methods are available for the measurement of content of AGE/ALE in body fluids and tissues: immunohistochemistry with polyclonal or monoclonal antibodies, high performance liquid chromatography (HPLC), and mass spectrography. A majority of these substances are auto-fluorescing even if not visible to the human eye, and so can be used for diagnostic purposes (Meerwaldt et al., 2005a,b). The fluorescence has its maximum at wavelengths between 350 and 440 nm (Meerwaldt, 2005b). Often-studied substances such as CML and CEL do not, unfortunately, show any fluorescence ability, nor do they have any colour. Despite this, measuring fluorescence is an excellent tool for clinical use, especially for screening of individuals with suspected high levels of AGE/ALE in the body, but also for screening of foods suspected to be rich in such dysfunctional proteins.

22.6 RAGE: a Master Switch and Key to Inflammation

RAGE is a prominent member of what has been called the immunoglobulin superfamily of cell-surface molecules. It is described as a ‘master switch’ with the ability to coordinate the inflammatory reaction in the body. RAGE induces a long-lasting activation of the pro-inflammatory transcription factor NF-κB and suppresses a series of endogenous auto-regulatory functions (Bierhaus et al., 2001, 2005a; Schmidt et al., 2001; Vlassara, 2005). Increased deposition of AGE/ALE in the tissues is suggested to be a key element in the development of the so-called metabolic syndrome (Koyama et al., 2005; Soldatos et al., 2005). Accumulation of AGE/ALE and subsequent activation of RAGE is reported to induce a significant down-regulation of leptin in adipose cells (Unno et al., 2004). RAGE activation induces effects on a great variety of tissues, but they are particularly pronounced in endothelial cells, where increased expression occurs of a long row of molecules such as VCAM-1, ICAM-1, E-selektin, eNOS and TGF-β. TNF-α, 11-6, PAI-1 and VEGF are seen (Bohlender et al., 2005). Strong RAGE-induced effects are often observed on immune cells, macrophages (Sunahori et al., 2006), and dendritic cells (de Leeuw et al., 2005; Ge et al., 2005), but also on smooth muscle, particularly in the walls of blood vessels, under the mucosa and in the skin (Aronson, 2003). These changes are associated with subsequent reduction in regenerative capacity and function of the cells, increased blood pressure, and development of chronic diseases and/or exacerbation of complications to chronic diseases (Monnier et al., 2005). However, the conditions vary from tissue to tissue, the most sensitive and vulnerable being those with low regenerative capacity and long-lived cells such as myelin- and collagen-rich structures, where the substances are likely to remain. Among these are brain, peripheral nerves, skeleton, muscles, tendons, joints, skin and eye, especially the lens.

Research in recent years has also demonstrated the existence of an endogenous soluble form of RAGE known as sRAGE, which has an important effect as a decoy for RAGE and has been shown to prevent accumulation of RAGE in body tissues (Bierhaus et al., 2005b). This suggests that chronic diseases are not only associated with increased levels of RAGE in the body, but also, and probably as important, with low levels of sRAGE.
22.7 Factors Underlying Enhanced Systemic Inflammation

The largest part of the immune system is, in contrast to what was earlier believed, to be found in the gastrointestinal (GI) system (Brandtzaeg et al., 1989) as 70–80% of the Ig-producing cells are located within the GI tract. This explains why the food we eat has such a profound influence on our well-being and health. Although AGE/ALE seem to play a major role, it is also clear that numerous other food-related factors will influence the degree of systemic inflammation in the body, the sensitivity to develop disease and our daily well-being. Increasing evidence suggests that all these factors are additive and collectively contribute to development of a sustained, long-lasting, often discrete and unrecognized, exaggerated stage of inflammation in the body, commonly seen before and when a chronic disease is manifest. Such factors are:

- **Low vitamin D status.** There is a strong correlation between the level of vitamin D in the body, degree of inflammation and incidence of chronic diseases. Individuals living at higher latitudes such as Canada, Russia and Scandinavia, but also countries in the Southern hemisphere, such as Argentina, New Zealand and Uruguay are reported to have generally lower levels of vitamin D in serum, especially during the winter season. This phenomenon is associated with higher incidence of coronary–vascular diseases, acute coronary events (Zittermann et al., 2005; McCarty, 2005) and other chronic diseases such as cancer (Mohr et al., 2006, 2007, 2008).

- **Low levels in the body of antioxidants such as folic acid and glutathione, and increased levels of homocysteine.** Increase in serum levels of homocysteine is regularly associated with increased levels of systemic inflammation and chronic diseases (Mattson, 2003).

- **Impaired hormonal homeostasis.** Ageing as well as chronic diseases are commonly accompanied by hormonal disturbances of various kinds, sometimes to the extent that ageing has been referred to as a state of 'hormonal chaos' (Hertoghe, 2005). Hormonal disturbances are often accompanied by increased oxidative stress/increased release of free radicals, intracellular accumulation of ‘waste products’, inhibition of apoptosis, disturbed repair mechanisms, reduced gene polymorphism, premature shortening of telomeres and reduced immune defence. Reduced resistance to disease is often observed in premature ageing as well as in several chronic diseases (Hertoghe, 2005). In particular, 17β-estradiol, plentiful in dairy products, is known to induce a strong activation of RAGE mRNA in endothelial cells. This effect is abolished if an anti-estradiol such as 4-OH tamoxifen is supplied (Yamagishi et al., 1998, Suzuma et al., 1999). An impaired hormonal homeostasis is suggested to explain why chronic diseases are often aggravated during pregnancy, frequently seen as vascular and eye complications to diabetes (Suzuma et al., 1999). Physical as well as mental stress also contributes to activation of RAGE, and increased release of noradrenaline is reported to reduce immune defence and increases sensibility to acquire infections by up to 4 logs (Cooper, 1946). Increased release of noradrenaline in the intestine will dramatically reduce the beneficial intestinal flora, and increase the virulence of potentially pathogenic micro-organisms (Kinney et al., 2000; Alverdy et al., 2003), changes, which most likely also contribute to increased RAGE activation. Permanently increased levels of noradrenaline are reported in chronic diseases such as Alzheimer’s disease and also found to correlate well with severity of the disease (Peskind et al., 1998). Parathyroid hormones constitute another example of hormones deeply involved in the inflammatory process. Significant elevations in IL-6 are observed in hyperparathyroidism as in other chronic conditions with increased systemic inflammation such as obesity (Flyvbjerg et al., 2004).

- **Angiotensin/renin.** Oxidative stress and increased systemic inflammation is also strongly associated with increased release
of angiotensin, increased levels of free fatty acids in serum, and with reduction in beta-cell function in diabetes (Flyvbjerg et al., 2004; Allen et al., 2005; Tikellis et al., 2006). The observation that blockage of the angiotensin receptor will reduce production and accumulation of AGE both in vitro and in vivo is of great interest (Allen et al., 2005).

- Larger intake of glutenoids. Glutenoids are increasingly regarded as pro-inflammatory in the body (Tlaskalová-Hogenová et al., 2005), and suggested to occur even in the absence of intestinal changes (Brady and Hoggan, 2002; Sbarbati et al., 2003).
- Low intake of plant antioxidants.
- High intake of carbohydrates.
- High intake of saturated and trans fatty acids. A strong association is repeatedly documented between the average content of fat in food and morbidity and/or mortality in chronic diseases in a country, as demonstrated for breast cancer (Carroll, 1975), as well as other cancers and also chronic diseases such as coronary heart disease (Artaud-Wild et al., 1993; Moss and Freed, 1999) and diabetes (Dahl-Jorgensen et al., 1991). More than three quarters of the saturated fat consumed is of bovine origin, and thus it is not surprising that the incidence of various chronic diseases also correlates well with the amount of dairy products consumed (Ganmaa et al., 2002).

22.8 Dietary Choice

The incidence of most chronic diseases has increased dramatically during the last 150 years, much of it in parallel with the significantly altered intake of foods which has occurred since the year 1850: a doubling of intake of saturated fat, 50% reduction in intake of omega-3 fatty acids, and a more than doubling in intake of omega-6 fatty acids (Leaf and Weber, 1988). The intake of refined sugar has during the same time period increased from approximately 0.5 kg to about 50–60 kg per person per year. Furthermore, the transition in use of carbohydrates with stronger pro-inflammatory effects such as high-fructose corn syrup (HFCS), seems to make the situation even worse. In the United States, the intake of HFCS in carbonated drinks and fast foods now exceeds that of sucrose (Gaby, 2005).

A recent study in mice is of particular interest. Over 4 months, RAGE knockout (KO) mice received either a standard diet (7% fat) or a Western ‘fast-food’-like diet (21% fat) and were compared to wild-type mice, receiving the same diet. The Western-food-like diet was associated with significant cardiac hypertrophy, inflammation, mitochondrial-dependent superoxide production and accumulation of AGE in both strains, but significantly less in the RAGE-KO mice. Both strains demonstrated reduced levels of inflammation and oxidative stress, in association with reductions in AGE as well as RAGE on supply of an AGE inhibitor (alagebrium chloride, 1 mg kg⁻¹ day⁻¹ (Tikellis et al., 2008).

Much can be learnt from studies of Japan, which has during the last 50–60 years made similar, although not as extensive, changes in food habits as the West. The incidence of several chronic diseases has increased dramatically during this time. As an example, the incidence of prostate cancer has increased 25-fold during the last 50 years, much in parallel with an increase in the consumption of industrially produced agricultural foods: 7 times more eggs, 9 times as much meat, and 20 times as much dairy product (Ganmaa et al., 2002, 2003).

22.9 Dairy in Focus

Commonly, 10–20% and sometimes up to 70% of the amino acid lysine is reported to be modified during the common industrial treatment of milk (including sterilization, pasteurization and irradiation). Fructoselysine is the dominating modified molecule, but CML and pyrraline are also produced during the processing of milk. Sugar content, the level and time of elevated temperature, and storage time are the main factors behind the increased production of AGE/ ALE in milk products.

Not only the industrial treatment of dairy products but also the feeds given to the cows
have changed dramatically during the 20th century – from mainly forage-based to starch-rich and fast-absorbed carbohydrates such as corn, maize grains, barley, molasses and dextrose. Intensive feeding of cows with carbohydrates induces insulin resistance in animals as well as in humans. Should the animals be allowed to live long enough they would also show the same symptoms of Western diseases including manifest diabetes. It has been demonstrated that milk- and lactose-fed calves show signs of insulin resistance at a young age (Hostettler-Allen et al., 1994).

High levels of pro-inflammatory cytokines and various stress hormones are regularly observed in intensively fed animals. No information is available in the literature, however, to support the notion that elevated inflammatory molecules are transferred to humans by meat and dairy products from such animals. Today’s dairy products come, much in contrast to the old days, up to about 80%, from pregnant cows, and consequently are rich in growth factors and various hormones, especially sex hormones (Malekinejad et al., 2006), some of which (like 17β-oestradiol) are potentially pro-inflammatory and carcinogenic. It is suggested that dairy-derived hormones and growth factors are important pathogenetic factors behind the development of hormone-dependent cancers, especially of the colon, prostate and breast (Outwater et al., 1997). These hormones follow the fat fraction and are thus more concentrated in condensed products such as butter, cheese, and powdered milk.

It has been demonstrated that vegans, in great contrast to meat-eaters and lacto-vegetarians, have lower levels of AGE/ALE. Lacto-vegetarians seem to have even higher levels of AGE/ALE than meat-eaters (Sebekova et al., 2001), which might be explained by a higher intake of dairy products, especially cheese, to compensate for not eating lean meat, but which might also be due to a higher intake of fructose. Significant health advantages are reported for vegans: lower levels of pro-inflammatory molecules, cytokines and acute phase proteins; lower systolic and diastolic blood pressure; lower total cholesterol; lower LDL-cholesterol; lower fasting blood sugar and triglycerides; and lower incidence of chronic diseases, especially diabetes and complications to diabetes (Barnard et al., 2009). It would be no surprise if the lowest levels of AGE/ALE are to be found in the group referred to as raw-eaters, especially if they avoid dairy-based foods, but unfortunately, this group has attracted few studies and none with regard to the content of AGE/ALE.

### 22.10 AGE/ALE and Disease

Increased accumulations of AGE/ALE in tissues have been reported in numerous chronic diseases, as detailed below. In addition, changes in the skin and oral cavity may serve as markers of health risks associated with AGE/ALE.

#### 22.10.1 Allergy and autoimmune diseases

Thermal processing, curing, and roasting of foods are known to often increase allergenicity of pre-existing allergens and also to introduce new antigens. Sometimes, however, reduced allergenicity has been reported (Davis et al., 2001; Sancho et al., 2005). Common foods such as milk, peanuts and soy are reported to induce significant increases in AGE levels and to severely affect the IgE-binding capacity (Chung et al., 2001; Franck et al., 2002; Rautava and Isolauri, 2004). Significantly elevated urinary levels of the AGE pentosine are observed in allergic children in association with signs of exacerbation of atopic dermatitis (Tsukahara et al., 2003).

#### 22.10.2 Alzheimer’s disease and other neurodegenerative diseases

Alzheimer’s disease (AD) is one of the most common chronic diseases, affecting approximately 5% of all individuals over 65 years of age and more than 35% of those over 80. Strong similarities exist between AD and type 2 diabetes (T2DM), to the extent that Alzheimer’s has been called ‘the diabetes of the brain’, or type 3 diabetes. The incidence of AD is reported to be increased two- to fivefold in T2DM (Nicolls, 2004). An approximate threefold
increase in content of AGE is observed in AD brains compared to age-matched controls (Moreira et al., 2005). A common feature of both diseases is accumulation of amyloid deposits, a process which progresses during the course of disease, and much relates to the stage of disease. Signs of amyloidosis, perturbation of neuronal properties and functions, amplification of glial inflammatory response, increased oxidative stress, increased vascular dysfunction, increased Aβ in the blood–brain barrier and induction of autoantibodies are regularly seen. Increased levels of AGE and signs of oxidative damage are almost regularly observed in the eyes, known to be early targets of AD (Moreira et al., 2005). Central to, if not the cause of AD, is the progressive oligomerization and deposition in the cells of amyloid β-peptides (Aβ), tau, prions and transthyretin, all glycated molecules with strong neurotoxic effects. Amyloid β-peptides accumulate extracellularly to form amyloid plaques, while tau protein deposits occur as neurofibrillary tangles within the cells. Increased levels of AGE/ALE are most often demonstrated with immunohistochemical methods in senile plaques, tau proteins, amyloid β-proteins, and in neurofibrillary tangles (see below (Vitek et al., 1994; Moreira et al., 2005)). Accumulation of AGE/ALE in brain tissues has also been observed in Parkinson’s disease (PD) (Castellani et al., 1996; Dalfo et al., 2005), and cytoplasmic proteinaceous inclusions composed of the protein α-synuclein (α-syn) and named Lewy bodies are regularly observed in PD. AGE/ALE are also implicated in the pathogenesis of other neurodegenerative diseases: amyotrophic lateral sclerosis (ALS) (Chou et al., 1998; Kikuchi et al., 2002; Kaufmann et al., 2004), Huntington’s disease (Ma and Nicholson, 2004), familial amyloidotic polyneuropathy (Gomes et al., 2005), and Creutzfeldt-Jakob disease (Sasaki et al., 2002). Early accumulation of AGE is also reported in Down’s syndrome, and early antiglycation treatment is suggested to reduce cognitive impairments (Odetti et al., 1998; Thiel and Fowkes, 2005). It has also been suggested that bovine spongiform encephalopathy (BSE) a disease with its significant similarities to AD, might be associated with increased glycation and lipoxidation (Frey, 2002). Involvement of glycation products and activation of prion proteins are also suggested by other authors (Boratynski and Gorski, 2002; Choi et al., 2004). AGE, amyloid fibrils, and prions all seem to have the same target, RAGE, and all activate the NF-κB pathway. Interaction between RAGE and Aβ is most likely to be the most important implication in the development of AD, enhancing inflammation in blood vessel endothelium, inducing increased response of NF-κB, mediating transport of Aβ across the blood–brain barrier, suppressing cerebral blood flow, and inducing cell death (apoptosis). RAGE is known to mediate Aβ-induced migration of monocytes across the thin brain endothelium and into the brain tissues.

Increased cholesterol is suggested to contribute to the production of AD by increasing generation of beta-amyloid (Aβ), and animal studies suggest that cholesterol co-localizes with fibrillar Aβ in the amyloid plaques (Burns et al., 2003).

### 22.10.3 Atherosclerosis and other cardiovascular disorders

Oxidative stress, lipid peroxidation and protein glycation are repeatedly associated with extensive arteriosclerosis. Significant increases in both chemical AGE (carboxymethyllysine) and fluorescent AGE (spectrofluorometry) were observed in 42 patients with atherosclerosis when compared to 21 healthy controls (p<0.001) (Kalousova et al., 2005). Increased levels of malondialdehyde, lipid peroxides and pentosidine were seen in a study of 225 haemodialysis patients and these also correlated significantly with the degree of coronary artery calcifications (Taki et al., 2006). Significant lipid oxidation, deposition of AGE/ALE in the arterial walls and development of atherosclerosis, are reported in rabbits fed a diet containing 1% cholesterol. Deposition is further enhanced when 10% fructose is added to the diet (Tokita et al., 2005). Structural modifications of high density lipoproteins (HDL), lipoxidation, glycation, homocysteinylation, or enzymatic degradation will make HDL lose its anti-inflammatory and cyto-protective ability (Ferretti et al., 2006). This emphasizes its
importance in the pathogenesis of atherosclerosis, as in neurodegenerative diseases, diabetes and other autoimmune diseases (de Leeuw et al., 2005). Supplementing AGE-modified serum albumin to experimental animals will significantly increase secretion of pro-inflammatory cytokines, maturation of dendritic cells, and augment the capacity to stimulate T-cell proliferation (Ge et al., 2005). The AGE CML in plasma was followed for six years in 1270 people aged 65 and older. In this period, 227 (22.4%) died during the period, 105 of cardiovascular disease. This mortality was significantly associated with high CML levels (Semba et al., 2009).

22.10.4 Cancer

Individuals with high levels of oxidative stress, such as those with type 2 diabetes and significantly increased accumulation in the body of AGE/ALE, suffer a significantly increased risk of developing cancer (Abe and Yamagishi, 2008). The receptor RAGE and its multiple ligands are shown to be involved in the pathogenesis of multiple tumours: brain, breast, colon, colorectal, lung, prostate, oral squamous cell carcinoma and ovarian cancer, as well as lymphoma and melanoma (Takada et al., 2004; Genkinger et al., 2006; Logsdon et al., 2007). In vitro and animal studies, as well as preliminary clinical observations, support the view of a direct link between RAGE activation and proliferation, migration, invasion of tumour cells and survival (Logsdon et al., 2007; Abe and Yamagishi, 2008). RAGE expression is reported to be elevated in human cells with high metastatic ability and low in tumour cells with low metastatic ability (Takada et al., 2004). A tumour-suppressive function of RAGE has also been reported for some distinct cell types (Gebhardt et al., 2008). It is suggested that cytokines produced by cells of the innate immune system play an indispensable role in tumour-promoting inflammation, while protective anti-tumour effects derive largely from adaptive immune cells, particularly T cells (Dougan and Dranoff, 2008). An up-regulation of the gene S100P, known to be involved in the activation of RAGE, has been reported for several tumour tissues including lung, breast, pancreas, prostate, and colon (Rehbein et al., 2008). The RAGE ligand sRAGE, highly expressed in healthy lung tissues especially at the site of alveolar epithelium, is significantly down-regulated in lung carcinomas (Jing et al., 2010), but also in pancreatic cancer (Krechler et al., 2010). The relationship between RAGE expression in surgical specimens of primary tumours and prognosis of the patient was studied recently in 216 patients with oesophageal squamous cell carcinoma (Tateno et al., 2008). Those with positive RAGE expression in tumour cells exhibited a significantly better prognosis than those with negative RAGE expression (5-year survival, 52% versus 32%, respectively) (Tateno et al., 2008).

22.10.5 Cataract and other eye disorders

AGE/ALE accumulate with age in all ocular tissues including lacrimal glands, and trigger pathogenic events, especially in diabetics, in all parts of the eye (Stitt, 2005). The lens contains abundant proteins, which undergo translational modifications throughout the lifespan, contributing to ageing and cataract formation. Kynurenines are diffusible components of the lens that absorb UVA and UVB radiation, and are believed to protect the retina from light damage. However, it is also unstable under physiological conditions and undergoes deamination, its half-life being approximately 7 days. The deaminated products, known to affects lens proteins and modify specific amino acids, are believed to contribute to AGE formation, ageing of the lens, and to development of cataracts (Nagaraj et al., 2010). Age-related macular degeneration (AMD) is also strongly associated with increased oxidative stress, and with increased deposition of AGE/ALE. A recent study found signs of systemic AGE accumulation in patients with AMD, implicating a role for AGE/ALE in the pathophysiology of AMD (Mulder et al., 2010). The AGE CML and pentosidine are also shown to be significantly increased in AMD patients relative to healthy controls: CML (~54%), and pentosidine (~64%) (p < 0.0001) (Ni et al., 2009). RAGE and
its ligands are also reported to be involved in retinal diseases (Barile and Schmidt, 2007) and in glaucoma (Tezel et al., 2007).

**22.10.6 Diabetes**

More than 6000 publications in PubMed deal with AGE/ALE and more than half of them particularly with their role in diabetes mellitus (DM). Several excellent reviews have been published recently (Meerwaldt et al., 2008; Orasanu and Plutzky, 2009; Yan et al., 2009). Over-consumption of fat and carbohydrates, not only of glucose, but also of other carbohydrates such as lactose and fructose, contribute in diabetics to a significantly increased accumulation of AGE/ALE in the tissues. The consumption of high-fructose corn syrup in the United States today exceeds that of sucrose. It is ten times more capable of producing AGE/ALE, and is suggested as a major contributor, not only to obesity and accumulation of fat in the liver, but specifically to development of type 2 diabetes as well as to severe complications of both type 1 and 2 diabetes (Gaby, 2005). Chronic hyperglycaemia is suggested to alter mitochondrial function through glycation of mitochondrial proteins. A direct relationship is demonstrated between excess intracellular formation of reactive species, intracellular formation of AGE from mitochondrial proteins, and decline in mitochondrial function (Rosca et al., 2005). Methylglyoxal (MGO), a highly reactive α-dicarbonil by-product of glycolysis, which readily reacts with arginine, lysine and sulphhydryl groups of both proteins and nucleic acids to form AGE, is significantly increased in diabetes (Ceriello, 2009). Diabetic complications such as retinopathy, nephropathy, and neuropathy are significantly associated with levels of AGE in the body. Increased levels of AGE in skin biopsies are found to be significantly associated with the outcome of micro-vascular complications (Genuth et al., 2005), and closely associated with incidence and severity of diabetic complications. Intensive control of glycaemia in insulin-dependent diabetes (IDDM) effectively delays the onset and slows down the progression of diabetic retinopathy, nephropathy, and neuropathy (DCCT, 1993). Five years of such treatment will significantly reduce various AGE/ALE in the body (30–32% lower furosine, 9% lower pentosidine, 9–13% lower CML), and increase the levels of soluble collagen (24% higher in acid-soluble collagen and 50% higher in pepsin-soluble collagen) (Monnier et al., 1999).

**22.10.7 Endocrine disorders**

Many, if not most, of the signs and symptoms of ageing, and age-associated diseases are strongly associated with multiple hormone deficiencies. Most consequences of ageing, such as excessive free radical formation, imbalance of the apoptosis systems, failure of repair systems, tissue accumulation of waste products, deficient immune system, poor gene polymorphisms and premature telomere shortening, are all associated, if not caused, by hormone deficiencies (Hertoghe, 2005). Up-regulation of putative pathological pathways, accumulation of advanced glycation end products, activation of the renin-angiotensin system, oxidative stress and increased expression of growth factors and cytokines are all intimately associated with ageing. However, little information is yet available about the content of AGE/ALE in endocrine organs and their influence on the body both in health or disease. With the exception of the ovaries, most of the endocrine organs – the pituitary gland, thyroids, parathyroids, adrenals and testes – are thus far almost totally unexplored. Increased serum AGE levels and increased activation of RAGE are reported in women with polycystic ovary syndrome (PCOS) (Diamanti-Kandarakis et al., 2005). A recent study reports that the content of AGE/ALE is twice as high in patients with PCOS as in healthy controls, and also strongly associated with signs of increased chronic inflammation; increases in homocysteine (Hcy), malonyldialdehyde (MDA), C-reactive protein (CRP) and with higher fasting insulin levels; and a higher homeostasis model assessment (HOMA) index (fasting glucose (mg dl−1) x fasting insulin (mU ml−1) x 0.055/22.5) (Kaya et al., 2009). Deposition of excess collagen in PCOS tissues that induce cystogenesis are suggested,
at least in part, to be due to stimulation by AGE (Papachroni et al., 2009).

**22.10.8 Gastrointestinal disorders**

Various gastrointestinal cancers and their ability to grow and produce metastases are associated to increased levels of AGE/ALE in the body and to increased activity of RAGE. Little, however, is known about an eventual association between accumulation in the body of these molecules or activity of RAGE and common inflammatory and ulcerative conditions in the gut. The only exception seems to be a recent study, which reports that the urinary concentration of pentosidine is significantly elevated in active compared to inactive IBD, ulcerative colitis (0.12 versus 0.021 mg mg⁻¹), and Crohn’s disease (0.071 versus 0.039 mg mg⁻¹) (Kato et al., 2008).

**22.10.9 Liver disorders**

Patients with liver cirrhosis demonstrate much increased AGE levels, which sometimes reach almost the same extent as in patients with end-stage renal disease (Sebekova et al., 2002). Serum levels of AGE (CML) are shown to be significantly affected by the stage of the disease in liver cirrhosis, and are closely associated with liver function capacity, as reported in a study of 110 patients with chronic liver disease (CLD) compared to 124 healthy controls (Yagmur et al., 2006). Furthermore, the level of AGE (CML) seemed to correlate well with levels of hyaluronic acid (HA) (r = 0.639, P < 0.0001). Glyoxal-derived adducts are suggested to be increased up to no less than 15 times in both portal and hepatic venous plasma of cirrhotic patients compared to healthy controls (Ahmed et al., 2005). A significant increase in glutathione and pro-regenerative cytokines TNF-α and IL-6 are observed, in addition to decreased hepatic necrosis and increased survival (Ekong et al., 2006). Much remains to be done to define the role of AGE/ALE and RAGE in the progression of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) and cirrhosis. However, recent observations of serum glyceraldehyde-derived AGE levels (U ml⁻¹) being significantly elevated in NASH patients (9.78) compared to those with simple steatosis (7, P = 0.018) and healthy controls (6.96, P = 0.003) are of significant interest (Hyogo et al., 2007). These authors also observed an inverse correlation to the level of adiponectin, an adipokine with insulin-sensitizing and anti-inflammatory properties. Immunohistochemistry of glyceraldehyde-derived AGE also showed increased staining in the livers of NASH patients.

**22.10.10 Lung disorders**

A variety of airway diseases such as asthma, acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis and idiopathic pulmonary fibrosis are all characterized by lack of homeostasis in the oxidant/antioxidant balance. Interaction of AGE/ALE and RAGE are well known to play a large, if not dominating, role in the depletion of antioxidants, particularly reduced glutathione (GSH) in lung epithelial lining and to play a key role in the pathogenesis of these disorders (Foell et al., 2003; Rahman et al., 2006a).

**22.10.11 Rheumatoid arthritis and other skeletomuscular disorders**

Among the highest levels of AGE in the body, and the strongest expression of RAGE, are found in inflamed tissues characterized for slow turnover, such as tendons, bone, cartilage, skin and amyloid plaques. These changes are associated with a slight change in colour towards yellow-brown and an increased fluorescence, all associated with
increased expression of pro-inflammatory cytokines and matrix metalloproteinases (MMP), especially MMP-1 and MMP-9. These manifestations are regarded as being responsible for the observed increased tissue stiffness and brittleness in structures such as intervertebral discs, bones, tendons, cartilages, synovial membranes and skeletal muscles, and are regarded as major pathogenic factors behind diseases such as osteoarthritis (DeGroot, 2004; Steenvoorden, 2006), rupture of inter-vertebral discs (Hornel and Eyre 1991), rupture of Achilles tendons (Reddy, 2004), menisci and also in rheumatoid diseases (Hein et al., 2005; Sunahori, et al., 2006), such as rheumatoid arthritis (Matsumoto et al., 2007) and fibromyalgia (Hein and Franke, 2002; Rüster et al., 2005). A significant increase in glycation of myosin occurs with age (Ramamurthy et al., 2001), and is most likely to contribute to age-associated muscular disorders. High levels of AGE/ALE in the body are also reported in patients with osteoporosis; significantly elevated levels of pentosidine and CML in serum (Hein et al., 2003) and significantly increased pentosidine in cortical bone (Odetti et al., 2005) being observed. It has also been observed that the remodelling of senescent bone is impaired by AGE, with both stimulation of bone-resorbing cytokines and enhancement of bone resorption by osteoclasts being observed (Miyata et al., 1996). A recent American study reports a significantly reduced bone density in older women consuming > 3 cola drinks per week when compared to matched controls consuming similar amounts of other carbonated soft drinks (Tucker et al., 2006). This information is especially interesting when one considers that cola drinks, much in contrast to other soft drinks, are considered rich in AGE. A recent in vitro study reports profound effects by cola-derived AGE: activation of platelets, an up to 7.1-fold increase in CD62 expression, and an increase of up to 2.2-fold in CD63 at the platelet surface membrane, also accompanied by increases in RAGE expression (Gawlowski et al., 2009).

The common belief that bovine milk prevents osteoporosis is today much questioned. Instead, increasing documentation suggests that it has quite the opposite effect, and that negative interactions of RAGE and AGE/ALE play a larger role in the pathogenesis of osteoporosis than lack of minerals.

### 22.10.12 Skin and oral cavity issues

The skin is one of, if not the largest, organ in the body. The health condition of the skin has a similar ability to the gingiva in the mouth, to reflect the total health of the body. Skin autofluorescence seems to be a good measure of cumulative metabolic stress and accumulation of advanced glycation end products in the body (Meerwaldt et al., 2005a,b). Accumulation of AGE/ALE in the skin relates to the content of these proteins in the body, and so is an expression of the risk of developing chronic diseases, in particular coronary heart disease. A recent study found a significant correlation between coronary calcifications and AGE/ALE in the body measured as skin fluorescence, suggesting such measurements could serve both as a marker of risk but also as a measurement of therapeutic success when patients are treated (Conway et al., 2010). Skin autofluorescence is especially suggested to be a method for prediction of the risk for progression of diabetic complications such as angiopathy, nephropathy and retinopathy, and the severity of disease and mortality in haemodialysis patients (Meerwaldt et al., 2005b).

The skin has a high density of RAGE receptors. AGE/ALE are known to accumulate in dermal elastine and in collagens, and to interact with dermal fibroblasts, inhibiting their proliferation capacity. A tenfold reduction in proliferation rate is described to occur normally in humans from the second to seventh decade (Stamatas et al., 2006), and is suggested as explaining the reduced healing capacity of age-related wounds, and especially chronic wounds, such as those on the diabetic foot. RAGE and AGE/ALE-induced apoptosis and enhanced loss of fibroblasts and osteoblasts are also regarded as major pathogenic factors in periodontal pathology, especially in chronic periodontitis (Holla et al., 2001). A 50% increase is observed compared with controls in RAGE mRNA in gingiva of diabetic patients (p<0.05) (Katz et al., 2005).
22.10.13 Urogenital disorders

Nephropathy is today more common than ever before and continues to increase, much in parallel to the increase in diabetes. It is the single most important cause of end-stage renal failure in the western world (Ostergaard et al., 2005). Consequently it receives great interest from scientists. Today no less than 1000 papers in PubMed deal with RAGE and AGE/ALE in renal diseases. Diabetic nephropathy today affects 15–25% of patients with type 1 diabetes, and as much as 30–40% of patients with type 2 diabetes. The kidney appears as both culprit and target of AGE/ALE, and it is well documented that RAGE is significantly activated, and advanced AGE/ALE markedly elevated in renal failure patients. Patients with mild chronic uraemic renal failure are reported to have plasma glycation-free adduct concentrations increased up to fivefold, patients with end-stage renal disease as much as 18-fold when on peritoneal dialysis, and up to 40-fold on haemodialysis (Agalou et al., 2005). A decrease in renal function and reduced clearance is observed much in parallel to increases in circulating AGE levels. AGE are involved in the structural changes observed in progressing nephropathies such as glomerulosclerosis, interstitial fibrosis and tubular atrophy (Bohlender et al., 2005). For detailed information, see recent excellent reviews (Vlassara et al., 2009; Daroux et al., 2010; Schepers et al., 2010). Kidney transplantation is reported to improve but does not fully correct the increased AGE/ALE levels in previously dialysed patients.

22.11 Foods Rich in AGE/ALE

It is a most interesting observation that increased accumulation of AGE/ALE in endothelial cells, and most likely also in some other tissues, can be significantly avoided or reduced by control of intake of foods known to contain these substances in large amounts. Thus far the information regarding content of AGE/ALE in foods is rather incomplete. Leading universities around the world are building institutions for studies of nutragenomics (how various food ingredients affect our health). However, from existing information it is clear that dysfunctioning proteins are especially rich in foods which have been subjected to industrial processing. The foods with the highest AGE content were animal-derived products exposed to high, dry heat such as broiling, frying and grilling. A detailed description of the database can be found in Goldberg et al. (2004). A brief summary is provided below.

- **Heated dairy products**: powdered milk (ice cream, baby and clinical nutrition formulas) cheese, especially when heated. High in pizza, tacos, nachos, salad dressings, fast food, sandwiches, sauces and brown cheeses.
- **Heated grain products**: bread (e.g. toasted bread, bread crusts, and crispbreads).
- **Heated meat, poultry, and fish**: especially bacon, sausages, fried and barbecued meat. The content of AGE/ALE increases as one goes from boiling to oven frying: boiling (1000 kU/serving) < roasting (4300 kU) < broiling (5250 kU) < deep frying (6700 kU) < oven frying (9000 kU/serving) (Goldberg et al., 2004).
- **Other heated foods**: egg yolk powder, lecithin powder, coffee (especially dark roasted), hard-cured teas, roasted and salted peanuts, dark and sugar-rich alcoholic beverages, broth, Chinese soy, balsamic vinegar, cola drinks, etc.

A recent study adds further and important information about dietary AGE (Uribarri et al., 2010). It should be observed that lean red meats and poultry contain high levels of dietary AGE. Even when cooked under dry heat, the explanation is that among the intracellular components of lean muscle there are highly reactive amino lipids, as well as reducing sugars such as fructose or glucose-6-phosphate. In the presence of heat, this combination rapidly accelerates new AGE formation (Uribarri et al., 2010). The highest AGE levels are observed in beef and cheeses followed by poultry, pork, fish, and eggs, while lamb ranked relatively low in AGE, at least when compared to other meats. Cheeses, butter, and different types of oils are AGE-rich, even in uncooked forms. High-fat spreads, including butter,
cream cheese, margarine and mayonnaise, are also among the foods containing the highest AGE, followed by oils and nuts. It should especially be observed that olive oil, for example, contains large amounts of AGE when heated to 100°C for 5 min (Uribarri et al., 2010). Carbohydrate-rich foods such as vegetables, fruits and whole grains contain relatively few AGE, even after cooking (Uribarri et al., 2010).

22.12 Prevention and Treatment of AGE/ALE Accumulation

22.12.1 Changing food preparation habits

It is clear that significant benefits will be obtained by reducing the intake of cheese, meats, powdered milk, other processed foods such as heated oils, and also of bread; and instead increasing the consumption of vegetables and fruits, especially when raw. These recommendations are in line with the policy of various expert organizations with the aim to reduce chronic diseases such as cancer, heart diseases and hypertension (cancer: American Cancer Society, 2006; heart: Lichtenstein et al., 2006; hypertension: US Department of Health and Human Services et al., 2006). AGE formation in food is reduced when cooking on surfaces that provide no direct contact with metal; when foods are immersed in acid solutions such as tomato sauce and ketchup; and when there is contact with aminoguanidine, a known inhibitor of AGE formation (He et al., personal communication, 2010). Eating foods raw or prepared at a low temperature (below 80°C), steam cooking or boiling, and minimal cooking are preferred over frying, grilling and microwaving, and also to roasting and salting. Recent information seems especially to warn against microwaving food, as this treatment dramatically accelerates the rate of AGE production (Visentin et al., 2010). A trial designed to compare the potential metabolic effects of two different diets, one based on mild steam cooking and another based on high-temperature cooking was recently reported (Birlouez-Aragon et al., 2010). A randomized crossover study assigned 62 volunteers (university students) to each of the two diets for four weeks. Consuming the steamed-cooked diet for 1 month induced significantly improved insulin sensitivity and also increased plasma levels of omega-3 fatty acids (217%, \( p = 0.002 \)), vitamin C (213%, \( p = 0.0001 \)), and vitamin E (28%, \( p = 0.01 \)), in comparison to the high temperature diet. Furthermore, reduced concentrations of plasma cholesterol (5%, \( p = 0.01 \)) and triglycerides (9%, \( p = 0.01 \)) were also reported.

A challenge for the future in the Western world is to find techniques to produce bread at 100°C or below as the Chinese have done for centuries. Marinating for some hours at room temperature with ingredients such as antioxidant-rich herbs, garlic, tea, red wine, onions, olive oil and beer are also known to significantly reduce the development of AGE/ALE, and this was also recently demonstrated for heterocyclic aromatic amines (Melo et al., 2008). Reduction in total intake of proteins (Uribarri and Tuttle, 2006), and most likely a particular reduction in methionine and other sulphur-containing amino acids, are additional issues of relevance (McCarty et al., 2009).

22.12.2 Energy restriction

Significant reduction in body content of AGE/ALE in comparison to controls (eating standard Western food) is observed in individuals, who for > 2 years practise what is called caloric restriction (CR). They eat only two thirds of what they would like to, and this is accompanied by significant health advantages compared to matched controls: lower blood pressure (102/61±7 versus 131/83 mmHg), and lower levels of markers of inflammation such as CRP (0.3 versus 1.9 mg l\(^{-1}\)), TNF-\(\alpha\) (0.8 versus 1.5 pg ml\(^{-1}\)), and TGF-\(\beta\) (29.4 versus 35.4 mg ml\(^{-1}\)) (Meyer et al., 2006). Elevated RAGE and low sRAGE is reported in patients with active rheumatoid arthritis (RA), but patients with RA practising CR for about 2 months demonstrated not only lower levels of pentosidine (an often-measured AGE) in urine, but also lower disease activity (Iwashige et al., 2004). Thirty-seven obese individuals (mean BMI of 28.3 ± 3.2) were treated with calorie restriction for 8 weeks. Reduction occurred in BMI (6.3%, \( p < 0.001 \)), waist
circumference (5.7%, \( p < 0.002 \)), triglycerides (11.9 % \( p < 0.002 \)), and AGE (7.21%, \( p < 0.001 \)) (Gugliucci et al., 2009). FEV1, an expression of respiratory capacity, almost doubled.

### 22.12.3 Antioxidants and vitamins

Provision of vitamins such as A, B (especially B₆ and B₁₂), C, D, E, and K, as well as glutathione and folic acid, is often emphasized. Many plant antioxidants, particularly those collectively defined as polyphenols, have documented oxidation-quenching properties up to ten times more powerful than conventional vitamins. They have also been shown to have great chemo-preventive properties, a marked ability to prevent accumulation in the body of AGE/ALE, and significant capacities to reduce inflammation in the body and to prevent reduction in organ function and premature ageing (Delmas et al., 2005; Osawa and Kato, 2005; Bengmark, 2006; Rahman et al., 2006b; Sun et al., 2010). Such plant antioxidants exist in nature in many thousands – most probably hundreds of thousands – of different compounds. More than 4000 flavonoids alone have been identified, and almost 1000 carotenoids. Here are the most studied: isothiocyanates in cruciferous vegetables; anthocyanins and hydroxycinnamnic acids in cherries; epigallocatechin-3-gallate (EGCG) in green tea; chlorogenic acid and caffeic acid in fresh coffee beans and also in fresh tobacco leaves; capsacin in hot chilli peppers; chalcones in apples; euginol in cloves; gallic acid in rhubarb; huperitin and naringenin in citrus fruits; kaempferol in white cabbage; hisperitin and naringenin in citrus fruits; kaempferol in white cabbage; myricetin in berries; rutin and quercetin in apples and onions; resveratrol and other procyanidin dimers in red wine and virgin peanuts; various curcumenoids, the main yellow pigments in turmeric curry foods; and daidzein and genistein from the soy bean.

### 22.12.4 Supplementing histidine, taurine, carnitine or carnosine

Supplementing the diet with histidine, taurine, carnitine or carnosine has also been reported to assist in protecting the body from AGE/ALE (Nandhini et al., 2004, 2005). No vegetarian food contains taurine, with the exception of certain algae. This important amino acid is only obtained from eating animal-derived foods – meat, poultry and fish.

### 22.12.5 Pharmaceuticals

Several pharmaceuticals, especially those used for treatment of diabetes, are reported to reduce the content of AGE/ALE in the body, at least in short-lived tissues; that is, those with high turnover.

### 22.13 Pro- and Synbiotics

Probiotics and synbiotics have a dual role in reduction of dietary AGE/ALE as they both metabolize these substances (Erbersdobler et al., 1970; Finot and Magnenat, 1981; Faist and Erbersdobler, 2001; Faist et al., 2001, Wiame et al., 2002) and also release important vitamins and antioxidants with documented preventive effects against AGE and ALE. A rich intestinal flora is regarded as necessary for the release and absorption of various important antioxidants. However, the increased intake of refined food and deficient intake of fresh fruits and vegetables among Westerners has led to a significant reduction in both density and diversity of the flora. This reduction is especially pronounced for strong fibre-fermenting lactic acid bacteria (LAB) such as Lactobacillus plantarum and L. paracasei. Seventy-five per cent of omnivorous Americans and 25% of vegetarians in the United States lack L. plantarum (Finegold et al., 1983). A more recent Scandinavian study found L. plantarum in only 52% and L. paracasei in only 17% of healthy individuals (Ahrné et al., 1998). This information is particularly interesting, as L. plantarum and L. paracasei belong to the small group of intestinal bacteria with ability to break down semi-resistant fibres such as inulin (Müller and Lier, 1994), reduce inflammation, reduce infection, and eliminate pathogenic bacteria such as Clostridium difficile (Naaber et al., 2004). Some specific LAB might well have the ability to
eliminate AGE/ALE from foods, in a way that is very similar to that demonstrated for gluten (di Cagno et al., 2005) and heterocyclic amines (Tavan et al., 2002). In vitro studies have shown that fructoselysine, the dominating AGE in heated milk, can be effectively eliminated when incubated with fresh intestinal flora (Erbersdobler et al., 1970).

22.14 Conclusions

There is growing consensus that the dietary intake or endogenous production of AGE/ALE is associated with a diverse range of disorders, although the underlying mechanisms remain largely obscure. This chapter has focused on the dietary sources of AGE/ALE and on the effects of heat treatment in the production of these dysfunctional adducts during the processing of foods. The accumulation of AGE has been documented in conditions such as Alzheimer’s disease, cardiovascular disease, cancer, diabetes, lung disease, liver disease and other disorders, and this research evidence has also been reviewed in this chapter.

It is increasingly clear that the intestinal microflora and its more than 2 million metagenes play a key role in health and disease. Western lifestyle is clearly associated with a deranged microbionta, with reduced diversity and an increased quotient between gram-negative and gram-positive bacteria, which in association with reduced barrier function seems to contribute to the observed elevated systemic inflammation. Patients with metabolic derangements such as obesity and with chronic diseases are known to have increased blood levels of endotoxin, a product of gram-negative bacteria. Recent in vitro observations suggest that human AGE-modified albumin and lipopolysaccharide (LPS) exhibit a synergistic effect on proinflammatory cytokine/chemokine interleukin-6, interleukin-8, and monochoeom ATTR activity protein-1 production in human endothelial cells (Liu et al., 2009). A link exists between fat intake and accumulation of endotoxin in the blood (endotoxaemia), and recent studies demonstrate that intake of emulsified fat in particular (water-in-oil emulsions such as butter; free oil or dispersed fat inclusions in cheeses, cookies, ice cream and dressings), which is known to affect the kinetics of lipid absorption, increases both endotoxaemia and inflammation (Laugerette et al., 2010).

It is now well documented in the literature that a healthy lifestyle has profound effects on health and well-being. Control of what we eat is an important component within such a programme. Studies suggest reductions of as much as 83% in coronary heart disease (Stampfer et al., 2000), 91% in diabetes (Hu et al., 2001), and 71% in colon cancer (Platz et al., 2000) in patients adhering to a ‘healthy lifestyle’ (such as no use of tobacco, moderate use of alcohol, regular physical exercise, and controlled food intake). To these four factors must be added control of stress. Numerous studies demonstrate that both physical and mental stress increases the degree of inflammation in the body and activates RAGE (Kjaer, 2004; Bierhaus et al., 2006; Chida et al., 2006). Control of intake and endogenously produced AGE/ALE, will, together with restrictions on the intake of fat and carbohydrate-rich foods, significantly improve health and well-being. However, only a fraction of consumers are willing to consider this option. A study in the United States (Reeves and Rafferty, 2005), suggests that only about 3% adhere to the principles advocated above.

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Modified Amino Acid-Based Molecules


23 Phenylketonuria: Newborn Identification Through to Adulthood

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23.1 Abstract

The discovery of phenylketonuria (PKU) in 1934 by Asbørn Følling has led to many advances involving identification, diagnosis, classification, and treatment not only for PKU, but for many other inborn errors of metabolism. It took 20 years after the discovery before the first amino acid mixture was introduced for treatment, and another ten years for newborn screening tests to become available in developed nations. The successful identification and treatment of PKU has reversed the profound mental retardation associated with this disorder, and those affected and treated can now lead essentially normal lives. It is remarkable to think that simple restriction of the amino acid phenylalanine (Phe) could have such an effect. However, the dietary treatment is not ideal and maternal PKU remains a problem. It is difficult for many women with PKU to achieve low blood Phe levels throughout the pregnancy. High levels are harmful to the baby and the diet is so restrictive it may be lacking in essential nutrients. However, the past ten years have seen a surge in research, new products, and new treatment modalities. There are many different forms of medical food products available that include flavoured powder, gels, bars, capsules, and tablets to make adherence easier. However, since much of the diet is synthetic there are reported nutrient deficiencies which affect growth and cognitive development. New treatment modalities are also available with the use of large neutral amino acids (LNAA) and glycomacropeptide. In 2007, Kuvan®, a new drug for the treatment of PKU, was introduced. This is the synthetic form of tetrahydrobiopterin (BH₄) and acts on the phenylalanine hydroxylase which stimulates enzyme activity, lowering the blood Phe in those who respond. Gene therapy continues to be researched and enzyme substitution is currently in clinical trials. Looking back over 75 years since the discovery of this disorder, we have come a long way. With continuing research, new products and treatment modalities, new guidelines will need to be implemented and perhaps a new paradigm of treatment strategy will emerge.

23.2 Introduction

Asbørn Følling was a Norwegian physician who fortunately also had a biochemical background. His discovery of 'imbecillitas
Phenylketonuria

phenylpyruvica' laid the co-groundwork for the successful accomplishments involving identification, diagnosis, classification, treatment, and progress for the next 75 years, bringing the PKU story to an apparent conclusion (Folling, 1934). It is remarkable to realize the significant progress that has been made in conquering a newly identified metabolic disorder that in the past caused profound mental retardation, in comparison to our present situation when a newborn baby with PKU can be expected to develop normally, attend school, marry, have children, and live a normal life. Dr Folling's accomplishment provided the opportunity and stimulus to lay the ground-work for an entirely new approach to the diagnosis and care of children with various other metabolic disorders. Archibald Garrod (1902) is to be honoured for initiating an interest in metabolic disorders; however, it is unlikely that Dr Folling was aware of the work of Garrod. Folling's discovery of PKU has resulted in the salvaging of many affected persons, allowing them to live normal lives.

23.3 Background

Prior to Folling's identification of PKU and even 20 years afterward, affected persons in the United States were eventually institutionalized in large facilities because no treatment was available. In 1954 Dr Horst Bickel, at the Manchester Children's Hospital in the United Kingdom (Bickel, et al., 1953), developed the first Phe-restricted diet, which became the foundation for the treatment of PKU. It was found that young children with PKU improved clinically and psychologically when treated with this diet; however, older severely mentally retarded persons usually did not benefit substantially.

In 1960 Robert Guthrie (Guthrie and Susi, 1963) developed a newborn screening blood test to identify babies with PKU. He was motivated after his wife's niece was identified to have PKU through the use of a ferric chloride test of the child's urine. Unfortunately, this child was already mentally delayed by the time the diagnosis was made. Dr Guthrie subsequently obtained a grant from the United States Children's Bureau, a Federal agency, to establish the accuracy of this test for PKU. Surprisingly, the development of newborn screening for PKU created controversy among members of the medical profession. It was thought that the test was not very accurate. Some physicians believed treatment for PKU would not be beneficial, while others thought this was the beginning of socialized medicine.

Our experience in treating late-diagnosed children with PKU using the Phe-restricted diet demonstrated that their intelligence did not degenerate, but improved over time with treatment. In 1964, one of us (R.K.) applied for a Federal grant to document these preliminary findings (Azen et al., 1991). The Collaborative Study of Children Treated for PKU was a prospective study of infants. Two hundred and eleven newborns identified in 14 states were enrolled and monitored for 12 years. It was shown clearly that the subjects who were maintained on the Phe-restricted diet for ten years had superior intellectual function than those who maintained the diet for only five years. The data were published by Azen et al. (1991).

23.4 Current Problem: Maternal PKU Therapy

In 1980 Harvey Levy MD, the head of the Harvard PKU programme at Boston Children's Hospital, and R. Lenke (Lenke and Levy, 1980) were the first physicians to do a worldwide assessment of maternal PKU (MPKU) problems. Initially it was thought that women with PKU could have normal children, regardless of whether or not they were on a diet restricted in Phe. However the Levy report clearly showed that women with PKU who have blood Phe levels greater than 1204umol 1⁻¹ during pregnancy have a high possibility of giving birth to severely mentally retarded children. Levy reported that over 90% of the infants born to these women suffered from microcephaly and 12-14% had congenital heart disease. The rate of abnormality depended
upon the blood Phe levels that were documented in the mothers. These data led physicians to advise women with PKU not to have their own children, but rather to adopt if they wanted a family.

At the Children’s Hospital of Los Angeles, dietary treatment was never discontinued; accordingly, during pregnancy, women with PKU were kept on the diet and blood Phe levels of 120–360 μmol l⁻¹ were recommended. Fortunately the rate of microcephaly in their infants was very low (2–3%) and congenital heart disease was 1%. As a result the National Institute of Child Health and Human Development decided to fund a long-term study on MPKU, which involved 430 pregnancies (Koch et al., 2003a). Unfortunately 90% of clinics were discontinuing dietary therapy between 1970 and 1980 because it was mistakenly thought that PKU patients could eat a normal diet because the brain growth was completed by the age of 6 years. (The reader is referred to Pediatrics 112 (6) December 2003 supplement for additional details.) Many of the mothers who were enrolled in the study had intelligence quotients between 70 and 90 and a significant number of them did not start the Phe-restricted diet until late in the first trimester. Four mothers did not actually join the study until they were in their third trimester. The overall results were disappointing. Twenty four percent of the offspring exhibited a head circumference of 32 cm or less and 7% of the women had babies with congenital heart disease. The study did demonstrate that women who planned their pregnancies and kept their blood Phe levels at 120–360 μmol l⁻¹ had babies with normal head circumferences of 33–36 cm. The rate of congenital heart disease in this group was 1–2%.

23.5 The Role of Tetrahydrobiopterin (BH₄) Treatment for Patients with PKU

In 1999 Professor Shigeo Kure in Japan published the first paper on treatment of five children with PKU who received BH₄ therapy during infancy and demonstrated significant improvement in blood Phe levels (Kure et al. 1999). Historically, of course, BH₄ was discovered at the National Institute of Neurological Disorders in Bethesda, Maryland (Kaufman 1963). However nearly all of the earlier studies were performed on animals and it was not tried on humans until the discovery of BH₄ metabolic defects in 1974 (Kaufman et al., 1975). BH₄ was found to have no toxicity and to be well tolerated. There are six biochemical steps in the human body resulting in biopterin production. Fortunately defects in biopterin production are rare. In the first author’s 55 years of caring for patients with PKU, the only patient we have seen with a biopterin defect died in early infancy. Thus it was not until Dr Kure published his initial results that BH₄ was used to treat patients with PKU. Why is BH₄ important in patients with PKU? BH₄ is a cofactor required by Phe hydroxylase (PAH) to convert Phe to tyrosine, which in turn is then converted into dopamine in human metabolism. It may be in some obscure way related to schizophrenia. In addition, BH₄ is a cofactor for nitric oxide synthase (NOS), an enzyme important in brain metabolism. NOS is involved in the transmission of signals between neurons. The reader is referred to Chapter 168 in Scriver et al. (2001) for further details regarding NOS metabolism.

At present we have only two years of experience with this new product, however it has created a great deal of excitement. Persons who have mild hyper Phe due to mild mutations of the PKU gene may actually go off diet restriction of Phe altogether. Persons with one mild mutation and one severe mutation may exhibit a significant reduction of the blood Phe levels – by as much as 30%. Persons with two severe mutations, such as R408W/R408W rarely show a reduction in blood Phe levels, but do feel better, have more energy, and display improvement in behaviour (first author’s personal observation). So we do not yet know the extent of the usefulness of BH₄ in the treatment of PKU. The first author would predict that all patients with PKU will eventually be taking BH₄ in addition to dietary restriction of Phe. In addition, the role of BH₄ in maternal PKU may become very important.
23.6 Dietary Therapy

The dietary management of PKU is a success story. Diet therapy implemented shortly after birth prevents the devastating outcomes associated with untreated PKU which includes severe cognitive delays, neurological deterioration, eczema, seizures and epilepsy, and progressive motor disorder. The mainstay of the treatment has been the Phe-restricted diet. Careful management and adherence to the dietary recommendations are essential for a good outcome. To date there is considerable variation on the recommended plasma blood Phe concentrations to be maintained at various ages. But most countries, including the United States, recommend blood Phe levels <360 μmol 1^{-1} under the age of six years (NIH, 2000; Schweitzer-Krantz and Burgard, 2000). Maintaining the blood Phe concentrations is based on tolerance, which is the amount of Phe ‘tolerated’ for blood Phe concentrations to remain in the recommended ranges. However, the Phe-restricted diet is not easy to maintain. The standard diet consists of an amino acid mixture that contains all the necessary nutrients for normal growth except for Phe. The diet restricts high protein foods such as meat, poultry, fish, pork legumes, nuts and dairy products. Fruits, vegetables and a limited amount of grains are used in combination with low protein foods to provide a regulated amount of Phe in order to keep blood Phe levels within the recommended range. For many individuals the taste of the amino acid mixture is unpalatable and the amount that must be consumed is unattainable. Because the majority of this diet is synthetic with very low amounts of natural food, the diet is suboptimal as impaired growth, bone density, and other nutritional deficiencies have been reported.

During the 1960s when the diet for PKU was being established, there were reports of poor growth which was attributed to ‘Phenylalanine deficiency syndrome’. In fact, two infants were reported to have been fed the Phe-restricted diet in error during the first few weeks of life and suffered failure to thrive, eczema, listlessness, and developmental delay (Rouse, 1966). Hanley et al. (1970) also reported malnutrition in the first year of life due to over-restriction of Phe as possibly contributing to mental retardation. In addition, the insufficiency of Phe may have led to growth deficiencies, as reported in a study in Germany where height and head circumference were decreased significantly during the first two years of life despite adequate weight gain and protein intake (Schaefer et al., 1994). The National PKU Collaborative Study conducted from 1967 to 1983 showed that normal physical growth was achievable using a protocol with prescribed amounts of protein and energy (Holm et al., 1979). In North America the growth status in PKU individuals on the Phe-restricted diet has been essentially normal compared to growth data from the National Center for Health Statistics (NCHS) in height and head circumference. However, PKU males and females weighed more and the growth curves suggested that this weight difference was related to diet adherence. High blood Phe concentrations were associated with higher weights, especially in females (McBurnie et al., 1991). Additionally, high blood levels of leptin, a hormone associated with obesity, has been reported in individuals with PKU and high blood Phe concentrations (Schulpis et al., 2000). Nutrient intakes and physical growth were assessed in PKU individuals using three different amino acid mixtures (Acosta et al., 2003). Outcome of this report revealed no statistical difference in mean z scores for height and body mass index (BMI) between the three groups. However, there is a suggestion of a trend towards obesity in those with PKU.

Most clinics in the United States use the Ross Protocols (Acosta and Yannicelli, 2001) for initiating the protein prescription which can be as much as 110–130% over the recommended dietary intakes (RDI). Huemer et al. (2007) reported the results of growth and body composition in children with classic PKU, showing that there were no statistical differences in growth or body composition or in fat-free mass in PKU subjects and age/sex-matched controls. Phe-free amino acid mixtures exceeded the RDI by a mean 20–40%. There was also a significant correlation between fat-free mass and natural protein intake (Huemer et al., 2007). A significant correlation between natural protein intake and
head circumference was also reported suggesting that improvement of protein quality may improve growth and body composition in PKU (Hoeksma et al., 2005).

There are also a number of reports indicating subtle cognitive deficits in individuals with PKU who are well treated (Waisbren et al., 1994; DeRoche and Welsh, 2008). A comprehensive review of executive abilities was performed in 2010 and reported a number of studies finding information-processing speed, fine motor control, perception, and visual-spatial abilities are compromised in those with PKU, with conflicting reports on assessing language, learning, and memory impairments (Jänzen and Nguyen, 2010). There is no clear delineation on the specific mechanisms for these impairments. However, dopamine deficiency and white matter abnormalities are highly suggestive (Christ et al., 2010). In contrast, the pathogenesis of cognitive dysfunction hypothesized by de Groot et al. (2010) is reduced cerebral neurotransmitter and protein synthesis, caused by impaired brain uptake of the non-Phe LNAA with elevated blood Phe concentrations. Looking at the dietary intake and nutrient deficiencies may provide some answers.

It is well known that docosahexaenoic acid (DHA) (22:6n-3) and arachidonic acid (ARA) (20:4n-6) are very important structural components of the central nervous system and are transferred across the placenta, accumulating in the brain and other organs during the development of the fetus (Martinez, 1992). Studies have shown that DHA is vital throughout pregnancy in the first few weeks of brain cell division and the last trimester, when the content of the cerebrum and cerebellum increase three- to five-fold. There is a significant rapid increase in brain DHA from birth to 2 years of age. DHA is also the most abundant omega-3 fatty acid in the retina of the eye, which has the highest DHA concentration of any other organ, and is important for visual acuity (Dobbing and Sands, 1973). DHA facilitates many functions in the body including the regulation of gene expression, regulation of synthesis of eicosanoids derived from ARA, maintenance of membrane fluidity, protection of neural cells from apoptotic death, and regulation of nerve growth factor and neuron size; it is also needed for dopaminergic and serotonergic neurotransmission (Sinclair et al., 2002). Deficiencies of this very important essential fatty acid have been reported in individuals with PKU.

As early as 1973 (Acosta et al., 1973) long-chain polyunsaturated fatty acids (LCPUFA) have been found to be deficient in individuals with PKU, both treated and untreated (Galli et al., 1991; Sanjurjo et al., 1994; Giovannini et al., 1995; Moseley et al., 2002). Since then there have been many reports in the literature documenting deficiencies (specifically in DHA) and normalization after supplementation. A randomized controlled trial was conducted showing that supplementation of LCPUFA in infants with PKU prevented the decline in DHA (Agostoni et al., 2006). It was also shown that supplementation of LCPUFA including DHA improved visual function in children with well-controlled blood Phe levels, as well as improvement in fine motor skill and coordination (Agostoni et al., 2000; Beblo et al., 2007; Koletzko et al., 2009). The study of essential fatty acids, especially DHA, is an emerging field of study and may contribute to the neurocognitive deficits that are seen in treated PKU individuals. It will be interesting to see over time if the individuals who are now supplemented in early infancy have the same deficits.

Since the primary source of the Phe-restricted diet is a synthetic medical food product many nutrients may not be absorbed or bioavailable. Deficiencies of iron, zinc, selenium, and Vitamin B₁₂ have also been found in individuals with PKU (Acosta, 1996, 2004; Lombeck et al., 1996; Van Bakel et al., 2000). Vitamin B₁₂ deficiency was also reported in a young female who was not taking her medical food product on a consistent basis yet eating very low protein foods. She presented with spastic paraparesis, tremor disorientation, slurred speech and deteriorating mental function with megaloblastic anaemia (Hanley et al., 1996). After routinely checking complete blood count, ferritin, MCV, B₁₂ and RBC folate, another 12 individuals were found with suboptimal B₁₂ levels. Supplementation reversed some of the symptoms in some individuals but not all. Deficiencies of Vitamin B₁₂ were also found in patients on an unrestricted or relaxed diet.
Phenylketonuria (Robinson et al., 2000). Additionally, PKU patients on a strict low Phe diet had low levels of vitamin $B_6$, vitamin $B_{12}$ and folate which resulted in high blood levels of homocysteine; this may contribute to coronary artery disease later in life (Schulpis et al., 2002; Hvas et al., 2006). All of these deficiencies may impair normal growth and cognitive development.

Another area of concern is the bone health of individuals with PKU on restricted diets. A recent study of 31 patients reported osteopaenia in 42% of the subjects. Compromised bone density with no clear cause was found in 42% of the subjects (Modan-Moses et al., 2007). Other studies also report decreased bone mineralization, increased excretion of bone resorption markers and high osteoclast activity with no clear cause in individuals with PKU (Hillman et al., 1996; Przyrembel and Bremer, 2000; Millet et al., 2005; Porta et al., 2008). Therefore, it is critical to monitor other nutritional markers that warrant supplementation in addition to blood Phe levels for normal growth and development.

Within the last ten years research in the area of PKU has surged as well as the availability of new products and treatment modalities. Phe-free amino acid mixtures are available in flavoured and unflavoured powdered form, gels, capsules, tablets, amino acid bars, and juice box drinks. They are also available fat free, low calorie, low volume, and high protein and tailored to each individual’s needs (see Table 23.1). A number of products are now also supplemented with omega-3 fatty acids and DHA. The low-protein foods available to be used in conjunction with the diet have increased greatly in variety. There are low-protein pizzas, pastas, burgers, hot dogs, tortillas, cheese, yogurt, egg substitutes, a large variety of breads (including bagels, rolls, and buns), as well as a variety of ready-to-eat foods. However, up until recently, these foods contained trans fatty acids and very few nutrients. Several new treatment modalities are now available with the large neutral amino acid therapy, glycomacropeptide and BH$_4$.

Table 23.1. Medical food manufacturers.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Products</th>
<th>Website</th>
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<tbody>
<tr>
<td>Nutricia</td>
<td>Powder, capsules, tablets, ready-to-drink, amino acid bars, add-ins, LNAA, low-protein food products</td>
<td><a href="http://www.nutricia-na.com">www.nutricia-na.com</a></td>
</tr>
<tr>
<td>SHS International</td>
<td>powders, add-ins, low-protein food products</td>
<td><a href="http://www.myspecialdiet.com">www.myspecialdiet.com</a></td>
</tr>
<tr>
<td>Abbott Nutrition</td>
<td>Powder</td>
<td><a href="http://www.abbottnutrition.com">www.abbottnutrition.com</a></td>
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<tr>
<td>Mead Johnson</td>
<td>Powder</td>
<td><a href="http://www.meadjohnson.com">www.meadjohnson.com</a></td>
</tr>
<tr>
<td>Applied Nutrition</td>
<td>Powder, amino acid bars, amino acid blends, LNAA, low-protein food products</td>
<td><a href="http://www.medicalfood.com">www.medicalfood.com</a></td>
</tr>
<tr>
<td>VitaFlo USA</td>
<td>Gel, powder, ready-to-drink (DHA added)</td>
<td><a href="http://www.vitafloweb.com">www.vitafloweb.com</a></td>
</tr>
<tr>
<td>Solace</td>
<td>Tablets, powder, LNAA</td>
<td><a href="http://www.solacenutrition.com">www.solacenutrition.com</a></td>
</tr>
<tr>
<td>Cambrooke Foods</td>
<td>Ready-to-drink, glycomacropeptide products</td>
<td><a href="http://www.cambrookefoods.com">www.cambrookefoods.com</a></td>
</tr>
<tr>
<td>Arla Food Ingredients</td>
<td>Glycomacropeptide</td>
<td><a href="http://www.arlafoodingredients.com">www.arlafoodingredients.com</a></td>
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<tr>
<td>Low-protein foods only</td>
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<td>Dietary Specialties</td>
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<td>Ener-G-Foods</td>
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<td>Specialty Food Shop (Canada)</td>
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<tr>
<td>Glutino (Canada)</td>
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<tr>
<td>Promin-PKU (United Kingdom)</td>
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<td><a href="http://www.promin-pku.com">www.promin-pku.com</a></td>
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</tbody>
</table>
23.6.1 Large neutral amino acids

The use of the large neutral amino acid (LNAA) therapy has increased within the last few years because adherence to the Phe-restricted diet has decreased in the adolescent and adult population (Walter et al., 2002). The LNAA are amino acids that share a common transporter into the brain and compete with one another. Since Phe is so high in the blood it overwhelms the carrier and other LNAA do not get on. By giving large doses of LNAA except Phe the concentrations of other amino acids is increased (Christensen, 1953; Pardridge and Olendorf, 1975; Andersen and Avins, 1976; Olendorf and Szabo, 1976; Pratt, 1980). Although the exact mechanism for the brain damage in PKU is not known, it is believed the cause is decreased brain protein synthesis due to the lack of other LNAA, which results in increased myelin turnover and abnormalities in amine neurotransmitter systems (Surtees and Blau, 2000). Many studies including magnetic resonance spectroscopy (MRS) that actually can measure Phe in the brain have shown that LNAA can block the entry of Phe into the brain thereby reducing brain Phe (Knudsen et al., 1995; Pietz et al., 1999). Our six-month study conducted on six individuals with PKU used LNAA with enhanced amounts of tyrosine and tryptophan, and was found to lower brain Phe and increase the blood levels of tyrosine, as well as reduce the Phe/Tyr ratio. However, there was no significant effect on blood Phe (Koch et al., 2003b). Another study conducted reported giving 0.5 g kg⁻¹ in three divided doses to eight subjects and 1 g kg⁻¹ to three patients for 1 week; blood Phe was reduced by 50% in both groups ((Matalon et al., 2006). He also conducted a double-blind placebo study in 20 patients for 1 week and reported an average 39% decline in blood Phe (Matalon et al., 2007). A double-blind placebo crossover study using LNAA and MRS was also performed showing no correlation between brain and blood Phe when the blood Phe was under 1200 μmol l⁻¹, but a positive effect on executive function (Schindeler et al., 2007).

The dietary aspects of the LNAA therapy are the opposite of the Phe-restricted diet. The amount of protein in the Phe-restricted diet is approximately 20% natural protein and 80% synthetic protein coming from the medical food product. In the LNAA therapy 20% comes from the medical food product and 80% comes from natural protein. We have used this therapy in our clinic for the past 7 years in adults and adolescents. However, it may be necessary to provide a protein source without Phe (medical food product) since many individuals may not eat enough natural protein to meet nutritional requirements. A list of manufacturers of the medical food products and low-protein foods is presented in Table 23.1.

23.6.2 Glycomacropeptide

Another new approach to dietary treatment is glycomacropeptide. This compound is a protein in cheese whey, contains 64 amino acids and the commercial product contains 2.5-5 mg Phe g⁻¹ of protein (Table 23.1). Its branched-chain amino acids compare to average dietary protein and it contains high amounts of threonine and isoleucine. In studies using glycomacropeptide, PKU mice showed significant decreases in both blood and brain Phe concentrations. In one case report a 29-year-old male completed a 15-week trial comparing glycomacropeptide to his usual medical food product. The glycomacropeptide products were an orange sports beverage, a pudding and a snack bar and were well accepted. The amino acid profile showed significant increases in plasma concentrations of LNAA and a 10% reduction in blood Phe with glycomacropeptide compared to the usual medical food product (Kyungwha et al., 2007; Ney et al., 2008, 2009). Because glycomacropeptide is an abundant food ingredient, naturally low in Phe, it may provide improved protein synthesis for growth due to the higher quality of protein. Long-term studies are needed to evaluate those parameters.

23.6.3 Tetrahydrobiopterin

Kuvan® is the name of the new BH₄ drug approved by the FDA in the United States in 2007. The dose ranges from 5–20 mg kg⁻¹ and
is shown to be well tolerated and effective in those who respond. Individuals who respond experience a drop in blood Phe concentrations and an increase in tolerance as well. A response is considered a drop in blood Phe concentrations of at least 30\% (Burton et al., 2007; Kuvan, 2007; Levy et al., 2007). The majority of individuals who respond have mild or moderate PKU and are able to increase their tolerance to allow more natural foods. A recent study reported that the use of BH$_4$ allowed the liberalization of the diet in individuals with mild and moderate PKU, and improved LCPUFA status (specifically DHA) (Vilaseca et al., 2010). The use of BH$_4$ in normalizing the diet may prevent many of the deficiencies now seen in the Phe-restricted diet. It may also be especially important in MPKU, as the diet is so restrictive and very difficult for many women to consume the medical food products. It is labelled as a ‘Pregnancy Category C’ by the FDA, indicated by animal reproduction studies showing adverse effects on the fetus at 600 mg kg$^{-1}$ which is 30 times the recommended dose. There are limited studies in the literature. Recently we reported the use of BH$_4$ on two women who were also following the Phe-restricted diet and both had a normal outcome (Moseley et al., 2009). The authors have followed six women with PKU who were taking small doses of BH$_4$ (300–600 mg) along with the Phe-restricted diet, with normal outcomes.

23.7 Future Research

Gene therapy research in PKU has been ongoing for the last two decades and when a therapeutic agent that is not harmful is introduced it perhaps will be the cure. However, much more research is needed. Currently, PEGylated recombinant phenylalanine ammonia lyase (PEG-PAL) is in Phase 2 clinical trials. This is enzyme-substitution therapy. PAL acts as a surrogate for the deficient PAH and converts the excess Phe to non-toxic trans-cinnamic acid and insignificant levels of ammonia. This new treatment could improve current therapy (no diet restriction) and increase quality of life (Sarkissian et al., 2009).

23.8 Conclusions

There is no question that Dr Folling’s discovery of PKU, Dr Bickel’s development of the Phe-restricted diet and Dr Guthrie’s discovery of the newborn screening test are significant events. The contributions of Dr Savio Woo for his discovery of the structure of the PAH gene; and those of Dr Charles Scriver for the development of the PKU gene map, cataloging the various mutations of the gene, have provided significant possibilities in understanding and studying PKU.

The new discovery that BH$_4$ can improve treatment for many people with PKU has been a significant development. More studies need to be performed for use in pregnancy, as the use of BH$_4$ may improve the outcomes due to the action on nitric oxide which may improve neuronal development in the fetal brain. Additionally, for those who respond, it will allow the consumption of more natural protein while maintaining low Phe blood concentrations.

The dietary treatment of PKU has progressed and many products and treatment options are now available. Individuals with PKU who were diagnosed in early infancy and followed the recommendations can have a positive outcome. However, as stated earlier, many adults and adolescents are not following the recommendations and many are doing very well. This may be due to many factors such as strict diet adherence during formative years, continuance of medical food products into adolescence and adulthood but no phe-restriction, or just the many variations of PKU. Therefore, more studies are needed about aging with PKU in order to develop sound recommendations. There are no clear guidelines as to how long the diet must be maintained and what the adult blood Phe concentrations should be. It has been 10 years since the National Institutes of Health (NIH) Consensus Conference for Phenylketonuria was conducted, and new information and treatments have emerged. Currently the NIH is putting together a task force and will be conducting another conference. Also, the Genetic Metabolic Dieticians International (GMDI) is collaborating with the Southeast Region Genetics Collaborative and the Health Resources and
Services Administration (HRSA) to develop nutrition guidelines. With the conglomeration of all reviews of the literature and our current knowledge of PKU in the development of these guidelines, perhaps a new paradigm of treatment strategy will be established.

References


Phenylketonuria


24 Principles of Rapid Tryptophan Depletion and its Use in Research on Neuropsychiatric Disorders

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24.1 Abstract

Serotonin (5-HT) is a neurotransmitter which plays an important role in many psychiatric disorders. Existing evidence on the central nervous effects of 5-HT relies to a great extent on pharmacological investigations. Many studies used the administration of selective serotonin reuptake inhibitors (SSRI), which allow the investigation of how an increase in central nervous 5-HT neurotransmission influences behavioural characteristics and neural functioning. However, in order to achieve a central nervous dysfunction in 5-HT neurotransmission in animals and humans, a different approach called rapid tryptophan depletion (RTD) can be used. The fundamental concept of RTD builds on the administration of a tryptophan-free diet within an amino acid drink lacking tryptophan, the physiological precursor amino acid of 5-HT. RTD allows a short-term depletion of 5-HT synthesis in the brain. Following this there is a close link between nutritional intake of essential large neutral amino acids (such as tryptophan) and serotonergic neurotransmission in humans. The depletion of central nervous 5-HT allows the study of behavioural and neural effects of this deficit by combining RTD with behavioural test procedures, genetic markers, and imaging techniques. The data obtained under depleted conditions in such studies can serve as a human model for a central nervous 5-HT deficit, which is thought to play a decisive role in a variety of neuropsychiatric disorders. The following chapter gives an overview on the basic principles of RTD and how it can be used in an experiment involving both healthy subjects and patient populations.

24.2 Introduction

The neurotransmitter serotonin (5-hydroxytryptamine (5-HT)) was shown to be involved in the underlying pathophysiology of a variety of neuropsychiatric disorders, including affective disorders (in particular depression), as well as behavioural constructs and symptoms such as impulsivity and aggression. A

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considerable amount of evidence on central nervous 5-HT functioning comes from psychopharmacological investigations, in particular research involving the acute and/or ongoing systematic administration of selective serotonin reuptake inhibitors (SSRI). SSRI are frequently used in order to treat depressive symptoms in humans. However, neuropsychopharmacological research also used SSRI administration in order to study a variety of processes in the human brain (in particular cognitive and affective processes), with the aim of providing a link between behavioural constructs and symptoms observed in humans, and serotoninergic neurotransmission (Ise and Bond, 2002; Harmer et al., 2003; Scoppetta et al., 2005). This approach is particularly relevant to mood disorders such as major depression, bipolar disorder and acute manic states (Zepf, 2009). The considerable literature available on SSRI administration and its effects on neural functioning in a variety of neuropsychiatric disorders mainly focuses on increasing central nervous 5-HT availability by inhibiting the reuptake of 5-HT into the pre-synaptic neuron (Loubinoux et al., 2002, 2005; New et al., 2004; Harmer et al., 2006; Lundberg et al., 2007; Marsteller et al., 2007; McClure et al., 2007; Wingen et al., 2008; Kim et al., 2009; Murphy et al., 2009; Simmons et al., 2009; McCabe et al., 2010; Windschberger et al., 2010). However, a considerable amount of research has studied a ‘reversed’ approach in terms of a reduction of central nervous 5-HT neurotransmission in humans (Young et al., 1988, 1996; Young and Teff, 1989; Pihl et al., 1995; Moore et al., 2000; Young and Leyton, 2002; Stadler et al., 2007; Zepf et al., 2008a,b; Zepf et al., 2009a,c,d; Zepf et al., 2010). One technique enabling such a lowering of central nervous 5-HT neurotransmission is the rapid tryptophan-depletion test (RTD), which allows a short-term reduction of 5-HT synthesis in the human brain. Other terms frequently used for this approach are ‘acute tryptophan depletion’ (ATD), ‘tryptophan depletion’ (TD) or simply ‘serotonin depletion’ (SD). In the following the term RTD is used as a synonym for all of the aforementioned items in order to describe fundamental principles of RTD in human neurobiological and neuropsychiatric research.

24.3 Basic Principles

The fundamental concept of RTD builds on the dietary administration of large neutral amino acids (so called LNAA). Of note, RTD can be used in both animals and humans. The LNAA administered compete with endogenous tryptophan (Trp), the physiological precursor amino acid of 5-HT, on the uptake over the blood–brain barrier into the central nervous system. The physiological uptake of amino acids into the brain uses the so-called L-1 transport system (Figure 24.1), which is saturated under physiological conditions. Tryptophan competes with other amino acids such as leucine, isoleucine, phenylalanine, tyrosine and valine on the unidirectional influx on L-1 into the central nervous system. Plasma concentrations of the LNAA which are administered influence the uptake of tryptophan over the blood–brain barrier (Wurtman et al., 1980). There are three different transport systems for managing the influx of amino acids into the central nervous system over the blood–brain barrier:

1. for neutral amino acids (L-1, ASC, A);
2. for cationic amino acids (y+);
3. for anionic amino acids (y-) (Oldendorf and Szabo, 1976; Pardridge, 1983, 2002; Zepf et al., 2008c).

Tryptophan is transported sodium-independently into the central nervous system using L-1. L-1 can be found on the luminal and abluminal membrane of capillary endothelial cells. It is saturated under physiological conditions and can be found in all cell types in the human body. However, the L-1 located on capillary endothelial cells of the blood–brain barrier has a 100–1000 times increased affinity to the relevant amino acids which use it as a shuttle into the central nervous system, which makes it different from the L-1 in other cell types (Oldendorf and Szabo, 1976). The influx into the central nervous system at L-1 uses Michaelis–Menten kinetics with competitive substrate inhibition (Smith, 1967; Oldendorf and Szabo, 1976; Smith and Takasato, 1986; Smith et al., 1987; Smith and Stoll, 1998). The influx rate for all LNAA was calculated at about 50 nmol min⁻¹ and per gram brain tissue (Smith and Takasato, 1986; for a summary see also Kewitz, 2002).
The diminished uptake of 5-HT into the central nervous system (which is achieved by competitive antagonism of the amino acids administered with endogenous tryptophan) leads in turn to a diminished substrate availability for the tryptophan hydroxylase 2 (TPH2), the rate-limiting enzyme in central nervous 5-HT synthesis (Fig. 24.1). This again results in reduced hydroxylation of tryptophan and a subsequent reduction in 5-hydroxytryptophan synthesis. The substrate availability in terms of cerebral tryptophan regulates brain 5-HT synthesis. Finally, the diminished availability of 5-hydroxytryptophan results in a reduced central nervous availability of 5-HT, as the 5-hydroxytryptophan decarboxylase also lacks sufficient substrate availability in analogy to the TPH2.

A further proportion of tryptophan uses passive diffusion in order to get into the central nervous system, which contributes at the level of about 10% to the overall influx of tryptophan under physiological conditions.

In humans the amino acid tryptophan is mostly protein-bound (about 95%), and a proportion of 5% relates to free tryptophan. This free proportion is essential for tryptophan availability in the central nervous system and regulates 5-HT synthesis in the brain (Tagliamonte et al., 1971, 1973; Gessa and Tagliamonte, 1974). The concept that 5-HT synthesis depends on tryptophan availability was proven in studies involving both animals and humans (Moreno et al., 2010; Tagliamonte et al., 1971, 1973, 1974; Biggio et al., 1974, 1975; Gessa et al., 1974, 1975; Moja et al., 1988; Young et al., 1988; Young and Teff, 1989; Delgado et al., 1990).

The following section will give a detailed description on the actual test protocol in order to provide a guide on how RTD studies can be conducted, in particular with respect to patient safety. The safety requirements mentioned should be met regardless of which RTD protocol is actually used, in accordance with Moja and colleagues (Moja et al., 1988), Moja-De (Zepf et al., 2008b), or with Young and co-workers (Young et al., 1989; Young and Teff, 1989).

### 24.4 RTD Protocol

Before RTD is used in both patients as well as in healthy populations, institutional board
review (IRB) should be obtained at the local ethics committee. Once IRB approval is available one should also think of having special insurance for the subjects enrolling in the study.

24.4.1 Before administration

Several safety requirements should be met before the amino acids are administered. Pregnancy tests in females as well as drug screenings (both immediately before amino acid administration) should be performed. Apart from the inclusion criteria for the particular study, the subjects should be screened (by interview) for several exclusion criteria, including developmental disorders, endocrine and metabolic disorders (diabetes, hypothyroidism, hyperthyroidism), schizophrenia, drug abuse and suicidal behaviour. There is currently no evidence that RTD endangers people using vehicles or large machines on the day of administration.

24.4.2 Administration of amino acids

The amino acids should be toxicologically tested before being mixed together in the required quantities with respect to the RTD procedure used. The creation of the amino acid mixtures for depletion and tryptophan-balanced placebo conditions should be done by a qualified person, in most cases a pharmacist. One should bear in mind that although the different composites of the amino acid drink can possibly be stored for almost a year before usage (if stored correctly, see below), once combined, the amino acid mixtures should be used within three months. It is advised that consultancy with a local pharmacist is sought before conducting RTD studies. Moreover, the amino acid mixtures should be stored in a refrigerator at approximately 5°C or below, in brown glass bottles with light-absorbing glass, until used. This is in order to prevent quality loss, because amino acids are rather sensitive to light. The fridge used for storage should be lockable in order to ensure that once the amino acids are stored no further components can be added or taken away. In addition, one should also ensure when using a tryptophan-balanced amino acid drink as a placebo condition that overall consistency, smell, and appearance should be as identical as possible. This can be achieved by mixing the amino acids of the RTD or placebo condition with other nutritional compounds (carbohydrates, salt) in order to achieve similar consistency and taste.

The amino acids should be administered in an aqueous suspension, and this is also the easiest way. An alternative could be capsules. However, because of the considerable amount of amino acids needed for sufficient depletion this would result in the intake of a relatively high number of capsules, which makes this an unsuitable method of administration, in particular when younger subjects are involved in the study. If the amino acids are administered in an aqueous suspension the amount of water should be kept to a minimum (approximately 200–300ml) in order to keep the overall volume of the drink small. This makes it easier for the subjects to drink the amino acid mixture. They should also be advised to drink the mixture rather quickly, for once suspended in water, the amino acids very often tend to clump. A milk frother can be used in order to create smaller particles and thus help to minimize such unfavourable effects. Additional flavouring such as vanilla, peppermint, or raspberry, can be used if necessary in order to achieve a better taste. Other compounds such as oat flakes can be added in order to achieve similar consistency which may be required when aiming to compare RTD effects with ‘real’ placebo conditions (i.e. a sugar tablet) instead of using a tryptophan balanced amino acid load.

24.5 Effects on Mood

The RTD technique has been used in many studies involving animals and human subjects, including patients and healthy controls. With respect to the serotonin hypothesis of depression, one might be worried if significant mood changes occur if RTD is administered to healthy subjects. Overall, as reviewed
by Moore and co-workers, the effects of RTD on mood ratings in healthy subjects are relatively small if measurable at all (Moore et al., 2000). As regards the use of RTD in children and adolescents, recent data showed no significant effects of RTD on mood in patients with attention deficit hyperactivity disorder (Zepf et al., 2009d). The picture changes when it comes to depressed patients; it is notable that it was shown that RTD leads to a depressive relapse in patients with major depressive disorder (Leyton et al., 2000).

### 24.6 Side Effects and Metabolic Complications

Side effects previously observed after the intake of conventional RTD protocols included nausea and vomiting in particular. Most of these events occurred in close temporal association with the intake of the RTD amino acids. The bad taste of some of the amino acids administered within the different RTD protocols was also a reason for drop-outs in some studies. Here the sulphuric side chain in methionine needs to be mentioned as a particular reason for the problems in taste just mentioned. Of note, recent developments have been promising as there is now a new RTD protocol available which has significantly fewer of the side effects outlined above. Known as Moja-De (a modification of the frequently used RTD protocol developed by Moja and colleagues), the protocol takes into account the body weight of the subjects. It evolved after the detection of a positive correlation between body weight and plasma concentrations of tryptophan in healthy adults (males and females) as well as in male children and adolescents (Demisch et al., 2002, 2004; Stadler et al., 2007; Zepf et al., 2008b). When administering the amino acids of the Moja-De protocol, they resemble approximately a single (or at the most, double) daily requirement of amino acids (Zepf et al., 2009b).

With respect to coenzyme metabolism one must note that RTD, when administered over a longer period of time (1 week or longer), may also have serious metabolic consequences. Niacin and NAD⁺ are both tryptophan derived. Following this, a prolonged lack of tryptophan can have an effect on coenzyme metabolism and oxidative stress, in particular as NAD⁺ is involved in a variety of redox reactions. A possible solution for such states evolving under RTD could be the supplementation of NAD⁺. However, this should only be considered with caution, in particular because even small dosages can result in flush symptoms. In line with this, supplementation of NAD⁺ requires extensive monitoring techniques as regards the assessment of the 5-HT metabolite 5-hydroxy-indole-acetic acid (5-HIAA) in the urine, as well as the serum concentrations of chromogranin-A. In patients with renal problems additional monitoring may also be required, since chromogranin-A can be falsely increased in such states. As a consequence, the assessment of additional renal parameters is highly recommended. An option for studies with single administration of RTD and/or a balanced amino acid drink for controlling purposes might be the administration of B₃ within a commercially available multivitamin tablet or drink (Zepf, 2008; Zepf et al., 2008a,b; Zepf et al., 2009a,b,c).

### 24.7 Positron Emission Tomography Studies

Several studies used positron emission tomography (PET) in order to study the central nervous effects of RTD in animals and humans. One study found a diminished activity in the ventral anterior cingulate, the caudate nucleus, and in the orbitofrontal cortex after RTD (Smith et al., 1999). Moreover, further studies found a diminished central nervous 5-HT synthesis after administration of RTD in animals and humans in various parts of the brain (Nishizawa et al., 1997, 1998; Shoaf et al., 1998; Diksic et al., 2000). One of these studies found that in males, mean 5-HT synthesis rate was about 52% higher compared to females (Nishizawa et al., 1997). Other measurements (H₂O¹⁵-PET) showed a co-variation of activity in the habenula and dorsal raphe nuclei following RTD (Morris et al., 1999). A PET study using¹⁸F-Septoperone in healthy women showed a decreased 5-HT₇-receptor binding in the left insula, the left
superior temporal, and superior frontal gyrus, as well as in the left fusiform gyrus (Yatham et al., 2001). However, the interpretation of such PET data obtained after administration of RTD is limited, because they are mainly related to the pre-synaptic serotonergic pool, and the results cannot provide information on overall serotonergic activity and synaptic 5-HT-release (Agren and Reibring, 1994).

24.8 Conclusions

It can be summarized that RTD is a physiological neurodietary interventional strategy in order to lower central nervous 5-HT synthesis, which allows one to gain insight on various processes in the human brain related to serotonergic neurotransmission. If the precautions mentioned are respected, RTD can be considered a safe procedure to lower central nervous neurotransmission in both healthy humans and many patient populations. Acutely ill patients (such as depressive patients, and those with schizophrenia) should not be included in these studies. The advantages of RTD are that it allows one to study a subject under a real central nervous 5-HT deficit, and that RTD can be combined with other measurements such as behavioural test batteries, genetic investigations and imaging techniques. Management of potential metabolic complications after prolonged depletion should be conducted by experienced clinicians. Future research using RTD will help to increase the understanding of neuropsychiatric disorders, building on significant changes in central nervous neurotransmission in humans.

References


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25 Excitatory Amino Acids in Neurological and Neurodegenerative Disorders

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25.1 Abstract

Excitatory amino acids (glutamate and aspartate) form the mainstay of synaptic transmission in the central nervous system. By the same token, dysfunctional, excitotoxic activity of excitatory amino acids can lead to and/or become instrumental in the progression of a number of neurological and neurodegenerative conditions. Dementia due to Alzheimer’s disease (AD) is characterized by extracellular plaques containing amyloid (Aβ peptide) which, together with its disruption of dendritic morphology, affects glutamate (AMPA and NMDA) receptor function to alter glutamatergic transmission. The progressive neurodegeneration of nigrostriatal neurons in Parkinson’s disease (PD) may in part arise as a result of overactivity of glutamatergic inputs from the cortex and subthalamic nuclei, presenting the utility of respective antagonism and agonism of stimulatory and inhibitory metabotropic glutamate receptors (mGlur) in PD therapeutics. Huntington’s disease (HD) manifests as atrophy of the corpus striatum and cortex, with neurons containing the mutant huntingtin protein perhaps being more susceptible to excitotoxicity from corticostrial inputs, as reflected by the NMDA receptor loss and interactions of huntingtin with facilitatory Group I mGlur. In schizophrenia, abnormalities in brain (dendritic) development and synaptic plasticity may precipitate the dysfunction of mesolimbic and mesocortical dopaminergic pathways. Here again, aberrations in glutamatergic transmission in the form of NMDA receptor hypofunction may underpin the pathophysiology, with inhibitory mGlur2/3 agonism presenting potential as a therapeutic recourse. Depression is classically attributed to defects in monoaminergic neurotransmission, but long-term changes in dendritic architecture in limbic areas arising from chronic stress may be subject to some influence of glucocorticoids on the glutamatergic input to hypothalamic neurons, and thus affect the hypothalamic/pituitary/adrenal axis and glucocorticoid secretion itself. Epilepsy is the perhaps the most clear example of excitatory transmission gone awry, with the manifest increases in cortical network activity during seizures. Increased glutamatergic activity is instrumental in the pathology, particularly

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given evidence of the convulsant associations of the kainate type glutamate receptors (KAR). Glutamatergic hyperactivity ultimately leads to excessive Ca$^{2+}$ influx which can initiate the sequelae of events leading to neuronal damage and death. Thus the Ca$^{2+}$-permeable NMDA plays a villain’s role in excitotoxic culling of motor neurons seen in amyotrophic lateral sclerosis (ALS) and indeed the necrotic death of neurons following stroke and cerebral ischaemia. However, it is now increasingly evident that AMPA receptors and KAR, with subunit compositions that permit Ca$^{2+}$-permeability, may contribute significantly to neurodegenerative chaos when overactivated. Addressing the excitotoxic aspects of excitatory amino acids therefore represents a major challenge in any potential therapeutic intervention with a number of neuropathologies.

25.2 Introduction

In a number of neurological conditions, damage to neurons may be induced, at least in part, by excessive activation of receptors for the excitatory amino acids including glutamate and aspartate (Table 25.1). In other studies, it has been concluded that glutamate excitotoxicity may also damage the myelin-producing cells of the central nervous system. Aberrations in the glutamate system of neurotransmission may be the underlying thread in many neurological conditions. Here, we review the published evidence on neuropathologies where excitotoxicity might play a role in the instigation, perpetuation or final consequences of the disorder.

### Table 25.1. Neuropathologies associated with excitatory amino acids: receptors and effects.

<table>
<thead>
<tr>
<th>Disease or neural state</th>
<th>Main receptors involved</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>NMDAR, AMPAR</td>
<td>No LTP; Inhibition</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>NMDAR, mGluR</td>
<td>Loss of glutamatergic synapses</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>NMDAR, GABAR</td>
<td>Loss of NMDA and GABAergic transmission</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>NMDAR, AMPAR, mGluR</td>
<td>Hypofunction of receptors</td>
</tr>
<tr>
<td>Depression</td>
<td>NMDAR</td>
<td>Loss of glutamatergic transduction</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>KAR, AMPAR, NMDAR</td>
<td>GABAergic inhibition</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>NMDAR, AMPAR</td>
<td>Motor neuron</td>
</tr>
<tr>
<td>Stroke</td>
<td>NMDAR</td>
<td>Excitotoxicity</td>
</tr>
</tbody>
</table>

25.3 Alzheimer’s Disease and Glutamate Receptors

Alzheimer’s disease (AD) is an age-related disorder characterized by the dysfunction and death of neurons in brain regions such as the hippocampus and frontal cortex, critical structures in learning and memory processes. This neurological disease is the most common cause of dementia among the elderly and has a heterogeneous aetiology, involving genetic and environmental factors. AD is characterized by two major neuropathological hallmarks: extracellular plaques composed of the 40–42 residues Aβ peptide and neurofibrillary tangles, consisting of abnormal phosphorylated Tau protein (for review see Bayer and Wirths, 2010). Several studies have shown that neuronal death is limited in normal ageing, whereas in AD there is considerable neuronal loss (for review see Dickstein et al., 2007). In addition, there is increasing evidence that Aβ peptide accumulates inside neurons, this being one of the mechanisms of neuronal degeneration (Gouras et al., 2000), with Aβ peptide being able to induce the loss or alteration of neuronal dendritic spines (Knobloch and Mansuy, 2008).

At the neuronal level, the cholinergic system is one of the most affected in AD. Pyramidal cells in cortical and hippocampal areas are severely degenerated as well as those of the nucleus basalis of Meynert (Samuel et al., 1994). The latter structure provides ~80% of the cholinergic neurons in the central nervous system (Samuel et al., 1994). Several studies have demonstrated that in AD and other dementias,
there are morphological changes in dendritic spine density, mainly observed in the prefrontal cortex (PFC) and the hippocampus (Uylings and de Brabander, 2002; Knobloch and Mansuy, 2008). The dendrites of AD brains show an increased curvature of processes, a decrease in dendritic length and spine density, and abruptly terminated dendritic endings (Dickstein et al., 2007, 2010). In addition, it has also been found that AD brains are characterized by reduced cell proliferation in the CA1 area of the hippocampus (Ferrer and Gullotta, 1990; Einstein et al., 1994; Scheff et al., 2007) and PFC (Shim and Lubec, 2002). Recently, alterations have been reported in the neuronal morphology of an AD mouse model (Aoki et al., 2007; Spires-Jones et al., 2007; Knafo et al., 2009). Taken together, AD brains are distinguished by the presence of dysmorphic dendrites in the pyramidal neurons of the hippocampus and PFC (for review see Dickstein et al., 2007).

It is known that Aβ protein binds to AMPA and NMDA receptors (AMPAR and NMDAR, respectively) to cause their internalization, leading to inhibition of long-term potentiation (LTP), which is an enhancement of synaptic strength that is correlated with memory (Snyder et al., 2005). In addition, a recent report has suggested that Aβ disruption of mitochondrial trafficking could contribute to AMPAR removal and trafficking defects, leading to synaptic inhibition (Rui et al., 2010). Moreover it has been suggested that it is possible to prevent Aβ-mediated synaptic plasticity disruption by using GluN2B subunit-containing NMDAR antagonists (Hu et al., 2009). Interestingly, Reelin, a signalling protein that is produced by interneurons in the brain, has an effect on synaptic function that is opposite to the Aβ protein, causing increased glutamatergic neurotransmission. Thus it is purported that Reelin signalling in glutamatergic synapses may restore normal synaptic plasticity, which is impaired by concentrations of oligomeric Aβ protein that lie well within the range present in the brains of AD patients (Durakoglugil et al., 2009). Finally, the treatment for AD has focused on symptomatic relief; however recent advances in molecular therapeutics have suggested specific new treatments, such as stem cell therapy, immunotherapy and neurotrophic factors with neuroprotection action that may alter the natural progression of this devastating illness.

25.4 Parkinson's Disease and Glutamate Receptors

Parkinson's disease is a progressive neurodegenerative disorder that is characterized by the degeneration of dopamine (DA) neurons of the substantia nigra pars compacta (SNc) (for review see Parent and Parent, 2010). The resultant degeneration of the nigrostriatal pathway is a main cause of the symptoms of PD, i.e. tremor, rigidity and bradykinesia (Deutch, 1993; Marsden, 1994). The main cells of the striatum, the medium spiny neurons, receive excitatory inputs from the cortex – especially from the PFC and thalamus (for review see Vertes, 2004; Pennartz et al., 2009). The striatum comprises the caudate-putamen (CPu) and nucleus accumbens (NAcc). Both of these structures are interconnected by cell bridges (Heimer et al., 1991) and are densely innervated by dopaminergic projections (Anden et al., 1966; Beckstead et al., 1979; Voorn et al., 1986). The NAcc receives DA fibres mainly from mesolimbic neurons of the ventral tegmental area (Deutch et al., 1988), whereas the DA neurons of the SNc send projections to the medium spiny neurons of the CPu (Anden et al., 1964). Therefore, denervation of dopaminergic terminals to the medium spiny neurons of the CPu is the main event in PD (Deutch, 1993; Marsden, 1994). Taking into account the dopamine theory of PD, L-DOPA and dopaminergic agonists are generally used in PD therapy. However, in recent years, the role of glutamate in the pathophysiology of PD has been studied. Recent data from Solis et al. (2007) suggest that 6-OHDA-lesioned rats, an animal model of PD, may develop altered dendritic morphology in the CPu, NAcc and PFC, which may have participated in the emergence of the behavioural changes observed in these animals. The glutamatergic inputs from the cortex make an asymmetric synaptic contact with the heads of dendritic spines, and critically regulate the
functions of the medium spiny neurons, the principal output neurons of the CPu (Ingham et al., 1989). DA afferents make synapses at the spine neck or dendritic shafts of the medium spiny neurons of the CPu, to regulate the excitatory drive of these neurons (for review see Arbuthnott et al., 2000). Thus it has been shown that alterations in dopaminergic inputs to the medium spiny neurons of the CPu may result in a reorganization of glutamatergic inputs into the CPu, and consequent changes in the functional properties of these neurons (Voulalas et al., 2005). An inappropriate functional interaction between SNc dopaminergic and cortical glutamatergic inputs on the medium spiny neurons of the CPu by the nigrostriatal dopaminergic disconnection may result in the selective loss of the glutamatergic synapse on the striatopallidal medium spiny neurons, as a recent report suggests (Day et al., 2006). In addition, the two CPu efferent systems, striatonigral (direct) and striato-pallido-subthalamo-nigral (indirect) pathways, oppositely regulate activity of the main output of the basal ganglia, the GABAergic nigrothalamic pathway. Interestingly, in the striato-pallido-subthalamo-nigral pathway, the subthalamic nucleus (STN) plays a critical role in the activity of the nigrothalamic projection. It is known that the glutamatergic neurons of the STN show an overactivity in the PD (for review see Blandini, 2001; Caudle and Zhang, 2009).

The pathophysiology of PD emphasizes that abnormalities of glutamatergic neurotransmission, especially the corticostriatal and STN hyperactivity, may be critical in local circuits that regulate the function of basal ganglia. Glutamatergic receptors are widely expressed in the basal ganglia, thus glutamatergic agents may modulate the activity of the medium spiny neurons of the CPu and STN neurons. Systemic or intrastratal administration of group I (mGluR) antagonists (mGluR5–MPEP; MTEP; mGluR1–AIDA) were found to inhibit Parkinsonian-like symptoms (catalepsy, muscle rigidity) in animal models of PD. Similarly, ACPT-1, a group III mGluR agonist, administered into the striatum, globus pallidus or substantia nigra, inhibited the catalepsy (for review see Osowska et al., 1994), emphasizing that targeting ionotropic glutamate receptor signaling may also be of therapeutic benefit in PD.

### 25.5 Huntington's Disease

Huntington's disease (HD) is an inherited autosomal-dominant genetic disorder, characterized by cognitive dysfunction and abnormal body movements (chorea or choreoathetosis), as well as by cognitive and emotional disturbances (depression, irritability, apathy and impulse control problems, for instance). Symptoms usually begin when patients are between 35 and 50 years old, although the onset may occur at any time. Death usually takes place 15–20 years after symptoms first appear. HD is also characterized by progressive neurodegeneration of the striatum but also involves other regions, basically the cerebral cortex. HD is considered to be an important disease for study, embodying many of the major themes in modern neuroscience, including molecular genetics, selective neuronal vulnerability, excitotoxicity, mitochondrial dysfunction, apoptosis, and transcriptional dysregulation.

Huntington's disease is caused by a mutation of a gene located on chromosome 4, which encodes a protein called huntingtin, in whose amino-terminal portion there is a repeated CAG triplet sequence, corresponding to a variable length string of polyglutamine, which in the normal population is between 12 and 36 residues in length. In patients with Huntington's disease there are an increased number of CAG triplets and therefore an increase in polyglutamine. This increase is variable and unstable, and the severity of the
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disease and age of onset correlate with and reverse, respectively, with the number of triplets. The number of triplets remains relatively stable, with variations smaller than ± 1 triplet in the mother–child transmission and in two thirds of parent–child transmission. In one third of the latter, however, there is an increase in the number of triplets that can be a few or several dozen. This explains why, while the children of a patient with Huntington’s disease often have clinical features very similar to those of their mothers, in the case of father–child transmission an increase in the severity of the disease is possible.

Certain lines of evidence (from mouse studies) indicate that the major pathogenic mechanisms of HD involve a toxic gain of function by the mutant protein; the abnormal length of the polyglutamine repeat gives huntingtin a toxic property not found in the wild-type protein:

1. Like all the other polyglutamine repeat disorders, huntingtin has a dominant mode of inheritance, which is typically the result of gain-of-function mutations.
2. The age of onset for homozygotes for the HD mutation generally is not markedly less than the age of onset for cases with only one copy of comparable repeat length, although this is not necessarily the case in the other glutamine repeat diseases.
3. No cases of HD or related polyglutamine disorders have been identified with deletions or point mutations in any of the causative genes. In contrast, the fragile X phenotype can be caused by a triplet repeat expansion leading to impaired transcription, a deletion, or a point mutation; all three types of mutations result in loss of normal protein function. Finally, mice with targeted deletions of the HD gene resulting in expression that is a small fraction of normal, demonstrate developmental abnormalities rather than a progressive neurologic disorder.

The size of the CAG trinucleotide expansion is not the only thing that makes possible a prognostic on HD. Other elements of genetic origin are the polymorphisms of the glutamate receptors. It is possible, but so far unknown, that other genetic or environmental factors, as well as biological or pharmacological factors, may play a modulating role in the speed of the disease progression.

Various hypotheses have been advanced to explain the pathogenic mechanisms of mutant huntingtin-induced neuronal dysfunction and cell death, but none of these has gained universal support at present. Anumber of recent reports have concluded that oxidative stress also plays a key role in HD pathogenesis. Although there is no specific treatment available to block disease progression, treatments are available to help in controlling the chorea symptoms.

The nature of the motor symptoms changes over time, but generally speaking the movement disorder of HD can be said to consist of two components: involuntary movements and abnormal voluntary movements. These include some abnormal eye movements, such as slow, hypometric saccades and catchy pursuit; uncoordinated, arrhythmic, and slow fine motor movements; dysphagia and dysarthria; dysdiadochokinesis; rigidity; and gait disturbances. The most common clinical manifestation of HD is chorea. Chorea is defined as quick, vermicular movement, which may be superimposed on a purposeful act.

In contrast to AD, patients with HD seem to have trouble with retrieval rather than storage of memories. They are more apt than patients with AD to recognize words from a previously memorized list or to respond to other cues to help them recall information. All of these cognitive losses accumulate progressively. In patients with late-stage HD deficits in memory, visuospatial abilities, and judgment are observed to develop. Although attention impairment, problem solving, and verbal fluency are also described, along with memory deterioration over time, aside from dysfunction of the vocal apparatus, expressive and receptive language abilities may remain relatively stable or show only minimal disruption over the first few years of the disease. Anomia and aphasia, for example, are rare in early stages; the disease generally spares language functions, including comprehension, vocabulary, and general knowledge. As the disease progresses, language abilities begin to decline and combine with more severe exacerbation of early impairments to produce a general intellectual state that further causes mental retardation. Another behavioural alteration of HD is
altered sexuality; the possible cause may be a
delicate imbalance of hormones in the brain.

The most prominent atrophy in HD is
found in the caudate nucleus and putamen,
which together comprise the corpus striatum
within the basal ganglia. Striatal atrophy
leads to hydrocephalus ex vacuo and marked
dilatation of the lateral ventricles. In addition,
there is overall atrophy of the brain. Within
the striatum, there is selective neuronal vul-
nerability, both in the anatomic pattern of
regions affected and in the particular neurons
lost. Loss of neurons in the caudate and puta-
men shows a gradient, with early and most
severe loss in the dorsal and medial regions,
and progressive loss of neurons in ventral
and lateral regions, as the disease progresses.
There is severe loss of medium spiny projec-
tion neurons, especially those synthesizing
enkephalin and γ-aminobutyric acid, but rel-
ative preservation of large and medium
aspyne interneurons. Neuronal loss is accom-
panied by reactive astrocytosis (gliosis). Other
areas of the basal ganglia also become
atrophic, especially the globus pallidus and
subthalamic nucleus, although less than the
striatum. Large cortical neurons appear to be
most severely affected, and there is laminar
specificity, with greatest loss in layer VI and
significant loss in layers III and V. The neu-
rons lost in the greatest numbers appear to
project to the thalamus, whereas most neu-
rons that project to the caudate and putamen
lie in more superficial regions of layer V. The neu-
rons lost in the greatest numbers appear to
project to the thalamus, whereas most neu-
rons that project to the caudate and putamen
lie in more superficial regions of layer V. In
addition, the extent of cortical degeneration
does not closely correlate to the severity of
striatal degeneration. This set of observations
indicates that the loss of neurons in the cortex
does not arise simply from retrograde changes
beginning in the striatum. Whereas the atro-
phy and neuronal cell loss of HD have been
extensively studied, less attention has focused
on the morphology of the surviving neurons.
Contrary to expectations, application of the
Golgi metal impregnation method to study
neuronal morphology in the caudate and cor-
text from HD cases revealed evidence, in the
surviving neurons, of ‘regenerative’ or ‘plas-
tic’ changes. Relative to neurons in these
regions from normal brains, surviving neu-
rons in HD cases had more dendrites, greater density, and
larger size of dendritic spines, and greater
somatic area. A complete understanding of
the pathogenesis of HD will need to encom-
pass an explanation of these regenerative
changes as well as neuronal death and brain
atrophy (Kowall et al., 1987; Sotrel et al., 1993;
Kim et al., 1999; Ross and Margolis, 2002).

25.5.1 Animal models

There are several animal models available for
HD, with a variety of advantages and limita-
tions; these models mimic a number of HD
symptoms to greater or lesser extent. There
are various cascades contributing to HD
pathogenesis and progression as well as drug
targets, such as dopaminergic, γ-aminobutyric
acid (GABA)ergic, glutamatergic, purinergic
(adenosine), peptidergic and cannabinoid
receptors, and adjuvant therapeutic drug tar-
gets such as oxidative stress and mitochon-
drial dysfunction that can be addressed in
future experimental studies.

One approach is the use of the transgenic
animal model. The first animal model for HD
was generated in mice using exon 1 of hunt-
ingtin with a very long expanded repeat.
These animals developed progressive neuro-
logic deficits strikingly similar to those of HD,
including incoordination, abnormal involun-
tary movements, seizures and weight loss.
However, unlike patients with HD, neuronal
cell loss is not prominent. These mice also
developed intranuclear inclusions containing
the truncated huntingtin transgene product,
but not the endogenous huntingtin protein.
The intranuclear inclusions are clearly dis-

tinct from the nucleolus, and no membrane
separates them from the rest of the nucleus.
The intranuclear inclusions are present at the
time, and perhaps before, the animals have
neurologic signs or brain or body weight loss
(Schilling et al., 1999).

Another HD mouse model has a truncated
N-terminal fragment of huntingtin driven by
the prion protein promoter, resulting in mice
with features such as hypoactivity, incoordina-
tion and weight loss, and, on neuropathologic
examination, show both intranuclear inclu-
sions and neuritic aggregates. A transgene
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consisting of a full-length huntingtin cDNA driven by the CMV promoter resulted in a line of mice with a rather different phenotype, characterized by early weight gain and hyperactivity, but followed later by hypoactivity. These mice have both intranuclear inclusions and some loss of neurons (Reddy et al., 1998).

Perhaps the most promising mouse model of HD involves the use of yeast artificial chromosome (YAC) constructs, so the transgene consists of the entire human HD gene, including the human HD promoter and all introns, with an expanded repeat. These mice develop neurologic signs, electrophysiologic abnormalities, and a shortened lifespan. A single founder with a long repeat had striking evidence of selective striatal neurodegeneration and nuclear localization of N-terminal epitopes of huntingtin in striatal neurons. If additional lines can be generated, this model may be the closest to the human disease of any model yet generated. Another model of potential utility was generated by inserting an expansion of polyglutamine into the mouse huntingtin gene, thus avoiding the confounding factor of the presence of the human transgene. So far, these mice have not developed neurologic signs, and no neuronal loss has been detected (Hodgson et al., 1999). There is evidence of translocation of huntingtin into the nucleus in striatal neurons. If additional lines can be generated, this model may be the closest to the human disease of any model yet generated. Another model of potential utility was generated by inserting an expansion of polyglutamine into the mouse huntingtin gene, thus avoiding the confounding factor of the presence of the human transgene. So far, these mice have not developed neurologic signs, and no neuronal loss has been detected (Hodgson et al., 1999). There is evidence of translocation of huntingtin into the nucleus in striatal neurons. Thus, these mice may model early aspects of HD pathogenesis and could provide a useful model for studying the initial features of the disease. The construction of an inducible mouse model of HD has yielded insight into HD pathogenesis. A transgene containing exon 1 of huntingtin with an expanded glutamine repeat under the control of the tet-off system was inserted so that the timing of transgene expression could be externally controlled by the presence or absence of an antibiotic in the animals' food (Yamamoto et al., 2000). With the transgene on, mice developed neurologic signs and neuropathologic changes including nuclear inclusions. Remarkably, when the expression of huntingtin was turned off, these abnormalities partially reversed. This surprising result suggests that the brain may have more restorative and plastic ability than previously appreciated, and that if the pathologic changes of HD could be halted, substantial repair would perhaps be possible. Apart from mice, some other models have been used, such as invertebrates, which offer the potential of using powerful genetic techniques to search for genetic factors that enhance or suppress an experimentally induced phenotype.

In reviewing HD, we can also consider alterations of neurotransmitter levels, especially glutamate, GABA and DA receptors. Altered expression of neurotransmitter receptors precedes clinical symptoms in transgenic mice and contributes to subsequent pathology. Inhibition of caspase activation prevents down-regulation of receptors, suggesting that caspases are mediators not only for cell death, but also for cell dysfunction. A faulty gene and excess glutamate may lead to damaging free radicals, which can harm the DNA of the nerve cell. Glutamate also may lead to detrimental Ca" influx, which can churn out its own supply of DNA-damaging free radicals. The free radicals also may injure neurofilaments, proteins that serve as the skeleton of the cell. In addition, the immune system may be involved in damaging neurons.

Referring to the role of glutamate, for instance, an 'excitotoxicity hypothesis' can be applied to HD. This hypothesis stipulates that increased glutamate release from cortical afferents and reduced uptake of glutamate by glia lead to excessive activation of glutamate receptors, or hypersensitivity of postsynaptic glutamate receptors on striatal projection neurons. This causes an alteration in intracellular Ca" homeostasis and mitochondrial dysfunction, resulting in neuronal dysfunction and death of striatal medium spiny neurons. Support for the excitotoxic hypothesis comes in part from radioligand binding studies in post-mortem HD brain tissue, which show a disproportionate loss of NMDAR from the striatum of patients in early symptomatic stages, and, in a few cases, presymptomatic stages of the disease. These studies suggest that striatal neurons with high NMDAR expression are the most vulnerable and are lost early during disease progression. Since NMDA receptors are intimately associated with excitotoxicity, they were one of the first glutamate receptors studied in mouse models of HD.
Early evidence suggests a decreased level of GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) in post-mortem HD brains. Whereas larger aspiny interneurons are unaffected in the early stages of HD, spiny neurons are severely diminished. The loss of striatal GABA receptors probably represents the loss of striatal neurons. However, the increase in GABA receptors in the GP external (GPe), an area that normally receives synaptic input from striatal projections, probably represents a measure of denervation supersensitivity. Disruptions in GABA systems are not limited to the striatum.

Glutamate release can be regulated by GABA receptors (GABAR) located on corticostriatal terminals. Activation of these receptors exerts a significant inhibitory effect. Although glutamate receptors are thought to contribute to excitotoxic neuronal loss in HD, it is still unclear whether group I mGluR activation could delay or accelerate HD, as different studies have reported contradictory data (DiFiglia, 1990; Beal et al., 1991; Nicoletti et al., 1996; Bruno et al., 2001; Zeron et al., 2002; Tang et al., 2003; Schiefer et al., 2004). Recently, it has been determined that group I mGluR interact with mutant huntingtin and that mGluR5 signalling was selectively uncoupled as a consequence of this interaction (Anborgh et al., 2005). The neuronal cell loss that takes place in the striatum and cortex of HD patients is considered to be the primary cause of HD symptoms and eventual death of HD patients (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). It is still not clear why mutant huntingtin protein leads to selective neuronal cell death, and why there is a delayed loss of neurons late in life. mGluR1/5 can signal to activate different pathways that can be either protective or exacerbate neuronal cell death. What is postulated is that mGluR1/5 can signal to activate different pathways that can be either protective or exacerbate neuronal cell death.

25.6 Schizophrenia and Glutamate Receptors

Schizophrenia is a complex disorder of thought, perception, and social interactions affecting 1% of the world’s population. The main diagnostics observed in schizophrenic patients comprise positive symptoms (e.g. thought disorder, delusions, hallucinations), negative symptoms (e.g. anhedonia, apathy, social withdrawal), and cognitive deficiencies, including attention deficit, impaired memory, and deficit in executive function. Various theories have been advanced to explain schizophrenia, and among these the dopamine theory postulates an overactive mesolimbic dopaminergic system in mediating some of the behavioural manifestations of the disorder. However, in recent years neuroimaging and neuropathological studies have provided strong evidence for structural and molecular changes in the PFC, hippocampal formation, and amygdala of schizophrenic brains, which are suggestive of abnormalities in brain development and plasticity. In addition, alterations in the cell pattern and orientation in the hippocampus of schizophrenic patients have been observed (Jacob and Beckman 1986). The volume of the amygdalo-hippocampal formation is decreased in schizophrenic patients in post-mortem brains (Bogerts et al., 1985; Brown et al., 1986; Jakob and Beckmann, 1986; Jeste and Lohr, 1989; Arnold et al., 1991) and by in vivo analysis using magnetic resonance imaging (MRI) (De Lisi et al., 1988; Suddath et al., 1989, 1990; Rossi et al., 1990).

Consistent with the hypothesis of altered cortical synaptic plasticity in schizophrenia, several reports have shown decreased dendritic arbour and spine density of prefrontal cortical pyramidal neurons in post-mortem
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schizophrenic brains (Garey et al., 1998; Glantz and Lewis, 2000; Broadbelt et al., 2002; Lewis et al., 2003). Animal studies clearly indicate roles for hippocampal, amygdala and/or PFC inputs in modulating the activity of the medium spiny neurons of the NAcc (for review see Marcotte et al., 2001; Lipska, 2004). For example, recent studies with neonatal lesions of the hippocampus or amygdala lesions in rats have revealed a reduction in the dendrites’ parameters such as spines density and dendritic length in the pyramidal neurons of the PFC and medium spiny neurons of the NAcc (Flores et al., 2005, Alquicer et al., 2008, Solis et al., 2009). Neuronal dendrites are instrumental in the formation and maintenance of neural networks, the regulation of synaptic plasticity and the integration of electrical inputs (for review, see Dickstein et al., 2007). In addition, dendritic spines are small protrusions which are distinguished along the body of the dendrites of certain neurons and represent a site of excitatory synapses. The heads of dendritic spines comprise proteins involved in excitatory synaptic transmission, such as NMDAR, AMPAR, mGluR and associated signalling proteins (for review, see Kitanishi et al., 2009). Therefore, pathophysiological theories of schizophrenia emphasize that abnormalities of glutamatergic neurotransmission, especially a hypofunction of NMDAR signalling, may be critical in local circuits that regulate the function of a given brain region, or control projections from one region to another (e.g. hippocampal-cortical projection) (for review, see Marek et al., 2010). Interestingly, AMPAR expression is abnormally decreased in the schizophrenic hippocampus, and similar changes have been reported for KAR expression in the hippocampus as well as NMDAR in some cortical regions (for review, see Meador-Woodruff and Healy, 2000). A few studies have investigated the level expression of metabotropic glutamate receptor 2 (mGluR2) in post-mortem human brain of schizophrenic subjects, but the results have been conflicting thus far. While Ghose et al. (2008) reported an increase in mGluR2 expression in the PFC using in situ hybridization, Gonzalez-Maeso et al. (2008) showed lower levels of expression of mGluR2 using PCR. The expression of mGluR3 is unaffected in schizophrenia. However, agonists decreased both positive and negative symptoms of schizophrenia, raising hopes that glutamatergic mechanisms may provide therapeutic potential in this disease (for review, see Marek et al., 2010).

25.7 Depression and Glutamate Receptors

Depression is a common, recurrent and chronic disorder that is a leading cause of functional impairment and disability. This disorder affects one in eight persons in the world and is projected to become the second leading cause of disability worldwide by the year 2020 (for review, see Gaynes et al., 2009). Depression is not merely attributed to the functional defect of monoaminergic neurotransmission (e.g. serotonin, norepinephrine, and dopamine), but is also due to the structural impairment of neuroplasticity. Interestingly, chronic stress decreases dendritic arbor and dendritic spine density in the prefrontal cortex, hippocampus, and nucleus accumbens (Silva-Gómez et al., 2003; Alquicer et al., 2008), and neurotrophin levels (for review, see Calabrese et al., 2009), precipitating or exacerbating depression. Conversely, antidepressant drugs increase expression of various neurotrophins (e.g. brain-derived neurotrophic factor). Therefore, effective treatments of depression should not be limited to their effects on the control of neurotransmitter release, but should seek to normalize defective mechanisms that sustain the impairment of neuronal plasticity (for review see Calabrese et al., 2009).

The mechanism by which chronic stress induces long-term changes in dendritic architecture of neurons in the limbic regions such as PFC, hippocampus and NAcc is not clear. However, several reports suggest that increases in glucocorticoid levels, especially cortisol, and loss of glutamatergic synaptic transmission during chronic stress, may participate in this process (for review see Prager and Johnson, 2009). Interestingly, glucocorticoids exert an opposing rapid regulation of glutamate and GABA synaptic inputs to hypothalamic neurons via the activation of postsynaptic
membrane-associated receptors (Di et al., 2009). In addition the hypothalamic-pituitary-adrenal axis is the key regulator of glucocorticoid levels. Dysregulation of this axis is thought to play a central role in the pathophysiology of depressive disorders (for review see Durand et al., 2008). Glutamate and its receptors (GluR) are found in all the hypothalamic areas critically involved in neuroendocrine functions. Similarly, the pituitary gland also expresses these excitatory amino acid receptors. In addition, several reports support the critical role of the GluR as regulators of hypothalamus-pituitary function (for review, see Durand et al., 2008). Therefore, accumulating evidence suggests that glutamatergic neurotransmission plays a critical role in the neurobiology of depression, and represents a key therapeutic target for this disease.

Clinical studies have demonstrated that the non-competitive NMDA receptor antagonist, ketamine, has rapid antidepressant effects in patients with depressive disorder, suggesting the role of glutamate in the pathophysiology of this disease (for review, see Hashimoto, 2010). Studies using animal models of depression have demonstrated that the agents which act at glutamate receptors such as NMDAR, AMPAR and mGluR might have antidepressant-like activities (for review see Hashimoto, 2010). Conversely, chronic treatment with antidepressants may modify the expression of the GluR in limbic regions (for review, see Machado-Vieira et al., 2009). For example, chronic desipramine treatment decreased AMPAR subunit GluR3 expression in PFC and hippocampus (Barbon et al., 2006), whereas chronic imipramine reduced the inhibitory properties of group II mGluR (Palucha et al., 2007). Neuropathological studies indicate that there is increased AMPA binding coupled with a decreased GluR1 subunit expression in the striatum of patients with mood disorder (Meador-Woodruff et al., 2001). In the PFC of patients with mood disorder, a reduction of GluR2 levels has also been reported (Scarr et al., 2003). Finally, several studies have reported intracellular protein changes associated with the postsynaptic density of the NMDA receptor complex (for review, see Machado-Vieira et al., 2009). For example, reduced expression of NR1 and NR2A subunits has been observed in hippocampus, striatum, and thalamus of patients with mood disorder (Clinton et al., 2004; Kristiansen and Meador-Woodruff, 2005; McCullumsmith et al., 2007).

25.8 Epilepsy and Glutamate Neurotransmission

Epilepsy affects 1–3% of people worldwide. Currently, there is little agreement as to the definition of the terms seizure and epilepsy. Nevertheless, the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE) have proposed definitions for these terms. Epilepsy is a disorder of the brain characterized by an enduring predisposition to the generation of epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition; an epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain. Such definitions are important for communication among professionals in many different fields (Fisher et al., 2005). Recently, the ILAE Commission on Classification and Terminology has revised concepts, terminology and approaches for classifying seizures and forms of epilepsy (Berg et al., 2010), but these will not be addressed here for reasons of space.

25.8.1 Excitatory amino acids in epilepsy

Little is known about the effects of seizures on brain amino acid metabolism. Altered release of amino acid and monoamine transmitters, caused by increased network activity during the initial stages of a seizure, can have a profound effect on the further course of that seizure. Increased release of glutamate can exacerbate or prolong pre-existing seizure activity, and may result in excitotoxicity. Increased γ-aminobutyric acid (GABA) release, on the other hand, may be a compensatory inhibitory mechanism in the epileptic focus and surrounding tissue, which limits
the progression and spread of seizure activity. These studies have often focused on the hippocampus because of its involvement in the pathophysiology of temporal lobe seizures, the most common type of seizure in adults. Increases in hippocampal extracellular glutamate and GABA have consistently been observed during spontaneous seizures, as well as during different types of chemically and electrically induced seizures in rats (Meurs et al., 2008).

Alterations in the metabolism of several amino acids, especially glutamate, aspartate, and GABA, have been reported in the genetically epilepsy-prone rat. Lehmann (1989) studied basal and high potassium-stimulated release of endogenous amino acids measured using brain dialysis in the hippocampus of urethane-anesthetized seizure-resistant (SR) and seizure-susceptible (SS) rats. The basal extracellular concentrations of amino acids did not differ between SR and SS rats; however, aspartate release was higher in SS rats during stimulation with 100 mM K⁺. In addition, aspartate was significantly elevated in the hippocampus, cortex and cerebellum of SS animals, concurrently with a depression in GABA concentrations in the hippocampus and cortex. Lasley (1991) compared seizure-naive and seizure-experienced genetically epilepsy-prone rats in order to distinguish transmitter amino acid changes related to seizure severity from those associated with seizure experience. GABA concentrations were lower in both moderate and severe seizures, compared to non-epileptic controls in each brain region examined. The low concentrations of GABA are consistent with a role for this amino acid in determination of seizure susceptibility. Aspartate content was elevated in brain areas in severe epilepsy compared to non-epileptic controls. Changes resulting from seizure experience consisted of increases in aspartate, glutamate, and glycine. Furthermore, the seizure-induced changes in aspartate and glutamate support the concept that these excitatory amino acids mediate changes in seizure predisposition.

Li et al. (2000), in a pentylenetetrazole (PTZ) model of epilepsy, studied the extracellular concentration of glutamate in the frontal cortex of freely-moving PTZ ‘kindled’ rats, using an in vivo microdialysis. A significant and sustained increase in glutamate levels was observed in the kindled rats. In contrast, a slight and delayed increase was observed in the non-kindled rats when the same grade seizure was induced by PTZ. Kindling is an experimental model of complex partial seizures followed by secondary generalization, which is characterized by the progressive development of electrographic after-discharge in response to repetitive and sub-threshold electrical stimulation of various brain structures, especially those from the limbic system including the cerebral cortex, hippocampus, and amygdala, where glutamate is the major excitatory neurotransmitter. The sub-threshold repetitive stimulation of these structures produces an enhancement of the cortical activity accompanied by the development of tonic-clonic seizures. The generalized seizures that are induced with this paradigm (Fig. 25.1) underlie a susceptibility state of the nervous system, which is permanently altered (Magdaleno-Madrigal et al., 2010).

Glutamate is the major fast excitatory amino acid transmitter in the central nervous system, and excessive glutamatergic neurotransmission has been considered an underlying factor in epilepsy. Glutamate exerts its action through receptors that function as ion channels such as NMDA receptors, AMPA receptors and KAR, and also through signaling cascades initiated by metabotropic receptors. KAR mediate most aspects of seizures pharmacologically induced by the neurotoxin and convulsant kainate, and thus are targets for antiepileptic drug action.

### 25.8.2 Kainate receptors in epilepsy

KAR are a family of glutamate receptors that participate in normal synaptic transmission. The actions of the defining agonist kainate (KA) at these receptors are mediated postsynaptically, to regulate excitatory synaptic transmission, and presynaptically, to modulate neurotransmitter release. In the latter context, KAR have been implicated in the modulation of both glutamate and GABA release in the hippocampus. The physiological properties of KAR and their roles in
Fig. 25.1. Representative polygraph recording showing a generalized convulsive seizure (stage VI of kindling) in the cat. (a) Arrow indicates amygdaloid stimulation (AK). Part of the frame in the top panel is expanded below. (b) L-Pf Cx, left prefrontal cortices; R-Pf Cx, right prefrontal cortices; L-Am, left amygdalae; R-Am, right amygdalae; L-LGB, left lateral geniculate bodies; R-LGB, right lateral geniculate bodies; L-Hip, left hippocampi; R-Hip, right hippocampi; EOG, electrooculography; EMG, electromyography (modified with authorization of Magdaleno-Madrigal et al. (2010)).

synaptic transmission have been discerned only recently, and have been carried out mainly in the hippocampus (see Huettner, 2003, and Lerma, 2003 for reviews).

KA is a potent convulsive agent that, when administered in vivo leads to the generation of seizures. It has also been established that certain brain areas such as the CA3 and the CA1 regions of the hippocampus are particularly susceptible to the depolarizing actions of KA. When the granule cells therein are destroyed, there is a parallel reduction in the number of KA-binding sites and a decrease in the susceptibility to epileptogenic activity induced by the convulsant (see Lerma, 2003 for review).

Rodriguez-Moreno and Sihra (2004) described an increase in glutamate release at mossy fibre-CA3 (MF-CA3) synapses in the rat hippocampus induced by low KA concentrations. This regulation was mediated by the activation of an adenylyl cyclase (AC) (cAMP)/PKA cascade, but independently of G protein involvement. On the other hand, Negrete-Diaz et al. (2006) showed that the activation of presynaptic KAR at MF terminals by higher KA concentrations results in a long-lasting inhibition of glutamate release. This action of KAR seems to be mediated by the activation of a PTX-sensitive G protein, suggesting the coupling of KAR to a second messenger cascade involving the regulation of PKA activity. These observations therefore extend the metabotropic actions of KAR in the brain to include the AC (cAMP)/PKA signalling cascade. In addition,
Rodríguez-Moreno et al. (1997, 2000), using microcultured neurons and hippocampal slices, suggested that KAR on presynaptic GABAergic terminals reduce transmitter release by a G protein-mediated activation of phospholipase C and PKC. In vivo experiments using brain dialysis demonstrated that KA reversibly abolished recurrent inhibition and induced an epileptic-like electroencephalogram (EEG) activity. These results indicate that KAR activation down-regulates GABAergic inhibition by modulating the reliability of GABA synapses. The decrease in the efficacy of normal inhibition induced by KAR therefore promotes excitation and development of seizures.

Diverse studies on the effects of KAR activation on evoked excitatory postsynaptic currents (eEPSC) in the hippocampus have reported both decreases and increases of eEPSC amplitudes. Figure 25.2 shows the bidirectionality of the effects of a KA in vitro experiment using evoked field excitatory postsynaptic potential (fEPSP) recordings of hippocampal mossy fibres.

Finally, an important body of evidence at the molecular level has demonstrated the importance of KAR in epileptic pathology. For example, it has been discovered that allelic variants of the human KAR in certain subunits confer an increased susceptibility to the development of juvenile absence seizures, and that the level of KAR mRNA seems to be altered in the hippocampus of patients suffering from temporal lobe epilepsy (see Lerma, 2003 for review).

Overall, the role of glutamate in the pathogenesis of epilepsy is complex and

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**Fig. 25.2.** KAR activation has a biphasic effect on glutamate release in hippocampal brain slices of the mouse. (a), Kainate receptors activation with low concentrations of KA (50 nM) enhanced the fEPSP amplitude and the excitability of mossy fibres in the hippocampus. Representative traces show single recordings of MF-CA3 synapses. A typical characteristic of the MF-CA3 synapse is its ability to display rapid and pronounced frequency facilitation with paired stimuli (1, 2), protocol at 40 ms interval (arrows). (b1), KAR activation with higher concentrations of KA (1 μM) produces a decrease in fEPSP amplitude in the mouse prefrontal cortex and (b2) lateral amygdalae (LA).
involves glutamate transporters, ionotropic, and metabotropic glutamate receptor activation; regulation of PKA and PKC; and may even be underpinned by inherited and acquired channelopathies (Bernard et al., 2004; Zhang et al., 2004; Negrete-Diaz et al., 2006; Fuortes et al., 2008; Karr and Rotecki, 2008). Crucially, the evidence suggests that KAR are excellent candidates as targets for future drug development in the treatment of epilepsy.

25.9 Amyotrophic Lateral Sclerosis and Excitatory Amino Acids

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease of middle and late life. The onset of ALS is age-related, with the highest rate of onset being between ages 55 and 75 years (Logroscino et al., 2008). A recent systematic review of ALS has found a relatively uniform incidence across the white populations of Europe and North America, and a lower incidence among African, Asian and Hispanic ethnicities. The degree to which these differences are due to lower ascertainment and/or lower life expectancy of the latter remains unclear. However, reduced mortality rates have been consistently observed in Hispanic and Asian groups compared with similar studies in white populations (Cronin et al., 2007). Existing evidence supports the concept of ALS as a complex genetic disease caused by multiple susceptibility genes interacting with a variety of environmental risks. European populations share common ancestral origins and, depending on the degree of relatedness, are likely to share a variety of rare ‘at-risk’ genes, combinations of which may increase susceptibility to the disease. Moreover, the population frequency of some rare at-risk genes may be higher in more homogenous populations, as has been demonstrated in Irish and Scottish ALS studies. Conversely, admixed populations, containing a much wider variety and different combinations of at-risk alleles, are therefore likely to experience a lower overall risk of developing the disease (Zaldivar et al., 2009). Although scant attention has been paid to variations in the frequency and natural history of neurodegenerative diseases outside ancestral European populations, there is evolving evidence that the prevalence of PD, for example, may be reduced in admixed populations, and that those of non-European or admixed origin with AD may differ from ancestral Europeans with respect to age at onset, phenotype, and survival (Prince et al., 2007; Miller et al., 2008).

ALS is characterized by a loss of motor neurons in the motor cortex, brainstem, and spinal cord. This results in progressive muscle weakness, and wasting and death—usually from respiratory failure—within 2–5 years. It has been recognized that motor neurons might be particularly susceptible to degeneration induced by toxicity because of their size, a somatic diameter of 50–60μm with long axonal processes, and the high energy requirements of such a large cell. Approximately 10% of ALS cases are familial (FALS), and within this group, about 20% show mutations in the copper–zinc superoxide dismutase 1 (SOD1) gene (Rosen et al., 1993). The clinical features and pathology of the sporadic and familial forms of ALS are very similar, suggesting common mechanisms of neurodegeneration. Phenotypes of rodent models with mutations in SOD1 closely resemble those of the human disease (Cleveland and Rothstein, 2001; Bruijn et al., 2004). Hence, a number of studies have been carried out by modelling toxicity in ALS using mutant SOD1 (mSOD1) to elucidate the mechanism underlying selective motor neuronal death.

Current evidence suggests that there may be a complex interplay between several pathogenetic mechanisms underlying motor neuron degeneration. Although there are a number of possible aetiologies for ALS, including environmental agents such as toxic metals and viral infection, autoimmunity, oxidative stress and alteration in neurotrophic factors (Salasar et al., 1995; Arsac et al., 1996; Cookson and Shaw, 1999), there are several lines of circumstantial evidence implicating a disturbance of glutamatergic neurotransmission and excitotoxic mechanisms in the pathogenesis of ALS.
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(Heath and Shaw, 2002). In fact, mitigation of the effects of glutamate excitotoxicity in motor neurons is one of the most intensely investigated areas for the treatment of ALS, as excessive motor neuronal excitation by glutamate through ionotropic glutamate receptors has been demonstrated.

25.9.1 Glutamate receptors and excitotoxicity in ALS

Recent studies have shed light on the involvement of NMDAR in ALS motor neuronal excitotoxicity. A specific, non-competitive NMDA receptor blocker, memantin, was demonstrated to delay disease progression and prolong lifespan in the mSOD1 mouse model, through both subcutaneous and oral administrations. These data suggested that NMDAR-mediated excitability is involved in ALS pathogenesis, at least in part (Sasabe and Aiso, 2010). The reason for the discrepancy between effectiveness of NMDAR antagonists, and the relatively low expression of NMDAR in ALS remains uncertain, but an essential requirement for a co-agonist for the activity of these receptors should be considered.

Because glutamate-induced Ca$^{2+}$ influx into neurons occurs mainly through NMDAR, they were initially considered to be uniquely responsible for the associated excitotoxicity. More recently, however, several lines of evidence demonstrate that some specific forms of AMPAR, lacking GluR2 subunits, also contribute to the Ca$^{2+}$ influx and excitotoxicity (Choi, 1988; Hollmann et al., 1991; Burnashev, 1992). In fact, excitotoxic motor neuron death is mediated by extensive Ca$^{2+}$ entry into the cell through Ca$^{2+}$-permeable AMPAR. The Ca$^{2+}$ permeability of AMPAR is determined by the presence or absence of the GluR2 subunit in the receptor complex. However, there are contrasting studies in which lowering or overexpression of GluR2 subunits, respectively, aggravated or slowed down the motor neuron loss in a model of mutant SOD1-induced motor neuron degeneration in mice (Foran and Davide, 2010). The question arises as to whether genetically-determined differences in GluR2 expression could make humans more susceptible to diseases in which excitotoxicity is pathogenically involved.

GluR2 mRNA is regulated by editing, splicing, and the mRNA transported to dendrites where it can be locally translated. Non-NMDA receptors activate and desensitize rapidly and are primarily responsible for fast excitatory synaptic transmission. Thus, modulation of AMPAR properties can profoundly alter motor neuronal excitability. Most AMPAR are permeable to Na$^+$ and K$^+$, but impermeable to Ca$^{2+}$. Ca$^{2+}$ impermeability is conferred by the presence of GluR2 subunits, because the subunit can undergo RNA editing to encode a positively charged arginine (R) at position 586 (Q/R site), while unedited subunits contain a glutamine (Q) residue at this position. In the context of ALS, this becomes particularly interesting as mutations in TDP-43 and FUS/TLS, which are RNA binding molecules, were recently discovered as genetic causes of the disease (Arai et al., 2006; Neumann et al., 2006; Lemmens et al., 2009).

Notwithstanding GluR2 mRNA editing as a developmentally regulated switch in AMPAR properties, the motor neuronal AMPA (and KA) receptor population is unusually Ca$^{2+}$-permeable and, therefore, vulnerable to excessive glutamate stimulation (Foran and Davide, 2010) because the expression of the GluR2 subunit is low or absent in motor neurons in any case. Thus, the assembly and incorporation of AMPAR complexes lacking the GluR2 into the synapse may be tightly regulated processes that could be disturbed in ALS and predispose patients to develop the disease. In addition, growth factors, secreted by cells surrounding motor neurons thought to play a dominant role in ALS pathogenesis, could also influence the expression level of the GluR2 subunit. For example, the vascular endothelial growth factor, secreted by astrocytes, is capable of inducing GluR2 expression levels. Consequently, such growth factors and other astrocytic mediators may modulate GluR2 expression in motor neurons and therefore determine their likely susceptibility to excitotoxicity.
25.9.2 Glutamate metabolism and transport in ALS

There is an underlying defect in glutamate metabolism in ALS. Studies of the fasting levels of glutamate in the plasma or serum have shown an increased level in ALS patients (Plaitakis et al., 1988) and oral loading with glutamate results in significantly greater elevations in plasma glutamate and aspartate in comparison with controls (Gredal and Moller, 1995; Plaitakis et al., 1988). Not all studies have confirmed these reports. A similar dichotomy exists in the studies of levels of glutamate in the cerebrospinal fluid (CSF) of ALS patients in comparison with controls. The level of glutamate in CSF has been reported to be raised in at least a subgroup of ALS patients, although not all laboratories have replicated this finding (Heath and Shaw, 2002).

There is evidence that some component in ALS plasma and CSF is toxic to neurons in culture. This toxicity appears to be mediated by non-NMDA glutamate receptors, suggesting that the toxic product is a non-NMDA receptor agonist. This concept is based on the evidence that the glutamate-release inhibitor, riluzole, is neuroprotective (Couratier et al., 1994). On the other hand, some studies have shown a decrease in glutamate levels in various regions of post-mortem CNS tissue in ALS patients in comparison with controls (Perry et al., 1987; Tsai et al., 1990). This suggests that there are some underlying disturbances in glutamate metabolism and transport in ALS. The failure to properly regulate glutamate may result in raised extracellular levels of glutamate and reduced levels within CNS tissue, but the abnormalities seen in levels of tissue or extracellular glutamate are not caused by derangement in the activity of the major synthesizing enzyme glutamate dehydrogenase. There is, however, evidence for altered expression and function of glial glutamate transporters in ALS, particularly the excitatory amino acid transporter 2 (EAAT2) (Heath and Shaw, 2002). Abnormal splice variants of EAAT2 have been detected in the human CNS. At present, it appears that alterations in the expression and function of EAAT2 do accompany the ALS disease state. Overexpression of EAAT2 in a rodent SOD1 model delays the onset of motor neuron dysfunction (Guo et al., 2003), and treatment of mSOD1 mice with a beta-lactam antibiotic, which potently stimulates EAAT2 expression, increased their lifespan significantly with the delayed onset of neurodegeneration (Rothstein et al., 2005), supporting the idea that the loss of EAAT2 contributes to ALS pathogenesis. Overall, however, it remains unclear whether these changes represent a primary defect or part of an ascendant cycle of motor neuron injury, or if they occur as a secondary result of motor neuron loss and an attempted compensatory response to a failing motor system.

25.10 Stroke

25.10.1 Background

Stroke and cerebral ischaemia are the leading causes of death and permanent disability (Asplund et al., 1995), with no effective treatment currently known. Transient global cerebral ischaemia occurs during cardiac arrest, cardiopulmonary bypass surgery, and other situations that deprive the brain of oxygen and glucose for short periods. In both humans and animals, ischaemia damages neurons in vulnerable regions of the brain, including the hippocampus, striatum, cerebral cortex and cerebellum (Radenovic et al., 2008). Cerebral infarct is defined as a necrotic area that develops in sites where an acute ischaemic event has taken place, and can be divided into two forms: ischaemic or haemorrhagic. Haemorrhagic infarct is a consequence of the post-ischaemic reperfusion and can be a product of the re-opening of the artery, a partial occlusion of the artery, or because the blood is flowing from adjacent arteries. Acute ischaemia is associated with areas of complete infarct with tissue necrosis, oedema, and inflammation (Arango-Davila et al., 2004).

In general, cerebral ischaemia occurs when the amount of oxygen and other nutrients supplied by blood flow is insufficient to meet the metabolic demands of brain tissue. In ischaemic stroke, the blood supply to the
brain is disrupted by cerebrovascular disease. For decades, extensive research and clinical approaches to combat stroke have focused on the vascular aspects of cerebral ischaemia. Therapeutic advances, including carotid endarterectomy, thrombolytic therapy, anti-coagulation for cardiogenic stroke, antiplatelet agents and the treatment of risk factors such as hypertension and hyperlipaemia, have had significant effects on the morbidity and mortality of stroke. The final event in cerebral ischaemia is the death of neurons that can be of two forms: the first, which is closely related with energetic deprivation, is necrotic death; and the second, corresponding with the cellular programmed cell death, is apoptosis, which requires an adequate energetic supply in the neuron. In either case the result is an irreversible loss of neurologic function (Arango-Davila et al., 2004).

The advent of animal and tissue culture models of ischaemia has led to many new insights into the mechanisms by which ischaemic neurons die. If ischaemia is complete and prolonged, neuronal death is inevitable. However, it has become increasingly clear that many secondary biochemical changes that exacerbate injury occur in response to the initial insult. In models of cerebral ischaemia in rodents, as much as 50% or more of ischaemic brain may be spared from infarction by preventing these secondary biochemical events. Understanding of the mechanisms by which neuronal cell death takes place has resulted in a number of therapeutic strategies that aim to prevent secondary biochemical changes and thus decrease the damage that results from cerebral ischaemia. These basic mechanisms may also have relevance to other neurodegenerative diseases associated with excessive neuronal death.

25.10.2 Glutamate and glutamate receptors in cerebral ischaemia

It is well known that under physiologic conditions, glutamate participates in many neurologic functions, including memory, movement, sensation, cognition and synaptic plasticity (Lipton and Kater, 1989; Gasic and Hollmann, 1992; Martin and Wang, 2010). However, glutamate can also have a pathologic effect. Glutamate-mediated toxicity was first demonstrated by Olney and co-workers (Olney et al., 1971) by peripheral administration of an agonist that selectively killed neurons in the arcuate nucleus of the hypothalamus. These neurons contain high concentrations of glutamate receptors. Choi and co-workers demonstrated that micromolar extracellular concentrations of glutamate produce rapid increases in intraneuronal cytosolic Ca\(^{2+}\) concentrations. In accord, other studies have indicated that hippocampal neurons exposed to in vitro ischaemia exhibit increased Ca\(^{2+}\) accumulation following the activation of AMPA receptors. This accumulation is prevented by Joro spider toxin, a selective blocker of Ca\(^{2+}\)-permeable AMPA receptors, and is associated with a higher vulnerability to AMPA-mediated excitotoxicity (Ying et al., 1997).

Blocking the translation of a gene that encodes a subunit of the NMDA receptor with intraventricular injection of antisense oligonucleotides also decreases infarction volume after middle cerebral artery occlusion in the rat (Wahlestedt et al., 1993). These data and many other studies support the hypothesis that excitotoxicity contributes to ischaemic injury in vivo. Extracellular glutamate may activate other receptors besides the NMDA channel. NMDAR facilitates an influx of both Na\(^{+}\) and Ca\(^{2+}\), whereas the non-NMDA receptors (AMPA and kainate receptors) primarily facilitate an influx of Na\(^{+}\). However, some of the KAR and AMPA receptors are comprised of subunits that allow Ca\(^{2+}\) permeability. This may be relevant to ischaemic injury, because after cerebral ischaemia, the GluR2 subunit in neurons, responsible for non-NMDA receptors maintaining low Ca\(^{2+}\) permeability, is relatively depleted (Pellegrini-Giampietro et al., 1992). Accordingly, these non-NMDA receptors may become Ca\(^{2+}\) permeable after ischaemia. Type I metabotropic glutamate receptors may also increase intracellular Ca\(^{2+}\) by mobilizing Ca\(^{2+}\) from stores in the endoplasmic reticulum. Studies with antagonists of mGluR show that, depending on their subunit specificity, some, but not all, drugs of this class are neuroprotective in models of focal...
ischaemia (Lam et al., 1998; Bond et al., 1999). Thus, in vivo, excitotoxicity may be ameliorated by additional strategies besides inhibition of NMDAR.

Treatment with antagonists that compete with glutamate for the receptor (competitive NMDA antagonists) or antagonists that bind to the ion channel itself (non-competitive antagonists) can block Ca\(^{2+}\) entry into neurons and prevent cell death induced by glutamate (Hartley and Choi, 1989; Choi, 1990). Glycine is a co-agonist that is required in addition to glutamate to open the NMDA-Ca\(^{2+}\) channel (Thomson, 1989). Antagonists that bind to the glycine site on the NMDAR also block excitotoxicity in vitro (Smith and Meldrum, 1992). Compelling evidence is also available to indicate that excitotoxicity mediated by the NMDAR contributes to injury from cerebral ischaemia in vivo. A rapid and large increase in the concentration of extracellular amino acids can be monitored by microdialysis after cerebral ischaemia (Benveniste et al., 1984). Although NMDA antagonists are not effective in global ischaemia models in which temperature is carefully controlled (Buchan et al., 1991), a large number of studies have found that they decrease infarction volume in both permanent and temporary middle cerebral artery occlusion models in rodents (McCulloch, 1992).

Recent studies (Hardingham et al., 2002; Liu et al., 2007) suggest that a preferential activation of synaptically localized NMDAR, which predominantly contain NR2A, have a pro-survival role via activation of a neuronal survival signalling complex that is coupled to these receptors. In contrast, activation of extra-synaptic NMDA receptors, which predominantly contain NR2B (Iovar and Westbrook, 2002; Liu et al., 2007), may have a cell death-inducing role through stimulation of a neuronal death signalling complex. After stroke insult, the NMDAR complex interacts with death-associated protein kinase 1 (DAPK1), a member of a serine/threonine kinase family well known for its role in cell death (Bialik and Kimchi, 2006). DAPK1 directly interacts with the NMDA receptor via an interaction in the carboxyl tail region of the NR2B subunit. Future research is therefore important to determine how DAPK1 binding to NR2B induces cell death. Some data suggest that the binding is upstream of c-Jun N-terminal protein kinase (JNK) activation, an important step previously implicated in induction of neuronal death, but whether this is a consequence of DAPK1-dependent potentiation of NR2B-containing NMDA receptors, or direct activation of the neuronal death signalling cascade, is unclear (Martin and Wang, 2010). It is worth noting that specific antagonists of NR2B-containing NMDA receptors have shown little promise as a post-stroke therapeutic, suggesting that changes in channel conductance may not be critical in neuronal death at a later stage of stroke (Liu et al., 2007).

Intraneuronal Ca\(^{2+}\) increases not only depend on the activation of glutamate receptors, but also on the activation of voltage-gated Ca\(^{2+}\) channels (Choi, 1990). Drugs that prevent prolonged opening of P- and Q-type Ca\(^{2+}\) channel antagonists are also neuroprotective in animal models of stroke (Goldin et al., 1995). Hyperexcitation leads to the peri-infarct depolarization with the subsequent increase in the energetic failure, especially when the membrane is undergoing repolarization (Choi, 1992; Schiene et al., 1996; Back, 1998). Subsequently, increases in intraneuronal Ca\(^{2+}\) concentrations, peri-infarct depolarization and acidosis, contribute to the beginning of the processes that continue with inflammation and the activation of apoptotic mechanisms (Banasiak et al., 2000; White et al., 2000), amplifying the area of lesion and the death of adjacent healthy neurons. Mitochondria can buffer the large Ca\(^{2+}\) loads, but they do so at the expense of triggering injurious reactive oxygen species production. However, the final and definitive pathway to neuronal death has not yet been elucidated.

In addition to the direct downstream effects of enzymes that are activated by elevation of intracellular Ca\(^{2+}\), a number of complex interactions and positive feedback loops augment the contribution of glutamate to ischaemic brain injury. For example, free arachidonic acid can potentiate NMDA-evoked currents in neurons and inhibit reuptake of glutamate by astrocytes (Miller...
et al., 1992; Volterra et al., 1992). In addition, platelet-activating factor, a phospholipase A2 metabolite, can stimulate the release of glutamate. Acidotic conditions favour the release of free iron, which can then participate in the metabolism of peroxide into the hydroxyl radical via the Fenton reaction. The pH sensitivity of the NMDA receptor has received increasing interest, since pH changes are extensively documented during ischaemia (Silver and Erecinska, 1992). The acidification associated with this latter pathological condition should serve to inhibit NMDA receptors, which may provide a negative feedback that minimizes their contribution to neurotoxicity. In addition, glutamate can interfere with the function of the cystine transporter. Inhibition of the cystine transporter results in decreased intracellular concentrations of glutathione and diminished intracellular endogenous antioxidant stores (Murphy et al., 1989), which contribute to the excitotoxic damage.

Our knowledge of the mechanisms by which ischaemic neurons die has increased considerably. It is now clear that the toxic effects of glutamate exacerbate injury resulting from ischaemia. Antagonizing excitotoxicity via a variety of approaches can ameliorate injury in animal models of ischaemia. However, these treatments appear to be too toxic and are effective for too short an interval after the onset of ischaemia to be of practical use in treatments in humans. When ischaemia is transient or less severe, programmed cell death is activated. These events occur hours or days after the onset of ischaemia and thus may be more practical targets for treatment. Since there are multiple mechanisms involved in the ischaemic process, it is reasonable to assume that the combination of several drugs can have synergistic effects by blocking different steps in excitotoxicity. These combinations may include new and existing drugs able to limit neuronal depolarisation and abnormal Ca\(^{2+}\) and Na\(^+\) loading of the neuron during energy deprivation. Further work is needed to determine the most effective and practical therapeutic strategies to prevent neuronal death after ischaemia.

### 25.11 Conclusions

Glutamatergic synaptic transmission is fundamental to the normal functioning of the central nervous system. Alterations to this normal functioning can precipitate diseases of the nervous system. Excitotoxicity resultant from the overactivity of the glutamatergic system is seen to contribute to the aetiology of a number of neuropathologies discussed here. The propensity for excitotoxicity arises in the first instance from the pathological increase in extracellular glutamate concentrations produced by excessive release or inadequate uptake of the amino acid. A reduction in the energetic status of the neuron may contribute to this, in that lowered ATP levels (following ischaemia, for instance) can result in the cytosolic efflux of glutamate by reversal of glutamate transporters, as well as evoke exocytotic release due to the failure of the compromised neuron to keep presynaptic cytosolic [Ca\(^{2+}\)] low. The excitotoxic sequelae that follow increased extracellular glutamate are underpinned to a large degree by the cytosolic [Ca\(^{2+}\)] issue, in that glutamate receptor-induced insults typically involve the pathophysiological activation of Ca\(^{2+}\)-permeable ionotropic glutamate receptors. This may be compounded by glutamate activation of type I mGluR, which instigate intracellular Ca\(^{2+}\) store mobilization. Altogether, the excessive cytosolic [Ca\(^{2+}\)] initiates Ca\(^{2+}\)-dependent pathways that set neurodegeneration in motion. Regaining control of a glutamatergic system gone awry is therefore a clear and key objective in therapeutic interventions to either forestall or delay neurodegenerative processes underpinning neurological disorders. Pharmacologically, this could take the form of antagonists for the ionotropic glutamate receptors in principle; however, given the prevalence of these receptors and the generally pleiotropic effects of drugs targeted at them, better prospects may be forthcoming with the therapeutic development of agonists for the inhibitory metabotropic glutamate receptors, both pre- and post-synaptically. The challenge comes in determining when such interventions would be most efficacious given the complexity of the neuropathologies confronting us.
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Excitatory Amino Acids in Disorders


26 Efficacy of L-DOPA Therapy in Parkinson’s Disease

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26.1 Abstract

L-3,4-dihydroxyphenylalanine (L-DOPA) is a classical example of an amino acid that is an immediate precursor to dopamine and has been developed as an effective therapeutic agent for a neurodegenerative disease. Dopamine deficiency is known to be a core pathogenic event in Parkinson’s disease (PD). It is the main neurotransmitter responsible for modulation of motor behaviour. Peripherally given, L-DOPA is transported into the brain and upon decarboxylation converted into dopamine. L-DOPA pharmacotherapy is used as a symptomatic treatment of PD as it restores dopamine neurotransmission. Since L-DOPA can also be decarboxylated in the periphery, and peripherally formed dopamine is not able to cross the blood–brain barrier, inhibition of the peripheral decarboxylation increases bioavailability of L-DOPA in the brain. An initial satisfactory clinical response to L-DOPA is invariably hampered later in the course of the disease, mainly due to progression of the neurodegenerative changes in the brain. In addition, side effects such as motor fluctuations and abnormal involuntary movements become a significant problem for patients with advanced disease. Moreover, it is thought that pulsatile administration of L-DOPA is one of the most important underlying trigger factors for induction and maintenance of these motor complications. This mode of stimulation is thought to lead to abnormal changes in basal ganglia neurons at the molecular level. These plastic changes can be reduced or avoided if dopaminergic replacement is done in a more continuous and physiologic manner. Currently, continuous dopaminergic stimulation can be achieved by using long-lasting drug formulations or continuous L-DOPA infusion pumps. In addition to these peripheral administration approaches, recently, viral-mediated intrastriatal 3,4-dihydroxyphenylalanine (DOPA) delivery strategies have been developed as an alternative treatment in Parkinson’s disease.

26.2 Introduction

PD is a neurodegenerative disorder, first described in a monograph by James Parkinson (1817). Clinical features at onset typically include asymmetric bradykinesia (slowness...
in movements), rigidity (stiffness in muscles), and tremor (shaking) at rest. The mean age of onset is 57 years and the disease affects 1-2% of the population over the age of 60 years, although it can also begin at a much younger age (Koller et al., 1987). The motor symptoms emerge when a significant proportion of dopamine-producing neurons in the substantia nigra have been lost and striatal dopamine has been reduced by 60-80% (Hornykiewicz, 1998; Lang, 2007). Other neuronal populations are also known to be lost in addition to the dopamine system, resulting in deficits in noradrenergic, serotonergic, and cholinergic neurotransmission. These are thought to underlie the non-motor symptoms including cognitive decline, sleep abnormalities, and depression, as well as gastrointestinal and genitourinary disturbances.

Dopamine deficiency in PD cannot be replaced by direct peripheral administration because dopamine has a very short half-life in the blood and does not cross the blood-brain barrier. Instead, dopaminergic drugs are used for replacement therapy. The uses of dopaminergic drugs improve motor function, significantly reduce both the morbidity and mortality of the affected individuals, and improve quality of life (Rajput, 2001).

26.3 L-DOPA

L-3,4-dihyroxyphenylalanine (L-DOPA), the naturally occurring isomer of aromatic amino acid 3,4-dihydroxyphenylalanine (DOPA), is a classical example of neurotransmitter replacement therapy in the brain. As a precursor of dopamine, L-DOPA is the mainstay of treatment in PD. Unlike dopamine, L-DOPA is transported into the brain by the large neutral amino acid transport system (Nutt and Fellman, 1984).

D,L-DOPA racemate was first synthesized in the laboratory in 1911. Two years later the L-isomer was isolated from legumes (seedlings of Vicia faba) by Guggenheim. Even though early pharmacological investigations suggested that L-DOPA was biologically inactive, in 1927 Hirai and Gondo found that it was an active compound causing hyperglycaemia and hypotension in rabbits. In 1938, Holtz and Credner demonstrated in mammalian tissue extracts that L-DOPA was decarboxylated by aromatic amino acid decarboxylase (AADC) (reviewed in Hornykiewicz, 2002).

Dopamine was identified in the brains of rats and other animals in 1957 (Montagu, 1957; Weil-Malherbe and Bone, 1957). In the same period the antipsychotic action of reserpine was discovered and Dr Arvid Carlsson (who was one of three to be awarded the Nobel Prize in Physiology or Medicine in 2000) started to study this potent drug that was able to deplete neurotransmitters. In 1957, Carlsson administered DOPA to reserpine-treated rabbits and mice in order to understand its action, and discovered that this amino acid had a central stimulant action and was able to reverse the akinetic and sedative actions of reserpine (Carlsson et al., 1957). Following this, Carlsson showed that dopamine was a normal brain constituent, and mapped its regional distribution (mainly in basal ganglia) (reviewed in Iversen and Iversen, 2007).

Based on Carlsson’s discovery of dopamine, striatal dopamine deficiency in PD was described by Ehringer and Hornykiewicz in 1960, and, one year later, Birkmayer and Hornykiewicz showed that intravenous injection of single dose L-DOPA resulted in marked resolution of akinesia in 2-3 hours for up to 24 hours in PD patients (reprinted in English in Ehringer and Hornykiewicz, 1998; Birkmayer and Hornykiewicz, 2001). Later, Cotzias showed that oral administration of D-L-DOPA at a dose of 3-16 g day⁻¹ was also effective, but four patients developed agranulocytopenia (Cotzias et al., 1964, 1967). Subsequently he found that the use of the L-form was less toxic than the racemic D, L-DOPA mixture (Cotzias, 1968). These clinical studies established the efficacy of L-DOPA therapy in PD, and the US Food and Drug Administration approved L-DOPA as a treatment for PD in 1970.

It was only in 1975 when post-mortem analysis of brain tissue from L-DOPA-treated PD patients were found to have elevated levels of dopamine compared to untreated patients, that the effectiveness of L-DOPA was found to result from its metabolism to dopamine in the brain (Lloyd et al., 1975).
26.4 Dopamine Biosynthesis in Physiological Conditions

Tyrosine is an essential amino acid that is abundant in dietary proteins. Blood-borne tyrosine is taken up into the brain by the neutral amino acid transport system and subsequently from brain extracellular fluid into dopaminergic neurons (Elsworth and Roth, 1997). In the appropriate neuronal compartment, tyrosine is converted to DOPA by the tyrosine hydroxylase (TH) enzyme (Fig. 26.1). This reaction is the rate-limiting step in the synthesis of dopamine. The rate of tyrosine hydroxylation is not influenced by tyrosine availability in vivo under normal conditions in most dopaminergic neurons; however when the enzyme is activated by phosphorylation, or in dopamine neuronal systems that have a relatively high basal firing rate, tyrosine levels can affect the rate of conversion to DOPA. The activity of the TH enzyme is dependent on the presence of tetrahydrobiopterin, which acts as an essential cofactor and is formed from guanosine triphosphate (GTP) in a three-step enzymatic reaction. GTP cyclohydrolase 1 (GCH1) is the first and rate-limiting enzyme in tetrahydrobiopterin biosynthesis. During hydroxylation tetrahydrobiopterin is reduced to dihydrobiopterin by the activity of the dihydropteridin reductase enzyme.

Cytosolic DOPA is rapidly converted into dopamine by the pyridoxine-dependent aromatic AADC enzyme. Thus, under normal circumstances, the levels of DOPA in the brain are very low, typically below the detection limits of high-performance liquid chromatography (HPLC) assays. Needless to say, all endogenously formed DOPA in tissue is the L-isomer, whereas the D-isomer of the molecule does not act as a substrate for synthesis of dopamine. Importantly, however, not only dopamine neurons but also serotonin neurons, glia, and endothelial cells possess AADC activity and are known to contribute to the formation of dopamine, especially upon exogenous administration of L-DOPA (Melamed et al., 1981; Mura et al., 1995; Arai et al., 1996).

Once dopamine is synthesized, it is transported from the cytoplasm to specialized storage vesicles by the help of vesicular monoamine transporter-2 (VMAT2). In the vesicular compartment dopamine is concentrated to approximately 0.1 M, much higher than its levels in the cytosol (Kelly, 1993; Peter et al., 1995). Activation of the dopaminergic neuron by excitatory input causes influx of calcium ions that in turn give rise to fusion of vesicles with the synaptic membrane. The release of dopamine into the synaptic cleft stimulates its specific receptors located at both pre-synaptic and post-synaptic sites. This action is limited in duration by reuptake to the pre-synaptic terminal via the dopamine transporter. In addition, there is some evidence that glia and non-dopaminergic neurons may, to a limited extent, take up and metabolize extracellular dopamine (Hitri et al., 1994).

Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) are the main enzymes responsible for the metabolism of dopamine (Fig. 26.1). 3,4-dihydroxyphenylacetic acid (DOPAC) is formed either intraneuronally (dopamine and serotonin neurons) or extraneuronally (glial cells) by the MAO enzyme. Homovanillic acid (HVA) is the major end product of dopamine metabolism in the brain and is formed exclusively in the non-neuronal compartments via the COMT enzyme (Kopin, 1985).

26.5 Basic Principles of L-DOPA Pharmacotherapy in Parkinson’s Disease

Although L-DOPA has the capability to cross the blood–brain barrier, only 1% of an orally administered dose of L-DOPA enters into the brain, because of the rapid conversion into dopamine in the periphery. After the discovery of the role of peripheral AADC in the metabolism of L-DOPA (Bartholini et al., 1967), the addition of peripheral AADC inhibitors that do not cross the blood–brain barrier (carbidopa or benserazide) was found to significantly enhance the therapeutic efficacy, reduce the total dose required by approximately tenfold, and in turn lower the peripheral side effect profile (Cedarbaum, 1987; Pletscher and DalPrada, 1993). The therapeutic efficacy and side effects of these two AADC inhibitors when administered with L-DOPA have been thoroughly studied and
Tyrosine, an essential amino acid obtained from dietary proteins, is converted to 3,4-dihydroxyphenylalanine (DOPA) by the tyrosine hydroxylase (TH) enzyme. Activity of the TH enzyme is dependent on the presence of tetrahydrobiopterin (BH4), which acts as a cofactor and is synthesized from guanosine triphosphate (GTP) in a three-step enzymatic reaction. GTP cyclohydrolase 1 (GCH1) is the first and the rate-limiting enzyme in BH4 biosynthesis. DOPA is either immediately converted into dopamine by the pyridoxine- (B6) dependent aromatic amino acid decarboxylase (AADC) enzyme, or methylated to form 3-O-methyl-DOPA (3-OMD). The enzyme known as catechol-O-methyltransferase (COMT) catalyzes this methylation and S-adenosylmethionine (SAM) serves as the donor compound for the methyl group. Dopamine is metabolized to form either 3,4-dihydroxyphenylacetic acid (DOPAC) by the monoamine oxidase (MAO) or 3-methoxytyramine (3-MT) by the COMT enzymes. The end product of this metabolic pathway is homovanillic acid (HVA). The arrows on the left and right sides of the figure represent other synthesis and/or metabolism pathways, e.g. tyramine from tyrosine, melanin from DOPA, or norepinephrine from dopamine.
were found not to be significantly different (Greenacre et al., 1976).

In the presence of an AADC inhibitor, L-DOPA is substantially inactivated in the periphery by COMT enzyme to form 3-O-methyl-DOPA (3-OMD) (Fig. 26.1). Under these circumstances about 5–10% of the administered L-DOPA reaches the brain (Kaakkola, 2000). The 3-OMD metabolite is a large neutral amino acid with a long half-life (15 h). It crosses the blood–brain barrier but does not bind to the dopamine receptor and has no antiparkinsonian activity. The efficacy of L-DOPA can therefore be also enhanced by co-administration of a COMT inhibitor, which reduces O-methylation in the gut, increases L-DOPA absorption, and prolongs its half-life (Nutt et al., 1994). The first available COMT inhibitor in the market was tolcapone, but its usage was limited due to hepatic toxicity. Today entacapone is the most widely used COMT inhibitor and, when combined with L-DOPA, increases ‘on’ time and reduces ‘off’ time (Heikkinen et al., 2001).

26.6 Clinical Pharmacokinetics and Pharmacodynamics of L-DOPA Therapy

In clinical practice, L-DOPA is only available in a fixed-combination formulation with an AADC inhibitor. Between 80% and 90% of L-DOPA is absorbed in the proximal part of the small intestine by an active saturable carrier system for large neutral amino acids (Wade et al., 1973). The stomach has limited capacity for absorption but may be an important site of decarboxylation of L-DOPA, therefore the rate of gastric emptying is the principal determinant in the bioavailability of L-DOPA. L-DOPA alters the pattern of gastric emptying in healthy volunteers, explaining its erratic absorption from the gastrointestinal tract (Robertson et al., 1990). Gastric emptying may be delayed by food (especially fat), resulting in a threefold increase in the $t_{max}$ of L-DOPA (Baruzzi et al., 1987). It should therefore be administered at least 30 min before meals.

Oral protein loads have shown to reverse the therapeutic effect of L-DOPA without reducing the plasma concentrations (Pincus and Barry, 1987). This is thought to be due to the competition between large neutral amino acids and L-DOPA for a common transport across the blood–brain barrier (Alexander et al., 1994). Dietary manipulations, including rescheduling of protein intake or protein restriction (e.g. 0.8 g of protein kg$^{-1}$), can be a simple and effective adjunct to the treatment, especially in the advanced PD patients (Karstaedt and Pincus, 1992).

In the early course of PD, L-DOPA therapy gives a sustained effect, which has been termed a long-duration response (LDR) (Zappia et al., 2000). This is a consequence of the ability of the nigrostriatal dopamine system to convert L-DOPA to dopamine, store it in the presynaptic vesicles, and release it in response to physiological stimuli. However, after a variable time period from months to years, this smooth clinical response is replaced with short-duration response (SDR), which provides an improvement in motor disability that lasts only a few hours after the administration of a single dose of L-DOPA. It is assumed that both type of responses are present when the treatment is initiated, but SDR may be unnoticed due to the masking effect of LDR (Nutt et al., 1992).

As the LDR is progressively lost and the clinical picture becomes dominated by the SDR, PD patients experience fluctuations in the clinical response to L-DOPA therapy. Motor fluctuations include delayed onset of L-DOPA’s therapeutic effect or loss of efficacy between doses, termed ‘wearing off’. L-DOPA benefit wearing off is characterized by the re-emergence of both the motor and various non-motor symptoms such as mood changes, anxiety, dysesthesias and diaphoresis (reviewed in Stocchi et al., 2010). L-DOPA-induced abnormal movements (dyskinesias) are the other motor complication of long-term L-DOPA therapy that has a significant impact on the quality of life of the patients.

26.7 L-DOPA-induced Dyskinesias

Dyskinesias are a central side effect of dopaminergic therapy and represent a major clinical problem in the management of patients with PD. Cotzias first observed ‘the reversible
induction of athetoid movements, which had been observed only in patients with PD and only when the therapeutic effect was significant (Cotzias et al., 1967). Later observations clearly identified dyskinesias as a side effect of L-DOPA treatment (Barbeau, 1971). Based on published series, it has been estimated that PD patients treated for less than 5 years have an 11% risk of developing dyskinesias, those treated for 6–9 years have a risk of 32%, whereas patients treated for more than 10 years have a risk of 89% (Fabbrini et al., 2007).

The underlying mechanisms of L-DOPA-induced dyskinesias are still not fully understood. It is generally recognized that L-DOPA does not induce dyskinesias in PD patients (or in experimental animals) that have not been treated with dopaminergic medications previously. The process by which the brain becomes sensitized, such that each administration of dopaminergic therapy modifies the response to subsequent dopaminergic treatments, is called priming. Following priming, the development of dyskinesias largely depends on two additional factors, the pulsatile administration of L-DOPA, and the degree of dopaminergic denervation in the striatum (reviewed in Del Sorbo and Albanese, 2008). Current views suggest that both pre-synaptic (i.e., production, storage, controlled release and reuptake of dopamine by nigrostriatal dopaminergic neurons) and post-synaptic (i.e. status of receptors and second messenger signalling pathways in striatal neurons) components are critical in induction and maintenance of dyskinesias (reviewed in Cenci and Lundblad, 2006).

As the pre-synaptic dopaminergic neurodegeneration progresses in the parkinsonian brain (both in patients and in experimental models), other cell types start to take part in handling exogenously administered L-DOPA and its conversion to dopamine. In particular, serotonin neurons have recently been identified as playing a critical role. Serotonergic cells contain AADC enzyme and VMAT-2 transporter, and therefore possess not only the capability to convert L-DOPA to dopamine, but also to store it in synaptic vesicles as a false neurotransmitter (Arai et al., 1996). However, because they do not express the dopamine D2 auto-receptors and dopamine transporter, which are essential for the normal auto-regulatory feedback control of dopamine release from the pre-synaptic terminal, their activity is thought to lead to uncontrolled swings in extracellular dopamine concentrations. Importantly, it has been recently shown that dopamine released from serotonin terminals was responsible for the appearance of L-DOPA-induced dyskinesias in parkinsonian rats (Carta et al., 2007). Furthermore, either a lesion of the serotonin system by specific toxins, or pharmacological silencing of these neurons by selective serotonin 5-HT1A and 5-HT1B agonists, dramatically reduced or even completely abolished L-DOPA-induced dyskinesias in 6-hydroxydopamine (6-OHDA) lesioned rats and MPTP-treated monkeys (Munoz et al., 2008). These studies point to the deterministic role of the pre-synaptic dopamine-releasing compartment on the occurrence of dyskinesias.

Moreover, by using a novel system allowing the functional depletion of striatal dopamine while maintaining the structural integrity of the pre-synaptic compartment, we have shown that post-synaptic plastic changes caused by dopamine receptor stimulation occur as a consequence of the lack of dopamine, and do not require structural damage to the pre-synaptic dopaminergic terminals (Ulusoy et al., 2010). Importantly, although dyskinesias may be elicited as a function of the intrinsic cellular changes of the post-synaptic compartment, the striatal neurons respond normally to regulate dopamine release from an adequate pre-synaptic compartment.

### 26.8 Gene Therapy-mediated Continuous DOPA Delivery in the Parkinsonian Brain

In the light of these observations, it is plausible to think that the dyskinetic side effects of L-DOPA could be overcome by continuous delivery of L-DOPA. Actually, the proof-of-principle has already been obtained in the clinics. Intragastric, duodenal or intravenous infusions of L-DOPA have been shown to effectively decrease L-DOPA-induced dyskinesias
(Kurth et al., 1993; Nilsson et al., 1998; Stocchi et al., 2005; Olanow et al., 2006). These approaches support the basis for the concept of viral vector delivery of genes that encode enzymes critical for the synthesis of DOPA locally in the brain, as a tool for obtaining a source of continuous and physiological dopamine delivery locally in the brain.

Continuous DOPA delivery using viral vector-mediated gene transfer relies on the presence of a residual pool of endogenous AADC enzyme for synthesis of dopamine in the brain. The dopamine and serotonin terminals are two major sources of AADC in the striatum, while at least one additional minor non-neuronal pool is thought to be present as well. In the parkinsonian brain, the remaining dopamine axons and the serotonergic terminals are the two most likely places where conversion to (and release of) dopamine takes place. As the disease progresses, it is anticipated that fewer and fewer dopamine terminals will remain. Nevertheless, the serotonergic denervation of the striatum is significantly less than the dopaminergic one in PD patients and thus may remain as a reliable long-term source in a majority of the patients (Kish et al., 2008).

It is now established that not only TH, but also tetrahydrobiopterin (or the GCH1 gene), has to be provided for optimal DOPA synthesis ectopically in striatal neurons after gene transfer. By utilizing a new generation of recombinant-adeno-associated virus (rAAV2) vectors, the combined TH-GCH1 strategy provides therapeutic levels of DOPA synthesis in parkinsonian rats. At these levels of continuous DOPA synthesis, the animals not only recover in drug-induced rotation tests, but also show improvements on spontaneous motor tests (Kirik et al., 2002; Bjorklund et al., 2010). In line with these findings, it was recently shown that rAAV5-mediated DOPA delivery could reverse previously manifested L-DOPA-induced dyskinesias in rats (Carlsson et al., 2005). In this study, rats with an intrastriatal 6-OHDA lesion with moderate-to-severe behavioural impairments received daily pulsatile L-DOPA treatment until they became stably dyskinetic. After striatal injection of rAAV5-TH and rAAV5-GCH1, the severity of the abnormal dyskinetic movements gradually decreased to about 15% of the initial scores at 12 weeks post injection. The behavioural improvements, as well as the reversal of dyskinesias, would suggest that dopamine synthesized after this gene therapy approach should reach the post-synaptic receptors on striatal neurons in a physiological manner, and that the therapeutic effects are correlated with normalization of the dopamine neurotransmission. Indeed, dopamine D2 receptor ligand imaging using $[^{13}C]$Raclopride in AAV-treated rats showed normalization of dopamine D2 receptor binding and affinity, confirming this interpretation (Leriche et al., 2009). The ratio between TH and GCH1 availability is also important for optimization of continuous in vivo DOPA synthesis, and it was recently shown that optimal TH enzyme functionality requires GCH1/TH ratios of between 1/3 and 1/7 (Bjorklund et al., 2009).

Taken together, these data show that viral vector-mediated, continuous DOPA delivery is an attractive strategy for enzyme replacement in PD patients, and should be investigated further with the ultimate goal being clinically tested for efficacy.

### 26.9 Summary and Concluding Remarks

L-DOPA is a classic example of brain neurotransmitter replacement therapy. In the early stages, it gives a 'honeymoon' life to patients with PD. However, long-term motor complications constitute a major limiting factor in the treatment of advanced disease. Although patients experience these disabling side effects, they need continuous dopaminergic replacement therapy to have symptomatic relief. Use of L-DOPA in PD has been developed and improved by never-ending intensive research in the field, and optimization efforts are still ongoing. As the underlying mechanisms of long-term complications of L-DOPA are understood, new solutions are being developed and investigated for an optimized use in PD. Some of these include extending its half-life and improving efficacy by concomitant use with peripheral AADC and COMT inhibitors,
attempts to develop oral long-lasting once-daily pills, transdermal formulations, and continuous infusion systems. Especially the latter approach is expected to have a big impact in quality of life for advanced PD patients. In this context, viral vector-mediated direct gene transfer techniques have been shown to provide continuous and stable dopamine synthesis in the experimental models of PD, and are currently being explored as a novel treatment modality in early clinical trials.

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27 Amino Acid Profiles for Diagnostic Applications

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27.1 Abstract

We describe how amino acid profiles could be used for clinical diagnosis through the generation of an Aminolndex, a function composed of multiple amino acid concentrations. Early studies on the correlation of amino acids indicated that the relationships between certain amino acids changed with their physiological state. Initial animal studies suggested that a fractional function composed of multiple amino acids could be used to separate different disease and physiological states. We describe the various problems regarding analytical speed, sample handling, and algorithm that had to be overcome in order to apply the methodology to clinical diagnoses. Finally, the analysis of clinical data by the Aminolndex method is presented for liver fibrosis in type C hepatitis, metabolic syndrome, colon cancer and breast cancer. The results so far suggest that the Aminolndex has a potential to be used in a number of clinical diagnostic applications.

27.2 Introduction

Since the recognition of inborn errors in metabolisms over 100 years ago, it has been known that extreme cases of metabolic imbalance can lead to profound consequences (Garrod, 1909). Since then, the understanding of amino acid metabolism and the ability to quantify free amino acids have progressed. The measurement of amino acid concentrations in biological fluids and tissues has provided us with important biochemical and nutritional information that enables the diagnosis of diseases, especially metabolic deficiencies (Armstrong and Stave, 1973a,b,c; Deyl, 1986).

Recently, there has been much progress in genomics, transcriptomics, proteomics, and metabolomics. Although there are comprehensive technologies for genomics, transcriptomics, and proteomics where universal methods are available to measure the relevant parameters, this is not yet the case in metabolomics.

One of the aims of metabolomics is to determine all, or as many as possible, of the relevant metabolites and to elucidate their relationships in a comprehensive manner. We have taken the view that useful information may be gained by the analysis of a metabolomic subset, such as the set of amino acids, and this type of approach has been called focused or targeted metabolomics (Kimura et al., 2008). Focused metabolomics has also been used in the analysis of the lipid metabolomic subset (German et al., 2007; Piomelli...
The amino acid metabolomic subset seemed to be especially relevant in studying one of our interests – the effects of excessive intakes of amino acids, and how to determine safe upper intake levels. The amino acid metabolomic subset was also a convenient model for a study on how to analyse metabolomic data.

It was clear from earlier studies that plasma amino acid levels did not simply reflect the net intake of dietary amino acids (Moundras et al., 1993; Forslund et al., 2000), and that the final plasma level for each amino acid is determined by a multitude of factors including catabolic rates and transport rates into and out of various organs. We realized that one key concept was that when considering the relationships among metabolites, it was important to note that the various metabolites constituted networks within cells, tissues, or the whole organism. Much information regarding the network of relationships among the various metabolites would be lost by the traditional approach of averaging results from different individuals within a treatment group. This was especially so when considering the potential for genetic variability and variations in gene expression levels among individuals within a treatment group (Noguchi et al., 2003).

### 27.3 Correlation-based Analysis of Amino Acids

Initial studies focused on data obtained from experiments where the protein contents of diets for rats were varied from deficiency to gross excess. Rats were fed for 2 weeks on diets where only the protein and carbohydrate contents were varied, with protein ranging from 5% to 70% of the diet. Two different types of protein, casein and purified egg protein, were used in parallel experiments. Blood samples were collected several hours after the end of the feeding period to minimize the effect of the postprandial rise in plasma amino acid levels. When analysing the data, it was found that when plasma amino acid concentrations from each rat were plotted against each other, most of the relationships were linear, or consisted of a combination of linear segments (Noguchi et al., 2006). From these observations, if was found that the analysis of correlations between plasma amino acid levels in individual animals was a promising direction in the analysis of amino acid profiles. We have suggested that the use of correlation analysis could be useful in analysing metabolomic and other ‘omics’ data, as correlation is without units and can accommodate pseudo-quantitative data, such as the peak heights of unidentified metabolites (Noguchi et al., 2003; Sakai et al., 2004). The analysis of correlation has been further extended to studying the network of relationships between plasma amino acid concentrations, and the expression levels of genes related to amino acid metabolism of different organs (Noguchi et al., 2008). As this type of analysis is totally data driven, we feel that correlation-based analysis will be extremely useful for the future development of the ‘-omics’ technologies as the data become more quantitative. However, correlation-based analysis requires many data points in order to obtain a pattern of relationships, and is not suited for use in determining the particular physiological state of an individual organism. In theory, multiple blood samples could be used to generate a correlation-based picture of the metabolic network of an individual, but this would seem impractical.

### 27.4 Development of Amino Acid Diagnostics

#### 27.4.1 Background

Amino acids, supplied as food intake or synthesized endogenously, play essential physiological roles both as basic metabolites and as regulators in many metabolic pathways (Felig, 1975; Brosnan, 2003; Cynober, 2004). These include protein synthesis, protein degradation, urea cycle, glucogenesis, NO production, as precursors for neurotransmitters in the central nervous system, and others. Since blood circulates as a medium linking all organ systems, the profiles of plasma amino acid concentration (aminogram) could be influenced by the metabolic variation of a particular organ system induced in a specific disease. Altered
profiles of amino acid concentrations have been observed in various diseases including liver disease, cancer, diabetes, renal failure, and so on (Felig et al., 1970; Watanabe et al., 1984; Hong et al., 1998; Holm et al., 1999). However, the difference in any single plasma amino acid concentration between a normal state and a particular disease state, except for inborn errors of metabolism, may not be sufficient for gaining statistical significance due to factors including individual variability and heterogeneous backgrounds. Historically, as a first and simplest example of an amino acid-based diagnostic index, Fischer’s ratio – the ratio between the branched-chain amino acids (BCAA) and aromatic amino acids (AAA), \((\text{Leu} + \text{Val} + \text{Ile}) / (\text{Tyr} + \text{Phe})\) – has been used for the diagnosis of hepatic encephalopathy and monitoring its drug treatment efficacy (Fischer et al., 1975, 1976). Although Fischer’s ratio has been used in practice for clinical diagnosis, the extensive search for an optimal diagnostic index from a dataset of amino acid concentrations has not been exploited.

### 27.4.2 Initial studies

Initially, an attempt was made using available data to separate animals at a low protein state from those at a high protein state. It was found that a single amino acid could not act as a parameter to separate the two groups, especially if data for the two different types of protein were used. However when simple fractional functions similar in form to Fischer’s ratio, and composed of the plasma amino acid concentrations, were screened by trial and error, it was found that a fractional function, \((\text{Thr} + \text{Lys} + \text{Leu}) / \text{Ser}\), could separate low-protein animals from high-protein animals. However, it could not be determined if this was the best function, as all possible combinations of amino acids had not been tested, so the expression was generalized and a computer programme was created to test all combinations of amino acids. When this program was applied to separate animals at a low-protein state from those at a high-protein state, the function, \(\text{Ile} / \text{Glu} + (\text{Thr} + \text{His} + \text{Tau}) / \text{Ser}\), was found to be the best index for separation.

The first algorithm used to generate a diagnostic index was as follows (Noguchi et al., 2006):

1. Amino acids are classified into two groups on the basis of their positive or negative correlation with the target parameter, whether continuous, ordinal, or dichotomous, that represents the disease stage or physiological condition.
2. Amino acids from the positive correlation group are assigned to the numerator, and amino acids from the negative correlation group are assigned to the denominator, and all possible combinations for the fractional function in the form \((A + B + C...) / (N + O + P...) + (D + E + F...) / (Q + R + T...)\), where letters A–M represent amino acids with a positive correlation to the target parameter, while letters N–X represent amino acids with negative correlation to the target parameter, are generated. The correlation with the target parameter is calculated for each combination. The fractional functions are selected for further processing by evaluation methods, such as the sum of squares due to error based on simple linear regression for continuous target parameters, or variance ratio for categorical target parameters.
3. The optimal fractional function is finally selected after cross-validation of a large set of candidate functions by using random sampling to attain robustness with respect to sample variability and noise (Hjourth, 1989; Lee et al., 2004). The resulting function is called an Aminolindex.

In order to validate the diagnostic potential of the Aminolindex, the program was applied in dichotomous mode to obtain functions that can discriminate diabetic model rats from normal rats. Goto-Kakizaki rats and streptozotocin-treated rats (Pain and Garlick, 1974) were used as the diabetic models, and it was found that the function \((\text{Tau} + \text{Cit} + \text{Lys}) / (\text{Asp} + \text{Ile}) + (\text{Thr} + \text{Iyr} + \text{His}) / \text{Glu}\) could successfully discriminate between the diabetic and normal rats with a sensitivity of 98.6% and a specificity of 97.6%. Long-term insulin treatment of the streptozotocin-induced diabetic rats resulted in a statistically significant shift of the amino index toward normal values.
For continuous target variables, the program can generate an amino index with the best linear fit to the target variables. In order to test this mode, dimethylnitrosamine-treated liver dysfunction model rats were used (Vendemiale et al., 2001). Liver hydroxyproline concentration, reported to be a marker for liver fibrosis (Rojkind and Kershenobich, 1976), was chosen as the target parameter. The linear regression to the hydroxyproline concentrations resulted in the amino index (Tau + Cit + Met + Arg) / (Ser + Leu) + (Phe + Orn) / (Glu + Trp) with a coefficient of determination (r²) of 0.85. This compared favourably to Fischer’s ratio which had a r² of 0.49 (Noguchi et al., 2006). The efficacy of this type of function may reflect the linear nature of the relationships of the amino acid concentrations found in the previously described correlation-based analysis. We have found it surprising that fitting to this type of simple fractional function, without coefficients, gives high correlations to biological parameters, and suspect that this type of function may be approximating some aspects of the true steady-state kinetics functions governing the behaviour of the amino acids. Given enough computing power it may be possible to derive the real function in a totally data-driven manner.

However, one drawback to the initial approach is that simple combinations of n variables give rise to 2^n-1 fractional functions; hence, calculation of all possible combinations of the amino acids to derive the optimal fractional function is time consuming. We have found that calculations with 30 amino acids would take all night on a personal computer, and although an increase in computing speed is effective in reducing the computing time for a given number of variables, it did not allow a great increase in the number of variables that could be handled, as the computing time increased exponentially with variable number.

In order to speed up analysis, various statistical methods have been incorporated in the analysis, and currently Aminolndex can be generated by a data-mining approach, through multi-variate statistical methods such as logistic regression, linear regression, linear discriminant analysis, support vector machine and Bayesian network (Hastie et al., 2001; Armitage et al., 2002), in addition to the fractional function approach (Fig. 27.1). Optimal variable selection and cross-validation methods are

![Flowchart from plasma amino acids to Aminolndex generation](image)

Fig. 27.1. Flowchart from plasma amino acids to Aminolndex generation. The Aminolndex generation proceeds from a dataset including amino acid profiles and target variables, through multi-variate statistical analysis, to the selection of Aminolndex with an optimal discriminating power.
27.4.3 Measurement of amino acids

Key historical developments in the analysis of amino acids was the use of paper chromatography (Martin and Synge, 1941), followed by the development of the amino acid analyser utilizing post-column ninhydrin reactions for detection (Spackman et al., 1958). Amino acid analysers currently available are being replaced with smaller and faster computerized operations and data processing, and advances in ion-exchanging resins has made it possible to analyse 41 plasma amino acids in 100 min. However, even though the analysis has been speeded up, at most only ten samples can be analysed in a day by one analyser. Because of such poor efficiency, it would not be suitable for handling large number of samples as required for routine diagnosis. In recent years, a wide variety of methods using pre-column derivatization of amino acids to replace the post-column ninhydrin reactions have increased the speed and sensitivity of amino acid analysis. We have developed new reagents for pre-column derivatization of amino acids so that separation can take place on reversed phase high-performance liquid chromatography (HPLC) for detection by mass spectrometry (Shimbo et al., 2009). This method can shorten the analysis time to about 7 min, which is less than one tenth of the post-column ninhydrin method.

27.5 Clinical Applications

27.5.1 Factors influencing stability of data

Early studies with clinical samples indicated that when collecting from different sites, the standardization of sample handling was essential. Otherwise, differences in amino acid concentrations reflecting differences in sample handling between different sites would give rise to Aminolndices that differentiated the clinical sites rather than the physiological conditions of the subjects. Factors which influence the stability of the data needed to be examined.

There is generally an increase in plasma amino acid levels after a meal containing amino acids (Ozalp et al., 1972; Ljungqvist et al., 1978; Hegarty, 1982). It was therefore important to choose a time to collect blood when there was the least influence of the preceding meal. We have found that blood collected in the morning without breakfast, between 7 a.m. and 10 a.m., was consistent for an individual on separate days. The red blood cells and plasma have very different amino acid profiles so it is also important that there is no haemolysis of the blood sample. The blood contains enzymes that catabolize amino acids, and the time and temperature after blood collection was found to influence certain plasma amino acid concentrations. In order to avoid artefacts, it was found that cooling the samples on ice water immediately after blood collection and separating the plasma from the blood cells within 4 hours of blood collection gave stable results. It was also found that ethylendiamine tetraacetic acid disodium salt (EDTA 2Na) was superior to heparin as an anti-coagulant as it partly inhibited enzymes catabolizing amino acids. The plasma should be stored at −70°C rather than at −20°C, as certain amino acid concentrations decrease with time at −20°C. We have found that samples stored at −70°C can be thawed and restored up to three times without significant effects on plasma amino acid concentrations. Samples can be transported under dry ice for work with multiple sites.

27.5.2 Application of Aminolndex to liver fibrosis

The initial fractional function AminoIndex algorithm was first applied to the study of liver fibrosis to evaluate its usefulness in clinical diagnosis (Zhang et al., 2006). This study aimed to develop a non-invasive and effective method for the diagnosis of liver fibrosis using the plasma amino acid profiles. The background shows that among patients with chronic hepatitis C infection, the progression of liver fibrosis leads to cirrhosis and increases the risk of hepatocellular carcinoma (Poynard
The grade of fibrosis influences the efficacy of current therapy, and therefore the accurate detection of the stage of fibrosis is needed for determining whether treatment is necessary, and what treatment is appropriate (Fried, 2002; Aspinall and Pockros, 2004; Shiffman, 2004). Although fibrosis grading by biopsy has been considered as a gold standard, there is a high demand for alternative effective and non-invasive methods (Imbert-Bismut et al., 2001). In this study the liver specimens were analysed histologically, and graded with the METAVIR scoring system (Bedossa, 1994), where F0 means no fibrosis, F1 means portal fibrosis without septa, F2 means fibrosis with rare septa, F3 means portal fibrosis with numerous septa, and F4 means cirrhosis. The distribution of the 23 plasma amino acids of all 53 patients exhibiting the range of fibrosis stages is shown in Fig. 27.2 (from Zhang et al., 2006). In the Kruskal-Wallis test for each amino acid, significant changes in the concentration of Phe, Val, Ile, Tyr, Gln, Leu, Met (p < 0.01) and

Fig. 27.2. The distribution of the plasma amino acids over different stages of liver fibrosis: F0/F1, F2, F3, and F4. The unit is μmol l⁻¹. The asterisks indicate the significance values by the Kruskal–Wallis test (** p<0.01, * p<0.05). Aba designates α-amino butyric acid.
α-ABA (p < 0.05) were observed between the different fibrosis stages. The dataset was analysed to give the AminoIndex, (Phe) / (Val) + (Thr + Met + Orn) / (Pro + Gly), that was optimized to be a surrogate marker to the liver stages obtained through biopsies. For the assessment of the surrogate AminoIndex, the area under the curve (AUC) of receiver operator characteristic (ROC) curve was used for evaluation. The AminoIndex showed high performance for discriminating advanced fibrosis (fibrosis stages F3 and F4) from the earlier stages F0-2, and also for discriminating cirrhosis (F4) from all other stages, with AUCs of 0.92 (95% CI 0.84–1.00) and 0.99 (0.96–1.00), respectively. Fischer’s ratio has been reported for its good performance in assessing chronic hepatitis (Kano et al., 1991), and the comparison between it and AminoIndex is shown in Fig. 27.3 (modified from Zhang et al., 2006). The AminoIndex was generated to have a positive correlation with the degree of fibrosis, showing an inverse pattern to Fischer’s ratio, and exhibited the larger values of ROC AUC than Fischer’s ratio. The AminoIndex is a combination of two molar ratios, (Phe) / (Val) + (Thr + Met + Orn) / (Pro + Gly), and and the analysis in detail revealed that the former ratio mainly contributed to the F4 discrimination whereas the latter ratio mainly contributed to discrimination of advanced fibrosis (F3, F4). This is partially supported by the fact that the ratio Phe/Val correlated well with the inverse of Fischer’s ratio (r = 0.95). Because BCAA had a tendency of mutual correlation, as did Tyr and Phe, it was confirmed that the substitution of Val with Leu or Ile or the substitution of Phe with Tyr showed similar discriminative power. These results suggested that the fibrosis AminoIndex based on plasma amino acid concentrations could be applied to evaluate liver fibrosis as an effective, less invasive method, than liver biopsy.

27.5.3 Application of AminoIndex to metabolic syndrome

Metabolic syndrome is a pathophysiological state consisting of hypertension, diabetes, and dyslipidaemia in combination, and is also a major risk factor for cardiovascular disease (Grundy et al., 2004; Després and Lemieux, 2006). With the high prevalence of metabolic syndrome in developed countries, where overeating and inactivity are common, metabolic syndrome is becoming a serious issue from the viewpoint of social medical care. The AminoIndex was also applied to the
study of metabolic syndrome (Takahashi et al., 2006) based on the observation that the plasma amino acids showed significant change associated with the metabolic syndrome subcomponents including obesity, dyslipidaemia, hypertension, and hyperglycaemia. From the viewpoint of metabolic networks, many amino acids play crucial roles as metabolic hubs, with highly linked metabolic nodes in all the metabolic pathways connecting glucose, lipid, and amino acids (Wagner and Fell, 2001). The elucidation of the metabolic state in metabolic syndrome is expected to lead to a deeper understanding of the physiological mechanism behind its aetiology.

In the study the criteria for extracting the metabolic syndrome group was based on the Examination Committee of Criteria for Metabolic Syndrome (2005), with the subcriteria:

1. waist circumference ≥ 85 cm for males and ≥ 90 cm for females;
2. systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg;
3. triglycerides ≥ 150 mg dl⁻¹ or HDL cholesterol < 40 mg dl⁻¹; and
4. fasting plasma glucose ≥ 110 mg dl⁻¹.

Metabolic syndrome is diagnosed if criterion 1 and at least two of criteria 2–4 are satisfied.

Plasma amino acids of 32 volunteers satisfying the metabolic syndrome criteria and 173 non-metabolic syndrome volunteers were analysed. When differences in individual amino acids were examined by t-test, significant increases in Ala, Glu, Tyr, Val and Trp, and significant decreases in Gly and Ser were seen in subjects with metabolic syndrome compared to non-metabolic syndrome subjects. Aminogram changes in glucogenic amino acids and branched-chain amino acids were similar to those in obesity and diabetes (Felig et al., 1969, 1970), and may reflect the metabolic modulation by insulin resistance, in which both the metabolic shift to protein catabolism and the up-regulation of gluconeogenesis in the liver would be induced (Chevalier et al., 2006). The optimal search of Aminolndex in the logistic regression analysis was performed for discriminating metabolic syndrome and gave the index, \(-1.043 - 0.028 \text{Gly} + 0.011 \text{Ala} + 0.023 \text{Thr} - 0.029 \text{Ser} + 0.020 \text{Glu},\) with which the value of ROC AUC was 0.82 (95% CI 0.75–0.89), and the sensitivity and specificity were 0.81 and 0.75, respectively (Fig. 27.4). These results suggested that the Aminolndex for metabolic syndrome could reflect the nutritional and metabolic state as induced by the physiological changes including insulin resistance.

![ROC curve](image)

**Fig. 27.4.** Profiles of the Aminolndex for metabolic syndrome (MetS) discrimination. (a) Receiver operator characteristic (ROC) curves for training data (black) and test data (grey); (b) Distribution plots for MetS(−) and MetS(+) groups in training data (left) and test data (right). Bars are mean ± SD.
27.5.4 Application of Aminolndex to colorectal and breast cancer

It is well known that the metabolism of cancer cells is notably altered and that their plasma amino acid profiles are changed. There has been accumulating evidence of important effects on Arg levels in association with activities of the immune system in cancer patients. Several reports on cancer have mentioned increased production of arginase I, which catalyses the conversion of Arg to Orn and urea, and increased production of nitric oxide synthase, which catalyses the conversion of Arg to Cit and nitric oxide (NO) (Rodriguez et al., 2004; Yamaguchi et al., 2005). Plasma level of Arg would be affected by the change of arginase I activity (Vissers et al., 2005). It is also demonstrated that the central metabolism of cancer cells is changed drastically in studies measuring intracellular metabolites using capillary electrophoresis–mass spectrometry (CE–MS) (Hirayama et al., 2009). Therefore identification of metabolic changes using amino acid profiles was seen as a promising approach for detecting the presence of cancer. On the other hand, cachexia and malnutrition, typical symptoms observed in most patients with advanced cancer, also cause changes in plasma amino acid profiles. However, changes in the plasma levels of several amino acids are observed in cancer patients with little or no weight loss (Vissers et al., 2005). Therefore the changes in plasma amino acid profiles might be due not only to malnutrition, but to cancer-specific alterations of amino acid metabolism that occur even in early-stage cancer patients without cachexia and malnutrition.

Colorectal cancer is a major cause of morbidity and mortality worldwide, and is one of the most common causes of cancer deaths in Japan (Saito, 1996), while breast cancer is the most common cancer among Japanese women (Ohnuki et al., 2006). If diagnosed at an early stage, these kinds of cancer can be eliminated by surgical or endoscopic excision, without recurrence or metastases, in most patients.

Aminolndex was applied for early detection of colorectal and breast cancer patients (Okamoto et al., 2009). Plasma amino acid profiles were compared between cancer patients (who had colorectal cancer or breast cancer without any apparent symptoms) and control subjects for each type of cancer. The plasma concentrations of several amino acids in the colorectal cancer patients were significantly different from those observed in the controls. The concentrations of Thr, Cit, Val, Met, Ile, Leu, Tyr and Phe were reduced in the colorectal patients, while that of Glu was increased. The alteration of the plasma amino acid profile in breast cancer differed from that in colorectal cancer, with fewer changes observed. The levels of Met, Ile, Phe, and Arg decreased in the breast cancer patients, while those of Thr, Ser, Glu, α-ABA and Orn increased.

Multiple logistic regression analyses with selected variables were made using each data set. For colorectal cancer, discriminant-1 consisted of the six amino acids Val, Glu, Thr, α-ABA, Gln and Pro. For breast cancer, discriminant-2 consisted of the six amino acids Thr, Ala, α-ABA, Ile, Orn and Arg. When using training data sets, 0.860 of ROC AUC for colorectal cancer and 0.906 for breast cancer were obtained, respectively (Fig. 27.5, solid lines). To confirm the performance of the indices obtained, ROC curves from the split test data were also calculated. These reproduced the similar diagnostic performance, with AUC of 0.910 for colorectal cancer, and 0.865 for breast cancer, respectively (Fig. 27.5, broken lines). In the case of colorectal cancer, about 60% of the patients were categorized as early stage. In the case of breast cancer, most of the patients were also categorized as early stage. In both cases, the Aminolndex score in the early stages was not significantly different from those in the late stages. These results indicate that the Aminolndex is equally predictive for early and later stage patients, and may be useful for the early detection of colorectal and breast cancer.

27.6 Future Perspectives

Although the published applications of Aminolndex are still limited, the foundations for its use for diagnostic purposes are in progress as described above. Animal studies indicate that the Aminolndex concept is valid and can be used to separate physiological
Amino Acid Profiles

Fig. 27.5. ROC curves of discriminant-1 for colorectal cancer (a) and discriminant-2 for breast cancer (b) of training data (solid line) and test data (dotted line).

states, while studies with clinical data indicate that even with individual variability, the AminoIndex can be used to separate certain disease and physiological states. The practical aspects of the use of the AminoIndex as a diagnostic tool are also being addressed, with the development of improved analytical methods for amino acid quantification allowing higher throughput, and the standardization of sample handling conditions to minimize inter-site differences in data.

There is further scope for development in the analysis, such as the inclusion of parameters other than amino acids in the calculations. We believe that there is potential benefit of the AminoIndex as a preliminary diagnostic screen for multiple diseases. By further accumulating data on multiple diseases and physiological conditions, it is hoped that multiple indices, each associated exclusively with a specific disease, can be generated without overlaps or crosstalk between the indices. It is also hoped that this type of analysis may be of great use in tailor-made medicine and nutrition, as it may be possible to discriminate populations for which certain pharmaceutical or nutritional interventions would be useful.

References


Armstrong, M.D. and Stave, U. (1973c) A study of plasma free amino acid levels. V. Correlations among the amino acids and between amino acids and some other blood constituents. Metabolism 22, 827–833.


Amino Acid Profiles


Part V

Conclusions
Emergence of a New Momentum

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28.1 Abstract

It is appropriate and instructive to review the evidence presented in the preceding chapters of *Amino Acids in Human Nutrition and Health*. The purpose here is to integrate the individual sets of conclusions in a manner that emphasizes not only the underlying complexities of interdependence in amino acid metabolism and utilization, but also the relationship to diverse disorders and the potential for development of novel therapeutics. Enzyme expression and activity have been selected as the principal exponents of the impact of amino acid metabolism on nutrition and health.

An important function of the enzymes considered in this volume is the biosynthesis of the nutritionally dispensable amino acids. As a consequence of the action of these and other enzymes, glutamate, aspartate, arginine, glutamine, glycine, serine, tyrosine and cysteine need not be supplied in the diet. Nevertheless, glutamine supplementation may be beneficial in particular clinical conditions. Thus, the distinction between nutritional dispensability and clinical efficacy justifies further definition not only in relation to glutamine, but also to arginine. In other disorders, nutritional support comprising administration of branched-chain amino acids has been associated with consistent and positive responses. The position of glycine, specifically as an immunonutrient, has also been suggested. As further research is conducted on fundamental issues such as dynamics and metabolic regulation, nutritional support should emerge as a more consistent form of clinical intervention. This effort may well be enhanced by current studies designed to quantify requirements and endogenous losses of amino acids, and to assess nutritional adequacy of food proteins.

Notwithstanding the foregoing developments, a defining feature of recent research is the recognition of the critical role of several enzymes in eliciting normal, synchronized, aminoacidergic, monoaminergic and nitric neurotransmission in the central and peripheral nervous systems. For example, glutamatergic function is modulated by the interdependent activities of several enzymes reviewed in this volume, while nitric neurotransmission is determined by the competitive action of two key enzymes. The role of amino acid transporters is also of profound importance in these processes. Disruption of the major neurotransmission systems has been implicated in the pathophysiology of a diverse range of disorders. Thus, aberrations of the glutamatergic...

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system may contribute to conditions such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, schizophrenia and some forms of epilepsy. Abnormalities of monoaminergic neurotransmission have been linked with cognitive impairment, psychiatric disorders and even obesity. Disruption of the nitricergic system has been implicated in multiple sclerosis and endothelial disorders. However, current research suggests that key enzymes and receptors associated with neurotransmission may emerge as potential targets for the development of novel therapeutics.

Progress in the nutritional and health sciences has been significantly enhanced by the development and application of innovative techniques, with the added benefit that even well-established syndromes, such as inborn errors of metabolism, are currently subject to reassessment. Another theme of contemporary work is the development of clinical methodologies for monitoring and risk assessment. Currently there is interest in employing homocysteine and post-translationally modified amino acid residues as indicators of cardiovascular disease and ornithine decarboxylase as a risk factor in carcinogenesis. Elevated plasma concentrations of aminotransferases may serve as markers for a variety of conditions including non-alcoholic fatty liver disease, insulin resistance, hazardous alcoholic consumption and atherosclerosis in rheumatoid arthritis.

There is thus unequivocal evidence of the emergence of a new momentum which should drive forward a comprehensive and dynamic agenda aimed at a more profound understanding of the functional metabolism and utilization of amino acids. Progress with this programme should enable the elucidation of the biochemical basis of a variety of amino acid-related disorders and the development of novel therapeutic and risk assessment strategies.

28.2 Rationale

The unprecedented scale of recent biochemical advances has provided the impetus for publication of this first edition of *Amino Acids in Human Nutrition and Health*. Even the most perfunctory inspection of the research literature will indicate a quantum shift in conceptual developments and published outcomes in virtually every discipline underpinning this title. New benchmarks have now been established across the breadth of the subject to provide new momentum for further research and clinical applications. Although occasional reviews have appeared before, it was deemed appropriate to formally acknowledge recent advances within a comprehensive volume. The recruitment of authors with exceptional merit constituted an integral part of this strategy.

The timing is opportune, as the programme embodying the concept of mammalian protein metabolism completes its full course. With a new ethos now emerging, focus has moved from nutritional questions associated with the indispensable amino acids to health and well-being implications of the dispensable amino acids. However, even traditional issues, such as protein-energy malnutrition, are being investigated in the light of kinetics of specific amino acids, with reduced emphasis on whole-body protein dynamics. Against such a background, it was considered appropriate to secure reviews that would reflect a modernizing and progressive agenda in amino acid research.

28.3 Objectives and Approach

The purpose of this chapter is to review the principal findings of individual chapters in this volume and to consider the conclusions within a general interpretation of contemporary issues in human nutrition and health. Such an approach entails cross-referencing not only to chapters within this volume, but also to other relevant publications, with annotation as appropriate.

This chapter is divided into several broad categories, reflecting recent developments in enzyme characterization, molecular interactions, nutritional support, and diverse disorders associated with amino acid metabolism. Future initiatives relating to novel therapeutics are also of relevance and the chapter will end with a summary of salient points and comments on the outlook for further developments in a rapidly expanding field.
28.4 Key Enzymes and Pathways

The evidence presented in Chapters 1–10 demonstrates how profoundly classical perceptions of the characteristics and roles of enzymes in amino acid utilization have evolved over recent decades. The view that enzymes merely provide substrates for protein synthesis or mediate the disposal of excess dietary amino acids is no longer tenable. In this section, recent advances in the expression, characterization, and regulation of key enzymes are considered within the context of physiological, nutritional and clinical issues.

Amino acids of nutritional or endogenous origin are extensively metabolized in human tissues. Catabolic activity is significantly greater and more diverse than anabolic processes, owing to the inability of mammals to synthesize the carbon skeletons (or keto acids) of the nutritionally indispensable amino acids. The major sites of amino acid metabolism are the gut, liver, muscle and brain. However, inter-organ flux of amino acids is important in determining the profile that reaches the ultimate sites of utilization and, in addition, membrane transport may influence availability of particular substrates. Amino acids cannot be retained indefinitely as free molecules and must follow anabolic routes to peptides, proteins, hormones and other bioactive compounds, or catabolic pathways to urea. In the breakdown of amino acids, the carbon skeletons are utilized in the synthesis of glucose and/or ketones. Thus, certain amino acids may serve as a source of energy.

A summary of the mammalian metabolism of amino acids, as depicted by a series of key enzyme-mediated reactions, is shown in Table 28.1. It will be apparent that diverse types of enzymes are involved in the metabolism of amino acids, including dehydrogenases, transferases, decarboxylases, synthetases and

Table 28.1. A selection of key enzymes and reactions associated with the mammalian metabolism of amino acids. The table provides a summary of enzymes and reactions that underpin several of the chapters in this volume.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>Synthesis/breakdown of glutamate</td>
</tr>
<tr>
<td>Specific aminotransferases</td>
<td>Synthesis of a variety of amino acids, including aspartate and alanine</td>
</tr>
<tr>
<td>Glutamate decarboxylase</td>
<td>Synthesis of GABA</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Synthesis of glutamine</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>Breakdown of glutamine with synthesis of glutamate</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>Initiation of polyamine synthesis</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>Synthesis of citrulline</td>
</tr>
<tr>
<td>Argininosuccinate synthetase</td>
<td>Synthesis of argininosuccinate</td>
</tr>
<tr>
<td>Argininosuccinate lyase</td>
<td>Arginine synthesis</td>
</tr>
<tr>
<td>Arginase</td>
<td>Breakdown of arginine to urea and ornithine</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td>Synthesis of nitric oxide</td>
</tr>
<tr>
<td>Histidine decarboxylase</td>
<td>Histamine synthesis</td>
</tr>
<tr>
<td>Serine racemase</td>
<td>Conversion of L- to D-serine</td>
</tr>
<tr>
<td>Phenylalanine hydroxylase</td>
<td>Synthesis of tyrosine</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Synthesis of DOPA</td>
</tr>
<tr>
<td>Aromatic decarboxylase</td>
<td>Dopamine synthesis</td>
</tr>
<tr>
<td>Tryptophan hydroxylase</td>
<td>Synthesis of 5-hydroxytryptophan</td>
</tr>
<tr>
<td>5-Hydroxytryptophan decarboxylase</td>
<td>Synthesis of 5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>Tryptophan 2,3-dioxigenase</td>
<td>Kynurenine synthesis</td>
</tr>
<tr>
<td>Methionine adenosyltransferase</td>
<td>Formation of S-adenosylmethionine</td>
</tr>
<tr>
<td>S-Adenosylhomocysteine hydrolase</td>
<td>Homocysteine synthesis</td>
</tr>
<tr>
<td>Methionine synthase</td>
<td>Remethylation of homocysteine to yield methionine</td>
</tr>
<tr>
<td>Cystathionine β-synthase</td>
<td>Cystathionine synthesis</td>
</tr>
<tr>
<td>Cystathionine γ-lyase</td>
<td>Formation of cysteine</td>
</tr>
</tbody>
</table>

The evidence presented in Chapters 1–10 demonstrates how profoundly classical perceptions of the characteristics and roles of enzymes in amino acid utilization have evolved over recent decades. The view that enzymes merely provide substrates for protein synthesis or mediate the disposal of excess dietary amino acids is no longer tenable. In this section, recent advances in the expression, characterization, and regulation of key enzymes are considered within the context of physiological, nutritional and clinical issues.

Amino acids of nutritional or endogenous origin are extensively metabolized in human tissues. Catabolic activity is significantly greater and more diverse than anabolic processes, owing to the inability of mammals to synthesize the carbon skeletons (or keto acids) of the nutritionally indispensable amino acids. The major sites of amino acid metabolism are the gut, liver, muscle and brain. However, inter-organ flux of amino acids is important in determining the profile that reaches the ultimate sites of utilization and, in addition, membrane transport may influence availability of particular substrates. Amino acids cannot be retained indefinitely as free molecules and must follow anabolic routes to peptides, proteins, hormones and other bioactive compounds, or catabolic pathways to urea. In the breakdown of amino acids, the carbon skeletons are utilized in the synthesis of glucose and/or ketones. Thus, certain amino acids may serve as a source of energy.

A summary of the mammalian metabolism of amino acids, as depicted by a series of key enzyme-mediated reactions, is shown in Table 28.1. It will be apparent that diverse types of enzymes are involved in the metabolism of amino acids, including dehydrogenases, transferases, decarboxylases, synthetases and...
hydroxylases. A number of enzymes exist in isoforms, associated with distinctive functional properties and cellular localization. This section provides an appropriate opportunity to summarize the isozymes of importance in the biochemistry of amino acids (Table 28.2). However, it is also important to recognize the neuronal role of a number of these enzymes (Table 28.3). The clinical significance of enzymes associated with amino acid metabolism is a recurring theme in this volume. However, relevant evidence will be considered in the section pertaining to disorders.

### 28.4.1 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) is a pivotal enzyme in amino acid metabolism due to its involvement in both the synthesis and metabolism of amino acids. It plays a crucial role in the metabolism of amino acids, particularly in the production of glutamate, a neurotransmitter.

#### Table 28.2. Isoforms of key enzymes catalysing the metabolism of amino acids. Sources of information are provided in the text of this chapter and elsewhere in this volume.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Isoform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase (GDH)</td>
<td>GDH 1 (housekeeping)</td>
</tr>
<tr>
<td></td>
<td>GDH 2 (nerve-specific)</td>
</tr>
<tr>
<td>Branched-chain aminotransferase (BCAT)</td>
<td>BCATm (mitochondrial)</td>
</tr>
<tr>
<td></td>
<td>BCATc (cytosolic; neuronal)</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT)</td>
<td>ALT 1 (cytosolic)</td>
</tr>
<tr>
<td></td>
<td>ALT 2 (mitochondrial)</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST)</td>
<td>ASTc (cytosolic)</td>
</tr>
<tr>
<td></td>
<td>ASTm (mitochondrial)</td>
</tr>
<tr>
<td>Glutamic acid decarboxylase (GAD)</td>
<td>GAD 65 (65 kDa isoform)</td>
</tr>
<tr>
<td></td>
<td>GAD 67 (67 kDa isoform)</td>
</tr>
<tr>
<td>Glutaminase (GA)</td>
<td>KGA (kidney isoform)</td>
</tr>
<tr>
<td></td>
<td>GAC (colon, heart, pancreas, placenta)</td>
</tr>
<tr>
<td></td>
<td>LGA (liver isoform)</td>
</tr>
<tr>
<td></td>
<td>GAB (brain, breast cancer cells, pancreas)</td>
</tr>
<tr>
<td>Arginase</td>
<td>Arginase I (cytosolic)</td>
</tr>
<tr>
<td></td>
<td>Arginase II (mitochondrial)</td>
</tr>
<tr>
<td>Nitric oxide synthase (NOS)</td>
<td>iNOS (inducible)</td>
</tr>
<tr>
<td></td>
<td>cNOS (constitutive)</td>
</tr>
<tr>
<td></td>
<td>eNOS (endothelial)</td>
</tr>
<tr>
<td></td>
<td>nNOS (neuronal)</td>
</tr>
<tr>
<td>Tryptophan hydroxylase (TPH)</td>
<td>TPH 1 (in peripheral tissues and pineal body)</td>
</tr>
<tr>
<td></td>
<td>TPH 2 (nerveonal form)</td>
</tr>
<tr>
<td>Methionine adenosyltransferase (MAT)</td>
<td>MAT I and MAT III (hepatic isoforms)</td>
</tr>
<tr>
<td></td>
<td>MAT II (extra-hepatic isoform)</td>
</tr>
</tbody>
</table>

#### Table 28.3. The neuronal role of enzymes involved in the metabolism of amino acids. A summary based on studies reviewed in this volume.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Neurobiochemical function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>Supply of neuronal glutamate</td>
</tr>
<tr>
<td>Glutamate decarboxylase</td>
<td>Synthesis of GABA</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Participant in the glutamate–glutamine cycle in the CNS</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>Participant in the glutamate–glutamine cycle in the CNS</td>
</tr>
<tr>
<td>Nitric oxide synthase</td>
<td>Astrocyte-derived NO implicated in multiple sclerosis</td>
</tr>
<tr>
<td>Histidine decarboxylase</td>
<td>Provision of neuronal histamine</td>
</tr>
<tr>
<td>Serine racemase</td>
<td>Yields D-serine, a modulator of glutamatergic neurotransmission</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Initiates pathway to dopamine biosynthesis</td>
</tr>
<tr>
<td>Tryptophan hydroxylase</td>
<td>Initiates pathway to serotonin biosynthesis</td>
</tr>
</tbody>
</table>
breakdown of glutamate in a reversible reaction (Chapter 1). Thus glutamate is broken down to and re-synthesized from α-ketoglutarate and ammonia by the action of mitochondrial GDH. When linked with aminotransferase reactions, GDH activity enables the synthesis of the nutritionally dispensable amino acids and the degradation of all amino acids.

Although the basic functions of GDH have long been recognized, research continues to demonstrate more diverse aspects of its characterization (Table 28.2), action and important attributes. It is sometimes assumed that GDH serves primarily to facilitate the metabolism of amino acids, whether by disposing of excess or ameliorating deficiencies through linkage with aminotransferase activity. The incidence of the novel hyperinsulinism-hyperammonaemia syndrome in hypoglycaemic children has provided the impetus for elucidating the expression (Anno et al., 2004), regulation (Herrero-Yraola et al., 2001; Fang et al., 2002) and other functional characteristics of GDH (Fujioka et al., 2001; Tanizawa et al., 2002; Choi et al., 2007; Stanley, 2009). In addition, possible interactions of GDH and glutamine synthetase in the prefrontal cortex of patients with psychiatric and neurodegenerative disorders should sustain further research (Burbaeva et al., 2003, 2005).

### Table 28.4. α-Keto acids readily transaminated in mammalian tissues.

<table>
<thead>
<tr>
<th>α-Keto acid</th>
<th>Amino acid product</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoglutarate</td>
<td>Glutamate</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>Aspartate</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Alanine</td>
</tr>
<tr>
<td>α-Ketoisocaproate</td>
<td>Leucine</td>
</tr>
<tr>
<td>α-Keto-β-methylvalerate</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>α-Ketoisovalerate</td>
<td>Valine</td>
</tr>
<tr>
<td>Phenylpyruvate</td>
<td>Phenylalanine</td>
</tr>
</tbody>
</table>

#### 28.4.2 Aminotransferases (transaminases)

Aminotransferases or transaminases catalyse the transfer of an amino group from one amino acid to a keto acid to form another amino acid (Chapter 2). These enzymes require pyridoxal phosphate as cofactor to maximize catalytic efficacy. In theory, all aminotransferase reactions should be reversible. However, within mammalian tissues only a limited number of α-keto acids are readily transaminated to their respective amino acids (Table 28.4).

The initial step in the degradation of most amino acids involves a transamination reaction, which, when coupled with the action of GDH, results in the production of ammonia. The liver is the primary site for coupled reactions of this type, enabling catabolism of all amino acids. The ammonia may be re-utilized or detoxified in the hepatic synthesis of urea prior to excretion via the kidneys. Skeletal muscle, however, is the major site for the transamination of the three branched-chain amino acids (BCAA), leucine, isoleucine, and valine (Harper et al., 1984). Adipose tissue may be another important site for BCAA metabolism, assuming that work with a mouse model is applicable to other mammalian systems. Herman et al. (2010) suggested that activity of catabolic enzymes in adipose tissue may be instrumental in the modulation of circulating levels of BCAA. Such a statement would imply a role for BCAA aminotransferase in this process.

The expression and metabolic significance of branched-chain aminotransferase (BCAT) in its two isozymes (Table 28.2) are reviewed in Chapter 2. The data of Sweatt et al. (2004) and Garcia-Espinosa et al. (2007) with reference to the mitochondrial isozyme (BCAT_m) and the cytosolic form (BCAT_c) in neurons (Table 28.3) are regarded as important contributions to current knowledge.

In the mammalian brain, kynurenine aminotransferase is a key enzyme in the breakdown of tryptophan, yielding the neuroactive metabolite kynurenic acid. The relationship between kynurenine aminotransferase and kynurenine 3-monoxygenase in the mammalian metabolism of tryptophan in the brain continues to attract attention (Amori et al., 2009). It is becoming increasingly clear that this pathway of tryptophan metabolism is capable of producing a number of neuroactive intermediates.
28.4.3 Glutamate decarboxylase

The conversion of glutamate into γ-aminobutyrate (GABA) typifies the physiologically-important decarboxylation reactions that lead to the synthesis of bioactive molecules such as neurotransmitters (Chapter 6). The direct reaction is catalysed by glutamic acid decarboxylase (GAD), a pyridoxal phosphate-dependent enzyme. GAD is expressed in two isoforms (Table 28.2) encoded by different genes (Korpershoek et al., 2007). GAD 65 refers to the 65 kDa form, while GAD 67 represents the 67 kDa isozyme. Based on work with the developing mouse lens, Kwakowsky et al. (2007) observed that GAD isoforms are subject to spatiotemporal expression. GAD 65 appeared in primary fibres, while GAD 67 expression predominated in the postnatal secondary fibres. More recent studies include the post-translational regulation of the functional characteristics of GAD in the brain, including protein phosphorylation and activity-dependent cleavage (Wei and Wu, 2008). Although of particular significance in neurotransmission, GAD is also expressed in the pancreas. GAD is an important auto-antigen in the development of type 1 diabetes. In non-obese diabetic mice, administration of GAD 65 isoform can prevent autoimmune degeneration of pancreatic beta-cells. The therapeutic potential of GAD in both intervention and prevention strategies for type 1 diabetic patients is currently under investigation (Ludvigsson, 2009).

28.4.4 Glutamine synthetase

The action of glutamine synthetase (GS) represents a second important mechanism of ammonia assimilation. Under the influence of GS, ammonia combines with glutamate to yield glutamine in a non-reversible reaction. Recent developments have placed GS in an enhanced perspective in terms of localization and function. Although GS is ubiquitous in mammalian tissues, its expression in the brain is predominantly within the astrocytes where it serves to modulate the glutamate-glutamine cycle (Table 28.3). This control is effected by the uptake of extracellular glutamate via specific transporters and metabolizing the amino acid to glutamine through the action of GS in the astrocytes (Lo et al., 2008).

28.4.5 Glutaminase

Glutaminase catalyses the breakdown of glutamate into glutamine and ammonia, enabling the enzyme to participate in the glutamate-glutamine cycle in the brain (Table 28.3). The synthesis of glutamate in the brain is primarily the result of the activity of glutaminase (Table 28.2), a point unequivocally underlined in Chapter 7 of this volume. The work of de la Rosa et al. (2009) and Marquez et al. (2009) have contributed to an evolving hypothesis of expression and metabolic functions. However, this synthesis of glutamate is sensitively regulated in order to avoid precipitation of excitotoxic lesions.

28.4.6 Ornithine decarboxylase

Ornithine decarboxylase (ODC) initiates the synthesis of polyamines, molecules that are essential in the regulation of cell growth and differentiation. ODC acts on ornithine to produce putrescine and CO₂. Inputs of decarboxylated 5-adenosylmethionine at two separate stages result in the synthesis of spermidine and spermine. Polyamine production appears to be an essential adjunct in all tissues that are actively synthesizing proteins. For example, polyamines act as mediators in the histological differentiation of specialized cells. Polyamine synthesis is also an important focal point for the action of antinutritional factors in legume seeds. In addition, the involvement of methionine in polyamine biosynthesis imposes competing metabolic demands, particularly when the tissue supply of cysteine may be critical. Currently there is considerable interest in different aspects of ODC localization, regulation, and expression due to its link with carcinogenesis. Regarding intracellular localization, Schipper et al. (2004) suggested that nucleocytoplasmic shuttling might be important in the regulation and function of ODC. This process may be facilitated by its regulatory protein, antizyme 1. Regulation of ODC
Emergence of a New Momentum

has been reviewed by Pegg (2006) who outlined key factors in this process at the levels of transcription, translation, and protein turnover.

28.4.7 Urea-cycle enzymes

Four enzymes participate in the urea cycle, namely ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase. The component reactions of this pathway are listed in Table 28.1. In the urea cycle the metabolism of ornithine, citrulline, argininosuccinate and arginine are linked in a pathway that allows mammals to dispose of excess N from amino acids that cannot be used for anabolic purposes. The liver is the primary site for this activity. Waste N enters the urea cycle as carbamoyl phosphate, synthesized from ammonia, carbon dioxide and ATP by the action of carbamoyl phosphate synthetase. Carbamoyl phosphate reacts with ornithine to form citrulline. However, waste N also enters the cycle directly, via aspartate which combines with citrulline to form argininosuccinate. This intermediate breaks down to arginine and fumarate. The action of arginase results in the production of urea and the regeneration of ornithine.

28.4.7.1 Arginase

The metabolic role of arginase has been given particular attention in order to reflect recent biochemical advances and clinical implications (Chapter 3). The expression of the cytosolic and mitochondrial forms (arginase I and arginase II, respectively) is a significant feature (Table 28.2). Arginase competes with nitric oxide synthase for their common substrate, arginine. Recent data suggest that arginase II is part of a system that modulates nitric oxide synthesis. Additionally, Munder (2009) claimed that arginase is a key participant in the mammalian immune system.

28.4.8 Nitric oxide synthase

Nitric oxide synthase (NOS) catalyses the synthesis of nitric oxide (NO) from arginine with the concomitant production of citrulline (Chapter 4). This reaction is facilitated by NOS isoforms expressed in specific compartments (Table 28.2). The constitutive isoform, cNOS, is always present and generates intermittent low levels of NO, whereas the inducible isoyme (iNOS) is activated by cytokines and endotoxins. Following induction, iNOS produces large and sustained quantities of NO. Barouch et al. (2002) referred to spatial confinement of neuronal and endothelial isoforms allowing NO signals to exert independent and even opposite effects on organ function.

There is growing evidence that NOS interacts with arginase since they share the same substrate, arginine. Thus, Topal et al. (2006) reported that endothelial NO synthesis depends on the activity of arginase II in the mitochondria. Subsequently, Durante et al. (2007) confirmed the role of arginase as a critical regulator of NO synthesis.

28.4.9 Histidine decarboxylase

Histidine decarboxylase (HDC) catalyses the synthesis of histamine from histidine with the release of carbon dioxide (Chapter 5). Histamine is credited with diverse pathophysiological functions, including the induction of allergic and other inflammatory responses, gastric acid secretion, bone loss and regulation of sleep and appetite. In addition, histamine has been attributed with transmitter and signalling functions (Haas et al., 2008). A role for histamine in cognition has also been proposed (Alvarez, 2009). In other studies, neuronal histamine and its receptors have been associated with the regulation of obesity (Masaki and Yoshimatsu, 2009). Furthermore, up-regulation of HDC expression has been observed in superficial cortical nephrons during pregnancy in mice and in women (Morgan et al., 2006).

28.4.10 Serine racemase

Serine, when presented as the L-isomer, is a nutritionally dispensable amino acid with
unique metabolic features. It has been consistently stated that L-amino acids are utilized in mammalian systems in preference to, or in certain instances to the exclusion of, the corresponding D-isomers. In cases where the D enantiomorphs are metabolized, these must be converted to the L-isomer prior to utilization. The incorporation of L-serine during protein and peptide synthesis is totally consistent with the above principles. Uniquely, however, this doctrine is reversed for serine in its neurotransmitter/modulatory role. Relatively substantial quantities of the D-isomer occur in mammalian brain and in neuronal ganglion cells of the retina (Chapter 8). D-serine is synthesized from its L-isomer by the action of serine racemase. Aspects recently under investigation include induction of serine racemase expression (Wu et al., 2004) and feedback inhibition (Mustafa et al., 2007). Of particular interest is the neuronal expression of D-serine and serine racemase in the vertebrate retina (Dun et al., 2008).

28.4.11 Hydroxylases

Phenylalanine hydroxylase catalyses the formation of tyrosine from the indispensable amino acid, phenylalanine. A genetic deficiency of phenylalanine hydroxylase results in the well-recognized condition, phenylketonuria. Tyrosine is used in protein and in thyroxine synthesis but, critically, also contributes to the formation of 3,4-dihydroxyphenylalanine (DOPA), dopamine, noradrenaline and adrenaline. This cascade is initiated by tyrosine hydroxylase, the rate-limiting enzyme in the biosynthetic pathway. Consistent with its biochemical and physiological importance, tyrosine hydroxylase continues to be the focus of extensive research. Recent investigations include tyrosine hydroxylase gene transcription in catecholaminergic neuronal subtypes (Rusnak and Gainer, 2005); mechanisms of activation (Gelain et al., 2007); and regulation of expression in the enteric nervous system (Chevalier et al., 2008).

Tryptophan hydroxylase catalyses the rate-limiting reaction in the pathway leading to the synthesis of 5-hydroxytryptamine (serotonin) from tryptophan (Chapter 9). It is now known that tryptophan hydroxylase (TPH) exists in two forms: TPH 1, expressed in peripheral tissues and in the pineal body; and TPH 2, the neuronal isoform (Table 28.2; Winge et al., 2008). However, TPH 1 may act specifically on the development of serotonin neurons and subsequently modulate behaviour (Nakamura et al., 2006).

28.4.12 Enzymes of methionine metabolism

Key enzymes of methionine metabolism are presented in Chapter 10 and summarized in Table 28.1 of this chapter. Methionine adenosyltransferase (MAT) is expressed in three isoforms: MAT I, MAT II and MAT III (Table 28.2; Finkelstein, 2006). Other enzymes include S-adenosylhomocysteine hydrolase, methionine synthase, cystathionine β-synthase and cystathionine γ-lyase.

The mammalian metabolism of methionine proceeds along well-established routes. The principal components of these pathways include activation, transmethylation, remethylation and transsulphuration. The first three of these reactions comprises the methionine cycle, a process which serves to minimize losses of key intermediates such as S-adenosylmethionine, S-adenosylhomocysteine, homocysteine and methionine itself. The transsulphuration reaction occurs as a result of the irreversible formation of cystathionine from homocysteine, a process which leads to the synthesis of cysteine. The turnover of intermediates in the methionine cycle and, in particular, the transsulphuration of homocysteine provides the sites for metabolic regulation of methionine dynamics in different tissues and organs. In this respect, Finkelstein (2007) has considered the potential of S-adenosylmethionine and S-adenosylhomocysteine in the regulation of the methionine cycle.

Relative rates of transmethylation and transsulphuration form the basis of recent research in a variety of metabolic and physiological circumstances. For example, under normal conditions higher rates of transsulphuration occur in early gestation in humans, while higher rates of transmethylation are seen in late pregnancy (Dasarathy et al., 2010).
Rates of transmethylation in both pre-term and full-term infants were observed to be high, and transsulphuration reactions were evident even in the immediate neonatal phase (Thomas et al., 2008). Polymorphisms of methionine metabolism have been implicated in meningioma formation, with the effects on transmethylation of DNA being postulated as the possible focal point (Semmler et al., 2008). However, it was pointed out that methionine kinetics are not only a function of genetic factors but are also subject to dietary modulation, for example, by B-vitamin intake. In laboratory models, alterations in components of the methionine cycle may be affected by acute valproate administration (Ubeda et al., 2002) or during copper accumulation (Delgado et al., 2008).

Homocysteine is an independent risk factor for vascular disorders (Wagner and Koury, 2007) and other conditions such as cognitive impairment in the elderly (Kim et al., 2007). The association is considered to be causal, although the pathophysiological mechanisms involved remain to be elucidated. Homocysteine status is influenced by a variety of nutritional factors, food intake, age, genetics and lifestyle (Chapter 21). A consistent relationship between plasma homocysteine levels and status of certain B vitamins is now emerging. In addition, dietary fat may be another relevant factor in determining plasma homocysteine concentrations.

Taurine is relevant here, not only by virtue of its derivation from cysteine, but also in recognition of its relationship with amino acids in human disorders. Although not a dietary essential, taurine is attributed with important physiological, modulatory and structural functions, with implications for human diseases. Thus uptake of taurine by the retinal pigment epithelium may exert consequences for the transport of small solutes between the choroid and the outer retina (Hillenkamp et al., 2004). According to Hayes et al. (1989), taurine modulates platelet aggregation in both cats and humans. The role of taurine as a constituent of mitochondrial tRNA has been examined and new proposals have been advanced as to its function and putative relationship with the development of human mitochondrial diseases (Suzuki et al., 2002). The association between plasma taurine and other amino acids in human sepsis has also been investigated (Chiarla et al., 2000). The kinetics of taurine in the metabolism of healthy adult humans has been published (Rakotoambinina et al., 2004).

28.5 Neurotransmitters

A number of amino acids, including glutamate, aspartate, proline, GABA, glycine, serine, β-alanine and taurine are attributed with direct neurotransmitter and/or modulatory functions; while arginine, glycine and cysteine are precursors of recognized and putative gaseous neurotransmitters including nitric oxide (NO), carbon monoxide (CO), hydrogen sulphide (HS) and sulphur dioxide (SO₂). In addition, tyrosine, tryptophan and histidine act as precursors of dopamine, serotonin and histamine, respectively. Several of the amino acids listed here fulfil the long-standing specific criteria required for classification as neurotransmitters. However, it is instructive to consider current evidence and, particularly, any implications for the pathophysiology of neurodegenerative and psychiatric disorders.

28.5.1 Glutamate

In nutritional classification, glutamate is identified as a dispensable amino acid, but its metabolic and neuronal roles place it in a unique category. Indeed, glutamate has been referred to as an amino acid of ‘particular distinction’ and detailed evidence for such a claim will be found in this volume. Of particular significance is the emergence of glutamate as the principal excitatory neurotransmitter in the mammalian central nervous system (CNS) (Chapter 25). The role of specific transporters, and ionotropic and metabotropic receptors, in the glutamatergic synapse is now well-established (Meldrum, 2000). Glutamate promotes normal synaptic transmission and long-term potentiation as well as long-term depression (Rodriguez-Moreno and Sihra, 2007). However, glutamatergic signalling in
non-neuronal cells, particularly in astrocytes, is an emerging issue awaiting elucidation in terms of biochemical function and molecular mechanisms (Nedergaard et al., 2002). The conversion of glutamate into GABA typifies the physiologically-important decarboxylation reactions that result in the synthesis of key bioactive molecules. The enzyme responsible for GABA production is glutamate decarboxylase.

28.5.2 Aspartate

Although aspartate has long been associated with neurotransmitter activity, various aspects are still under investigation, particularly in relation to mechanisms of depolarization-induced release from cerebrocortical synaptosomes. The conclusions of Cavallero et al. (2009) are an appropriate representation of current views. Evidence was advanced to confirm that aspartate is released from nerve terminals by Ca-dependent exocytotic mechanisms. While the status of aspartate as an neurotransmitter is undisputed, other work emphasizes that in certain conditions, for example in the ventilatory response to hypoxia, the balance between excitatory and inhibitory amino acids may be of importance (Hehre et al., 2008).

28.5.3 Proline

The case for proline as an amino acid neurotransmitter was emphasized by Shafqat et al. (1995) who claimed that several of the classical criteria had been fulfilled for such a function. Two of these conditions included the presence of a brain-specific high-affinity L-proline transporter and the efflux of this amino acid from brain slices and synaptosomes after appropriate depolarization. Subsequently, the expression of the proline transporter in subpopulations of excitatory nerve terminals in rat forebrain indicated a role for this carrier in neurotransmission (Renick et al., 1999). The candidature of proline as a neurotransmitter was further supported by observations that L-proline (but not D-proline) induces its depressor and bradycardic activities via ionotropic excitatory amino acid receptors in the nucleus tractus solitarii of the anesthetized rat (Takemoto, 2001). In a further study, Takemoto and Semba (2006) used immunohistochemical evidence for the localization of neurons containing proline, and concluded that this amino acid may function as a neurotransmitter or neuromodulator in the brain. In the Second Proline Symposium, Phang (2008) advanced the concept of proline acting as a neurotransmitter by suppressing glutamatergic neurons.

28.5.4 γ-Aminobutyrate and glycine

It is widely acknowledged that GABA is the major inhibitory neurotransmitter in the mammalian CNS. Its modus operandi is primarily via the ligand-gated channel, the GABA_A receptor (Wegner et al., 2008). The functioning of these receptors is determined by age, mediating excitatory effects in early development to facilitate differentiation of the brain, and inhibitory effects in more advanced stages and in adults. The physiology and function of GABA_A receptors in the brain also change in subjects with epilepsy (Galanopoulou, 2008). Several anti-epileptic drugs currently in use target components of the GABAergic system, for example receptors, transport and degradation. The paper of Madsen et al. (2010) provides an insight into the potential of targeting GABA transporters in the development of anti-epileptic drugs.

The multifunctional attributes of glycine are widely recognized. It is involved in the synthesis of purines, creatine, and haem, and participates in the well-established interrelationship with serine. Glycine is also attributed with anti-inflammatory, cytoprotective and immunomodulatory properties.

Glycine is the primary inhibitory neurotransmitter in the spinal cord, brain stem and retina (Gundersen et al., 2005; Bowery and Smart, 2006). Glycine-gated chloride channels are sited in neurons of the central nervous system. Composite proposals for the extensive action of glycine have been reviewed by Gundersen et al. (2005). The localization of glycine receptors has now been accomplished with immunohistochemical techniques
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(Baer et al., 2009). Furthermore, the co-release of glycine and GABA from the single synaptic terminal is an emerging issue (Katsurabayashi et al., 2004). Hernandes and Tronsone (2009) maintain that this concomitant release may be an important process to elucidate the role of glycine in forebrain neural transmission. Other studies point to the role of glycine and GABA in stimulating the release of glucagon-like peptide-1 (Gameiro et al., 2005).

28.5.5 D-Serine

Substantial quantities of D-serine occur in the brain, where it serves as an important modulator of glutamatergic neurotransmission (Chapter 8; see also Mustafa et al., 2004; Bauer et al., 2005). The case for D-serine as a neurotransmitter/modulator has been advanced by Mustafa et al. (2004) for a number of reasons:

1. synthesis of D-serine from its L-isomer occurs in astrocytic glia associated with synapses in regions of the brain that are well-endowed with NMDA receptors;
2. D-serine is more potent than glycine in the activation of certain sites at these receptors;
3. selective degradation of D-serine reduces NMDA neurotransmission;
4. glutamate initiates the release of D-serine to facilitate joint action at NMDA receptors;
5. D-serine is also implicated in neural development.

28.5.6 β-Alanine and taurine

It is widely acknowledged that β-alanine exerts a modulatory or neurotransmitter role in the mammalian CNS. β-Alanine may also act as a neurotransmitter in the visual system (Sandberg and Jacobson, 2008).

Junyent et al. (2009) attribute taurine with a number of significant properties. It is one of the most abundant free amino acids in the mammalian CNS with the capacity to reduce or prevent epileptic seizures. It is associated with neuroprotectant roles in experimental conditions, and its levels in the brain are subject to modulation by astrocytes.

28.5.7 Gases

Important signalling gases are now known to be synthesized by both animals and humans (Li et al., 2009). Reference has already been made to NO in Chapter 4, and attention is now being directed at CO, H2S and SO2. The sources of these gases are, respectively, glycine (haem) and cysteine. At physiological concentrations, NO, CO and SO2 facilitate the production of cGMP which then induces diverse reactions including vascular smooth muscle relaxation, neurotransmission and cellular metabolism. H2S controls neurological function and also acts as a vasorelaxant (Yang et al., 2008). In addition, according to Li et al. (2009), NO, CO and H2S impart cytoprotective and immunomodulatory functions. They further suggest that arginine, glycine, and cysteine act as both precursors and regulators of the synthesis of these gases in a cell-specific manner.

This review would be incomplete without reference to the diverse interactions involving NO, CO and sub-cellular components. There is convincing evidence that CO acts as a neurotransmitter and thus fulfils a similar function as NO (Lin et al., 2004). Furthermore, the haem-oxygenase-2 (H2O2)/CO axis is thought to serve in various physiological roles including regulation of vascular tone. Lin et al. (2004) developed the concept in more detail by demonstrating interactions between CO and group II and III metabotropic glutamate receptors in central cardiovascular regulation.

Of particular relevance to this chapter is the NO–CO interaction. While the function of both neurotransmitters in the brain is well-recognized, Xue et al. (2000) concluded that they are also important in the enteric nervous system. The evidence for their claim was based first on the co-localization of their respective biosynthetic enzymes (neuronal NOS and HO2) and second on altered intestinal
function of mice with genomic deletion of these enzymes. Another manifestation of the NO–CO interaction is seen in the work of Wang and Wu (2003). Their results demonstrated that NO and CO act on different amino acid residues of K_{ca} channel proteins. It was further stated that the interactions of NO and CO determine the functional status of K_{ca} channels in vascular smooth muscle cells.

All mammals are endowed with the capacity to produce and to salvage metabolic ammonia (NH_{3}). Thus NH_{3} is ubiquitous, and readily takes part in key assimilation reactions catalysed by glutamate dehydrogenase, glutamine synthase and carbamoyl phosphate synthetase. Another reaction of NH_{3} may be of relevance in hepatic encephalopathy, a condition with multifactorial aetiology. Jones (2002) suggested that NH_{3} may act on the GABA system to enhance inhibitory neurotransmission and thus contribute to the pathogenesis of hepatic encephalopathy. Basile (2002) indicated that NH_{3} is able to modulate GABAergic neurotransmission via direct and indirect mechanisms, and considered the implications for the pathogenesis of hyperammonaemic syndromes. An updated review of the role of NH_{3} in the glutamate/GABA-glutamine cycle and neurotransmitter homeostasis has been published by Bak et al. (2006).

28.6 Molecular Interactions

Interactions comprise the very essence of amino acid metabolism with profound implications for nutrition, health, and therapeutic interventions. However, the full extent of these interrelationships is often underestimated or, in certain cases, yet to be determined. This volume contains references to several types of molecular interactions at various levels of intricacy. As stated in Chapter 13, hormonal, neuronal and nutritional factors may exert complex effects in the regulation of gene expression. Another example is illustrated by the action of mitochondrial arginase II which modulates NO synthesis in endothelial cells, with consequent implications for vascular disease (Topal et al., 2006).

The interactions within the aminoacidergic and monoaminergic systems, involving intricate and coordinated activities of enzymes, neurotransmitters, transporters and receptors, exemplify a higher order of complexity. In addition, other factors may impact on such activity. For example, D-serine functions as an important modulator of glutamatergic neurotransmission. Other observations indicate that glutamate may modulate the growth rate and branching of dopaminergic axons (Schmitz et al., 2009). As stated in Chapter 5, histamine interacts with other neurotransmitters including dopamine, serotonin and acetylcholine. The multifactorial nature of these interactions, although of immense scientific relevance, implies that drug development may require the targeting of multiple sites within and between aminoacidergic and monoaminergic systems. Barone (2010) concurs, advocating the development of agents that interact with several of the affected neurotransmission systems in Parkinson’s disease.

28.6.1 Transport

Membrane transport of amino acids has been investigated at four principal sites: small intestine, kidney, brain and placenta. In essence, and as might be predicted, similar mechanisms operate in all mammalian systems. Typically, such mechanisms involve the interaction of transporters with the corresponding amino acids (Sengers et al., 2010). The intestinal absorption and transport of amino acids (and peptides) has been exhaustively reviewed by Krehbiel and Matthews (2003). Interest in renal transport is sustained by continuing research in the inherited disorder, cystinuria, as discussed later in this chapter (Aydogdu et al., 2009). Efforts are now directed towards the role of the blood–brain barrier in determining availability of amino acids to the brain for metabolism within astrocytes and neurons (Chapter 11). The practical implications of this research are now emerging. Thus, the use of large neutral amino acids (LNAA) to impede brain transport of phenylalanine has been considered for phenylketonuria (PKU) patients (Chapter 23). Competition between LNAA and tryptophan
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at the blood–brain barrier forms the basis for current neuropsychiatric investigations (Chapter 24). There is also increasing awareness of the potential importance of placental amino acid transport in the regulation of fetal growth (Bajoria et al., 2002), including effects in pregnancies complicated by diabetes (Jansson et al., 2002). Desforges et al. (2010) emphasized the importance of system A-mediated transport for the provision of neutral amino acids for fetal growth. An autocrine/paracrine role for leptin in regulating system A amino acid transport in the human placenta has recently been proposed (von Versen-Hoynck et al., 2009).

28.6.2 Leucine signalling

Reference to Chapter 13 will indicate the case for amino acids as signalling molecules with critical effects in the regulation of gene expression and physiological functions. It is apparent that mammals are able to modulate changes in amino acid availability by regulating expression of numerous genes.

It is worthwhile reviewing other evidence relating specifically to leucine. Studies with animal models indicate that leucine is the dominant amino acid in metabolic and nutritional antagonisms with isoleucine and valine (Chapter 19). In addition, leucine is possibly unique among the BCAA in its ability to act as a transduction molecule in the stimulation of muscle protein synthesis. This property is attributed to enhanced availability of specific eukaryotic initiation factors. It is suggested that leucine stimulates protein synthesis in skeletal muscle by enhancing both the activity and synthesis of proteins involved in mRNA translation (Anthony et al., 2001). This effect is thought to be mediated partly via the mammalian target of a rapamycin (mTOR) signalling pathway where both insulin and leucine act in concert to maximize protein synthesis. In addition, leucine may suppress protein catabolism. Leucine also regulates glucose oxidation in skeletal muscle by stimulating recycling via the glucose–alalanine mechanism. Recognition of these interactions has stimulated interest in the potential use of leucine in the treatment of obesity (Layman and Walker, 2006). Studies with animal models suggest that increasing leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism via diverse mechanisms (Zhang et al., 2007). The potential of leucine to counteract the effects of muscle-wasting manifestations of AIDS, sepsis, kidney failure and bed rest has also been considered (Drummond and Rasmussen, 2008).

28.6.3 Hormonal modulation

The anabolic effects of insulin and its relationship with leucine are well recognized, but a brief account of recent developments is appropriate here. According to Layman and Walker (2006), leucine interacts with the insulin-signalling process to enhance downstream regulation of protein synthesis. Others maintain that leucine, when consumed with glucose synergistically stimulates insulin secretion in healthy adults (Kalogeropoulou et al., 2008), while Moriyama et al. (2008) observed increased insulin and leptin secretion following amino acid infusion during off-pump coronary arterial bypass surgery. The overall efficacy of amino acids may be determined by the relative concentration of leucine (Katsanos et al., 2006; Drummond and Rasmussen, 2008). The mechanism of action of leptin may reside in its capacity to regulate amino acid transport (von Versen-Hoynck et al., 2009).

Insulin secretion may also be stimulated in patients with GDH gene defects (Anno et al., 2004; Stanley, 2009; Chapter 1). Overexpression of constitutively activated GDH induces insulin secretion by oxidizing glutamate to α-ketoglutarate, thus providing substrate for the tricarboxylic acid cycle.

Other evidence, obtained with animal models, suggests that gonadotropin and growth hormone secretion may be modulated by interactions between GABAergic and aminoacidergic pathways (Pinilla et al., 2002; Aguilar et al., 2005).

Hormonal interactions with aminoacidergic systems may operate within mutually controlled mechanisms. There is limited clinical evidence to suggest that some GABAergic neurological conditions in humans may be linked with thyroid dysfunction. Indeed,
Weins and Trudeau (2006) concluded that there is strong support for the concept of reciprocal regulation between thyroid hormone and GABA systems in vertebrates. Another view of this aspect is reflected in the observations of Leret et al. (2007), demonstrating the role of maternal corticosterone levels in the development and maturation of aminoacidergic systems in the rat brain.

A complex picture of amino acid–endocrine interactions is now emerging, but much more research is still required to fully elucidate the implications for human health.

28.6.4 Umami flavour

The characteristic feature of the umami flavour appears to reside in the synergy between glutamate and purinic ribonucleotides. This effect is mediated via interactions with heteromeric macromolecules of the class-C G protein-coupled receptors. The molecular mechanisms are reviewed in Chapter 20. The interaction between taste and olfactory pathways in the human brain with respect to the umami flavour has been elaborated by Rolls (2009). In addition, GABA may also act as a mediator in a taste-transduction pathway (Chapter 6).

28.6.5 Post-translational adducts

Amino acids may retain reactivity, post-translationally, to confer unique properties to proteins. Two examples are highlighted below to underline evolving implications for nutrition and health.

28.6.5.1 Advanced glycation end-products

Under particular circumstances, post-translational modification of amino acid residues of proteins may occur, resulting in the synthesis of advanced glycation end-products (AGE) (Chapter 22). The residues regularly implicated are N\textsuperscript{\textpm}-(carboxymethyl) lysine (CML) and N\textsuperscript{\textpm}-(carboxyethyl) lysine (CEL) (Hartog et al., 2007). In a nutritional context, Uribarri and Tuttle (2006) indicate that there is 'compelling' evidence from experimental models and human investigations to associate excess dietary protein with the incidence of progressive kidney lesions. The effects were attributed to increased production of AGE. However, enhanced AGE synthesis has also been observed in cardiovascular disorders, diabetes and intestinal inflammation. The formation of AGE has been attributed to the accretion of reducing sugars under certain physiological conditions such as diabetes and ageing (Quintero et al., 2010). The clinical and prognostic significance of recent observations are discussed later in this chapter.

28.6.5.2 Proline-rich proteins

A number of mammalian species, including humans, are endowed with the capacity to secrete salivary proteins rich in proline, as a defence mechanism against the anti-nutritional effects of dietary tannins (see Griffiths, 1991). For example, young rats fed sorghum tannins develop hypertrophy of the parotid glands, and subsequently a marked increase in the output of salivary proline-rich proteins (PRP). It is suggested that PRP may also serve to facilitate the provision of a protective milieu for the teeth by restricting bacterial adhesion to oral surfaces. The primary structures of six human salivary PRP have been elucidated by Hay et al. (1988) who indicated the importance of configuration on the functional properties of these proteins. The different isoforms and post-translational variants of human PRP have been further investigated by Inzitari et al. (2005). The abnormally high proline content of PRP confers an open type of structure with a distinctive affinity for condensed tannins. Consequently, salivary PRP are highly effective as precipitators of tannins and provide a first line of defence against such polymers (Shimada, 2006). The interaction of tannins with human salivary PRP has been investigated (Charlton et al., 1996; Lu and Bennick, 1998). In particular, it has been observed that tannin interactions with full-length PRP display a higher affinity than with single proline-rich repeats (Charlton et al., 1996). Furthermore, an extra-oral function of PRP has been suggested by Bennick (2007) who proposed that PRP may act as scavenger molecules preventing intestinal absorption of
tannins but allowing the assimilation of other beneficial compounds such as antioxidants.

28.7 Clinical Support

28.7.1 Biochemical considerations

In common with other mammals, humans are unable to synthesize eight specific keto acids, a feature normally expressed as a dietary requirement for the corresponding amino acids. Accordingly, leucine, isoleucine, valine, phenylalanine, lysine, methionine, threonine and tryptophan are designated as nutritionally indispensable (or essential) amino acids. Dietary provision of these amino acids is therefore obligatory. On the other hand, the potential for the synthesis of other amino acids is extensive and well documented. Since α-ketoglutarate, oxaloacetate and pyruvate are ubiquitous and readily transaminated, the corresponding amino acids are considered to be nutritionally dispensable (or non-essential). However, it is important to acknowledge that although glutamate is readily synthesized in mammalian tissues and is widely distributed in foods, it exerts a critical, even unique, role in metabolic processes. Thus ‘nutritional dispensability’ and ‘metabolic efficacy’ should be recognized as distinct attributes, particularly with respect to glutamate, but also to other non-essential amino acids.

In theory, glutamine may be regarded as a nutritionally dispensable amino acid, by virtue of the action of GS. However, there is evidence that glutamine may confer benefits in particular clinical conditions, consistent with the role of a ‘pharmaconutrient’. Although open to wide interpretation, this term probably encapsulates the paradoxical concept of nutritional dispensability and clinical efficacy. Normal processing within the urea cycle means that all intermediates, and arginine in particular, are nutritionally dispensable. However, Nijveldt et al. (2004) indicated that when degradation and/or utilization is enhanced, as for example in wound healing, trauma and sepsis, arginine assumes the role of an indispensable amino acid. The term ‘conditionally indispensable’ is generally used to describe the nutritional classification of arginine. Citrulline status also declines in critical illness, although levels increase spontaneously during recovery.

The transsulphuration pathway ensures that cysteine is a nutritionally dispensable amino acid as long as dietary methionine intake is adequate. However, in severe childhood undernutrition, cysteine production may be impaired due to decreased mobilization from tissue protein. This effect is exacerbated in the oedematous form of undernutrition (Jahoor et al., 2006).

28.7.2 Supplements

Food constitutes the ultimate source of amino acids for all species, including humans. Systematic studies are now in progress to assess the amino acid requirements (Chapter 16) and availability in foods (Chapter 15) using modern methodology. In healthy individuals consuming a balanced diet, there is normally no need for supplements of amino acids. Indeed, there is no evidence that such supplements are more effective than high-quality food proteins in stimulating muscle protein synthesis or skeletal muscle mass (Chapter 17). Foods such as eggs, meat and milk are readily digested, inducing minimal losses of endogenous amino acids (Chapter 14). As such these foods should provide near-optimal levels of the essential and non-essential amino acids to satisfy requirements. However, there is increasing evidence (Table 28.5) that administration of particular amino acids, individually or as mixtures, may confer benefits for pre-term infants or patients with conditions such as Duchenne’s dystrophy and chronic obstructive pulmonary disease (COPD). Amino acid supplements may also be of use in critical illness and in the care of the elderly.

The general strategy underlying the use of supplemental amino acids is to enhance the overall metabolic and nutritional status of vulnerable subjects. It is not intended that such measures should act as specific antidotes, but rather to contribute to well-being and management of patients.
Table 28.5. Amino acids in clinical support.

<table>
<thead>
<tr>
<th>Amino acid(s)</th>
<th>Clinical condition</th>
<th>Efficacy/conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>Duchenne muscular dystrophy</td>
<td>Inhibition of whole-body protein degradation</td>
</tr>
<tr>
<td></td>
<td>Chronic obstructive pulmonary disease</td>
<td>Increased plasma concentrations of citrulline and arginine</td>
</tr>
<tr>
<td></td>
<td>Burn care</td>
<td>Favourable prospects</td>
</tr>
<tr>
<td></td>
<td>Surgical patients</td>
<td>Attenuation of plasma interleukin-6 levels</td>
</tr>
<tr>
<td></td>
<td>Infectious morbidity in critically ill surgical patients</td>
<td>Ineffective at dose levels used</td>
</tr>
<tr>
<td></td>
<td>Major abdominal surgery for cancer</td>
<td>Outcome not affected</td>
</tr>
<tr>
<td></td>
<td>Inflammatory bowel disease</td>
<td>Ineffective</td>
</tr>
<tr>
<td></td>
<td>Chronic obstructive pulmonary disease</td>
<td>Increased glutamate availability</td>
</tr>
<tr>
<td></td>
<td>Radiofrequency ablation therapy for hepatocellular carcinoma</td>
<td>Enhanced whole-body protein synthesis</td>
</tr>
<tr>
<td></td>
<td>Surgical management for hepatocellular carcinoma</td>
<td>Improved liver function</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis and hepatocellular carcinoma</td>
<td>Favourable prospects</td>
</tr>
<tr>
<td></td>
<td>Hepatic encephalopathy; liver regeneration; hepatic cachexia</td>
<td>Reduced frequency of complications</td>
</tr>
<tr>
<td></td>
<td>Septic encephalopathy</td>
<td>Benefits depend on type of liver disease and on the presence of inflammatory reactions</td>
</tr>
<tr>
<td></td>
<td>Alcoholic liver disease</td>
<td>Beneficial effects</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>Effective as a therapeutic immuno-nutrient</td>
</tr>
<tr>
<td>Arginine</td>
<td>Hepatic failure</td>
<td>Potentially harmful effects</td>
</tr>
<tr>
<td></td>
<td>Transplantation</td>
<td>Attenuation of donor lung injury associated with haemorrhagic shock</td>
</tr>
<tr>
<td></td>
<td>Post-surgical head and neck cancer</td>
<td>Improved local wound complications and decreased length of stay</td>
</tr>
<tr>
<td>β-alanine</td>
<td>Neuromuscular fatigue in elderly subjects</td>
<td>Improved muscle endurance</td>
</tr>
<tr>
<td>Special mixture</td>
<td>Sarcopenia in elderly subjects</td>
<td>Enhanced whole-body lean mass and insulin sensitivity</td>
</tr>
</tbody>
</table>

Following encouraging results with experimental models, glutamine emerged as one of the important candidate amino acids in nutritional therapy. The general rationale for the clinical use of glutamine is based on the proposal that endogenous provision may become inadequate, for example during critical illness. The insufficiency may be a consequence of declining muscle mass which is unable to maintain normal export of glutamine. The shortage may be reflected in decreased plasma concentrations of glutamine (Rutten et al., 2006a). The complexities of inter-organ fluxes of glutamine are considered in detail in Chapter 12 of this volume. It is suggested that the efficacy of glutamine administration in critical illness is due to its role as metabolic fuel and in its action in the attenuation of the inflammatory response and oxidant stress (Wischmeyer, 2007).

The effects of glutamine in children with Duchenne muscular dystrophy are reviewed in Chapter 18. However, there is currently interest in the use of glutamine and glu-
mate in COPD. Rutten et al. (2006a) observed that repeated ingestion of glutamine, but not glutamate, increased plasma concentrations of citrulline and arginine. Nevertheless, in a separate study Rutten et al. (2006b) concluded that glutamate supplementation might be a suitable option to increase whole-body glutamate turnover in COPD patients and in healthy elderly individuals who are likely to be dependent on dietary sources of this amino acid.

Windle (2006) reviewed the efficacy of enteral and parenteral glutamine supplementation in burn care. It was concluded that the prospects for glutamine therapy in burns are favourable. On the other hand, Gianotti et al. (2009) indicated that perioperative intravenous glutamine supplementation did not influence outcome in major abdominal surgery for cancer. Others question the effectiveness of enteral glutamine in reducing infectious morbidity (Schulman et al., 2006) and whether the evidence supports the case for glutamine supplementation in humans (Alpers, 2006). Glutamine-enriched total parenteral nutrition conferred no biochemical or clinical advantage in patients with inflammatory bowel disease (Ockenga et al., 2005), although surgical patients did benefit (Lin et al., 2005). Responses to glutamine may, however, depend upon overall nutritional status of patients (Gianotti et al., 2009).

The evidence for the use of BCAA supplements appears to be more consistent (Table 28.5). Thus, Engelen et al. (2007) concluded that BCAA supplements to soy protein enhanced whole-body protein synthesis in patients with COPD. Others observed benefits in patients under surgical management (Okabayashi et al., 2008) or after radiofrequency ablation therapy (Ishikawa et al., 2009) for hepatocellular carcinoma. Holeczek (2010) has suggested three potential targets for BCAA use, namely hepatic encephalopathy (see also Chapter 19), liver regeneration and hepatic cachexia, although the benefits might depend upon the type of disease and on the development of inflammatory reactions. In clinical amino acid imbalance caused by liver disease and in the management of cirrhosis and hepatocellular carcinoma, BCAA supplementation may be associated with decreased frequency of complications (Charlton, 2006; Lam and Poon, 2008). Glycine may also be used for chemoprevention and treatment of hepatocellular carcinoma, according to Yamashina et al. (2005), who concluded that the amino acid is a potent therapeutic immunonutrient.

According to Nijveldt et al. (2004), the case for arginine supplementation (Table 28.5) requires careful assessment, as it is unlikely to be suitable for critically-ill patients with hepatic failure. Elevated plasma arginine concentrations occur in these patients, implying potentially harmful effects of supplementation. On the other hand, Preissler et al. (2009) observed that arginine attenuates donor lung injury associated with haemorrhagic shock; while De Luis et al. (2009) concluded that in post-surgical cancer patients, an arginine-enriched formula elicited beneficial outcomes in terms of local wound complications and duration of stay.

Recent data suggest that the elderly may also respond positively to arginine-enriched supplements of indispensable amino acids. Improvements may be seen in terms of lean body mass, strength and physical function (Borsheim et al., 2008), as well as reduced plasma and liver triacylglycerols (Borsheim et al., 2009). Furthermore, elderly individuals may respond positively to supplements of β-alanine (Stout et al., 2008). It is suggested that enhanced tissue carnosine status improves pH regulation, thereby facilitating muscle endurance. Oral administration of an amino acid mixture enhanced whole-body lean mass and insulin sensitivity in elderly patients with sarcopenia (Solerte et al., 2008). Methionine kinetics may also be altered in the elderly. Mercier et al. (2006) indicated that cysteine demands may increase during ageing in order to supplement host defence mechanisms against oxidative stress through the provision of glutathione.

Nutritional support is not merely a question of supplementation with amino acids. As indicated below, consumption of vegetables
rich in nitrates, nitrites and antioxidants may complement the effects of arginine in ischaemic conditions. Furthermore, B-vitamin fortification may prevent incident stroke episodes and neural tube defects, presumably by reducing risks associated with elevated homocysteine levels (Chapter 21). B-vitamin deficiencies also tend to occur in PKU patients on Phe-restricted diets (Chapter 23).

28.8 Food Toxicology

28.8.1 Nitrate and nitrite

Nitrates and nitrites are widely distributed in vegetables and are also used in the production of cured meats. Epidemiological evidence of an association between high intake of cured meats and the incidence of certain forms of cancer is persuasive, but Eichholzer and Gutzwiller (2003) discounted any consistent effect of nitrates or nitrites per se. Indeed, for nitrate in vegetables, an inverse relationship was observed with the incidence of stomach cancer. Further support for the positive effects of nitrates and nitrites of plant origin is provided in Chapter 4 of this volume. The authors conclude that arginine on its own is ineffective in restoring NO bioavailability under ischaemic conditions. Provision of vegetables rich in nitrates, nitrites and antioxidants in combination with arginine may complement NO biosynthesis and homeostasis, and may thus represent a rescue or protective pathway for individuals at risk of cardiovascular disorders.

28.8.3 Monosodium glutamate

Glutamate is arguably the most ubiquitous of all amino acids present naturally in foods (Simon and Ishiwata, 2003). In addition, the sodium form of glutamate (MSG) is routinely used in South-east Asian cuisine as a flavour-enhancing agent imparting a specific taste attribute known as ‘umami’ (Chapter 20). There are suggestions that dietary MSG supplementation may improve food intake in the elderly and in patients with chronic inappetence. MSG is currently regarded by the US Food and Drugs Agency as safe. Nevertheless, disquiet over its use continues, following its association with ‘Chinese restaurant syndrome’ (Kwok, 1968) and widespread recognition of the neuronal function of glutamate. The adverse reaction is characterized by burning sensation, headache, nausea, and chest pains. The present-day approach in the scientific community is to largely set aside safety issues and instead focus on molecular mechanisms and physiology. Thus, the 100th anniversary symposium of umami discovery published in The American Journal of Clinical Nutrition in 2009 included, among others, reviews on subjects such as taste receptors (Li, 2009), functional neuroimaging (Rolls, 2009), glutamate metabolism (Stanley, 2009) and the blood-brain barrier, in relation to glutamate (Hawkins, 2009). Updated versions of two of these titles appear in Chapters 11 and 20.

28.8.4 Maillard products

In the heat-processing of common foods, non-enzymatic reactions may occur between amino acid residues in proteins and the carbonyl groups of sugars (Chapter 22). The resulting Maillard products, while enhancing organoleptic properties of such foods, are also associated with deleterious effects including allergy and autoimmune diseases, and neurodegenerative and cardiovascular disorders. Two major groups of Maillard products include AGE as defined above and advanced lipoxidation end-products (ALE). The concentrations are dependent upon processing temperature as well as duration of storage at
elevated temperatures. Thus AGE arises in food processing, but may also occur in body fluids and tissues with increased levels of sugars as, for example, in diabetics.

Recent investigations indicate the formation of the potential carcinogen, acrylamide, in certain plant products following cooking at relatively high temperatures as in frying, grilling and baking. Acrylamide synthesis is attributed to Maillard reactions between sugars and amino acids such as asparagine, methionine and cysteine. Highest concentrations have been found in potato crisps. In model systems, proline is highly effective in reducing acrylamide production (Koutsidis et al., 2009).

### 28.8.5 Lysinoalanine

The unusual amino acid, lysinoalanine, may also arise during heat processing of foods. Its occurrence is indicative of the extent of thermal damage in foods. The feeding of proteins containing lysinoalanine has been associated with reduced dietary biological values and the incidence of renal lesions in rats. However, Langhendries et al. (1992) concluded that exposure of pre-term infants to lysinoalanine and Maillard reaction products in heat-processed milk formulas did not affect kidney function.

### 28.9 Disorders

This volume contains numerous references to a wide range of adverse effects and specific conditions linked by complex mechanisms to the metabolism of amino acids (Tables 28.6 and 28.7). Enzyme studies have provided intriguing insights into the biochemical changes in a variety of these disorders. The hypothesis advanced here is that the metabolism of amino acids is (a) associated with, or (b) modulated by a diverse array of disorders which, in certain instances, may provide markers for risk assessment (Fig. 28.1). This model has been simplified to facilitate clarity in the presentation of the main effects, rather than the interactions of multifactorial systems. Those well-versed in disorders of amino acid metabolism will be aware of the complexities of such interactions. Thus the dopamine theory for schizophrenia, and the GABA mechanism for anxiety disorders, have not been included in Fig. 28.1. The aim here is to collate the evidence for different aspects of this model within an integrated account.

#### 28.9.1 Clinical amino acid imbalance

The concept of clinical amino acid imbalance has been advanced to accommodate the incidence of adverse ratios of groups of amino acids in specific disease conditions of diverse aetiology (Chapter 19). In patients with septic encephalopathy or with chronic liver disease, plasma ratios of BCAA to aromatic amino acids are consistently reduced. This imbalance may be observed within 12h of the onset of septic encephalopathy. Furthermore, it has been suggested that clinical amino acid imbalance may serve as a diagnostic marker in the assessment of the severity of the septic syndrome. With regard to hepatic encephalopathy, Romero-Gomez (2005) investigated a putative role for phosphate-activated glutaminase in the pathogenesis of this condition. As stated previously, hepatic encephalopathy is a disorder of complex aetiology, with amino acid imbalance as a contributory factor (Chapter 19). Other forms of clinical amino acid imbalance have been observed in patients with chronic renal failure, Huntington’s disease, and lung and breast cancer, as detailed in Chapter 19. Clinical amino acid imbalance is distinct in aetiology from the nutritional versions, as exemplified in depletion studies with humans (Chapter 24), and in contrived experiments with animal models (D’Mello, 2003). Nevertheless, nutritional interventions involving administration of BCAA-enriched supplements are beneficial in the management of septic patients or those with cirrhosis and hepatocellular carcinoma.

#### 28.9.2 Obesity

The notion that amino acids may exert a role in the regulation of food intake and obesity is gaining support. Specifically, and not
Table 28.6. Clinical significance of enzymes involved in the metabolism of amino acids. (Disorders caused by genetic deficiencies of enzymes are presented in Table 28.7.)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase (GDH)</td>
<td>Linked with glutamine synthetase (see below); marker of alcohol dependence</td>
</tr>
<tr>
<td>Aminotransferases</td>
<td>Ornithine carbamoyltransferase to alanine aminotransferase (ALT) ratio potent indicator for hepatocellular carcinoma; obesity and elevated ALT associated with insulin resistance and cardiovascular risk in rheumatoid arthritis</td>
</tr>
<tr>
<td>Glutamate decarboxylase (GAD)</td>
<td>Proposed therapy with GAD 65 in diabetes; GABA and mood disorders</td>
</tr>
<tr>
<td>Glutamine synthetase (GS)</td>
<td>Loss of GS in human epileptogenic hippocampus proposed as possible mechanism for raised extracellular glutamate in mesial temporal epilepsy; levels of GS and GDH altered in the prefrontal cortex of patients with schizophrenia and Alzheimer’s disease</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>Co-expression of glutaminase K and L isozymes in human tumour cells; modulation of glutamine-glutamate cycle in schizophrenia; pathogenesis of hepatic encephalopathy</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>Over-expression in a variety of cancers</td>
</tr>
<tr>
<td>Arginase</td>
<td>Endothelial dysfunction; allergic asthma</td>
</tr>
<tr>
<td>Nitric oxide synthase (NOS)</td>
<td>Expression of inducible NOS in multiple sclerosis lesions</td>
</tr>
<tr>
<td>Histidine decarboxylase (HDC)</td>
<td>Histamine involved in allergy and other inflammatory responses; HDC is potential target to attenuate histamine production in certain pathological states</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Dopamine neurotransmission: implications for psychological function</td>
</tr>
<tr>
<td>Tryptophan hydroxylase</td>
<td>Serotonin neurotransmission: implications for psychiatric disorders</td>
</tr>
<tr>
<td>Tryptophan 2,3-dioxygenase</td>
<td>Key kynurenine-synthesizing enzyme: up-regulation of kynurenine pathway in patients with major psychotic disorders, including schizophrenia and bipolar disorder</td>
</tr>
<tr>
<td>Methionine adenosyltransferase</td>
<td>S-Adenosylmethionine synthesis and depression</td>
</tr>
<tr>
<td>S-Adenosylhomocysteine hydrolase</td>
<td>Homocysteine synthesis: independent risk factor for cardiovascular disease</td>
</tr>
<tr>
<td>D-Amino acid oxidase</td>
<td>Expression and activity increased in schizophrenia</td>
</tr>
</tbody>
</table>

unexpectedly, there is interest in the potential role of leucine in the treatment of obesity and the metabolic syndrome. The basis for such an expectation is the interaction of leucine with the insulin transduction pathway and regulation of glucose utilization (Layman and Walker, 2006). Studies with mice suggest that leucine reduces diet-induced obesity and improves glucose and cholesterol metabolism via multiple mechanisms (Zhang et al., 2007). In Chapter 5 of this volume, it is suggested that the histamine system in the brain might be a potential target for the treatment of eating disorders leading to obesity.

28.9.3 Neuropathologies

Derangements of aminoacidergic and/or monoaminergic neurotransmission have been implicated as predisposing or as secondary factors in the aetiology of a wide range of neurological conditions. Three major categories of CNS disorders are currently under investigation in this respect:

1. the group of neurodegenerative conditions including Alzheimer’s disease, Parkinson’s disease, Huntington's disease, amyotrophic lateral sclerosis, stroke and multiple sclerosis are consistently linked with glutamatergic dysfunction;
Table 28.7. Genetic disorders associated with defective enzymes of amino acid metabolism or with other aberrant mechanisms. Citation of recent references is designed to emphasize continuing research even in well-established syndromes.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Enzyme* or mechanism</th>
<th>Clinical presentation and effects</th>
<th>Reference update</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperinsulinism/ hyperammonaemia</td>
<td>Glutamate dehydrogenase</td>
<td>Excessive insulin and ammonia synthesis</td>
<td>Chapter 1</td>
</tr>
<tr>
<td>Glutamine synthetase deficiency</td>
<td>Glutamine synthetase</td>
<td>Glutamine absent in physiological fluids; multi-organ failure; neonatal mortality</td>
<td>Haberie <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Carbamoyl phosphate synthetase 1 deficiency</td>
<td>Carbamoyl phosphate synthetase 1</td>
<td>Two forms: lethal-neonatal and less severe delayed-onset; brain damage; seizures</td>
<td>Ono <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Ornithine transcarbamylase deficiency</td>
<td>Ornithine transcarbamylase</td>
<td>Classical neonatal and late-onset types; acute hyperammonaemic encephalopathy; fatal outcomes</td>
<td>Ben-ARI <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Citrullinaemia</td>
<td>Argininosuccinate synthetase</td>
<td>Three clinical presentations: neonatal, infantile and late-onset; increased citrulline levels in physiological fluids; hyperammonaemic encephalopathy; mental retardation</td>
<td>Albayram <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Argininosuccinate lyase deficiency</td>
<td>Argininosuccinate lyase</td>
<td>Hyperammonaemia; accumulation of argininosuccinate; arginine depletion; systemic hypertension; seizures</td>
<td>Brunetti-Pieri <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Hyperargininaemia</td>
<td>Arginase</td>
<td>Hyperammonaemia; spastic quadriplegia; seizures; mental impairment</td>
<td>Hertecant <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>Phenylalanine hydroxylase</td>
<td>Vomiting; cognitive impairment</td>
<td>Chapter 23</td>
</tr>
<tr>
<td>Albinism</td>
<td>Tyrosinase</td>
<td>Lack of pigmentation due to reduced melanin synthesis; reduced visual acuity</td>
<td>Kirkwood (2009)</td>
</tr>
<tr>
<td>Tyrosinaemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>Fumarylacetoacetate hydrolase</td>
<td>Vomiting; diarrhoea; jaundice; liver failure; fatal outcomes</td>
<td>Croffie <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>Type 2</td>
<td>Tyrosine aminotransferase</td>
<td>Oculocutaneous symptoms; corneal opacity, keratitis with photophobia; mental impairment</td>
<td>Pasternack <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>Type 3</td>
<td>4-Hydroxyphenylpyruvate dioxygenase</td>
<td>Autism; mental impairment; neurological abnormalities</td>
<td>D'Eufemia <em>et al.</em> (2009)</td>
</tr>
</tbody>
</table>

*Continued*
Table 28.7. Continued.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Enzyme(^1) or mechanism</th>
<th>Clinical presentation and effects</th>
<th>Reference update</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaptonuria (defect of Tyr catabolism)</td>
<td>Homogentisate 1,2-dioxygenase</td>
<td>Dark pigmentation of urine; destruction of connective tissue; back pain; late-onset arthritis</td>
<td>Watts and Watts (2007)</td>
</tr>
<tr>
<td>Hypermethioninaemia</td>
<td>Methionine adenosyltransferase</td>
<td>Methionine accumulation; most affected individuals asymptomatic</td>
<td>Finkelstein (2006)</td>
</tr>
<tr>
<td>S-Adenosylhomocysteine hydrolase deficiency</td>
<td>S-Adenosylhomocysteine hydrolase</td>
<td>Psychomotor delay; periporal fibrosis</td>
<td>Finkelstein (2006)</td>
</tr>
<tr>
<td>Homocystinuria</td>
<td>Cystathionine β-synthase</td>
<td>Mental impairment</td>
<td>Skovby et al. (2010)</td>
</tr>
<tr>
<td>Cystinuria</td>
<td>Renal transport of cystine and dibasic amino acids</td>
<td>Urinary stones</td>
<td>Aydogdu et al. (2009)</td>
</tr>
<tr>
<td>Maple syrup urine disease (inborn error of branched-chain amino acid degradation)</td>
<td>Branched-chain α-keto acid dehydrogenase complex</td>
<td>Brain damage; seizures; mental impairment</td>
<td>Zinnanti et al. (2008); Amaral et al. (2010)</td>
</tr>
<tr>
<td>Familial hyperlysinaemia</td>
<td>Lysine-ketoglutarate reductase and/or saccharopine dehydrogenase</td>
<td>Mental impairment</td>
<td>Sacksteder et al. (2000)</td>
</tr>
<tr>
<td>Glutaric aciduria type 1 (inborn error of Lys and Trp degradation)</td>
<td>Glutaryl-coenzyme A dehydrogenase</td>
<td>Brain damage</td>
<td>Kurul et al. (2004)</td>
</tr>
<tr>
<td>Propionic acidaemia (inborn error of Met, Ileu, Thr and Val degradation)</td>
<td>Propionyl-CoA carboxylase</td>
<td>Severe neonatal-onset and late-onset presentations; vomiting, loss of appetite, heart abnormalities, stroke-like episodes; seizures, coma and possibly death</td>
<td>Scholl-Burgi et al. (2009)</td>
</tr>
<tr>
<td>Histidinaemia</td>
<td>Histidine α-deaminase</td>
<td>Original description: speech defects; mental impairment. Recent studies: most patients with normal intelligence</td>
<td>Kawai et al. (2005)</td>
</tr>
<tr>
<td>Hyperprolinaemia Type 1</td>
<td>Proline dehydrogenase</td>
<td>Neurological, renal and/or auditory defects; some asymptomatic individuals</td>
<td>Mitsubuchi et al. (2008)</td>
</tr>
<tr>
<td>Type 2</td>
<td>Δ1-pyruroline-5-carboxylic acid dehydrogenase</td>
<td>Mental Impairment; seizures</td>
<td>Baslow and Guilloyle (2009)</td>
</tr>
<tr>
<td>Canavan disease (inborn error of N-acetylaspartate degradation)</td>
<td>Aspartoacylase</td>
<td>Early onset; psychomotor retardation; mental impairment</td>
<td>de Koning (2006)</td>
</tr>
<tr>
<td>Serine deficiency</td>
<td>3-Phosphoglycerate dehydrogenase</td>
<td>Congenital microcephaly; psychomotor retardation; seizures</td>
<td></td>
</tr>
<tr>
<td>Non-ketotic hyperglycinaemia</td>
<td>Glycine cleavage system</td>
<td>Severe neonatal-onset; intractable epilepsy; convulsive seizures</td>
<td>Rossi et al. (2009)</td>
</tr>
</tbody>
</table>

\(^1\)Defective inhibition of glutamate dehydrogenase; all other enzyme-related disorders attributed to deficiencies.
2. among the neurochemical syndromes of relevance are affective disorders, schizophrenia and anxiety. Aggression and attention deficit /hyperactivity disorder (ADHD) are also of importance in this context; and
3. within the neurophysiological classification, epilepsy is widely regarded as a manifestation of complex aminoacidergic dysfunction.

28.9.3.1 Conditions associated with glutamate excitotoxicity

The concept of excitotoxicity is invoked almost exclusively in the context of glutamatergic dysfunction. As is well known, glutamate is the principal excitatory neurotransmitter in the CNS. The precise functioning of the glutamatergic system requires the synchronized action of the glutamine–glutamate cycle and associated enzymes (Table 28.6) and receptors. Disruption of this system has been associated with a wide range of adverse effects (Chapter 25). It is generally acknowledged that prolonged exposure to, and excess concentrations of, glutamate are toxic to neurons. According to Martel et al. (2009), during an ischaemic event, extracellular glutamate accumulates to cause excessive activation of the N-methyl D-aspartate (NMDA) subclass of glutamate receptor, which mediates Ca²⁺-dependent cell death. The concept that
glutamate excitotoxicity may prejudice health and well-being is not new. Lipton and Rosenberg (1994) proposed that glutamate and aspartate excitotoxicity represents the ‘final common pathway’ in the pathogenesis of neurological disorders. Glutamatergic dysfunction, mediated via excessive NMDA receptor activation, has been associated with acute neurodegenerative disorders such as stroke (Liu et al., 2007) as well as chronic forms like Parkinson’s disease and Alzheimer’s disease (Chapter 25). Burbaeva et al. (2005) reported perturbations in the metabolism of glutamate in the brain of patients with Alzheimer’s disease, based upon data for GDH and GS. Lack of synchronization between the enzyme contributors of the glutamate-glutamine cycle may be implied. However, pathological changes in the histaminergic system have also been observed in the brain of patients with Alzheimer’s disease. There is now speculation as to whether some of the cognitive symptoms of such patients might be ameliorated after treatment with histamine-related drugs (Chapter 5). In addition, neurological and psychiatric disorders may represent other manifestations of glutamate excitotoxicity (Rammes et al., 2008).

There is universal consensus now that disruption of glutamatergic neurotransmission is an underlying feature of the pathophysiology of schizophrenia (Kantrowitz and Javitt, 2010; Chapter 25), although the dopamine hypothesis is still cited in textbooks. Burbaeva et al. (2003) observed significant differences in GS and GDH levels in the prefrontal cortex of patients with schizophrenia relative to controls. It was concluded that glutamate metabolism was impaired, with the abnormal functioning of the glutamate-glutamine cycle, in the prefrontal cortex of patients with schizophrenia. A study by Maeshima et al. (2007) investigated increased plasma glutamate by antipsychotic medication and its relationship to glutaminase 1 and 2 genotypes in schizophrenia. It was concluded that glutaminase genes were not risk factors for schizophrenia, although plasma glutamate status might reflect the clinical course of the condition. Gaisler-Salomon et al. (2009) confirmed the role of disturbed glutamatergic neurotransmission in the pathophysiology of schizophrenia and further suggested that modulation of glutaminase activity might provide the basis for novel therapies. Furthermore, the neurobiology of D-amino acid oxidase and its implications in schizophrenia are now emerging on the basis of genetic, biochemical, and behavioural studies (Verrall et al., 2010).

Overstimulation of glutamate receptors may also damage the myelin-producing cells of the CNS and contribute to lesions in multiple sclerosis (MS) (Pitt et al., 2000). The action of iNOS in the production of NO has been implicated in the pathogenesis of MS (Table 28.6). Liu et al. (2001) concluded that iNOS is induced in different cell types in MS lesions and that astrocyte-derived NO may influence the manifestation of inflammatory reactions, particularly at the blood–brain barrier.

Deficiency of GS in the astrocytes has been proposed as a possible molecular mechanism for elevated extracellular glutamate in seizure development in mesial temporal lobe epilepsy (Eid et al., 2004). Subsequent data with a rat model reinforced the notion that deficiency in hippocampal GS causes recurrent seizures (Eid et al., 2008). Therapeutic interventions targeting this relative deficiency of GS in the epileptogenic hippocampus are envisaged.

It follows that targeting the glutamatergic system might be part of a therapeutic strategy for conditions such as focal cerebral ischaemia (Muir and Lees, 1995), MS (Pitt et al., 2000; Sarchielli et al., 2003) and depression (Yoon et al., 2009).

Two other glutamate-related human disorders are relevant here. Both BOAA and BMAA are attributed with neurotoxic properties due to their action as potent glutamate receptor agonists (Chapter 19). BOAA is associated with neuroathyrixism, a disorder characterized by muscular rigidity, paralysis of leg muscles and, in extreme cases, death. More controversially, BMAA has been implicated in Guam dementia (amyotrophic lateral sclerosis/Parkinson dementia complex).

28.9.3.2 Psychological and cognitive impairments: emerging methodology

Depletion of the amino acid precursors of monoamine neurotransmitters is emerging as
a popular means of investigating psychological performance and behaviour. The technique involves the administration of amino acid mixtures devoid of the appropriate precursors.

The dopaminergic neurotransmission system has been implicated in the pathogenesis of unipolar depression and in motivated behaviour (McLean et al., 2004). Acute tyrosine and phenylalanine depletion has been employed to transiently reduce dopamine neurotransmission in both animal models and humans. Ellis et al. (2005) examined the effects of acute tyrosine depletion in healthy men, and observed that under these conditions, working memory performance was not impaired. However, stimulation of D₁/D₂ receptors during acute tyrosine depletion induced a subtle impairment in spatial working memory performance. Ellis et al. (2005) questioned the reliability of this methodology as a modulator of dopamine status and function in humans. Similarly, Lythe et al. (2005) indicated the lack of behavioural effects following acute tyrosine depletion in healthy volunteers. Thus, acute tyrosine depletion did not influence mood, while measures of memory, attention and behavioural inhibition were also unaltered. Lythe et al. (2005) were somewhat uncertain about how robust or consistent the effects of depletion were on the assessment of psychological function. The scepticism was reinforced by the paper entitled ‘Acute tryptophan or tyrosine depletion test: time for reappraisal’ (Badawy, 2005).

Notwithstanding these doubts, others have used the technique with interesting results. For example, Vrshek-Schallhorn et al. (2006) demonstrated that healthy individuals showed affective bias and response modulation after tyrosine depletion, in sensitivity tests to positively and negatively valenced words. Furthermore, McTavish et al. (2001) concluded that reduced tyrosine supply to the brain attenuated the pathological increases in dopamine neurotransmission after administration of the psychostimulant drug methamphetamine.

Acute tryptophan-depletion techniques have also been applied to ascertain any association between serotonin and psychiatric conditions (Chapter 24). Studies of this type have led to the conclusion that there is an inverse relationship between serotonin status and aggression in children with ADHD.

There is increasing evidence that the kynurenine pathway of tryptophan degradation may be up-regulated in patients with schizophrenia and bipolar disorder. A key enzyme in this sequence is tryptophan 2,3-dioxygenase (Miller et al., 2008; Barry et al., 2009).

Results with animal models show that histamine status affects cognitive functioning. The studies of van Ruitenbeck et al. (2009) with human subjects suggest that histidine depletion might serve as a promising technique in the assessment of histamine-based cognitive impairment. However, validation of the methodology is necessary.

### 28.9.4 Cardiovascular disease

Increased arginase activity is widely associated with vascular dysfunction, for example in inflammatory bowel disease (Horowitz et al., 2007) and in endothelial cell oxidative stress (Sankaralingam et al., 2010; Chapter 3). NO is a well-recognized modulator of cardiac relaxation. Hence the conclusion of Silberman et al. (2010), that uncoupled cardiac NOS mediates diastolic dysfunction, is in accordance with that concept. In infants and children with pulmonary hypertension and congenital heart disease, Hoehn et al. (2009) reported up-regulation of eNOS as well as iNOS at an early stage. It was hypothesized that such an enhancement may be a compensatory reaction to restrict the rise in pulmonary artery pressure. There is general consensus that formation of AGE is associated with the development and progression of chronic heart failure, typified by the comments of Hartog et al. (2007). Accumulation of AGE is enhanced in the cerebral vessels of diabetic patients (van Deutekom et al., 2008).

### 28.9.5 Diabetes

It is worth summarizing, on the basis of limited evidence, that diabetes may directly or indirectly impact on transport, metabolism and post-translational reactivity of amino
acids. For example, alterations in placental activity of amino acid transporters may contribute to enhanced fetal growth rates in pregnancies complicated by diabetes, according to Jansson et al. (2002). Romero et al. (2008) indicated that diabetes-induced coronary vascular dysfunction involves enhanced arginase activity (see also Chapter 3). Experimental data imply that BCAA catabolism may be down-regulated in type 2 diabetes (Kuzuya et al., 2008). Furthermore, in type 2 diabetics with nephropathy, homocysteine metabolism is impaired, reflecting changes in the dynamics of the methionine cycle and transsulphuration reactions (Tessari et al., 2005). In addition, long-term metformin administration to patients with type 2 diabetes increases risk of vitamin B12 deficiency, which results in elevated homocysteine concentrations. Other studies point to the accumulation of lysine adducts (as AGE) in greater concentrations in diabetic patients. Increased levels were recorded for these subjects with renal dysfunction (Lieuw-A-Fa et al., 2004) and in the cerebral blood vessels of diabetics compared with control values (van Deutekom et al., 2008).

28.9.6 Cancer

The association between cancer and expression of amino acid-metabolizing enzymes is the subject of considerable research. In human tumour cells, Perez-Gomez et al. (2005) identified two glutaminase isoforms, namely K (kidney-type) and L (liver-type). Since glutamine is an important source of energy in neoplastic tissues, recent studies have focused on the expression and roles of glutaminases in this process (Szeliga and Obara-Michlewska, 2009).

Arginase expression and activity are enhanced in breast, colon, and prostate cancers (Chapter 3). In human prostate cancer, co-expression of both arginase II and NOS occurs in tumour cells. In lung cancer, arginase II expression neither induces immune suppression nor influences progression of the disease (Rotondo et al., 2008). The lack of effect may be attributed to the absence of NOS expression in that study.

More consistent evidence is available for ODC activity which is elevated in a variety of malignant states. Brabender et al. (2001) reported an up-regulation of ODC mRNA expression in Barrett's-associated adenocarcinoma of the oesophagus. It was suggested that high ODC mRNA expression is an early event in the initiation and progression of this condition and may be a clinically useful marker for incident adenocarcinoma. ODC is also overexpressed in both benign prostatic hyperplasia and in neoplastic tissues, and again, Young et al. (2006) indicated that enhanced activation of the enzyme appears to be an early indicator for prostate carcinogenesis. It had previously been demonstrated that ODC was induced by androgens in human prostatic epithelial cells (Visvanathan et al., 2004). Elevated activity of ODC has been linked with other forms of cancer. Thus, Zell et al. (2009) and Brown et al. (2009) published observations on polymorphism in the ODC gene and the risk of colorectal and breast cancer, respectively. In the latter paper, increased ODC activity in breast cancer tissue, compared with benign and normal tissues, was emphasized. Work with laboratory models reinforces the above observations and conclusions. Thus susceptibility to tumour development is enhanced in transgenic mice expressing elevated levels of ODC, and diminished in those with reduced levels of this enzyme (Pegg, 2006; Young et al., 2006). Expression of non-mast cell HDC in tumour-associated microvessels might exert a regulatory role in human oesophageal squamous cell carcinoma (Li et al., 2008). The presence of HDC in neuroendocrine tumours has also been revealed using immunohistochemical and gene expression techniques (Uccella et al., 2006).

The relationship between abnormal methionine metabolism and hepatocellular cancer has been considered in Chapter 10 of this volume. S-Adenosylmethionine appears to be a key intermediate in this respect. The question now being addressed is the mechanism by which a defect or excess of hepatic S-adenosylmethionine might initiate cancer.
28.9.7 Genetic defects

Examination of Table 28.7 will confirm the wide range of genetic conditions associated with the metabolism and transport of amino acids. The disorders listed relate to virtually all the important amino acids in human metabolism. Thus, the biochemical fate of glutamate, glutamine, urea cycle intermediates, phenylalanine, tyrosine, sulphur amino acids, branched-chain amino acids, lysine, tryptophan, threonine, histidine, proline, glycine and serine may be influenced by deleterious enzyme mutations. In addition, an inherited transport disorder may affect renal function with respect to cystine and dibasic amino acids. A number of these syndromes, for example, PKU (Chapter 23), albinism, alkaptonuria and maple syrup urine disease are long-established and well-characterized. However, as shown in Table 28.7, research continues on virtually all genetic disorders, as efforts are directed at the application of modern techniques to investigate gene mutations or provide non-invasive clinical assessments of affected patients. The prospects of new products and treatment modalities, including gene therapy, provide additional impetus for ongoing investigations. The incidence of these enzyme and transport deficiencies is generally quite low, only 8 per 100,000 births for PKU, 3 per 100,000 births for albinism, and significantly lower for the remaining disorders listed in Table 28.7.

A summary of the salient features may be discerned in the light of recent advances (Table 28.7). Although the disorders relate almost exclusively to enzymes of amino acid catabolism, at least one condition, namely serine deficiency disorder, is caused by a biosynthetic enzyme. The hyperinsulinism/hyperammonaemia syndrome has been attributed to mutations that cause a loss of inhibition of GDH (Chapter 1). Anno et al. (2004) attributed hyperinsulinism to over-expression of constitutively activated GDH. Although considerable emphasis has been placed on neonatal screening, and justifiably so, it is salutary to note the recurring theme of late-onset presentation of disorders. Prolonged survival may be observed in the latter group, but this is not a consistent feature for all conditions. In the interests of clarity, less emphasis has been placed on metabolic changes concerning the accumulation of intermediates in tissues and physiological fluids as a result of genetic enzyme deficiencies. These observations are well documented in basic biochemistry texts. Thus, it is widely appreciated that argininosuccinate lyase deficiency is accompanied by accumulation of argininosuccinate. Similarly, in PKU, abnormally high concentrations of phenylalanine in plasma are commonly observed in affected individuals (Chapter 23). A considerable number of the congenital disorders listed in Table 28.7 are associated with a common underlying theme of brain damage, seizures and mental impairment. In many instances, gross observations are accompanied by data obtained at sub-cellular and molecular levels. As might be anticipated, hyperammonaemia occurs commonly in deficiencies of urea cycle enzymes.

Again, as might be predicted, the arginine–NO pathway has been attributed with a role in the pathophysiology of congenital urea cycle disorders, although the precise molecular mechanisms await elucidation (Brunetti-Pierri et al., 2009; Nagasaka et al., 2009). In addition, however, increased NO synthesis by neutrophils of a patient with tyrosinaemia type 3 has been reported (D'Eufemia et al., 2009).

28.9.8 Risk factors

Recent developments highlighted in this volume indicate the potential for identifying risk factors associated with certain disorders. Elevated plasma concentrations of aminotransferases have been suggested as markers for a variety of conditions (Chapter 2; Table 28.6) including non-alcoholic fatty liver disease (Lu et al., 2009), insulin resistance (Burgert et al., 2006; Chen et al., 2009) and hazardous alcoholic consumption (Chaudhry et al., 2009). Another study indicated that serum aminotransferases are associated with insulin resistance and atherosclerosis in rheumatoid arthritis (Dessein et al., 2007). Genetic
factors, however, may contribute to the variation in circulating aminotransferase concentrations (Makkonen et al., 2009).

As indicated earlier in this chapter, it has been observed that high ODC mRNA expression is an early event in the initiation and progression of diverse forms of cancer. It is, therefore, logical to consider ODC up-regulation as a clinically useful risk factor in carcinogenesis (Young et al., 2006).

As stated previously, homocysteine is an independent risk factor for vascular disease and other conditions such as cognitive impairment in the elderly. The association is considered to be causal, although the pathophysiological mechanisms involved remain to be elucidated (Chapter 21).

Post-translational modification of proteins may be associated with the synthesis of advanced glycation end products (AGE) (Chapter 22). The most comprehensive set of investigations implicate AGE in vascular disease. Baidoshvili et al. (2006) indicate that CML accretion in intramyocardial blood vessels in patients with acute myocardial infarction might reflect increased risk rather than a consequence of this disorder. Hartog et al. (2007) suggested that AGE synthesis is a factor in the pathogenesis of chronic heart failure and may also reflect severity of the condition. Increased depositions of CML have also been observed in the cerebral blood vessels of diabetic patients compared with age-matched controls (van Deutekom et al., 2008). Furthermore, elevated AGE accretion has been reported in diabetic subjects with renal dysfunction (Lieuw-A-Fa, et al., 2004). In addition, the AGE burden may correlate with clinical outcomes in acute lung injury (Calfee, 2008) and there are also implications for dental implant osseointegration and stability (Quintero et al., 2010).

28.9.9 Therapeutics

As stated in Chapter 26, L-DOPA has now become the classical example of brain neurotransmitter therapy and current efforts are being directed at a more effective treatment modality for Parkinson’s disease. There is much anticipation about future developments in prophylactic measures for other neurodegenerative disorders (Chapter 25). However, much work at the fundamental level remains to be accomplished.

A significant factor driving amino acid research is the quest to identify appropriate targets for therapeutic intervention. In addition to the potential use of amino acid supplements in clinical support (Iable 28.5), alternative strategies are now under active consideration. The following account indicates the nature of recent work in this area and is not designed to be fully comprehensive, but as illustrative of the diversity of approaches now being considered. Furthermore, the importance of interactions should be recognized in the development of novel therapeutic agents. Thus, although aberrations in glutamatergic neurotransmission have been implicated in the aetiology of Parkinson’s disease (Rodriguez-Moreno and Sihra, 2007; Chapter 25), the characteristic motor symptoms have been attributed to degeneration of dopaminergic neurons. Barone (2010) implies that agents designed to interact with several of the affected neurotransmission systems would enhance therapeutic efficacy in this disease. Nevertheless, the challenge in developing appropriate pharmaceuticals, particularly for CNS disorders, is formidable. Thus the choice of agonists as opposed to antagonists of glutamatergic neurotransmission shows the ongoing dilemma (Chapter 25). On the other hand, tetrahydrobiopterin (BH4) therapy may enhance outcomes in patients with PKU (Chapter 23) or with vascular conditions (Silberman, 2010). In addition, the role of BH4 as a cofactor for tyrosine hydroxylase (Chapter 26) raises further issues regarding therapeutic potential.

28.9.10 Dietary modulation

In Chapter 1 of this volume, the case is presented for the development of alternative strategies for the treatment of GDH-mediated insulin disorders. The prevailing consensus is that the epigallocatechin gallate component of green tea might offer some therapeutic value by virtue of its specific inhibition of GDH. The authors further imply that anti-cancer treatment which combines GDH inhibitors with
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Those that suppress glucose utilization might be effective against tumours (Chapter 1). Since elevated ODC, polyamine, and NO levels are commonly associated with tumour initiation and development, there has been some speculation as to dietary strategies to control risk. For example, Zell et al. (2007) suggested important roles for arginine and meat consumption in the control of colorectal tumours. Recent observations suggest that arginine on its own is ineffective in restoring NO bioavailability under ischaemic conditions. Consumption of vegetables rich in nitrates, nitrites and antioxidants in combination with arginine may serve as a rescue or protective mechanism for individuals at risk to cardiovascular disorders (Chapter 4).

Dietary folate appears to be effective in reducing homocysteine concentrations in adolescents and adults. However, in clinical trials folate, vitamin B₆ and vitamin B₁₂ supplementation failed to reduce the risk of recurrent coronary heart disease, stroke and venous thromboembolism. Although folic acid supplementation has been associated with reduced incidence of neural tube defects, other maternal factors should be considered in efforts to further minimize congenital risks (Chapter 21).

Gaenslen et al. (2008) point to some positive effects of nutrition on the development of Parkinson’s disease, suggesting that dietary polyunsaturated fatty acids may decrease glutamate release and activate NMDA binding sites as part of the mechanism. Reduced serotonin synthesis has been linked, in part at least, with the onset of depression (Porter et al., 2007). As a consequence of these responses, there is now interest in the use of tryptophan-rich proteins to enhance serotonin status by nutritional means (Markus et al., 2008).

28.9.11 Non-protein amino acids in cancer prevention

The potential for specific non-protein amino acids in cancer therapy (Chapter 19; D’Mello, 1991) is worth reiterating here. For example, ester derivatives of canavanine appear to be markedly more effective than the parent amino acid in suppressing the growth of cultured pancreatic carcinoma cells. The development of specific analogue inhibitors is gaining momentum (Wu et al., 2007; Bailey et al., 2010). In the latter study, for example, difluoromethylornithine was examined in skin cancer prevention in subjects with a previous history of this form of malignancy. Preclinical studies demonstrated that inhibition of ODC by this analogue decreased tissue concentrations of polyamines and prevented neoplastic growth in many tissue types. It was suggested that difluoromethylornithine therapy might form part of a preventative strategy in subjects with a previous history of skin cancer.

Se-Methylselenocysteine is most effective against mammary tumours and is also highly effective in potentiating the efficiency of anti-cancer drugs and in protecting against drug-induced toxicity. Indeed, it has been suggested that the increased therapeutic efficacy of certain anti-cancer drugs may be dependent upon the dose of the amino acid. Se-Allylselenocysteine is more effective than a number of other selenoamino acids for chemoprevention of mammary cancer in a rat methylnitrosourea model. Limited work points to the potential therapeutic use of selenomethionine, but a cautious approach is recommended to minimize cytotoxic effects. The efficacy of S-methylcysteine sulphone and its metabolite, methyl methane thiosulphinate, on mouse genotoxicity suggests that these two organosulphur compounds may contribute to the anti-carcinogenic properties of brassica vegetables. However, epidemiological evidence only supports the case for prevention of gastric and lung cancers through consumption of these vegetables. Finally, interest in mimosine as a potential anti-cancer agent is typified by its action in blocking cell-cycle progression, inhibiting DNA replication and inducing apoptosis.

28.9.12 Molecular targets

28.9.12.1 Enzymes

Much emphasis has been placed on the potential for the development of novel therapeutics by targeting enzymes associated with specific
disorders. The following is a summary of selected examples of recent research based on evidence surveyed and referenced earlier in this chapter. Modulation of GAD in both intervention and prevention strategies for type 1 diabetes has been suggested. Deficiency of GS in the epileptogenic hippocampus may represent a new focus for treatment of seizures. Disturbed glutamatergic neurotransmission in schizophrenia may be responsive to modulation of glutaminase activity. Preclinical investigations suggest that inhibition of ODC by specific analogues may prevent neoplastic growth in diverse tissue types. Similarly, it has been suggested that tyrosine kinase blockers may provide the basis for new approaches in cancer therapy (Pytel et al., 2009). Arginase inhibition may represent a mechanism for the treatment of acute and chronic asthma (Maarsingh et al., 2009). As might be predicted, HDC is a potential target to attenuate histamine release in a number of pathological conditions (Table 28.6). To this end, Wu et al. (2008) examined inhibitory and structural properties of coenzyme-substrate analogues relative to the activity of human HDC. Employing computer-based modelling, Moya-Garcia et al. (2009) attempted to elucidate structural features of HDC that might provide the basis for specific inhibition. This approach represented an extension of the ‘structure to function’ theme proposed in an earlier paper (Moya-Garcia et al., 2005).

### 28.9.12.2 Aminoacidergic and monoaminergic receptors

Another strategy under active investigation relates to modulation of aminoacidergic and monoaminergic receptors for therapeutic purposes. The evidence presented in Table 28.8 indicates that a wide range of receptors is currently being targeted for drug development. In summary, research has focused on receptors of the following classes and subtypes:

1. \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA);
2. \(\text{GABA}_\text{A}\) and \(\text{GABA}_\text{C}\);
3. glycine;
4. histamine H1 and H3;
5. dopamine D2 and D3;
6. serotonin; and
7. serotonin/glutamate complex.

Modulation of the NMDA and metabotropic subclasses of glutamate receptors may

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Disorder/condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>Schizophrenia</td>
<td>Yang and Svensson (2008)</td>
</tr>
<tr>
<td>Metabotropic (glutamate)</td>
<td>Alzheimer's disease</td>
<td>Kotermanski and Johnson (2009)</td>
</tr>
<tr>
<td>Ca-permeable AMPA</td>
<td>Schizophrenia</td>
<td>Conn et al. (2009)</td>
</tr>
<tr>
<td>GABA_A</td>
<td>Neurons in Parkinsonian state</td>
<td>Kobylecki et al. (2010)</td>
</tr>
<tr>
<td>GABA_A</td>
<td>Huntington's disease</td>
<td></td>
</tr>
<tr>
<td>GABA_C</td>
<td>Fear and anxiety</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>Inflammatory pain; spasticity; epilepsy</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>Human sterile disease</td>
<td></td>
</tr>
<tr>
<td>Histamine H3</td>
<td>Cognitive</td>
<td>Xue et al. (2010)</td>
</tr>
<tr>
<td>Histamine H3</td>
<td>Psychosis</td>
<td>Twelvetrees et al. (2010)</td>
</tr>
<tr>
<td>Histamine H1 and H3</td>
<td>Atypical antipsychotic weight gain</td>
<td>Cunha et al. (2010)</td>
</tr>
<tr>
<td>Dopamine D2</td>
<td>Management of neuroendocrine tumours</td>
<td>Gilbert et al. (2009)</td>
</tr>
<tr>
<td>Dopamine D3</td>
<td>Drug addiction</td>
<td>Davies et al. (2010)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Irritable bowel syndrome</td>
<td>Ito (2009)</td>
</tr>
<tr>
<td>Serotonin/glutamate complex</td>
<td>Psychosis</td>
<td>Esbenshade et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deng et al. (2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribeiro-Oliveira et al. (2008)</td>
</tr>
</tbody>
</table>

Table 28.8. Aminoacidergic and monoaminergic receptors as potential targets in the development of molecular therapeutics.
constitute a potential mechanism for the treatment of schizophrenia and related disorders. In addition, NMDA receptor selectivity to the Alzheimer’s drug memantine may be an useful model for future research. Targeting AMPA receptors might be of therapeutic value in L-DOPA-induced dyskinesia in Parkinson’s disease. GABA receptors are considered as possible targets for the treatment of a variety of disorders including Parkinson’s disease and Huntington’s disease, as well as fear and anxiety. The glycine receptor mediates inhibitory neurotransmission and is currently emerging as a potential drug target for inflammatory pain, spasticity and epilepsy. Other defects of the glycinerergic system, implicated in human startle disease, have been linked to mutations in the post-synaptic glycine receptor. The latter is a potential target for therapeutic purposes. Receptors of the histaminergic system have been proposed as potential focal points for the treatment of cognitive disorders, psychosis and atypical antipsychotic-induced weight gain. The potential role of dopamine receptors as targets in the management of neuroendocrine tumours and for drug addiction pharmacology has been suggested. In other studies, serotonin receptor modulators are being considered as prospective drugs for the treatment of irritable bowel syndrome. Of particular interest is the association of a serotonin/glutamate receptor complex with psychosis. Further studies should reveal any potential for modulating this axis by specific inhibitors.

28.9.12.3 Transporters

It is conceivable that further advances in molecular pharmacology may well emerge as a result of contemporary investigations with CNS transporters (Chapter 11). The expression of brain-specific high-affinity L-proline transporter in subpopulations of putative glutamatergic neurons implies a role in excitatory activity and a possible focus for modulation in drug development (Rennick et al., 1999). The work of Chen et al. (2003) suggesting that glycine transporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons both in vitro and in vivo is another example of innovative approaches now being pursued. On a more definitive note, Madsen et al. (2010) proposed that neuronal and non-neuronal GABA transporters may serve as putative targets for anti-epileptic drugs.

28.10 Innovation

There is universal consensus that recent advances reviewed in this volume have largely been the result of developments in molecular and non-invasive techniques. The refinement and exploitation of these methodologies should contribute to further progress in the future. One example is the use of amino acid profiles in clinical diagnosis (Chapter 27). Further studies may well include measures of monoaminergic function. However, other forms of innovation are now under scrutiny.

28.10.1 Modelling

Modelling is perceived as an essential technique in a wide range of biological disciplines, including nutrition and health sciences. In general, there is widespread belief that more rapid progress might be achieved by the adoption of modelling techniques. It is suggested that only simulation processes can provide the capacity to embody complex biochemical pathways within dynamic and flexible models. In support of computational modelling, Sengers et al. (2010) point out that placental amino acid flux involves the interaction of 15 or more transporters for 20 amino acids. It is also claimed that the elaborate requirements of drug development demand the use of modelling methodologies (Moya-Garcia et al., 2009). Nevertheless, even in such cases, validation through determination of clinical efficacy would still be necessary. Furthermore, it is argued that the identification of future research priorities is best accomplished through logic generated within simulation protocols. However, it is patently clear that virtually all the advances recorded in this volume have been the result of deliberate and
arduous empirical research. On balance, it has to be conceded that a combination of approaches may be necessary in the future; in other words, the two procedures are not mutually exclusive. Indeed, the promulgation of contrived schismatic positions may well prove to be a retrogressive step in the long term.

28.11 Summary

Notable developments have recently emerged to enhance our understanding of the functional role of amino acids in human nutrition and health. The advances have unquestionably been the result of expansionary approaches adopted in diverse research programmes at a global level. The advent and innovative application of sophisticated techniques have contributed markedly to progress in virtually every aspect of this endeavour. Substantive features, ordered in a series of thematic statements with appropriate cross-referencing, are presented below.

28.11.1 Underlying theme

- Specific enzymes have been selected for review in this volume to exemplify the impact of amino acid metabolism on nutrition and health issues, including disorders, identification of risk factors and targets for development of molecular therapeutics.

28.11.2 Metabolism

- Major developments have been published on the expression, molecular characterization and regulation of key enzymes of amino acid metabolism (Chapters 1–10; Table 28.1). The localization and functional properties of isoforms of a number of these enzymes are a particular feature of recent research (Table 28.2).
- The enzymes associated with amino acid metabolism enable the synthesis of a diverse range of physiologically-important intermediates and end-products. In addition, a number of these enzymes ensure the disposal of amino acids present in excess of immediate requirements.
- A neuronal role for enzymes has now been extended to include glutamate dehydrogenase, glutamine synthetase, glutaminase, nitric oxide synthase, histidine decarboxylase, serine racemase, tyrosine hydroxylase and tryptophan hydroxylase. As such these enzymes exert a constitutive or supportive role in aminoacidergic, monoaminergic or nitricergic neurotransmission systems.
- A much more diverse range of amino acids is now attributed with neurotransmitter functions compared to a few years ago.
- The emergence of gaseous neurotransmitters and their mutual interactions is another key development indicating the need to recognize the complexity of signalling mechanisms.
- Thus, the classical view that amino acids serve merely as structural components and that associated enzymes are largely for re-distribution or disposal of surpluses has been substantially revised. For example, glutamate dehydrogenase exerts a pivotal role in insulin homeostasis and neurotransmission, in addition to ureagenesis (Chapter 1).

28.11.3 Nutrition

- A major feature of the enzymes considered in this volume is the biosynthesis of the nutritionally dispensable amino acids. As a consequence, glutamate, glutamine, aspartate, arginine, glycine, serine, tyrosine and cysteine need not be supplied in the diet, providing precursor levels are adequate. Nevertheless, glutamate, glutamine and arginine have been considered as potential supplements in particular clinical conditions.
- The distinction between nutritional dispensability and clinical efficacy of amino acids may thus need further amplification in the evaluation of interventions for vulnerable individuals.
The consensus now emerging is that the nutritional classification of glutamate, glutamine and arginine as dispensable grossly underestimates the key metabolic and clinical significance of these amino acids.

- Existing protocols derived from animal models have been applied to determinations of digestibility, availability and requirements of key indispensable amino acids (Chapters 14–16).
- The potential for nutritional interventions with amino acids (Table 28.5) will only be fully realized following further advances in fundamental issues such as transport (Chapter 11), inter-organ fluxes (Chapter 12), metabolic regulation and availability (Chapters 13, 14 and 15), and clinical imbalance and safety (Chapters 19 and 20). This effort should be enhanced by employing measurements of oxidation to quantify amino acid requirements and to assess nutritional adequacy of food proteins.
- Nevertheless, the attributes of glutamine and branched-chain amino acids in nutritional support are now emerging (Table 28.5). In addition, there may be potential in the administration of tryptophan-rich proteins and hydrolysates for the treatment of affective disorders.
- There are indications that dietary monosodium glutamate supplementation may improve food intake in the elderly and in patients with chronic inappetence.
- The dietary inclusion of vegetables rich in nitrate, nitrite and antioxidants combined with arginine may represent an optimal balance of substrates to restore and maintain nitric oxide bioavailability under conditions of ischaemia or oxidative stress (Chapter 4).
- B-vitamin fortification may reduce risks associated with elevated homocysteine levels (Chapter 21). B-vitamin deficiencies also tend to occur in PKU patients on Phe-restricted diets (Chapter 23).
- The oral administration of amino acid mixtures devoid of tryptophan (Chapter 24) or histidine or phenylalanine plus tyrosine, in order to deplete brain concentrations of the respective neurotransmitters, is emerging as a worthwhile technique in psychopharmacology.

### 28.11.4 Food safety

- The toxicity and safety of non-protein amino acids in food have been reviewed together with their physiological and molecular interactions (Chapters 19–20).
- There is some disquiet over the use of nitrates and nitrites in the production of cured meats. However, recent research indicates that consumption of vegetables rich in nitrates, nitrites and antioxidants may confer benefits that outweigh any perceived cancer risks (Chapter 4).
- Monosodium glutamate is currently regarded by the US Food and Drugs Agency as safe. The protective functions of the blood–brain barrier have been emphasized (Chapter 11).
- The occurrence of Maillard reaction products (Chapter 22) in processed foods is associated with adverse health effects and reductions in nutritional value.

### 28.11.5 Health and disease

- The structural functions of amino acids underline the requirements of these nutrients for optimal health and well-being. However, it is the neurotransmission roles in normal and, by imputation, in diseased states (Chapters 5, 7, 24, and 25) that encapsulate the modern perspective of amino acids in human health.
- Amino acid metabolism has been implicated in, or is affected by a diverse range of disorders (Fig. 28.1). In addition, metabolic dysfunction may provide markers for several clinical conditions.
- Consistent evidence is available to demonstrate that ornithine decarboxylase (ODC) activity is elevated in a variety of malignant conditions including Barrett’s oesophagus and Barrett’s-associated adenocarcinoma, prostate cancer, colorectal cancer and breast cancer.
Up-regulation of the kynurenine pathway of tryptophan degradation has been observed in patients with major psychotic disorders, including schizophrenia and bipolar disorder (Table 28.6).

Neuropsychiatric conditions and behaviour are influenced by serotonin neurotransmission as elucidated in tryptophan-depletion tests (Chapter 24).

Dopaminergic factors appear to be involved in the pathogenesis of unipolar depression and in motivated behaviour.

Disruption of glutamatergic function and ensuing excitotoxicity have been implicated in the pathophysiology of psychiatric and neurodegenerative disorders (Chapters 7 and 25). Detailed consideration has been given to current evidence for depression, schizophrenia, Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, stroke and epilepsy, attributed principally to loss of glutamatergic transmission or to excitotoxicity (Chapter 25).

Inducible NO synthase is expressed in different cell types in multiple sclerosis lesions, and astrocyte-derived NO may influence the manifestation of inflammatory reactions, particularly at the blood–brain barrier (Table 28.6).

In ischaemic conditions or oxidative stress, NO homeostasis may be impaired, requiring supplementation via dietary nitrate and nitrite (Chapter 4).

The resolution of interactions is critical for further advances in disease characterisation and treatment. Thus, both glutamate excitotoxicity and NO synthesis are implicated in the pathogenesis of multiple sclerosis. Similarly, Parkinson’s disease is attributed to disruption of glutamatergic neurotransmission (Chapter 25), but the characteristic motor manifestations arise with degeneration of dopaminergic neurons (Chapter 26).

Histamine is associated with several pathological conditions including allergic reactions, inflammation, atherosclerosis, cancer and epilepsy. There is substantial evidence linking the histaminergic system in the brain with cognitive impairments observed in patients with Alzheimer’s disease (Chapter 5).

The regulation of obesity by histamine (Chapter 5) and by leucine should provide impetus for further research to elucidate the underlying mechanisms involved in this effect. The modulation of insulin signalling may represent one line of enquiry.

Limited evidence indicates that diabetes may directly or indirectly impact on transport, metabolism and post-translational reactivity of amino acids.

The evidence presented in Table 28.7 confirms the wide range of genetic conditions associated with the metabolism and transport of amino acids. An emerging issue is the late-onset presentation in certain disorders, permitting prolonged survival. A considerable number of the congenital disorders, including PKU (Chapter 23), are associated with a common underlying theme of brain damage, seizures and mental impairment. Novel non-invasive technologies have been employed to revisit long-established genetic disorders.

Aminotransferases, ODC, homocysteine and post-translational lysine adducts may serve as markers or mediators of risk in a variety of disorders (Chapters 2, 21, and 22).

The quest to identify appropriate targets for therapeutic intervention provides an important impetus for continuing research. Targets under consideration include enzymes, neurotransmitter receptors (Table 28.8) and transporters implicated in specific neurodegenerative, cognitive and psychotic disorders. However, the development of therapeutic agents that interact with several of the affected regulatory mechanisms might contribute to enhanced efficacy. A number of non-protein amino acids have been screened for anti-cancer properties (Chapter 19).

### 28.12 Outlook

It was always inevitable that support for the ethos of mammalian protein metabolism...
would decline sooner rather than later. The limitations inherent in the underlying concepts of nitrogen balance and protein turnover, for example, have long been recognized. Questions concerning protein-energy malnutrition have now been replaced by an awareness of the functional and molecular roles of individual amino acids and associated enzymes in a wide range of other disorders. Amino Acids in Human Nutrition and Health has been designed to formalize this transition towards a more dynamic and comprehensive approach. There is now considerable optimism that recent developments, reviewed in this volume, should provide significant impetus for continuing progress in several of the disciplines underpinning this title.

Further developments in fundamental areas of dynamics, as presented in this volume, should yield valuable data to enhance the potential of amino acids in nutritional support. Current studies designed to measure endogenous losses, requirements and availability of amino acids should ensure that nutritional support emerges as a more consistent and routine form of clinical intervention. Integral to this and other applications will be the need to evaluate the potential impact of amino acid–endocrine interactions. The role of amino acids as immune modulators should also be addressed in a more comprehensive approach.

A diverse range of disorders is now attributed to, or reflected in, the abnormal metabolism of amino acids. Disruption of the neurotransmission mechanisms has been implicated in several neurodegenerative, affective, cognitive and cardiovascular conditions, but there is clearly a need for more definitive evidence that would underpin the development of novel therapeutics. Specific components of signalling systems provide potential focal points for targeting such disorders with novel pharmaceuticals. However, the multifactorial aspects of interactions within and between these systems may well determine the efficacy of any programme of drug development. Future efforts will inevitably be directed at evaluating the clinical significance of changes in amino acid metabolism in diabetes, cancer and genetic disorders.

The role of amino acids, post-translationally modified residues and enzymes as risk factors or mediators for disease should provide the basis for future developments in diagnostic applications. There is scope for a more broad-based metabolomic approach in risk assessment.

Future advances will be critically dependent upon the refinement of existing protocols and development of innovative technologies. At the leading edge of this effort will be the application of non-invasive imaging devices and molecular methodologies. It is imperative that any strategy is not polarized or confounded by preconceptions concerning empirical and modelling approaches.

In conclusion, the prospects for enhanced use of amino acids in nutritional support should improve with further advances in kinetics and metabolic regulation. Moreover, current initiatives should establish the mechanisms whereby other nutrients and dietary constituents may complement critical properties, or mitigate adverse effects, of amino acids. It is also clear that the dysfunctional metabolism of amino acids is associated with or reflected in a diverse array of disorders and, in certain instances, may provide markers for risk assessment. In view of a more progressive agenda now emerging, there is renewed confidence that elucidation of the complex interactions underlying diverse disorders should yield the molecular basis for the development of novel therapeutics and diagnostic methodologies.

References


Emergence of a New Momentum


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Li, X. (2009) T1R receptors mediate mammalian sweet and umami taste. The American Journal of Clinical Nutrition 90, 73S–737S.


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