Nitrogen Metabolism in *Ostertagia (Teladorsagia) circumcincta*

A thesis presented
In partial fulfilment of the requirements
For the degree of
DOCTOR OF PHILOSOPHY
In Physiology

at Massey University, Palmerston North
New Zealand

NOORZAID MUHAMAD
2006

Table of Contents

Chapter 4 Metabolism of Alanine and Aspartate in *Ostertagia circumcincta*

Abstract

The aim of the experiments was to investigate some key areas of nitrogen metabolism in adult and third-stage larval *Ostertagia (Teladorsagia) circumcincta*, to seek enzymes either not present in mammals or with distinctive kinetic properties, which clearly differentiated the nematode and host metabolic systems. The study encompassed excretion and uptake in intact worms and determining the kinetic properties of eleven enzymes involved in the metabolism of arginine, urea, alanine aspartate and glutamate.

The metabolism of *O. circumcincta* was different from that in mammals and more like that of microorganisms and plants. Ammonia was the main excretory product, with a little urea, both apparently crossing the cuticle through specific permeases. The excretion rate increased with temperature, but decreased as the external ammonia concentration increased, suggesting that ammonia may be a source of nitrogen additional to amino acids, which were taken up by adult worms. Ammonia could be incorporated directly into glutamate and other amino acids through the glutamine synthetase-glutamate synthase pathway, which was more active in adult worms. Glutamate dehydrogenase was able to use either NADH or NADP in the deaminating direction, which would be the predominant direction because of the low affinity of GDH for ammonia. In the aminating direction, there was greater activity with NADH than NADPH.

Creatinase and arginase were probably the sources of excreted urea. There was no urease activity to convert urea to ammonia. No role could be assigned to creatinase other than to degrade host creatine, perhaps to supply sarcosine for metabolism. The unusual feature of aspartate metabolism was aspartase activity in addition to aspartate aminotransferase, which, in larvae, had the highest activity of all enzymes studied. In adult worms, which are

believed to have a more anaerobic metabolism than larvae, aspartase would allow aspartate to be formed directly from fumarate in association with only a partial TCA cycle.

Perhaps the most important finding was the identification in the parasites of three enzymes, creatinase, aspartase and glutamate synthase, which are not believed to be expressed in the sheep host or other mammals, making them possible candidates for developing novel anthelmintic therapies.

Acknowledgements

This thesis would not have materialised without the firm nudging and assistance with writing of my supervisor Professor Heather Simpson, whose advice, criticism and encouragement have been most invaluable. More than simply guiding me in my experimental work, she quickly became a mentor to indoctrinate me into the world of the research scientist.

Also, I would like to take this opportunity to express my heartfelt thanks to Associate Profesor Kevin Pedley for his supervision of the uptake studies and active participation in my PhD project and thesis. On both a professional and personal level, I really appreciated the support that he has given to me during my PhD down times.

I would also like to thank Dr David Simcock and Dr Simon Brown for their advice and assistance in using spectrophotometric techniques for enzyme assays. I would especially like to thank Dr Simcock for his assistance with infecting sheep, collecting adult worms and helping me carry out multiple assays to make the best use of adult worms. I am also grateful to him and Lisa Walker for providing me with unpublished data for use in discussion. There is no way I would have been able to finish the bench work on time if it weren't for their assistance. I would like to thank my fellow students for their team work in maintaining the supply of parasites for the laboratory.

I would like to thank Lisa Walker, Lois Taylor, Juliet Sutherland, and Alexandra Huber for their assistance in the laboratory and Mat Levin for making sure that the network ran smoothly. I am very grateful for the encouragement of Dr Gordon Reynolds, Miria Busby, everyone in the PTC building, postgraduate students in the PD hut and Portaloo building, the Malaysian Students Association, especially Zul, Lani, Daniel, Nik, Pica, Clayton, Alex and Nandoo and Sylvia Hooker and staff of ISO. To Dr Bruce

Simpson thanks for your cheery motivational advice, I really appreciate it. Not to forget those in IFNHH especially Professor Geoffrey Annison, former HOI of IFNHH, for his concern and support.

My thanks go to UNIMAS for personal support and Meat and Wool New Zealand for providing the financial support for my research work.

I would like to dedicate my love and thanks to my mom, my dad, brother and sisters. Thank you for your support, encouragement and your never-ceasing prayers.

To my beautiful wife Resni who has to endure dinner conversations focused solely on metabolism and parasite for more than a year, thanks for your patience. To Sarah and Imran, as promised you can now use the computer. To Armin sorry for not being able to give you the attention you need as daddy was fully occupied.

"The man who removes a mountain begins by carrying away small stones"

List of Figures

List of Tables

List of Abbreviations

2D	two dimensional
A. aegypti	*Aedes aegypti*
A. galli	*Ascaridia galli*
A. lumbricoides	*Ascaris lumbricoides*
A. marina	*Arenicola marina*
A. suum	*Ascaris suum*
aa	amino acids
ADC	arginine decarboxylase
AGAT	arginine-glycine amidinotransferase
AK	arginine kinase
AlaAT	alanine aminotransferase
AMP	adenosine monophosphate
APC	acid-polyamine-choline
AS	asparagine synthetase
AspAT	aspartate aminotransferase
ATF1	amino acid transporter superfamily 1
B. malayi	*Brugia malayi*
B. mori	*Bombyx mori*
B. pahangi	*Brugia pahangi*
BCAT	branched chain aminotransferases
C. briggsae	*Caenorhabditis briggsae*
C. elegans	*Caenorhabditis elegans*
C. emasculans	*Cercaria emasculans*
C. lingua	*Cryptocotyle lingua*
C. oncophera	*Cooperia oncophera*
cAlaAT	cytosolic alanine aminotransferase

cAspAT	cytosolic aspartate aminotransferase
CCBL	cysteine *S*-conjugate β-lyase
cGDH	cytosolic glutamate dehydrogenase
Ci	curie
CK	creatine kinase
cNOS	constitutive nitric oxide synthase
CoA	Coenzyme A
CPS	carbamoyl phosphate synthetase
D. immitis	*Dirofilaria immitis*
D. melanogaster	*Drosophila melanogaster*
D. polymorpha	*Dreissena polymorpha*
DFMO	difluoromethylornithine
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
e.p.g.	eggs per gram
ES	excretory/secretory
Expt	experiment
F. hepatica	*Fasciola hepatica*
Fd	ferrodoxin
g	gram
g	gravitational force
G. intestinalis	*Giardia intestinalis*
G. lamblia	*Giardia lamblia*
GABA	γ-aminobutyric acid
GABA-T	4-aminobutyrate:2-oxoglutarate aminotransferase
GAMT	*S*-adenosyl-L-methionine:N-guanidinoacetate methyltransferase

GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
GSH	glutathione
GST	glutathione *S*-transferase
GTP	guanosine triphosphate
h	hour
H. alvei	*Hafnia alvei*
H. citelli	*Hymenolepis citelli*
H. contortus	*Haemonchus contortus*
H. diminuta	*Hymenolepis diminuta*
H. nana	*Hymenolepis nana*
H. polygyrus	*Heligmosomoides polygyrus*
H. pylori	*Helicobacter pylori*
IMP	inosine monophosphate
iNOS	inducible nitric oxide synthase
kg	kilogram
L. carinii	*Litomosoides carinii*
L3	third stage larva
L4	fourth stage larva
LASPO	L-aspartate oxidase
M	molar
M. expansa	*Moniezia expansa*
M. similis	*Microphallus similis*
mAlaAT	mitochondrial alanine aminotransferase
mAspAT	mitochondrial aspartate aminotransferase
mCi	millicurie
MDH	mitochondrial malate dehydrogenase

MFS	major facilitator superfamily
mg	milligram
mGDH	mitochondrial glutamate dehydrogenase
min	minute
ml	millilitre
mM	millimollar
MW	molecular weight
n	number
N. americanus	*Necator americanus*
N. brasiliensis	*Nippostrongylus brasiliensis*
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAG	N-acetylglutamate
nl	nanolitre
nm	nanometre
nmole	nanomole
NOS	nitric oxide synthase
O. circumcincta	*Ostertagia circumcincta*
O. cuniculi	*Obeliscoides cuniculi*
O. volvulus	*Onchocerca volvulus*
OAA	oxaloacetate
OAT	ornithine aminotransferase
ODC	ornithine decarboxylase
OTCase	ornithine transcarbamylase
OUC	Ornithine-Urea Cycle
p	probability

P. crassipalpis	*Parasarcophaga crassipalpis*
P. freudenreichii	*Propionibacterium freudenreichii*
P. islandicum	*Pyrobaculum islandicum*
P. pacifica	*Pista pacifica*
P. putida	*Pseudomonas putida.*
P. redivivus	*Panagrellus redivivus*
P5C	Δ^1-pyrroline-5-carboxylic acid
P5CDH	pyrroline-5-carboxylate dehydrogenase
P5CR	pyrroline-5-carboxylate reductase
P5CS	pyrroline-5-carboxylate synthase
RO	reverse osmosis
PBS	phosphate buffer saline
PC	pyruvate carboxylase
PEP	Phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate
POT	proton oligopeptide transporter
RNA	ribonucleic acid
S. sclerotiorum	*Sclerotinia* sclerotiorum
S. bibionis	*Steinernema bibionis*
S. cerevisiae	*Saccharomyces cerevisiae*
S. cynthia ricini	*Samia cynthia ricini*
S. frugiperda	*Spodoptera frugiperda*
S. japonicum	*Schistosoma japonicum*
S. mansoni	*Schistosoma mansoni*
S. solida	*Semele solida*
S. typhimurium	*Salmonella typhimurium*

SAMdc	*S*-adenosyl methionine decarboxylase
SDS	sodium-dicarboxylate symporters
SEM	standard error of the mean
SL	sheathed larva
T. colubriformis	*Trichostrongylus colubriformis*
T. cruzi	*Trypanosoma cruzi*
T. spiralis	*Trichinella spiralis*
TCA	tricarboxylic acid
µCi	microcurie
µg	microgram
µl	microlitre

Introduction

Parasitism of farmed animals reduces the weight gain of young animals, wool quality may be poor and reproductive performance and milk production may be lower in chronically infected animals. Mortality can occur in severe cases. Farmers depend heavily on anti-parasitic drugs to control internal parasites, however, many of these anthelmintics are losing their efficacy as the parasites acquire resistance to them. Novel methods of controlling nematode parasites of sheep and cattle are urgently needed to augment current chemical anthelmintics and perhaps slow the rate at which drench resistance is developing. Alternative strategies to drenching include breeding for resistent and resilient animals, stock management to minimise larval intake, the use of plants containing natural anthelmintics as fodder, trapping the free-living stages of nematodes by fungi and the development of vaccines against the parasites.

Nematode parasites are a major health problem affecting sheep in New Zealand, particularly the abomasal parasite *Ostertagia (Teladorsagia) circumcincta* and the intestinal parasite *Trichostrongylus colubriformis*. *Haemonchus contortus* can cause severe disease in sheep in warmer areas. A goal of the Laboratory for Biochemical Parasitology at Massey University, supported by funding from Meat and Wool New Zealand, is to identify new targets for anthelmintic control of abomasal parasites of sheep, particularly *O. circumcincta*, which is a common parasite. Enzymes forming the metabolic pathways in this nematode must provide several targets which can be exploited, yet relatively little is known about these enzymes.

Most chemical control is directed at the parasitic stages in the sheep, but the free-living stages on the pasture may also be vulnerable to chemicals. In the experiments reported in this thesis, nitrogen metabolism has been studied in both the infective third-stage larva (L3) and adult worms collected from infected sheep. As the L3 are more readily available, enzyme assays and other methodology were developed using this life-cycle stage and

subsequently extended to the smaller number of adult worms that could be recovered quickly from sheep.

A review of the literature (Chapter 1) revealed how little was known about nitrogen metabolism in *O. circumcincta* or even in other parasitic nematodes of sheep. It was decided that the study should encompass only some key areas of nitrogen metabolism, given that it was such a large area and almost no data were available. It was hoped that some novel enzymes would be identifed which were absent in mammals or that kinetic studies would reveal properties which clearly differentiated the nematode and host enzymes. In addition to identifying potential anthelmintic targets, the study would provide a basis for improving the *in vitro* culture conditions for both L3 and adult worms being carried out in the Laboratory.

The experiments reported below were designed to investigate the excretion and uptake of nitrogenous compounds (Chapter 2), and metabolism based around arginine and urea (Chapter 3), alanine and aspartate (Chapter 4) and glutamate and glutamine (Chapter 5).

Chapter 1

LITERATURE REVIEW

Nitrogen is present in all cells as a component of DNA, RNA, amino acids, structural proteins and enzymes. The nitrogen metabolism of an organism broadly encompasses processes involved in breakdown of ingested nutrients, their absorption, metabolism and excretion. Nitrogen metabolism has received much less attention than has energy metabolism in helminths, and animal parasitic nematodes are underrepresented in these studies compared with plant parasitic and free-living nematodes and other helminth groups.

Early studies of substrate usage and resulting end-products in the nematode *Caenorhabditis briggsae* (Rothstein and Tomlinson, 1961, 1962; Rothstein and Mayoh, 1964a,b, 1966; Rothstein, 1965; Liu and Rothstein, 1976) indicated that there were differences between nematode metabolism and that in vertebrates, notably in the ability to synthesise the essential amino acids, the presence of isocitrate lyase (a glyoxylate cycle enzyme) and the likelihood of additional pathways. In similar experiments with the human trematode parasites *Schistosoma mansoni* and *Schistosoma japonicum*, glutamate, aspartate, alanine, arginine and proline were the main exogenous amino acids metabolised *in vitro* (Bruce *et al.*, 1972). As there is little direct information available on nitrogen metabolism in sheep nematode parasites, the amino acids listed above are the focus of the present study, which considers broad areas relating to:

(1) uptake and excretion

(2) urea and arginine metabolism

(3) alanine and aspartate metabolism and the role of transamination

(4) glutamate metabolism.

Where there are limited data for sheep nematodes, the most relevant models are likely to be other invertebrates and helminths, particularly plant parasitic and free-living nematodes, although similar systems in other classes of organisms are also considered for comparison.

1.1 SOURCES OF NITROGEN

Living organisms absorb nitrogen in both organic and inorganic forms. Most nitrogen exists in the atmosphere as N_2, where it is primarily fixed by the action of lightning to form oxides and eventually nitrate and nitrite (Nna Mvondo *et al.*, 2001). The ability to fix atmospheric N_2 into organic compounds is limited to prokaryotes (Silver and Postgate, 1973; Oelze, 2000). A small number of plant species that have symbiotic relationships with nitrogen-fixing bacteria are, therefore, also able to utilise atmospheric nitrogen indirectly (Siddiqui and Mahmood, 1995). Plants generally use nitrate and ammonium as their main sources of nitrogen (Pate, 1973) and, as in bacteria (Richardson and Watmough, 1999), the reduction of nitrate is the first step in generating ammonium for the synthesis of amino acids and other nitrogenous compounds (Miflin and Lea, 1976). Organic nitrogen may make a significant contribution to plant nitrogen metabolism in environments where free amino acids are more plentiful in the environment, such as arctic areas and marshland (Henry and Jeffries, 2003; Thornton and Robinson, 2005).

Unlike plants, bacteria and yeasts, which are able to use a variety of inorganic and organic nitrogen compounds, including ammonia, urea, peptides and amino acids (Jørgensen *et al.*, 1987, 1999), most animals are unable to synthesise their entire amino acid requirements and must include these "essential amino acids" in their diet. Higher animals obtain most of their nitrogen from protein, which is degraded in the digestive tract before absorption in the intestine as peptides (Ziv and Bendayan, 2000) or amino acids (Christensen, 1990; Wagner *et al.*, 2001).

The sources of nitrogen for *Ostertagia circumcincta* are unknown and could include mucins, cells, components of leaked interstitial fluid or the abomasal digesta, which includes bacteria. Proteolytic enzymes are present on the intestinal brush border and/or are secreted by many parasitic nematodes of plants (Vanholme *et al.*, 2004) and animals (reviewed by Sajid

and McKerrow, 2002), including L3, L4 and adult *O. circumcincta* (Young *et al.*, 1995), *Haemonchus contortus* (Cox *et al.*, 1990; Redmond *et al.*, 1997; Knox *et al.* 2003; Williamson *et al.*, 2003), *Ostertagia ostertagi* (Geldof *et al.*, 2000), *Necator americanus*, *Ancylostoma caninum* (Williamson *et al.* 2003b) and *Toxocara canis* (Loukas *et al.*, 1998). These species are, therefore, capable of degrading protein externally or in the gut as a source of nutrients. Gut membrane proteases are considered good candidates for antiparasitic vaccines, particularly against blood-feeding species (Andrews *et al.*, 1995; Skuce *et al.*, 1999a; Knox *et al.*, 2003).

1.2 UPTAKE OF NITROGENOUS COMPOUNDS

The transport mechanisms for nitrogenous compounds have been the subject of extensive study in unicellular organisms through to mammals. Uptake of ammonia, urea, peptides, protein and amino acids occurs through both active and passive processes involving a large number of transporters in prokaryotes, animals and plants, whereas nitrate transport takes place in plants, bacteria, fungi and yeasts, but not higher animals.

Ammonia can cross membranes either by diffusion of the unprotonated form (NH_3) or by uptake of NH_4^+ by specific transporters (Kleiner, 1981). Ammonium transporters are present in all classes of organisms (Howitt and Udvardi, 2000; Williams and Miller, 2001; Javelle *et al.*, 2003; Khademi *et al.*, 2004). Four homologues of high affinity ammonium transporters are present in the *Caenorhabditis elegans* genome (Howitt and Udvardi, 2000).

Urea permeability varies in different tissues, some of which possess specific urea transporters, particularly mammalian liver, kidney tubules and erythrocytes. These transporters translocate urea by facilitated diffusion, which is not dependent on either Na^+ or Cl^- and is not saturable up to 200 mM urea (Shayakul and Hediger, 2004). There are also urea transporters in the frog bladder (Couriaud *et al.*, 1999), dogfish kidney (Smith and Wright, 1999) and fish gill (Walsh *et al.*, 2001).

Nitrate transporters belonging to the Major Facilitator Superfamily of transporters are associated with the ability to fix inorganic nitrogen in bacteria, yeasts fungi and plants (Pao *et al.*, 1998; Richardson and Watmough, 1999; Forde, 2000; Williams and Miller, 2001).

Facing page 4

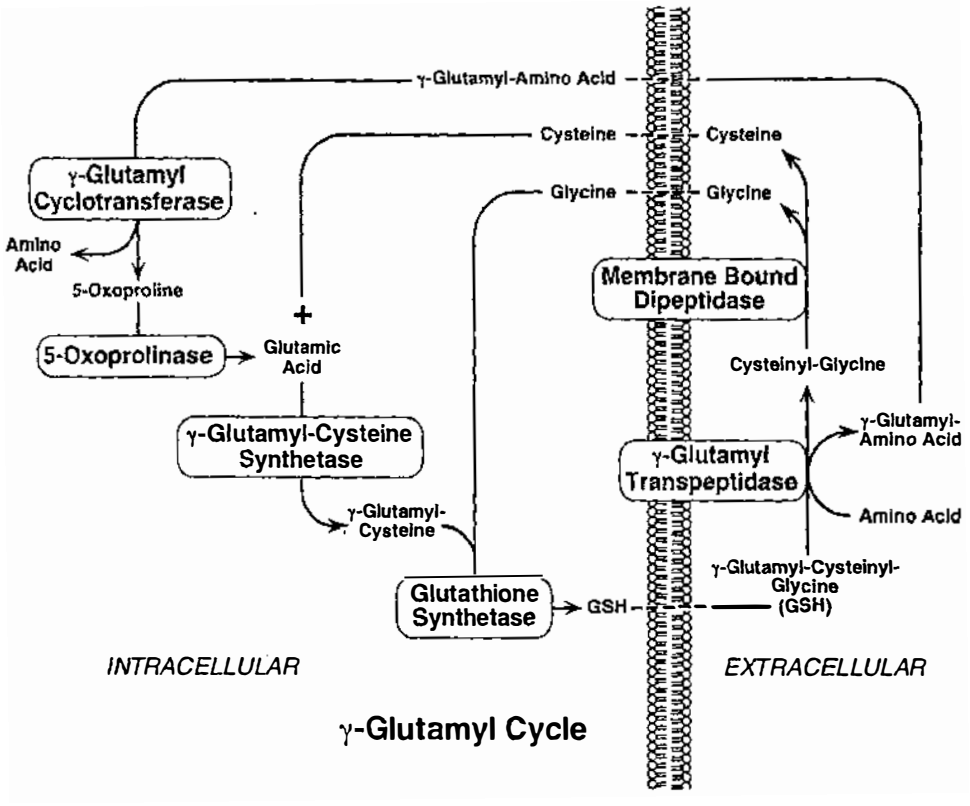


Figure 1.1. The γ-glutamyl cycle which acts as an amino acid transporter. (From Lieberman *et al.*, 1995).

Table 1.1. Classification of amino acid transport systems in the brush border membrane (top) and basolateral membrane (bottom) of mammalian enterocytes. Table adapted from Mircheff *et al.* (1980).

Transport system	Substrates	Dependence on Na^+ gradient	Involvement of others ions
B	Dipolar α-aa	Yes	None
$B^{0,+}$	Dipolar α-aa Basic aa Cystine	Yes	None
$b^{0,+}$	Dipolar α-aa Basic aa Cystine	No	None
y^+	Basic aa	No	None
IMINO	Imino acids	Yes	Cl^-
β	β-aa	Yes	Cl^-
X^-_{AG}	Acidic aa	Yes	K^+
A	Dipolar α-aa Imino acids	Yes	
ASC	Three and four carbon dipolar aa	Yes	
Asc	Three and four carbon dipolar aa	No	
L	Bulky, hydrophobic, dipolar aa	No	
y^+	Basic aa	No	

Peptides are co-transported with protons by the POT (proton oligopeptide transporter) superfamily in plants (Williams and Miller, 2001), bacteria, yeasts and animals (Meredith and Boyd, 2000), including *C. elegans* (Fei *et al.*, 1998; Meissner *et al.*, 2004). The peptide transporters PEPT1 and PEPT2 are widely distributed in mammalian tissues and are important for dietary nitrogen absorption from the intestine, reabsorption of filtered peptides in the kidney and re-uptake of neurotransmitters (Meredith and Boyd, 2000). Deletion of the *C. elegans* intestinal peptide transporter orthologue of *pep-1* (*pep-2*) abolished peptide uptake and severely retarded worm growth, development and reproduction (Meissner *et al.*, 2004).

Protein absorption occurs in the intestine of mammals (Ziv and Bendayan, 2000) by a process of transcytosis involving the cytoskeleton (reviewed by Tuma and Hubbard, 2003). Casartelli *et al.* (2005) have demonstrated a similar mechanism of protein uptake in the insect intestine.

Amino acid transporters in animals, plants and fungi fall into at least five superfamilies: amino acid-polyamine-choline (APC) transporters, sodium-dicarboxylate symporters (SDS), the neurotransmitter superfamily, amino acid transporter superfamily 1 (ATF1), and the amino acid transporters within the major facilitator superfamily (MFS) (reviewed by White, 1985; Horak, 1986; Williams and Miller, 2001; Wipf *et al.*, 2002; Verry *et al.*, 2004). In mammalian cells, transporters may be sodium independent or dependent, with the driving force commonly being Na^+- or H^+-coupled cotransport, whereas K^+-cotransport can also occur in invertebrates (Castagna *et al.*, 1997; Giordana *et al.*, 1998; Wolfersberger, 2000). An overview of the characteristics of the major groups of transporters in mammalian enteroctyes, based on Mircheff *et al.* (1980), is presented in Table 1.1.

The γ-glutamyl cycle (Figure 1.1) may function as a transporter of amino acids through the interaction of amino acids with glutathione (L-γ-glutamyl–L-cysteinylglycine) to form γ-glutamyl amino acids and their subsequent release inside the cells (Orlowski and Meister, 1970; Lieberman *et al.*, 1995). Glutathione (GSH) is a tripeptide made up of glutamate, cysteine and glycine, which is found in most organisms and has numerous cellular functions, particularly as an antioxidant. The synthesis and degradation of GSH involves six enzymes, two for synthesis and four for catabolism as shown in Figure 1.1

(Lieberman *et al.*, 1995). The first step of glutathione catabolism involves the enzyme γ-glutamyl transpeptidase, which transfers the γ-glutamyl moiety to an amino acid to form a γ-glutamyl amino acid, in which form amino acids can be transported into the cell. Gamma-glutamyl transpeptidase is present in the brush border of rat kidney tubules (Orlowski and Meister, 1970) and many other mammalian secretory and absorptive surfaces (Hanigan and Frierson, 1996), in the protozoan *Trypanosoma cruzi* (Repetto *et al.*, 1987), in the Malpighian tubules and midgut of *Musca domestica* larvae (Bodnaryk *et al.*, 1974) and in helminths (Dass and Donahue, 1986; Abidi and Nizami, 1995).

1.2.1 UPTAKE OF NITROGENOUS COMPOUNDS IN HELMINTHS

Research on uptake of nitrogen in helminths has focused on amino acid transport (reviewed by Pappas and Read, 1975; Pappas, 1988) and most studies are restricted to only a few species, particularly *Ascaris suum* and *Ascaridia galli* amongst the parasitic nematodes. Helminths include cestodes (tapeworms), acanthocephalans (spiny-headed worms), trematodes (flukes and schistosomes) and nematodes, four sub-groups with different anatomical structure of the gut and outer surface. Amino acid uptake may not be comparable in the different helminths. Cestodes and acanthocephalans lack a digestive tract, so absorption can take place only across the tegument, an external structure resembling an intestinal brush border (Pappas *et al.*, 1973). Trematodes have both an absorptive tegument and an incomplete gut (Asch and Read, 1975; Hanna, 1980; Pappas, 1988), whereas nematodes have digestive tracts and cuticles, which are unlike typical absorptive surfaces (Bird and Bird, 1981; Page, 2001), except in a few species, such as the insect parasite *Bradynema* sp, which has microvilli on the cuticle (Riding, 1970).

Uptake of amino acids in helminths involves both diffusion and a variety of transporters, some very specific, others accepting a number of amino acids (Isseroff *et al.*, 1976; Jeffs and Arme, 1985, 1987). Uptake may also occur though the γ-glutamyl cycle (Dass and Donahue, 1986; Abidi and Nizami, 1995). Pappas (1988) reviewed the data available at that time and concluded that absorption did involve the nematode cuticle, but was secondary to the nutrient absorption though the intestine. *In vitro* experiments may not allow the relative rates to be determined accurately, as parasites may stop feeding,

which would bias the results towards cuticular uptake (Pappas and Read, 1975).

Veljkovic *et al.* (2004a) identified nine genes coding for homologues of HAT transporters in the *C. elegans* genome. They expressed two proteins (AAT-1 and AAT-3), which were characterised as surface-located transporters of neutral amino acids, particularly alanine and serine. An aromatic amino acid transporter AAT-9 is expressed in excitable cells in the *C. elegans* head and pharynx, where it may provide the precursors of serotonin or dopamine neurotransmitters (Veljkovic *et al.*, 2004b). Identification of genes involved in the transport of amino acid neurotransmitters has been a focus in nematodes (reviewed by Strange, 2003), because the nematode nervous system has already been successfully used as the target of anthelmintic drugs.

1.3 EXCRETION OF NITROGENOUS COMPOUNDS

Ammonia, urea and uric acid are the major nitrogenous excretory products of animals, the principal one depending upon the environment in which the animal lives (reviewed by Wright, 1995; Walsh, 1997; Singer, 2003). Aquatic animals, both vertebrate and invertebrate, generally excrete ammonia. Terrestrial animals prevent ammonia becoming toxic by converting it to either urea or uric acid for excretion in a small volume of urine, thereby also conserving water. Minor excretory products are creatine, creatinine, amino acids, trimethylamine oxide and guanine. Mammals predominantly excrete urea, birds and insects excrete uric acid, most fish excrete ammonium, while reptiles excrete both urea and uric acid.

1.3.1 AMMONIA EXCRETION

Ammonia is generated during amino acid catabolism, which is predominantly via transamination, in which the amino group is transferred to an acceptor such as 2-oxoglutarate (Section 1.6.11), followed by deamination by glutamate dehydrogenase (GDH) to form NH_4^+ and 2-oxoglutarate (Section 1.7.1). Glutaminase also releases ammonia from glutamate (Section 1.7.2.). Other enzymes producing ammonia, such as adenylate kinase and serine dehydratase, make appreciable contributions in some invertebrates (Fellows and Hird, 1979). Another source of ammonia is the breakdown of urea, which requires the enzyme urease. Although urease is not usually present in animal

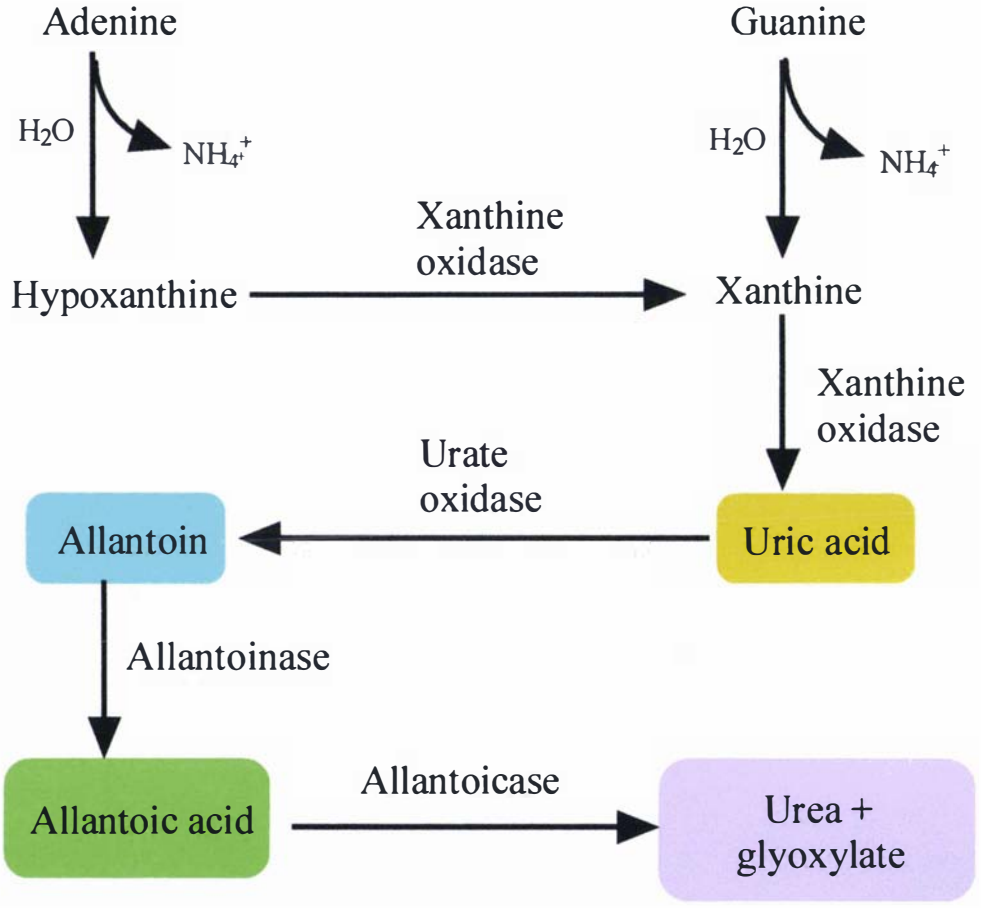


Figure 1.2. Degradation of purines to uric acid and other excretory products. Primates, birds, reptiles and insects mainly excrete uric acid, which is formed by deamination to hypoxanthine, oxidation by xanthine oxidase to xanthine and oxidation to uric acid. In teleost fish, uric acid is excreted as allantoic acid. Elasmobranchs and amphibians degrade allantoic acid to urea and glyoxylate. (Adapted from Reginald and Charles, 1999).

tissues, the microorganisms in symbiotic relationships in the gut or tissues do usually contain the enzyme and the ammonia that they produce can be absorbed by the host (Whitehead *et al.*, 1992; Stevens and Hume, 1998).

Ammonia is very soluble in water and diffuses readily across cell membranes (Kleiner, 1981), preventing ammonia accumulating to toxic levels inside the animal, provided the volume of water in which it lives is great enough to prevent external concentrations rising too high (Wright, 1995). Although only 1% of ammonia is present as a free base (NH_3) at physiological pH, the conversion of NH_4^+ to NH_3 is instantaneous and is not rate limiting for excretion. NH_4^+ can also be transported across cell membranes by specific transporters (reviewed by Kleiner, 1981; Weihrauch *et al.*, 2004). High ammonia levels in body fluids have multiple toxic effects (Visek, 1984; Wright, 1995), which include altered pH gradients leading to membrane collapse (Kleiner, 1998) and the formation of toxic compounds, such as chloramines, which may oxidise membrane and intracellular components (Thomas *et al.*, 1983).

1.3.2 URIC ACID EXCRETION

The metabolism of purines generates uric acid. This is the major excretory product, rather than urea or ammonia, in the "uricotelic" species (Wright, 1995; Singer, 2003), which include insects (Hopkins and Lofgren, 1968; Buckner *et al.*, 1980; Cochran, 1981), birds (Tsahar *et al.*, 2005) and some reptiles and amphibians. In these species, uricase activity is absent and uric acid is not metabolised further. Uric acid formation from purines involves deamination to hypoxanthine, oxidation by xanthine oxidase to xanthine and oxidation to uric acid. While uric acid is the end product of purine metabolism in humans, in most mammals other than primates, uric acid is converted to allantoin as the excretory product (Varela-Echavarria *et al.*, 1988). Some fish hydrolyse allantoin to allantoic acid, which is then excreted, however, most fish further hydrolyse allantoic acid to glyoxylate and urea; urea is then hydrolysed to ammonia for excretion. The different excretory products of purines are summarised in Figure 1.2.

1.3.3 Urea excretion

Terrestrial animals most commonly excrete excess nitrogen as urea, which passes through cell membranes either by diffusion through aqueous pores or by membrane transporters (Shayakul and Hediger, 2004). Urea is generated either from NH_4^+ or the amino-group of glutamine by the ornithine-urea cycle (OUC) or from the catabolism of uric acid or arginine (Meijer *et al.*, 1990). Although most vertebrates have the genes for the OUC enzymes (Withers, 1998), these are not always expressed throughout the entire life cycle e.g. in some species of fish, these enzymes have significant activity only at particular times in the life cycle (Wright *et al.*, 1995; Chadwick and Wright, 1999; Barimo *et al.*, 2004) or during the stress of air exposure (Chew *et al.*, 2003a) or crowding (Wood *et al.*, 2003).

1.3.4 Excretion of Nitrogenous Compounds in Nematodes

Experiments with free-living and parasitic nematodes have shown that they are predominantly ammonotelic (Rogers, 1952; Rothstein, 1963; Wright, 1975a,b). *In vitro*, *A. galli* excreted very little urea and *Nematodirus filicollis* and *Nematodirus spathiger* excreted 11-17% of nitrogen as urea in aerobic, but not anaerobic, culture (Rogers, 1952). Other nitrogenous compounds excreted by nematodes are proteins, peptides and amino acids, however, these may be the result of incomplete digestion or secretions from glands and reproductive apertures (Haskins and Weinstein, 1957; Rothstein, 1963; Wright, 1975a; Wright and Newall, 1976). Another possibility is the passive leakage of metabolically formed amino acids through the body wall and gut (Rothstein, 1970). It has been suggested that the appearance of amino acids in the medium is a sign of stress in nematodes; this was the case in the incubation of *Panagrellus redivivus*, as amino acid accumulation in the incubation medium was associated with environmental stress (Wright, 1975b).

Proteins and other compounds "excreted" by parasites may be deliberate "secretions" designed to modify the host response or to obtain nutrients (reviewed by Coombs and Mottram, 1997; Williamson *et al.*, 2003a,b; McKerrow *et al.*, 2006). The excretory/secretory (ES) products of nematodes and other parasites are known to include collagen (Rhoads *et al.*, 2001) or other components of the surface coat (Lopez de Mendoza *et al.*, 1999), polypeptides (Schallig *et al.*, 1994), proteases (Cox *et al.*, 1990; Young *et al.*,

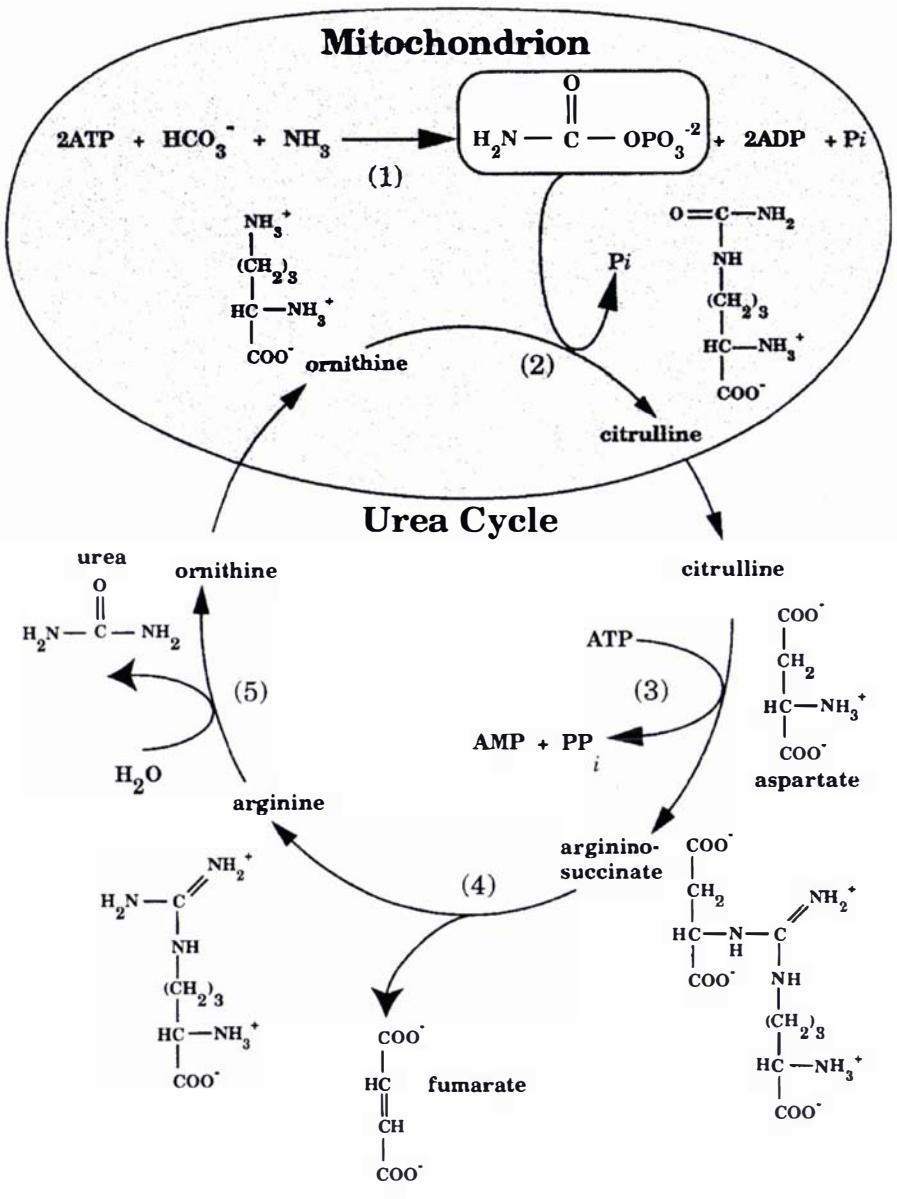


Figure 1.3. Ornithine-Urea cycle. The five enzymes are: (1) carbamoyl phosphate synthetase (2) ornithine transcarbamylase (3) argininosuccinate synthetase (4) argininosuccinate lyase (5) arginase. Diagram based on Holden *et al.* (1999).

1995; Gamble and Mansfield, 1996; Redmond *et al.*, 1997; Sajid and McKerrow, 2002), glycosidases (Gamble and Mansfield, 1996; Irwin *et al.*, 2004), elastase (Knox and Jones, 1990), acetylcholinesterase (Lee and Hodsden, 1963; Ogilvie *et al.*, 1973; Knox and Jones, 1990; Lee, 1996) and a stimulant of cell proliferation (Huby *et al.*, 1995, 1999).

1.4 UREA METABOLISM

Terrestrial vertebrates generate urea in the liver via the OUC and in other tissues as a product of arginase activity. Microorganisms possess enzymes to metabolise creatinine to creatine and thence to urea and sarcosine, but these pathways are not generally believed to operate in vertebrates (reviewed by Wyss and Kaddurah-Daouk, 2000). Another pathway, which generates urea, is the hydrolysis of agmatine by agmatinase to form putrescine and urea (Section 1.5.4).

1.4.1 THE ORNITHINE-UREA CYCLE

The ornithine-urea cycle (OUC) operates in the liver of ureotelic vertebrates to generate urea from ammonia (reviewed by Morris, 2002). The cycle is illustrated in Figure 1.3, based on Holden *et al.* (1999). The OUC consists of five enzymes: (1) carbamoyl phosphate synthetase (CPS) (2) ornithine transcarbamylase (OTCase) (3) argininosuccinate synthetase (4) argininosuccinate lyase and (5) arginase I. Two of these enzymes are mitochondrial and three are cytosolic. In addition, the mitochondrial ornithine/citrulline transporter and the enzymes glutaminase (to generate ammonia) and N-acetyl glutamate synthetase (to generate N-acetyl glutamate, a co-factor/regulator of CPS) are needed to support the cycle.

The CPS isoform involved in the OUC in mammals is CPS-I, which uses ammonia as the nitrogen donor. The urea cycle operates only in those fish which air-breathe or can tolerate air exposure, including the lungfish (Chew *et al.*, 2003a), toadfish (Anderson and Walsh, 1995; Barimo *et al.*, 2004), the snakehead (Chew *et al.*, 2003b), catfish (Kharbuli *et al.*, 2006) and sleeper (Anderson *et al.*, 2002). Rainbow trout (Wright *et al.*, 1995) and Atlantic cod (Chadwick and Wright, 1999) excrete urea and express the OUC enzymes during early lifecycle stages. In these fish, the isoform expressed is not CPS-I, but CPS-III, which uses glutamine as the nitrogen donor. CPS-III has a site to

generate ammonia from glutamine and a second site where the synthetase activity is located (Rubino *et al.*, 1986; Raushel *et al.*, 1998). The third isozyme, glutamine-utilising CPS-II, is expressed in most organisms as part of the pathway for pyrimidine nucleotide synthesis (Saeed-Kothe and Powers-Lee, 2002).

The urea cycle enzymes are not universally present in invertebrates, as argininosuccinate synthase and lyase are usually absent and only arginase and OTCase have significant activity. Urea formation may be associated with arginase (and OCTase) activity and not necessarily with a full OUC, as these may be the only two enzymes detected; this is the case for the giant tubeworm (De Cian *et al.*, 2000), *S. mansoni* (Senft, 1966), *Hymenolepis diminuta* (Campbell, 1963), silkmoths and cockroaches (Reddy and Campbell, 1969). The full complement of enzymes have been detected, usually only with very low activity, in a few species including a terrestrial snail (Linton and Campbell, 1962), a carnivorous dipteran insect (Pant and Kumar, 1978), the free-living nematode *P. redivivus* (Wright 1975a), some trypanosomes (Yoshida *et al.*, 1978) and the trematode *Fasciola gigantica* (Mohamed *et al.*, 2005).

Campbell (1963) suggested that the incorporation of ^{14}C-bicarbonate into urea by *H. diminuta* indicated a functional OUC, although this is not now believed to be the only pathway to account for this observation. Measurement of enzyme activities supports the opposite view. Janssens and Bryant (1969) investigated the OUC in species from all four subgroups of helminths, and agree with other studies that urea is only a minor excretory product and that the full complement of OUC enzymes is rarely present and if so, only in low activity. This was confirmed in a later study of the nematodes *Heligmosomoides polygyrus* and *P. redivivus*, neither of which had detectable activities of all enzymes of the OUC (Grantham and Barrett, 1986a).

1.4.2 Creatinase

Microorganisms, including some species of the microflora resident in vertebrate digestive tracts, possess enzymes to metabolise creatinine, including creatininase (which degrades creatinine to creatine) and creatinase (which degrades creatine to form urea). Creatinase catalyses the reaction of creatine with water to form urea and sarcosine; sarcosine is further degraded by sarcosine dehydrogenase to glycine and formaldehyde (Wyss and

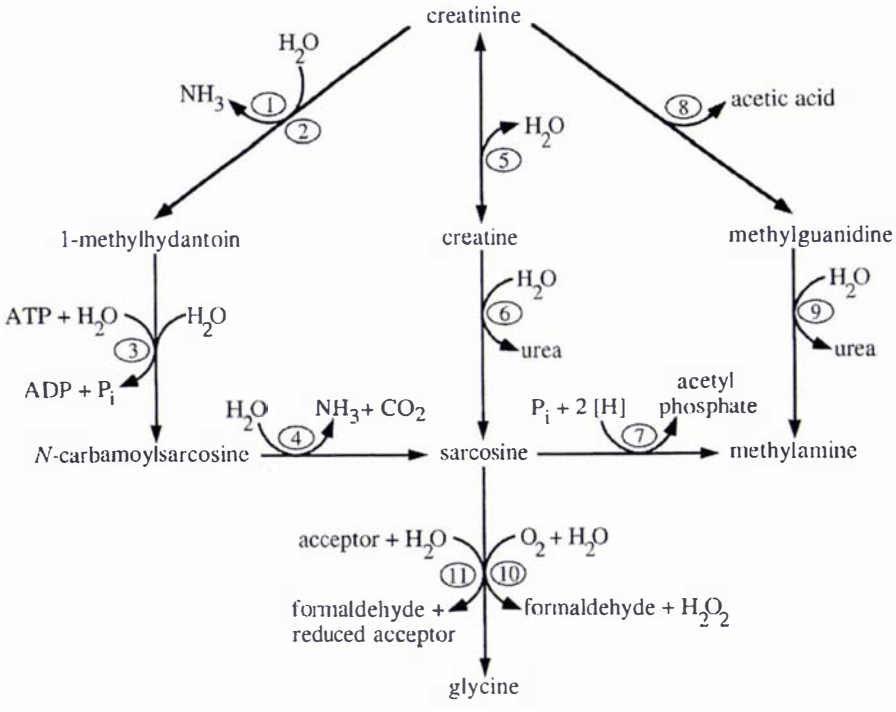


Figure 1.4. Pathways by which creatinine and creatine may be degraded in microorganisms. The enzymes involved are (1) creatinine iminohydrolase (creatinine deaminase; EC 3.5.4.21), (2) cytosine aminohydrolase (cytosine deaminase; EC 3.5.4.1), (3) 1-methylhydantoin amidohydrolase [ATP dependent (EC 3.5.2.14) or non-ATP dependent], (4) *N*-carbamoylsarcosine amidohydrolase (EC 3.5.1.59), (5) creatinine amidohydrolase (creatininase; EC 3.5.2.10), (6) creatine amidinohydrolase (creatinase; EC 3.5.3.3), (7) sarcosine reductase (EC 1.4.4.-); (8) not characterized, (9) methylguanidine amidinohydrolase (EC 3.5.3.16), (10) sarcosine oxidase (EC 1.5.3.1), (11) sarcosine dehydrogenase (EC 1.5.99.1) or dimethylglycine dehydrogenase (EC 1.5.99.2). (From Wyss & Kaddurah-Daouk, 2000).

Kaddurah-Daouk, 2000). There are also other pathways of creatine degradation in microorganisms (Figure 1.4). It is generally believed that there is no creatinase activity in vertebrates, although there is an unconfirmed report of its detection in human muscle by Miyoshi *et al.* (1980a,b) (cited by Wyss and Kaddurah-Daouk, 2000). Because of the absence of creatinase activity, creatinine is excreted unchanged in vertebrates and can be used as a marker to estimate renal glomerular filtration rate. Patients with renal failure may have an apparent metabolism of creatine, which is attributed to diffusion into the intestine, where it is metabolised by the gut microflora (Jones and Burnett, 1974).

1.4.3 UREASE

Urease is a plant, algal, fungal and microbial enzyme which converts urea and water to ammonia and CO_2 (reviewed by Mobley and Hausinger, 1989). Many pathogenic organisms generate ammonia to counteract acidic conditions (reviewed by Audia *et al.*, 2001; Cotter and Hill, 2003). *Helicobacter pylori* is a notable example of an infectious agent which uses urease to liberate ammonia from urea (Mobley and Hausinger, 1989; Mégraud *et al.*, 1992), while others use the dihydrolase pathway to convert arginine to ammonia, ornithine and CO_2 (Section 1.5.2). A second strategy to cope with low pH involves the import of an extracellular substance, which is metabolised with the incorporation of a proton, and export of the more neutral product. Two examples of this latter process are the conversion of arginine to agmatine using the enzyme arginine decarboxylase (ADC) (Section 1.5.4) and the metabolism of glutamate to γ-amino butyric acid (GABA) using glutamate decarboxylase (GDC) (Section 1.7.6).

Although urease is generally not considered to be present in animal tissues, it has been detected in cestodes (Simmons, 1960) and in the intestine of the nematodes *Nematodirus* spp and *Ascaris lumbricoides* (with less in other tissues), but was not present in *H. contortus* (Rogers, 1952). There are also numerous earlier reports (1920-1950) of urease activity in invertebrates (cited by Simmons, 1960). Urease has also been identified in insects, such as the bruchis beetle larva, which feeds on the toxic arginine analog L-canavanine, which it catabolises using arginase and urease (Rosenthal *et al.*, 1982). In the silkworm *Bombyx mori*, the enzyme appears to have been acquired from ingested plant material, as it is present in the haemolymph

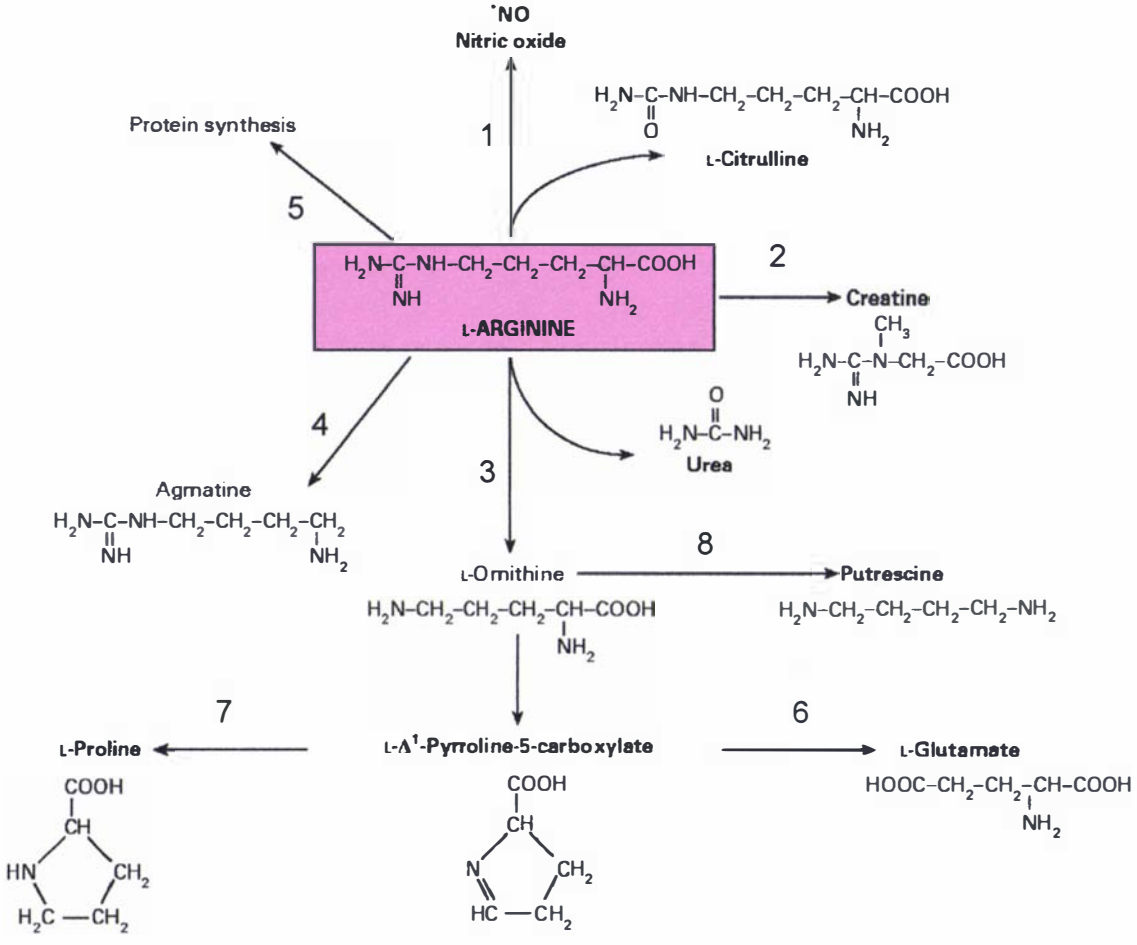


Figure 1.5. Arginine metabolism in mammals. The enzymes are: (1) nitric oxide synthase (NOS) (2) arginine:glycine amidinotransferase (3) arginase (4) arginine decarboxylase (5) arginyl tRNA synthetase (6) Δ^1-pyrroline-5-carboxylic acid dehydrogenase (P5CDH) (7) Δ^1-pyrroline-5-carboxylic acid reductase (P5CR) (8) ornithine decarboxylase (ODC). (Diagram based on Wu and Morris, 1998).

the larvae have been reared on mulberry leaves, but not on an artificial diet (Sumida *et al.*, 1995). The mulberry root or leaf urease binds to the microvilli of the epithelium of the midgut, is transported into the cells and appears in the haemolymph (Hirayama *et al.*, 2000; Sugimura *et al.*, 2001; Kurahashi *et al.*, 2005). Urease is also active in the gut lumen and appears to make a significant contribution to nitrogen incorporation into silk proteins (Hirayama *et al.*, 1999).

1.5 ARGININE METABOLISM

L-arginine is involved in numerous reactions (reviewed by Wu and Morris, 1998; Morris, 2002, 2004). Five of these reactions which are carried out in mammalian cells are summarised in Figure 1.5 (Wu and Morris, 1998). Arginine is also linked to pyrimidine synthesis through carbamoyl phosphate. In non-mammalian cells, there is another pathway which generates ornithine from arginine, the arginine deiminase pathway (reviewed by Zuniga *et al.*, 2002), which anaerobically generates ATP, ornithine, ammonia and CO_2 in microorganisms and protozoon parasites

Arginine catabolic enzymes are expressed in specific cell types and are highly regulated. Co-localisation of particular enzymes either in the cytosol or mitochondria allows the fate of arginine to be regulated e.g. the co-localisation in mitochondria of arginase II and ornithine aminotransferase (OAT) may preferentially direct arginine to glutamate and proline synthesis, whereas in the cytosol, arginase I and ornithine decarboxylase (ODC) may direct arginine to polyamine synthesis (Li *et al.*, 2001).

Arginine can be synthesised through the OUC in higher organisms, or in lower eukaryotes, plants and microorganisms, from glutamate via the intermediate N-acetylglutamate (NAG) in an eight-step pathway (reviewed by Caldovic and Tuchman (2003). NAG can be generated from glutamate either by ornithine acetyltransferase or by NAG synthase. The latter enzyme is used by ureotelic species to generate NAG, an essential cofactor of CPS I in the OUC. In mammals, additional arginine is synthesised by the small intestine-kidney to supplement uptake in the diet. Arginine is synthesised by arginino succinate synthase and lyase in the kidney using L-citrulline generated by the intestine from glutamine/glutamate (Morris, 2004; Boelens *et al.*, 2005).

1.5.1 SYNTHESIS OF ORNITHINE BY ARGINASE

Arginase, the enzyme which catalyses the hydrolysis of arginine to ornithine and urea (Figure 1.5), is present in bacteria, yeasts, plants, invertebrates and vertebrates (reviewed by Jenkinson *et al.*, 1996). Trematodes in which arginase activity has been studied include many free-living and parasitic species including *Fasciola hepatica* (Campbell and Lee, 1963), *S. mansoni* (Senft, 1966), *F. hepatica* (Kurelec, 1975a,b) and *F. gigantica* (Mohamed *et al.*, 2005). Campbell and Lee (1963) also detected arginase in eight parasitic cestodes, including *H. diminuta* and *Hymenolepis citelli*. Arginase is present in the nematodes *A. lumbricoides* (Rogers, 1952; Paltridge and Janssens, 1971), *A. galli* and *Nematodirus* spp. (Rogers, 1952) and there is low activity in *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1986a).

Because of the number of competing pathways using arginine as a substrate, arginase activity may be a significant regulatory point in arginine metabolism. This is the case for the generation of NO, an important cell signalling molecule and neurotransmitter and toxic molecule used against some pathogens (Section 1.5.3), which can be regulated by the consumption of arginine by arginase. Some parasites protect themselves from host defences by strategically using their arginase to compete with host NO synthase (NOS) for the common substrate arginine (Vincendeau *et al.*, 2003).

The two isoforms of arginase in mammals have been traditionally believed to have separate functions: the liver type arginase I as the final enzyme forming urea by the OUC (Figure 1.3) and the widely expressed extrahepatic isoform responsible for many of the functions shown in Figure 1.5, including the synthesis of ornithine, polyamines, proline, creatine, glutamate and agmantine (Wu and Morris, 1998). Yu *et al.* (2003) have shown that this tissue distribution is not so clear cut and that arginase I is present in the highest activity in the liver, but also in many other tissues, whereas arginase II is absent from many tissues which lack a complete urea cycle, such as lactating mammary gland, gastro-intestinal tract, kidney, brain, prostate gland and activated macrophages. The two isoforms are products of separate genes and have differences in their functional properties as well as being immunologically distinct and having different locations in the cell, arginase I in the cytosol and arginase II in the mitochondria (Cederbaum *et al.*, 2004).

1.5.2 Synthesis of ornithine by arginine deiminase

The arginine deiminase pathway is an alternative pathway to the arginase-catalysed reaction for generating ornithine from arginine. It is the most common anaerobic pathway in microorganisms by which arginine is catabolised, generating ATP, ornithine, ammonia and CO_2. This pathway is also present in the amitochondrial anaerobic parasites *Giardia intestinalis* (Scholfield *et al.*, 1990; Edwards *et al.*, 1992), *Trichomonas vaginalis* (Linstead and Cranshaw, 1983; Yarlett *et al.*, 1994) and *Tritrichomonas foetus* (Yarlett *et al.*, 1994), but has not been reported to be present in the cells of higher animals (Zuniga *et al.*, 2002). Arginine catabolism is used as a major source of ATP by *G. intestinalis* using this pathway (Scholfield *et al.*, 1990; Edwards *et al.*, 1992).

The pathway involves three steps, using (1) arginine deiminase to convert arginine to citrulline and ammonia; (2) ornithine transcarbamoylase (OTC) to convert citrulline to ornithine and generate carbamoyl phosphate; (3) carbamate kinase to convert carbamoyl phosphate to ammonia and CO_2, and ADP to ATP (Zuniga *et al.*, 2002).

1.5.3 Synthesis of nitric oxide

Nitric oxide (NO) is generated by nitric oxide synthase (NOS) during the catabolism of arginine to citrulline (reviewed by Wu and Morris, 1998). There are three isoforms of mammalian NOS, which are either produced constitutively (cNOS) or are inducible by proinflammatory cytokines or bacterial components (iNOS). NO has in recent years been recognised both as a neurotransmitter and as a signalling molecule through the production of the second messenger cGMP, particularly in the cardiovascular system (reviewed by Boucher *et al.*, 1999; Cary *et al.*, 2006). NO is used as a signalling molecule by many nematodes, like most organisms, including *S. mansoni*, *S. japonicum* (Long *et al.*, 2004), *Dirofilaria immitis, Brugia pahangi* (Kaiser *et al.*, 1998) and *A. suum* (Bascal *et al.*, 2001).

Lower concentrations of NO are anti-inflammatory but overproduction of NO is believed to contribute to the pathology of a number of diseases. NO is toxic to parasites (Taylor-Robinson and Looker, 1998; Colasanti and Venturini, 2001), bacteria and tumour cells and is a host defence to bacterial pathogens

of vertebrates (Vincendeau *et al.*, 2003; Nioche *et al.*, 2004) and invertebrates (Rivero, 2006). The competition between arginase and NOS for their substrate arginine provides a possible means of controlling NO levels (Boucher *et al.*, 1999). As a strategy to limit NO production, some parasites deplete host arginine (Vincendeau *et al.*, 2003) or suppress its expression. An example is the rapid down-regulation of inducible NOS in the jejunum in response to a *Trichinella spiralis* infection, when the parasite arginase begins to compete for arginine (Bian *et al.*, 2001).

1.5.4 Synthesis of agmatine

Agmatine is formed by the decarboxylation of arginine by the enzyme ADC (Figure 1.4). Although known for many years to be an enzyme in bacteria, plants and invertebrates, only recently has ADC been recognised as a mammalian enzyme (reviewed by Zhu *et al.*, 2004) and the human gene cloned (Mistry *et al.*, 2002). Agmatine is a neurotransmitter and a regulator of cell proliferation and inflammation.

Agmatine can be used as an alternative precursor for the polyamines to the usual arginase-ODC route (Section 1.5.5). Agmatine is generated by ADC then hydrolysed by agmatinase to form putrescine and urea (Slocum *et al.*, 1984; Tabor and Tabor, 1985. Mammalian ADC and ODC can use either ornithine or arginine as substrate, leading to the proposal that they may be isoforms of the same enzyme (Gilad *et al.*, 1996), although Regunathan and Reis (2000) have characterised two mammalian enzymes with distinct properties. The *C. elegans* and *H. contortus* ODC/ADC were described as "atypical" in being membrane-bound (like the mammalian enzymes), may be both arginine and ornithine decarboxylases and have extremely low K_m for ornithine (Schaeffer and Donatelli, 1990). Macrae *et al.* (1995) reported that ADC activity was not detectable in their study of polyamines in *C. elegans*, as was also the case for homogenates of adult *Nippostrongylus brasiliensis* (Walker and Barrett, 1991a).

1.5.5 Synthesis of polyamines

The polyamines putrescine, spermidine and spermine are regulators of cell growth and differentiation in microorganisms, plants and eukaryotes (reviewed by Slocum *et al.*, 1984; Tabor and Tabor, 1985; Pegg, 1986). In

animals, arginine is converted to urea and ornithine by arginase; putrescine is then generated from ornithine by ODC (Pegg, 1986). In microorganisms and plants, in addition to the ODC pathway, putrescine (and urea) can be formed from agmatine, which has been generated from arginine by ADC (Slocum *et al.*, 1984; Tabor and Tabor, 1985). Putrescine is further metabolised to spermidine by the enzyme spermidine synthase and thence to spermine by spermine synthase. Other polyamines are also produced in some microorganisms (Tabor and Tabor, 1985). The proportions of the three main polyamines vary in different organisms: prokaryotes generally have more putrescine than spermidine and have no spermine; eukaryotes have all three, with putrescine the least, while protozoa appear to be intermediate between these two groups (White *et al.*, 1983; Tabor and Tabor, 1985; Pegg, 1986).

Polyamines have attracted the attention of parasitologists as potential targets for novel control methods of protozoa and nematodes (Henderson and Fairlamb, 1987; Zhang, 1987; Walter, 1988; Yarlett, 1988; Klein *et al.*, 1997; Da'dara *et al.*, 1998). Trypanosomes are particularly sensitive to the ODC inhibitor difluoromethylornithine (DFMO), which has been used successfully as a therapeutic agent (Van Nieuwenhove *et al.*, 1985; Bacchi *et al.*, 1994). ODC inhibition is not effective in nematodes, since polyamines are not generally synthesised from arginine, but are reliant on uptake and interconversion (Walter, 1988). In four filarial nematodes (*D. immitis, Onchocerca volvulus, Brugia patei and Litomosoides carinii*) ODC and ADC activities were undetectable, putrescine levels were low, spermidine predominated and spermine levels were high (Wittich *et al.*, 1987). Similar observations were made in the dog hookworm *Ancylostoma ceylanicum* and rat parasite *N. brasiliensis*, although the ratio of spermidine: spermine was about 4:1 (Sharma *et al.*, 1989). ODC activity was undetectable in *N. brasiliensis* (Walker and Barrett, 1991a). Consistent with these polyamine patterns, either transporters, the rate limiting enzyme *S*-adenosylmethionine decarboxylase (*S*AMdc) (which provides the amino-propyl group for the synthesis of spermidine and spermine), or the enzymes involved in polyamine catabolism (the novel diamine acetylase and polyamine oxidase) may be more useful anti-parasitic targets in nematodes (Walter, 1988; Wittich and Walter, 1989, 1990; Müller and Walter, 1992; Da'dara *et al.*, 1998).

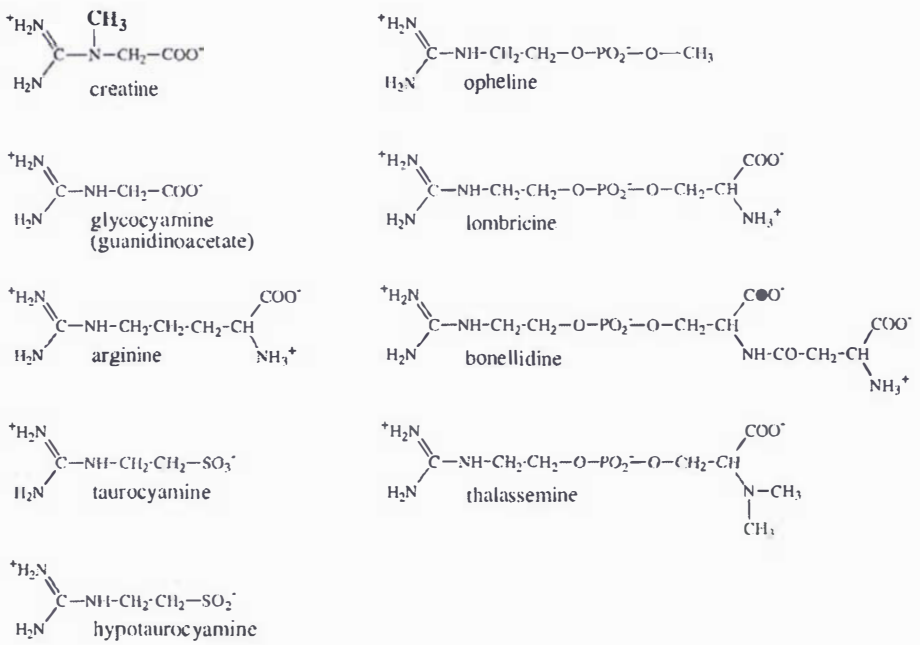


Figure 1.6. Invertebrate phosphagen precursors which form the corresponding phosphagen by covalent attachment of a phosphate group to the guanidino moiety at the left of the molecule. (Diagram from Wyss and Kaddurah-Daouk, 2000).

Schaeffer and Donatelli (1990) characterised *C. elegans* and *H. contortus* ODCs as membrane–bound and having different kinetic properties from ODCs from other organisms, whereas Niemann *et al.* (1996) described the properties of the *P. redivivus* enzyme as more closely resembling other eukaryote ODCs. The ODC gene has been cloned from *P. redivivus* (von Besser *et al.*, 1995; Niemann *et al.*, 1996), *C. elegans* (Macrae *et al.*, 1995), and (Klein *et al.*, 1997) and shown in the latter two species to be dispensible for growth. Another nematode gene to be cloned is spermidine synthase from *C. elegans* (Dufe *et al.*, 2005).

1.5.6 SYNTHESIS OF CREATINE

Arginine is a precursor of creatine and phosphocreatine. In vertebrates, phosphocreatine is the only member of the phosphagen family, phosphorylated guanidine compounds which act as stores of energy from which phosphoryl groups can be transferred to ATP by phosphagen kinases. The synthesis of creatine involves the kidney and liver, followed by transport of creatine into the muscles, where it is a major source of energy for muscle contraction. Creatine synthesis requires two reactions: first, a transamination catalysed by arginine-glycine amidinotransferase (AGAT), which generates glycocyamine and ornithine from arginine and glycine; secondly, the reaction of glyocyamine with S-adenosyl-L-methionine to form creatine and S-adenosyl-L-homocysteine, catalysed by the enzyme S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT) (reviewed by Ennor and Morrison, 1958; Wyss and Kaddurah-Daouk, 2000).

Most invertebrate tissues do not contain phosphocreatine and creatine kinase (CK), so related phosphagens and their kinases fulfil the same role (Ennor and Morrison, 1958; Van Pilsum *et al.*, 1972). Phosphoarginine and arginine kinase (AK), the most common invertebrate guanidino phosphagen and phosphagen kinase respectively, are present in arthropods, echinoderms molluscs and nematodes (Ennor and Morrison, 1958; Wallimann and Eppenberger, 1973; Dumas and Camonis, 1993; Suzuki *et al.*, 1997; Platzer *et al.*, 1995, 1999; Kotlyar *et al.*, 2000). AK has also be identified in the protozoan parasite *T. cruzi* (Pereira *et al.*, 2000). Glycocyamine is one of at least seven other guanidino phosphagens reported from invertebrates (shown in Figure 1.6, Wyss and Kaddurah-Daouk, 2000). Stein *et al.* (1990) cloned a *S. mansoni* gene encoding an ATP:guanidine kinase which was expressed

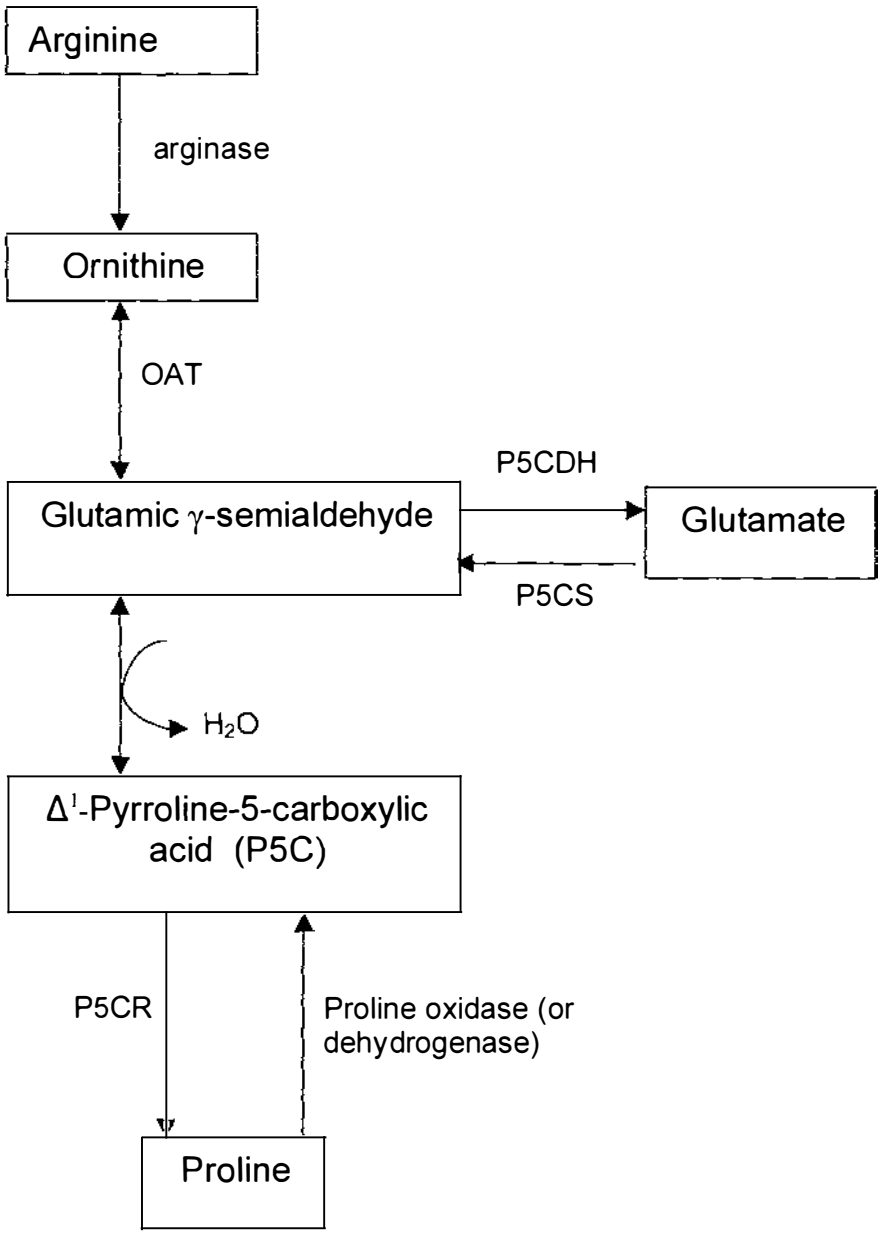


Figure 1.7. Enzymes involved in the interconversion of arginine, glutamate and proline. Abbreviations: OAT: ornithine aminotransferase; P5CR: pyrroline-5-carboxylate reductase; P5CDH: pyrroline-5-carboxylate dehydrogenase; P5CS: pyrroline-5-carboxylate synthase. Glutamic γ-semialdehyde spontaneously hydrates to form P5C.

maximally in the infective cercarial stage of the life cycle. As they detected only weak CK activity and no AK activity in *S. mansoni*, they designated it a CK, however, as the gene has greatest homology to AKs (Dumas and Camonis, 1993), the enzyme may well be a member of that family, not a CK. AK and phosphoarginine have been identified in the entomopathogenic nematode *Steinernema carpocapsae* (Platzer *et al.*, 1999) and in *H. contortus* L3 (Platzer *et al.*, 1995). Barrett (1973) could not detect phosphagens in *A. lumbricoides* muscle, but did find a very active non-specific adenylate kinase. Barrett and Lloyd (1981) were unable to detect phosphagens in *F. hepatica*, *H. diminuta* or *Moniezia expansa*, but found considerable taurocyamine phosphotransferase activity, but not its phosphagen, in the cestode *Schistocephalus solidus*.

Invertebrate AKs have been shown to be important causes of allergy in humans. The shrimp enzyme has been identified as a seafood allergen (Yu *et al.*, 2003) and IgE from allergic patients showed that the AK from the Indian meal moth, a widespread food pest in cereals and dry foods, cross reacted with AKs from a number of invertebrates (dust mites, mussels, lobsters, king prawns and cockroaches) (Binder *et al.*, 2001). The *S. mansoni* phosphagen kinase described in the cercarial stage of the life cycle (Stein *et al.*, 1990; Shoemaker, 1994) may be similarly antigenic and useful in a protective vaccine in humans.

1.5.7 SYNTHESIS OF GLUTAMATE AND PROLINE

Arginine and ornithine are precursors of glutamate and proline via the intermediate Δ^1-pyrroline-5-carboxylic acid (P5C) (Figure 1.5). Interconversion of ornithine, proline and glutamate proceeds in most organisms through the reactions shown in Figure 1.7, but there is an alternative pathway from ornithine to proline via α-keto-δ-aminovaleric acid (not via Δ^1-P5C) in plants (Mestichelli *et al.*, 1979). In mammals, P5C released into plasma is taken up by cells such as erythrocytes, fibroblasts and glial cells, transferring oxidising potential (via subsequent NADPH oxidation during conversion to proline) and regulates the pentose phosphate shunt (Mixson and Phang, 1988).

P5C is generally synthesised from ornithine by the enzymes OAT and pyrroline-5-carboxylate reductase (P5CR) (Figure 1.7). P5C is synthesised in higher eukaryotes from glutamate by a single multifunctional enzyme pyrroline-

5-carboxylate synthase (P5CS) and by two enzymes in lower organisms (a γ-glutamyl kinase and γ-glutamyl phosphate reductase). Proline is oxidised to P5C and glutamate by different enzymes from those in the synthetic pathway (Figure 1.7): the first step is proline oxidation by the enzyme proline dehydrogenase (also called proline oxidase or proline:O_2 oxidoreductase) and the second step uses the enzyme pyrroline-5-carboxylate dehydrogenase (P5CDH).

Proline is an important amino acid in many organisms as a source of energy and as an osmoprotectant. It is used as an energy source in parasitic trypanosomes (Obungu *et al.*, 1999) and for flight by insects (Auerswald *et al.*, 1998; Auerswald and Gäde, 1999; Scaraffia and Wells, 2003). Marine crustaceans accumulate proline during osmotic stress (Burton, 1992; Bishop and Burton, 1993; Willett and Burton, 2002) using P5CS and P5CR, the genes for which have been cloned in a copepod by Willett and Burton (2002). Bacteria also use proline as an osmoprotectant, usually obtaining it from the medium through a proline porter (Milner *et al.*, 1988; Lee *et al.*, 2003). Proline accumulates in plants as a reaction to (and protectant against) stresses as diverse as pathogens, osmotic stress (salinity or drought), nutrient deprivation, UV light, or temperature (Song *et al.*, 2005; Yamada *et al.*, 2005).

The nematode cuticle consists mainly of collagen-like molecules, which require proline as a precursor. This can either be obtained by synthesis or by uptake, perhaps after digestion of host mucins. Highly conserved cuticular collagen genes have been cloned from nematodes, including *O. circumcincta* (Johnstone *et al.*, 1996), *H. contortus* (Shamansky *et al.*, 1989), *T. spiralis* (Fu *et al.*, 2005) and *C. elegans* (Cox *et al.*, 1981). Parasite proline is responsible for collagen deposition in bile ducts infected with *F. hepatica* (Wolf-Spengler and Isseroff, 1983; Modavi and Isseroff, 1984) and in the liver during *S. mansoni* infection (Dunn *et al.*, 1977, 1978). Not surprisingly, the enzymes OAT and P5CR of the proline synthetic pathway from ornithine (Figure 1.7) are very active in *F. hepatica* and *S. mansoni* (Ertel and Isseroff, 1974; Isseroff and Ertel, 1976; Goldberg *et al.*, 1980; Isseroff *et al.*, 1983). The OAT activities of *F. hepatica* adults and *S. mansoni* ova were over seven times, and in *S. mansoni* adults about three times that in rat liver. P5CR was over 35 times more active in ova and 17 times more active in adult *S. mansoni* and 5 times in *F. hepatica* homogenates than in rat liver. Despite the high proline

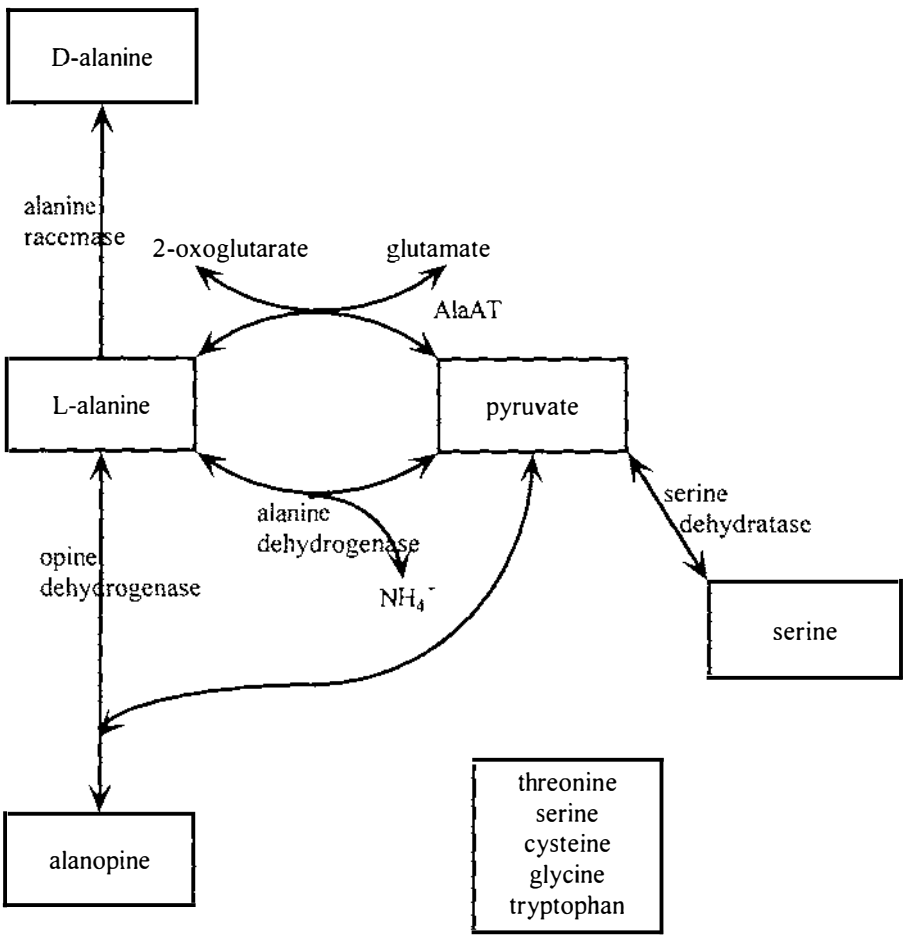


Figure 1.8. The major reactions and pathways for which alanine is a substrate.

production and excretion by these parasites, inhibition of OAT and P5CR *in vitro* did not cause *F. hepatica* deterioration, nor did proline analogues and enzyme inhibitors prevent parasite establishment in rats (Sheers *et al.*, 1982).

Use of proline requires the enzymes proline oxidase (dehydrogenase) and P5CDH. There is considerable interest in P5CDH in human medicine as an inherited mutation leads to hyperprolinaemia and neurological symptoms (Geraghty *et al.*, 1998). Both proline catalytic enzymes were present in *H. polygyrus* and *P. redivivus*, as were P5CR and OAT (Grantham and Barrett, 1986b). OAT activity is present in *S. mansoni* (Goldberg *et al.*, 1979) and *N. brasiliensis* (Walker and Barrett, 1991a). Neither Kuralec (1975b) nor Isseroff and Ertel (1976) detected P5CDH activity in *F. hepatica* homogenates, perhaps because of the dominant proline synthetic pathway which results in excretion of large amounts of proline into host tissues.

1.6 ALANINE AND ASPARTASE METABOLISM: TRANSAMINATION

Alanine and aspartate are central amino acids linking carbohydrate and nitrogen metabolism and are important for the interconversion of amino acids needed to maintain the pool of amino acids required for protein synthesis. In mammals, alanine and glutamine are the principal compounds transporting nitrogen between the gut, muscle and the liver and alanine is the most important amino acid contributing to gluconeogenesis (Brosnan *et al.*, 2001; Brosnan, 2003). The aminotransferases alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) are responsible for reversible transamination of alanine and aspartate to their respective keto acids pyruvate and oxaloacetate (OAA). Pyruvate is derived from sugars through glycolysis, from lactate and from amino acids by transamination in mammals (Garber *et al.*, 1976), fish (Walton and Cowey, 1982), invertebrates (Osanai *et al.*, 2000) and plants (de Sousa and Sodek, 2003). Alanine is a metabolic product of cultured insect cells, (Drews *et al.*, 2000) and the protozooan parasites *Giardia lamblia* and *G. intestinalis* (Edwards *et al.*, 1989; Paget *et al.*, 1990; Nygaard *et al.*, 1994) and is the main amino acid that accumulates under anaerobic conditions in plants (Streeter and Thompson, 1972; Ricard *et al.*, 1994; de Sousa and Sodek, 2003). Figure 1.8 summarises the major enzymatic reactions involving alanine.

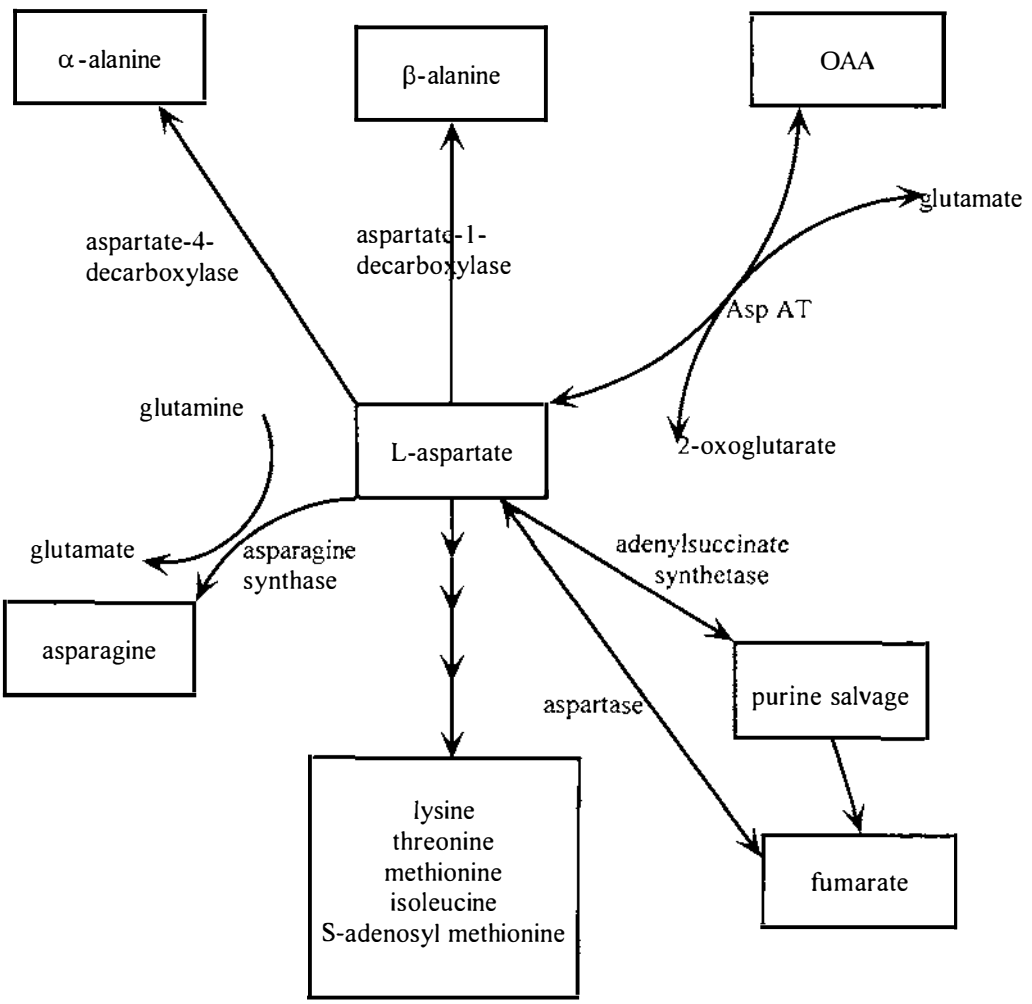


Figure 1.9. The major reactions and pathways for which aspartate is a substrate.

Aspartate contributes carbon skeletons to the TCA cycle by transamination to OAA and the malate/aspartate shuttle transports into the mitochondria the reducing equivalents required for respiration. Depending on the organism, aspartate is the precursor of asparagine, β-alanine and the "essential" amino acids threonine, methionine and lysine. Figure 1.9 summarises the major enzymatic reactions involving aspartate, some of which occur only in microorganisms and plants, while others, such as AspAT, are universally present.

1.6.1 ALANINE RACEMASE

Alanine racemase, which converts L-alanine to D-alanine, is important for bacterial survival, as D-alanine is an essential component of the cell wall (Badet and Walsh, 1985; Shaw *et al.*, 1997). Although Shaw *et al.* (1997) described alanine racemase as being unique to bacteria, with the one exception being a fungal enzyme (Hoffmann *et al.*, 1994), there are several reports of its presence in marine invertebrates, including crayfish (Shibata *et al.*, 2000), prawns (Yoshikawa *et al.*, 2002), a sipunculid (Low *et al.*, 1996) and molluscs (Matsushima and Hayashi, 1992; Yamada and Matsushima, 1992; Nomura *et al.*, 2001) and in the protozoon parasite *Leishmania amazonensis* (Panizzutti *et al.*, 2006). Initially, D-amino acids were not considered to be biologically important, however, this is now known not to be the case, particularly in invertebrates (Corrigan, 1969), in which they appear to be osmolytes (Fujimori and Abe, 2002). It is not clear whether alanine racemase is present in nematodes, as the only report appears to be that it was undetectable in adult *N. brasiliensis* (Walker and Barrett, 1991a).

1.6.2 L-ALANINE DEHYDROGENASE

Amino acid dehydrogenases catalyse the $NAD(P)^+$ linked oxidative deamination of L-amino acids to their corresponding keto acids. Alanine dehydrogenase [L-alanine:NAD^+ oxidoreductase] is a microbial enzyme which catalyses the conversion of alanine to pyruvate. In *Bacillus subtilis*, the main function is believed to be the generation of pyruvate to provide energy during sporulation (Siranosian *et al.*, 1993), to allow alanine as a sole nitrogen source in *Mycobacterium smegmatis* (Feng *et al.*, 2002b) and in *Bilophila wadsworthia* it is involved in taurine metabolism (Laue and Cook, 2000). In other bacteria, the predominant direction deduced from V_{max} and K_m values is the reverse

direction to assimilate nitrogen (Caballero *et al.*, 1989). In *Rhizobia*, which are symbionts of legumes, alanine dehydrogenase is the principal enzyme involved in providing nitrogen for the plant host and blockage of the rhizobial enzyme reduces plant growth. Ammonia is the usual product released to the plant, however, alanine is also secreted in soybean and pea nodules (Allaway *et al.*, 2000; Lodwig *et al.*, 2004).

1.6.3 OPINE DEHYDROGENASES

Many marine invertebrates, including molluscs (Fields *et al.*, 1980; Livingstone *et al.*, 1981), sponges (Barrett and Butterworth, 1981; Kan-no *et al.*, 2005) and sea anemones (Ellington, 1979) have unique metabolic responses to anoxia. Rather than accumulating lactate, in addition to succinate and alanine production, reversible reductive condensation of pyruvate with an amino acid generates opines: octopine, tauropine, strombine, alanopine (reviewed by de Zwaan and Wijsman, 1976; de Zwaan and Putzer, 1985; Gäde and Grieshaber, 1986). The opines octopine, strombine and alanopine are products of opine dehydrogenases, which catalyse the reaction in the presence of NADH of pyruvate with arginine, glycine or alanine respectively. Two further enzymes have been reported: tauropine dehydrogenase and β-alanopine dehydrogenase (Kan-no *et al.*, 2005). Preference for octopine formation correlates with high levels in muscle of phosphoarginine (de Zwaan and Wijsman, 1976). Plant tumours also make opines using enzymes encoded by genes which are inserted into the genome of plant cells by invading bacteria. These bacteria rely on the opines as growth substrates (reviewed by Dessaux *et al.*, 1993).

1.6.4 PYRUVATE SYNTHESIS

In addition to alanine, serine, glycine, threonine, cysteine and tryptophan may enter the TCA cycle as pyruvate and used for respiration. Serine is deaminated to pyruvate by the PLP-dependent enzyme L-serine dehydratase. In mammals, its activity is highest in the liver (Kashii *et al.*, 2005; Sun *et al.*, 2005) and there is an active enzyme in *N. brasiliensis* (Walker and Barrett, 1991a), *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1986a). L-serine hydroxymethyltransferase, in the pathway for generation of N^5,N^{10}-methanyltetrahydrofolate from dihydrofolate, is present in *N. brasiliensis* (Walker and Barrett, 1991a), *B. pahangi* and *D. immitis* (Barrett, 1983).

Threonine is a substrate for L-serine dehydratase in some organisms, as one of the many groups of enzymes which catabolise threonine: (1) by threonine dehydrogenase to generate 2-amino-3-oxobutyrate; (2) by threonine aldolase to form glycine and acetaldehyde or aminoacetone; (3) by threonine dehydratase (or L-serine dehydratase) to form 2-oxobutanoate (α-ketobutyrate) and ammonia (Bird and Nunn, 1983; Bird *et al.*, 1984). There is an active threonine dehydratase in *N. brasiliensis* (Walker and Barrett, 1991a), *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1986a).

1.6.5 ALANINE SYNTHESIS BY ASPARTATE-4-CARBOXYLASE

Aspartate can be irreversibly β-decarboxylated to α-alanine by aspartate-4-decarboxylase, an enzyme not dependent on PLP as a cofactor. It is present in microorganisms (Meister *et al.*, 1951; Seaman, 1960; Kakimoto *et al.*, 1969), chicken liver (Rathod and Fellman, 1985a) and mammalian liver (Rathod and Fellman, 1985a,b), kidney and brain (Wong, 1985). Rathod and Fellman (1985b) have proposed that aspartate-4-decarboxylase, together with pyruvate carboxylase, regulates the fate of OAA and the efficiency of carbohydrate metabolism. Since aspartate-4-decarboxylase is inhibited by acetyl-CoA and stimulated by 2-oxoglutarate, they propose that an abundance of TCA intermediates and decreased acetyl-CoA levels as fatty acids are synthesised promotes aspartate channelling into alanine. In the opposite state of lipid usage, acetyl-CoA levels rise, pyruvate carboxylase is stimulated and OAA is generated for energy production. There may be similar effects on phosphoenolpyruvate carboxylase as on pyruvate carboxylase.

1.6.6 β-ALANINE SYNTHESIS

There are several pathways for the synthesis of β-alanine, the only naturally occurring β-amino acid, which is a component of muscle dipeptides (Boldyrev and Severin, 1990), insect cuticles (Hodgetts, 1972; Rawls, 2006) and pantothenic acid, the precursor of coenzyme A (Maas, 1952; Genschel, 2004). In microorganisms, β-alanine is synthesised from aspartate by L-aspartate-1-decarboxylase (Williamson and Brown, 1979). This enzyme (also called panD) is considered a possible target to control tuberculosis (Chopra *et al.*, 2002). Another method of producing β-alanine by the degradation of uracil is used by animals (Fink *et al.*, 1953; Fritzson and Pihl, 1957), plants (Evans and Axelrod, 1961) and some microorganisms (Campbell, 1957). The final

step in that pathway is catalysed by the enzyme β-alanine synthase (Gojkovic *et al.*, 2001). The yeast *Saccharomyces cerevisiae* has an unusual pathway for β-alanine synthesis from spermine, which may be more widely used by fungi and plants (White *et al.*, 2001).

1.6.7 ASPARAGINE SYNTHESIS AND CATABOLISM

Asparagine is synthesised from aspartate by a reaction which uses ATP and is catalysed by an amidotransferase, glutamine-dependent asparagine synthetase (AS) (Richards and Schuster, 1998). The human AS gene is activated when cells are deprived of glucose or amino acids (Barbarosa-Tessmann *et al.*, 1999). As well as AS activity in mammals (Cuistea *et al.*, 2005), it is present in plants (Sieciechowicz *et al.*, 1988), bacteria (Schwartz *et al.*, 1966) and in *P. redivivus* (Grantham and Barrett, 1988). Asparagine is one of the amino acids excreted in low concentrations by *H. diminuta in vitro* (Zavras and Roberts, 1984). Asparagine is an important nitrogen transporter within plants (reviewed by Sieciechowicz *et al.*, 1988).

A chemotherapeutic treatment for acute lymphoblastic leukaemia in humans is based on depleting tumour asparagine, which is needed for protein synthesis and cell survival. If AS activity is inadequate, asparagine deprivation leads to glutamine and glutamate depletion, followed by cell cycle arrest and apoptosis (Bussolati *et al.*, 1995). Bacterial asparaginase is used to reduce the supply of asparagine to the tumour (Schwartz *et al.*, 1966; Pieters *et al.*, 1997), however, patients may be refractory to asparaginase if their AS activity is elevated (Pieters *et al.*, 1997).

Asparagine can be catabolised by asparaginase or by transamination: asparaginase breaks down asparagine to aspartate and ammonium, whereas the transamination requires a keto acid as the amino group acceptor and produces 2-oxosuccinamate. Asparagine transaminase from rat liver has broad substrate specificity for amino acids and keto acids, although the most active amino acids were asparagine, L-phenylalanine and S-methyl-L-cysteine (Cooper, 1977). Asparagine transaminase activity is present in the tapeworms *H. diminuta* and *H. citelli* (Wertheim *et al.*, 1960) and the nematodes *P. redivivus* and *H. polygyrus* (Grantham and Barrett, 1986a). Asparaginase activity was localised to the cuticle of the nematode *D. immitis* using immune sera (Tsuji *et al.*, 1999).

1.6.8 ASPARTATE KINASE

Aspartate can be used by plants and microorganisms to synthesise *S*-adenosyl-methionine as well as lysine, threonine, methionine and isoleucine (reviewed by Azevedo *et al.*, 1997), which are considered "essential" in animals. There are 16 enzymes in the complete pathway, the first step of which is catalysed by aspartate kinase. Inhibitors of enzymes in the pathway have been developed to control bacteria, fungi and weeds (LaRossa and Scloss, 1984; Whitcombe, 1999), as this pathway is not present in mammals. Deregulation of the pathway may lead to overproduction of essential amino acids in plants destined for animal and human food (Paris *et al.*, 2002).

There do not appear to be reports of any of these enzymes in helminths, however, Rothstein and Tomlinson (1961) reported that *C. briggsae* was capable of synthesising several essential amino acids, including threonine and lysine from labelled acetate, glucose and glycine. They believed that neither bacterial contamination nor symbionts were responsible, which suggests that the aspartate pathway may be present in nematodes. Kapur and Sood (1984) described similar biosynthetic processes in *H. contortus*.

1.6.9. PURINE SALVAGE PATHWAY

Purines needed for DNA or RNA synthesis can be supplied either by purine salvage or *de novo* synthesis. Many helminths are unable to synthesise purine, whereas salvage pathways are present (reviewed by el Kouni, 2003). In vertebrates, the purine nucleotide cycle regenerates AMP from IMP by reactions which require GTP and are catalysed by the enzymes adenylsuccinate synthetase and adenylsuccinate lyase. Aspartate is converted to fumarate with the release of ammonia. The cycle is completed by the reduction of AMP to IMP by AMP deiminase (Magasanik and Karibian, 1960; Borza *et al.*, 2003). During exercise, rat skeletal muscle converts aspartate to fumarate with the release of ammonia as a result of this pathway. This is associated with reduced output of alanine and glutamine (Tornheim and Lowenstein, 1972; Goodman and Lowenstein, 1977).

Many helminths have either low *de novo* synthesis (Wong and Yeung, 1981) or only take up exogenous adenosine and use the purine salvage pathway (MacInnis *et al.*, 1965; Heath, 1970; Senft *et al.*, 1972; Levy and

Read, 1975; Dovey *et al.*, 1984). Adenosine deaminase, but not adenine deaminase, was present in *H. diminuta* (Gamble and Pappas, 1981). There are also pathways for interconversion of nucleotides. In *A. lumbricoides* muscle, there was no measurable AMP deaminase activity, but there were very active adenylate kinase to rephosphorylate ADP to ATP and nucleosidediphosphate kinase to transfer phosphate between nucleotides (Barrett, 1973).

1.6.10 ASPARTASE

Aspartase [L-aspartate ammonia-lyase] catalyzes the reversible deamination of L-aspartate to produce fumarate and ammonium. Viola (2000) has comprehensively reviewed the structure and unusual kinetic properties of aspartase, notably the differences in kinetics and co-factor requirements at alkaline pH. Most interest in this enzyme has been in microorganisms because of its commercial application in the production of aspartic acid and the sweetener aspartame. Aspartase is also believed to be involved in the bacterial nitrogen mineralisation of soils (Senwo and Tabatabai, 1999). Although usually not considered an enzyme of higher organisms, aspartase has been reported from plants and lower vertebrates: frog embryos (Kurata, 1962), teleost fish (Salvatore *et al.*, 1965) and elasmobranchs (Salvatore *et al.*, 1965; Cutinelli *et al.*, 1972).

Whether the role of aspartase in nitrogen metabolism is catabolic or anabolic is uncertain. Halpern and Umbarger (1960) suggested that the primary function of aspartase is to degrade amino acids, releasing ammonia from L-aspartate to produce fumarate, which then enters the TCA cycle. On the other hand, there was evidence of an anabolic function in *Escherichia coli* mutants lacking glutamate dehydrogenase, which were still capable of growth with glutamate as a carbon source; this was accompanied by elevated aspartase activity (Vender and Rickenberg, 1964).

1.6.11 TRANSAMINATION

Aminotransferases (formerly called transaminases) are amongst the large group of enzymes which are dependent on pyridoxal 5'-phosphate (PLP) as a cofactor. The structure, gene sequences and functions of PLP-dependent enzymes have been reviewed by Mehta *et al.* (1989), Alexander *et al.* (1994),

John (1995), Jensen and Gu (1996), Jansonius (1998) and Toney (2005). Aminotransferases catalyse the transfer of an amino group from a donor α-amino acid to acceptor α-keto acid, which is usually 2-oxoglutarate, thus converting one amino acid into another. During the catalytic cycle, the cofactor shuttles between the PLP and the pyridoxamine 5'-phosphate (PMP) forms. There are many aminotransferases, often accepting multiple substrates, but the most active and universally present are AlaAT and AspAT.

1.6.11.1 Alanine aminotransferase

AlaAT catalyzes the reversible transamination between 2-oxoglutarate and L-alanine, to form glutamate and pyruvate respectively. Through its forward reaction (formation of pyruvate), it is involved in gluconeogenesis, and ATP production via the Krebs cycle, while the reverse reaction (formation of alanine) is involved in the synthesis of protein. Cytoplasmic and mitochondrial AlaAT are distinct molecular and genetic forms (Astrin *et al.* 1982; Ruščák *et al.*, 1982; Kielty *et al.*, 1982; Sohocki *et al.*, 1997; Yang *et al.*, 2002). It has been proposed that the kinetics of the two enzymes are consistent with the mitochondrial enzyme being involved in the conversion of alanine to pyruvate, while the reverse reaction is preferred by the cytosolic enzyme (DeRosa and Swick, 1975).

In mammals, the highest AlaAT activity is in the liver, muscle, heart, kidney, brain and adipose tissue (Kamoda *et al.*, 1980; Ruščák *et al.*, 1982; Rajamohan *et al.*, 2006). When the liver or other tissues of vertebrates are damaged, a large amount of aminotransferase is released into the circulation so that elevated AlaAT activity is considered one of the most sensitive indicators of hepatobiliary cellular damage from helminth parasites (Vengušt *et al.*, 2003), nutritional stress (Pan *et al.* 2003), toxins (Tiwari and Singh, 2003) or pathogens (Agrawal *et al.*, 1996).

AlaAT activity has been demonstrated in numerous helminths: the cestodes *H. diminuta* and *H. citelli*, but not in *Hymenolepis nana* (Wertheim *et al.*, 1960), in *M. expansa* (Rasero *et al.*, 1968); the Digeneans *Cryptocotyle lingua* and *Cercaria emasculans* (Watts, 1970) and the nematodes *A. lumbricoides* (Rasero *et al.*, 1968), *Cooperia oncophera*, *H. contortus*, *N. brasiliensis*, *O. circumcincta*, *Trichostrongylus colubriformis*, *P. redivivus*, *Steinernema bibionis* (Walker and Barrett, 1991b) and *H. polygyrus* (Grantham

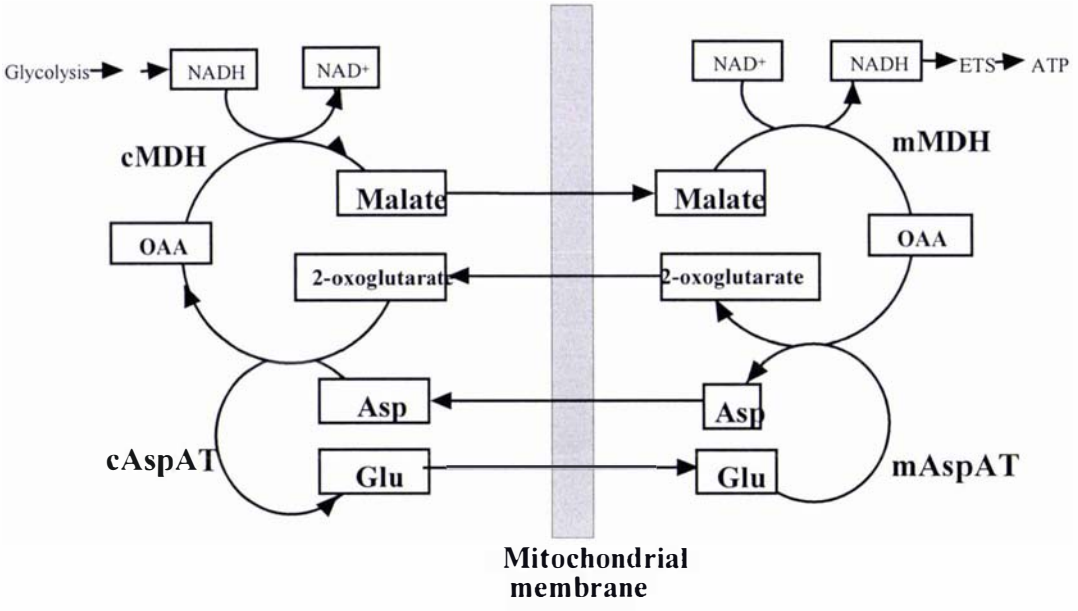


Figure 1.10. Diagram of the malate-aspartate shuttle for the transport of reducing equivalents between the cytosol and mitochondria in the electron transport system of the mammalian cell. Abrreviations: ETS electron transport system; cMDH cytosolic malate dehydrogenase; mMDH mitochondrial malate dehydrogenase; cAspAT cytosolic aspartate aminotransferase; mAspAT mitochondrial aspartate aminotransferase; OAA: oxaloacetate. (From Setoyama *et al.*, 1990).

and Barrett, 1986a). Comparison of enzyme activities in the forward (L-alanine catabolism) and reverse directions showed greater activity for alanine formation (Walker and Barrett, 1991b). D-alanine was transaminated at extremely low rates only by the abomasal parasites *H. contortus* and *O. circumcincta*. *H. contortus* and *N. brasiliensis* mitochondrial (mAlaAT) and cytosolic (cAlaAT) enzymes were studied in more detail by Walker and Barrett (1991b). In *N. brasiliensis*, cAlaAT was responsible for 80% of activity but for only 54% in *H. contortus*, with 22% associated with the cell debris and cuticle fractions. *H. contortus* cAlaAT differed from the rat liver enzyme in being less tolerant of temperatures of 45°C and having different responses to protective agents.

1.6.11.2 Aspartate aminotransferase

Aspartate aminotransferase (AspAT), previously also called glutamate oxaloacetate transaminase, catalyses the reversible transamination of L-aspartate and 2-oxoglutarate to form oxaloacetate and glutamate. The cytosolic and mitochondrial isoforms have different substrate specificities and are coded by distinct nuclear genes (Martinez-Carrion *et al.*, 1967; Michuda and Martinez-Carrion, 1969; Reed and Hess, 1975; Panteghini, 1990; Morin *et al.*, 1992; Mattingly *et al.*, 1995; Verleur *et al.*, 1997). Each of the two main isoforms also exists in several forms (Bertland and Kaplan, 1968, 1970; Rej, 1981).

AspAT is present in most organisms; mammals (Van Leuven, 1976; Teranishi *et al.*, 1978), birds (Shrawder and Martinez-Carrion, 1973), fish (Srivastava *et al.*, 1999), invertebrates (Mordue and Goldsworthy, 1973; Felbeck and Grieshaber, 1980), plants (Reed and Hess, 1975; Turano *et al.*, 1990), protozoa (Cazzulo *et al.*, 1977) and microorganisms (Alfano and Kahn, 1993). AspAT has been reported from helminths: the cestodes *H. diminuta*, *H. nana* and *H. citelli* (Wertheim *et al.*, 1960), and *M. expansa* (Rasero *et al.*, 1968), the Digeneans *C. lingua* and *C. emasculans* (Watts, 1970) and the nematodes *A. lumbricoides* (Rasero *et al.*, 1968) and *N. brasiliensis* (Walker and Barrett, 1991a).

An important function of mammalian cytosolic (cAspAT) and mitochondrial AspAT (mAspAT) is the transport into the mitochondria of the reducing equivalents required for respiration by the malate/aspartate shuttle (Setoyama *et al.*, 1990), shown in Figure 1.10. NADH is principally

synthesised in the cytoplasm by glycolysis, and as the mitochondrial membrane is impermeable to NADH (Lehninger, 1951), the malate/aspartate shuttle, which involves cAspAT and mAspAT and cytosolic and mitochondrial malate dehydrogenase (MDH) activities and the aspartate-malate exchanger in the mitochondrial membrane.

AspAT (and AlaAT) are considered candidates for anti-parasitic drugs, because they also have cysteine *S*-conjugate β-lyase (CCBL) activity, which generates cell toxins (Adcock *et al.*, 1996). Glutathione conjugates toxic compounds (catalysed by glutathione *S*-transferase (GST) (Section 1.8.7); subsequently these are metabolised to cysteine *S*-conjugates, which are then subjected to CCBL activity. During this process, toxic cysteine *S*-conjugates preferentially target mitochondria (Cooper *et al.*, 2002).

1.6.11.3 Other aminotransferases

Helminth aminotransferases are able to transaminate a large number of L-amino acids, usually using the glutamate 2-oxoglutarate system. In addition to the highly active AspAT and AlaAT, Wertheim *et al.* (1960) demonstrated asparagine transaminase activity in *H. diminuta*, *H. citelli* and *H. nana.* Rothstein and Mayoh (1964a,b) followed the metabolism of ^{14}C-aspartate in *C. briggsae* and deduced that there must be isocitrate lyase activity and the glyoxylate transaminase system to account for the production of ^{14}C-glycine as a major product. As nematodes have isocitrate lyase activity (Rothstein and Mayoh (1964a,b; Barrett *et al.*, 1970; Khan and McFadden, 1980), their interpretation is probably correct and glycine and serine could both be synthesised from TCA cycle intermediates by this pathway.

Branched chain aminotransferases (BCAT) catalyse the transamination of leucine, isoleucine and valine to their keto-acids (reviewed by Hutson, 2001). In mammals, which cannot synthesise these amino acids, BCATs have a catabolic function. In lower eukaryotes and bacteria, BCATs have an anabolic function catalysing the first step of the synthesis of branched chain amino acids. BCATs have been identified in *N. brasiliensis* (Walker and Barrett, 1991a) and in *F. hepatica* (Lee *et al.*, 1983).

GABA, an inhibitory neurotransmitter in the vertebrate central nervous system and nematode neuromusculature acts through receptors located on inhibitory chloride channels (Feng *et al.*, 2002a; Schuske *et al.*, 2004). GABA

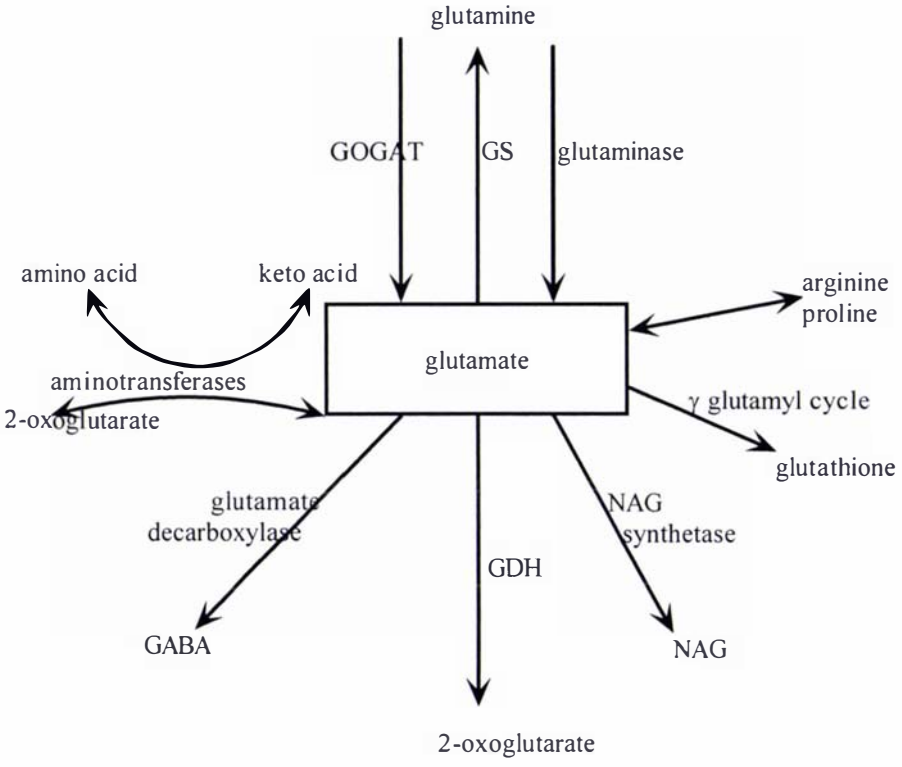


Figure 1.11. The major reactions and pathways for which glutamate is a substrate.

catabolism by the enzyme 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA-T) was described in *A. lumbricoides* and *M. expansa* by Rasero *et al.* (1968). *N. brasiliensis* GABA-T is strongly inhibited by NaCl and four amino acids can substitute for GABA as substrate (Watts and Atkins, 1983, 1984). Rat β-alanine:oxoglutarate aminotransferase, which catalyses the transamination of β-alanine in the brain and liver (Maître *et al.*, 1975; Fujimoto *et al.*, 1986; Ohyama *et al.*, 2004), also transaminates GABA (Ohyama *et al.*, 2004).

1.7 GLUTAMATE METABOLISM

Glutamate has a central position in both anabolic and catabolic pathways of nitrogen metabolism in microorganisms, plants, vertebrates and invertebrates, although pathways vary in importance between organisms. Glutamate is maintained as an intracellular amino acid because high concentrations are toxic to nerves (Anderson and Swanson, 2000). It is readily converted to glutamine, which then becomes a major transporter of organic nitrogenous compounds between organs and is the amino acid with the highest concentration in extracellular fluids of both mammals (Hamilton, 1945; Tapiero *et al.*, 2002) and invertebrates (Hirayama and Nakamura, 2002).

Key reactions involving glutamate are shown in Figure 1.11, some of which have been discussed earlier. The interconversion of glutamate, proline, ornithine and arginine have been described in Section 1.5.7 and illustrated in Figure 1.7. Many aminotransferases catalyse the transfer of an amino group from a donor α-amino acid to 2-oxoglutarate, forming glutamate, as discussed above in Section 1.6.11. This allows utilisation of excess amino acids, by removing and excreting the amino group and contributing the carbon skeleton to energy metabolism. The transfer of amino groups between carbon backbones also allow synthesis of non-essential amino acids and maintains the correct pool of amino acids from which tissues produce proteins. The metabolism of glutamate to glutathione and the conjugation of glutathione to toxic compounds and drugs are discussed later in Section 1.8.7. The ability of the γ-glutamyl cycle, by which glutathione is synthesised and degraded, to act also as an amino acid transporter has been discussed in Section 1.2.

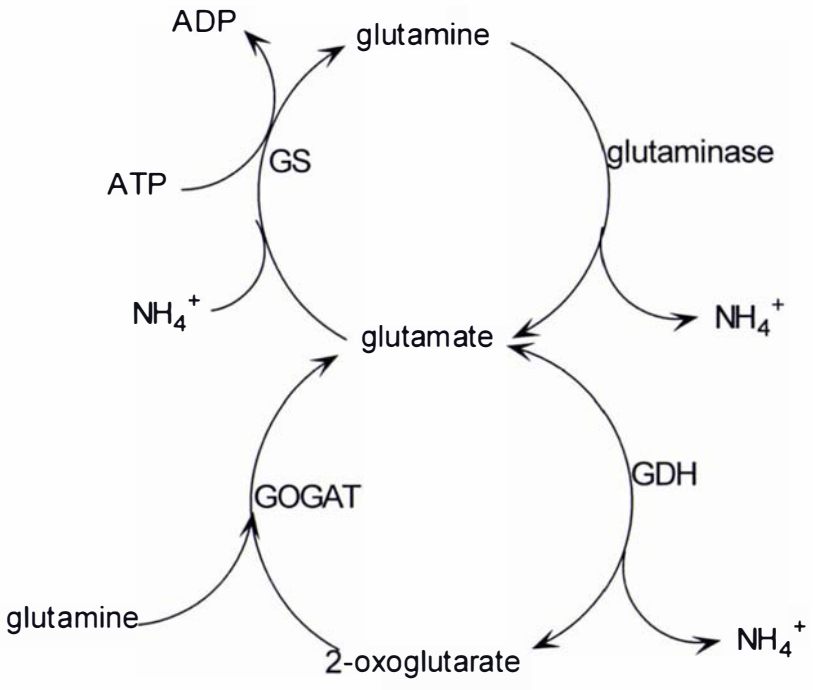


Figure 1.12. The reactions and enzymes catalysing the interconversions of glutamine, glutamate and 2-oxoglutarate. GS: glutamine synthetase; GOGAT: glutamate synthetase; GDH: glutamate dehydrogenase.

Mammalian glutamate metabolism is well characterised, including the mechanisms of the reactions, the importance of individual enzymes in different organs and how they are regulated. In mammals, GDH, glutamine synthetase (GS) and glutaminase, which together with the aminotransferases, are involved in the interconversions of glutamate, glutamine and 2-oxoglutarate (Figure 1.12) are located primarily in the mitochondria. The most significant differences in non-vertebrates are in the anabolic reactions which incorporate ammonia to form amino acids. In mammals, the principal route involves GDH acting in the synthetic direction together with aminotransferases, but in plants, microorganisms and some lower animals, including some insects, there is the alternative GS-GOGAT pathway (Section 1.2.3-4). The most appropriate model for nematode glutamate metabolism may therefore not be the mammalian system, but rather those of plants, microorganisms or other invertebrates.

Although there are alternative pathways to generate the same end-product, there may be physical separation of enzymes in different organs, cells or compartments within cells. This is the case in the mammalian liver, where periportal cells express glutaminase and OUC enzymes, but the smaller number of perivenous cells express GS. As a result, ammonia is generated by glutaminase and converted to urea in periportal cells and the excess ammonia is subsequently converted to glutamine by GS in perivenous cells and released as glutamine. On subsequent re-circulation to the liver, glutamine can then be converted to urea by the periportal cells, or alternatively can be used by the kidney to buffer excreted ammonia (Brosnan, 2000). The need to remove glutamate quickly from synapses requires separation of glutamine-glutamate metabolism between neurons and glial cells (Waagepetersen *et al.*, 2005). Astrocytes take up glutamate after it is released at synapses, convert it to glutamine using GS (and GDH to a lesser extent) and release glutamine which is then taken up by neurons for resynthesis of glutamate using phosphate-dependent glutaminase.

Glutamate is an important source of nitrogen for excretion. In most organisms, GDH operating in the catabolic direction is the main source of ammonia. Other pathways have been suggested to make a contribution in some invertebrates, such as AMP deaminase (Bishop and Barnes, 1971), urease (Hanlon, 1975), D-amino acid oxidases (D'Aniello *et al.*, 1993) and

specific deaminases (Grantham and Barrett, 1986a). Ammonia is either excreted directly by aquatic animals or enters the OUC for conversion to urea in the liver of ureotelic species (Section 1.4.1). Ammonia, produced by glutaminase (from glutamine), allows the vertebrate kidney to produce an acid urine in which H^+ is buffered by ammonia and excreted as NH_4^+ (Section 1.7.2). GDH activity correlated positively with ammonia excretion in some invertebrates, but not others, e.g. there was a strong relationship in the crustacean *Praunus flexuosus* (Bidigare and King, 1981), but only weak in the polychaete *Arenicola marina* (Batrel and Le Gal, 1984). GDH has also been proposed to participate in osmoregulation by generating an increased pool of free amino acids (particularly alanine, glycine, proline and taurine), since both GDH and aminotransferase activities increased with adaptation to increasing salinity and decreased in reduced salinity (Gerard and Gilles, 1972; Gilles, 1973; Wickes and Morgan, 1976; Batrel and Le Gal, 1984; Reitze *et al.*, 1989; Matsushima and Hayashi, 1992). Plants also accumulate amino acids, particularly proline, during osmotic stress (Section 1.5.7).

There are major differences in nitrogen assimilation in animals, plants and bacteria. In addition to the vertebrate pathway of glutamate synthesis by the aminating reaction of GDH, plants and microorganisms use also an alternative pathway via GS and GOGAT (reviewed by Miflin and Lea, 1976; Miflin and Habash, 2002; Hodges *et al.*, 2003; Magasanik, 2003). GS-GOGAT first incorporates ammonia to form glutamine, which then reacts with 2-oxoglutarate to form two glutamate molecules. Nitrogen assimilation via GDH does not require ATP, but is usually limited by the high K_m for ammonia of GDH, whereas the GS-GOGAT pathway requires ATP but has a higher affinity for ammonia. In higher plants, the GS-GOGAT pathway has been established as the main pathway, based on labelling, inhibitor and genetic studies (reviewed by Miflin and Habash, 2002). In both plants and bacteria, the GS-GOGAT pathway is used if ATP is readily available, whereas the GDH pathway operates when ammonia concentrations are high, energy is limited or the organism is stressed (Fisher and Sonenshein, 1991; Osuji and Madu, 1996; Helling, 1998; El-Shora and Abo-Kassem 2001).

1.7.1 GLUTAMATE DEHYDROGENASE

The glutamate dehydrogenases are a group of enzymes catalysing the reversible oxidative deamination of L-glutamate to 2-oxoglutarate and

ammonia, using either NAD^+ or $NADP^+$ as the cofactor. The structure, physical and chemical properties of these enzymes and their regulation have been well characterised, particularly GDH from bovine liver (reviewed by Goldin and Frieden, 1971; Hudson and Daniel, 1993). Cofactor requirements separate the GDHs into three broad groups: NAD^+ specific, $NADP^+$ specific or enzymes with dual cofactor-specificity. A single enzyme may have dual specificity, as in vertebrates, or there may be two enzymes with differing specificity. In general, the aminating direction is catalysed by NAD-GDH and the deaminating direction by NADP-GDH.

Whether the enzyme is functioning in the formation or deamination of glutamate will depend on the concentrations of activators and inhibitors and the K_m for the substrates, in particular for ammonia, which is high in many species. The regulation of the dual specificity mammalian enzymes is very complex, being strongly regulated by purine nucleotides, particularly activated by ADP, AMP and some amino acids and inhibited by GTP (Goldin and Frieden, 1971; Hudson and Daniel, 1993). These activators and inhibitors also affect the formation of multienzyme complexes of GDH with aminotransferases, 2-OG dehydrogenase, citrate synthase and MDH, which allow the direct transfer of 2-OG, GTP and NADH between enzymes (Fahien and Smith, 1974; Fahien *et al.*, 1985, 1989). The kinetics of GDH *in vitro* may therefore not reflect the situation *in vivo*, where there is substrate channelling.

GDH activities in invertebrates are far less uniform than in mammals and do not always have the characteristic activation by ADP and inhibition by GTP and may be very different in their co-factor requirements and favoured direction. The correlation between GDH activity and ammonia excretion in several invertebrates suggests an excretory role for GDH (Bidigare and King, 1981). On the other hand, an osmoregulatory role has been proposed for GDH and aminotransferases, whose activities increase with adaptation to increasing salinity and decrease in reduced salinity (Gerard and Gilles, 1972; Gilles, 1973; Wickes and Morgan, 1976; Batrel and Le Gal, 1984; Reitze *et al.*, 1989; Matsushima and Hayashi, 1992).

There is GDH activity in helminths including *H. contortus* (Rhodes and Ferguson, 1973; Skuce *et al.*, 1999b), *A. suum* (Langer, 1972), *D. immitis* (Langer and Jiamperpoon, 1970; Turner *et al.*, 1986), *H. polygyrus*, *P.*

redivivus (Grantham and Barrett, 1986a), *Obeliscoides cuniculi* (Hutchinson and Fernando, 1975), *L. carinii* (Davies and Köhler, 1990), adult *F. hepatica* (Prichard and Schofield, 1968; Thorpe, 1968), the Digenean *Microphallus similis* (McManus and James, 1975) and four cestodes (Rothman and Lee, 1963). A cytosolic NAD-GDH was demonstrated in the cestode *H. diminuta* (Mustafa *et al.*, 1978), in *F. hepatica* mGDH was more active than cGDH activity (Prichard and Schofield, 1968). Unlike bovine liver GDH, AMP, ADP, ATP, GDP, GTP and amino acids had little effect on *H. diminuta* GDH, whereas the TCA intermediates fumarate, malate and succinate were inhibitory in high, probably not physiologically relevant concentrations (Mustafa *et al.*, 1978). Turner *et al.* (1986) found both mitochondrial and cytosolic GDH in *D. immitis* and the properties of purified *H. contortus* GDH were compared with those of ovine and bovine liver GDHs by Rhodes and Ferguson (1973). GDH was mainly mitochondrial in both *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1986a). In the direction of glutamate formation, activity with NADH was twice the rate with NADPH in both nematodes, but in the reverse direction, activity with NAD^+ was three and eight times greater than with $NADP^+$ in *P. redivivus* and *H. polygyrus* respectively. The role of GDH may be more variable in helminths according to whether their metabolism is more or less anaerobic than other species, probably a function of their size and whether parasitic or free-living.

1.7.2 GLUTAMINASE

Glutamine transports organic nitrogen between organs of animals and after transport into the cells much is catabolised to glutamate by glutaminase. Vertebrate liver- and kidney-type glutaminases are isoforms of glutaminase I, also called phosphate-activated glutaminase, to distinguish it from glutaminase II, which is phosphate-independent and either a glutamine aminotransferase or a γ-glutamyltransferase. The two isoforms are products of different genes and have different kinetic properties, most notably the inhibition of kidney-type glutaminase by glutamate and lack of inhibition of the liver isoform (reviewed by Curthoys and Watford, 1995; Kvamme *et al.*, 2000). Both isoforms are expressed in the brain and in some tumour cells (Márquez *et al.*, 2006).

Liver-type glutaminase is expressed along with OUC enzymes in periportal hepatocytes, where ammonia released by glutaminase is immediately incorporated into carbamoylphosphate by CPS I. Kidney-type

glutaminase is expressed in the kidney, intestine, brain and lymphocytes, pancreatic islets, where glutamine is used as a respiratory fuel, as well as for the biosynthesis of other amino acids, purines, pyrimidines and glucosamine. In the kidney, glutaminase supplies the ammonia needed for excretion of acid, the enzyme activity increasing with increased demands of ammonia production (Curthoys *et al.*, 1976; Margolis and Lifschitz, 1985; Curthoys, 2001). Glutamine is the main respiratory fuel for the small intestine (Windmueller and Spaeth, 1980) and also provides substrates for biosynthesis of nucleic acids and other compounds (reviewed by McCauley *et al.*, 1999). Glutamatergic neurons use glutaminase to synthesise glutamate from the glutamine provided by the astrocytes (Waagepetersen *et al.*, 2005).

The glutaminases of microorganisms fall into a number of classes, few of which have similar properties to the mammalian phosphate-dependent enzymes (reviewed by Nandakumar *et al.*, 2003). While some are specific for glutamine, others are also active asparaginases and used in chemotherapy of leukaemia (Section 1.6.7). Others are salt tolerant and of interest to the food industry. The enzyme in *Bacillus pasteurii* is phosphate-activated, unlike other microbial glutaminases (Klein *et al.*, 2002). Bacterial glutaminase A becomes more active as growth slows and has been suggested as a regulator of growth by decreasing cell glutamine levels (Hartmann and Stochaj, 1978). Glutaminase B is reciprocally regulated with GS by ADP and ATP, which are believed to prevent futile cycling of glutamate-glutamine, which would waste energy (Pruisner and Stadtman, 1971, 1976; Huerta-Saquero *et al.*, 2004).

There are few reports of glutaminase activity being investigated in nematodes. As glutamate is also a neurotransmitter in nematodes (Section 1.8.5), it is likely that similar enzymes to those of the mammalian brain glutamine-glutamate cycle, GS and glutaminase, are associated with nematode neuromuscular tissue. Both glutaminase and asparaginase activities were identified in *H. polygyrus* and *P. redivivus* at very much lower levels than in rat liver (Grantham and Barrett, 1986a), but no glutaminase activity was detectable in *L. carinii* (Davies and Köhler, 1990). It is not known whether invertebrate glutaminase (Downton and Kennedy, 1986; Jabbar *et al.*, 1987) is of the mammalian or bacterial type, but may be either the latter, as glutaminase II, but not type I was present in the fleshfly *Parasarcophaga crassipalpis* (Downton and Kennedy, 1986).

1.7.3 GLUTAMINE SYNTHETASE

Glutamine synthetase (GS), which catalyses the reaction of ATP, glutamate and ammonium to form ADP, phosphate, H^+ and L-glutamine, requires either Mg^{2+} or Mn^{2+} for activity. While it is reversible, the biosynthetic or forward reaction is the only physiological direction. GS, which is present in all organisms, including fossils (Kumada *et al.*, 1993) is of three types: GSI found only in prokaryotes, GSII present in eukaryotes and some bacteria and GSIII expressed in a rumen anaerobe (Amaya *et al.*, 2005) and in cyanobacteria (Pesole *et al.* 1995), particularly during nitrogen starvation (García-Domínguez *et al.*, 1997). GS has been identified in the nematodes *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1988).

GS is usually the enzyme responsible for ammonia assimilation when there is adequate ATP, in a pathway with GOGAT in plants and bacteria, whereas GDH provides an alternative route when energy is limited. GS in the brain is involved in ammonia detoxification and neurotransmitter recycling (Suárez *et al.*, 2002), in perivenous cells of the liver in converting excess ammonia to glutamine and in the kidney to buffer excreted acid (Brosnan, 2000). GS is inhibited by "cumulative feedback inhibition" of nine different end-products of glutamine metabolism, which worked best in combination to give 90% inhibition (Woolfolk and Stadtman, 1967). When the enzyme is adenylated, it is more susceptible to feedback inhibition, providing an extra level of control of GS. Cumulative feedback inhibition ensures that accumulation of single end-products of the many pathways using glutamine do not individually inappropriately inhibit GS activity (Stadtman, 2001).

Mycobacterium tuberculosis GS has been identified as an antibacterial target, as the inhibitor L-methionine-(*S*)-sulfoximine (MetSox), which competes for binding with glutamate (Maurizi and Ginsberg, 1982), protected guinea pigs from the infection (Harth and Horwitz, 2003).

1.7.4 GLUTAMATE SYNTHASE

Glutamate synthase (GOGAT), which catalyses the glutamine-dependent reductive amination of 2-oxoglutarate to form two molecules of glutamate, was first identified in *Aerobacter (Klebsiella) aerogenes* by Tempest *et al.* (1970). Plant GOGAT is either the form which uses reduced ferrodoxin

as the electron donor (Fd-GOGAT) or one that uses NADH as the electron donor (NADH-GOGAT), while a third form, NADPH-GOGAT, is present in bacteria (reviewed by Vanoni and Curti, 1999; Suzuki and Knaff, 2003; van den Heuvel *et al.*, 2004; Vanoni *et al.*, 2005).

The two-reaction pathway formed by GS and GOGAT is the most important for ammonia assimilation in plants (reviewed by Miflin and Habash, 2002) and bacteria, the GS-GOGAT pathway is used if ATP is readily available, whereas the GDH pathway operates when ammonia concentrations are high, energy is limited or the organism is stressed (Fisher and Sonenshein, 1991; Osuji and Madu, 1996; Helling, 1998; El-Shora and Abo-Kassem 2001).

GOGAT may be an important enzyme in invertebrates. GOGAT activity has been identified by direct assay or labelling studies in the tissues of the silkworms *B. mori* (Hirayama and Nakamura, 2002) and *Samia cynthia ricini* (Osanai *et al.*, 2000), in a *Spodoptera frugiperda* Sf9 insect cell line (Doverskog *et al.*, 2000; Drews *et al.*, 2000) and in the mosquito *Aedes aegypti* (Scaraffia *et al.*, 2005). The GOGAT inhibitor azaserine significantly increased glutamine levels and decreased proline concentrations in the haemolymph of the mosquito after a blood meal, supporting an important role for GOGAT. It was located in the fat body, but not the intestine (Scaraffia *et al.*, 2005). Genes for NADH-GOGAT have been identified during sequencing of the genome of *C. elegans* (Vanoni and Curti, 1999), suggesting GOGAT activity may also be present in nematodes.

1.7.5 Synthesis of N-acetylglutamate

Glutamate is the precursor of N-acetylglutamate (NAG), which can be generated from glutamate either by ornithine acetyltransferase or by NAG synthetase. The latter enzyme is used by ureotelic species to generate NAG, an essential cofactor and allosteric regulator of the urea cycle enzyme CPS I (Brosnan, 2000). The hepatic cell glutamate concentration determines the rate of NAG synthesis, hence of urea formation. In lower eukaryotes, plants and microorganisms, ornithine and arginine can be synthesised from glutamate via N-acetylglutamate (NAG) in an eight-step pathway involving N-acetylated intermediates (reviewed by Caldovic and Tuchman (2003).

1.7.6 GLUTAMATE DECARBOXYLASE

Gamma-aminobutyrate (GABA), which is synthesised from glutamate by glutamate decarboxylase, is an important inhibitory neurotransmitter in the vertebrate central nervous system (reviewed by Mody *et al.*, 1994). There is an excitatory GABA receptor involved in defaecation in nematodes (Schuske *et al.*, 2004). GABA also acts on receptors on inhibitory chloride channels in the neuromusculature (Feng *et al.*, 2002a; Schuske *et al.*, 2004), which are a possible site of action of avermectins in *H. contortus* (Blackhall *et al.*, 2003). Plants overexpressing glutamate decarboxylase are resistant to nematode parasites, presumably because of the toxicity of the high levels of GABA (McLean *et al.*, 2003). Singh *et al.* (1983) located glutamate decarboxylation in *A. galli* in the mitochondrial fraction of the cuticle. The degrading enzyme GABA-T (Section 1.6.11.3) is present in *A. lumbricoides, M. expansa* (Rasero *et al.*, 1968) and *N. brasiliensis* (Watts and Atkins, 1983, 1984; Walker and Barrett, 1991a).

The GABA shunt (the γ-aminobutyrate bypass), which allows the metabolism of 2-oxoglutarate to succinate, consists of glutamate decarboxylase, GABA-T and succinic semialdehyde dehydrogenase. The GABA shunt operates in plants (Shelp *et al.*, 1999) and in bacteria such as *Rhizobia* and *E. coli* during anaerobic conditions when 2-oxoglutarate dehydrogenase is repressed (reviewed by Dunn, 1998). The GABA shunt operates in both *O. volvulus* and *B. pahangi* to produce succinate (MacKenzie *et al.*, 1989) and may be important in nematodes with low 2-oxoglutarate dehydrogenase activity.

1.8 OTHER NEMATODE ENZYMES

A number of enzymes which metabolise nitrogenous compounds have been identified in nematodes in addition to those discussed above.

1.8.1. CHITIN METABOLISM

Chitin is a straight polymer of β-1,4 linked N-acetyl glucosamine residues, which are synthesised from fructose-6-phosphate by the hexosamine biosynthetic pathway. The first step is catalysed by L-glutamine:D-fructose-6-phosphate amidotransferase to form glucosamine 6-phosphate. The highest

activity of the amidotransferase in *A. suum* eggs was immediately after fertilisation of mature oocytes and was least in the zygotes in the eggs in the last part of the uterus (Dubinský *et al.*, 1985). The second enzyme, glucosaminephosphate isomerase, appeared to play no part in chitin formation in *A. suum* eggs, unlike the situation in other organisms.

Chitin is present in the nematode eggshell (Bird and Bird, 1991), the cuticle of *A. suum* (Hill *et al.*, 1991) and in the feeding apparatus in the pharynx of *Oesophagostamum dentatum* (Neuhaus *et al.*, 1997) and *C. elegans* (Veronico *et al.*, 2001). Chitin is formed by chitin synthase, which adds residues to the polymer. There appear to be two genes for the enzyme in nematodes, one related to eggshell chitin formation and the other in the pharynx where chitin is present in the feeding apparatus. The eggshell-related chitin synthase gene is expressed in the reproductive tract of egg-laying adults of *C. elegans* and *Meloidogyne artiella* (Veronico *et al.*, 2001) and *Brugia malayi* (Harris *et al.*, 2000), whereas the other gene is expressed just before a moult in the pharynx of *C. elegans* (Veronico *et al.*, 2001).

Nematodes also express chitinases, which are believed to be needed for hatching, moulting and perhaps also tissue invasion (Gooday *et al.*, 1988; Adam *et al.*, 1996; Wu *et al.*, 2001; Geng *et al.*, 2002) and can be antigens recognised by the host (Fuhrman *et al.*, 1992; Adam *et al.*, 1996).

1.8.2 Amino acid catabolism

Amino acid oxidases were active with 14 amino acids in *P. redivivus* but with only 12 in *H. polygyrus* (not with L-alanine and proline) (Grantham and Barrett, 1986a). For *P. redivivus*, enzyme activity in descending order was with histidine, lysine, aspartate, glycine, tyrosine, isoleucine, threonine and proline and arginine. In general, the activity was much higher in *H. polygyrus*, activity in descending order being with methionine, glycine, aspartate, threonine, isoleucine and histidine, GABA which were all greater than in *P. redivivus* (Grantham and Barrett, 1986a). The presence of aspartate oxidase activity is of interest as it may indicate that nematodes use the bacterial pathway for NAD^+ synthesis via L-aspartate oxidase (LASPO) (Mattevi *et al.*, 1999) rather than the eukaryote pathway by oxidation of tryptophan.

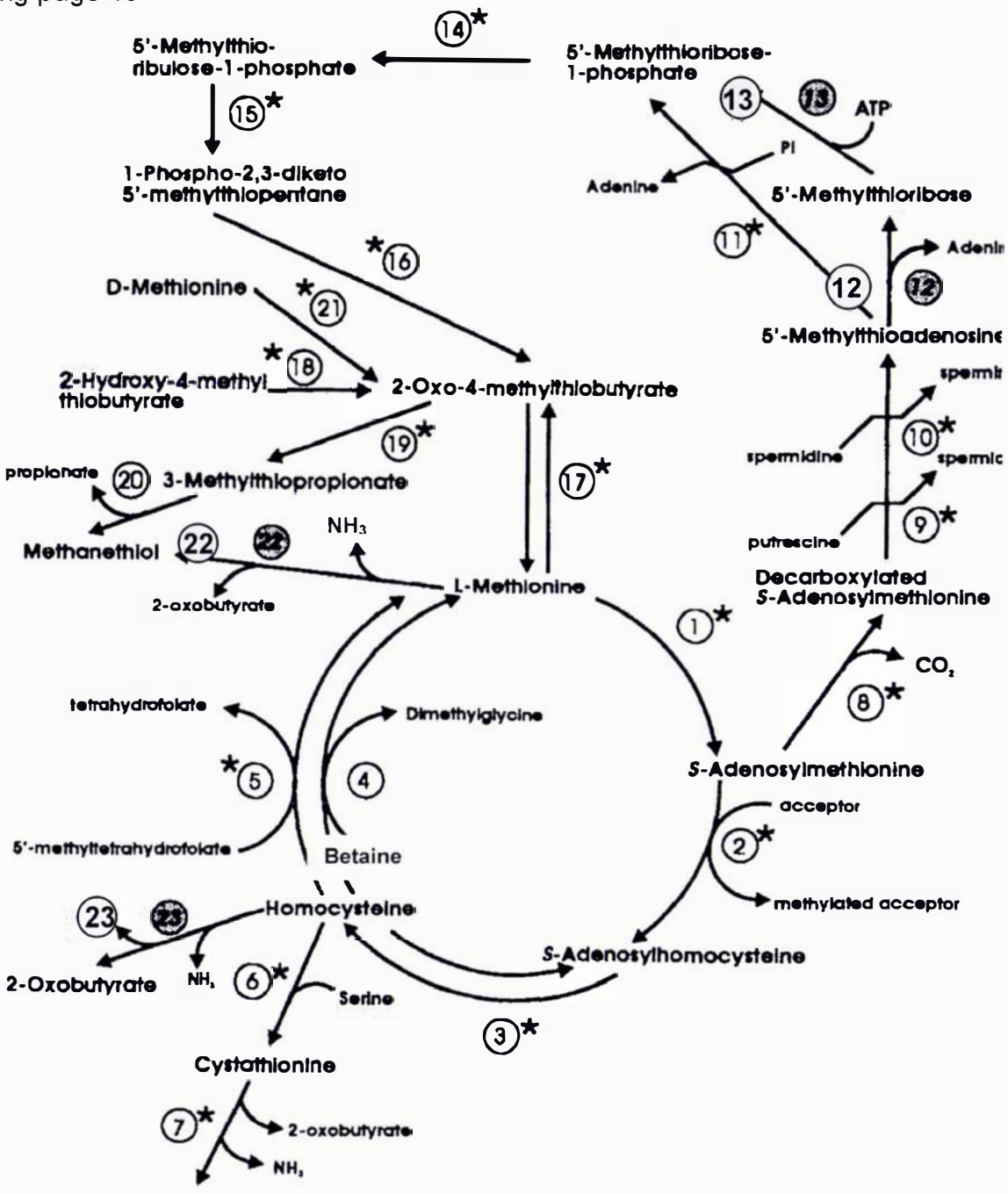


Fig. 1.13. Generalised scheme of methionine metabolism in mammals and parasites. Pathways and enzymes demonstrated or implicated in parasitic protozoa and helminths are indicated by *. Pathways/enzymes present in parasitic protozoa but absent from their mammalian hosts are shaded. (from Walker and Barrett, 1997).

Key to enzymes: 1. Methionine adenosyltransferase (AdoMet synthetase) (EC 2.5.1.6). 2. Various 5-adenosylmethionine methyltransferases (EC 2.1.1.?). 3. S-Adenosylhomocysteine hydrolase (EC 3.3.1.1). 4. Betaine:homocysteine methyl-transferase (EC 2.1.1.5). 5. 5'-Methyltetrahydrofolate:homocysteine methyltransferase (methionine synthase) (EC 2.1.1.13). 6. Cystathionine β-synthase (EC 4.2.1.22). 7. β-Cystathionase (EC 4.4.1.1). 8. 5-Adenosylmethionine decarboxylase (EC 4.1.1.50). 9. Spermidine synthase (putrescine:propylamine transferase) (EC 2.5.1.16). 10. Spermine synthase (sper-midinerpropylamine transferase) (EC 2.5.1.22). 11. 5'-Methylthioadenosine phosphorylase (EC 2.4.2.28). 12. 5'-Methyl-thioadenosine nucleosidase (EC 3.2.2.16). 13. 5'-Methylthioribose kinase (EC 2.7.1.100). 14-16. Intermediates identified, but enzymes/reaction mechanisms not fully elucidated. 17. Unidentified 2-oxoglutarate-linked aminotransferase, possibly glutamine aminotransferase (EC 2.6.1.15) in mammals. 18. 2-Hydroxyacid dehydrogenase (EC 1.1.99.6). 19. Branched-chain 2-oxo acid dehydrogenase (EC 1.2.4.3). 20. Dethiomethylation; substrate/product identified, but enzyme not elucidated. 21. D-Amino acid oxidase (EC 1.4.3.3). 22. Methionine γ-lyase (EC 4.4.1.11). 23. "Homocysteine desulphurase" reaction, catalysed by methionine γ-lyase (certain protozoa) or γ-cystathionase (mammals).

Decarboxylation of the amino acids serine, leucine and valine, in addition to alanine, aspartate and glutamate, was observed in the intestines, ovaries and cuticle of *A. galli* (Singh *et al.*, 1983). There were complete pathways for the catabolism of the branched chain amino acids isoleucine, leucine and valine to TCA cycle intermediates in *P. redivivus* and *H. polygyrus* (Grantham and Barrett, 1986b). There was low deiminase activity towards histidine (histidase) in *P. redivivus*, but in *H. polygyrus* it was 50 times higher and was similar to the levels in rat liver (Grantham and Barrett, 1986a).

1.8.3 SHIKIMATE PATHWAY

The precursors of tyrosine, tryptophan and phenylalanine are synthesised in plants and microorganisms by the shikimate pathway, which consists of seven steps, the final one of which is catalysed by chorismate mutase (Romero *et al.*, 1995). Chorismate mutase is expressed in the plant parasitic nematodes *Meloidogyne javanica* (Lambert *et al.*, 1999), *Meloidogyne arenaria* (Long *et al.*, 2006), *Meloidogyne incognita* (Rosso *et al.*, 1999; Huang *et al.*, 2005). Its expression in the *Globodera pallida* (Jones *et al.*, 2003) suboesophageal gland (Jones *et al.*, 2003; Long *et al.*, 2006), suggests it is a secreted protein. Based on comparisons with bacterial genes, Yan *et al.* (1998) proposed that plant parasitic nematodes acquired the enzyme by horizontal gene transfer from bacteria. As all enzymes of the shikimate pathway are present in a number of parasitic protozoa and are essential for survival (Roberts *et al.*, 1998, 2002; Campbell *et al.*, 2004), these enzymes are considered potential therapeutic targets.

1.8.4 SULPHUR AMINO ACID METABOLISM

The metabolism of methionine and cysteine by parasitic helminths has been reviewed by Walker and Barrett (1997) (summarised in Figure 1.13). They concluded that the basic metabolism of sulphur amino acids in parasitic nematodes resembled that of the host and that any differences were too minor to encourage the development of novel therapies targeting these enzymes. There were, however, some unusual properties of nematode enzymes.

Methionine is a precursor of *S*-adenosylmethionine, which is required for polyamine synthesis (Section 1.5.5). Since nematodes are believed to take up polyamines from the host rather than synthesise them, it has been

suggested that the methionine salvage pathway might be a better anthelmintic target than the enzymes involved in methionine synthesis (Walter, 1988; Wittich and Walter, 1989; Müller and Walter, 1992; Da'dara *et al.*, 1998). Only the final part of the salvage pathway has been confirmed in the nematode *A. suum*. Conversion of methionine to cysteine involves trans-sulphuration reactions catalysed by the enzymes cystathione β-synthase and γ-cystathionase. Walker *et al.* (1992) identified a novel form of cystathione β-synthase in *N. brasiliensis* which had different substrate specificities from the enzyme in the liver of the rat host.

1.8.5 NEUROTRANSMITTERS

In invertebrates, amino acids and amines act as neurotransmitters and neurohormones (reviewed by Monastirioti, 1999). GABA is predominantly an inhibitory neurotransmitter (Section 1.7.6). Glutamate-gated channels are excitatory in vertebrates (Alagarsamy *et al.*, 2001; Mayer and Armstrong, 2004), but in invertebrates there is an unusual group of inhibitory glutamatergic chloride channels which are activated by the avermectin anthelmintics to paralyse nematode muscle, particularly in the pharynx (Cook *et al.*, 2006). Glutamate is the agonist for receptors on these channels on the nematode pharyngeal muscles and is the neurotransmitter released by inhibitory motor neurones in *A. suum* (Adelsberger *et al.*, 1999) and *C. elegans* (Avery, 1993).

Amino acids are the precursors of important amine neurotransmitters in nematodes: serotonin is derived from tryptophan, histamine from histidine and dopamine, catecholamines, tyramine and octopamine from tyrosine (reviewed by Blenau and Baumann, 2001). In addition, about 20 FMRF-amide peptides with the sequence x-x_o-Arg-Phe-NH_2 have been identified in nematodes (reviewed by Brownlee and Walker, 1999; Kimber and Fleming, 2005; McVeigh *et al.*, 2006) and have diverse actions on muscle in the body and reproductive tract.

Octopamine has many similar roles to those of catecholamines in vertebrates (reviewed by Roeder, 1999; Roeder *et al.*, 2003). It inhibits pharyngeal muscle pumping and egg laying and acts as an antagonist to serotonin (Horvitz *et al.*, 1982). Octopamine is synthesised from tyrosine by conversion to tyramine (by tyrosine decarboxylase) then to octopamine by the

enzyme tyramine β-hydroxylase. Tyramine inhibits egg laying in *C. elegans* by an action independent of that of octopamine (Alkema *et al.*, 2005).

1.8.6 TRANSGLUTAMINASE

Transglutaminases catalyse posttranslational modifications of proteins through the formation of isopeptide bonds between protein-bound glutamine residues and primary amines, such as polyamines and protein-bound lysine. If the amine is a protein-bound lysine, the proteins become cross-linked (reviewed by Greenberg *et al.*, 1991). Amongst the numerous activities of transglutaminases are involvements in cell signalling, apoptosis and providing resistance of tissues to degradation by chemicals, enzymes and physical damage. Mehta and co-workers have characterised transglutaminases from *D. immitis* (Singh *et al.*, 1995) and *B. malayi* (Mehta *et al.*, 1992; Singh and Mehta, 1994) and have established their role in growth, maturation and development (reviewed by Chandrashekar and Mehta, 2000), particularly formation of the sheath and cross-linking of host proteins into developing microfilariae (Mehta *et al.*, 1996). Transglutaminase activity has also been demonstrated in *C. elegans* (Mádi *et al.*, 2004) and there are high levels of lysine cross-linking in the sheath of the rat filarial parasite *L. carinii* (Tarcsa *et al.*, 1992).

1.8.7 GLUTATHIONE *S*-TRANSFERASE

Glutamate is converted to the tripeptide glutathione (L-γ-glutamyl–L-cysteinylglycine) by successive additions of cysteine and glycine by the enzymes γ-glutamylcysteine and GSH synthetases, then degraded by four enzymes, which together make up the γ-glutamyl cycle (Figure 1.1) (reviewed by Meister, 1981, 1988). The ability of the γ-glutamyl cycle to act as an amino acid transporter has been discussed in Section 1.2.

GSH has many cellular functions, particularly as an antioxidant and in leukotriene, steroid and prostaglandin metabolism, and more recently has been proposed to be a neuromodulator and neurotransmitter (Janáky *et al.*, 1999; Njålsson and Norgrem, 2005). There is considerable interest in parasite GSH and also in the glutathione *S*-transferases (GSTs), enzymes which conjugate toxic compounds and xenobiotics to glutathione and protect against the host immune response. They are also seen as potential targets for

antiparasitic vaccines. There are currently seven cytosolic classes and one mitochondrial family of GSTs recognised in mammals and there are other classes of GSTs in plants, prokaryotes and invertebrates (reviewed by Clark, 1989; Mahajan and Atkins, 2005).

GSTs have been identified, characterised or the encoding genes sequenced in many parasitic helminths: in the nematodes *L. carinii* (Bhargava *et al.*, 1983), *H. contortus* (Kawalek *et al.*, 1984), *D. immitis* and *B. pahangi* (Jaffe and Lambert, 1986), *A. suum* (Liebau *et al.*, 1997), *Onchocerca gutterosa* (Pemberton and Barrett, 1989), *H. polygyrus* (Brophy *et al.*, 1994) and *O. volvulus* (Liebau *et al.*, 1994; Salinas *et al.*, 1994); in the trematodes *F. hepatica* (Salvatore *et al.*, 1995; Rossjohn *et al.*, 1997), *S. japonicum* (Walker *et al.*, 1993; Milhon *et al.*, 1997; Jao *et al.*, 2006) *S. mansoni* (Walker *et al.*, 1993; Mei and LoVerde, 1997; *Paragonimus westermani* (Hong *et al.*, 2000) and *Clonorchis sinensis* (Hong *et al.*, 2001; Kang *et al.*, 2001); and the cestodes *M. expansa* (Brophy *et al.*, 1989) and *Echinococcus multilocularis* (Morello *et al.*, 1882; Liebau *et al.*, 1996). Because of differences in nematode and host GSTs (Campbell *et al.*, 2001), vaccines against GSTs have been used to reduce parasite burdens and fecundity (Balloul *et al.*, 1987; Sexton *et al.*, 1994; Morrison *et al.*, 1996; Salinas *et al.*, 1996; Da Costa *et al.*, 1999).

1.9 CONCLUSIONS

Compared with the information on nitrogen metabolism in other organisms, relatively little is known about the functioning of these enzymes in nematodes and almost no data exist for parasites of sheep, particularly *O. circumcincta*. Given the rapid growth of larval nematodes and the enormous requirements for protein to support growth and moulting in the larval stages and reproduction in adults, nitrogen metabolism may present opportunities for novel control strategies. A survey of the literature suggests pathways are present in nematodes which are different from those in mammalian hosts and have resemblances to those in plants and lower organisms. Therefore, a series of experiments has been undertaken to identify the patterns of nitrogen uptake, excretion and metabolism in larval *O. circumcincta* and to a lesser extent in adult worms. These studies are reported in the following Chapters.

Chapter 2

UPTAKE AND EXCRETION OF NITROGENOUS COMPOUNDS BY *OSTERTAGIA CIRCUMCINCTA*

2.1 INTRODUCTION

Although the uptake and excretion of nutrients have been extensively studied in many living organisms, this is not the case in parasitic nematodes of ruminants, despite their economic significance. The sources of nitrogen are likely to vary at different times in the life cycle. The nutrient requirements of the free-living stages of *O. circumcincta* are provided by the microbes in the sheep faeces, but are not known for the parasitic stages. The L3 enter the gastric gland lumen, where they develop and moult, leaving the glands as L4 or immature adults and subsequently living in close association with the mucus overlying the abomasal mucosa (Sommerville, 1954, 1963; Armour *et al.*, 1966). Possible sources of nitrogen for the luminal stages are the mucins, epithelial cells or cell debris, substances in leaked interstitial fluid or components of the abomasal digesta.

2.1.1 UPTAKE OF NITROGENOUS COMPOUNDS

Nitrogen may be taken in as ammonia, urea, peptides or amino acids, the major source depending on the species and its environment (Chapter 1, 1.1). Transporters for these different compounds have been reviewed in Chapter 1, 1.2. In higher animals, most nitrogen is derived from proteins which are degraded in the digestive tract before absorption in the intestine as peptides or amino acids (reviewed by Christensen, 1990; Ziv and Bendayan, 2000; Wagner *et al.*, 2001). This may also be the case in nematode parasites, as proteolytic enzymes are present on the intestinal brush border and/or secreted by many parasitic nematodes of plants (Vanholme *et al.*, 2004) and animals (reviewed by Sajid and McKerrow, 2002), including *O. circumcincta* (Young *et al.*, 1995).

Most uptake studies in nematodes have focused on amino acid uptake, although not at the cellular level, as has been the case in model organisms and the mammalian gastro-intestinal tract. Five major superfamilies of amino acid transporters have been well characterised in numerous organisms and are described in Chapter 1, 1.2. The γ-glutamyl cycle involving the enzyme γ-glutamyl transpeptidase, has also been proposed as a transporter of amino acids and shown to operate in helminths (Dass and Donahue, 1986; Abidi and Nizami, 1995).

Amino acid uptake by the four different groups of helminths has been comprehensively reviewed by Pappas and Read (1975) and Pappas (1988). There are few studies in nematodes (Weatherly *et al.*, 1963; Chen and Howells, 1979; Singh *et al.*, 1983) and acanthocephalans (Uglem *et al.*, 1973) in contrast to the large number in cestodes (Pappas *et al.*, 1973; Jeffs and Arme, 1985, 1987; McCracken and Lumsden, 1975; Webb, 1986) and trematodes (Pappas, 1971; Chappell, 1974; Asch and Read, 1975; Isseroff *at al.*, 1976; Hanna, 1980; Mercer and Chappell, 1985; Abidi and Nizami, 1995).

Of particular interest has been comparing the relative contributions of the nematode cuticle and gut to absorption (Pappas, 1988), as the cuticle does not appear to have the structure of an absorptive surface (Bird and Bird, 1981; Page, 2001). Pappas (1988) concluded that this question was unresolved because of technical difficulties associated with ligation of the intestine and the failure of the parasites to feed *in vitro* (Chen and Howells, 1979), as feeding may not occur and experiments may be biased towards cuticular uptake (Pappas and Read, 1975). The metabolism of labeled amino acids (particularly serine and alanine) to $^{14}CO_2$ (Singh *et al.*, 1983) may also be a confounding effect in uptake studies. Weatherly *et al.* (1963) found that the cuticle of *A. galli* was permeable to ^{14}C-alanine and Chen and Howells (1979) concluded that ^{14}C-leucine was taken up by *B. pahangi* only through the cuticle, as the worms did not ingest dye into the intestine.

2.1.2 Excretion of nitrogenous compounds

Both free-living and parasitic nematodes are predominantly ammonotelic with urea as a minor component (Rogers, 1952; Rothstein, 1963, 1970; Wright, 1975a,b). Urea excretion is usually not greater than about 20% of the total nitrogen excretion. Other excretions by nematodes are proteins,

peptides and amino acids, however, these have been suggested to be secretions from glands or excreted through the gut (Haskins and Weinstein, 1957; Rothstein, 1963; Wright, 1975a,b; Wright and Newall, 1976). In some experiments significant amounts of amino acids were recovered in incubation media, but whether this results from leakage through the cuticle or true excretion from the gut or excretory system is unknown. The appearance of amino acids in the medium in which *P. redivivus* was being incubated was associated with environmental stress (Wright, 1975b).

Proteins appearing in the incubation media may not be excretions but secretions of the parasites, which alter the host response or are proteases (Cox *et al.*, 1990; Young *et al.*, 1995; Gamble and Mansfield, 1996; Redmond *et al.*, 1997; Sajid and McKerrow, 2002) or glycosidases (Gamble and Mansfield, 1996; Irwin *et al.*, 2004) used to supply nutrients. Other proteins in these excretory/secretory (ES) products of nematodes may be components of the gut cells or cuticle (Rhoads *et al.*, 2001). The number of proteins in ES products is very large, e.g. incubates of adult *H. contortus* in RPMI contained in excess of 100 proteins on 2D gel electrophoresis (Yatsuda *et al.*, 2003).

There are two series of experiments reported in this Chapter. The first addressed the excretion of nitrogenous substances of adult and L3 *O. circumcincta* during *in vitro* incubation under various conditions. The second was a preliminary study to determine the permeability of the L3 cuticle and the adult cuticle and digestive tract to amino acids. The method used for the uptake study was to incubate *O. circumcincta* with radiolabeled amino acids in medium, with and without, competing unlabeled amino acids.

2.2 MATERIALS AND METHODS

All chemicals were supplied by Sigma Chemical Co. (Mo, USA) unless specified.

2.2.1 WORM CULTURE

L3 were cultured from the faeces of infected sheep (Appendix 1, 1.1) and stored at 4°C until used. Prior to each experiment, L3 were Baermannized to remove inactive worms, counted (Appendix 1, 1.5) and suspended in medium.

Exsheathed L3 were prepared by incubation of Baermannized L3 with sodium hypochlorite as described in Appendix 1, 1.3.

Adult worms were recovered from the abomasa of infected sheep as described in Appendix 1, 1.4.

2.2.2 EXCRETION OF NITROGENEOUS COMPOUNDS

The general method was to suspend parasites (sheathed or exsheathed L3 or adult worms) in a series of eppendorf tubes each containing 1 ml of 0.8 mM phosphate buffer (Appendix 3, 3.1) at pH 7.0 and temperature 37°C. Tubes containing only phosphate buffer were used as controls. The concentration of the compound of interest was then determined after a designated incubation period.

2.2.2.1 Ammonia excretion

Ammonia excretion was monitored according to the general method described above. The ammonia concentrations were determined by reacting ammonia with hypochlorite and phenol to produce indophenol, which were monitored spectrophotometrically at 635 nm (Appendix 2, 2.1).

Variations of the general method were made as follows:

(1) Effect of pH: 50,000 sheathed L3 were incubated for 5 hours in 1 ml 0.8 mM phosphate buffer at pH 6.0, 6.5, 7.0 or 7.5 at 37°C.

(2) Effect of incubation temperature: 50,000 sheathed L3 were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 for 5 hours at 4°C, 20°C or 37°C.

(3) Effect of parasite density: 5,000, 10,000, 50,000, 70,000 or 100,000 sheathed L3 were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 2.5 hours.

(4) Time course of ammonia production: 50,000 sheathed L3 were incubated for 48 hours in 1 ml of 0.8 mM phosphate buffer pH 7.0 at 37°C. The resulting media were assayed for ammonia after 0.8, 1, 2, 3, 4, 5, 6, 17, 19, 40 and 48 hours.

(5) Effect of external ammonia: 50,000 sheathed L3 were incubated for 5 hours in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C with or without the addition of 60 μM NH_4Cl to the incubation medium.

(6) Comparison of sheathed and exsheathed L3: 50,000 exsheathed and sheathed L3 from the same population were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 5 hours.

(7) Adult worms (~6 mg wet weight) were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 6 hours.

2.2.2.2 Urea excretion

50,000 sheathed or exsheathed L3 were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 1, 2, 3 or 4 hours. Tubes containing only phosphate buffer were used as controls. At the end of the incubation period, one unit of urease was added to 0.5 ml of the incubation medium. This was followed by a 30 minute incubation to allow the conversion of urea to ammonia before total ammonia concentration was measured. Another 0.5 ml of the incubation medium was taken for ammonia estimation without the addition of urease. Urea concentration was obtained by subtraction of the ammonia concentration without urease from the ammonia concentration with urease (Appendix 2, 2.2).

2.2.2.3 Uric acid excretion

50,000 sheathed or exsheathed L3 or adult worms (~6 mg wet wt) were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 5 hours. Tubes containing only phosphate buffer were used as controls. The concentration of uric acid in the incubation media was determined by measuring the absorption at 290 nm (Appendix 2, 2.5).

2.2.2.4 Amino acid excretion

50,000 sheathed or exsheathed L3 or adult worms (~6 mg wet wt) were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 5 hours. Tubes containing only phosphate buffer were used as controls. The concentrations of total amino acids for each of the incubation media were determined by the ninhydrin method (Magne and Larher, 1992) (Appendix 2, 2.4).

2.2.2.5 Protein excretion

50,000 sheathed or exsheathed L3 or adult worms (~6 mg wet weight) were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 5 hours. Tubes containing only phosphate buffer were used as controls. The concentrations of protein in the incubation media were determined by the Bradford assay (Bradford, 1976) (Appendix 2, 2.3).

2.2.3 UPTAKE OF AMINO ACIDS

The principle of the method was incubation of *O. circumcincta* with radiolabeled amino acids in a medium based on PBS (Appendix 3, 3.3), with or without, the addition of unlabeled amino acids, which would act as a competitor for uptake. After a specified incubation period, the parasites were separated from the medium and their radioactivity counted in a scintillation counter. Worm volume was estimated from the volume of distribution of $^{3}H_2O$ based upon the specific activity (dpm/µl) of the incubation medium. Separate incubations of parasites in PBS containing ^{14}C-mannitol were used to estimate the volume of extracellular water trapped with the worms.

2.2.3.1 Incubation media

The isotope stock solutions were: (1) [U-^{14}C]-protein hydrolysate (Amersham), 250 µCi, made up in PBS to specific activity 50 µCi/ml (2) [U-^{14}C]-mannitol (Amersham) in PBS, specific activity 1 mCi/ml (3) $^{3}H_2O$ (Amersham) specific activity 5 mCi/ml, made up in PBS to specific activity 25 µCi/ml. Unlabeled amino acids used were alanine, aspartate and glutamine, singly and in combinations, made up as a 10 mM solution in PBS.

2.2.3.2 Uptake in adult worms

In 3 experiments, amino acid uptake in adult worms was determined by incubating them either in medium containing [U-^{14}C]-protein hydrolysate and $^{3}H_2O$, or in medium containing [U-^{14}C]-protein hydrolysate, $^{3}H_2O$ and unlabeled amino acids for 30, 60 or 90 minutes. Worms were also incubated in medium containing [U-^{14}C]-mannitol and $^{3}H_2O$. Tubes were prepared with incubation media containing:

(1) 125 µl [U-^{14}C]-protein hydrolysate in PBS, 1.25µl $^{3}H_2O$, 123.75 µl PBS

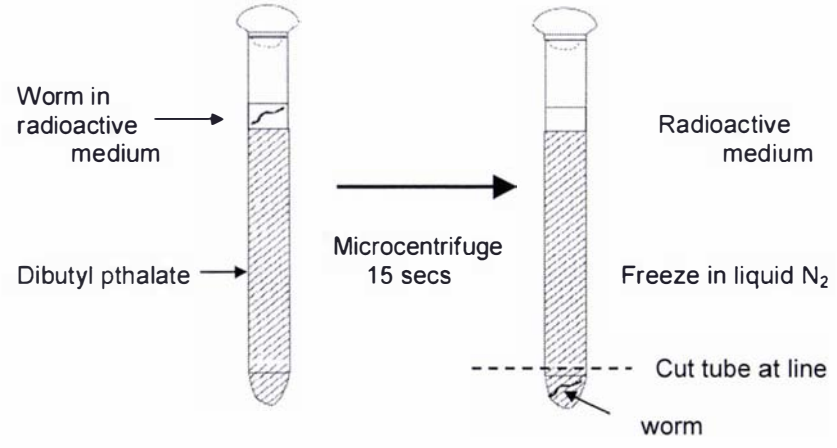


Figure 2.1. Diagrammatic representation of the procedure for separating *O. circumcincta* adult worms from residual medium by centrifugation through a dibutyl pthalate solution.

(2) 125 μl [U-^{14}C]-protein hydrolysate in PBS, 1.25 μl $^{3}H_2O$, 6.25 μl unlabeled amino acid, 117.5 μl PBS

(3) 6 μl ^{14}C-mannitol, 1.25 μl $^{3}H_2O$, 247.5 μl PBS

About 20 adult worms were centrifuged for a few seconds and as much fluid as possible removed. Incubation medium was added and the tubes were incubated at 37°C for the times specified below in individual experiments

Experiment 1: The incubation times were 30 and 90 minutes in [U-^{14}C]-protein hydrolysate plus $^{3}H_2O$ (medium 1), 90 minutes in tubes containing also unlabeled glutamine (medium 2) and 90 minutes in ^{14}C-mannitol (medium 3).

Experiment 2: The incubation time was 60 minutes. The unlabeled amino acids were glutamine, aspartate or glutamine + aspartate + alanine.

Experiment 3: The incubation time was 60 minutes. The unlabeled amino acids were glutamine, alanine or aspartate + glutamine + alanine.

After the incubation period, 10 female worms were individually removed from the medium and placed in separate tubes containing dibutyl pthalate solution for separation and counting as shown in Figure 2.1. Tubes were centrifuged for 15 seconds at 13,400 *g* then immersed in liquid nitrogen for 5 seconds to freeze the contents of the tube. The tip of the tube was cut near the bottom to release the parasites into a scintillation vial, while retaining the frozen liquid above it in the tube. Five ml of scintillation fluid (PCS Scintillation cocktail, Amersham) was added to the vial and the radioactivity determined in a liquid scintillation counter (Tri-Carb 2900TR, PerkinElmer).

Duplicate 2 μl aliquots of incubation medium were also retained after the incubation for determination of the specific radioactivity of the medium. Samples were also included containing only $^{3}H_2O$ or only ^{14}C so that the isotope crossover between channels in the scintillation counter could be calculated. Radioactivity was determined during 5 minutes for each sample on a dual-label programme for ^{3}H and ^{14}C.

2.2.3.3 Uptake in sheathed L3

A similar experiment was carried out on sheathed L3, with modifications because of their small size. About 50,000 L3 in 0.5 ml were placed in tubes, centrifuged briefly and as much fluid as possible removed. Media were added and the tubes incubated at 37°C. One tube from each series was removed at

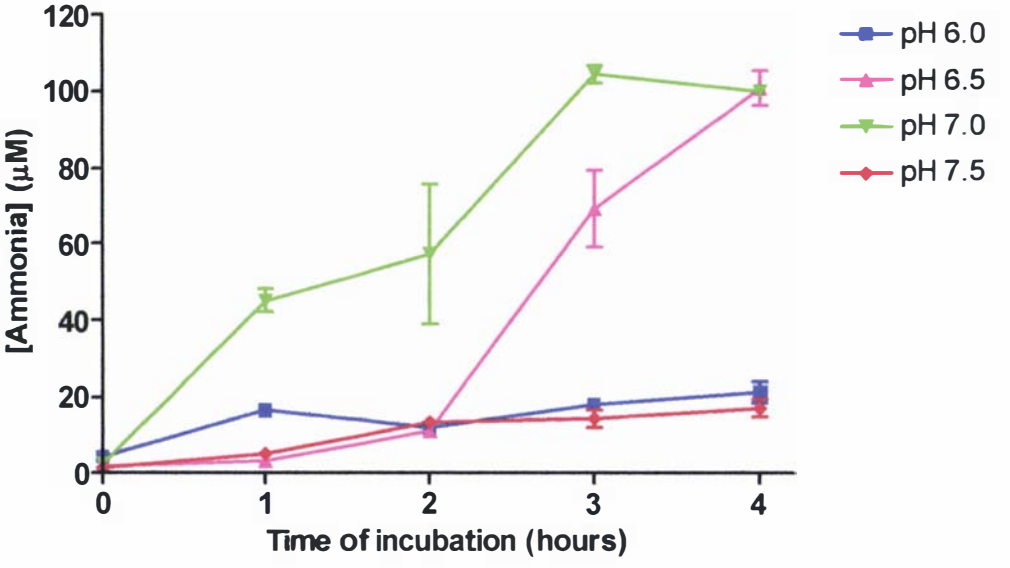


Figure 2.2. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed L3 in 1 ml 0.8 mM phosphate buffer of pH 6.0, 6.5, 7.0 and 7.5 at 37°C for 4 hours.

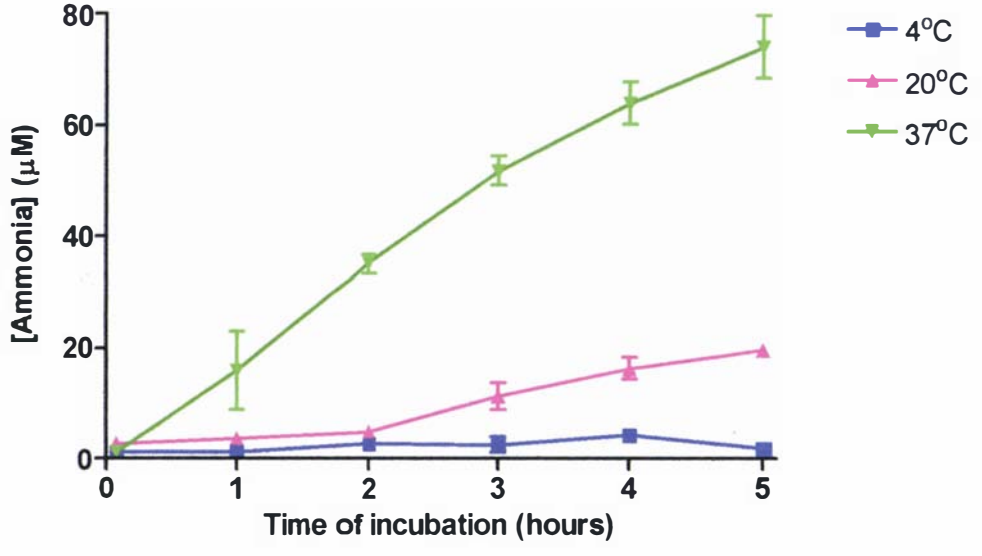


Figure 2.3. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed L3 in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 4°C or 20°C or 37°C for 4 hours.

each of 10, 30 and 90 minutes. L3 were incubated either in medium containing [U-^{14}C]-protein hydrolysate and [U-^{14}C]-mannitol or in medium containing only $^{3}H_2O$ for 10, 30 or 90 min. Tubes containing the two media were prepared:

(1) 125 µl [U-^{14}C]-protein hydrolysate, 1.25µl $^{3}H_2O$, 123.75 µl PBS

(2) 6 µl ^{14}C-mannitol, 1.25 µl $^{3}H_2O$, 247.5 µl PBS

For separation of the L3, 20 µl of the incubation medium containing the parasites were placed on top of a dibutyl pthalate solution and subsequently treated as for adult worms.

2.2.3.4 Calculation of amino acid uptake

The worm volume was calculated according to the formula:

Worm volume (nl) = (^{3}H dpm in worm / ^{3}H dpm in 1µl medium) x 1000

Amino acid uptake was corrected for differing amounts of ^{14}C-amino acids in each medium:

Uptake = ^{14}C-aa dpm in worm x (dpm in control medium/dpm in medium used)

2.2.3.5 Statistics

Uptakes in different media or at different times were compared using unpaired two-tailed student's t-test in Excel.

2.3 RESULTS

2.3.1 AMMONIA EXCRETION

2.3.1.1 pH of medium

The ammonia concentrations of the incubation media in which 50,000 L3 were incubated in 1 ml 0.8 mM phosphate buffer of pH 6.0, 6.5, 7.0 and 7.5 for 4 hours at 37°C are shown in Figure 2.2. The concentrations after 4 hours were 21.3 ± 2.7 µM at pH 6.0, 100.7 ± 4.5 µM at pH 6.5, 100.1 ± 0.5 µM at pH 7.0 and 17.2 ± 2.4 µM at pH 7.5 (all mean ± SEM, n = 3).

2.3.1.2 Incubation temperature

The ammonia concentrations of the incubation media in which 50,000 L3 were incubated in 1 ml 0.8 mM phosphate buffer, pH 7.0 for 5 hours at 4°C, 20°C and 37°C are shown in Figure 2.3. The concentrations after 5 hours

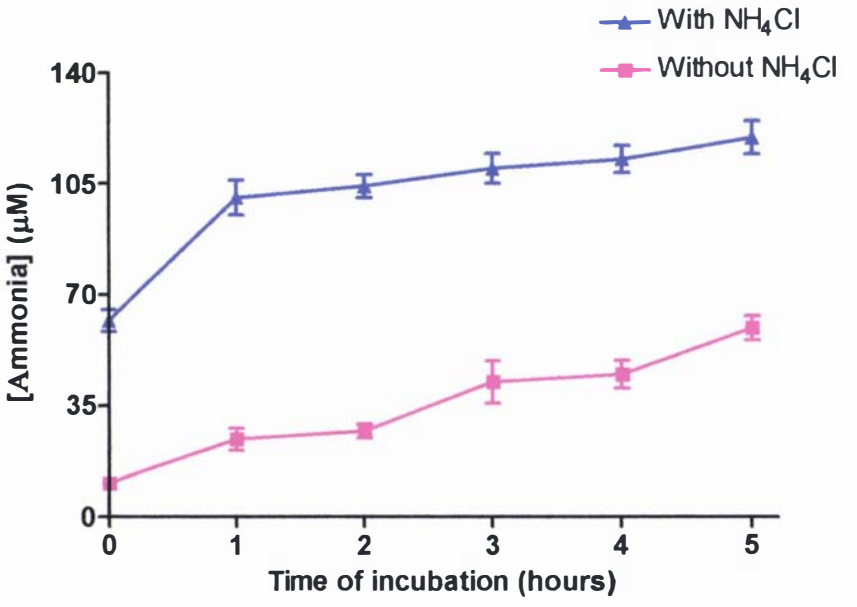


Figure 2.6. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed L3 in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 37°C for 5 hours, with and without the addition of 60 μM NH_4Cl.

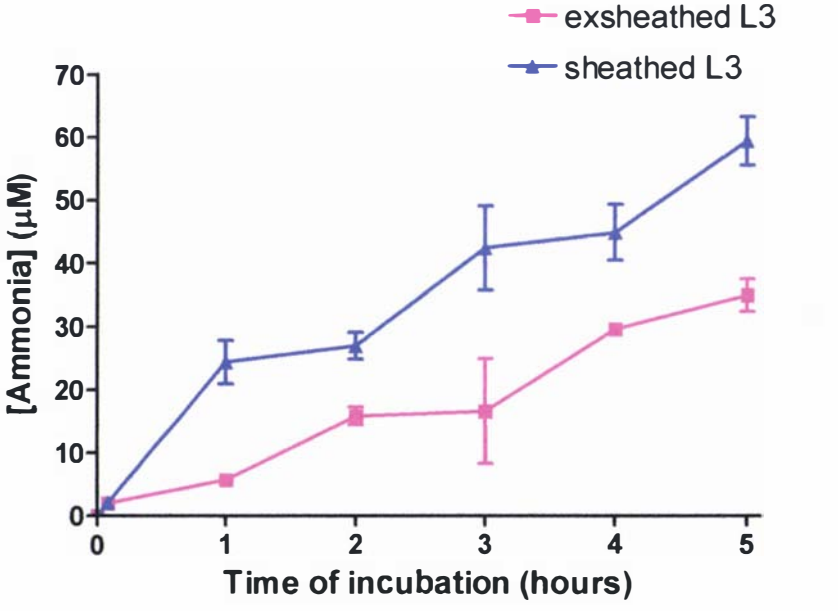


Figure 2.7. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed or exsheathed L3 in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 37°C for 5 hours.

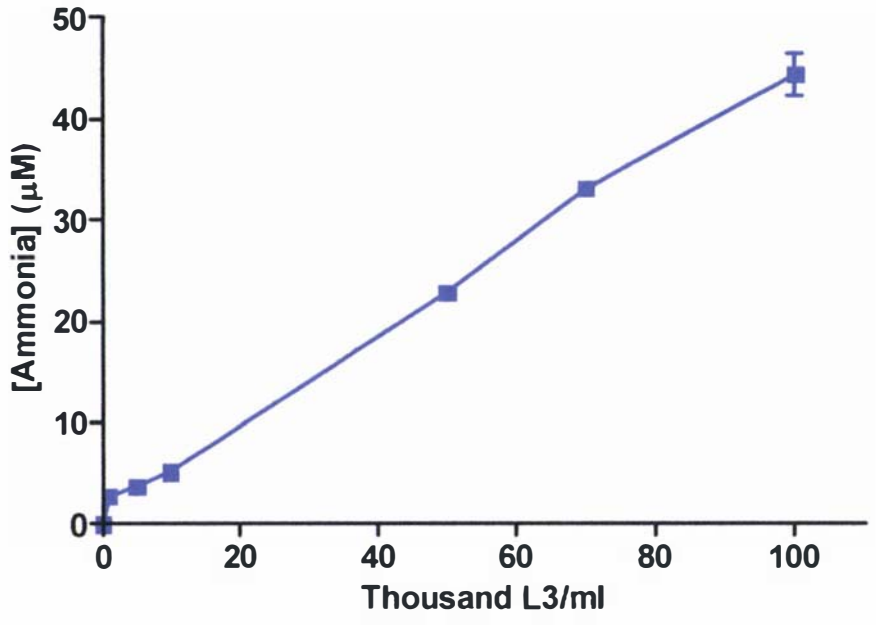


Figure 2.4. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) after the incubation for 2.5 hours of 5,000,10,000, 50,000, 70,000 and 100,000 sheathed L3 in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 37°C.

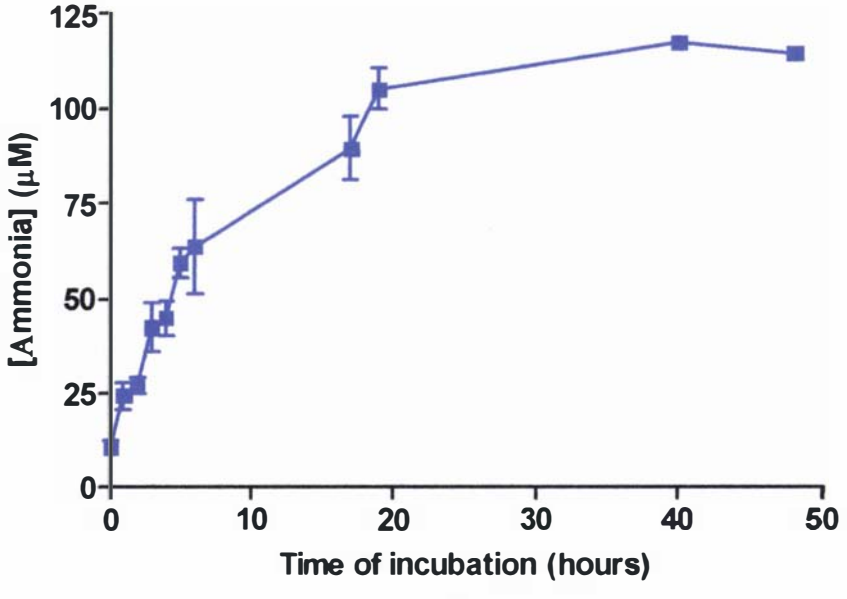


Figure 2.5. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed L3 in 1 ml 0.8mM phosphate buffer, pH 7.0 at 37°C.

were 4.6 ± 0.4 μM at 4°C, 19.7 ± 1.0 μM at 20°C and 74.0 ± 7.4 μM at 37°C (all mean ± SEM, n = 3).

2.3.1.3 Parasite density

The ammonia concentrations of the incubation media in which 1,000, 5,000, 10,000, 50,000, 70,000 or 100,000 L3 was incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 2.5 hours are shown in Figure 2.4. The concentrations after 2.5 hours were 2.8 ± 0.9 μM for 1,000 L3, 3.7 ± 0.7 μM for 5,000 L3, 5.2 ± 0.5 μM for 10,000 L3, 22.8 ± 0.9 μM for 50,000 L3, 33.1 ± 0.8 μM for 70,000 L3 and 44.3 ± 2.9 μM for 100,000 L3 (all mean ± SEM, n = 3).

2.3.1.4 Time of incubation

The ammonia concentrations of the incubation media in which 50,000 L3 were incubated in 0.8 mM phosphate buffer, pH 7.0 at 37°C for 48 hours are shown in Figure 2.5. The maximum concentration was 117 ± 0.7 μM (mean ± SEM, n = 3) after 40 hours. The rate of ammonia excretion was approximately linear at 0.2 nmoles $hour^{-1}$ $kworms^{-1}$ over the first 5 hours, after which it declined and became very low after 24 hours.

2.3.1.5 External ammonia concentration

The ammonia concentrations of the incubation media in which 50,000 L3 were incubated in 1 ml 0.8 mM phosphate buffer, pH 7.0 for 5 hours at 37°C, with and without the addition of 60 μM NH_4Cl, are shown in Figure 2.6. The overall increases in concentration in the medium after 5 hours were 58.0 ± 2.5 μM and 59.5 ± 3.8 μM (both mean ± SEM, n = 3), with and without NH_4Cl, respectively, however, the time courses differed. Starting at 60 μM NH_4Cl, the ammonia concentration in the medium rapidly rose to approximately 100 μM and slowly to 120 μM over 5 hours of incubation. With no ammonia in the initial buffer, the concentration in the medium rose steadily over the 5 hour period.

2.3.1.6 Exsheathed L3

Ammonia concentrations of incubation media in which 50,000 sheathed or exsheathed L3 from the same population were incubated for 5 hours in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 37°C are shown in Figure 2.7. The

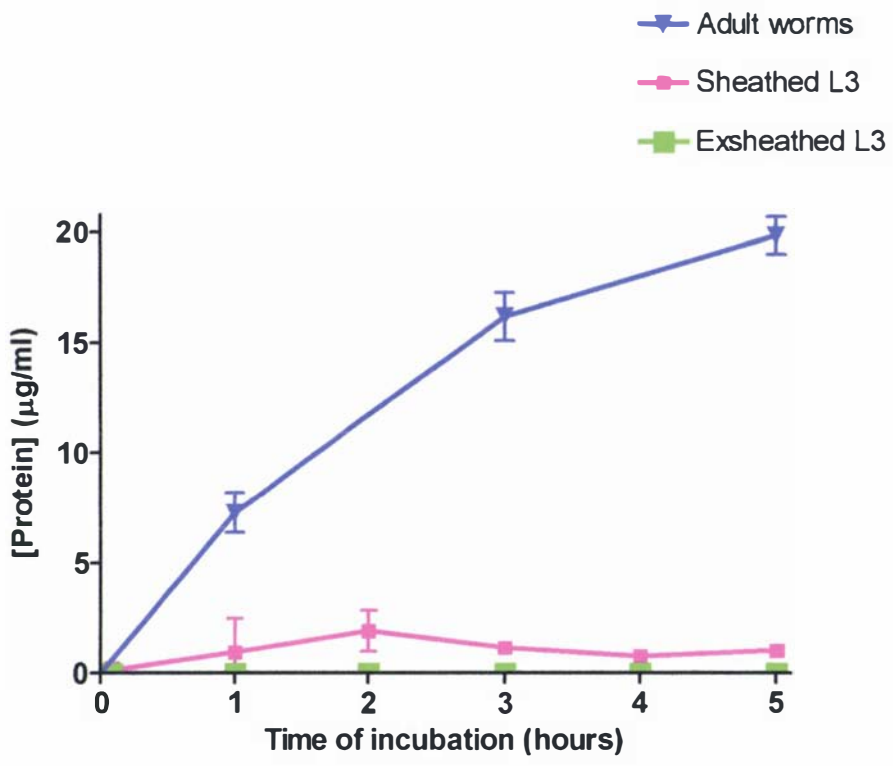


Figure 2.10. Protein concentrations in the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed L3 or adult worms (~6 mg wet weight) in 1ml 0.8 mM phosphate buffer, pH 7.0 at 37°C for 5 hours.

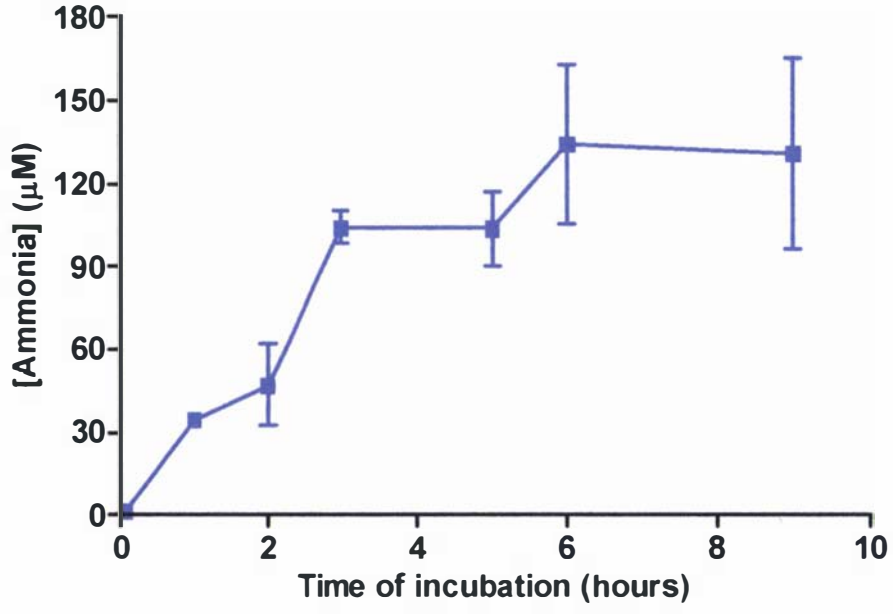


Figure 2.8. Ammonia concentrations of the incubation media (mean ± SEM, n = 3) during the incubation of adult worms (~ 6 mg wet weight) in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 37°C for 9 hours.

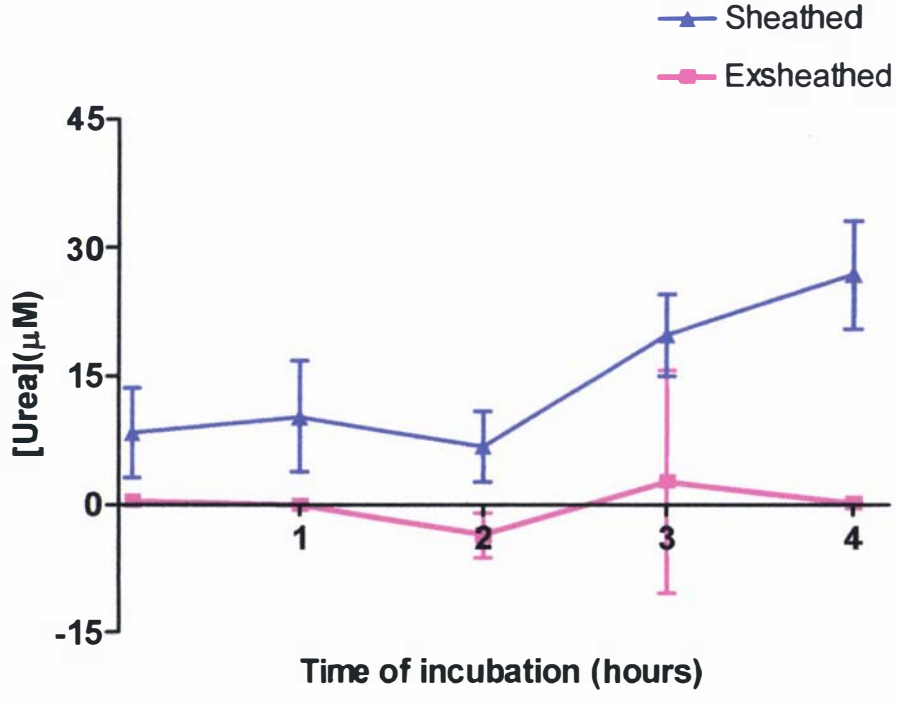


Figure 2.9. Urea concentrations in the incubation media (mean ± SEM, n = 3) during the incubation of 50,000 sheathed or exsheathed L3 in 1ml 0.8 mM phosphate buffer, pH 7.0 at 37°C for 4 hours.

concentrations after 5 hours were 59.5 ± 4.2 μM and 35.0 ± 2.5 μM (mean ± SEM, n = 3) for sheathed and exsheathed L3 respectively.

2.3.1.7 Adult worms

The ammonia concentrations of the incubation media in which adult worms (~6 mg wet weight) were incubated in 1 ml 0.8 mM phosphate buffer, pH 7.0, for 9 hours at 37°C are shown in Figure 2.8. The ammonia concentration after 6 hours was 130.7 ± 34.5 μM (mean ± SEM, n = 2).

2.3.2 UREA EXCRETION

The urea concentrations of the incubation media in which 50,000 sheathed or exsheathed L3 were incubated in 1 ml 0.8 mM phosphate buffer, pH 7.0 at 37°C for 4 hours are shown in Figure 2.9. The urea concentrations after 4 hours were 26.8 ± 6.2 μM and 0.2 ± 0.6 μM (both mean ± SEM, n = 3) for sheathed and exsheathed L3 respectively.

2.3.3 URIC ACID EXCRETION

No uric acid excretion was detected either by sheathed L3 (n = 3) or adult worms (n = 3).

2.3.4 AMINO ACID EXCRETION

No amino acid excretion was detected either by sheathed L3 (n = 3) or adult worms (n = 3).

2.3.5 PROTEIN EXCRETION

The protein concentrations of the incubation media in which 50,000 sheathed L3 or adult worms (~6 mg wet weight) were incubated in 1 ml 0.8 mM phosphate buffer pH 7.0 at 37°C for 4 hours are shown in Figure 2.10. The concentrations after 4 hours were 1.0 ± 0.1 μg/ml and 19.9 ± 6.6 μg/ml (both mean ± SEM, n = 3) for sheathed L3 and adult worms respectively. No protein excretion was detected for exsheathed L3.

2.3.6 AMINO ACID UPTAKE

In none of the experiments was there significant uptake of amino acids from the [U-^{14}C]-protein hydrolysate at the 5% level, although mean values suggested there was a trend towards uptake. Uptakes by adult worms in the 3

Table 2.1. Uptake of amino acids (mean ± SEM, n), expressed as adjusted disintegrations per minute, by adult *O. circumcincta* in three experiments in which they were incubated with a [U-^{14}C]-protein hydrolysate in PBS. All tubes contained $^{3}H_2O$ in addition to the labeled amino acids. Media in which unlabeled 0.2 mM amino acids were included are indicated.

Expt	Unlabeled amino acid	Time (min)	^{14}C-aa uptake (mean ± SEM (n))	% control	*p* value
1	-	30	14.2 ± 3.0 (7)	100	
	-	90	29.8 ± 8.9 (7)	211	0.12 (vs 1)
	glutamine	90	38.9 ± 12.2 (9)	196	0.58 (vs 2)
2	-	60	56.5 ± 19.5 (7)	100	
	glutamine	60	30.9 ± 4.8 (8)	55	0.20
	aspartate	60	43.8 ± 9.0 (7)	75	0.51
	gln, ala, asp	60	38.4 ± 5.9 (8)	68	0.56
3	-	60	98.1 ± 14.6 (9)	100	
	alanine	60	39.5 ± 12.6 (9)	40	0.23
	glutamine	60	38.2 ± 9.0 (9)	39	0.36
	gln, ala, asp	60	43.7 ± 17.1 (7)	45	0.33

experiments are summarised in Table 2.1. In Expt 1, uptake at 90 minutes was about 2.4 times that at 30 minutes (p = 0.11). Unlabeled amino acids caused a moderate, but again not significant, decrease. In Expt 2, all incubations were for 60 minutes. There was no indication that glutamine affected uptake, but uptake was reduced to 75% of control by the addition of unlabeled aspartate and to 68% of control by a mixture of aspartate, glutamine and alanine. In Expt 3, glutamine appeared to reduce the uptake of ^{14}C-aa to 39% of control, as did alanine (40% of control). Uptake was similar, but not significantly different, when aspartate, alanine and glutamine were added simultaneously (45% of control).

The single experiment on sheathed L3 gave no indication of amino acid uptake. The uptakes (mean ± SEM (n)) for 10 minutes were 3386 ± 833 dpm (6), 3150 ± 402 dpm (6) for 30 minutes and 3127 ± 814 dpm (5) for 90 minutes respectively. Relative to uptake at 10 minutes, uptake at 30 minutes was 135% and at 90 minutes was 97%, p = 0.80 and 0.83 respectively.

Worm volumes calculated from the volume of distribution of $^{3}H_2O$ were 73 ± 7.6 nl (23), 54 ± 6.6 nl (24) and 42 ± 3.1 nl (30) (mean ± SEM (n)) for the adult worms in Expts 1-3 respectively. The volumes of individual L3 were calculated to be 0.6 ± 0.06 nl (mean ± SEM, n = 20). The data resulting from incorporation of group worm volumes (estimated from $^{3}H_2O$ distribution) into the calculation of ^{14}C-aa uptake had an equally large SEM as earlier data, therefore were not included.

2.4 DISCUSSION

The principal finding in the excretion experiments was that the main nitrogenous product which accumulated in the phosphate buffer in which either L3 or adult *O. circumcincta* were incubated was ammonia, with urea as a minor component. No uric acid or amino acids were detectable in the incubation medium. Very preliminary studies of uptake of radiolabeled amino acids from a protein hydrolysate suggested that adult worms, but not sheathed L3, are able to take up amino acids. During the uptake studies, the volumes of adult worms and L3 were estimated from the volumes of distribution of $^{3}H_2O$. Adult worm volumes varied considerably in the three populations measured (means of 42, 54 and 73 nl), approximately 100 times that of the L3 (0.6 nl). These

values are consistent with estimates from worm measurements of length and radius (Assoc Prof K.C. Pedley, personal communication).

2.4.1 AMINO ACID UPTAKE

The amino acid uptake studies reported here were inconclusive, possibly due to the large SEM of the counts in each experiment, however, in some experiments large differences in mean values may be indicative of uptake of amino acids, at least in adult worms. Comparison of mean counts in Expt 1 (Table 2.1) showed the count at 90 minutes was 211% that at 30 minutes and decreased to 196% by the inclusion of cold glutamine. The availability only of a mixture of labeled amino acids, rather than individual labeled amino acids, was not ideal for these studies. Also, contributions to the variability would have been caused by variation in worm size and perhaps by the time delay in placing the last worms in incubation tubes. Estimation of worm volume did show there were marked differences in size of individual worms both within and between populations, however inclusion of volume in the calculation did not reduce the SEM. A possible cause of this variation might be the accessibility of the gut. In future experiments, it might be more accurate to measure simultaneously the extracellular volume of each worm with [^{3}H]-mannitol and uptake with ^{14}C-aa.

Reports of amino acid uptake in other nematode parasites would suggest that amino acid permeases are likely also to be present in *O. circumcincta*. Given the huge number of amino acid transporters which has been identified in all types of organisms (reviewed by White, 1985; Horak, 1986; Williams and Miller, 2001; Wipf *et al.*, 2002; Verrey *et al.*, 2004) and the range of amino acids carried on individual transporters, an exhaustive study was beyond the scope of this project.

The routes of absorption and forms in which nitrogenous substances are taken in by the free-living and adult stages of *O. circumcincta* are likely to be varied and may depend on availability in the environment and may not be identical in free-living and parasitic nematodes. Possible sources of nutrients for the parasitic forms include mucins, cells, leaked interstitial fluid, abomasal digesta, including microorganisms, whereas the free-living larval stages feed on bacteria and their products. The presence of proteases in the ES products of L3, L4 and adult *O. circumcincta* (Young *et al.*, 1995) supports the

breakdown of protein to peptides and/or amino acids to provide nutrients for all life-cycle stages.

Absorption may occur across the cuticle and through the intestine in adult worms. The cuticle of *A. galli* adult worms, in which body openings were occluded by celloidin, was permeable to both ^{14}C-alanine and ^{14}C-glucose and additional amounts were ingested and absorbed by non-coated worms (Weatherly *et al.*, 1963). [^{3}H]-leucine and [2-^{3}H]-glucose were taken up across the cuticle of larval *B. pahangi*, which did not feed during the experiment (Chen and Howells, 1979). Although these studies have shown parasitic nematodes have the capacity to transport amino acids, in *C. elegans*, the intestinal peptide transporter PEP-2 (but not PEP-1) was essential for normal growth, stress tolerance and reproduction and provision of amino acids in the medium could not restore these functions in PEP-2 deficient mutant worms (Meissner *et al.*, 2004).

2.4.2 NITROGEN EXCRETION

The observations on excretion/secretion are in agreement with previous reports that both free-living and parasitic nematodes are predominantly ammonotelic and that urea excretion is usually not greater than about 20% of the total nitrogen excretion (Rogers, 1952; Rothstein, 1963, 1970; Wright, 1975a,b). There were appreciable concentrations of protein in the medium in which adult *O. circumcincta* that had been incubated (mean 20 µg/ml after 5 hours), but very little protein appeared to be released by sheathed L3 (1 µg/ml after 5 hours) and undetectable amounts by bleach-exsheathed L3 (Figure 2.10).

The solutions in which nematodes have been incubated are usually designated excretory/secretory (ES) products because of the difficulty in determining whether the individual chemical components are true excretory products, secretions, lost cuticular components or substances which have leaked across the cuticle. *A. suum* releases cuticular collagen *in vitro* during development from L3 to L4 (Rhoads *et al.*, 2001). ES products are antigenic and both provoke and modify the host immune response (Lee and Hodsden, 1963; Ogilvie *et al.*, 1973; Cox *et al.*, 1990; Young *et al.*, 1995; Gamble and Mansfield, 1996; Redmond *et al.*, 1997; Sajid and McKerrow, 2002). Proteases (Skuce *et al.*, 1999; Sajid and McKerrow, 2002; Williamson *et al.*,

2003) and glycosidases (Gamble and Mansfield, 1996; Irwin *et al.*, 2004) may provide nutrients for the parasite. Peptides and proteins in adult worm ES products may be secretions from glands or excreted through the gut as well as of cuticular origin, whereas the small amount of protein lost by the non-feeding L3 is likely to be released from the cuticle.

There was no evidence of amino acid excretion over five hours of incubation, suggesting that the parasites were not stressed by the experimental conditions, at least for that time. The appearance of amino acids in the medium in which *P. redivivus* was being incubated was associated with environmental stress (Wright, 1975b). Recovery of significant amounts of amino acids from incubation media was attributed by Rothstein (1970) to passive leakage of amino acids through the body wall and gut.

Ammonia, and to a lesser extent urea, appear to be the forms in which nitrogen is excreted by *O. circumcincta*, whereas protein loss probably represents secretion and not excretion. Excretion of ammonia by sheathed L3 was linearly related to worm density (Figure 2.4) and was temperature dependent: very low at 4°C and much greater at 37°C than at 20°C (Figure 2.3). Excretion appeared to have an optimal medium pH of 7.0 (Figure 2.2). Temperature and pH may affect either the metabolic rate of the parasite or the excretory process. There are examples of both modes of action. Metabolism is temperature dependent and slows at low temperatures, as nematodes cannot regulate their internal temperature. Ammonia excretion is affected by environmental pH in the marine worm *Sipunculus nudus* as pH plays an important role in the selection of amino acids catabolised (Langenbuch and Pörtner, 2002). On the other hand, branchial ammonia excretion is decreased in rainbow trout with increases in pH of the gill boundary layer (Salama *et al.*, 1999).

An interesting observation was the approach towards a maximum concentration in the medium of approximately 120-140 µM after 24 hours incubation of 50,000/ml sheathed L3 (Figure 2.5) and after 6 hour incubation of 6 mg/ml adult worms (Figure 2.8). It is difficult to compare the excretion rates in L3 and adults because of the 100-fold difference in volumes, however, adult worms appear to excrete ammonia at a higher rate. The external ammonia concentration did not exceed 120 µM, even when the initial external concentration in which L3 were incubated was 60 µM (Figure 2.6). The

ammonia concentration in the medium rapidly rose to approximately 100 µM and slowly to 120 µM over 5 hours of incubation, in contrast to the situation for the L3 placed in ammonia free buffer, where the concentration in the medium rose steadily over the 5 hour period to 60 µM (Figure 2.6).

This maximum concentration of ammonia in incubation media is very low in comparison to the concentration of ammonia in arterial blood (200 µM), portal blood (487 µM) (Parker *et al.*, 1995), the rumen (around 5 mM) and abomasal fluid (1 mM) of the sheep to which the parasitic stages are exposed (Harrop, 1974; Harrop and Phillipson, 1974). The concentrations will vary with the diet of the sheep (Harrop and Phillipson, 1974). The explanation for the low concentration of ammonia in the incubation medium may be that ammonia may either absorbed or excreted according to the external concentration, which *in vivo* could frequently result in ammonia absorption for parasitic stages of *O. circumcincta*.

Whereas mammals have an obligatory urinary excretion and those in positive nitrogen balance excrete large amounts of urea, excretory rates may be very low in dormant L3 or rapidly growing and reproducing parasitic stages of nematodes. The observed excretion of ammonia by both L3 and adult *O. circumcincta* into 0.8 mM phosphate buffer could result from the reversal of the normal ammonia gradient and therefore excretion rather than absorption. High affinity ammonia permeases in plants, bacteria and yeasts are believed to scavenge ammonia from the environment, including recovering ammonia lost by diffusion when growing on sources of nitrogen other than ammonia (Marini *et al.*, 1997). Even in high external ammonia concentrations, plants and microorganisms maintain low cytoplasmic ammonia levels (Streeter, 1989; Roberts and Pang, 1992), either by incorporation into glutamine via glutamine synthetase (GS) or by trapping ammonia in organelles e.g. plants trap ammonia in vacuoles, so that they maintain a very low cytoplasmic ammonia concentration (<150 µM) at an external concentration of 1 mM (Roberts and Pang, 1992). GS has been proposed to fulfill a similar role during early development in rainbow trout to prevent toxic levels of ammonia building up by converting it to glutamine (Essex-Fraser *et al.*, 2005).

The reduced rate of ammonia excretion (Figure 2.7) and negligible urea excretion (Figure 2.9) by bleach-exsheathed L3 compared with sheathed L3 probably is the result of damage to the cuticle caused by the chemically

induced exsheathing process. This reduction in excretion may indicate that, as in fresh water fish, ammonia is excreted both via passive NH_3 diffusion and NH_4^+ permeases (Wilkie, 2002). Since ammonia can cross membranes either as the unprotonated form or be carried by ammonium transporters (Kleiner, 1981), the lower rate may be caused by loss of functioning transporters for ammonium ions, as diffusion would not to be affected by damage to the membrane. The reduced movement through the damaged cuticle would support urea being transported through specific channels and ammonium transport by permeases making a significant contribution to total ammonia loss. In the non-feeding L3, all excretion is via the cuticle or specialised excretory cell (Buechner *et al.*, 1999; Buechner, 2002), whereas in adult worms, both cuticular and intestinal exchange is likely to contribute to excreted urea and ammonia.

2.4.3 AMMONIA AND UREA PRODUCTION

Ammonia can be released from amino acids and other compounds by many enzymes including glutamate dehydrogenase, glutaminase, urease and aspartase, whereas urea can be generated by the full ornithine-urea cycle or by arginase, agmatinase or creatinase. Some of these enzymes are not generally seen in animals, but are worthy of investigation, as there are reports of unusual features of metabolism in nematodes. An example is urease activity, which was detected in *Nematodirus* spp and *A. lumbricoides* intestine (less from other tissues), but not in *H. contortus* (Rogers, 1952).

The possible sources of excreted ammonia and urea will be examined in enzyme studies to be reported in the following Chapters. In Chapter 3, activities of some of the enzymes which generate urea have been measured as part of a study on arginine and urea metabolism in adult and L3 *O. circumcincta*.

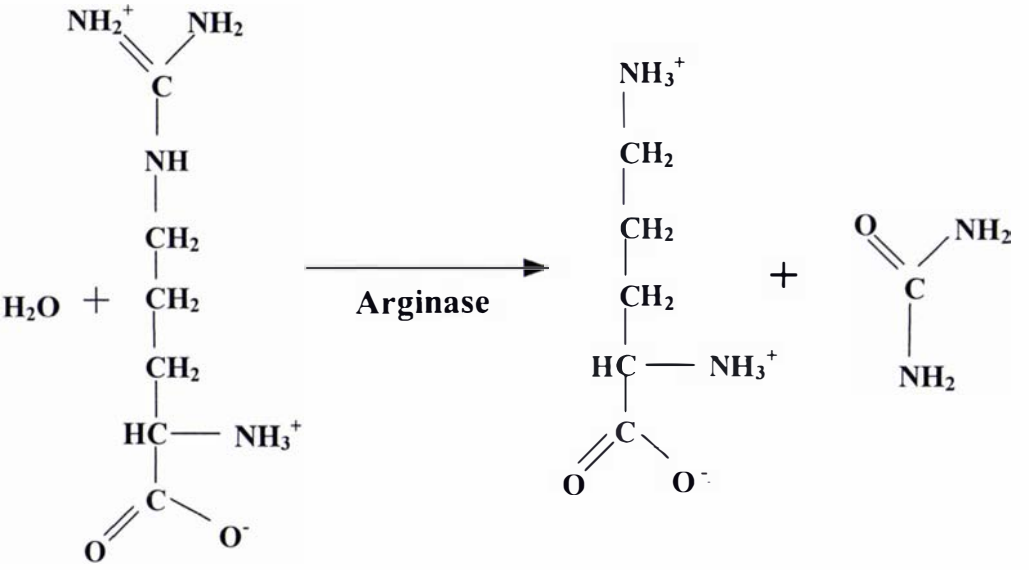
Chapter 3

METABOLISM OF ARGININE AND UREA BY *OSTERTAGIA CIRCUMCINCTA*

3.1 INTRODUCTION

O. circumcincta has been shown in the experiments reported in Chapter 2 to excrete predominantly ammonia and some urea. There are many possible sources of the ammonia, some of which will be discussed in Chapters 4 and 5. Ammonia is generated mainly by the removal of amino groups from amino acids by transamination followed by oxidative deamination, (principally by GDH), but also by deiminases or amino acid oxidases. Grantham and Barrett (1986a) demonstrated all classes of these enzymes in the rat parasite *H. polygyrus* and the free living nematode *P. redivivus*, activities of some of which are reported in later Chapters. In this Chapter, the activities in *O. circumcincta* of some of the enzymes involved in urea and arginine metabolism are reported.

The small amount of urea excreted by *O. circumcincta* does not necessarily indicate a functioning *O*UC, but rather could be produced by mitochondrial arginase, or other enzymes such as creatinase, which degrades creatine to urea and sarcosine (Chapter 1, 1.4.2), or agmatinase, which hydrolyses agmatine to form putrescine and urea (Chapter 1, 1.5.4). The urea cycle enzymes are not universally present in invertebrates, as argininosuccinate synthetase and lyase are usually absent and only arginase and *O*TCase have significant activity (Janssens and Bryant, 1969; De Cian *et al.*, 2000). Based on previous studies in nematodes, it is unlikely that *O. circumcincta* excretes urea generated by the OUC. Grantham and Barrett (1986a) failed to find a complete urea cycle in *H. polygyrus* and *P. redivivus*, as was also reported for other helminths (Janssen and Bryant, 1969). Even when all enzymes were present, the activities of some were very low. This was the case in the nematode *P. redivivus* (Wright, 1975a) and the trematode *F. gigantica* (Mohamed *et al.*, 2005).



arginine ornithine urea

Figure 3.1. The reaction catalysed by arginase.

As well as its involvement in the OUC, L-arginine is a central amino acid for protein synthesis, synthesis of creatine, generating ornithine for polyamine metabolism and formation of NO (Section 1.5). Arginine can also be interconverted with glutamate and proline (Figure 1.7). Proline is likely to be in heavy demand for collagen synthesis in growing and moulting nematodes and it may also be readily available to parasites living in association with gastrointestinal mucus. This suggests that the arginine-proline-glutamate pathway might be important in *O. circumcincta*, but whether it functions predominantly in the proline catabolic or anabolic direction is uncertain. Both proline catalytic enzymes were present in *H. polygyrus* and *P. redivivus*, as were P5CR and OAT (Grantham and Barrett, 1986b). OAT activity is present in *N. brasiliensis* (Walker and Barrett, 1991a) as well as in the trematode *S. mansoni* (Goldberg *et al.*, 1979).

3.1.1 ARGINASE (EC 3.5.3.1)

Arginase, the enzyme which catalyses the hydrolysis of arginine to urea and L-ornithine (Figure 3.1), is present in bacteria, yeasts, plants, invertebrates and vertebrates (Jenkinson *et al.*, 1996). In higher vertebrates, the two isoforms are products of separate genes and have differences in their functional properties, as well as being immunologically distinct and having different locations in the cell: arginase I in the cytosol and arginase II in the mitochondria (Ash, 2004; Cederbaum *et al.*, 2004). In mammals, the liver type arginase I is the final enzyme in the OUC (Figure 1.3), whereas arginase II is responsible for the synthesis of ornithine, polyamines, proline, creatine, glutamate and agmatine (Wu and Morris, 1998).

Jenkinson *et al.* (1996) have published an extensive review of the structure and properties of arginases from organisms in all kingdoms. Arginases have a bivalent metal ion requirement for catalytic activity, usually the physiological activator Mn^{2+}, although Co^{2+} and Ni^{2+}, and in some instances Fe^{2+}, VO^{2+} and Cd^{2+} will suffice (reviewed by Ash, 2004). The *H. pylori* arginase has optimal activity with cobalt as the metal cofactor and an acidic pH optimum of 6.1, proposed as adaptations to living in the acidic gastric contents and availability of Co^{2+} in cobalamin (McGee *et al.*, 2004). The Mn^{2+} required for stability and activation of arginase is often removed by dialysis during the purification process, so that the enzyme needs activation by preincubation with $MnCl_2$ to form a Mn^{2+}-enzyme complex (Hirsch-Kolb *et al.*,

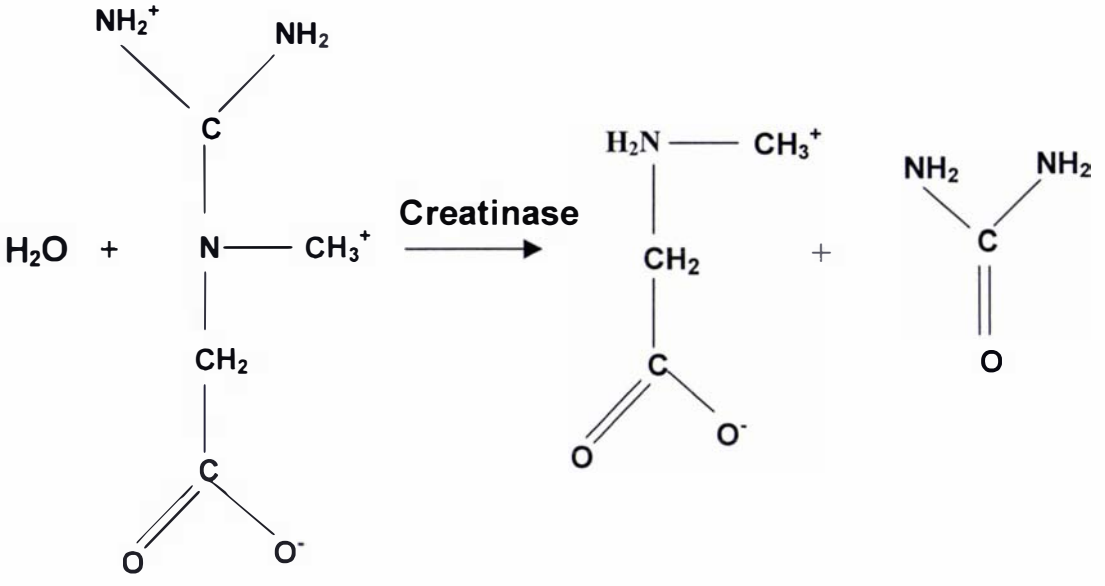


Figure 3.2. The reaction catalysed by creatinase.

1971). The Mn^{2+} is tightly bound to the enzyme and not always lost in crude preparations, nor displaced by chelators (Reczkowski and Ash, 1994).

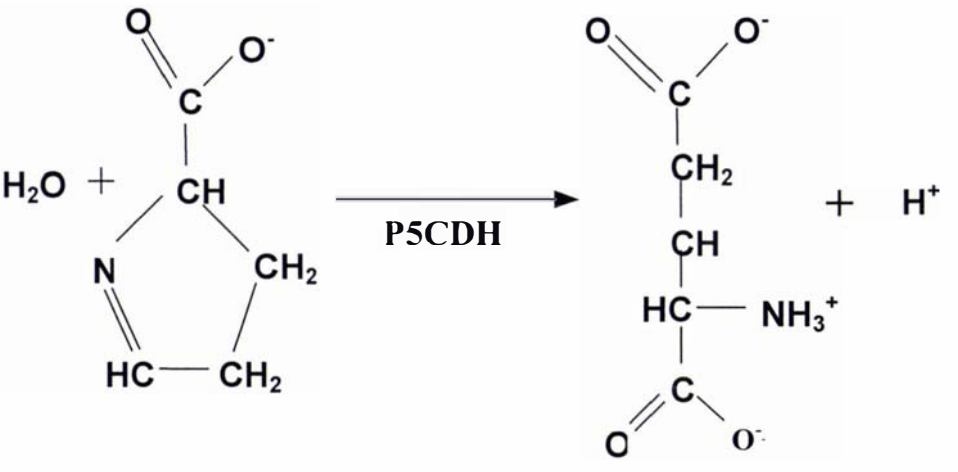
Many arginases have pH optima between pH 9 and 10, although a small number have lower pH optima: pH 6.1 for *H. pylori* (McGee *et al.*, 2004), pH 5.9 for bovine liver, 6.5-7.2 for rabbit liver and pH 7 for rat kidney (Jenkinson *et al.*, 1996). The *F. hepatica* arginase has been recently purified and characterised by Mohamed *et al.* (2005): the pH optimum was 9.5, Mn^{2+} was the best activator and the K_m for arginine was 6 mM. For many species, the values for the K_m for arginine varied from very low to very high: invertebrate arginases from 2-160 mM and mammalian enzymes from 0.5 to 45 mM. Jenkinson *et al.* (1996) concluded that an ionisable group at the catalytic site is responsible for the very basic pH optima and consequent high K_m values. Measurement of enzyme activities at around pH 7.4 resulted in values of 1-2 mM for rat liver arginase, lower by a factor of 2-3 times that at high pH (Maggini *et al.*, 1992).

3.1.2 CREATINASE (EC 3.5.3.3)

Microorganisms have creatinases, enzymes which catalyse the reaction of creatine with water to produce sarcosine with urea as the by-product (Wyss and Kaddurah-Daouk, 2000). (Figure 3.2). Despite the general belief that this enzyme is not present in vertebrates, there are two reports from Miyoshi *et al.* (1980a,b) that creatinase was present in human muscle and displayed altered properties in Duschenne muscular dystrophy patients. Most kinetic data are available from bacteria, particularly *Pseudomonas putida.* This enzyme is most active at pH 8, stable at pH 6-8 and has maximal activity at a temperature of 30°C and pH of 7.5-8.0 (Yoshimoto *et al.*, 1976). K_m values for creatine were 1.3-25 mM for *Pseudomonas* and 17.2 mM for *Alcaligenes*, in contrast with the data from the human enzyme, which had a pH optimum of 6.2 and K_m of 0.08 and 0.36 mM for normal subjects and muscular dystrophy patients respectively (Miyoshi *et al.* 1980b). Creatinase is not metal dependent.

3.1.3 UREASE (EC 3.5.1.5)

Urease is a plant, algal, fungal and microbial enzyme which converts urea and water to ammonia and CO_2 (reviewed by Mobley and Hausinger, 1989). The gastric pathogen *H. pylori* uses urease to liberate ammonia from



Δ[1]-pyrroline-5-carboxylate glutamate

Figure 3.3. Reaction catalysed by Δ[1]-pyrroline-5-carboxylate dehydrogenase (P5CDH).

urea to neutralise gastric acid (Mobley and Hausinger, 1989; Mégraud *et al.*, 1992). Although urease is generally not considered an animal enzyme, it has been reported to be present in cestodes (Simmons, 1960) and in the intestine of the nematodes *Nematodirus* spp and *A. lumbricoides* (with less in other tissues) (but not in *H. contortus*) (Rogers, 1952) and in invertebrates (cited by Simmons, 1960). Dietary urease is taken into the haemolymph in a functional form in the silkworm (Sumida *et al.*, 1995; Hirayama *et al.*, 2000; Sugimura *et al.*, 2001; Kurahashi *et al.*, 2005), where it maintains a low level of urea.

3.1.4 Δ^1-PYRROLINE-5-CARBOXYLIC ACID DEHYDROGENASE (EC 1.5.1.12)

Proline is catabolised to P5C by the enzyme proline oxidase (dehydrogenase); P5CDH catalyses the reaction by which P5C is converted to glutamate (Figure 3.3). Both of these proline catalytic enzymes were present in the nematodes *H. polygyrus* and *P. redivivus*, as were the enzymes in the ornithine-proline pathway, OAT and P5CR (Grantham and Barrett, 1986b). The expression of proline catabolic enzymes and/or synthetic enzymes will vary according to whether proline is being predominantly degraded or synthesised. Trematodes may differ from nematodes in this respect, as proline synthesis dominates and large amounts of proline are excreted into host tissues, hence neither Kurelec (1975) nor Ertel and Isseroff (1976) were able to detect P5CDH activity in *F. hepatica* homogenates. The latter study was conducted at pH 7.2–8.3 and used 0.28 mM P5C and 0.12 mM NAD^+, conditions under which enzyme activity was present in rat liver.

The experiments reported in this Chapter investigated the properties of arginase in adult and L3 *O. circumcincta*, as arginase activity may be a significant regulatory point for the competing pathways using arginine as a substrate. In addition to urea being generated by arginase, another possible source is creatinase an enzyme not reported from animals. Urease has been detected in some invertebrates and, if present, might degrade urea to ammonia, the main nematode excretory product. Therefore, the possibility of either or both of these enzymes was examined. Because of the demand for proline for cuticle formation in the growing and moulting nematode and the rich source of proline in gastro-intestinal mucus, the activity of P5CDH was determined.

3.2 MATERIALS AND METHODS

Parasites for enzyme assays were provided as detailed in Appendix 1. L3 were cultured from the faeces of infected sheep, stored at 4°C and, prior to each experiment, were Baermannized to remove inactive worms (Appendix 1, 1.5). Adult worms were recovered from the abomasa of infected sheep (Appendix 1, 1.4).

All chemicals were supplied by Sigma Chemical Co. (Mo, USA) unless specified.

3.2.1 HOMOGENATE PREPARATION

Enzyme activities were determined on homogenates prepared from sheathed L3 or adult *O. circumcincta* (Appendix 2, 2.6). The amount of homogenate used for the enzymes assays was based on its protein content, which was determined by the Bradford method (Bradford, 1976) (Appendix 2, 2.3), so that an amount of homogenate containing 50 µg protein was used for each assay.

3.2.2 ARGINASE

Arginase was assayed according to the general protocol for continuous assays described in Appendix 2, 2.7. The activity was monitored through coupled reactions with urease and glutamate dehydrogenase (GDH):

$$\text{arginine} + H_2O \xrightarrow{\text{arginase}} \text{ornithine} + \text{urea}$$

$$\text{urea} + H_2O \xrightarrow{\text{urease}} 2\,NH_4^+ + CO_2$$

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{GDH}} \text{glutamate} + NAD^+ + H_2O$$

The assay for arginase was a coupled assay with urease and GDH as used by Özer (1985). A preliminary assay to determine the optimum pH was conducted in duplicate at 30°C on L3 homogenate SL1. The reaction mixture contained 100 mM 2-oxoglutarate, 1 unit of urease, 1 unit of GDH and 50 µg homogenate protein prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 5.5 to 9.0 in a total volume of 1 ml. Subsequently,

0.2 mM NADH was added and the reaction was initiated by adding 5 mM arginine. Arginase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH, which was monitored spectrophotometrically at 340nm.

3.2.2.1 Kinetic parameters

To determine K_m and V_{max}, homogenates of sheathed L3 (n = 3) or adult worms (n = 2) were assayed in a reaction mixture containing 10 mM 2-oxoglutarate, 1 unit of urease, 1 unit of GDH and 50 µg homogenate protein prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 8.0 at 30°C in a total volume of 1 ml. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding arginine. Arginase activity was monitored with concentrations of arginine from 0-5 mM for L3 homogenates and 0-20 mM for adult worm homogenates.

3.2.2.2 Effectors/inhibitors

Each of the following was investigated in duplicate for effects on the rate of arginase activity of sheathed L3 homogenate SL4:

(1) 0.1 mM Fe^{2+} as $FeSO_4$

(2) 0.1 mM or 1.0 mM Mn^{2+} as $MnSO_4$

(3) 0.1 mM or 1.0 mM Cu^{2+} as $CuSO_4$

(4) 0.1 mM or 1.0 mM EDTA

Reaction mixtures described in Section 3.2.2.1 were made up with each of the test compounds. After the addition of 0.2 mM NADH, 5 mM arginine was added to initiate the reaction.

3.2.3. CREATINASE

Creatinase was assayed according to the general protocol for continuous assays described in Appendix 2, 2.7. The activity was monitored through coupled reactions with urease and GDH:

$$\text{creatine} + H_2O \xrightarrow{\text{creatinase}} \text{sarcosine} + \text{urea}$$

$$\text{urea} + H_2O \xrightarrow{\text{urease}} 2\,NH_4^+ + CO_2$$

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{GDH}} \text{glutamate} + NAD^+ + H_2O$$

The assay for creatinase was a coupled assay with urease and GDH as used by Özer (1985). A preliminary assay to determine the optimum pH was conducted in duplicate at 30°C on L3 homogenate SL5. The reaction mixture contained 50 mM 2-oxoglutarate, 2 mM EDTA, 1 unit of urease, 1 unit of GDH and 50 µg homogenate protein prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 5.5 to 9.0 in a total volume of 1 ml. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM creatine. Creatinase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH, which was monitored spectrophotometrically at 340nm.

3.2.3.1 Kinetic parameters

To determine K_m and V_{max}, homogenates of sheathed L3 (n = 3) or adult worms (n = 2) were assayed in a reaction mixture containing 50 mM 2-oxoglutarate, 2 mM EDTA, 1 unit of urease, 1 unit of GDH and 50 µg homogenate protein prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5 at 30°C in a total volume of 1 ml. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding creatine. Creatinase activity was monitored for concentrations of creatine from 0-20 mM for sheathed L3 and 0-8 mM for adult worms. An aliquot of one homogenate (SL6) was also assayed after storage for 24 hours at 4°C.

3.2.3.2 Effectors/inhibitors

Each of the following was investigated in duplicate for effects on the rate of creatinase activity of sheathed L3 homogenate SL9:

(1) 0.5 mM Fe^{2+} as $FeSO_4$

(2) 0.5 mM Fe^{3+} as $FeCl_3$

(3) 0.5 mM Cu^{2+} as $CuSO_4$

(4) 0.5 mM Mg^{2+} as $MgSO_4$

(5) 0.5 mM ADP

(6) 0.5 mM ATP

(7) 2.0 mM EDTA

Reaction mixtures described in Section 3.2.3.1 were made up with each of the test compounds. After the addition of 0.2 mM NADH, 5 mM creatine was added to initiate the reaction.

3.2.4 UREASE

Urease was assayed according to the general protocol for continuous assays described in Appendix 2, 2.7. The activity was monitored by a coupled reaction with GDH adapted from Özer (1985):

$$\text{urea} + H_2O \xrightarrow{\text{urease}} 2\,NH_3 + CO_2$$

$$2\text{-oxoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{GDH}} \text{glutamate} + NAD^+ + H_2O$$

To determine K_m and V_{max}, homogenates of sheathed L3 (n = 3) or adult worms (n = 2) were assayed in a reaction mixture containing 20 mM 2-oxoglutarate, 1 unit of GDH and 50 µg homogenate protein prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5 at 30°C in a total volume of 1 ml. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding urea in concentrations from 0-10 mM. Urease activity was calculated from the rate of NADH utilisation, through the oxidation of NADH, which was monitored spectrophotometrically at 340nm.

3.2.5 PYRROLINE-5-CARBOXYLATE DEHYDROGENASE

P5CDH was assayed according to the general protocol for continuous assays described in Appendix 2, 2.7. The assay, adapted from Small and Jones (1990), monitored the rate of NAD^+ utilisation in the reaction:

$$P5C + NAD^+ + H_2O \xrightarrow{\text{P5CDH}} \text{glutamate} + \text{NADH} + H^+$$

To determine K_m and V_{max}, homogenates of sheathed L3 (n = 3) were assayed in a reaction mixture containing 100 µg homogenate protein prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5 at 30°C in a total volume

Table 3.2. Arginase activities of adult *O. circumcincta* homogenates with increasing concentration of arginine. Arginase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Arginine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A1a	0.8	124
A1b	0.7	130
mean ± SEM, n = 2	0.7 ± 0.1	126 ± 3

Table 3.3. Arginase activities (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL4 in the presence of metal ions or EDTA. Arginase activity in the absence of added compounds was set at 100%.

Effector/inhibitor	Concentration (mM)	Activity (% control)
-	-	100
Mn^{2+}	0.1	106 ± 1
Mn^{2+}	1.0	113 ± 11
Fe^{2+}	0.1	46 ± 1
Cu^{2+}	0.1	117 ± 16
Cu^{2+}	1.0	72 ± 6
EDTA	0.1	111 ± 6
EDTA	1.0	78 ± 4

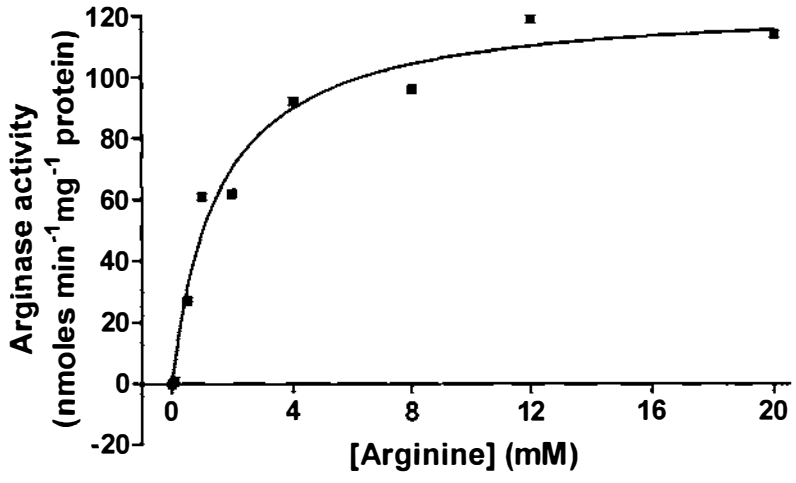


Figure 3.6. Arginase activity of adult *O. circumcincta* homogenate A1a with increasing concentration of arginine. Arginase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340 nm.

Table 3.1. Arginase activities of sheathed L3 *O. circumcincta* homogenates with increasing concentration of arginine. Arginase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in a coupled assay with urease and GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Arginine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL2a	0.4	26
SL2b	0.3	28
SL3	0.5	41
mean ± SEM, n = 3	0.4 ± 0.1	32 ±4

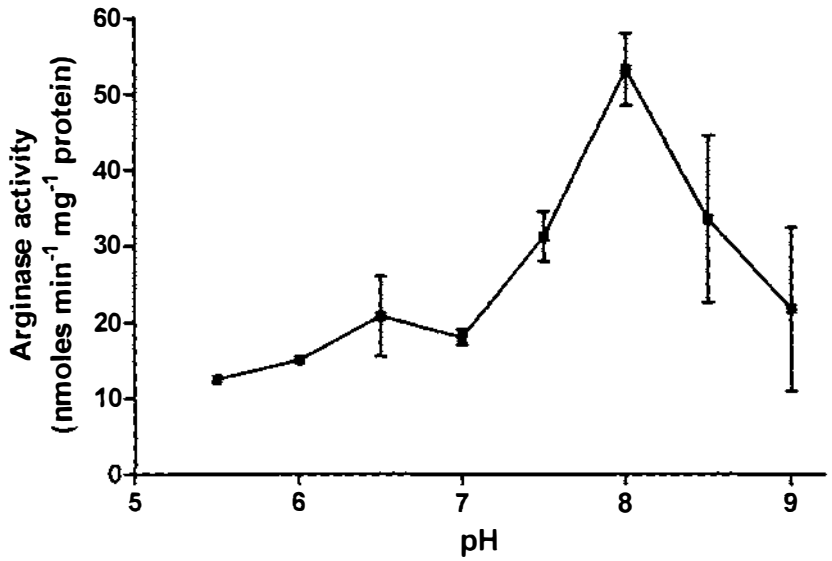


Figure 3.4. Effect of pH on arginase activity (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL1. The concentration of arginine used was 5 mM. Arginase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340 nm.

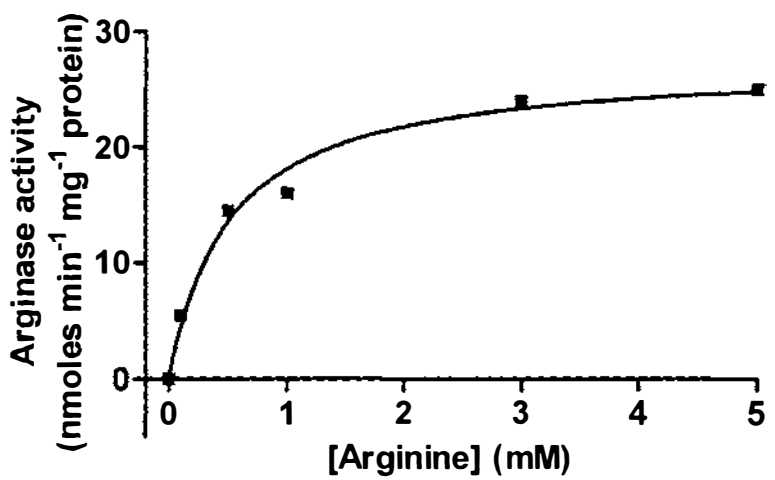


Figure 3.5. Arginase activity of sheathed L3 *O. circumcincta* homogenate SL2a with increasing concentration of arginine. Arginase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340 nm.

of 1 ml. Subsequently, 0.2 mM NAD^+ was added and the reaction was initiated by adding 1-pyrroline-5-carboxylate in concentrations from 0-10 mM. The activity of P5CDH was estimated from the rate of NADH production, which was monitored spectrophotometrically at 340nm.

3.3 RESULTS

3.3.1 ARGINASE

3.3.1.1 Effect of pH

The effect of pH on the arginase activity (mean ± SEM, n = 2) of sheathed L3 homogenate SL1 is shown in Figure 3.4. The optimum pH was pH 8 at which the activity was 53 ± 5 nmoles min^{-1} mg^{-1} protein.

3.3.1.2 Kinetic parameters

The arginase activities of homogenates of sheathed L3 (SL2a) and adult worms (A1a) are shown in Figures 3.5 and 3.6 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 3.1 and 3.2. For the substrate arginine, K_m values were 0.4 ± 0.1 mM and 0.7 ± 0.1 mM (mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2). The V_{max} were 32 ± 4 and 126 ± 3 nmoles min^{-1} mg^{-1} protein (mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively.

3.3.1.3 Effectors/inhibitors

Arginase activities of a homogenate of sheathed L3 (SL4) in the presence of various concentrations of metal ions and EDTA are shown in Table 3.3. Arginase activity was expressed as a percentage of the value in the absence of added compounds, which was set at 100%.

The addition of 0.1 mM Fe^{2+} had the greatest inhibitory effect, reducing the activity to 46 ± 1% (mean ± SEM, n = 2). The addition of 0.1 and 1.0 mM Mn^{2+} had slight effects with activities measured of 106 ± 1% and 113 ± 11% (both mean ± SEM) respectively. Copper ions were slightly stimulatory at 0.1 mM but had the opposite effect at 1 mM: the activities were 117 ± 16%and 72 ± 6% of control (both mean ± SEM, n=2) respectively. Activities with 0.1 and 1 mM EDTA were 111 ± 6% and 78 ± 4% of control (both mean ± SEM, n = 2) respectively.

Table 3.5. Creatinase activity of adult *O. circumcincta* homogenate A2 with increasing concentration of creatine. Creatinase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340nm.

Homogenate	Creatine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A2a	0.3	24
A2b	0.4	23
mean ± SEM, n = 2	0.3 ± 0.1	23 ± 1

Table 3.6. Creatinase activities (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL9 in the presence of metal ions, ADP, ATP or EDTA. Creatinase activity in the absence of added compounds was set at 100%.

Effector/Inhibitor	Concentration (mM)	Activity (% control)
-	-	100
Fe^{2+}	0.5	96 ± 2
Fe^{3+}	0.5	17 ± 2
Cu^{2+}	0.5	11 ± 1
Mg^{2+}	0.5	30 ± 2
ATP	0.5	94 ± 4
ADP	0.5	101 ± 2
EDTA	2.0	123 ± 2

Table 3.4. Creatinase activities of sheathed L3 *O. circumcincta* homogenates with increasing concentration of creatine. Creatinase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340nm.

Homogenate	Creatine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL6a	0.1	21
SL6b*	0.7	17
SL7	0.3	22
SL8	0.1	23
mean ± SEM, n = 3	0.2 ± 0.07	22 ± 1

* Assayed after storage at 4°C for 24 hours, not included in mean.

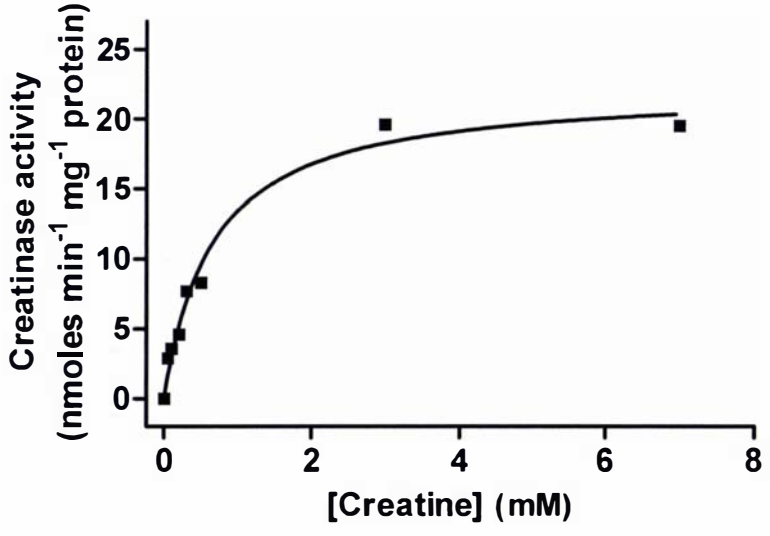


Figure 3.9. Creatinase activity of adult *O. circumcincta* homogenate A2a with increasing concentration of creatine. Creatinase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340nm.

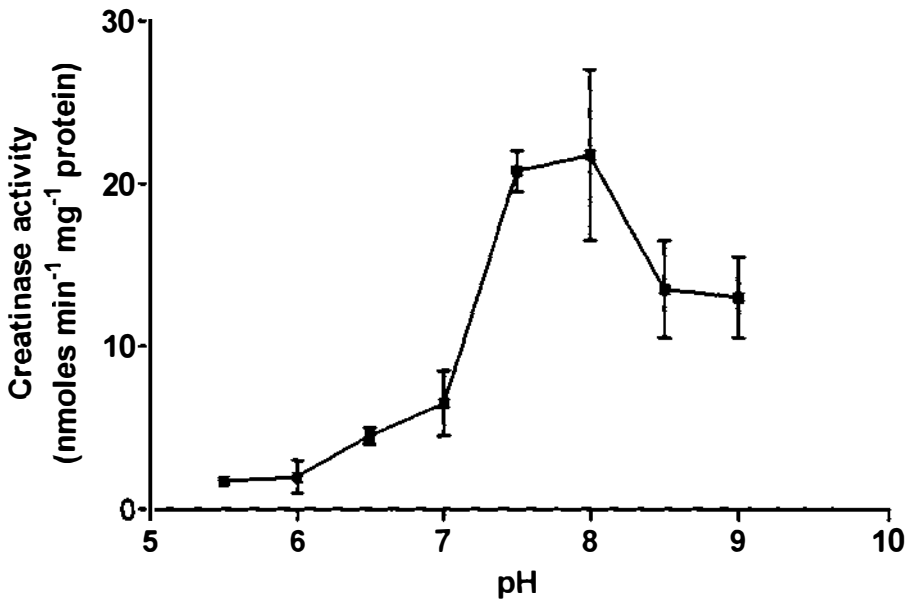


Figure 3.7. Effect of pH on creatinase activities (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL5. Creatinase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340 nm.

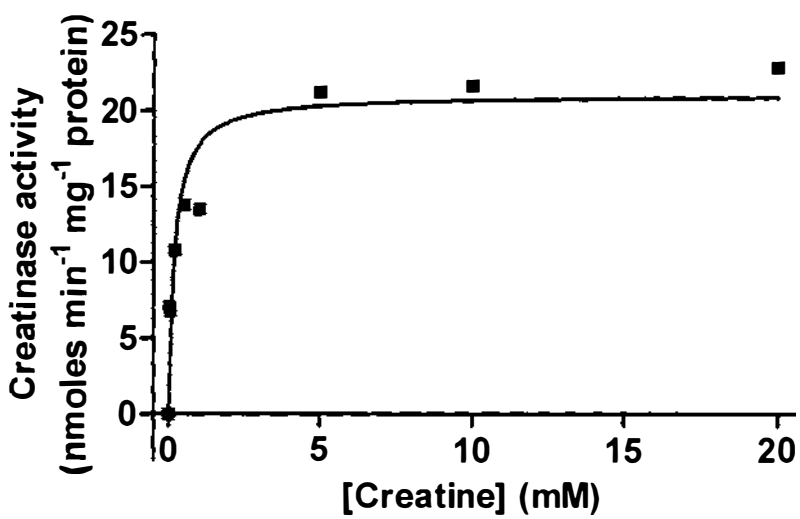


Figure 3.8. Creatinase activity of sheathed L3 *O. circumcincta* homogenate SL6 with increasing concentration of creatine. Creatinase activity was calculated from the rate of NADH utilisation, through the oxidation of NADH in coupled assays with urease and GDH, which was monitored spectrophotometrically at 340nm.

3.3.2 CREATINASE

3.3.2.1 Effect of pH

The effect of pH on the creatinase activity (mean ± SEM, n = 2) of a homogenate of sheathed L3 (SL5) is shown in Figure 3.7. The optimum pH was pH 7.5-8 at which the activity was 23 ± 6 nmoles min^{-1} mg^{-1} protein (mean ± SEM).

3.3.2.2 Kinetic parameters

The creatinase activities of homogenates of sheathed L3 (SL6) and adult worms (A2a) are shown in Figures 3.8 and 3.9 respectively. Values were similar for adult worms and L3. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 3.4 and 3.5. For the substrate creatine, the K_m values were 0.2 ± 0.07 mM and 0.3 ± 0.1 mM (mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively. The V_{max} were 22 ± 1 and 23 ± 1 nmoles min^{-1} mg^{-1} protein (mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively.

Storage of one homogenate at 4°C for 24 hours did not alter the V_{max} but the calculated K_m was slightly greater at 0.7 mM.

3.3.2.3 Effectors/inhibitors

Creatinase activities of homogenates of sheathed L3 in the presence of metal ions, ATP and ADP are shown in Table 3.6. Creatinase activity was expressed as a percentage of the value in the absence of added compounds, which was set at 100%.

Three metal ions were inhibitory: 0.5 mM Fe^{3+} in the form of $FeCl_3$ reduced the activity to 17 ± 2%, 0.5 mM Cu^{2+} in the form of $CuSO_4$ to 11 ± 1% and 0.5 mM Mg^{2+} in the form $MgSO_4$ to 30 ± 2% (all mean ± SEM, n = 2).

3.3.3 UREASE

No urease activity was detected in homogenates of L3 (SL10-12) or adult worms (A3-4).

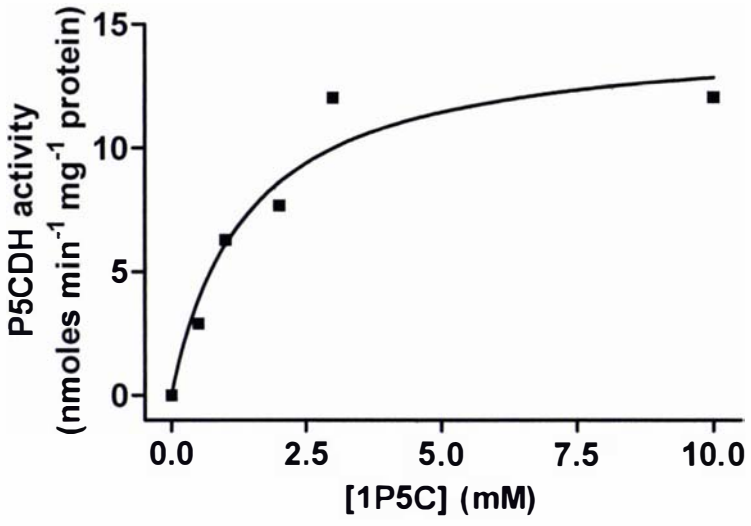


Figure 3.10. Pyrroline-5-carboxylate dehydrogenase (P5CDH) activity of sheathed L3 *O. circumcincta* homogenate SL13b with increasing concentration of 1-pyrroline-5-carboxylate. P5CDH activity was calculated from the rate of NAD^+ utilisation, which was monitored spectrophotometrically at 340nm.

Table 3.7. Pyrroline-5-carboxylate dehydrogenase activities of sheathed L3 *O. circumcincta* homogenates with increasing concentration of 1-pyrroline-5-carboxylate. P5CDH activities were calculated from the rate of NAD^+ utilisation, which was monitored spectrophotometrically at 340nm.

Homogenate	1-P5C K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL13a	1.4	15
SL13b	2.5	16
SL14	3.0	18
mean ± SEM, n = 3	2.3 ± 0.5	16 ± 0.8

3.3.4 PYRROLINE-5-CARBOXYLATE DEHYDROGENASE

The P5CDH activity of a homogenate of sheathed L3 (SL13b) is shown in Figure 3.10 and the K_m and V_{max} of the three homogenates in Table 3.7. For the substrate 1-pyrroline-5-carboxylate, the K_m was 2.3 ± 0.5 mM and V_{max} was 16 ± 0.8 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n = 3).

3.4 DISCUSSION

Arginase and creatinase activities have been demonstrated in both L3 and adult *O. circumcincta*, P5CDH was present in L3 (but not examined in adult worms), but no urease activity was found in either life-cycle stage. Kinetic data were collected to determine the importance of the enzymes being expressed in different life cycle stages as well as to predict the possible direction of metabolic pathways. The properties of these enzymes will be compared with those reported from other species, particularly mammals, to determine if there are major differences in the nematode enzymes which would identify them as potential targets to be exploited for drug development. Differences in metabolic pathways between nematodes and other species may also be useful in understanding parasite biology.

3.4.1 ARGINASE

Arginase activity was approximately 4 times higher in homogenates of adult *O. circumcincta* than in L3 homogenates (mean ± SEM of 126 ± 3 and 32 ± 4 nmoles min^{-1} mg^{-1} protein respectively) (Tables 3.1 and 3.2). It is not clear whether this represents upregulation of the enzyme in adult worms or reflects changing structure of the parasites. It would be reasonable for upregulation of arginase in parasitic stages to reduce the availability of arginine to host NOS, a common pathogen strategy to limit NO production (Vincendeau *et al.*, 2003). However, it probably is not valid to compare enzyme activities per mg protein of whole parasite homogenate in different life cycle stages. If the activity of a particular enzyme in both L3 and adult worms is high only in one tissue, such as the gut, anatomical changes as the parasite develops from one stage to another may change the activity per mg of protein in the homogenate of the whole parasite. Most notable are the development of the reproductive tract in adults and the relative reduction in cuticle mass, both of which would contribute additional protein but would dilute the gut enzyme.

There appears to be no change in the isoforms (if more than one) during development, as the K_m for arginine was similar in adult worms and L3 (0.7 ± 0.1 and 0.4 ± 0.1 mM respectively) (Tables 3.1 and 3.2). The most likely isoform is arginase II, which is usually a mitochondrial enzyme responsible for agmatine formation (Wu and Morris, 1998; Ash, 2004; Cederbaum *et al.*, 2004). Arginase II differs from arginase I, the cytosolic isoform found mainly in the liver of vertebrates, where it is the final enzyme of the OUC. These isoforms have different properties, such as a requirement for a lower concentration of Mn^{2+} and lower pH optimum for human arginase II than arginase I (Iyer *et al.*, 1998; Cederbaum *et al.*, 2004) and induction by different stimuli in mouse macrophages (Louis *et al.*, 1999). The pH optimum of 8.0 for the *O. circumcincta* enzyme (Figure 3.4) is consistent with it being the arginase II form. Most non-vertebrates appear to have only one isoform of arginase, e.g. the polychaete *Pista pacifica* (O'Malley and Terwilliger, 1974), the mollusc *Semele solida* which has a cytosolic enzyme (Carvajal *et al.*, 1994) and *Drosophila melanogaster* (Samson, 2000), although there are multiple forms of arginase in *Neurospora crassa*, which is unusual for a microorganism (Marathe *et al.*, 1998) and there were both a minor multimeric form of *F. hepatica* arginase and a major monomeric form (Mohamed *et al.*, 2005). In an earlier study, arginase was determined to be cytosolic in *F. hepatica* (Kurelec, 1975).

Maintenance of the quaternary structure and catalytic activity of arginases are dependent on the binding of a metal ion, usually Mn^{2+} (Green *et al.*, 1990; Jenkinson *et al.*, 1996; Ash, 2004). The *H. pylori* enzyme is Co^{2+} dependent (McGee *et al.*, 2004), which is suggested to be an adaptation to the availability of cobalamin in the gastric juice. In crude homogenates, arginase activity is not always dependent on additional Mn^{2+}, as the enzyme appears to retain bound Mn^{2+}, although activity is not maximal. This was the case for *H. diminuta* and several other cestodes and trematodes (except *H. citelli*) (Campbell and Lee, 1963), crustaceans (Hird *et al.*, 1986), insect fat body (Reddy and Campbell, 1969), the polychaete *P. pacifica* (O'Malley and Terwilliger, 1974) and the mollusc *Dreissena polymorpha* (Tormanen, 1997). In the experiments on *O. circumcincta* arginase, no added Mn^{2+} was required for activity and the increase in activity when 1 mM Mn^{2+} was added was very minor at only 13% (Table 3.3).

Table 3.8. K_m values for arginases of different organisms.

Source	K_m (mM)	Reference
Human (cytosol)	18.5	Basch *et al.* (1997)
Human (mitochondria)	49.5	Basch *et al.* (1997)
Human (arginase II)	0.3	Colleluori *et al.* (2001)
Rat liver	1.6	Maggini *et al.* (1992)
Buffalo liver	42	Dabir *et al.* (2005)
Genypterus maculatus (fish) liver	9.1 (pH 7.5) 11.5 (pH 9.5)	Carvajal *et al.* (1987)
Vajna catjang (plant)	2	Dabir *et al.* (2005)
S. cerevisiae	15.7	Green *et al.* (1990)
N. crassa	131	Borkovich and Weiss (1987)
H. pylori	1.8	McGee *et al.* (2004)
Pheretima communissima (earthworm) gut	12 (pH 9.5) 54 (pH 7.5)	Iino and Shimadate (1986)
S. solida (mollusc) (cytosol)	8.5 (pH 9.5) 20 (pH 7.5)	Carvajal *et al.* (1994)
P. pacifica (polychaete)	155-160	O'Malley and Terwilliger (1974)
Insect fat body (3 species)	2.4-9.1	Reddy and Campbell (1969)
Plasmodium falciparum	13	Müller *et al.* (2005)
F. hepatica	6	Mohamed *et al.* (2005)

O. circumcincta arginase activity was reduced by 22% by 1 mM EDTA (Table 3.3), but clearly retained adequate bound metal ion for activity. Tormanen (1997) reported a similar inhibition of 30% by 1 mM EDTA of arginase from *D. polymorpha*. Both Kuhn *et al.* (1991) and Recznowski and Ash (1994) used assays in which no added Mn^{2+} was required to measure arginase activity in rat and mouse liver respectively, and chelators, including EDTA, did not inhibit the enzymes. This was believed to be the result of tightly bound metal, which was inaccessible to added chelators. In studies of purified enzymes, to obtain maximum activity, or sometimes any activity, the enzyme must be pre-activated with $MnCl_2$, because the metal is removed by dialysis or treatment with solvents during the purification. These activated enzymes usually have pH optima in the range pH 9-10. The properties of these activated enzymes are different from those of enzymes studied at pH 7-8 and higher Mn^{2+} concentration (Kuhn *et al.*, 1991) and could be responsible for the very high V_{max} and K_m reported in some studies (Jenkinson *et al.*, 1996). The activity and K_m measured at pH 8 are more likely to be physiologically relevant, but lower than at very basic pH. Measurement of enzyme activities at physiological pH resulted in values of 1-2 mM for rat liver arginase, lower by a factor of 2-3 times that at high pH (Maggini *et al.*, 1992).

Comparison of the kinetic properties of *O. circumcincta* arginase with those of other organisms (Table 3.8) is very difficult because of the great variability of published values, even in the same species, for the reasons discussed above (Jenkinson *et al.*, 1996). *O. circumcincta* arginase activity was pH dependent, being 5-fold higher at the optimal pH of 8 compared with those at pH 5.5 or 9. Only a few reported enzymes have an optimum pH in the physiological range, e.g. pH 6.8 for human arginase II (Iyer *et al.*, 1998; Cederbaum *et al.*, 2004) and 6.1 for *H. pylori* (McGee *et al.*, 2004). Many kinetic studies of arginases are carried out in the presence of high added Mn^{2+}, which generally raises the pH optimum and V_{max}. It is not clear whether added Mn^{2+} or measurement at unphysiologically high pH (which both alter the enzyme stability) would always affect the K_m; it was altered in some studies (see Table 3.8), but not others, e.g. in arginase from *D. polymorpha* the K_m was similar with and without metal ions, although not the same with each ion (Tormanen, 1997).

Table 3.9. Effects of various inhibitors on *P. putida* creatinase activity (Yoshimoto *et al.*, 1976). Abbreviations: DFP: diisopropylfluorophosphate; EDTA: ethylenediaminetetraacetate sodium salt; NBS: *N*-bromosuccinimide; PCMB: *p*-chloromercuribenzoate.

Inhibitor	Concentration (mM)	Inhibition (%)
DFP	1	30
EDTA	2	10
NBS	1	30
PCMB	0.5	100
$HgCl_2$	1	100
$ZnCl_2$	1	100
$CuSO_4$	1	100
$CoCl_2$	1	71
$MgSO_4.7H_2O$	1	31
$CaCl_2$	1	32
Pb acetate	1	15
$FeCl_2$	1	15

The effects on arginase activity of other metal ions are also species dependent, e.g. cadmium can be either stimulatory (Reddy and Campbell, 1969; Patchett *et al.*, 1991; Caraval *et al.*, 1994) or inhibitory (Caraval *et al.*, 1984, 1996; Tormanen, 1997, 2006). The two metal ions tested in the present study were both inhibitory: Fe^{2+} inhibited the enzyme by 54% and Cu^{2+} by 28% at 1 mM (Table 3). Fe^{2+} was also inhibitory to *F. hepatica* arginase, along with five other metal ions (Mohamed *et al.*, 2005) and Cu^{2+} was more inhibitory to rat arginase I than arginase II (Tormanen, 2001) but completely inhibited human arginase II (Colleluori *et al.*, 2001). Overall, the properties of *O. circumcincta* arginase were those expected of an arginase II isoform, particularly requiring a small amount of metal ion for activity and a pH optimum around neutral.

3.4.2 CREATINASE

The identification of creatinase activity in both L3 and adult *O. circumcincta* was unexpected, as this enzyme has not previously been described in nematodes and is considered a microbial enzyme. The possibility of this enzyme, or any other found in worm homogenates, being of microbial origin cannot be definitely excluded until the gene is identified in the nematode genome, but is considered very unlikely as both L3 and adult worms were cleaned before homogenisation. Adult worms actively migrated out of a solid agar block, which removes most bacterial contamination. L3 moved through a paper filter during the Baermannization process and were subsequently washed before homogenisation, which would remove microbes in the storage fluid and any loosely adherent to the nematode cuticle. Any remaining bacteria would be expected to make an insignificant contribution to the homogenate. Since urease is also a typical bacterial enzyme, but no activity could be detected in adult or L3 homogenates, it is considered unlikely that enzyme activities are other than of nematode origin.

Relatively few reports contain kinetic data for creatinase, although the enzyme has been studied in *P. putida* (Yoshimoto *et al.*, 1976; Coll *et al.*, 1990; Chang *et al.*, 1992), *Bacillus* (Suzuki *et al.*, 1993) and *Actinobacillus* (Padmanabhan *et al.*, 2002). The K_m values for creatine were particularly variable. Yoshimoto *et al.* (1976) reported a K_m for creatine of a purified *P. putida* enzyme of 1.33 mM, greatest activity at pH 8, stability between pH 6 and 8 and an optimal temperature of 30°C. The enzyme was completely

inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+} and by the thio-blocking agent PCMB (Table 3.9). Others found a much higher K_m for creatine: 14.3 mM for the *P. putida* enzyme (Schumann *et al.*, 1993), 46 mM for an *Arthrobacter* sp enzyme (Nishiya *et al.*, 1998), 17.2 mM for *Alcaligenes* (Wyss and Kaddurah-Daouk, 2000) and 26.4 mM for a *Paracoccus* sp enzyme (Wang *et al.*, 2006). The human enzyme had a pH optimum of 6.2 and K_m of 0.08 and 0.36 mM for normal subjects and Duchenne muscular dystrophy patients respectively (Miyoshi *et al.* 1980b), much closer to the values for the *O. circumcincta* enzyme.

Overall, the *O. circumcincta* enzyme showed similar properties to those of bacterial enzymes, despite the low K_m for creatine of 0.2 ± 0.07 mM and 0.3 ± 0.1 mM (mean ± SEM) for sheathed L3 (n = 3) (Figure 3.8) and adult worms (n = 2) (Figure 3.9) respectively. Divalent metal ions were inhibitory, as for purified *P. putida* creatinase (Table 3.9, Yoshimoto *et al.*, 1976): 0.5 mM Fe^{3+}, Cu^{2+} and Mg^{2+} reduced the activity to 17%, 11% and 30% respectively. The pH optimum of pH 8 was similar to the pH 7.5-8 for bacterial creatinases (Wyss and Kaddurah-Daouk, 2000).

An interesting question is what role creatinase activity might play in a parasitic nematode and to speculate whether the enzyme may have been acquired from bacteria in the soil or in the sheep gastrointestinal tract. The most obvious advantage of the ability to degrade creatine to urea and sarcosine would be nutritional. Creatine obtained either from the host or bacteria could become a source of nitrogen from the degradation of urea to ammonia (which requires urease activity) or breakdown of sarcosine by sarcosine oxidase or dehydrogenase, both of which are present in mammals (Wittwer and Wagner, 1981; Reuber *et al.*, 1997) and may well also be present in nematodes. However, urease activity was not detected in *O. circumcincta*.

Blood feeding parasites would ingest large amounts of creatine in the erythrocytes, which is especially high in young cells (Jiao *et al.*, 1998; Okumiya *et al.*, 2004), whereas *O. circumcincta* may ingest creatine in fluid exudates or intact or sloughed gut cells. Nematodes, including *H. contortus* (Platzer *et al.*, 1995), do not use phosphocreatine as an energy buffer, but instead use phosphoarginine in the same role. Presumably this is the phosphagen also in *O. circumcincta*, which may allow creatinase activity in *O. circumcincta* without

the danger of breaking down its own phosphagen, should the two be co-located in tissues.

3.4.3 UREASE

No urease activity could be detected in either L3 or adult worm homogenates. This enzyme was examined for two reasons: firstly, there are several earlier reports of urease in cestodes (Simmons, 1960) and in the nematodes *Nematodirus* spp. and *A. lumbricoides* (Rogers, 1952) and secondly, *O. circumcincta* inhabits the stomach, as does *H. pylori*, which uses urease to neutralise gastric acid (Mobley and Hausinger, 1989; Mégraud *et al.*, 1992).

Although generally not present in animals, urease has been recorded in a number of invertebrates, including nematodes (cited by Simmons, 1960). Many of these studies were conducted decades ago, but the methodologies appear to be sound. More recently there have been additional reports of animal urease activity in insects and invertebrates. Urease activity has been identified both in worm tissue and in the large population of symbiotic bacteria harboured in a special internal structure in a deep sea giant tube worm *Riftia pachyptila* (Minic and Hervé, 2003) and in the bruchis beetle (Rosenthal *et al.*, 1982). The urease activity in silkworm haemolymph is not an insect protein, but is obtained from mulberry leaves by uptake through the microvilli in the mid-intestine (Sugimura *et al.*, 2001; Kurahashi *et al.*, 2005). Haemolymph urease allows recycling of urea, conserving nitrogen for silk formation, rather than losing it by excretion (Hirayama *et al.*, 1999).

Because there is both creatinase and arginase activity in *O. circumcincta*, it was considered possible that urease might also be present to make use of the urea generated by metabolism or the reasonably high concentrations of exogenous urea in abomasal fluid (Harrop and Phillipson, 1974). Bacteria, including *H. pylori*, use urease for amino acid synthesis (Williams *et al.*, 1996), however, this would not seem to be the case for *O. circumcincta*. Another reason that the parasitic stages of *O. circumcincta* might possess urease could be to generate ammonia to neutralise gastric acid, as does the gastric bacterium *H. pylori*, however, other bacteria facing acid challenge use a variety of other strategies (reviewed by Bearson *et al.*, 1997; Audia *et al.*, 2001; Cotter and Hill, 2003). The dihydrolase pathway is used by

Table 3.10. K_m values for pyrroline-5-carboxylate in the reaction catalysed by pyrroline-5-carboxylate dehydrogenase in different organisms.

Source	K_m (mM)	Reference
Rat liver	0.09	Small and Jones (1990)
Human liver	0.17	Forte-McRobbie and Pietruszko (1986)
S. typhimurium	11	Menzel and Roth (1981)
S. typhimurium	8	Muro-Pastor and Maloy (1995)

some organisms to generate ammonia from arginine, while others use amino acid decarboxlyses to incorporate a proton and export a more neutral compound. Examples include the import of extracellular arginine, glutamate or lysine and conversion and export of agmatine (using arginine decarboxylase), GABA (using glutamate decarboxylase) or cadaverine (using lysine decarboxylase) respectively. Abomasal nematodes may employ similar processes to survive the pH of about 2.5 in the sheep stomach (Lawton *et al.*, 1996).

3.4.4 PYRROLINE-5-CARBOXYLATE DEHYDROGENASE

The low P5CDH activity in homogenates of sheathed L3 *O. circumcincta* of 16 $\pm$ 0.8 nmoles min^{-1} mg^{-1} protein (Figure 3.10 and Table 3.7), suggests that the conversion of proline (or ornithine) to glutamate may be of minor importance relative to proline synthesis. The K_m for P5C of L3 homogenates was 2.3 $\pm$ 0.5 mM, intermediate between values for mammalian and bacterial enzymes (Table 3.10). A reason for the high K_m for many bacterial enzymes is substrate channelling in a multifunctional enzyme (Surber and Maloy, 1998). Whereas there are two enzymes in eukaryotes, in *Salmonella typhimurium* (Menzel and Roth, 1981; Surber and Maloy, 1998), *Bradyrhizobium japonicum* (Krishnan and Becker, 2005) and *P. putida* (Vílchez *et al.*, 2000), proline dehydrogenase (oxidase) and P5CDH activities reside in a single polypeptide. The intermediate P5C directly transfers between the two enzymatic functions via a leaky channel, so that higher concentrations of exogenous P5C are needed to compete with endogenously produced P5C, resulting in a higher K_m in assays (Surber and Maloy, 1998).

Nematodes appear to have all the enzymes for interconversion of ornithine, proline and glutamate (Grantham and Barrett, 1986b; Walker and Barrett, 1991a), but the predominant direction may change during the life cycle. The data from *O. circumcincta* suggests that in L3 the direction is not predominantly in the direction of glutamate formation. Proline may be being formed from ornithine, or alternatively an adequate supply of proline may be available. While sheathed L3 would not be actively forming cuticle, the moulting cycle would create a demand for cuticle synthesis. Proline may also be in demand as an energy and nitrogen source as it is in bacteria (Vílchez *et al.*, 2000), insects (Auerswald *et al.*, 1998; Auerswald and Gäde, 1999;

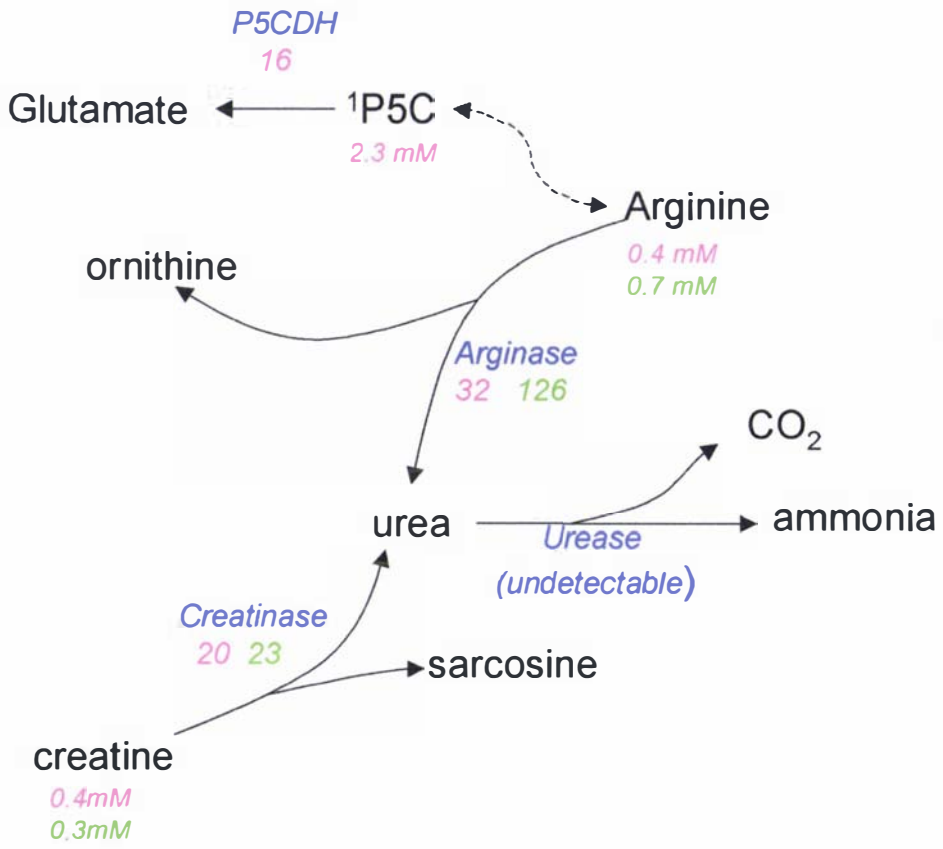


Figure 3.11. Metabolic map of enzymes of urea and arginine metabolism identified in L3 or adult *O. circumcincta* homogenates. Enzymes are shown in blue; K_m values (mM) for the substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reactions are shown in pink for L3 homogenates and green for adult worm homogenates.

Scaraffia and Wells, 2003) and parasitic trypanosomes (Obungu *et al.*, 1999) or as an osmoprotectant, as in crustaceans (Burton, 1992; Bishop and Burton, 1993; Willett and Burton, 2002), bacteria (Milner *et al.*, 1988; Lee *et al.*, 2003) and plants (Song *et al.*, 2005; Yamada *et al.*, 2005).

3.4.5. UREA AND ARGININE METABOLISM

A metabolic map linking the enzymes identified in L3 or adult *O. circumcincta* homogenates is shown in Figure 3.11. The K_m values for the substrates and the V_{max} for the reactions are indicated.

Urea excreted by *O. circumcinta* is probably generated by arginase and possibly also by creatinase. Agmatinase activity could also be a source of urea. *O. circumcincta* would be expected to be similar to trematodes and other nematodes in not having an active OUC (Janssen and Bryant, 1969; Wright, 1975a; Grantham and Barrett, 1986a; Mohamed *et al.*, 2005). Urea may merely be a by-product of these enzymes rather than a preferred method of excreting nitrogen. The absence of urease activity suggests that urea cannot be used as a source of nitrogen unless associated microorganisms in the abomasal fluid or in the parasite gut participate in recycling urea.

Arginine may be an essential amino acid for *O. circumcincta*, depending on the activity of OTCase needed to convert ornithine to arginine. In spite of nematodes having an incomplete OUC, arginase and OTCase are the enzymes reported to be active in some studies (Wright, 1975a). Kapur and Sood (1984) using ^{14}C-precursors (glucose, acetate, CO_2 and palmitic acid) found all amino acids to be labelled in *H. contortus*, including arginine.

Arginine catabolism is significant in both L3 and adult *O. circumcincta*, but based on enzyme activities, little ornithine is being metabolised to glutamate via P5CDH, suggesting proline synthesis predominates over catabolism. Arginine and ornithine are required for synthesis of phosphagens (either phosphoarginine or phosphocreatine), nitric oxide and the polyamines putrescine, spermine and spermidine. The 4-fold increase in arginase activity per mg protein in adult worms compared with sheathed L3 may be a genuine reflection of an increased metabolic rate in adult worms. Drawing such conclusions must be done with caution, as discussed previously, because an enzyme may be very active in the reproductive tract, but activity is unchanged

in the other organs where the enzyme is located. Changes in whole worm enzyme activity may more reflect the changing composition of the parasite and organs differing in their metabolic activities. However, in contrast to this apparent increase in arginase activity, creatinase activity was unchanged. Increased arginase activity in the parasitic stage may instead be a protective mechanism, as It has been suggested that parasites increase their arginase activity or induce increased host arginase activity to deplete NOS of substrate to reduce the production of toxic NO by the host (Giordanengo *et al.*, 2002; Vincendeau *et al.*, 2003). As in other parasites, arginase could therefore be used in the future as a target to eliminate the parasites (Olds *et al.*, 1980).

Arginine metabolism is linked via P5C and OAT to glutamate, a central amino acid in nitrogen metabolism. Synthetic enzymes of glutamate metabolism are reported in Chapter 5 and links to alanine and aspartate, mainly focussing on transamination, in Chapter 4.

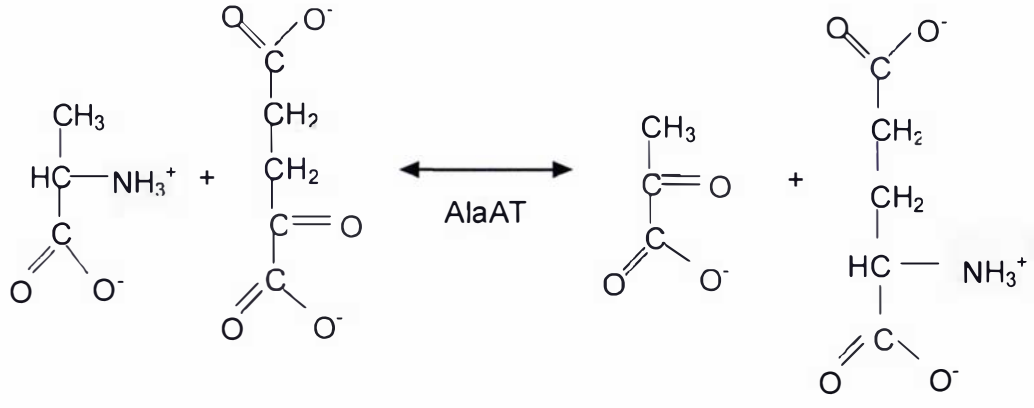


Figure 4.1. Reaction catalysed by alanine aminotransferase. The amino group from alanine is transferred to 2-oxoglutarate to form pyruvate and glutamate respectively.

Chapter 4

METABOLISM OF ALANINE AND ASPARTATE BY *OSTERTAGIA CIRCUMCINCTA*

4.1 INTRODUCTION

Alanine and aspartate are involved in the interconversion of amino acids and also in contributing carbon skeletons to the TCA cycle, principally through transamination by alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) to pyruvate and OAA respectively. Other reactions in which alanine and aspartate participate have been described in Chapter 1, 1.6 and are summarised in Figures 1.8-1.10, some of which are believed to be restricted to microorganisms and plants, while others, such as the transaminations are very active in most species studied. Several aminotransferases have been detected in nematodes (Rasero *et al.*, 1968; Grantham and Barrett, 1986a,b; Walker and Barrett, 1991a,b), including AlaAT in *O. circumcincta* (Walker and Barrett, 1991b).

4.1.1 ALANINE AMINOTRANSFERASE (ALAAT) (EC 2.6.1.2)

AlaAT is a PLP-dependent enzyme which catalyses the reversible transamination between 2-oxoglutarate and L-alanine to form glutamate and pyruvate respectively (Figure 4.1). The isoforms cAlaAT and mAlaAT are distinct molecular and genetic forms (Astrin *et al.* 1982; Ruščák *et al.*, 1982; Kielty *et al.*, 1982; Sohocki *et al.*, 1997; Yang *et al.*, 2002). The kinetics of the two forms are consistent with the mitochondrial enzyme mainly being involved in the forward reaction (formation of pyruvate) and the cytosolic mainly in the reverse reaction (formation of alanine) (DeRosa and Swick, 1975). Pyruvate generation is involved in gluconeogenesis and ATP production via the Krebs cycle, while alanine formation is involved in the synthesis of protein.

AlaAT has been identified in the cestodes in *M. expansa* (Rasero *et al.*, 1968), *H. diminuta* and *H. citelli* (but was not present in *H. nana*) (Wertheim *et al.*, 1960) and in the Digeneans *C. lingua* and *C. emasculans* (Watts, 1970).

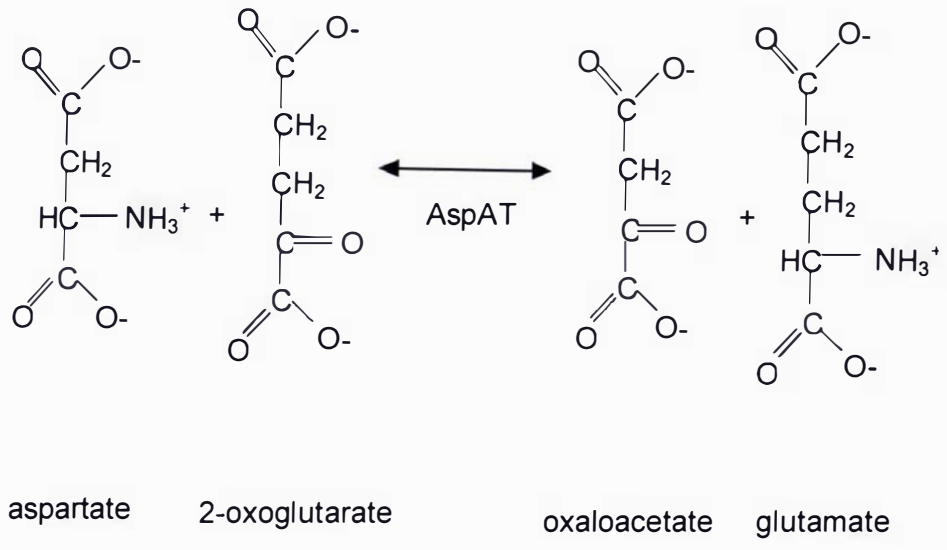


Figure 4.2. Reaction catalysed by aspartate aminotransferase. The amino group of aspartate is transferred to 2-oxoglutarate to form oxaloacetate and glutamate.

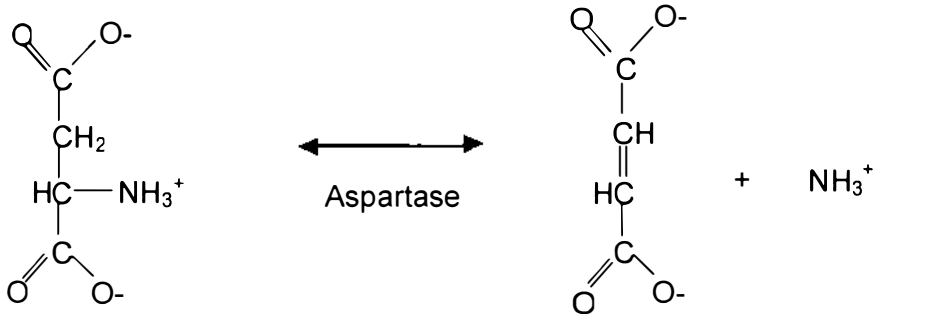
AlaAT has been reported from the nematodes *A. lumbricoides* (Rasero *et al.*, 1968), *C. oncophera*, *H. contortus*, *N. brasiliensis*, *O. circumcincta*, *T. colubriformis*, *P. redivivus, S. bibionis* (Walker and Barrett, 1991b) and *H. polygyrus* (Grantham and Barrett, 1986a). In contrast to the rat liver enzyme, in most nematode species, including *O. circumcincta*, there was greater AlaAT activity in the direction of alanine formation, but equal rates for the forward and reverse reactions in *H. contortus* (Walker and Barrett, 1991b). In *N. brasiliensis*, cAlaAT was responsible for 80% of activity but for only 54% in *H. contortus*, with 22% of total activity was associated with the cell debris and cuticle fractions.

4.1.2 ASPARTATE AMINOTRANSFERASE (ASPAT) (EC 2.6.1.1)

Aspartate aminotransferase, formerly called glutamate oxaloacetate transaminase, catalyses the reversible transamination between L-aspartate and 2-oxoglutarate to form glutamate and oxaloacetate respectively (Figure 4.2). It has an important role in transporting reducing equivalents into the mitochondria by the malate/aspartate shuttle, shown in Figure 1.10 (Setoyama *et al.*, 1990). Like other aminotransferases, AspAT is PLP dependent.

The cytosolic (cAspAT) and mitochondrial (mAspAT) isoforms have different substrate specificities and are coded by distinct nuclear genes (Martinez-Carrion *et al.*, 1967; Michuda and Martinez-Carrion, 1969; Reed and Hess, 1975; Panteghini, 1990; Morin *et al.*, 1992; Mattingly *et al.*, 1995; Verleur and Wanders, 1997) and there are also multiple subforms of cAspAT and mAspAT (Martinez-Carrion *et al.*, 1967; Bertland and Kaplan, 1968, 1970; Michuda and Martinez-Carrion, 1969; Rej, 1981). The pH optima of cAspAT and mAspAT differed in the rat brain isozymes (Recasens *et al.*, 1980). The cytosolic form had a pH optimum from 8.5-10, while the mitochondrial form had a pH optimum of 8.7 and at physiological pH 7.4, cAspAT activity was 60% and m-AspAT 95% of maximal activity. The cytosolic isozyme was more thermosensitive and there were differences in substrate specificity.

AspAT is present in most organisms and has been reported from helminths: cestodes *H. diminuta*, *H. nana* and *H. citelli* (Wertheim *et al.*, 1960) and the sheep parasite *M. expansa* (Rasero *et al.*, 1968) and the nematodes *A. lumbricoides* (Rasero *et al.*, 1968), *P. redivivus*, *H. polygyrus* (Grantham and Barrett, 1986a) and *N. brasiliensis* (Walker and Barrett, 1991a).



aspartate fumarate ammonia

Figure 4.3. Reaction catalysed by aspartase.

4.1.3 ASPARTASE (EC 4.3.1.1)

The enzyme aspartase (L-aspartate ammonia-lyase) catalyzes the reversible deamination of the amino acid L-aspartic acid to produce fumarate and ammonium (Figure 4.3). Aspartase activity is well recognised in bacteria, but has also been reported from frog embryos (Kurata, 1962), teleost fish (Salvatore *et al.*, 1965) and elasmobranchs (Salvatore *et al.*, 1965; Cutinelli *et al.*, 1972).

Aspartase is very specific for L-aspartate in the direction of fumarate formation (Giorgianni *et al.*, 1997). The enzyme has unusual kinetic properties, particularly the requirement for divalent cations (Mn^{2+} or Mg^{2+}) at alkaline pH but not below pH 7 and variation in the substrate K_m with pH because of conformational changes in the enzyme (Viola, 2000). Allosteric activation of the enzyme by L-aspartate is needed for both the deamination and amination reactions. Both the cation and aspartate bind to an activator-binding site, which is different from the active site (Fujii *et al.*, 2003).

The experiments reported below have focussed on obtaining kinetic data for AlaAT and AspAT, which are known to be active in *O. circumcincta* and other nematodes (Rasero *et al.*, 1968; Grantham and Barrett, 1986b; Walker and Barrett, 1991a,b). To examine whether there might be unusual aspartate metabolism, the activity of aspartase was measured and found to be considerable in L3 *O. circumcincta*.

4.2 MATERIALS AND METHODS

Parasites for enzyme assays were provided as detailed in Appendix 1. L3 were cultured from the faeces of infected sheep, stored at 4°C and prior to each experiment, were Baermannized to remove inactive worms. Adult worms were recovered from the abomasa of infected sheep (Appendix 1, 1.4).

All chemicals were obtained from the Sigma Chemical Co. (Mo, USA) unless specified.

4.2.1 HOMOGENATE PREPARATION

Enzyme activities were determined on homogenates prepared from sheathed L3 or adult *O. circumcincta* (Appendix 2, 2.6). The amount of homogenate used for the enzyme assays was based on its protein content,

which was determined by the Bradford method (Appendix 2, 2.3), so that an amount of homogenate containing 50 µg protein was used for each assay.

4.2.2 ALANINE AMINOTRANSFERASE

Alanine aminotransferase (AlaAT) was assayed both in the direction of alanine utilisation and formation:

4.2.2.1 Effect of pH and PLP concentration

The assay was an adaptation of that of de Sousa and Sodek (2003). Preliminary assays to determine the optimum pH and pyridoxal phosphate (PLP) concentration were carried out at 30°C in the direction of alanine formation and alanine utilisation.

To determine the optimal pH in the direction of alanine formation, Tris buffer (Appendix 3, 3.2) pH 5.5 to 8.5 was used in a reaction mixture (total volume of 1 ml) containing 2 mM glutamate, 0.2 mM PLP, 1 Unit of GDH, 40 mM NH_4Cl and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM pyruvate. AlaAT activity was calculated from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

To determine the optimal pH in the direction of alanine utilisation, Tris buffer pH 5.5 to 8.5 was used in a reaction mixture (total volume of 1 ml) containing 5 mM alanine, 0.2 mM PLP, 1 Unit of LDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 1 mM 2-oxoglutarate.

To determine the optimal PLP concentration in the direction of alanine utilisation, Tris buffer pH 7.5 was used in a reaction mixture (total volume of 1 ml) containing 5 mM alanine, 1 Unit of LDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5 with [PLP] from 0 to 0.5 mM. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 1 mM 2-oxoglutarate. AlaAT activity was calculated from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

The optimum values of pH 7.5 and PLP concentration of 0.2 mM were used in subsequent assays.

4.2.2.2 Kinetic parameters in the direction of alanine utilisation

AlaAT activity was estimated at 30°C in homogenates of sheathed L3 (n = 3) or adult worms (n = 2), using the protocol for continuous assays described in Appendix 2, 2.7. Enzyme activity was monitored using a coupled reaction with lactate dehydrogenase (LDH):

$$\text{L-alanine} + \text{2-oxoglutarate} \xrightarrow{\text{AlaAT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

The reaction mixture (total volume of 1 ml) contained 10 mM alanine, 0.2 mM PLP, 1 Unit of LDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer (Appendix 3, 3.2) pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 2 mM 2-oxoglutarate.

The K_m and V_{max} were determined for AlaAT activity for both the substrates 2-oxoglutarate and alanine.

(1) For 2-oxoglutarate, the reaction was initiated by the addition of 2-oxoglutarate in concentrations from 0 to 5 mM.

(2) For alanine, reaction mixtures were prepared with 10 mM 2-oxoglutarate, instead of 10 mM alanine, and the reaction was initiated by the addition of alanine in concentrations from 0 to 25 mM.

4.2.2.3 Kinetic parameters in the direction of alanine formation

AlaAT activity was estimated at 30°C in homogenates of sheathed L3 (n = 3) or adult worms (n = 1) using the protocol for continuous assays described in Appendix 2, 2.7. Enzyme activity was monitored with a coupled reaction with glutamate dehydrogenase (GDH):

$$\text{L-glutamate} + \text{pyruvate} \xrightarrow{\text{AlaAT}} \text{L-alanine} + \text{2-oxoglutarate}$$

$$\text{2-oxoglutarate} + \text{NH}_4^+ + \text{NADH} \xrightarrow{\text{GDH}} \text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

The assay for AlaAT was adapted from de Sousa and Sodek (2003). The reaction mixture (total volume of 1 ml) contained 5 mM glutamate, 0.2 mM

PLP, 1 Unit of GDH, 40 mM NH_4Cl and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 2 mM pyruvate.

K_m and V_{max} were determined for both substrates glutamate and pyruvate:

(1) For glutamate, reaction mixtures were prepared with 10 mM pyruvate instead of 5 mM glutamate. The reaction was initiated by the addition of glutamate in concentrations from 0 to 2 mM.

(2) For pyruvate, the reaction was initiated by the addition of pyruvate in concentrations from 0 to 10 mM.

4.2.3 ASPARTATE AMINOTRANSFERASE

Aspartate aminotransferase (AspAT) was assayed both in the direction of aspartate utilisation and formation:

$$\text{L-aspartate} + \text{2-oxoglutarate} \xleftrightarrow{\text{AspAT}} \text{L-glutamate} + \text{oxaloacetate}$$

4.2.3.1 Effect of pH, PLP concentration, ADP and ATP

Preliminary assays to determine the optimum pH, the optimum pyridoxal phosphate (PLP) concentration and the effect of ATP and ADP were conducted at 30°C using the reaction mixture described in Section 4.2.3.2.

To determine the optimal pH in the direction of aspartate utilisation, 100 mM Tris buffer pH 5.5 to 8.5 was used in a reaction mixture (total volume of 1 ml) containing 5 mM aspartate, 0.2 mM PLP, 1 unit MDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 1 mM 2-oxoglutarate. AspAT activity was calculated from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

To determine the optimal PLP concentration in the direction of aspartate utilisation, 100 mM Tris buffer pH 7.5 was used in a reaction mixture (total volume of 1 ml) containing 3 mM aspartate, 1 unit MDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5 with [PLP] from 0 to 1 mM. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 1 mM 2-oxoglutarate. AspAT activity was calculated from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

The optimum pH of 7.5 and [PLP] of 0.25 mM were used for subsequent experiments described in Section 4.2.3.2.

To determine the effects of ATP and ADP on AspAT activity, 1 mM of ATP or ADP was included in the reaction mixture (total volume of 1 ml) containing 100 mM Tris buffer pH 7.5, 5 mM aspartate, 0.2 mM PLP, 1 unit MDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 1 mM 2-oxoglutarate.

4.2.3.2 Kinetic parameters in the direction of aspartate utilisation

AspAT activity was estimated at 30°C in homogenates of sheathed L3 (n = 3) or adult worms (n = 2 or n = 3) using the protocol for continuous assays described in Appendix 2, 2.7. The activity was monitored through a coupled reaction with malate dehydrogenase (MDH):

$$\text{L-aspartate} + \text{2-oxoglutarate} \xrightarrow{\text{AspAT}} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

The assay for AspAT was adapted from Reed and Hess (1975). The reaction mixture (total volume of 1 ml) contained 10 mM aspartate, 0.2 mM PLP, 1 unit MDH and 50 µg protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 0.2 mM 2-oxoglutarate.

The K_m and V_{max} were determined for AspAT activity for both the substrates aspartate and 2-oxoglutarate:

(1) For 2-oxoglutarate, reaction mixtures were prepared as described above and 2-oxoglutarate was added in concentrations from 0 to 5 mM to initiate the reaction.

(2) For aspartate, reaction mixtures were prepared with 5 mM 2-oxoglutarate, instead of 10 mM aspartate, and aspartate was added in concentrations from 0 to 20 mM to initiate the reaction.

4.2.3.3 Kinetic parameters in the direction of aspartate formation

AspAT activity was estimated at 30°C in homogenates of sheathed L3 (n = 3) using the protocol for continuous assays described in Appendix 2, 2.7. Enzyme activity was monitored using a coupled reaction with glutamate dehydrogenase (GDH):

$$\text{L-glutamate} + \text{oxaloacetate} \xrightarrow{\text{AspAT}} \text{L-aspartate} + \text{2-oxoglutarate}$$

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADH} \xrightarrow{\text{GDH}} \text{L-glutamate} + NAD^+ + H_2O$$

The assay for AspAT was adapted from Alekhova *et al.* (2001). The reaction mixture (total volume of 1 ml) contained 1 mM glutamate, 0.2 mM PLP, 1 unit of GDH, 40 mM NH_4Cl and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 2 mM oxaloacetate.

The K_m and V_{max} were determined for AspAT activity for both the substrates glutamate and oxaloacetate:

(1) For glutamate, reaction mixtures were prepared with 1 mM oxaloacetate, instead of 1 mM glutamate, and glutamate in concentrations from 0 to 1 mM was added to initiate the reaction.

(2) For oxaloacetate, reaction mixtures were prepared as described above and oxaloacetate was added in concentrations from 0 to 0.75 mM to initiate the reaction.

4.2.4. ASPARTASE

Aspartase was assayed both in the direction of fumarate utilisation and formation:

$$\text{fumarate} + NH_3 \xleftrightarrow{\text{Aspartase}} \text{aspartate}$$

4.2.4.1 Kinetic parameters in the direction of fumarate utilisation

Aspartase activity was estimated at 30°C in homogenates of sheathed L3 (n = 2) and adult worms (n = 1) using the protocol for continuous assays described in Appendix 2, 2.7. The assay was adapted from Murase *et al.*

(1991) and Wang *et al.* (2000). The reaction mixture (total volume of 1 ml) contained 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Either fumarate was added subsequently and the reaction was initiated by adding NH_4Cl as the source of ammonia, or NH_4Cl was added and the reaction started by adding fumarate. Aspartase activity was monitored by measuring the disappearance of fumarate, which absorbs strongly at 260 nm.

The K_m and V_{max} were determined for aspartase activity for both the substrates fumarate and ammonia:

(1) For ammonia, reaction mixtures were prepared with 1.8 mM fumarate and NH_4Cl in concentrations from 0 to 20 mM was added to initiate the reaction.

(2) For fumarate, reaction mixtures were prepared with 20 mM NH_4Cl and fumarate, in concentrations from 0 to 0.8 mM for L3 homogenates or from 0 to 2 mM for the adult homogenate, was added to initiate the reaction. The concentration of fumarate added is limited by the ability to measure accurately concentrations over 2 mM on the spectrophotometer.

4.2.4.2 Kinetic parameters in the direction of fumarate formation

Aspartase activity was estimated at 30°C in homogenates of sheathed L3 (n = 3) using the protocol for continuous assays described in Appendix 2, 2.7. Enzyme activity was monitored through a coupled reaction with glutamate dehydrogenase (GDH):

$$\text{Aspartate} \xrightarrow{\text{Aspartase}} \text{Fumarate} + NH_3$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GDH}} \text{glutamate} + NAD^+ + H_2O$$

The assay was adapted from Özer (1985). The reaction mixture (total volume of 1 ml) contained 20 mM 2-oxoglutarate, 1 unit of GDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by the addition of aspartate. Aspartase activity was monitored at 340 nm for concentrations of aspartate from 0 to 10 mM.

4.2.4.3 Effect of pH

The effect of varying the pH of the incubation medium on aspartase activity in the direction of fumarate formation was investigated using sheathed L3 homogenate SL46. The reaction mixture (total volume of 1 ml) contained 20 mM 2-oxoglutarate, 1 unit of GDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer of pH from 5.5 to 9. Subsequently, 0.2 mM NADH was added and the reaction was initiated by the addition of 1 mM aspartate.

4.2.4.4 Effectors/inhibitors

Each of the following was investigated for effects on aspartase activity in the direction of fumarate formation of sheathed L3 homogenate SL47:

(1) 1 mM or 10 mM Ca^{2+} as $CaCl_2$

(2) 1 mM ATP

(3) 1 mM ADP

(4) 1 mM or 5 mM EDTA

(5) 1 mM Mn^{2+} as $MnCl_2$

(6) 1 mM K^{+} as KCl

(7) 1 mM or 10 mM Mg^{2+} as $MgCl_2$

Reaction mixtures (total volume 1 ml) contained 20 mM 2-oxoglutarate, 1 unit of GDH and 50 µg homogenate protein, prepared in 100 mM Tris buffer pH 7.5 and one of the test compounds. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM aspartate. Aspartase activity without test compounds was used as the control and set at 100%.

An aliquot of the homogenate was stored at 4°C and aspartase activity was determined after 24 hours to assess the stability of the enzyme.

4.3 RESULTS

4.3.1 ALANINE AMINOTRANSFERASE

4.3.1.1 Effect of pH and PLP concentration

The effects of pH on the AlaAT activity of a homogenate of sheathed L3 (SL15) in the direction of alanine utilisation and of SL16 in the direction of

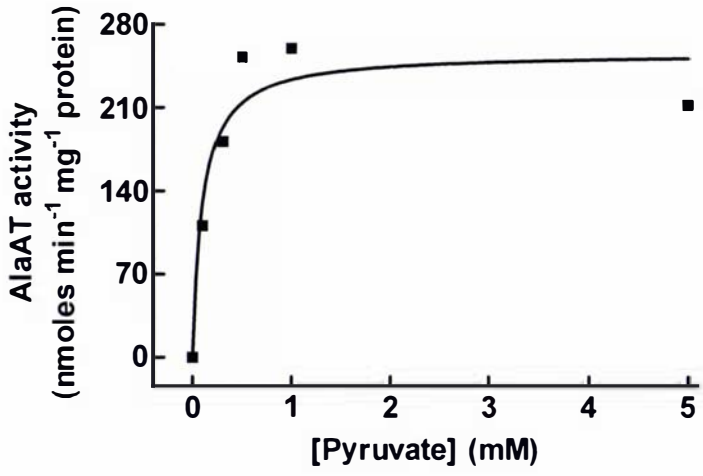


Figure 4.11. AlaAT activity of adult *O. circumcincta* homogenate A6 monitored in the direction of alanine formation with increasing concentration of pyruvate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 4.6. AlaAT activities of an adult *O. circumcincta* homogenate monitored in the direction of alanine formation with increasing concentration of pyruvate. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Pyruvate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A 6	0.1	256

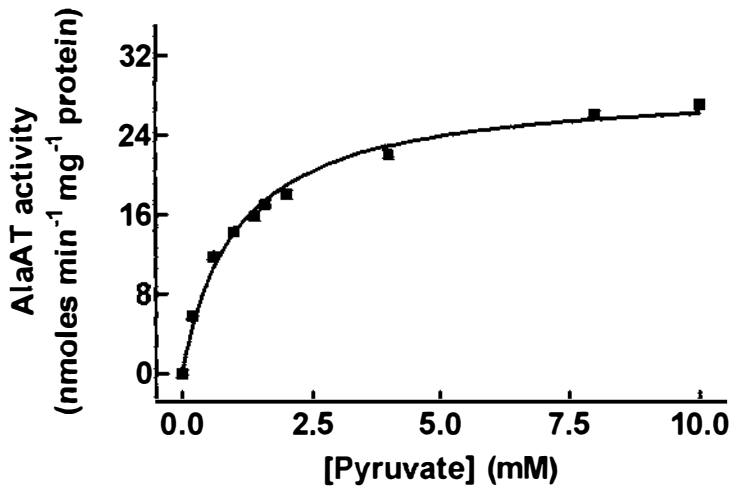


Figure 4.10. AlaAT activity of sheathed L3 *O. circumcincta* homogenate SL28 monitored in the direction of alanine formation with increasing concentration of pyruvate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 4.5. AlaAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of alanine formation with increasing concentration of pyruvate. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Pyruvate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 26	1.1	30
SL 27	1.6	27
SL 28	1.0	29
mean ± SEM, n = 3	1.2 ± 0.2	29 ± 1

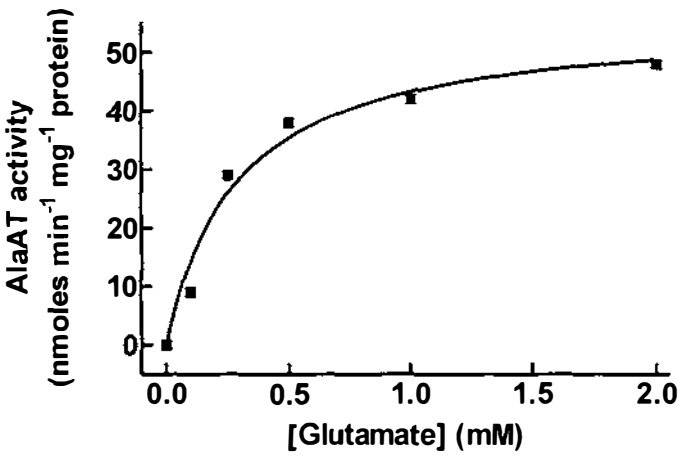


Figure 4.9. AlaAT activity of sheathed L3 O. *circumcincta* homogenate SL23 monitored in the direction of alanine formation with increasing concentration of glutamate. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 4.4. AlaAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of alanine formation with increasing concentration of glutamate. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 23	0.4	56
SL 24	0.6	74
SL 25	0.4	72
mean ± SEM, n = 3	0.5 ± 0.1	67 ± 7

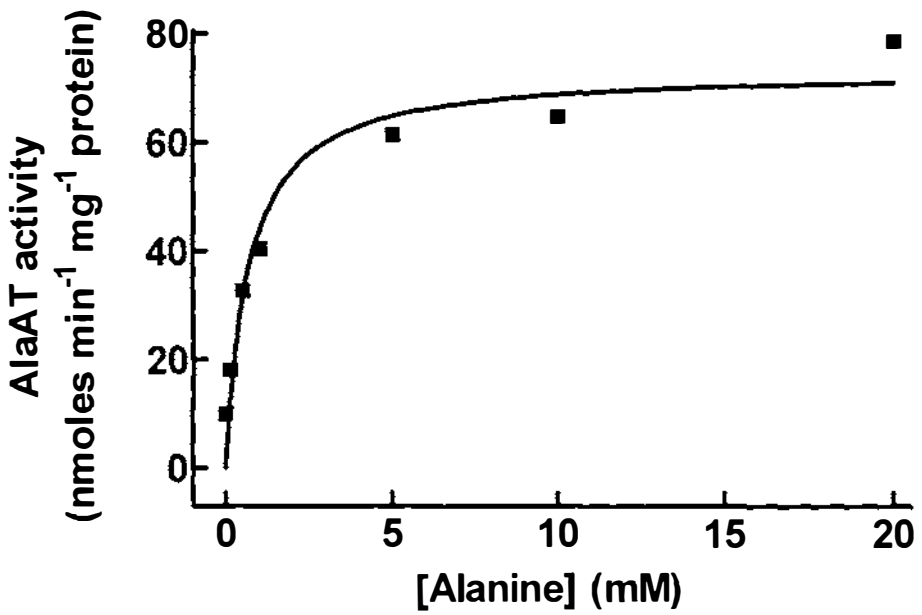


Figure 4.8. AlaAT activity of adult *O. circumcincta* homogenate A5a monitored in the direction of alanine utilisation with increasing concentration of alanine. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340nm.

Table 4.3. AlaAT activities of adult *O. circumcincta* homogenates monitored in the direction of alanine utilisation with increasing concentration of alanine. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340nm.

Homogenate	Alanine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A 5a	0.6	73
A 5b	0.4	85
mean ± SEM, n = 2	0.5 ± 0.1	79 ± 6

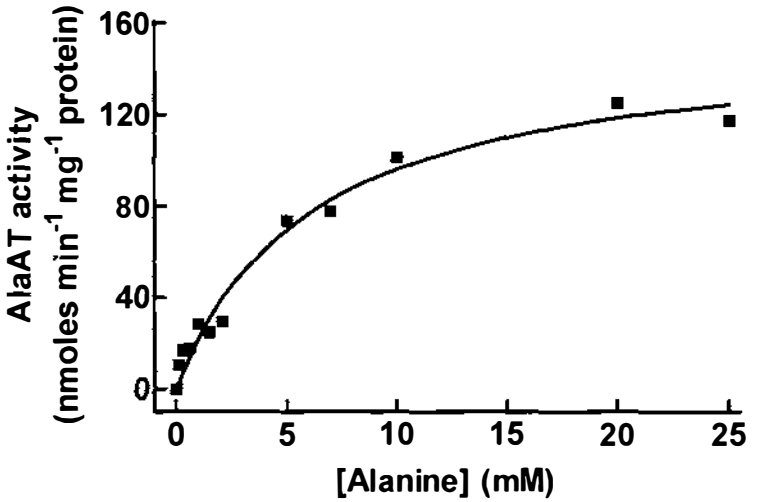


Figure 4.7. AlaAT activity of sheathed L3 *O. circumcincta* homongenate SL21b monitored in the direction of alanine utilisation with increasing concentration of alanine. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340 nm.

Table 4.2. AlaAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of alanine utilisation with increasing concentration of alanine. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Alanine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 21a	6.1	155
SL 21b	5.2	137
SL 22	4.4	55
mean ± SEM, n = 3	5.2 ± 0.5	115 ± 30

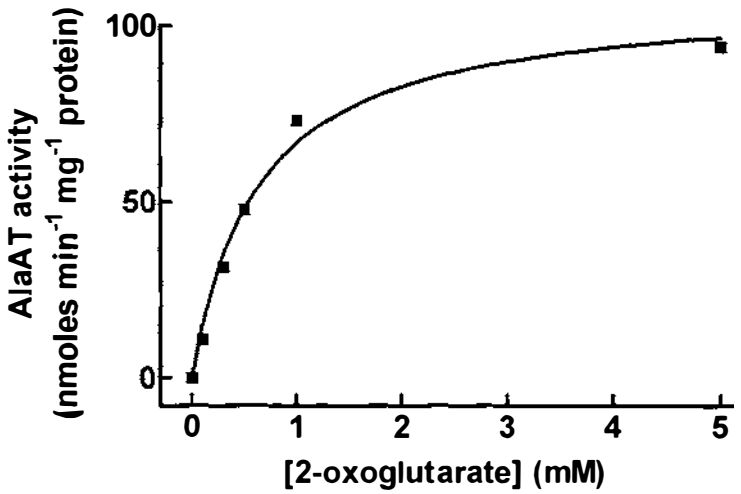


Figure 4.6. AlaAT activity of sheathed L3 *O. circumcincta* homogenate SL20 monitored in the direction of alanine utilisation with increasing concentration of 2-oxoglutarate. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340 nm.

Table 4.1. AlaAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of alanine utilisation with increasing concentration of 2-oxoglutarate. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	2-oxoglutarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 18	0.6	109
SL 19	0.3	97
SL 20	0.6	89
mean ± SEM, n = 3	0.5 ± 0.1	98 ± 6

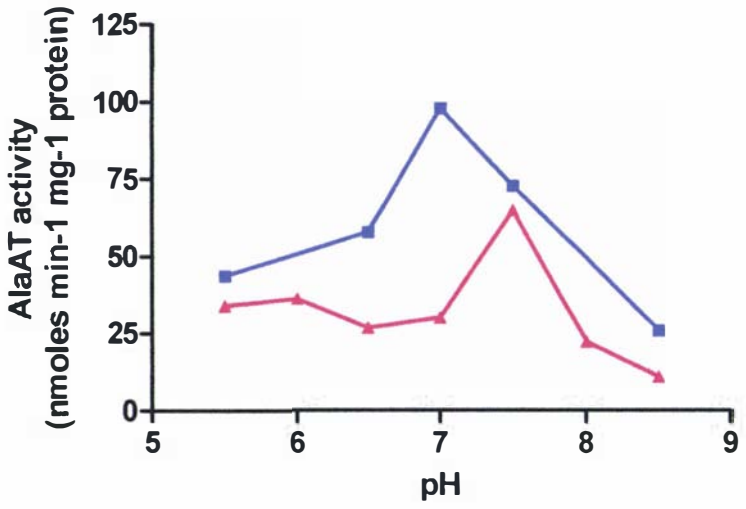


Figure 4.4. Effect of pH on AlaAT activities of sheathed L3 *O. circumcincta* homogenates in the direction of alanine utilisation (SL15) (▲) and formation (SL16) (■). Concentrations of 5 mM pyruvate and 2 mm glutamate were used for alanine formation and 5 mM alanine and 1 mM 2-oxoglutarate for alanine utilisation. AlaAT activity was calculated for alanine utilisation from the rate of NADH oxidation in a coupled assay with LDH and for alanine formation in a coupled assay with GDH, monitored spectrophotometrically at 340nm.

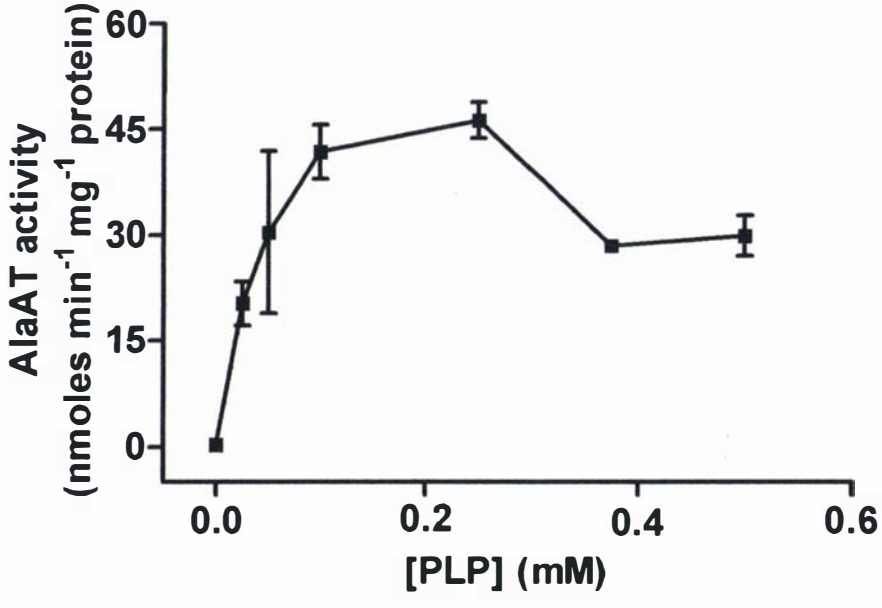


Figure 4.5. Effect of PLP concentration on AlaAT activity (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL17 in the direction of alanine utilisation. Concentrations of 5 mM alanine and 1 mM 2-oxoglutarate were used. AlaAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with LDH, which was monitored spectrophotometrically at 340nm.

alanine formation are shown in Figure 4.4. The pH optima were pH 7.5 and pH 7.0 respectively for alanine utilisation and formation.

The effects of [PLP] on the AlaAT activity (mean ± SEM, n = 2) of a homogenate (SL17) of sheathed L3 in the direction of alanine utilisation are shown in Figure 4.5. The optimum [PLP] was 0.25 mM.

4.3.1.2 Kinetic parameters in the direction of alanine utilisation

For the substrate 2-oxoglutarate, the AlaAT activity of a homogenate of sheathed L3 (SL20) is shown in Figure 4.6 and the K_m and V_{max} of the homogenates of sheathed L3 (n = 3) are shown in Table 4.1. The K_m and V_{max} were 0.5 ± 0.1 mM and 98 ± 6 nmoles min^{-1} mg^{-1} protein (both mean ± SEM).

For the substrate alanine, the AlaAT activities of homogenates of sheathed L3 (SL21b) and adult worms (A5a) are shown in Figures 4.7 and 4.8 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 4.2 and 4.3. The K_m values for alanine were 5.2 ± 0.5 mM and 0.5 ± 0.1 mM (both mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively. The V_{max} were 115 ± 30 and 79 ± 6 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively.

4.3.1.3 Kinetic parameters in the direction of alanine formation

For the substrate glutamate, the AlaAT activity of a homogenate of sheathed L3 (SL23) is shown in Figure 4.9 and the K_m and V_{max} of the homogenates of sheathed L3 (n = 3) are shown in Table 4.4. The K_m and V_{max} were 0.5 ± 0.1 mM and 67 ± 7 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) respectively.

For the substrate pyruvate, AlaAT activities of homogenates of sheathed L3 (SL28) and adult worms (A6) are shown in Figures 4.10 and 4.11 respectively. The K_m and V_{max} of the homogenates of sheathed L3 (n = 3) and adult worms (n = 1) are shown in Tables 4.5 and 4.6. The K_m values for pyruvate were 1.2 ± 0.2 mM (mean ± SEM, n = 3) for sheathed L3 and 0.1 mM (n = 1) for adult worms. The V_{max} were 29 ± 1 (mean ± SEM, n = 3) and 256 nmoles min^{-1} mg^{-1} protein for sheathed L3 and adult worms respectively.

Table 4.12. AspAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of aspartate formation with increasing concentration of glutamate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 36	0.12	380
SL 37a	0.09	433
SL 37b	0.14	411
mean ± SEM, n = 3	0.12 ± 0.02	408 ± 15

Table 4.11. AspAT activities of adult *O. circumcincta* homogenates monitored in the direction of aspartate utilisation with increasing concentration of aspartate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in coupled assay with MDH, which was monitored spectrophotometrically at 340nm.

Homogenate	Aspartate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A 8	1.5	170
A 9a	3.0	93
A 9b	3.4	90
mean ± SEM, n = 3	2.6 ± 0.6	118 ± 26

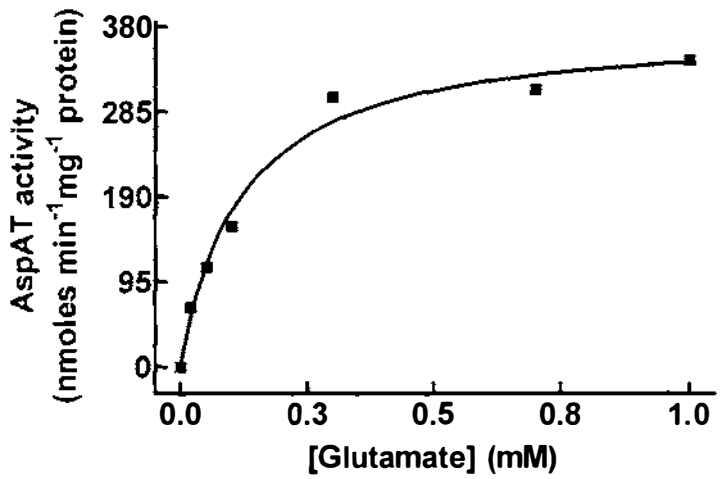


Figure 4.18. AspAT activity of sheathed L3 *O. circumcincta* homogenate SL36 monitored in the direction of aspartate formation with increasing concentration of glutamate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 4.10. AspAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of aspartate utilisation with increasing concentration of aspartate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Aspartate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 32	1.8	940
SL 34	2.5	1356
SL 35	2.2	1211
mean ± SEM, n = 3	2.1 ± 0.2	1170 ± 122

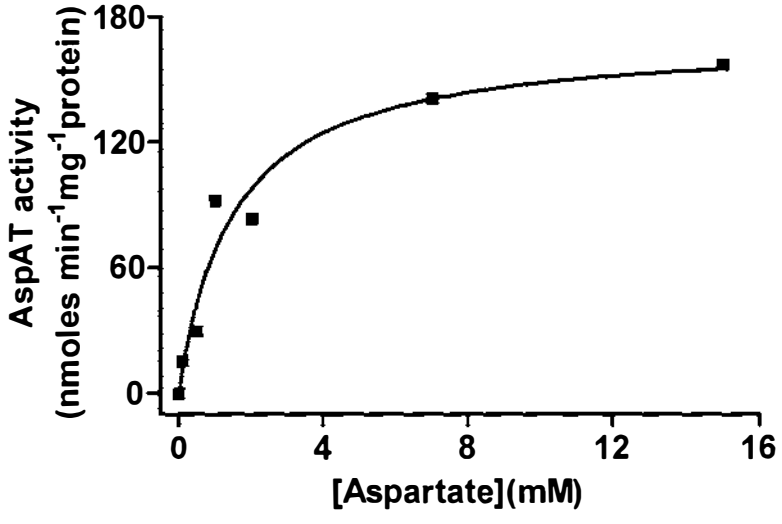


Figure 4.17. AspAT activity of adult *O. circumcincta* homogenate A8 monitored in the direction of aspartate utilisation with increasing concentration of aspartate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340nm.

Table 4.9. AspAT activities of adult *O. circumcincta* homogenates monitored in the direction of aspartate utilisation with increasing concentration of 2-oxoglutarate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340nm.

Homogenate	2-oxoglutarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A 7a	0.1	204
A 7b	0.3	370
mean ± SEM, n = 2	0.2 ± 0.1	287 ± 83

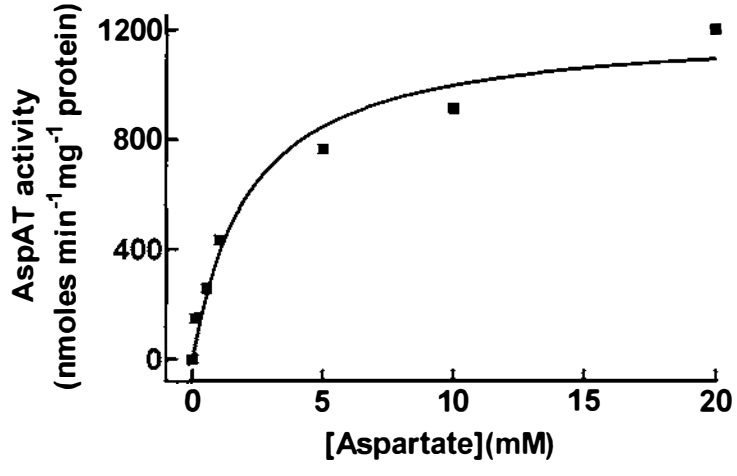


Figure 4.16. AspAT activity of sheathed L3 *O. circumcincta* homogenate SL35 monitored in the direction of aspartate utilisation with increasing concentration of aspartate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340 nm.

Table 4.8. AspAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of aspartate utilisation with increasing concentration of 2-oxoglutarate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	2-oxoglutarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 32	0.2	1007
SL 33	0.3	1258
SL 34	0.2	914
mean ± SEM, n = 3	0.2 ± 0.02	1060 ± 103

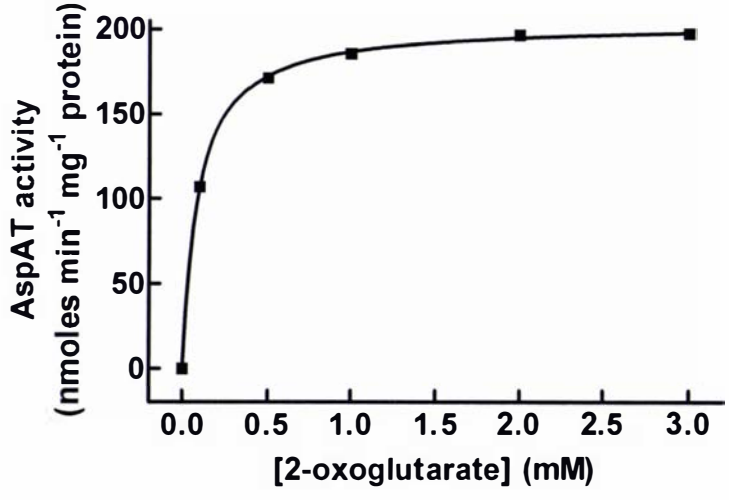


Figure 4.15. AspAT activity of adult *O. circumcincta* homogenate A7a monitored in the direction of aspartate utilisation with increasing concentration of 2-oxoglutarate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340nm.

Table 4.7. Effects of 1 mM ATP or ADP on the activities of AspAT (mean ± SEM, n = 3) of sheathed L3 *O. circumcincta* homogenate SL31 in the direction of aspartate utilisation. Concentrations of 5 mM aspartate and 1 mM 2-oxoglutarate were used. AspAT activities without the addition of these compounds were set at 100%.

Homogenate	1 mM ADP (%)	1 mM ATP (%)
SL 31a	78	64
SL 31b	94	76
SL 31c	94	77
mean ± SEM, n = 3	89 ± 6	73 ± 8

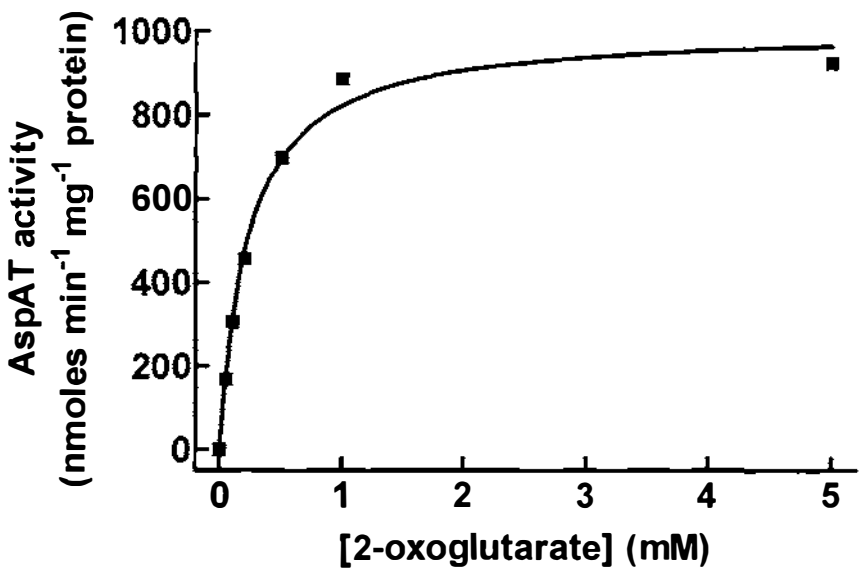


Figure 4.14. AspAT activity of sheathed L3 *O. circumcincta* homogenate SL32 monitored in the direction of aspartate utilisation with increasing concentration of 2-oxoglutarate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340 nm.

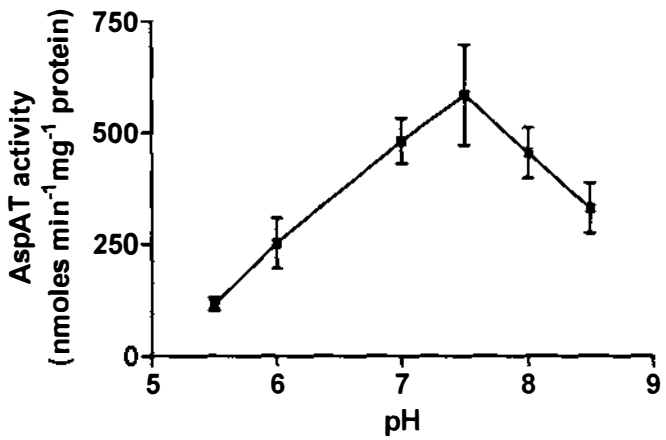


Figure 4.12. Effect of pH on AspAT activities (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL29 in the direction of aspartate utilisation. Concentrations of 5 mM aspartate and 1 mM 2-oxoglutarate were used. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340nm.

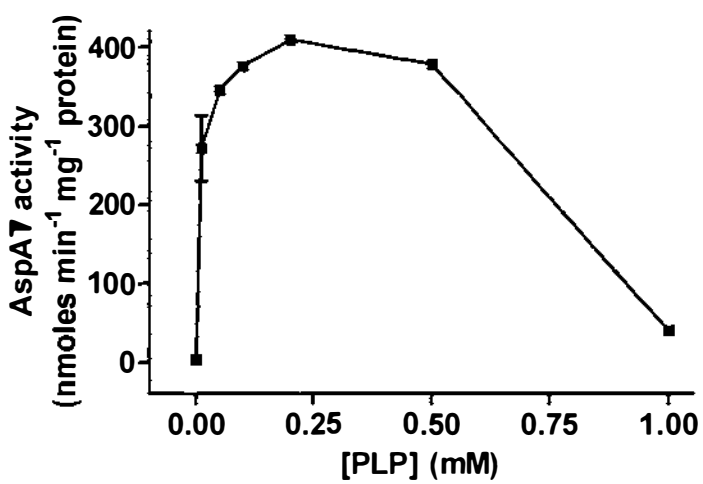


Figure 4.13. Effect of PLP concentration on AspAT activities (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenate SL30 in the direction of aspartate utilisation. Concentrations of 3 mM aspartate and 1 mM of 2-oxoglutarate were used. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with MDH, which was monitored spectrophotometrically at 340nm.

4.3.2 ASPARTATE AMINOTRANSFERASE

4.3.2.1 Effect of pH, PLP concentration, ADP and ATP

The effects of pH and [PLP] on the AspAT activity (mean ± SEM, n = 2) of homogenates of sheathed L3 (SL29 and SL30 respectively in duplicate) in the direction of aspartate utilisation are shown in Figures 4.12 and 4.13 respectively. The optimum pH and the optimum PLP concentration were pH 7.5 and 0.2 mM respectively.

The effects of 1 mM ADP and ATP on AspAT activity of a homogenate of sheathed L3 (SL31) are shown in Table 4.7. Activities decreased to 89 ± 6% and 73 ± 8% (mean ± SEM, n = 3) in the presence of 1 mM ADP and ATP respectively.

4.3.2.2 Kinetic parameters in the direction of aspartate utilisation

For the substrate 2-oxoglutarate, the AspAT activities of homogenates of sheathed L3 (SL32) and adult worms (A7a) and the K_m and V_{max} for these homogenates are shown in Figures 4.14 and 4.15 and Tables 4.8 and 4.9 respectively. The K_m values for 2-oxoglutarate were 0.2 ± 0.02 and 0.2 ± 0.1 mM (both mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively. The V_{max} were 1060 ± 103 and 287 ± 83 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2).

For the substrate aspartate, the AspAT activities of homogenates of sheathed L3 (SL35) and adult worms (A8) are shown in Figures 4.16 and 4.17 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 4.10 and 4.11. The K_m values for aspartate were 2.1 ± 0.2 and 2.6 ± 0.6 mM (both mean ± SEM, n = 3) for sheathed L3 and adult worms respectively. The V_{max} were 1170 ± 122 and 118 ± 26 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n = 3) for sheathed L3 and adult worms.

4.3.2.3 Kinetic parameters in the direction of aspartate formation

For the substrate glutamate, the AspAT activity of a homogenate of sheathed L3 (SL36) is shown in Figure 4.18 and the K_m and V_{max} of the homogenates of sheathed L3 (n = 3) are shown in Table 4.12. The K_m and

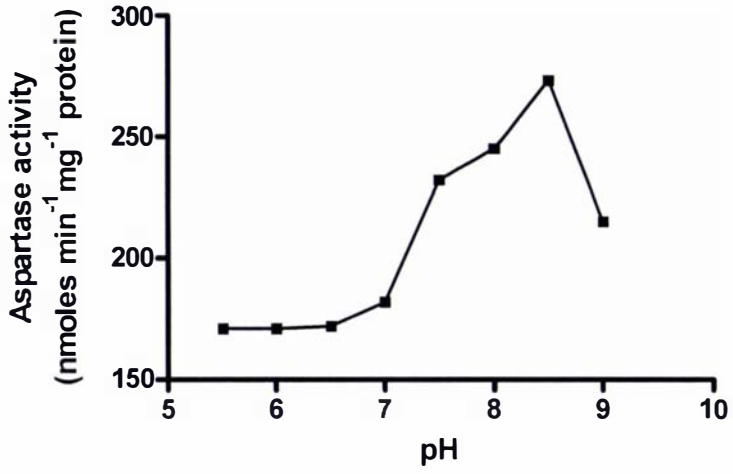


Figure 4.24. Effect of pH on aspartase activities of sheathed L3 *O. circumcincta* homogenate SL46 in the direction of fumarate formation. Concentrations of 1 mM aspartate and 20 mM 2-oxoglutarate were used. Aspartase activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340nm.

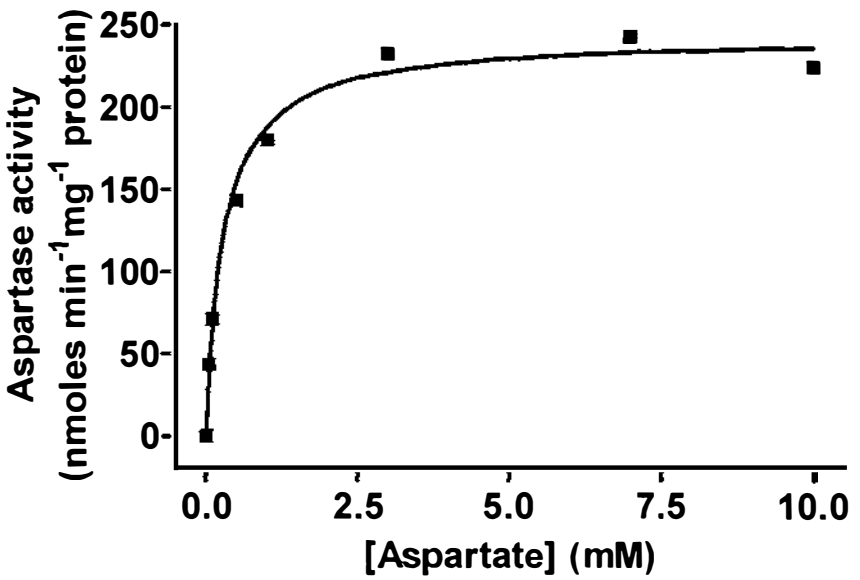


Figure 4.23. Aspartase activity of sheathed L3 *O. circumcincta* homogenate SL43 monitored in the direction of fumarate formation with increasing concentration of aspartate. Aspartase activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340nm.

Table 4.17. Aspartase activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of fumarate formation with increasing concentration of aspartate. Aspartase activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340nm.

Homogenate	Aspartate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 43	0.3	243
SL 44	0.2	232
SL 45	0.1	58
mean ± SEM, n = 3	0.2 ± 0.06	178 ± 60

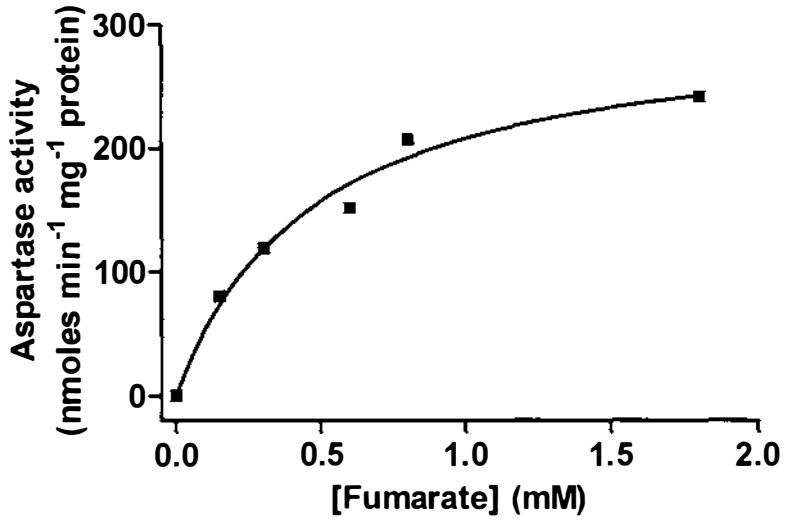


Figure 4.22. Aspartase activity of sheathed adult *O. circumcincta* homogenate A10 monitored in the direction of fumarate utilisation with increasing concentration of fumarate. Aspartase activity was calculated from the rate of fumarate utilisation, which was monitored spectrophotometrically at 260 nm.

Table 4.16. Aspartase activities of adult *O. circumcincta* homogenate A10 monitored in the direction of fumarate utilisation with increasing concentration of fumarate. Aspartase activity was calculated from the rate of fumarate utilisation, which was monitored spectrophotometrically at 260 nm.

Homogenate	Fumarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A10	0.5	307

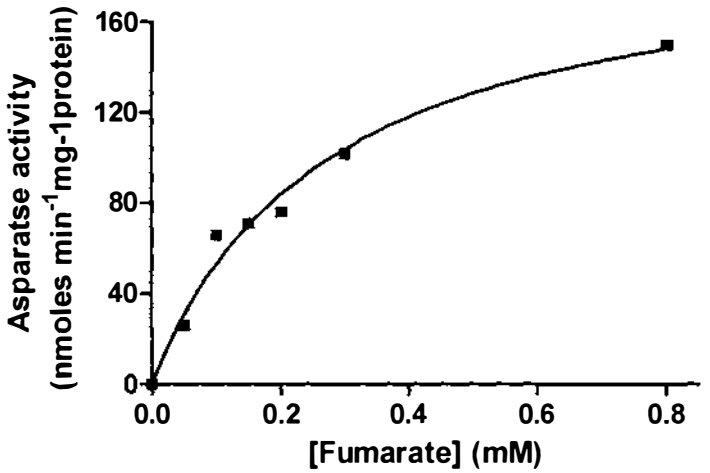


Figure 4.21. Aspartase activity of sheathed L3 *O. circumcincta* homogenate SL41 monitored in the direction of fumarate utilisation with increasing concentration of fumarate. Aspartase activity was calculated from the rate of fumarate utilisation, which was monitored spectrophotometrically at 260 nm.

Table 4.15. Aspartase activities of L3 *O. circumcincta* sheathed L3 homogenates monitored in the direction of fumarate utilisation with increasing concentration of fumarate. Aspartase activity was calculated from the rate of fumarate utilisation, which was monitored spectrophotometrically at 260 nm.

Homogenate	Fumarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 41	0.15	209
SL 42	0.30	198
mean ± SEM, n = 2	0.2 ± 0.08	204 ± 6

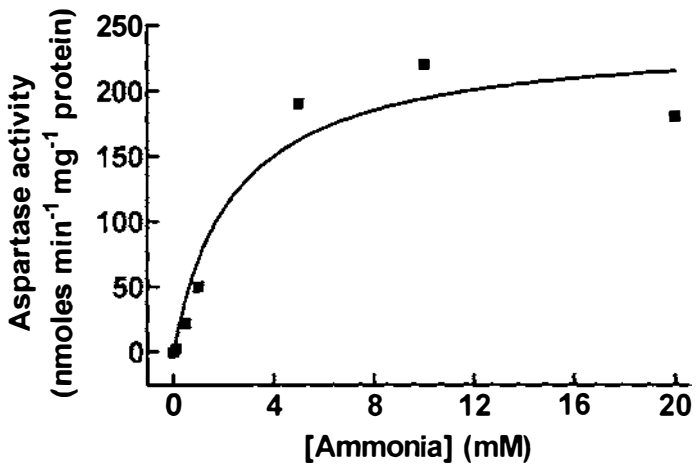


Figure 4.20. Aspartase activity of sheathed L3 *O. circumcincta* homogenate SL39 monitored in the direction of fumarate utilisation with increasing concentration of ammonia. Aspartase activity was calculated from the rate of fumarate utilisation, which was monitored spectrophotometrically at 260 nm.

Table 4.14. Aspartase activities of L3 *O. circumcincta* sheathed L3 homogenates monitored in the direction of fumarate utilisation with increasing concentration of ammonia. Aspartase activity was calculated from the rate of fumarate utilisation, which was monitored spectrophotometrically at 260 nm.

Homogenate	Ammonia K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 39	2.5	242
SL 40	1.5	142
mean ± SEM, n = 2	2.0 ± 0.5	192 ± 50

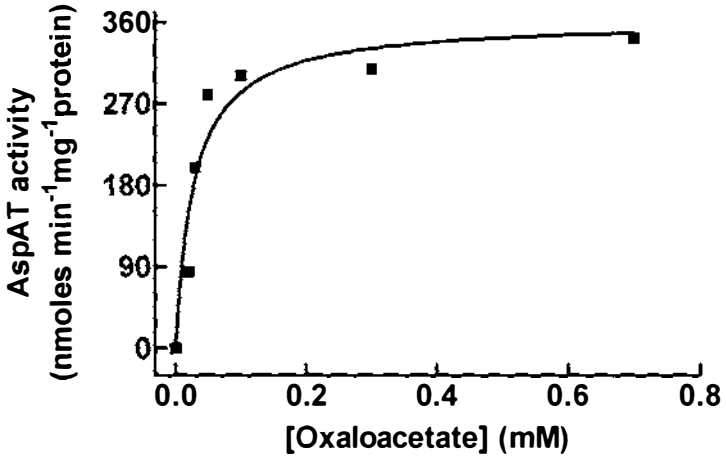


Figure 4.19. AspAT activity of sheathed L3 *O. circumcincta* homogenate SL37c monitored in the direction of aspartate formation with increasing concentration of oxaloacetate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 4.13. AspAT activities of sheathed L3 *O. circumcincta* homogenates monitored in the direction of aspartate formation with increasing concentration of oxaloacetate. AspAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Oxaloacetate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL 37c	0.03	363
SL 38a	0.12	300
SL 38b	0.12	223
mean ± SEM, n = 3	0.09 ± 0.03	295 ± 40

V_{max} were 0.1 ± 0.02 mM and 408 ± 15 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) respectively.

For the substrate oxaloacetate, the AspAT activity of a homogenate of sheathed L3 (SL37c) is shown in Figure 4.19 and the K_m and V_{max} of the homogenates of sheathed L3 (n = 3) are shown in Table 4.13. The K_m and V_{max} were 0.09 ± 0.03 mM and 295 ± 40 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) respectively.

4.3.3 ASPARTASE

4.3.3.1 Kinetic parameters in the direction of fumarate utilisation

For the substrate ammonia, aspartase activity of a homogenate of sheathed L3 (SL39) is shown in Figure 4.20. The K_m and V_{max} of the homogenates of sheathed L3 (n = 2) are shown in Table 4.14. The K_m was 2.0 ± 0.5 mM and the V_{max} was 192 ± 50 nmoles min^{-1} mg^{-1} protein (both mean ± SEM; n = 2).

For the substrate fumarate, the aspartase activity of a homogenate of sheathed L3 (SL41) and adult worms (A10) are shown in Figures 4.21 and 4.22 respectively. The K_m and V_{max} of the homogenates of sheathed L3 (n = 2) and adult worms (n = 1) are shown in Tables 4.15 and 4.16. The K_m were 0.2 ± 0.08 mM (mean ± SEM, n = 2) and 0.5 mM (n = 1) for sheathed L3 and adult worms. The V_{max} were 204 ± 6 (mean ± SEM, n = 2) and 307 nmoles min^{-1} mg^{-1} protein for sheathed L3 and adult worms respectively.

4.3.3.2 Kinetic parameters in the direction of fumarate formation

For the substrate aspartate, the aspartase activity of a homogenate of sheathed L3 (SL43) is shown in Figure 4.23 and the K_m and V_{max} of the homogenates of sheathed L3 (n = 3) are shown in Table 4.17. The K_m and V_{max} were 0.2 ± 0.06 mM and 178 ± 60 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) respectively.

4.3.3.3 Effect of pH

The effects of pH on the aspartase activity in the direction of fumarate formation of a homogenate of sheathed L3 (SL46) are shown in Figure 4.24. The optimum pH was pH 8.5.

Table 4.18. Aspartase activities in the direction of fumarate formation of sheathed L3 *O. circumcincta* homogenate SL47 in the presence of ions, ATP, ADP or EDTA. Aspartase activity in the absence of added compounds was set at 100%.

Effector/inhibitor	Conc (mM)	Activity (nmoles min^{-1} mg^{-1} protein)	Activity (% control)
Control	-	216	100
Ca^{2+}	1	180	83
Ca^{2+}	10	163	76
ATP	1	197	91
ADP	1	199	92
EDTA	1	208	97
EDTA	5	201	93
Mn^{2+}	1	164	76
K^{+}	1	191	88
Mg^{2+}	1	200	93
Mg^{2+}	10	175	81

4.3.3.3 Effectors/inhibitors

Aspartase activities in the direction of fumarate formation of a homogenate of sheathed L3 (SL47) in the presence of various concentrations of ions, EDTA ATP and ADP are shown in Table 4.18. Aspartase activities were also expressed as a percentage of the value in the absence of added compounds, which was set at 100%. The largest effects were with 10 mM Ca^{2+} or 1 mM Mn^{2+} (24% lower). Aspartase activity was reduced by 80% in an aliquot of the same homogenate which had been stored at 4°C for 24 hours.

4.4 DISCUSSION

The metabolism of alanine and aspartate is involved in the interconversion of amino acids to provide the mixture needed for protein synthesis and in contributing the carbon skeletons of amino acids to the TCA cycle for energy production. Aspartate also has a special role in aerobic metabolism as part of the malate-aspartate shuttle which transports NADH into mitochondria for oxidation (Figure 1.10). Central to the metabolism of both amino acids are the aminotransferases AlaAT and AspAT, which were very active, particularly AspAT, in L3 *O. circumcincta*. These aminotransferases have previously been reported in nematodes (Rasero *et al.*, 1968; Grantham and Barrett, 1986b; Walker and Barrett, 1991a,b), including *O. circumcincta* (Walker and Barrett, 1991b) (Chapter 1, Section 1.6.11). In contrast, the presence of aspartase activity is unexpected in an animal, and, if confirmed by identification of an aspartase gene and an important role established for the enzyme, *O. circumcincta* aspartase may be considered a potential target to be exploited for drug development against the parasite.

4.4.1 ALANINE AMINOTRANSFERASE (ALAAT)

AlaAT activity can be compared in the forward and reverse directions and between L3 and adult *O. circumcincta*. The kinetic properties of L3 *O. circumcincta* AlaAT were generally consistent with those of the enzymes of other species. AlaAT activity was PLP-dependent, with an optimum concentration (0.25 mM) and inhibition at higher concentration (Figure 4.5). The pH optimum of 7.5 (Figure 4.4) was similar to that of pig heart enzymes (Swick *et al.*, 1965; Saier and Jenkins, 1967).

Table 4.19. K_m values for the substrates alanine (Ala), 2-oxoglutarate (2-OG), pyruvate (Pyr) and glutamate (Glu) for the reactions catalysed by alanine aminotransferases of different organisms.

Enzyme source	K_m (mM)				Reference
	Ala	2-OG	Pyr	Glu	
O. circumcincta L3	5.2	0.5	1.2	0.5	Tables 4.1, 4.2, 4.4, 4.5
O. circumcincta adult worms	0.5		0.1		Table 4.3, 4.6
Atriplex spongiosa	0.25	0.30	0.45	0.55	Hatch (1973)
form 2	3.0	0.09	0.04	1.15	
form 3	3.1	0.03	0.02	0.8	
Corbicula japonica	5.6	0.12	0.04	9.5	Hayashi (1993)
cAlaAT					
mAlaAT	7.4	0.16	0.03	35.6	
Panicum miliaceum	6.7	0.15	0.33	5.0	Son *et al.* (1991)
Glossina morsitans fat body	7.7	0.4	0.53	20	Konji *et al.* (1984)
Flight muscle	35.7	2.5	0.22	12.5	
Dolphin muscle	8.2	0.45	0.87	15	Owen and Hochachka (1974)
Rat liver (cyt 1)	0.51	0.12			Vedavathi *et al.* (2004)
Rat liver (cyt 2)	1.14	0.17			
Chlamydomonas reinhardtii	2.7	0.05	0.24	0.52	Lain-Guelbenzu *et al.* (1991)
Pyrococcus furiosus	2.8	0.9	5.4	4.3	Ward *et al.* (2000)

AlaAT activity was moderate in L3 in the directions of alanine utilisation and formation (Tables 4.1-4.2 and 4.4-4.6), although lowest with pyruvate as the variable substrate. In contrast, for the two adult worm homogenates, activity was much lower with alanine (79 nmoles min^{-1} mg^{-1} protein) than with pyruvate (256 nmoles min^{-1} mg^{-1} protein), comparable to the 122 and 288 nmoles min^{-1} mg^{-1} protein respectively reported for adult *O. circumcincta* by Walker and Barrett (1991b). In the other species they studied, activity was equal in *H. contortus* (864 and 840 nmoles min^{-1} mg^{-1} protein respectively), but lower in the direction of alanine utilisation in *T. colubriformis* (47 and 59), *C. oncophora* (47 and 136), *N. brasiliensis* (361 and 727), *P. redivivus* (1060 and 2381) and *S. bibionis* (385 and 280 nmoles min^{-1} mg^{-1} protein).

The AlaAT activity in adult *O. circumcincta* may be higher than in L3. As discussed earlier for other enzymes (Chapter 3, 3.4.1), it is difficult to compare enzyme activities in homogenates of whole parasites at different life-cycle stages because of differences in body composition, however, it does seem likely that adult worms have greater AlaAT activity than L3. A possible difficulty in measuring AlaAT activity in the direction of alanine formation is endogenous LDH activity, which also uses pyruvate as substrate. Generally, LDH activity is only moderate in *O. circumcincta* L3 (D.C. Simcock and L. Walker, personal communication) and the enzyme is not as active as the 256 nmoles min^{-1} mg^{-1} protein which was recorded for the reverse direction of AlaAT in the adult worm homogenate tested (Table 4.6).

The comparison of K_m values for the four substrates of *O. circumcincta* AlaAT with published values of the isozymes of a number of plants, microorganisms and animals in Table 4.19 shows that the K_m for pyruvate and 2-oxoglutarate are fairly consistent across species, whereas the K_m values for alanine are either within the range 3-7 mM or 0.3-0.5 mM. A marked difference between AlaAT in L3 and adult homogenates was the lower K_m in adults for both alanine and pyruvate by a factor of 10 (5.2 ± 0.5 mM and 1.2 ± 0.2 mM for L3; 0.5 ± 0.1mM and 0.1 mM for adult worms respectively), which may indicate an increased proportion of the mitochondrial isoform. In rat liver, mAlaAT has a fourfold lower K_m for alanine than the cytosolic enzyme (Swick *et al.*, 1965), constitutes only about 8% of total activity and is more unstable *in vitro* (Swick *et al.*, 1965; Saier and Jenkins, 1967; DeRosa and Swick, 1975). Pyruvate formation is favoured by the mitochondrial enzyme and alanine

formation by the cytosolic enzyme (DeRosa and Swick, 1975). The cytosolic enzyme also predominates in nematodes: in adult *N. brasiliensis*, cytosolic AlaAT was responsible for 80% of activity, whereas in *H. contortus* 54% was cytosolic and 22% was associated with the cell debris and cuticle fractions (Walker and Barrett, 1991b).

Some of the differences between reported total AlaAT activities in parasitic nematodes may be influenced by host nutritional status, particularly vitamin B_6 levels. This has been shown to be the case for the rat filarial parasite *L. carinii* which has decreased AlaAT activity when parasitising vitamin B_6-deficient cotton rats (Beg *et al.*, 1995). The requirement for vitamin B_6 by the cestode *H. diminuta* was reported by Addis and Chandler (1944) and Roberts and Platzer (1967) and correlated with a 66% decline in AlaAT activity by Platzer and Roberts (1970).

4.4.2 Aspartate aminotransferase (AspAT)

AspAT activity was the highest of any enzyme measured in homogenates of L3 *O. circumcincta* in the present studies and 10-fold higher than AlaAT activity. The properties were consistent with those of AspAT from other organisms. The L3 enzyme was pH dependent, with markedly reduced activity outside the range pH 7-8.5 (Figure 4.12), therefore assays were carried out at pH 7.5. AspAT from sheep (Orlacchio *et al.*, 1979) and *T. cruzi* (Cazzulo *et al.*, 1977) had similar broad pH optima between pH 6.5 and 8.5. Typically for aminotransferases, it required PLP as a co-factor with an optimal concentration of about 0.25 mM (Figure 4.13). Activity was low below 0.15 mM and above 0.5 mM PLP concentration, with almost complete inhibition at 1 mM. There were only minor inhibitory effects of ADP (about 25%) and no significant effect of ATP (Table 4.7), consistent with the lack of effect of ATP on dolphin muscle AspAT (Owen and Hochachka, 1974).

Comparison of activity in crude homogenates in the directions of aspartate utilisation and formation is probably unreliable, as it is difficult to measure AspAT activity with OAA as substrate in the presence of high endogenous MDH activity (of the order of 8000 nmole $min^{-1}mg^{-1}$ protein in phosphate buffer or about half that in Tris buffer, which is used in the AspAT assay; Dr D.C. Simcock, personal communication). Although background enzymatic activity is corrected in the continuous method, there is more

Table 4.20. K_m values for the substrates aspartate (Asp), 2-oxoglutarate (2-OG), oxaloacetate (OAA) and glutamate (Glu) for the reactions catalysed by aspartate aminotransferases of different organisms.

Enzyme source	K_m (mM)				Reference
	Asp	2-OG	OAA	Glu	
O. circumcincta L3	2.1	0.2	0.09	0.1	Tables 4.8, 4.10, 4.12, 4.13
O. circumcincta adult worms	2.6	0.2			Tables 4.9, 4.11
Phormidium lapideum	5.0	0.2	0.03	5.7	Kim *et al.* (2003)
Rhizobium meliloti	5.3	0.87			Alfano and Kahn (1993)
Oat leaves form 1	4.1	0.22	0.06	32.9	Reed and Hess (1975)
Oat leaves form 2	2.3	0.3	0.03	13.7	
Daucus carota carrot (form 1)	2.8	0.22	0.05	23.6	Turano *et al.* (1990)
Pig heart (cyt)	4	0.4	0.02	14	Michuda and Martinez-Carrion (1969)
Pig heart (mit)	0.47	0.7	0.01	12.4	
Sheep liver (cyt)	3.0	0.09			Orlacchio *et al.* (1979)
Sheep liver (mit)	0.4	0.98			
Chicken liver (cyt)	1.2	0.03			Quiroga *et al.* (1985)
Chicken (cyt)	2	0.3	0.04	5	Shrawder and Martinez-Carrion (1973)
Chicken (mit)	0.6	1.2	0.02	11	
Human heart (cyt)	1.1	0.1			Teranishi *et al.* (1978)
Human heart (mit)	0.2	0.67			

likelihood of inaccuracy in this case. However, the apparent AspAT activity in the direction of aspartate formation was 300-400 nmole $min^{-1}mg^{-1}$protein (Tables 4.12 and 4.13), considerably less than in the opposite direction of 1000 nmole $min^{-1}mg^{-1}$ protein with either aspartate or 2-oxoglutarate as substrate (Tables 4.8 and 4.10). Assuming the activities recorded are at least indicative, L3 enzyme activity would appear to be greater in the direction of aspartate utilisation than formation. The affinity of AspAT for the substrates aspartate (2.1 mM) and OAA (0.09 mM) suggests aspartate formation might be favoured *in vivo*.

Adult worm homogenates had a 5-fold lower AspAT activity than L3 homogenates, but similar substrate affinities for both aspartate and 2-oxoglutarate (Tables 4.8-4.11). This may represent a real decrease in enzyme activity despite differences in the proportions of body tissues, since the difference in AlaAT activity between adult worms and L3 (79 compared with 115 nmole $min^{-1}mg^{-1}$protein respectively) was much less than the difference for AspAT activity.

AspAT has been identified in many organisms including the cestodes *H. diminuta*, *H. nana* and *H. citelli* (Wertheim *et al.*, 1960), and *M. expansa* (Rasero *et al.*, 1968), the Digeneans *C. lingua* and *C. emasculans* (Watts, 1970) and the nematodes *A. lumbricoides* (Rasero *et al.*, 1968) and *N. brasiliensis* (Walker and Barrett, 1991a). Reported K_m values for the four substrates for AspATs from several different types of organisms presented in Table 4.20 show the values for the *O. circumcincta* enzyme to be generally similar, although the K_m for glutamate was lower than commonly reported.

Where values are available for cAspAT and mAspAT from the same species, the pattern is for the K_m for aspartate to be lower and the K_m for 2-oxoglutarate to be higher in the mitochondrial isoform by a factor of 5-10. If there were similar differences in the nematode isozymes, *O. circumcincta* AspAT would appear to be predominantly the cytosolic form. In vertebrates, cAspAT is increased in gluconeogenic states (Horio *et al.*, 1988), along with phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate carboxylase (PC), which together generate the required phosphoenolpyruvate (PEP) from pyruvate and OAA (Nordlie *et al.*, 1965; Taylor *et al.*, 1971). A predominantly cAspAT in *O. circumcincta* may reflect a similar metabolic pattern.

Table 4.21. K_m values for the substrate aspartate (Asp) for the reactions catalysed by aspartases of different organisms.

Enzyme source	K_m (mM)	Reference
O. circumcincta L3	0.2	Table 4.17
Pseudomonas fluorescens	2.3	Kawata *et al.* (1999)
P. freudenreichii subsp *shermanii*	9.1	Crow (1987)
H. alvei (with Mn^{2+})	0.7	Nuiry *et al.* (1984)
(with Mg^{2+})	2.5	
Bacterium cadaveris	20	Williams and McIntyre (1955)
Bacillus sp	28.5	Kawata *et al.* (1999)
E. coli	0.33	Depue and Moat (1961)
	4	Rudolph and Fromm (1971)
	1.0	Suzuki *et al.* (1973)
	1.8	Jayasekera *et al.* (1997)
	1.2	Wang *et al.* (2000)

4.4.3 ASPARTASE

The aspartase activity in L3 *O. circumcincta* (Tables 4.14-4.15) was unexpected, as it is usually considered an enzyme of microorganisms (Viola, 2000), although it has been found in frog embryos (Kurata, 1962), teleost fish (Salvatore *et al.*, 1965) and elasmobranchs (Salvatore *et al.*, 1965; Cutinelli *et al.*, 1972). It is possible that the apparent aspartase activity was due to another enzyme. The most likely candidate would be AspAT, however, AspAT is a PLP-dependent enzyme and no PLP was added to the reaction mixture. AspAT could theoretically form fumarate sequentially: by converting aspartate to OAA (AspAT); conversion of OAA to malate (MDH); then conversion of malate to fumarate by fumarase. Although this route cannot be discounted, the kinetics of the reaction supports the reaction being direct via aspartase. The K_m for aspartate of 0.2 ± 0.06 mM for the L3 *O. circumcincta* enzyme was generally in the range of reported values for aspartases from microorganisms (Table 4.21) and 10-fold lower than the K_m for aspartate for L3 AspAT of 2.1 ± 0.2 mM (Table 4.10). The purine nucleotide cycle enzyme adenylosuccinate synthetase also has aspartate as a substrate (Chapter 1, 1.6.9), but the lack of added IMP and GTP would most likely rule out that reaction being responsible for the formation of fumarate.

The reverse reaction between fumarate and ammonia to form aspartate apparently also took place and K_m values were obtained for ammonia and fumarate for L3 homogenates and for fumarate for an adult worm homogenate. The K_m for ammonia of 2.0 ± 0.5 mM (Table 4.14) was considerably lower than the K_m for *E. coli* of 20 mM (Suzuki *et al.*, 1973) and for *Hafnia alvei* of 112 mM (Nuiry *et al.*, 1984). The K_m for fumarate of 0.2 ± 0.08 mM for L3 and 0.5 for adult worm homogenates are similar to the 0.2 mM for *E. coli* aspartase (Suzuki *et al.*, 1973). There are limitations in estimating the K_m for fumarate and ammonia in the reverse reaction, because the reaction is monitored by the disappearance of fumarate, the concentration of which is limited to 2 mM by the ability to read the absorbance reliably. The ammonia in *O. circumcincta* homogenates of 10-15 µM is accounted for in the continuous assay. Despite the limited starting concentration of fumarate, the V_{max} of the reaction was around 200-300 nmoles $min^{-1}mg^{-1}$protein in both directions.

There is also the possibility of competition for the common substrate fumarate between aspartase, fumarase or fumarate reductase/succinate dehydrogenase. Fumarate reductase/succinate dehydrogenase requires anaerobic conditions (Kita, 1992) and is not likely to be important. Fumarase, an enzyme structurally related to aspartase, catalyses the hydration of fumarate to form malate (Woods *et al.*, 1988), however, the addition of ammonia would be expected to favour aspartase activity. Therefore, measurement of aspartase activity based on fumarate disappearance could overestimate fumarase activity. In contrast, aspartase activity in the direction of fumarate formation might be underestimated if fumarate were removed by fumarase or fumarate reductase/succinate dehydrogenase.

Aspartase requires Mn^{2+} or Mg^{2+} for activity at alkaline pH, but not neutral or acidic pH (Suzuki *et al.*, 1973; Viola, 2000). There appeared to be adequate divalent cations in the *O. circumcincta* homogenates without including more in the assays, which were carried out at pH 7.5, although the V_{max} measured here may not be the maximum possible rates for the enzyme. Further support for there being adequate Mn^{2+} bound to the enzyme was the lack of activation by 1 mM Mn^{2+} in the inhibitor and effector study (Table 4.18). None of the substances tested had a marked effect on aspartase activity and there was only one experiment carried out. The 19% decrease in activity recorded for 10 mM Mg^{2+} may be a real inhibition, however, as this concentration showed a similar degree of inhibition of *Propionibacterium freudenreichii* aspartase (Crow, 1987). The only other changes of any magnitude were the lower activities by 24% with 10 mM Ca^{2+} and with 1 mM Mn^{2+}, perhaps similar to the 18% inhibition of *H. alvei* aspartase by Ca^{2+} reported by Yoon *et al.* (1998). EDTA was without effect on *O. circumcincta* aspartase, but is inhibitory to *E. coli* aspartase (Suzuki *et al.*, 1973).

Overall, the properties of *O. circumcincta* aspartase are similar to those of the microbial enzymes and, as was discussed previously for creatinase activity (Chapter 3, Section 3.4.2), there is the possibility that microbial contamination in the nematode homogenate is responsible for aspartase activity. This would best be resolved by a gene encoding aspartase being identified in the nematode genome.

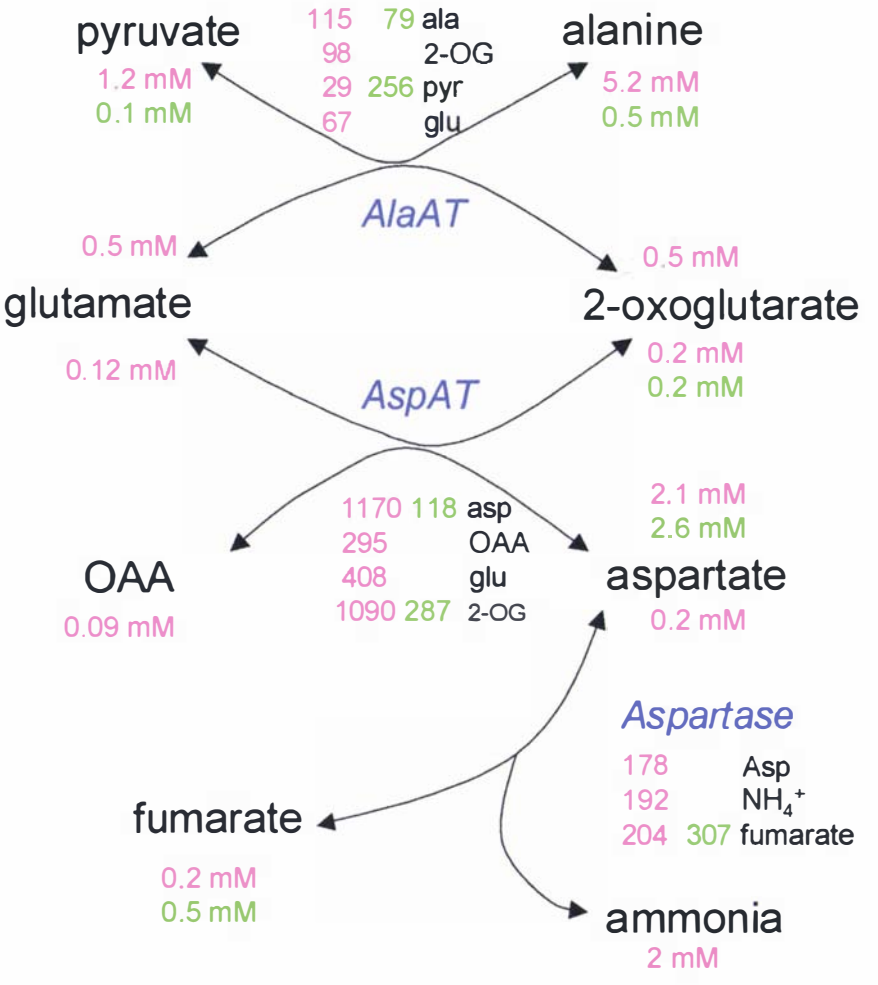


Figure 4.25. Metabolic map of enzymes of alanine and aspartate metabolism identified in L3 or adult *O. circumcincta* homogenates. Enzymes are shown in blue; K_m values (mM) for the substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reactions for the variable substrate are shown in pink for L3 homogenates and green for adult worm homogenates.

4.4.4 ALANINE AND ASPARTATE METABOLISM

A metabolic map linking the enzymes identified in L3 or adult *O. circumcincta* homogenates is shown in Figure 5.25. The K_m values for the substrates and the V_{max} for the reactions are indicated.

The metabolism of alanine and aspartate and the role of aminotransferases in nematodes are probably to link energy and amino acid metabolism, as in vertebrates. In those more complex animals, dietary protein is broken down to amino acids or small peptides, which are absorbed and metabolised in the intestine, releasing amino acids, predominantly alanine and glutamine into the circulation to be used by the liver and other organs (reviewed by Walton and Cowie, 1982; Brosnan, 2003). After a meal, excess amino acids are excreted as urea or used for gluconeogenesis. In the opposite case of starvation, body proteins, particularly in those in muscle, are broken down largely to alanine and glutamine and used for energy after removal of amino groups, mainly by transamination. Under these conditions, aminotransferase activity increases (Horio *et al.*, 1988; Srivastava *et al.*, 1999). In the non-feeding L3, contributing amino acid carbon skeletons to the TCA may be an important function, although the high K_m for alanine argues against this. In adult worms, the shift in K_m would suit this role.

Differences in the overall energy metabolism of parasites and their mammalian hosts and between free-living and parasitic stages may change the role of aminotransferases. It is generally considered there is a shift from aerobic to anaerobic metabolism during the transition to parasitism (Komunieki and Vanover, 1987). This may not be as marked in the smaller nematodes, such as *O. circumcincta*, because their higher surface area to volume ratio may allow more oxygen diffusion than is possible in the large ascarids, cestodes and trematodes which have been more frequently studied. The rate of oxygen delivery is likely to be less in adult worms than in L3, which are about 100 times less in volume (Chapter 2, 2.4).

Generalisation across species may be erroneous, e.g. filarial nematodes generally rely heavily on glycolysis and produce lactate as an end-product under both aerobic and anaerobic conditions (reviewed by Köhler, 1991). *L. carinii* was considered a model for the group, but has now been found to have a much more aerobic metabolism than other related parasites.

Table 4.22. Activities and substrate K_m of AlaAT, AspAt and aspartase compared with those of some TCA cycle enzymes in homogenates of L3 *O. circumcincta*. (Unpublished data for pyruvate kinase generously provided by Ms L. Walker and for phosphoenolpyruvate carboxykinase, malate dehydrogenase, fumarase, fumarate reductase and succinate dehydrogenase by Dr D.C. Simcock, IFNHH, Massey University).

Enzyme	Substrate	K_m (mM) mean ± SEM (n)	V_{max} (nmoles min^{-1} mg^{-1} protein)
AlaAT	Alanine	5.2 ± 0.5 (3)	115 ± 30 (3)
	Pyruvate	1.2 ± 0.2 (3)	29 ± 1 (3)
AspAT	Aspartate	2.1 ± 0.2 (3)	1170 ± 122 (3)
	OAA	0.09 ± 0.03 (3)	295 ± 40 (3)
Aspartase	Aspartate	0.2 ± 0.06 (3)	178 ± 60 (3)
Pyruvate kinase	PEP	0.15 ± 0.03 (8)	343 ± 53 (8)
Phosphoenol-pyruvate carboxykinase	PEP	0.02 ± 0.005 (6)	100 ± 21 (11)
Malate dehydrogenase	Malate	2.85 ± 0.54 (4)	759 ± 86 (7)
	OAA	0.03 ± 0.54 (4)	7728 ± 641 (4)
Fumarase	Fumarate	0.26 ± 0.05 (4)	448 ± 158 (4)
	Malate	2.45 ± 0.33 (4)	292 ± 67 (4)
Fumarate reductase	Fumarate	0.15 ± 0.04 (3)	9.5 ± 1.5 (3)
Succinate dehydrogenase	Succinate	0.32 ± 0.04 (4)	27 ± 2.3(4)

Within the gastro-intestinal nematode parasites, Ascarids are probably the most extensively studied, but probably have the most anaerobic metabolism (reviewed by Kurelec, 1975; Saz, 1981; Köhler, 1985). This parasite has little pyruvate kinase (PK) activity, so that PEP is carboxylated to OAA by PEPCK; OAA is then converted to malate by high cMDH activity. Malate enters the mitochondria and is converted to fumarate and succinate by fumarate reductase/succinate dehydrogenase. Another important end-product is acetate, formed by malic enzyme and pyruvate decarboxylase. Adult *O. circumcincta* metabolism appears to be more aerobic than that in *Ascaris* (D.C. Simcock, personal communication).

Amino acids are considered to contribute to nematode energy metabolism (Bruce *et al.*, 1972; Davies and Köhler, 1990), as some species are able to survive *in vitro* with glutamine or alanine as the sole carbon source. In filarial nematodes, alanine was degraded to lactate, acetate and CO_2 and the nitrogen of glutamine was partly excreted also as ammonia and alanine (Davies and Köhler, 1990). Alanine is a metabolic end-product in vertebrate tissues (skeletal muscle) and in invertebrate tissues (cultured insect cells), although its production in the latter ceases if glucose is limiting (Drews *et al.*, 2000). Alanine is also the main amino acid that accumulates under anaerobic conditions in plants (Streeter and Thompson, 1972; Ricard *et al.*, 1994; de Sousa and Sodek, 2003) and in *G. lamblia* and *G. intestinalis* (Edwards *et al.*, 1989; Paget *et al.*, 1990; Nygaard *et al.*, 1994).

Activities and substrate K_m of L3 *O. circumcincta* AlaAT, AspAT and aspartase are compared with those of enzymes sharing their substrates or products are presented in Table 4.22 (D.C. Simcock and L. Walker, unpublished data). The highest activities were for AspAT and MDH, which are required for the malate-aspartate shuttle (Figure 1.10), a transporter of NADH into the mitochondria for oxidation during aerobic metabolism (Chapter 1, 1.6.11.2). This suggests an active malate-aspartate shuttle. There was an apparent decrease in AspAT activity in adult worms, perhaps because of a shift towards more anaerobic metabolism as worm size increased. The K_m for aspartate is more typical of cAspAT, which in vertebrates is high in gluconeogenic states (Horio *et al.*, 1988), along with PEPCK and PC (Nordlie *et al.*, 1965; Taylor *et al.*, 1971). PEPCK activity is high in L3 *O. circumcincta* (Table 4.22).

It is difficult to assign a role for aspartase activity, other than as an alternative route to AspAT between aspartate and the TCA cycle. The activity was considerable and the K_m favourable to compete with AspAT for aspartate. Alternatively, it may operate in the opposite direction, as fumarase is presumed to operate in the direction of malate to fumarate in an anaerobic parasite TCA cycle, rather than in the normal direction. If aspartase activity can be confirmed by molecular biological studies, it may be easier to define its role in *O. circumcincta*.

Alanine, aspartate and glutamate metabolism are closely linked. Aminotransferases interconvert amino acids by catalysing the transfer of an amino group from a donor α-amino acid to acceptor α-keto acid, which is usually 2-oxoglutarate. In addition to this role in maintaining the balance of non-essential amino acids needed to synthesis proteins, in lower organisms, glutamate is also involved in incorporating ammonia into amino acids. This is possible in nematodes, as genes for the necessary enzymes have been identified in the *C. elegans* genome. Properties of enzymes involved in glutamate metabolism in *O. circumcincta* are reported in Chapter 5.

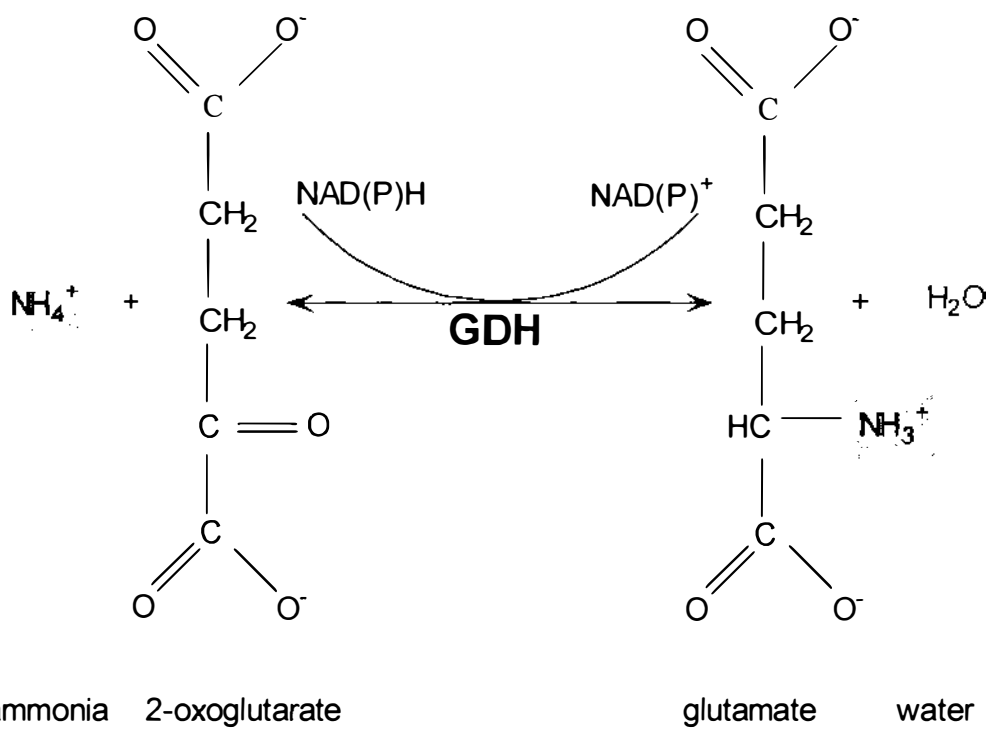


Figure 5.1. The reaction catalysed by glutamate dehydrogenase (GDH) by which ammonia is reversibly incorporated into 2-oxoglutarate.

Chapter 5

METABOLISM OF GLUTAMATE BY *OSTERTAGIA CIRCUMCINCTA*

5.1 INTRODUCTION

Glutamate is involved in numerous pathways: in anabolic pathways which incorporate ammonia into amino acids; in the catabolism of amino acids to produce ammonia for excretion; as a neurotransmitter; a substrate for protein synthesis and the precursor of glutamine, GABA and other compounds (summarised in Figure 1.11). Of the four main enzymes involved in glutamate synthesis and degradation, GDH, glutaminase, GS and GOGAT, only GDH has been examined in any detail in nematodes (Langer and Jiamperpoon, 1970; Langer *et al.*, 1972; Rhodes and Ferguson, 1973; Turner *et al.*, 1986; Skuce *et al.*, 1999b) and in a few other helminths. Given the central role of glutamate in nitrogen metabolism, the enzymes involved are likely to contain some novel features different from those of the mammalian host.

5.1.1 GLUTAMATE DEHYDROGENASE (GDH) (EC 1.4.1.2-4)

GDH catalyses the reversible oxidative deamination of L-glutamate to 2-oxoglutarate and ammonia (Figure 5.1), using either NAD^+ or $NADP^+$ as the cofactor (reviewed by Goldin and Frieden, 1971; Hudson and Daniel, 1993). There are three GDH groups based on cofactor requirements: NAD^+ specific (EC 1.4.1.2), $NADP^+$ specific (EC 1.4.1.4) or dual cofactor specific (EC 1.4.1.3) which can use either cofactor, the enzyme present in higher organisms. The dual-specificity activity may be due to a single enzyme or separate enzymes.

GDH activity has been identified in *H. contortus* (Rhodes and Ferguson, 1973; Skuce *et al.*, 1999b), *A. suum* (Langer, 1972), *D. immitis* (Langer and Jiamperpoon, 1970; Turner *et al.*, 1986), *H. polygyrus*, *P. redivivus* (Grantham and Barrett, 1986a), *O. cuniculi* (Hutchinson and Fernando, 1975), *L. carinii* (Davies and Köhler, 1990), *F. hepatica* (Prichard and Schofield, 1968; Thorpe, 1968) and *M. similis* (McManus and James, 1975). The properties of the

GDHs in helminths, as in many invertebrates, varied in subcellular location, cofactor requirements and sensitivities to activators and inhibitors from those of mammals, which are mitochondrial, have dual co-factor specificity and are activated by AMP and ADP and inhibited by GTP and ATP (Goldin and Frieden, 1971; Hudson and Daniel, 1993). GDH activity in the cytosol in helminths (Hutchinson and Fernando, 1975; Mustafa *et al.*, 1978) could be caused by disruption of mitochondria during the experiment, although mitochondria appeared to be intact in the latter study. It may also be a genuine cytosolic enzyme in *D. immitis* in which the mitochondrial and cytosolic GDH differed in almost all properties (Turner *et al.*, 1986), similar to *O. cuniculi* GDH (Hutchinson and Fernando, 1975). Mitochondrial GDH was the predominant form in *F. hepatica* (Prichard and Schofield, 1968), *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1986a).

Although not universally true, in many species, the aminating direction is catalysed by NAD-GDH and the deminating direction by NADP-GDH. The co-factor requirements were somewhat variable in helminths. The *H. diminuta* GDH was specific for NAD^+ (Mustafa *et al.*, 1978). *O. cuniculi* cGDH and mGDH were more active with NADH than NADPH in the direction of glutamate formation (Hutchinson and Fernando, 1975), the *F. hepatica* cGDH had 12 times higher activity with NADH and 4 times higher with NADPH than rat liver (Prichard and Schofield, 1968), whereas the *D. immitis* mGDH was equally reactive with NAD^+ and $NADP^+$. In both *H. polygyrus* and *P. redivivus*, in the direction of glutamate formation, GDH activity with NADH was twice the rate with NADPH and in the reverse direction, activity with NAD^+ was three and eight times greater than with $NADP^+$ in *P. redivivus* and *H. polygyrus* respectively (Grantham and Barrett, 1986a).

The properties of invertebrate GDHs were also variable. *D. melanogaster* GDH was located in the mitochondria and generally had similar properties to bovine liver GDH, but with a very low affinity for NADPH and lower activity with NADPH than with NADH (Prezioso *et al.*, 1985). The GDH of the muscle of the polychaete *A. marina* had no aminating activity with either NADH or NADPH (Batrel and Le Gal, 1984). In contrast, mGDH activity in several tissues of the mollusc *Modiolus demissus* was predominantly in the glutamate-forming direction and assigned the role of forming glutamate when ammonia levels were high (Reiss *et al.*, 1977).

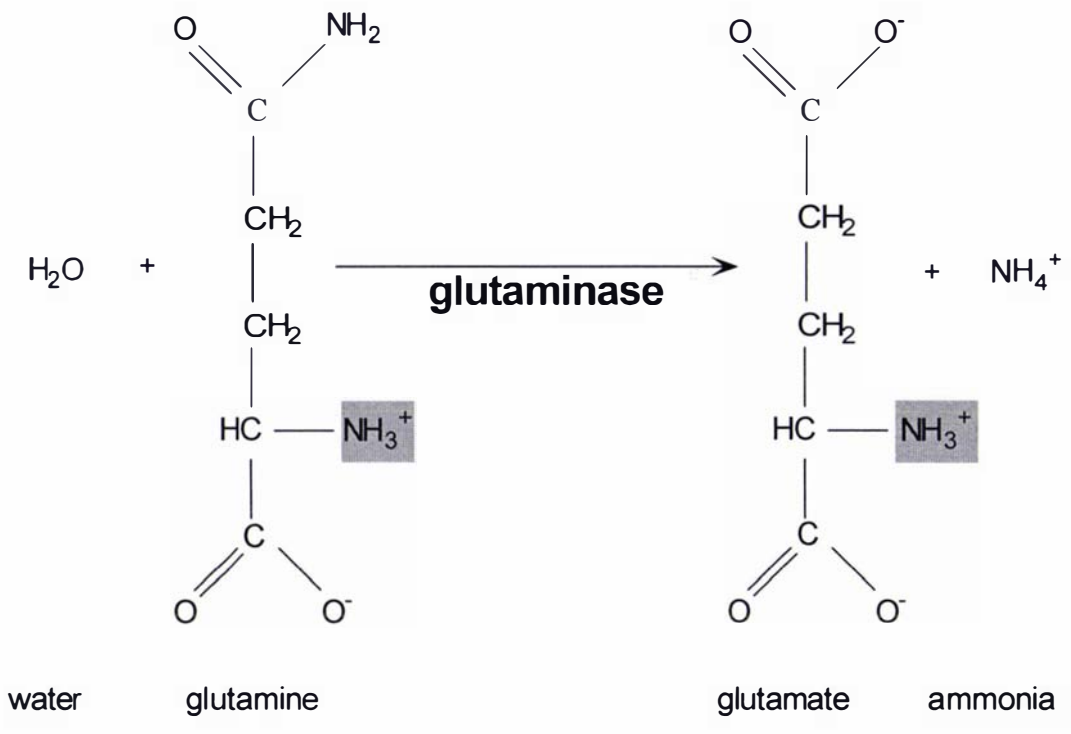


Figure 5.2. The reaction catalysed by glutaminase. Glutamine is hydrolysed to glutamate and ammonium.

In the direction of glutamate deamination, the pH optima for mammalian GDHs are pH 8-10 and for reductive amination about 0.5-2 pH units lower (Goldin and Frieden, 1971). There was little increase in activity of purified *H. contortus* GDH above 27°C, the pH optimum was 8.8 for NAD^+ reduction (similar to bovine and ovine liver GDH) and pH 8 for NADH oxidation, the latter higher than the pH 7.5-7.6 they recorded for ovine and bovine liver GDH (Rhodes and Ferguson, 1973). The mGDH of *D. immitis* had a pH optimum of 8.4 and the cytosolic cGDH a narrow pH optimum of 7.8-8 (Turner *et al.*, 1986). The binding of 2-oxoglutarate, but not glutamate, was pH dependent in *H. diminuta* GDH (Mustafa *et al.*, 1978).

The regulation of the dual specificity mammalian enzymes is very complex, being strongly regulated by purine nucleotides, particularly activated by ADP, AMP and some amino acids and inhibited by GTP (Goldin and Frieden, 1971; Hudson and Daniel, 1993). Unlike bovine liver GDH, AMP, ADP, ATP, GDP, GTP and amino acids had little effect on *H. diminuta* GDH, whereas the TCA intermediates fumarate, malate and succinate were inhibitory in high, probably not physiologically relevant, concentrations (Mustafa *et al.*, 1978). It was believed that the purine nucleotides were tested in physiological concentrations for nematodes, based on the calculated values of 0.7-2 mM (Barrett and Beis, 1973). Grantham and Barrett (1986a) found differences in the effects of purine nucleotides on *H. polygyrus* and *P. redivivus* mGDH. Whereas 1 mM ADP stimulated mGDH in both species in the direction of glutamate formation with NADH, with NADPH, ADP reduced the activity from 2.5 to 0.8 nmol glutamate/min/mg protein in *P. redivivus*, but there was little difference with and without ADP (0.8 and 0.5 nmol glutamate/min/mg protein respectively) for *H. polygyrus*. Also notable was the lack of effect of ATP in both species, but unusual stimulation by GTP for *P. redivivus* GDH with NADH. ADP failed to activate the GDH of the polychaete *A. marina* (Batrel and Le Gal, 1984), the protozooan *T. cruzi* (Cazzulo *et al.*, 1977) and the water mould *B. emersonii* (LéJohn, 1968).

5.1.2 GLUTAMINASE (EC 3.5.1.2)

Glutaminase catalyzes the hydrolysis of L-glutamine to glutamate and ammonia (Figure 5.2). Vertebrate liver- and kidney-type glutaminases are isoforms of phosphate-activated glutaminase (glutaminase I), whereas

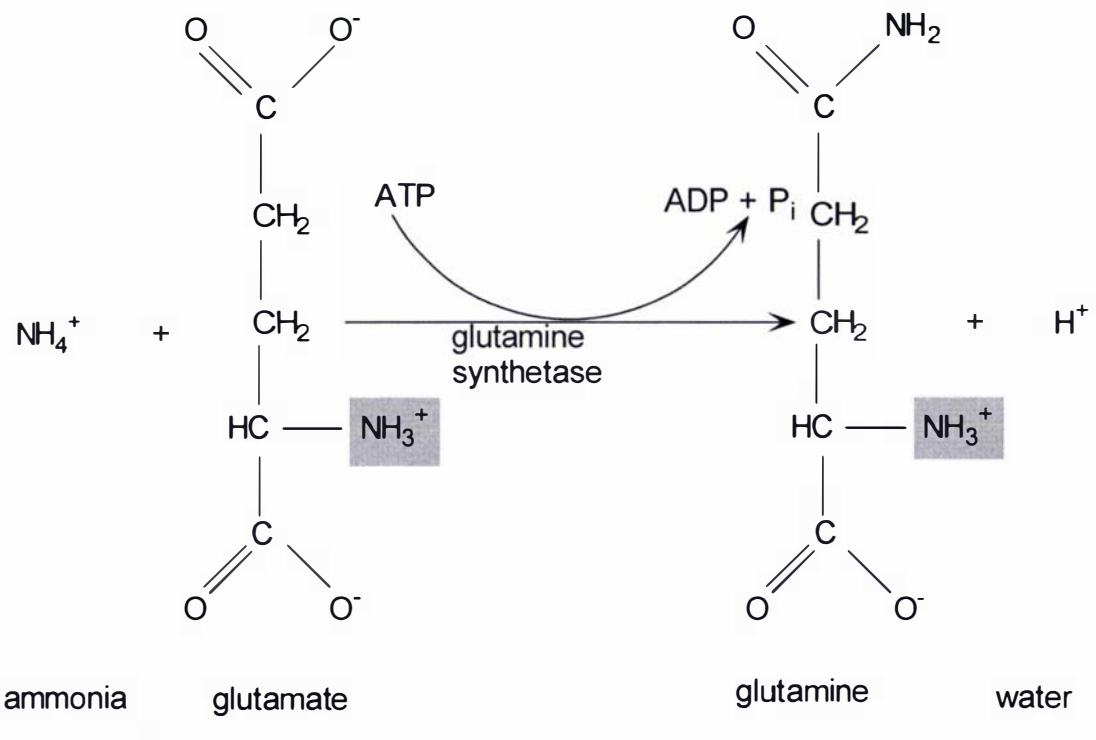


Figure 5.3. The reaction catalysed by glutamine synthetase. In an ATP-requiring reaction, glutamate is aminated to glutamine.

glutaminase II is phosphate-independent glutamine aminotransferase or a γ-glutamyltransferase (reviewed by Curthoys and Watford, 1995; Kvamme *et al.*, 2000). There are several classes of glutaminases of microorganisms which are not phosphate-activated and have different properties from the mammalian phosphate-dependent enzymes (reviewed by Nandakumar *et al.*, 2003). While some are specific for glutamine, others are also active asparaginases.

There are few reports of glutaminase activity in nematodes. Both glutaminase and asparaginase activities were identified in *H. polygyrus* and *P. redivivus* at very much lower levels than in rat liver (Grantham and Barrett, 1986a), but no glutaminase activity was detectable in *L. carinii* (Davies and Köhler, 1990). Nematode glutaminase may not be similar to the mammalian isozymes, but may be like a bacterial glutaminase or vertebrate type II, based on the report that glutaminase II, but not type I, was present in the insect *P. crassipalpis* (Downton and Kennedy, 1986).

Mammalian liver- and kidney-type glutaminases are distinguished by differences in the pH optima and sensitivity to inhibition by the end-product glutamate. The kidney-type glutaminase requires a polyvalent anion (usually phosphate with a $K_{0.5}$ of 20-30 mM), has a low K_m for glutamine (2-5 mM) and is strongly inhibited by glutamate. The liver-type has a higher K_m for glutamine (17 mM or 6 mM depending on association with the mitochondrial membrane), a lower $K_{0.5}$ for phosphate activation, a flat pH curve with an optimum between 7.8 and 8.2, is not inhibited by glutamate (up to 50 mM) or malate (Curthoys and Watford, 1995) and has a requirement for activation by ammonia (McGivan and Bradford, 1983). The kinetics depend on whether the enzyme is membrane associated or not, as the reaction towards glutamine is hyperbolic when membrane associated, but becomes sigmoidal when solubilised and the K_m for glutamine increases (McGivan *et al.*, 1980).

5.1.3 GLUTAMINE SYNTHETASE (GS) (EC 6.3.1.2)

Glutamine synthetase (GS), catalyses the reaction of ATP, glutamate and ammonium to form ADP, phosphate, H^+ and L-glutamine (Figure 5.3). It requires either Mg^{2+} or Mn^{2+} for activity. While it is reversible, the biosynthetic or forward reaction is the only physiological direction. GS is involved in ammonia detoxification and neurotransmitter recycling in the brain (Suárez *et al.*, 2002), converting excess ammonia to glutamine in perivenous cells of the

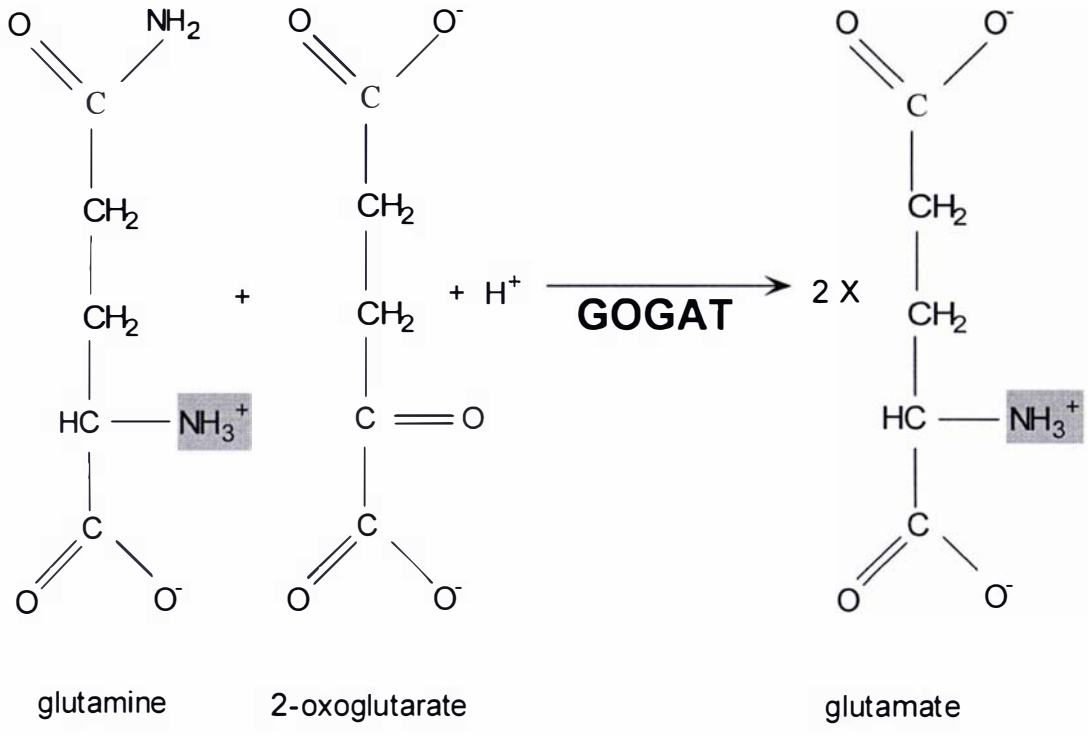


Figure 5.4. The reaction catalysed by glutamate synthase (GOGAT or Glts). The amino group of glutamine is transferred to 2-oxoglutarate to form two molecules of glutamate.

liver or used by the kidney to buffer excreted ammonia (Brosnan, 2000). GS and GOGAT are usually responsible in plants and bacteria for ammonia assimilation when there is adequate ATP, whereas GDH provides an alternative route when energy is limited.

GS is present in all organisms studied, including fossils (Kumada *et al.*, 1993) and, not unexpectedly, GS activity has been identified in the nematodes *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1988).

5.1.4 GLUTAMATE SYNTHASE (GOGAT OR GLTS) (EC 1.4.1.14)

Glutamate synthase (GOGAT) catalyses the glutamine-dependent reductive amination of 2-oxoglutarate to form two molecules of glutamate (Figure 5.4). Plant GOGAT is present in a form which uses reduced ferrodoxin as the electron donor (Fd-GOGAT) and one that uses NADH as the electron donor (NADH-GOGAT), while a third form, NADPH-GOGAT, is present in bacteria (reviewed by Vanoni and Curti, 1999; Suzuki and Knaff, 2003; van den Heuvel *et al.*, 2004; Vanoni *et al.*, 2005).

The GS-GOGAT pathway formed by GS and GOGAT is the most important for ammonia assimilation in plants (reviewed by Mifkin and Habash, 2002) and bacteria (Fisher and Sonenshein, 1991; Osuji *et al.*, 1996; Helling, 1998; El-Shora and Abo-Kassem 2001), provided there is adequate ATP. As the K_m for ammonia is low for GS compared with that for GDH, the GS-GOGAT pathway predominates when ammonia levels are low.

Recently it has been suggested that GOGAT may be an important enzyme in invertebrates. The enzyme has been identified either by an activity assay or deduced from labelling studies in the silkworms *B. mori* (Hirayama *et al.*, 1998; Hirayama and Nakamura, 2002) and *S. cynthia ricini* (Osanai *et al.*, 2000), in a *S. frugiperda* Sf9 insect cell line (Doverskog *et al.*, 2000; Drews *et al.*, 2000) and in the mosquito *A. aegypti* (Scaraffia *et al.*, 2005). Genes for NADH-GOGAT have been identified during sequencing of the genome of *C. elegans* (Vanoni and Curti, 1999), suggesting GOGAT activity may also be present in nematodes.

The aim of the experiments reported in this Chapter was to determine the presence and properties of key enzymes involved in the metabolism of

glutamate. The enzymes examined were glutamate dehydrogenase (GDH), glutamine synthetase (GS), glutamate synthase (GOGAT) and glutaminase.

5.2 MATERIALS AND METHODS

Parasites for enzyme assays were provided as detailed in Appendix 1. L3 were cultured from the faeces of infected sheep, stored at 4°C and prior to each experiment, were Baermannized to remove inactive worms (Appendix 1, 1.5). Adult worms were recovered from the abomasa of infected sheep (Appendix 1, 1.4).

All chemicals were obtained from the Sigma Chemical Co. (Mo, USA) unless specified otherwise.

5.2.1 HOMOGENATE PREPARATION

Enzyme activities were determined on homogenates prepared from sheathed L3 or adult *O. circumcincta* (Appendix 2, 2.6). The amount of homogenate used for the enzymes assays was based on its protein content, which was determined by the Bradford method (Appendix 2, 2.3), so that the amount of homogenate containing 50 :g protein was used for each assay.

5.2.2 GLUTAMATE DEHYDROGENASE

Glutamate dehydrogenase (GDH) was assayed both in the direction of glutamate utilisation and formation:

$$\text{L-glutamate} + NAD^+ + H_2O \xleftrightarrow{\text{GDH}} \text{2-oxoglutarate} + NH_4^+ + NADH$$

5.2.2.1 Effect of pH

Assays were conducted at 30°C on homogenates of sheathed L3 in the direction of glutamate formation (SL48-49) and glutamate utilisation (SL50-51) to determine the optimum pH for GDH activity. In the direction of glutamate utilisation, the reaction mixtures (total volume of 1 ml) were prepared containing 50 µg homogenate protein in 100 mM phosphate buffer (Appendix 3, 3.1) of variable pH from 5 to 10. Subsequently, 0.2 mM NAD^+ was added and the reaction was initiated by adding 5 mM glutamate. GDH activity was calculated from the rate of formation of NADH, which was monitored spectrophotometrically at 340 nm.

In the direction of glutamate formation, the reaction mixtures (total volume of 1 ml) were prepared containing 50 µg homogenate protein in 100 mM phosphate buffer of variable pH from 5 to 10. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM 2-oxoglutarate. GDH activity was calculated from the rate of utilisation of NADH, monitored spectrophotometrically at 340 nm.

5.2.2.2 Effect of temperature

Assays were conducted on 2 homogenates of sheathed L3 (SL52-53) in the direction of glutamate formation to determine the effect of temperature on GDH activity in the direction of glutamate formation. Reaction mixtures (total volume of 1 ml) were prepared containing 40 mM NH_4Cl and 50 µg homogenate protein in 100 mM phosphate buffer pH 7.5 (Appendix 3, 3.2) of variable temperature from 25°C to 45°C. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM 2-oxoglutarate. GDH activity was calculated from the rate of utilisation of NADH, monitored spectrophotometrically at 340 nm.

5.2.2.3 Effect of ATP and ADP

The effects of ATP or ADP on GDH activity at 30°C in the direction of glutamate formation and utilisation were studied on 2 homogenates of sheathed L3 (SL 54-55).

In the direction of glutamate utilisation, reaction mixtures (total volume of 1 ml) were prepared containing 50 µg homogenate protein in 100 mM phosphate buffer (Appendix 3, 3.2) pH 7.5, with and without 1 mM ATP or ADP. Subsequently, 0.2 mM NAD^+ was added and the reaction was initiated by adding 5 mM glutamate. The effect on GDH activity of added compounds was compared with activities without these compounds, set at 100%.

In the direction of glutamate formation, reaction mixtures (total volume of 1 ml) contained 5 mM EDTA, 40 mM NH_4Cl and 50 µg homogenate protein, prepared in 100 mM phosphate buffer (Appendix 3, 3.2) pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM 2-oxoglutarate. The effects on GDH activity of added compounds were compared with activities without these compounds, which were set at 100%.

5.2.2.4 Kinetic parameters in the direction of glutamate utilisation

GDH activity at 30°C was estimated in the direction of glutamate utilisation in homogenates of sheathed L3 or adult worms using the protocol for continuous assays described in Appendix 2, 2.7. The reaction mixture (total volume of 1 ml) contained 50 µg homogenate protein in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5. Subsequently, 0.2 mM NAD^+ was added and the reaction was initiated by adding 5 mM glutamate.

The K_m and V_{max} were determined for GDH activity for the substrate glutamate and the cofactor NAD^+ (or $NADP^+$):

(1) For the substrate glutamate, for homogenates of sheathed L3 (n = 3), varying concentrations of glutamate from 0 to 20 mM were added to the reaction mixture to initiate the reaction. For homogenates of adult worms (n = 2), varying concentrations of glutamate from 0 to 5 mM were added to the reaction mixture to initiate the reaction.

(2) For the cofactor NAD^+ (or $NADP^+$), for homogenates of sheathed L3 (n = 3), varying concentrations of NAD^+ or $NADP^+$ from 0 to 3 mM were added to initiate the reaction.

5.2.2.5 Kinetic parameters in the direction of glutamate formation

GDH activity at 30°C was estimated in the direction of glutamate formation in homogenates of sheathed L3 or adult worms using the protocol for continuous assays described in Appendix 2, 2.7. The reaction mixture (total volume of 1 ml) contained 5 mM EDTA, 40 mM NH_4Cl and 50 µg homogenate protein, prepared in 100 mM phosphate buffer (Appendix 3, 3.2) pH 7.5. Subsequently, 0.2 mM NADH was added and the reaction was initiated by adding 5 mM 2-oxoglutarate.

The K_m and V_{max} were determined for GDH activity for the substrates 2-oxoglutarate and ammonia and the cofactor NADH (or NADPH):

(1) For the substrate 2-oxoglutarate, for homogenates of sheathed L3 (n = 3), varying concentrations of 2-oxoglutarate from 0 to 5 mM were added to the reaction mixture to initiate the reaction. For the homogenates of adult worms (n = 3), varying concentrations of 2-oxoglutarate from 0 to 0.5 mM were added to the reaction mixture to initiate the reaction.

(2) For the substrate ammonia, for the homogenates of sheathed L3 (n = 3), varying concentrations of ammonia from 0 to 150 mM were added to the reaction mixture to initiate the reaction. For the homogenates of adult worms (n = 3), varying concentrations of 2-oxoglutarate from 0 to 250 mM were added to the reaction mixture to initiate the reaction.

(3) For the cofactor NADH (or NADPH), for homogenates of sheathed L3 (n = 3), varying concentrations of NADH or NADPH from 0 to 0.45 mM were added to initiate the reaction.

5.2.3 GLUTAMINASE

Glutaminase activity was monitored using a coupled reaction with glutamate dehydrogenase (GDH):

$$\text{glutamine} + H_2O \xrightarrow{\text{glutaminase}} \text{L-glutamate} + NH_4^+$$

$$\text{L-glutamate} + NAD^+ + H_2O + \xrightarrow{\text{GDH}} \text{2-oxoglutarate} + NH_4^+ + NADH$$

5.2.3.1 Kinetic parameters

Glutaminase activity at 30°C was estimated in homogenates of sheathed L3 (n = 3) or adult worms (n = 1) using the protocol for continuous assays described in Appendix 2, 2.7. The reaction mixture (total volume of 1 ml) contained 10 mM 2-oxoglutarate and 50 µg homogenate protein, prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5. Subsequently, 0.2 mM NAD^+ was added and the reaction was initiated by adding 5 mM glutamine. The K_m and V_{max} were determined for glutamine by initiating the reaction with glutamine in concentrations from 0 to 20 mM for the homogenates of sheathed L3 and from 0 to 40 mM for the homogenate of adult worms. Glutaminase activity was calculated from the rate of formation of NADH, monitored spectrophotometrically at 340 nm.

5.2.3.2 Effectors and inhibitors

Each of the following was investigated for effects on the rate of glutaminase activity of sheathed L3 homogenates (SL73-74) (n = 2):

(1) 0.1 mM arginine

(2) 0.1 mM Fe^{2+} in the form of $FeSO_4$

(3) 0.1 mM Cu^{2+} in the form of $CuSO_4$

(4) 0.1 mM Mg^{2+} in the form of $MgSO_4$

(5) 0.1 mM EDTA

(6) 0.1 mM ATP or ADP

(7) 0.1 mM azaserine

Reaction mixtures described in Section 5.2.3.1 were made up with each of the test compounds. After the addition of 0.2 mM NAD^+, 5 mM glutamine was added to initiate the reaction.

5.2.4 GLUTAMINE SYNTHETASE

Glutamine synthetase (GS) activity was assayed in the "biosynthetic" direction of glutamine formation:

$$\text{glutamate} + \text{ATP} + NH_4^+ \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + P_i$$

5.2.4.1 Kinetic parameters

Glutamine synthetase (GS) activity of sheathed L3 *O. circumcincta* homogenate SL75 was estimated in duplicate at 30°C in the direction of glutamate utilisation with increasing concentration of ammonia from the rate of utilisation of NADH in a coupled reaction with pyruvate kinase (PK) and lactate dehydrogenase (LDH), monitored spectrophotometrically at 340 nm using the protocol for continuous assays described in Appendix 2, 2.7:

$$\text{glutamate} + \text{ATP} + NH_4^+ \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + P_i$$

$$\text{PEP} + \text{ADP} \xrightarrow{\text{PK}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

The reaction mixture (total volume of 1 ml) contained 10 mM glutamate, 0.1 mM ATP, 10 mM PEP, 2 units of PK, 2 units of LDH and 50 µg homogenate protein, prepared 100 mM phosphate buffer (Appendix 3, 3.1).

0.2 mM NADH was added subsequently and the reaction was initiated by adding ammonium as NH_4Cl in concentrations of 0-10 mM. GS activity was calculated from the rate of utilisation of NADH, monitored spectrophotometrically at 340 nm.

GS activity of sheathed L3 *O. circumcincta* homogenates SL76-78) (n = 3) was estimated at 30°C by a colorimetric method (Appendix 2, 2.8) (Vorhaben *et al.*, 1973). Glutamate reacts with hydroxylamine (NH_2OH) to form γ-glutamyl hydroxamate; a brown colour develops after addition of $FeCl_3$,

$$\text{glutamate} + \text{ATP} + NH_2OH \xrightarrow{\text{GS}} \gamma\text{-glutamyl hydroxamate} + \text{ADP}$$

The assay for GS was adapted from Hirayama and Nakamura (2002). With glutamate as the variable substrate, reaction mixtures (final volume 0.5 ml) containing 5 mM ATP, 20 mM NH_4Cl, 500 mM NH_2OH and 0 to 5 mM L-glutamate were prepared in 100 mM Tris buffer (Appendix 3, 3.2) pH 8.5 and 0.5 ml of buffer containing 50 μg homogenate protein was subsequently added to initiate the reaction. After incubation for 0, 1, 5, 15 or 30 minutes, the reaction was terminated by the addition of 1.5 ml of a mixture of 3.5% $FeCl_3$, 2% trichloroacetic acid (TCA) and 0.25 N HCl. After centrifugation, the supernatant was read against a blank to which no homogenate had been added at 500 nm in a Pharmacia LKB Novaspec II. A standard curve was constructed with γ-glutamyl hydroxamate concentrations from 0-0.6 mM. Assays without ATP and glutamate were used as controls. GS activities were calculated from the equation in Appendix 2.8.

5.2.5 GLUTAMATE SYNTHASE

Glutamate synthase (GOGAT) activity was monitored in the direction of glutamine utilisation:

$$\text{glutamine} + \text{NADH} + \text{2-oxoglutarate} \xrightarrow{\text{GOGAT}} 2\ \text{glutamate} + NAD^+$$

5.2.5.1 Kinetic parameters

GOGAT activity at 30°C was estimated in homogenates of sheathed L3 (n = 3) or adult worms (n = 1) using the protocol for continuous assays described in Appendix 2, 2.7. To determine the K_m and V_{max} for the substrate

glutamine, the reaction mixture (total volume of 1 ml) contained 20 mM 2-oxoglutarate and 50 µg homogenate protein, prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5. 0.2 mM NADH was added subsequently and the reaction was initiated by the addition of glutamine in concentrations from 0 to 30 mM. GOGAT activity was calculated from the rate of utilisation of NADH, monitored spectrophotometrically at 340 nm.

To determine the K_m and V_{max} for the substrate 2-oxoglutarate in adult worm homogenate A13, the reaction mixture (total volume of 1 ml) contained 10 mM glutamine and 50 µg homogenate protein, prepared in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5. 0.2 mM NADH was added subsequently and the reaction was initiated by the addition of 2-oxoglutarate in concentrations from 0 to 20 mM. GOGAT activity was calculated from the rate of utilisation of NADH, monitored spectrophotometrically at 340 nm.

5.2.5.2 Comparison of GOGAT activities in sheep muscle and adult worms

GOGAT activity was monitored in sheep muscle and subsequently in adult worm homogenate A14. The protein content of each homogenate was determined by the Bradford method (Appendix 2, 2.3) and the volume containing 50 µg protein was calculated for each. The reaction mixture (total volume of 1 ml) containing 20 mM 2-oxoglutarate and 5 mM glutamine was prepared in a quartz cuvette in 100 mM phosphate buffer (Appendix 3, 3.2) pH 7.5 at 30°C and the reaction monitored at 340 nm. 0.2 mM NADH was added at 30 seconds, followed by 50 µg sheep muscle homogenate protein at 240 seconds and the GOGAT activity was monitored. At 320 seconds, 50 µg adult worm homogenate protein was added and GOGAT activity was monitored. At 540 seconds, 20 mM NH_4Cl was added to the reaction mixture to initiate the GDH reaction, which served as a control. Both GOGAT and GDH activities were observed from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

5.2.5.3 Effect of azaserine

The effect of azaserine on GOGAT activity at 30°C was tested on adult worm homogenate. The reaction mixture (total volume of 1 ml) contained 20 mM 2-oxoglutarate and 50 µg homogenate protein, prepared in 100 mM

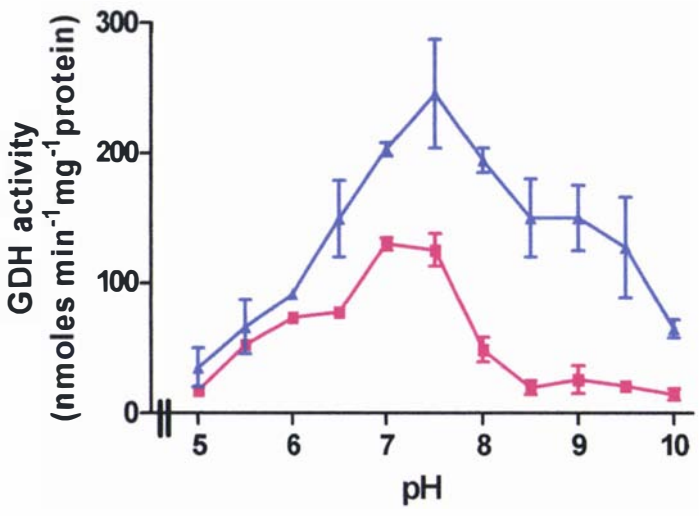


Figure 5.5. Effects of pH on glutamate dehydrogenase (GDH) activities (mean ± SEM, n = 2) at 30°C of sheathed L3 *O. circumcincta* homogenates in the direction of glutamate formation (SL48-49) (▲) and glutamate utilisation (SL50-51) (■). Concentrations of 5 mM 2-oxoglutarate or glutamate were used respectively. GDH activity was calculated from the rate of NAD^+ reduction or NADH oxidatione monitored spectrophotometrically at 340 nm.

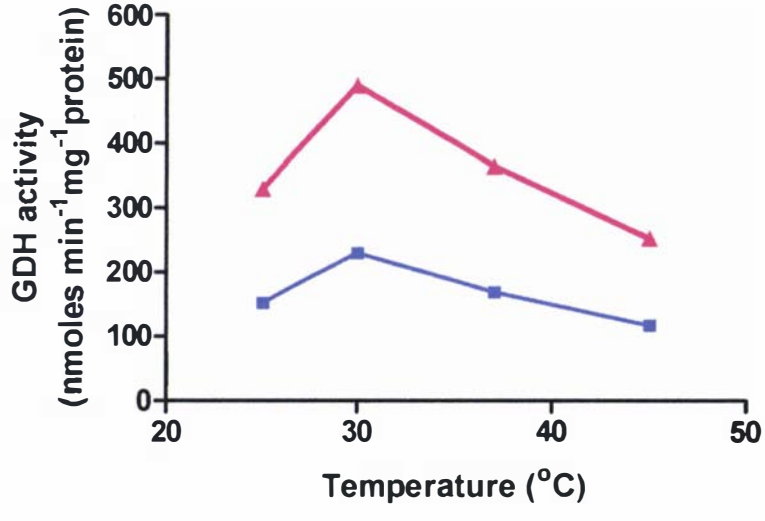


Figure 5.6. Effects of temperature on glutamate dehydrogenase (GDH) activities (mean ± SEM, n = 2) of sheathed L3 *O. circumcincta* homogenates SL52 (▲) and SL53 (■) in the direction of glutamate formation. Concentrations of 5 mM 2-oxoglutarate were used. GDH activity was calculated from the rate of oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

phosphate buffer (Appendix 3, 3.1) pH 7.5. 0.2 mM NADH was added subsequently. The reaction was initiated by the addition of 5 mM glutamine and the activity was monitored for 320 seconds. Azaserine (final concentration 0.1 mM) was then added to the reaction mixture. GOGAT activity was observed from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

5.2.5.4 Comparison of GOGAT and GDH activities

Adult worm homogenate A16 was assayed for both GOGAT and GDH activity at 30°C. The reaction mixture (total volume 1 ml) containing 20 mM 2-oxoglutarate and 50 µg homogenate protein was prepared in a quartz cuvette in 100 mM phosphate buffer (Appendix 3, 3.1) pH 7.5 and the reaction monitored at 340 nm. 0.2 mM NADH was added at 30 seconds. GOGAT activity was initiated by adding 5 mM glutamine at 180 seconds. 20 mM NH_4Cl was added at 580 seconds to initiate the GDH reaction and the absorbance at 340 nm was further monitored. Both GOGAT and GDH activities were observed from the rate of NADH utilisation, which was monitored spectrophotometrically at 340 nm.

5.3 RESULTS

5.3.1 GLUTAMATE DEHYDROGENASE

5.3.1.1 Effect of pH

The effect of pH on GDH activity in the directions of glutamate formation and utilisation at 30°C in homogenates of sheathed L3 (n = 2) are shown in Figure 5.5. In both directions, the optimum pH was 7-7.5, but activity was very little different from that at pH 7.5, the standard pH used to assay GDH. Activity in the direction of glutamate utilisation decreased to much lower levels above pH 8 than it did in the reverse direction.

5.3.1.2 Effect of temperature

The effects of temperature on GDH activity of two homogenates of sheathed L3 (SL52-53) in the direction of glutamate formation are shown in Figure 5.6. The optimal temperature for each was 30°C.

Table 5.6. Glutamate dehydrogenase (GDH) activities at 30°C of adult *O. circumcincta* homogenates, monitored in the direction of glutamate formation, with increasing concentration of 2-oxoglutarate. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	2-oxoglutarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A1c	0.02	735
A9d	0.14	563
A11b	0.02	86
mean ± SEM, n = 3	0.06 ± 0.04	461 ± 194

Table 5.5. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenates, monitored in the direction of glutamate formation, with increasing concentration of 2-oxoglutarate. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	2-oxoglutarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL62a	0.06	304
SL62b	0.06	303
SL63	0.05	301
mean ± SEM, n = 3	0.06 ± 0.03	303 ± 5

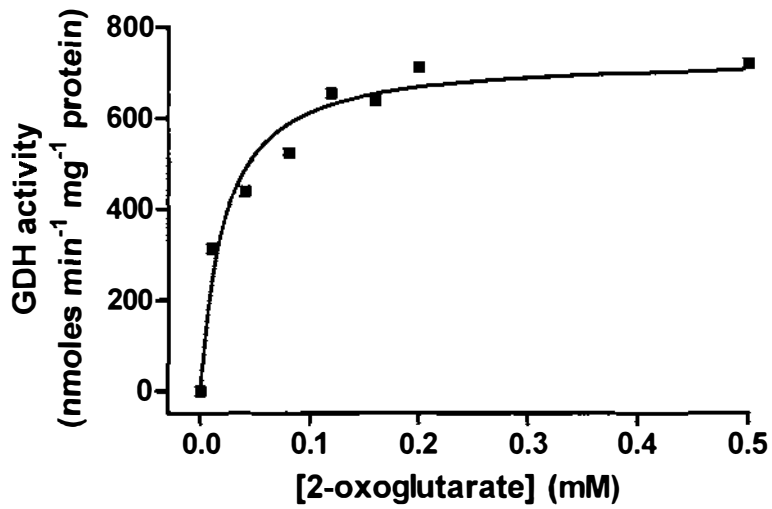


Figure 5.11. Glutamate dehydrogenase (GDH) activity at 30°C of adult *O. circumcincta* homogenate A1c, monitored in the direction of glutamate formation, with increasing concentration of 2-oxoglutarate. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.4. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenates, monitored in the direction of glutamate utilisation, with increasing concentration of NAD^+ or $NADP^+$. GDH activity was calculated from the rate of NADH or NADPH formation through the reduction of NAD^+ or $NADP^+$, which was monitored spectrophotometrically at 340 nm.

Homogenate	NAD^+		$NADP^+$	
	K_m (mM)	* V_{max}	K_m (mM)	* V_{max}
SL59	0.6	183	1.0	42
SL60	0.8	195	1.7	61
SL61	0.7	191	1.9	70
mean ± SEM, n = 3	0.7 ± 0.1	190 ± 4	1.5 ± 0.3	58 ± 8

* V_{max} in nmoles min^{-1} mg^{-1} protein

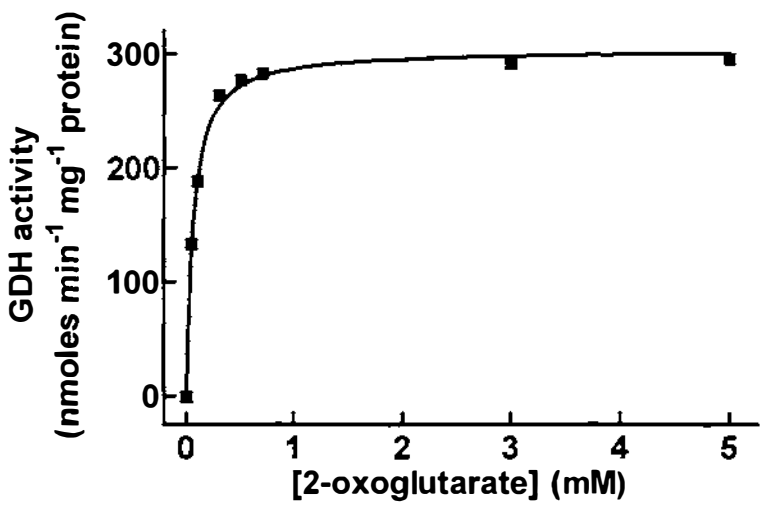


Figure 5.10. Glutamate dehydrogenase (GDH) activity at 30°C of sheathed L3 *O. circumcincta* homogenate SL62a, monitored in the direction of glutamate formation, with increasing concentration of 2-oxoglutarate. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.3. Glutamate dehydrogenase (GDH) activities at 30°C of adult *O. circumcincta* homogenates, monitored in the direction of glutamate utilisation, with increasing concentration of glutamate. GDH activity was calculated from the rate of NADH formation through the reduction of NAD^+, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A9c	0.10	72
A11a	0.15	68
mean ± SEM, n = 2	0.13 ± 0.03	70 ± 2

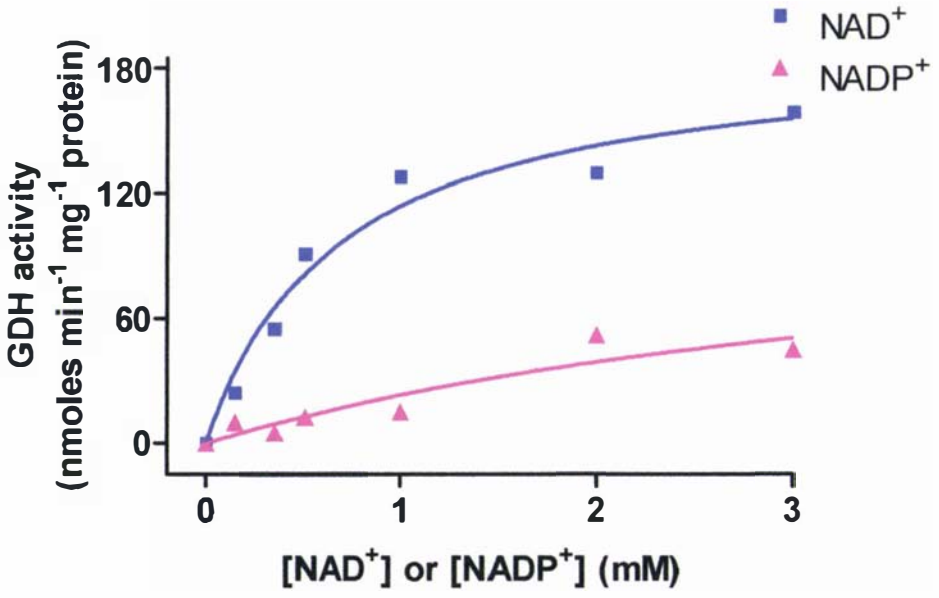


Figure 5.9. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenate SL60, monitored in the direction of glutamate utilisation, with increasing concentration of NAD^+ or $NADP^+$. GDH activity was calculated from the rate of NADH formation through the reduction of NAD^+ or $NADP^+$, which was monitored spectrophotometrically at 340 nm.

Table 5.2. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenates, monitored in the direction of glutamate utilisation, with increasing concentration of glutamate. GDH activity was calculated from the rate of NADH formation through the reduction of NAD^+, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL56	0.7	125
SL57	0.6	114
SL58	1.1	124
mean ± SEM, n = 3	0.8 ± 0.1	121 ± 4

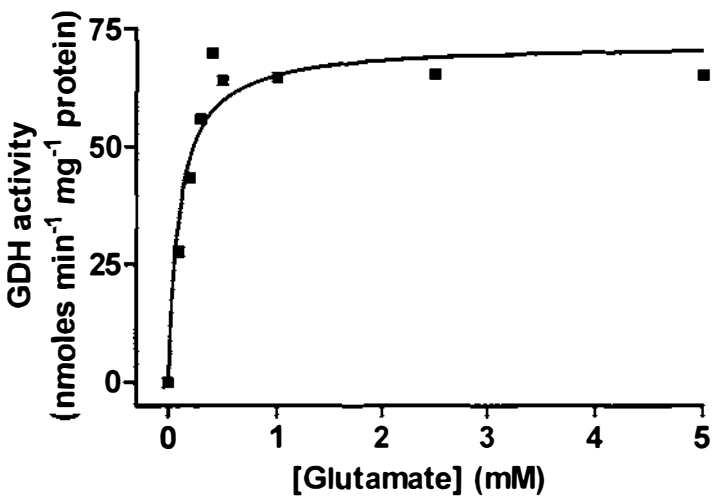


Figure 5.8. Glutamate dehydrogenase (GDH) activity at 30°C of adult *O. circumcincta* homogenate A9c, monitored in the direction of glutamate utilisation, with increasing concentration of glutamate. GDH activity was calculated from the rate of NADH formation through the reduction of NAD^+, which was monitored spectrophotometrically at 340 nm.

Table 5.1. Glutamate dehydrogenase (GDH) activities (mean ± SEM, n = 2) at 30°C of sheathed L3 *O. circumcincta* homogenates (SL 54-55) in the directions of glutamate formation and utilisation with 1 mM ATP or ADP added to the reaction mixture. GDH activities without the addition of these compounds were set at 100%. Concentrations of 5 mM 2-oxoglutarate or glutamate were used for glutamate formation and utilisation respectively.

ATP or ADP	Glutamate formation Activity (%)	Glutamate utilisation Activity (%)
-	100	100
1 mM ADP	212 ± 5	107 ± 10
1 mM ATP	545 ± 12	94 ± 7

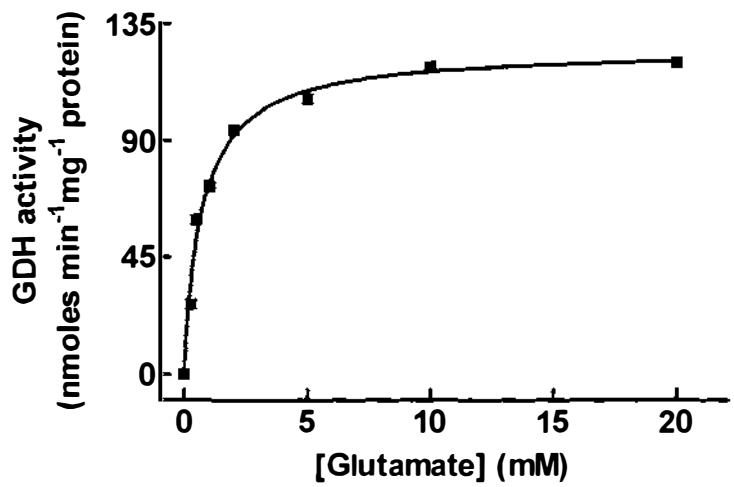


Figure 5.7. Glutamate dehydrogenase (GDH) activity at 30°C of sheathed L3 O. *circumcincta* homogenate SL56, monitored in the direction of glutamate utilisation, with increasing concentration of glutamate. GDH activity was calculated from the rate of NADH formation through the reduction of NAD^+, which was monitored spectrophotometrically at 340 nm.

5.3.1.3 Effect of ATP and ADP

GDH activities at 30°C in the directions of glutamate formation and utilisation with the addition of 1 mM ADP or ATP for homogenates of sheathed L3 (SL54-55) are presented in Table 5.1. The major effect was in the direction of glutamate formation: in both cases GDH was increased, approximately two-fold with ADP and over five-fold with ATP. In the deaminating direction, the effects were around 7% for each of ATP and ADP, with a large SEM.

5.3.1.4 Kinetic parameters in the direction of glutamate utilisation

For the substrate glutamate, the GDH activities at 30°C of a homogenate of sheathed L3 (SL56) and of a homogenate of adult worms (A9c) are shown in Figures 5.7 and 5.8 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 5.2 and 5.3 respectively. The K_m values for glutamate were 0.8 ± 0.1 and 0.13 ± 0.03 mM (both mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively. The V_{max} were 121 ± 4 and 70 ± 2 nmoles min^{-1} mg^{-1} protein (both mean ± SEM) for sheathed L3 (n = 3) and adult worms (n = 2) respectively.

For the cofactors NAD^+ and $NADP^+$, the GDH activities at 30°C of a homogenate of sheathed L3 (SL60) are shown in Figure 5.9 and the K_m and V_{max} of the homogenates of sheathed L3 (SL59-61) are shown in Table 5.4. GDH activity was about three times higher in the presence of NAD^+ than with $NADP^+$ as the cofactor. The K_m for NAD^+ was 0.7 ± 0.1 mM and for $NADP^+$ was 1.5 ± 0.3 mM (both mean ± SEM, n = 3) and the corresponding V_{max} were 190 ± 4 and 58 ± 8 nmoles min^{-1} mg^{-1} protein.

5.3.1.5 Kinetic parameters in the direction of glutamate formation

For the substrate 2-oxoglutarate, the GDH activities at 30°C of a homogenate of sheathed L3 (SL62a) and of a homogenate of adult worms (A1c) are shown in Figures 5.10 and 5.11 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 5.5 and 5.6 respectively. The K_m for 2-oxoglutarate were similar: 0.06 ± 0.03 and 0.06 ± 0.04 mM (both mean ± SEM, n = 3) for sheathed L3 and adult worms respectively. The V_{max} were 303 ± 5 and 461 ± 194 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n = 3) for sheathed L3 and adult worms respectively.

Table 5.12. Glutaminase activities (mean ± SEM, n = 2) at 30°C of sheathed L3 *O. circumcincta* homogenates (SL73-74) in the presence of metal ions, arginine or EDTA. Glutaminase activities without added compounds were set at 100%.

Effector/inhibitor	Concentration (mM)	Activity (% control)
-	-	100
arginine	0.1	115 ± 12
Fe^{2+}	0.1	106 ± 8
Cu^{2+}	0.1	133 ± 10
Mg^{2+}	0.1	121 ± 5
EDTA	0.1	119 ± 20
ATP	0.1	117 ± 8
ADP	0.1	111 ± 10
azaserine	0.1	98± 5

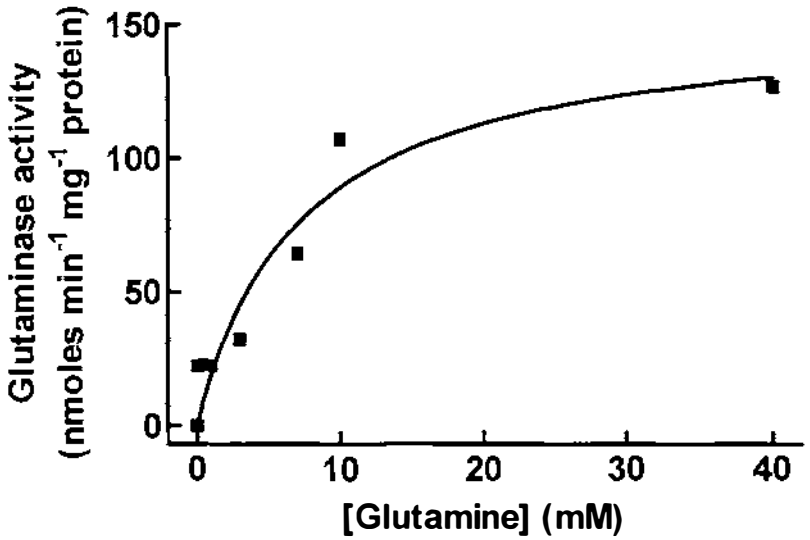


Figure 5.16. Glutaminase activity at 30°C of adult *O. circumcincta* homogenate A9e with increasing concentration of glutamine. Glutaminase activity was calculated from the rate of NADH formation through the reduction of NAD^+ in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 5.11. Glutaminase activity at 30°C of an adult *O. circumcincta* homogenate with increasing concentration of glutamine. Glutaminase activity was calculated from the rate of NADH formation through the reduction of NAD^+ in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A9e	7.2	154

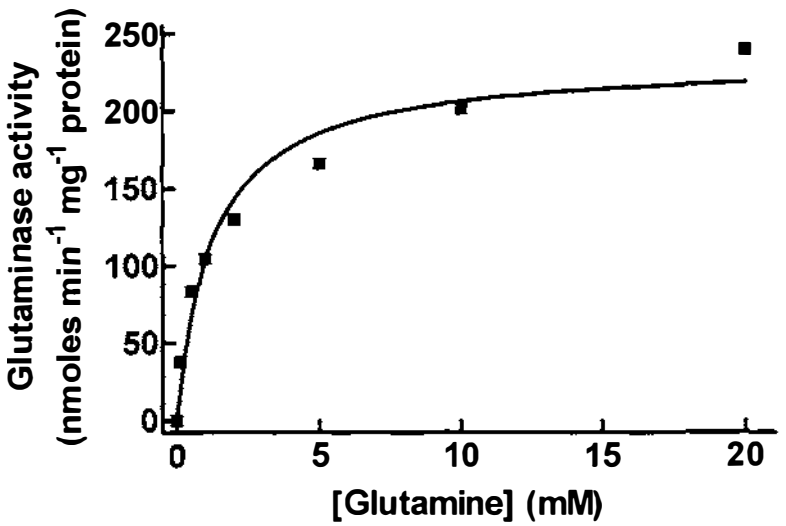


Figure 5.15. Glutaminase activity at 30°C of sheathed L3 *O. circumcincta* homogenate SL70 with increasing concentration of glutamine. Glutaminase activity was calculated from the rate of NADH formation through the reduction of NAD^+ in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Table 5.10. Glutaminase activities at 30°C of sheathed L3 *O. circumcincta* homogenates with increasing concentration of glutamine. Glutaminase activity was calculated from the rate of NADH formation through the reduction of NAD^+ in a coupled assay with GDH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL70	1.3	232
SL71	2.1	68
SL72	1.0	182
mean ± SEM, n = 3	1.5 ± 0.3	161 ± 50

Facing page 115

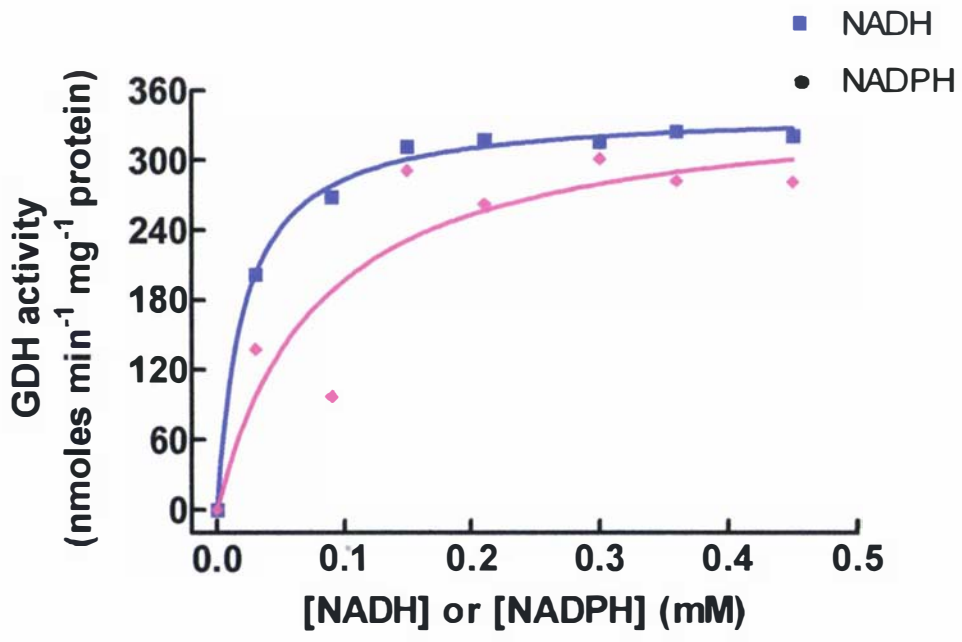


Figure 5.14. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenate SL68, monitored in the direction of glutamate formation, with increasing concentration of NADH or NADPH. GDH activity was calculated from the rate of NADH or NADPH utilisation through the oxidation of NADH or NADPH, which was monitored spectrophotometrically at 340 nm.

Table 5.9. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenates, monitored in the direction of glutamate formation, with increasing concentration of NADH or NADPH. GDH activity was calculated from the rate of NADH or NADPH utilisation through the oxidation of NADH or NADPH, which was monitored spectrophotometrically at 340 nm.

Homogenate	NADH		NADPH	
	K_m (mM)	* V_{max}	K_m (mM)	* V_{max}
SL67	0.01	312	0.08	354
SL68	0.02	343	0.07	362
SL69	0.07	355	0.15	422
mean ± SEM, n = 3	0.03 ± 0.02	337 ± 13	0.1± 0.02	379 ± 21

* V_{max} in nmoles min^{-1} mg^{-1} protein

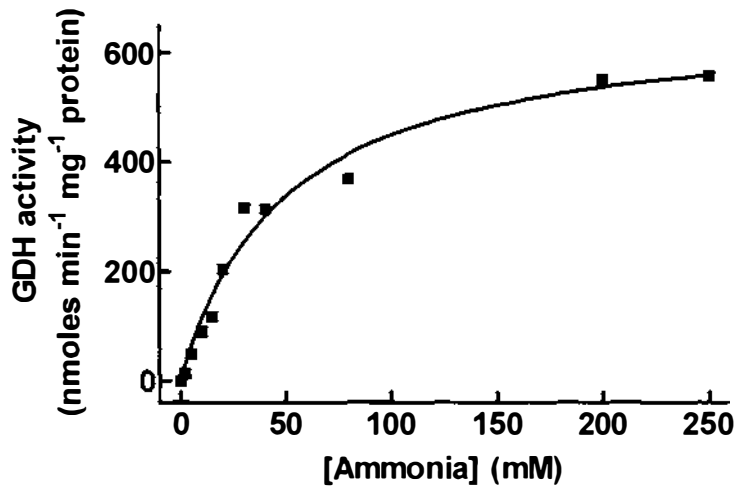


Figure 5.13. Glutamate dehydrogenase (GDH) activity at 30°C of adult *O. circumcincta* homogenate A9d, monitored in the direction of glutamate formation, with increasing concentration of ammonia. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.8. Glutamate dehydrogenase (GDH) activities at 30°C of adult *O. circumcincta* homogenates, monitored in the direction of glutamate formation, with increasing concentration of ammonia. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Ammonia K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A9d	49	669
A11c	34	232
A11d	40	352
mean ± SEM, n = 3	41 ± 4	418 ± 130

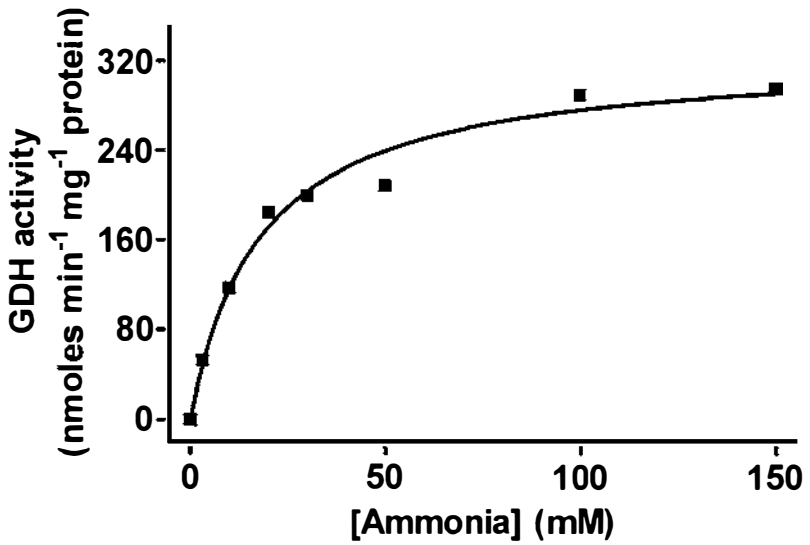


Figure 5.12. Glutamate dehydrogenase (GDH) activity at 30°C of sheathed L3 *O. circumcincta* homogenate SL64, monitored in the direction of glutamate formation, with increasing concentration of ammonia. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.7. Glutamate dehydrogenase (GDH) activities at 30°C of sheathed L3 *O. circumcincta* homogenates, monitored in the direction of glutamate formation, with increasing concentration of ammonia. GDH activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Ammonia K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL64	18	325
SL65	16	358
SL66	20	411
mean ± SEM, n = 3	18 ± 12	365 ± 25

For the substrate ammonia, the GDH activities at 30°C of a homogenate of sheathed L3 (SL64) and of a homogenate of adult worms (A9d) are shown in Figures 5.12 and 5.13 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 5.7 and 5.8. The K_m values for ammonia were 18 ± 12 and 41 ± 4 mM (both mean ± SEM, n = 3) for sheathed L3 and adult worms respectively. The V_{max} were 365 ± 25 and 418 ± 130 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n = 3) for sheathed L3 and adult worms respectively.

For the cofactors NADH and NADPH, the GDH activities at 30°C of a homogenate of sheathed L3 (SL68) are shown in Figure 5.14. The K_m and V_{max} of the homogenates of sheathed L3 are shown in Table 5.9. The K_m values were 0.03 ± 0.02 mM for NADH and 0.1 ± 0.02 for NADPH (both mean ± SEM, n = 3) respectively. The corresponding V_{max} were 337 ± 13 and 379 ± 21 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n = 3).

5.3.2 GLUTAMINASE

5.3.2.1 Kinetic parameters

For the substrate glutamine, the glutaminase activities at 30°C of a homogenate of sheathed L3 (SL70) and of a homogenate of adult worms (A9e) are shown in Figures 5.15 and 5.16 respectively. The K_m and V_{max} of the homogenates of sheathed L3 and adult worms are shown in Tables 5.10 and 5.11 respectively. The K_m values for glutamine were 1.5 ± 0.3 mM (mean ± SEM, n = 3) for sheathed L3 and 7.2 mM for the adult worm homogenate. The V_{max} were 161 ± 50 nmoles min^{-1} mg^{-1} protein (mean ± SEM, n = 3) for sheathed L3 homogenates and 154 nmoles min^{-1} mg^{-1} protein for the adult worm homogenate.

5.3.2.2 Effectors and inhibitors

Glutaminase activities at 30°C of homogenates of sheathed L3 (n = 2) in the presence of 0.1 mM metal ions, EDTA, ADP and ATP or azaserine are shown in Table 5.12. Glutaminase activity was expressed as a percentage of the value in the absence of added compounds, which was set at 100%. None of the added substances was inhibitory and the addition of 0.1 mM Cu^{2+} (as $CuSO_4$) was the most stimulatory at 133 ± 10% of control (mean ± SEM). 0.1 mM azaserine had no effect on glutaminase activity.

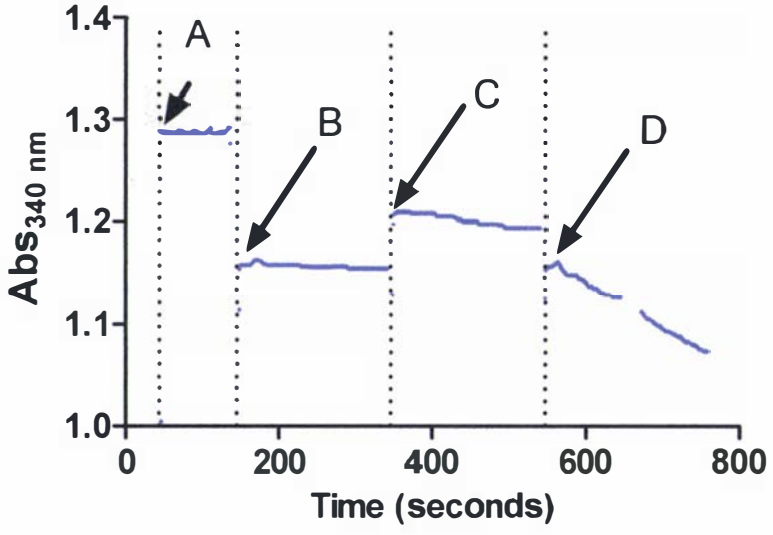


Figure 5.22. Assay of glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) activity at 30°C in a sheep muscle homogenate and adult *O. circumcincta* homogenate A14. There was no GOGAT activity in muscle (B), GOGAT activity with the adult worm homogenate (C) and GDH activity. Enzyme activity was determined from the rate of NADH utilisation monitored spectrophotometrically at 340 nm. The assay protocol was: A: reaction mixture was placed in the cuvette at time zero; B: muscle homogenate added; C adult worm homogenate added; D: 20 mM NH_4Cl added to initiate the GDH reaction.

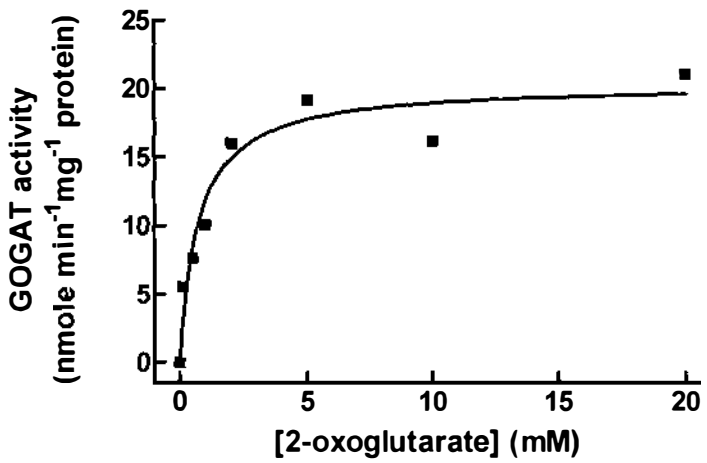


Figure 5.21. Glutamate synthase (GOGAT) activity at 30°C of adult *O. circumcincta* homogenate A13 with increasing concentration of 2-oxoglutarate. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.17. Glutamate synthase (GOGAT) activity at 30°C of an adult *O. circumcincta* homogenate with increasing concentration of 2-oxoglutarate. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	2-Oxoglutarate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A13	0.7	20

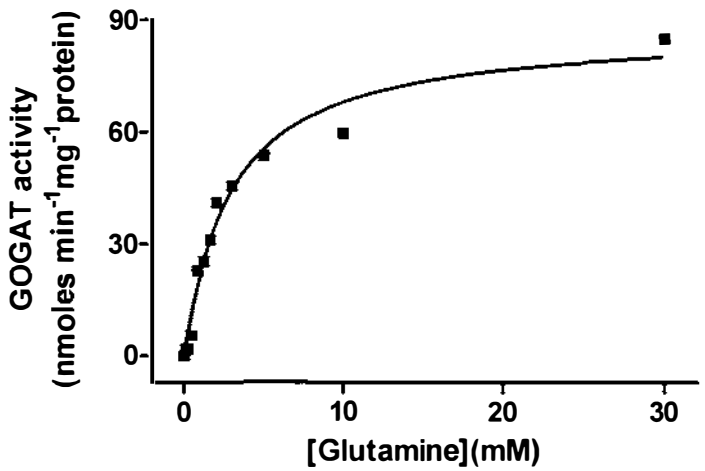


Figure 5.20. Glutamate synthase (GOGAT) activity at 30°C of adult *O. circumcincta* homogenate A12 with increasing concentration of glutamine. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.16. Glutamate synthase (GOGAT) activity at 30°C of adult *O. circumcincta* homogenate with increasing concentration of glutamine. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
A12	2.9	88

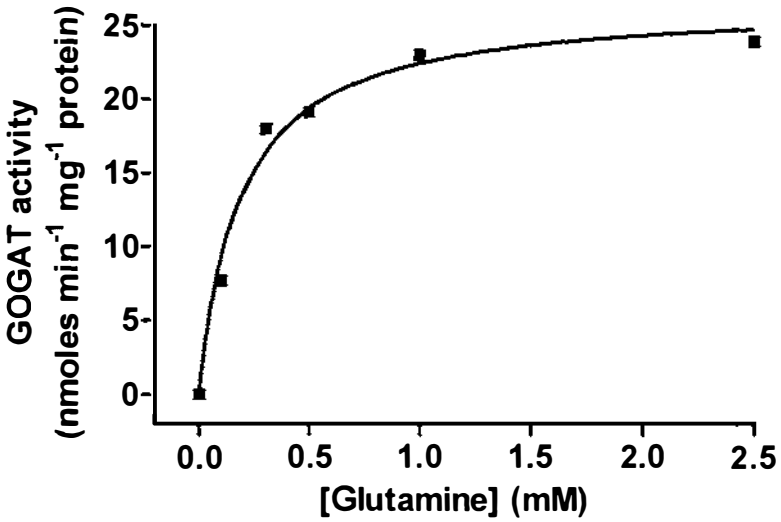


Figure 5.19. Glutamate synthase (GOGAT) activity at 30°C of sheathed L3 *O. circumcincta* homogenate SL81 with increasing concentration of glutamine. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Table 5.15. Glutamate synthase (GOGAT) activity at 30°C of sheathed L3 *O. circumcincta* homogenates with increasing concentration of glutamine. GOGAT activity was calculated from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm.

Homogenate	Glutamine K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL80	0.2	27
SL81	0.4	27
SL82	0.5	22
mean ± SEM, n =3	0.4 ± 0.1	25 ± 2

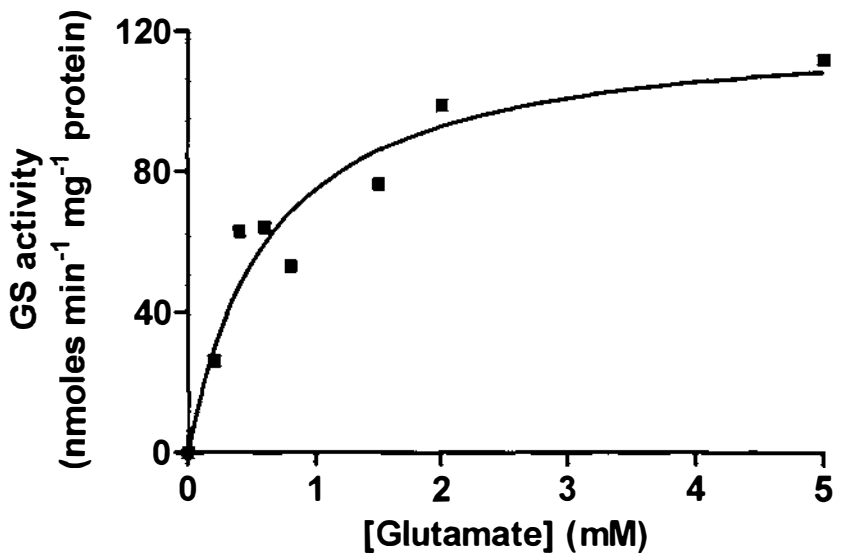


Figure 5.18. Glutamine synthetase (GS) activity of sheathed L3 *O. circumcincta* homogenate SL77, monitored at 30°C in the direction of glutamate utilisation, with increasing concentration of glutamate. GS activity was estimated from the rate of formation of γ-glutamyl hydroxamate from hydroxylamine.

Table 5.14. Glutamine synthetase (GS) activities of sheathed L3 *O. circumcincta* homogenates, monitored at 30°C in the direction of glutamate utilisation, with increasing concentration of glutamate. GS activity was estimated from the rate of formation of γ-glutamyl hydroxamate from hydroxylamine.

Homogenate	Glutamate K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL77	0.55	119
SL78	0.63	122
SL79	0.60	46
mean ± SEM, n =3	0.60 ± 0.02	95 ± 25

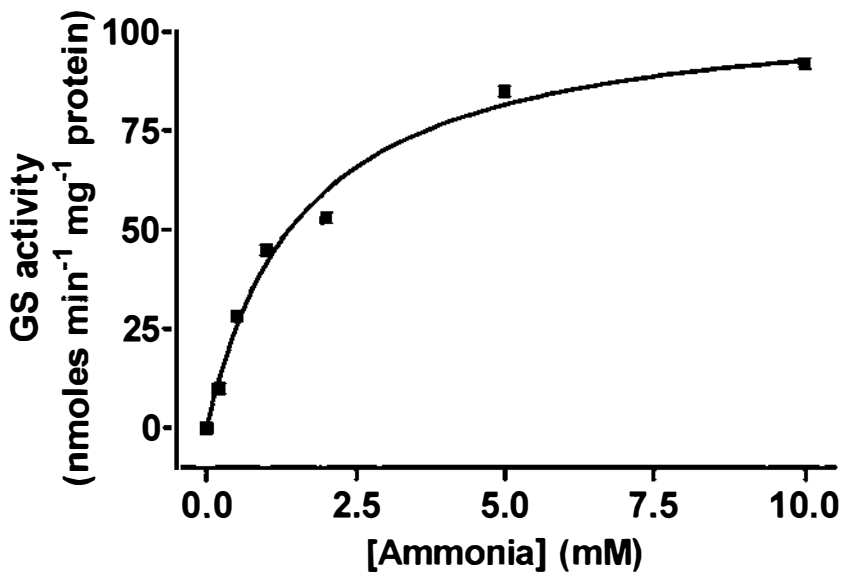


Figure 5.17. Glutamine synthetase (GS) activity of sheathed L3 *O. circumcincta* homogenate SL75a, monitored at 30°C in the direction of glutamate utilisation, with increasing concentration of ammonia. GS activity was estimated from the rate of utilisation of NADH in a coupled reaction with PK and LDH, monitored spectrophotometrically at 340 nm.

Table 5.13. Glutamine synthetase (GS) activity of sheathed L3 O. *circumcincta* homogenates monitored in the direction of glutamate utilisation with increasing concentration of ammonia. GS activity was estimated from the rate of utilisation of NADH in a coupled reaction with PK and LDH, monitored spectrophotometrically at 340 nm.

Homogenate	Ammonia K_m (mM)	V_{max} (nmoles min^{-1} mg^{-1} protein)
SL75a	1.9	101
SL75b	1.6	108
mean ± SEM, n = 2	1.8 ± 0.2	104 ± 4

5.3.3 GLUTAMINE SYNTHETASE

5.3.3.1 Kinetic parameters

GS activity at 30°C of duplicate measurements on a homogenate of sheathed L3 (SL75a) is shown in Figure 5.17 and the K_m for ammonia and V_{max} are shown in Table 5.13. The K_m and V_{max} were 1.8 ± 0.2 mM and 104 ± 4 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n = 2) respectively.

GS activity at 30°C of a homogenate of sheathed L3 (SL77) is shown in Figure 5.18 and the K_m for glutamate and V_{max} of the homogenates of sheathed L3 (SL77-79) are shown in Table 5.14. The K_m and V_{max} were 0.60 ± 0.02 mM and 95 ± 25 nmoles min^{-1} mg^{-1} protein (both mean ± SEM, n =2) respectively.

5.3.4 GLUTAMATE SYNTHASE

5.3.4.1 Kinetic parameters

For the substrate glutamine, the GOGAT activity at 30°C of a homogenate of sheathed L3 (SL81) and of a homogenate of adult worms (A12) are shown in Figures 5.19 and 5.20 respectively. The K_m for glutamine and V_{max} of the homogenates of sheathed L3 (SL80-82) are shown in Tables 5.15 and 5.16 respectively. The K_m values for glutamine were 0.4 ± 0.1 mM (mean ± SEM, n = 3) for sheathed L3 and 2.9 mM for the adult worm homogenate. The V_{max} were 25 ± 2 nmoles min^{-1} mg^{-1} protein (mean ± SEM, n = 3) for sheathed L3 and 88 nmoles min^{-1} mg^{-1} protein for the adult worm homogenate.

For the substrate 2-oxoglutarate, the GOGAT activity at 30°C of a homogenate of adult worms (A13) is shown in Figures 5.21 and the K_m for 2-oxoglutarate and V_{max} are shown in Table 5.17. The K_m for 2-oxoglutarate was 0.7 mM and the V_{max} was 20 nmoles min^{-1} mg^{-1} protein.

5.3.4.2 Comparison of GOGAT activities in sheep muscle and adult worms

An experiment to compare the GOGAT activity of a homogenate of sheep muscle with adult worm homogenate A14 is illustrated in Figure 5.22. When the sheep muscle homogenate was added at point B, there was no

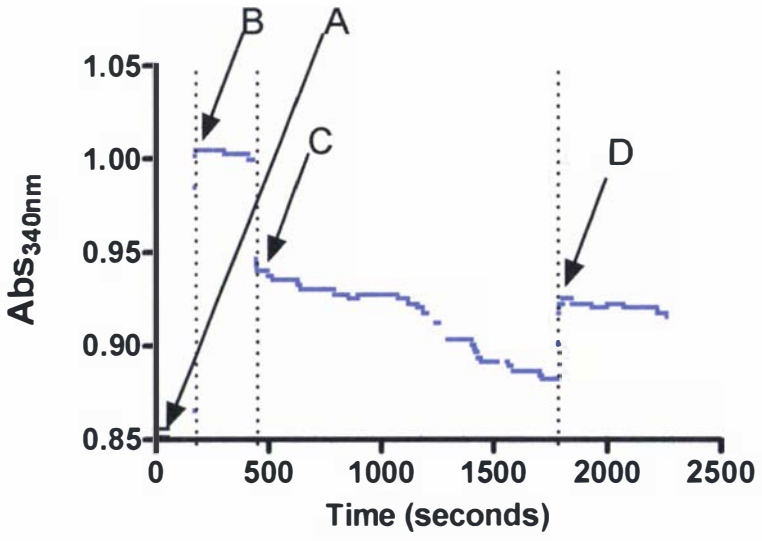


Figure 5.23. Assay of glutamate synthase (GOGAT) activity at 30°C of adult *O. circumcincta* homogenate A15 showing inhibition by 2 mM azaserine (added at D). Enzyme activity was determined from the rate of NADH utilisation through the oxidation of NADH, which was monitored spectrophotometrically at 340 nm. The assay protocol was: A: 20 mM 2-oxoglutarate in 100 mM phosphate buffer was placed in the cuvette at time zero; B: 0.1 mM NADH was added at 200 seconds; C: 5 mM glutamine was added at 480 seconds to initiate the GOGAT reaction; D: 2 mM azaserine was added at 1800 seconds.

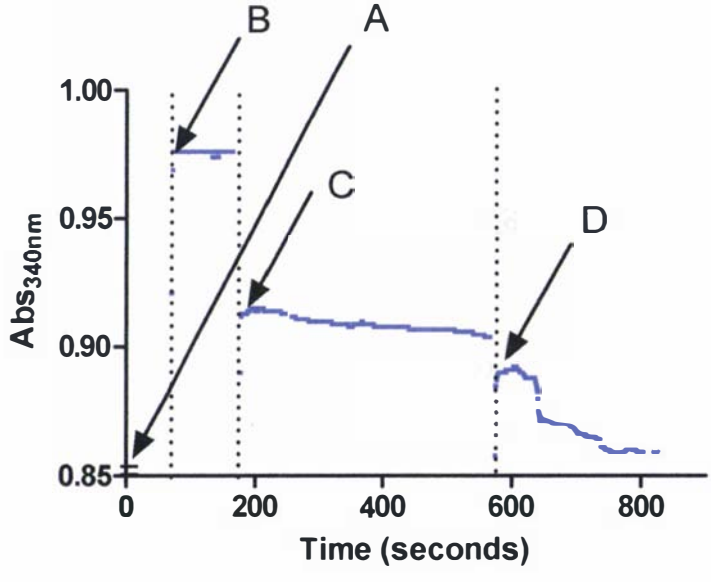


Figure 5.24. Experiment to distinguish activities of glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) at 30°C in adult *O. circumcincta* homogenate A16. Enzyme activity was determined from the rate of NADH utilisation which was monitored spectrophotometrically at 340 nm. The assay protocol was: A: 20 mM 2-oxoglutarate in 100 mM phosphate buffer was placed in the cuvette; B: 0.2 mM NADH was added; C: 5 mM glutamine was added to initiate the GOGAT reaction; D: 20 mM NH_4Cl was added to initiate the GDH reaction.

change in Abs_{340} due to GOGAT activity, whereas GOGAT activity was apparent on subsequent addition of the adult worm homogenate (C). The rate of NADH utilisation increased when 20 mM NH_4Cl added to initiate the GDH reaction.

5.3.4.3 Effect of azaserine

The inhibition by azaserine of GOGAT activity at 30°C in adult worm homogenate A15 is shown in Figure 5.23. GOGAT activity was initiated by the addition of 5 mM glutamine at point C. After 320 seconds, azaserine (final concentration 0.1 mM) was added at D and reduced the rate of NADH utilisation to zero.

5.3.4.4 Comparison of GOGAT and GDH activities

An experiment to compare GOGAT and GDH activity at 30°C in adult worm homogenate A16 is shown in Figure 5.24. GOGAT activity was initiated by adding 5 mM glutamine at point C. Subsequently, 20 mM NH_4Cl was added at point D to initiate the GDH reaction. Both GOGAT and GDH activities were observed from the utilisation of NADH, the rate increasing when both enzymes were active.

5.4 DISCUSSION

The most interesting aspect of this study of glutamate metabolism in *O. circumcincta* was the demonstration of GOGAT activity, which had previously been known in nematodes only from the sequence identified in the *C. elegans* genome. This further differentiates the metabolism *O. circumcincta* from that of its host and may have identified an enzyme which could become an anthelmintic target in the parasite. It also provides an alternative pathway to GDH for the assimilation of nitrogen into amino acids.

5.4.1 GLUTAMATE DEHYDROGENASE

GDH catalyses a reversible reaction, either deaminating glutamate or incorporating ammonia into 2-oxoglutarate (Figure 5.1), although the affinity of the enzyme for ammonia usually determines which direction predominates. The *O. circumcincta* GDH had a high K_m for ammonia (18 mM in L3 and 41 mM in adult worm homogenates), similar to values for the GDH from other organisms (Table 5.18). Despite relatively high enzyme activity, this very low

Table 5.18. K_m values for substrates ofr glutamate dehydrogenases from different organisms.

	Km (mM)							Reference
Source	**2-oxo**	**NH_4^+**	**NADH**	**NADPH**	**Glu**	**NAD^+**	**$NADP^+$**	
O. circumcincta L3	0.06	18	0.03	0.1	0.8	0.7	1.5	Tables 5.2, 5.4, 5.5, 5.7, 5.9
adult	0.06	41			0.13			Tables 5.3, 5.6, 5.8
H. contortus	0.74	42	0.03		3.3	0.31		Rhodes and Ferguson (!973)
A. suum	0.01	0.006	0.0007	0.01	0.24	0.002	0.001	Langer (1972)
T. cruzi	3.6	16	0.17		23.5	2.9		Urbina and Azavache (1984)
	3.8	18	0.16		11	1.4		Cazzulo *et al.* (1979)
Ovine liver	1.2	20	0.03		1.2	0.07		Rhodes and Ferguson (!973)
Bovine liver	0.12	57	0.02		1.9	0.02		Strecker (1953)
	0.57	105	0.04	0.067	2.9	0.13		Prezioso *et al.* (1985)
D. melanogaster	1.42	242	0.04	>10	5.4	0.16		Prezioso *et al.* (1985)
Mytilis edulis	0.38	20	0.03		5	0.55		Ruiz Ruano *et al.* (1985)
Anthopleura anthogrammica	0.59	61		0.009	4.7		0.005	Male and Storey (1983)
Brassica napus	0.87	6.6	0.02		3.23	0.12		Watanabe *et al.* (1999)
White turnip	2.0	22	0.09		28.6	0.25		Itagaki *et al.* (1988)
Ruminococcus flavefasciens	0.41	19			62			Duncan *et al.* (1992)
P. islandicum	0.06	9.7	0.005		0.17	0.03		Kujo and Ohshima (1998)

affinity for ammonia would suggest that in the reversible amination of 2-oxoglutarate to glutamate, the predominant direction is likely to be glutamate deamination, and not incorporation of ammonia.

Although GDH activity has been identified in many helminths, including *H. contortus* (Rhodes and Ferguson, 1973; Skuce *et al.*, 1999b), *A. suum* (Langer, 1972), *D. immitis* (Langer and Jiamperpoon, 1970; Turner *et al.*, 1986), *H. polygyrus*, *P. redivivus* (Grantham and Barrett, 1986a), *O. cuniculi* (Hutchinson and Fernando, 1975), *L. carinii* (Davies and Köhler, 1990), *F. hepatica* (Prichard and Schofield, 1968; Thorpe, 1968), *M. similis* (McManus and James, 1975), only a few reports contain kinetic data. A feature of data from a diverse selection of organisms included in Table 5.18 is the variation. Overall, the species with enzymes with similar properties to those of *O. circumcincta* are invertebrates, particularly in co-factor specificity.

The activity of GDH in the aminating direction was about 30% higher in adult worm homogenates than in L3 homogenates with both ammonia and 2-oxoglutarate as the variable substrate (Tables 5.5-5.8), but in the deaminating direction, which is more likely to operate *in vivo*, the reverse was the case, with higher activity in L3 (V_{max} of 70 and 121 nmoles min^{-1} mg^{-1} protein for adult worms and L3 respectively). Differences between adults and L3 were the lower K_m for glutamate in adult worms (0.13 mM and 0.8 mM in adult worms and L3 respectively) but higher Km for ammonia in adults (41 mM and 18 mM for adult worms and L3 respectively). Some GDHs exhibit complex kinetics which deviate from Michaelis-Menton kinetics (Hudson and Daniels, 1993), although this was not the case in the present study. K_m values may be affected by pH in many species (Bond and Sang, 1968; Itagaki *et al.*, 1988; Hudson and Daniels, 1993), e.g. the binding of 2-oxoglutarate, but not glutamate, was pH dependent in *H. diminuta* GDH (Mustafa *et al.*, 1978).

Cofactor requirements differentiate GDHs into three broad classes according to whether they are NAD^+ specific (EC 1.4.1.2), $NADP^+$ specific (EC 1.4.1.4) or dual enzyme specific (EC 1.4.1.3) or have dual coenzyme-specificity (reviewed by Goldin and Frieden, 1971; Hudson and Daniel, 1993). Although not universally true, in many microorganisms, the aminating direction is catalysed by NAD-GDH and the deminating direction by NADP-GDH (Hudson and Daniel, 1993). The *O. circumcincta* GDH belongs to the dual

coenzyme-specific group, functioning with both $NAD(P)^+$ and NAD(P)H, although the activities were not equal. Dual co-factor specificity may result from two isozymes or a single enzyme being present (Hudson and Daniel, 1993), but this cannot be resolved in the present experiments.

The *O. circumcincta* L3 enzyme did not fit a pattern of the aminating direction being catalysed by NAD-GDH and the deaminating direction by NADP-GDH. GDH was about four times more active with NAD^+ than with NADP in the deaminating direction and the K_m for NAD^+ was about half that for NADP (Figure 5.9, Table 5.4). In the aminating direction, the activity was similar with either co-factor, but the K_m for NADH was 2-3 times lower than for NADPH (Figure 5.14 and Table 5.9). Many other helminth GDHs seem to be more active with NAD+ or NADH than with NADP, e.g. *H. diminuta* (Mustafa *et al.*, 1978), *F. hepatica* (Prichard and Schofield, 1968) and *O. cuniculi* (Hutchinson and Fernando, 1975). In both *H. polygyrus* and *P. redivivus*, GDH activity with NADH was twice the rate with NADPH in the aminating direction and in the reverse direction, activity with NAD^+ was three and eight times greater than with $NADP^+$ in *P. redivivus* and *H. polygyrus* respectively (Grantham and Barrett, 1986a). In contrast, the *D. immitis* mGDH was equally reactive with NAD^+ and $NADP^+$.

The pH optima of the L3 *O. circumcincta* GDH with NAD^+/NADH as cofactor were pH 7-8 in both directions, with much reduced enzyme activity above or below that pH range, particularly in the deaminating direction (Figure 5.5). Relative to the properties of mammalian GDHs, which generally have pH optima of pH 8-10 in the deamination direction and 0.5-2 pH units lower in the aminating direction, the *O. circumcincta* pH optima are similar in the aminating direction, but lower in the opposite direction. These values are similar to those recorded for ovine liver GDH by Rhodes and Ferguson (1973), rather than for purified *H. contortus* GDH, which had pH optima of 8.8 for NAD^+ reduction (similar to bovine and ovine liver GDH) and pH 8 for NADH oxidation, the latter higher than the pH 7.5-7.6 the mammalian enzymes. The *O. circumcincta* GDH pH optima are consistent with those for *D. immitis*, in which mGDH had a pH optimum of 8.4 and the cytosolic cGDH a narrow pH optimum of 7.8-8 (Turner *et al.*, 1986).

The temperature optimum for the L3 enzyme was 30°C in the direction of glutamate formation (Figure 5.6), similar to the 27°C for purified *H. contortus* GDH (Rhodes and Ferguson, 1973). This may relate to living at lower environmental temperatures, since a temperature optimum (and optimum K_{cat}) of 30°C has also been reported for three species of Antarctic fish (Ciardiello *et al.*, 1997) and *D. melanogaster* (Prezioso *et al.*, 1985), while the opposite is the case in the hyperthermophilic bacterium *Pyrobaculum islandicum*, which has a temperature optimum of 90°C (Kujo and Ohshima, 1998). Different isoforms may not have the same optima, e.g. the *D. immitis* cGDH had lower activity at 37°C than at 25°C, but the opposite was the case for the mGDH (Turner *et al.*, 1986) and the mealworm (*Tenebrio molitor*) fat body GDH had a temperature optimum just below 30°C for deamination with NAD^+ but at 37°C with NADH in the aminating direction (Teller, 1988a).

The regulation of the dual specificity mammalian enzymes is very complex, being strongly regulated by purine nucleotides, particularly activated by ADP, AMP and some amino acids and inhibited by GTP (Goldin and Frieden, 1971; Hudson and Daniel, 1993). However, in humans, there is a nerve-specific GDH which is much more dependent on ADP activation than the "housekeeping" enzyme and is unresponsive to GTP (Mastorodemos *et al.*, 2005). These activators and inhibitors also affect the formation of multienzyme complexes of GDH with aminotransferases, 2-oxoglutarate dehydrogenase, citrate synthase and MDH, which allow the direct transfer of 2-2-oxoglutarate, GTP and NADH between enzymes (Fahien and Smith, 1974; Fahien *et al.*, 1985, 1989). The kinetics of GDH *in vitro* may therefore not reflect the situation *in vivo*, where there is substrate channelling.

The *O. circumcincta* GDH differed from the mammalian enzyme in the regulation by purine nucleotides: in the deaminating direction, it was unresponsive to both ATP and ADP, but in the deaminating direction, activity was doubled by 1 mM ADP, but surprisingly increased 5-fold by 1 mM ATP (Table 5.1). GDH from some species of invertebrates show the characteristic activation by ADP and inhibition by GTP or ATP (Prezioso *et al.*, 1985; Teller, 1988b) but others are unresponsive (Bishop *et al.*, 1978; Hoffmann *et al.*, 1978). Unlike bovine liver GDH, AMP, ADP, ATP, GDP, GTP and amino acids had little effect on *H. diminuta* GDH, whereas the TCA intermediates fumarate,

Table 5.19. K_m values for glutamine for the reaction catalysed by glutaminases in different organisms.

Source	K_m (mM) glutamine	Reference
O. circumcincta L3	1.5	Table 5.10
O. circumcincta adults	7.2	Table 5.11
Lactobacillus rhamnosus	4.8	Weingand-Ziade *et al.* (2003)
Micrococcus luteus		Moriguchi *et al.* (1994)
glutaminase I	4.4	
Glutaminase II	6.5	
B. pasteurii	9.5	Klein *et al.* (2002)
Debaryomyces spp.	4.5	Dura *et al.* (2002)
Rattus norvegicus Pancretic islets	2.6	Michalik *et al.* (1992)
Rat liver	6	McGivan *et al.* (1980)
	17	Smith and Watford (1988)
Rat heart	4	Nelson *et al.* (1992)
Rat kidney	5	Curthoys *et al.* (1976)
Pig brain membrane-bound form	1.5	Nimmo and Tipton (1981)

malate and succinate were inhibitory in high, probably not physiologically relevant concentrations (Mustafa *et al.*, 1978).

The 5-fold stimulation of the O. *circumcincta* GDH in the aminating direction by ATP is very unusual, although Grantham and Barrett (1986a) described similar stimulation by GTP, but not ATP, in the reaction of *P. redivivus* GDH with NADH. ATP also activated the GDH of a bacterium from high salt environments (Bonete *et al.*, 2003). Whether GDH is functioning in the formation or deamination of glutamate under particular cellular conditions will depend on the concentrations of activators and inhibitors as well as the K_m for the substrates particularly for ammonia.

5.4.2 GLUTAMINASE

There were similar glutaminase activities in L3 homogenates and an adult worm homogenate (Tables 5.10-5.11), although the mean value for the L3 homogenates was reduced by the particularly low activity of homogenate SL71. Glutaminase has previously been identified in nematodes, in *H. polygyrus* and *P. redivivus* at very much lower levels than in rat liver (Grantham and Barrett, 1986a), whereas Davies and Köhler (1990) did not detect any in the filarial nematode *L. carinii*. The glutaminase activity in O. *circumcincta* was quite high in most homogenate in the range of 150-250 nmoles min^{-1} mg^{-1} protein.

The O. *circumcincta* glutaminase could resemble a number of different types of the enzyme found in vertebrates or lower organisms. There are liver- and kidney-types isoforms of both vertebrate phosphate-activated glutaminase I and glutaminase II, which is a phosphate-independent glutamine aminotransferase or a γ-glutamyltransferase (reviewed by Curthoys and Watford, 1995; Kvamme *et al.*, 2000). There are several classes of glutaminases of microorganisms which are not phosphate-activated and have different properties from the mammalian phosphate-dependent enzymes (reviewed by Nandakumar *et al.*, 2003). While some are specific for glutamine, others are also active asparaginases. In the insect *P. crassipalpis*, the glutaminase was described as like glutaminase II and there was no type I enzyme present (Downton and Kennedy, 1986). The K_m for glutamine is the only property of the *O. circumcincta* enzyme available for comparison with enzymes from other species (Table 5.19). The mammalian kidney-type has a

low K_m for glutamine (2-5 mM), whereas the liver-type has a higher K_m for glutamine (17 mM or 6 mM depending on association with the mitochondrial membrane) (Curthoys and Watford, 1995).

The K_m for glutamine was much higher for the adult worm homogenate (7.2 mM) compared with 1.5 mM for the L3 homogenate. The closest K_m to the low value estimated for L3 homogenates was for the membrane-bound pig brain glutaminase (Nimmo and Tipton, 1981), whereas the higher K_m for the adult worm homogenate was similar to most values for other mammalian glutaminases and the enzymes from microorganisms. The effectors and inhibitors used for the L3 homogenates had very little effect and with the high variability no conclusions can be reached (Table 5.12). It was expected that Mg^{2+} might be stimulatory, as Mg^{2+} stimulated rat liver glutaminase, although the effect of Mg^{2+} was very sensitive to small changes in pH: the increase was 7-fold at pH 7.1, 1-5-fold at pH 7.4 and there was virtually no effect at pH 7.7 (Szweda and Atkinson, 1990). Therefore, the 21% higher activity in *O. circumcincta* L3 at pH 7.5 may possibly be due to Mg^{2+} activation of glutaminase (Table 5.12).

Given that glutamine is the substrate for a number of enzymes other than glutaminase, such as glutamine:fructose-6-phosphate amidotransferase and GOGAT, there is some question whether the enzyme activity demonstrated in *O. circumcincta* is glutaminase. It is unlikely that the other substrates for the amidotransferase, the first and rate limiting step in the hexosamine pathway (Section 1.8.1), would be present in sufficient quantities for significant enzyme activity to occur. The failure of the GOGAT inhibitor azaserine to inhibit glutaminase activity (Table 5.13), whereas it was inhibitory to GOGAT activity (Figure 5.23) is good evidence for glutaminase and GOGAT being separate enzymes.

5.4.3 GLUTAMINE SYNTHETASE

Predictably, glutamine synthetase (GS) activity was present in L3 *O. circumcincta* (Tables 5.13-5.14), as GS has been described as present in all organisms, including fossils (Kumada *et al.*, 1993). It has been previously reported from the nematodes *H. polygyrus* and *P. redivivus* (Grantham and

Table 5.20. K_m values for glutamate and ammonia for the reaction catalysed by glutamine synthetase in different organisms.

Source	K_m (mM) glutamate	K_m (mM) NH_3	Reference
O. circumcincta L3	0.6	1.8	Tables 5.13, 5.14
Human	3.5	0.15	Listrom *et al.* (1997)
Dog	1.1		Shin and Park (2004)
Tuber borchii (fungus)	54	0.75	Montanini *et al.* (2003)
S. cerevisiae	6.3		Mitchell and Magasanik (1983)
Marine alga	9.7	0.11	Rees *et al.* (1995)
Laccaria laccata (fungus)	3.2	0.02	Brun *et al.* (1992)
Arabidopsis thaliana	3.8	2.4	Ishiyama *et al.* (2004)
Plants (7 species) GS II	4.1-9.8		Acaster and Weitzman (1985)
Barley GS I	9		
Maize GS II	5.2		
E. coli	2.6	0.5	Singh *et al.* (2004)
S. typhimurium	2.1	0.46	Miller and Brenchley (1981)
Cyanobacteria GSIII	0.9	0.19	García-Domínguez *et al.* (1997)
GSII	2.3	1.33	
Synecoccus RF-1	5	0.2	Yuan *et al.* (2001)
Anabaena sp	2.1	0.02	Blanco *et al.* (1989)
			Orr and Haselkorn (1981)
Clostridium pasteurianum	3.7		Krishnan *et al.* (1986)

Barrett, 1988). GS activity was about 100 nmoles min^{-1} mg^{-1} protein with either ammonia or glutamate as the variable substrate.

In addition to ATP, GS activity requires either Mg^{2+} or Mn^{2+} for activity, the preferred cation varying with the species and isoform (Eisenberg *et al.*, 2000). The metal binding site is separate from the adenylation site where the enzyme activity is largely controlled (Stadtman, 2001). The cation can be tightly bound to the enzyme, particularly to the unadenylated form, e.g. the metal could not be removed by dialysis from the *Azospirillium brasilense* GS (Antonyuk *et al.*, 2001) and the GS of the sugar cane endophyte *Acetobacter diazotrophicus* was reported to be unresponsive to Mg^{2+} in some adenylation states (Ureta and Nordlund, 2001). The *O. circumcincta* enzyme was initially assayed without added cation and found to be active, therefore this method was continued.

The K_m for glutamate was at the low end of the values for other organisms in Table 5.20, which includes the three types of GS: GSI in prokaryotes, GSII in eukaryotes and some bacteria and GSIII expressed in a rumen anaerobe (Amaya *et al.*, 2005) and cyanobacteria (Pesole *et al.*, 1995; García-Domínguez *et al.*, 1997). Whereas the K_m for glutamate was determined in the commonly used "biosynthetic reaction" in which hydroxylamine replaced ammonium, the K_m for ammonia was determined in a coupled assay, which was very difficult to stabilise, so that only duplicate estimates from one homogenate are presented (Table 5.13). These are probably only approximate values, but are of interest in relation to the K_m for GDH, the competing pathway for ammonia assimilation. Notably the K_m of ammonia of 1.8 mM for GS was much lower than the K_m for ammonia of 18 mM for GDH in L3 homogenates. Together with the presence of GOGAT activity, it would seem that ammonia is assimilated in *O. circumcincta* via the GS-GOGAT pathway and not normally by GDH.

5.4.4 GLUTAMATE SYNTHASE (GOGAT)

The demonstration of GOGAT activity in both L3 and adult *O. circumcincta* (Tables 5.15-5.17) was unexpected, as the enzyme is often described as not present in animals. Prior to the present experiments, the gene sequences for NADH-GOGAT in the genome of *C. elegans* (Vanoni and Curti, 1999) was the only indication that GOGAT might be present in

Table 5.21. K_m values for glutamine for the reaction catalysed by glutamate synthase (GOGAT) in different organisms.

Source	K_m (mM) glutamine	Reference
O. circumcincta L3	0.4	Table 5.15
O. circumcincta adult	2.9	Table 5.16
B. mori	0.22	Hirayama *et al.* (1998)
S. sclerotiorum	2.6	Rachim and Nicholas (1985)
S. cerevisiae	0.29	Cogoni *et al.* (1995)
A. brasilense	0.45	Ratti *et al.* (1985)
Nocardia mediterranei	0.05	Mei and Jiao (1988)
Bacillus megaterium	0.2	Hemmilä and Mäntsälä (1978)
Medicago sativa root nodules	0.47	Anderson *et al.*(1989)

nematodes as well as in some insects. A search of BlastP using GI:15375027 from *Klebsiella aerogenes* revealed matches with unpublished sequences from *C. briggsae* and *Trichinella britovi*. GOGAT activity has been identified in the silkworms *B. mori* (Hirayama and Nakamura, 2002) and *S. cynthia ricini* (Osanai *et al.*, 2000), the mosquito *A. aegypti* (Scaraffia *et al.*, 2005) and in the *S. frugiperda* Sf9 insect cell line (Doverskog *et al.*, 2000; Drews *et al.*, 2000). It may be recognised as more widespread in invertebrates than at present, when more species are investigated.

GOGAT activity was fairly low but consistently detectable in homogenates of L3 *O. circumcincta* with glutamine as the variable substrate (mean 25 nmoles min^{-1} mg^{-1} protein) and in an adult worm homogenate with 2-oxoglutarate (20 nmoles min^{-1} mg^{-1} protein), but considerably higher with glutamine as the variable substrate (88 nmoles min^{-1} mg^{-1} protein). As the GOGAT activity may depend on the metabolic state of the organism, there may be a difference in activity of the two adult worm homogenates, or it may relate to less than saturating concentration of glutamine in the 2-oxoglutarate experiment. Adult worm, not L3, homogenates were used for subsequent experiments with azaserine and comparison of worm GOGAT with sheep muscle GOGAT and worm GDH (Figures 5.22-5.24).

The K_m for glutamine for L3 and adult *O. circumcincta* GOGAT are compared with those of the silkworm, plants, yeasts and bacteria in Table 5.21. The L3 enzyme had a typical K_m for glutamine (0.4 mM), whereas the K_m of the adult enzyme (2.9 mM) was more in the range of the enzyme of the fungus *Sclerotinia sclerotiorum* (Rachim and Nicholas, 1985). Values in Table 5.21 are for the forms of the enzyme which use NADH or NADPH as the electron donor and not Fd-GOGAT, which uses ferrodoxin as the electron donor in photosynthetic tissues in plants (reviewed by Vanoni and Curti, 1999; Suzuki and Knaff, 2003; van den Heuvel *et al.*, 2004; Vanoni *et al.*, 2005).

Experiments were carried out to validate that the enzyme activity was GOGAT. As a negative control, to show that the apparent GOGAT activity in *O. circumcincta* homogenates was not due to a different enzyme, a sheep muscle homogenate (which would not contain GOGAT) was added to the standard reaction mixture to initiate GOGAT activity (Figure 5.22). When no NADH utilisation occurred, an aliquot of a homogenate of adult *O. circumcincta*

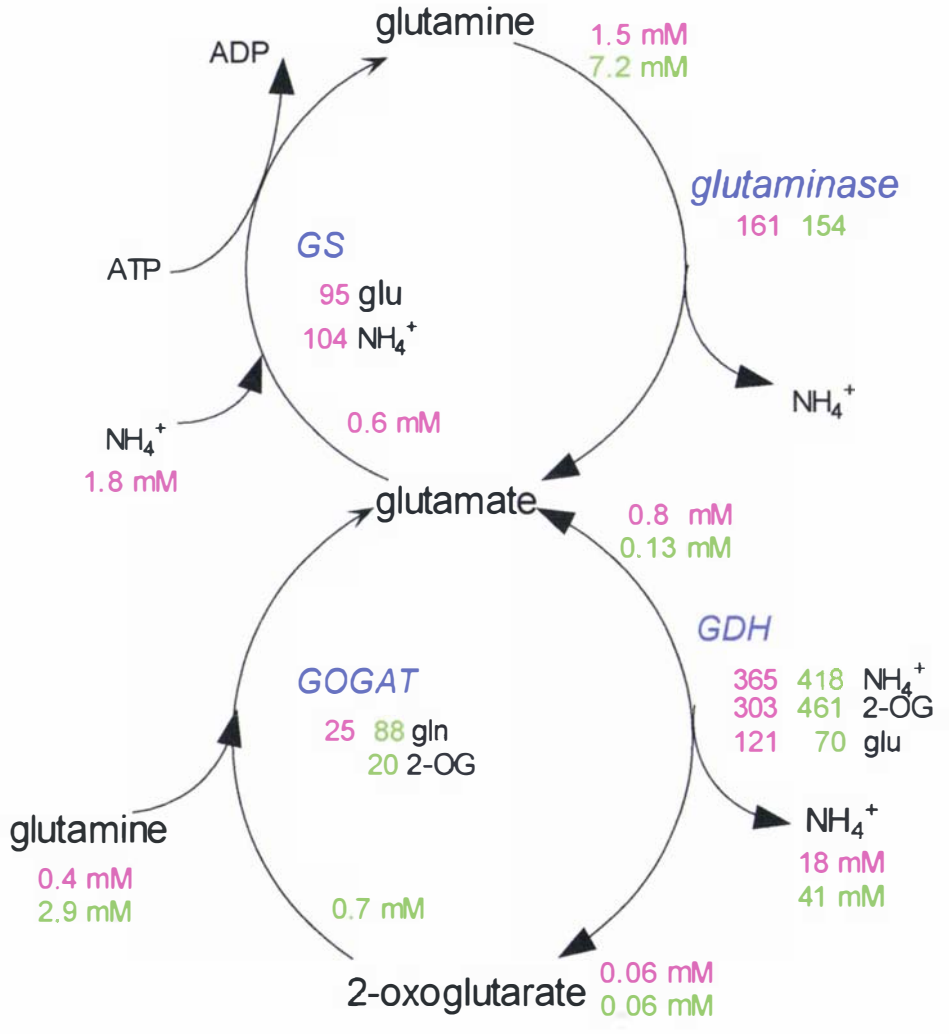


Figure 5.25. Metabolic map of enzymes of glutamate metabolism identified in L3 or adult *O. circumcincta* homogenates. Enzymes are shown in blue; K_m values (mM) for the substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reactions for the variable substrate are shown in pink for L3 homogenates and green for adult worm homogenates.

was added to the cuvette and low GOGAT activity was initiated. Finally NH_4^+ was added and the resulting GDH activity served as a control to show the homogenates had not been inactivated during preparation. As GDH and GOGAT share the substrate 2-oxoglutarate, the two enzymes were distinguished by the greater rate of NADH utilisation by GDH (adding NH_4^+) than by GOGAT (adding glutamine) (Figure 5.24).

The most convincing evidence of GOGAT activity was the inhibition by azaserine (Figure 5.23), a potent inhibitor of GOGAT, but a very weak inhibitor of glutamine:fructose-6-phosphate amidotransferase in *S. typhimurium* (Chmara *et al.*, 1985), *N. crassa*, *E. coli* and rat liver (Ghosh *et al.*, 1960). Azaserine has been used to block GOGAT in *A. aegypti* (Scaraffia *et al.*, 2005), *S. frugiperda* insect cells (Doverskog *et al.*, 2000) and *B. mori* (Hirayama *et al.*, 1998). The importance of GOGAT to mosquito metabolism was demonstrated when azaserine fed with a blood meal increased glutamine levels and decreased proline concentrations in the haemolymph (Scaraffia *et al.*, 2005). GS was low and GDH activity was very high in the mosquito midgut, whereas, in the fat body, GDH was comparatively low, and GS was twice as high as in the midgut. GOGAT was exclusively located in the fat body. This suggests that oxidation of the amino acids in the meal takes place in the midgut, but the fat body is the site of detoxification and incorporation of ammonia into glutamate by GS-GOGAT (Scaraffia *et al.*, 2005). As there is no equivalent of the fat body in nematodes, the site of GOGAT activity in *O. circumcincta* may be in the intestine.

5.4.5 GLUTAMATE METABOLISM

A metabolic map linking the enzymes identified in L3 or adult *O. circumcincta* homogenates is shown in Figure 5.25. The K_m values for the substrates and the V_{max} for the reactions are indicated.

Glutamate metabolism in *O. circumcincta* is clearly different from that in mammals and strongly resembles metabolism in insects, plants and microorganisms. The most significant differences between vertebrates and lower organisms are the anabolic reactions by which ammonia is incorporated into amino acids. The principal route in mammals is the formation of glutamate by GDH, then aminotransferases use the glutamate-2-oxoglutarate system to form several other amino acids from glutamate. In *O. circumcincta*, and

possibly in other nematodes, there is the alternative GS-GOGAT pathway used by plants, microorganisms and some insects to incorporate ammonia first into glutamine then to form two glutamate molecules from glutamine and 2-oxoglutarate (Miflin and Lea, 1976; Miflin and Habash, 2002; Hodges *et al.*, 2003; Magasanik, 2003). The GS-GOGAT pathway, which requires ATP but has a higher affinity for ammonia, is the preferred pathway in these species if ATP is readily available (Heller, 1994, 2002).

Nitrogen assimilation via GDH does not require ATP, but is limited by the high K_m for ammonia, which is the case for the O. *circumcincta*. In species with the GS-GOGAT pathway, GDH can operate in the aminating direction when ammonia concentrations are high, energy is limited or the organism is stressed (Fisher and Sonenshein, 1991; Osuji and Madu, 1996; Helling, 1998; El-Shora and Abo-Kassem, 2001). GDH probably functions in O. *circumcincta* as a catabolic enzyme and, as in many organisms, is likely to be the main source of excreted ammonia (Bidigare and King, 1981; Batrel and Le Gal, 1984). In invertebrates, GDH may also be involved in osmoregulation, as the activities of GDH and aminotransferases increase or decrease with changes in salinity (Gerard and Gilles, 1972; Gilles, 1973; Wickes and Morgan, 1976; Batrel and Le Gal, 1984; Reitze *et al.*, 1989; Matsushima and Hayashi, 1992).

As the tissues in which glutaminase are located in O. *circumcincta* are unknown, its specific role can only be the subject of speculation. One role may be to generate glutamate from glutamine, a non-toxic compound which is used to transport organic nitrogen between organs in vertebrates and some invertebrates, particularly to skeletal muscle where it is readily converted to glutamate by glutaminase. The other end-product is ammonia, which is used in the mammalian kidney to buffer an acid urine. Glutaminase may be used by adult *O. circumcincta* to produce ammonia to buffer the acid environment of the abomasum, whereas bacteria use either urease or the dihydrolase pathway to generate ammonia (Mobley and Hausinger, 1989; Mégraud *et al.*, 1992; Audia *et al.*, 2001; Cotter and Hill, 2003).

Glutamine may be important in the recycling of glutamate in the neuromuscular system, as it is in vertebrates. Glutamate is mainly an inhibitory neurotransmitter acting on chloride channels on the nematode pharyngeal muscles (Avery, 1993; Adelsberger *et al.*, 1999), whereas in

vertebrates, glutamate is an excitatory neurotransmitter (Alagarsamy *et al.*, 2001; Mayer and Armstrong, 2004). Rapid removal of glutamate from the vertebrate synapse is achieved by the separation of glutamine-glutamate metabolism between glial cells, which convert glutamate to glutamine using mainly GS, and neurons, which resynthesise glutamate using phosphate-dependent glutaminase (Waagepetersen *et al.*, 2005). The homologous nematode enzymes may have similar roles.

The present *in vitro* studies of glutamate metabolism in *O. circumcincta* have been carried out on homogenates of whole parasites, which mixes together enzymes from a number of anabolic and catabolic pathways for which glutamate is a substrate. *In vivo*, enzymes which potentially compete for a substrate or use the end-product of a reaction are often physically separated in different organs, cells or compartments within cells. This separation is evident in the mammalian liver, where periportal cells express glutaminase and OUC enzymes, but perivenous cells express GS. There are also some examples in invertebrates. GDH activity was very high in the mosquito midgut and comparatively low in the fat body; GS activity was low in the midgut and twice as high in the fat body; GOGAT was exclusively located in the fat body (Scaraffia *et al.*, 2005). GDH activity was located only in the intestinal cells of *H. contortus* and almost exclusively in the blood-feeding stages (Skuce *et al.*, 1999b). The specific roles of the key enzymes in glutamate metabolism will probably not be clarified until the location of these enzymes in *O. circumcincta* is known.

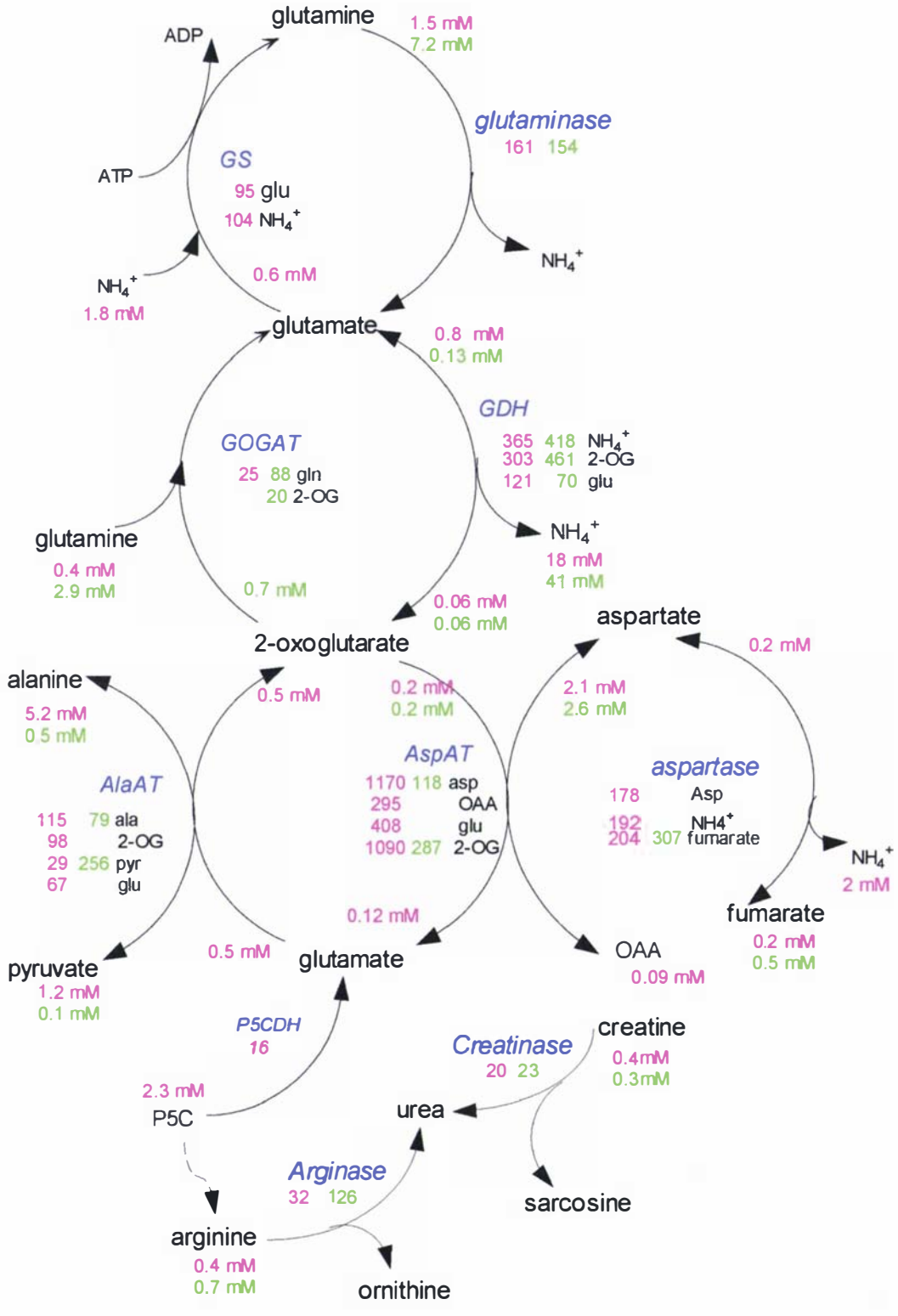


Figure 6.1. Metabolic map of enzymes of nitrogen metabolism identified in L3 or adult *O. circumcincta* homogenates. Enzymes are shown in blue; K_m values (mM) for the substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reactions for the variable substrate are shown in pink for L3 homogenates and green for adult worm homogenates.

Chapter 6

GENERAL DISCUSSION

The aim of the experiments reported in this thesis was to investigate some key areas of nitrogen metabolism, in anticipation of finding novel enzymes not present in mammals or with kinetic properties which clearly differentiated the nematode and host enzymes. An additional outcome would be the identification of aspects of nematode metabolism which would lead to improvements in *in vitro* culture of L3 and adult worms.

A metabolic map linking all enzymes of nitrogen identified in L3 or adult *O. circumcincta* homogenates is shown in Figure 6.1. The K_m values for the substrates and the V_{max} for the reactions are indicated. The most interesting findings were the presence in *O. circumcincta* homogenates of three enzymes, creatinase, aspartase and GOGAT, which are believed not to be expressed in the sheep host. Confirmation of the activities found in whole worm homogenates will be needed in the future, preferably by identifying their genes and localising their expression to particular tissues in the parasite. These enzymes will only be useful targets if their functioning is proven to be essential for worm survival.

Nitrogen metabolism has received much less attention than energy metabolism in nematodes, particularly the parasites of ruminants. The present studies of absorption, metabolism and excretion in L3 and adult *O. circumcincta* have revealed differences from these processes in their mammalian hosts. This is also true of energy metabolism, which in the larger parasites, such as Ascarids, cestodes and trematodes, is considered to change from aerobic to more anaerobic during the transition to parasitism (Komunieki and Vanover, 1987). There may not be as great a change in smaller nematodes like *O. circumcincta*, because their higher surface area to volume ratio may allow more oxygen diffusion. Nevertheless, progression from free-living to parasitic stages may affect oxygen delivery, as there is a 100-fold difference in worm volume between L3 and adult *O. circumcincta* (Chapter 2,

2.4). Oxygen availability and changes in nutrient supply may be key factors underlying some of the differences in nitrogen metabolism.

Both L3 and adult *O. circumcincta* excrete ammonia as the principal nitrogenous compound. This is consistent with previous observations in free-living and parasitic nematodes (Rogers, 1952; Rothstein, 1963; Wright, 1975a,b). The rate of ammonia excretion in L3 was temperature dependent, linking ammonia generation with the overall metabolic rate. Excretion would be expected to be low in relatively dormant L3 and probably equally low in rapidly growing and reproducing parasitic stages of nematodes with their great demands for amino acids.

Of note was the plateau in ammonia excretion when the concentration in the incubation medium reached 120-140 µM, which suggests that ammonia excretion may be limited by external ammonia concentrations. The concentrations of ammonia in the vicinity of the parasitic stages in the rumen fluid, gland lumen or free in the abomasum are all likely to exceed this concentration, based on published values of arterial blood (200 µM), portal blood (487 µM) (Parker *et al.*, 1995), rumen contents (5 mM) and abomasal fluid (1 mM) in sheep (Harrop, 1974; Harrop and Phillipson, 1974). Ammonia may, therefore, either be absorbed or excreted according to the external concentration, which for parasitic stages of *O. circumcincta in vivo* may frequently be ammonia absorption. The presence of GOGAT would allow ammonia to be used to generate glutamate and then other amino acids.

Movement of ammonia across the cuticle may involve ammonium transporters and perhaps also diffusion of ammonia. Less ammonia and no urea were excreted by bleach-exsheathed L3, probably because of damage to cuticular ammonium and urea permeases, as diffusion is not likely to be reduced, or could even increase. Some excretion may also be through the nematode excretory cell (Buechner *et al.*, 1999; Buechner, 2002). Ammonium transporters are present in all classes of organisms (Howitt and Udvardi, 2000; Williams and Miller, 2001; Javelle *et al.*, 2003; Khademi *et al.*, 2004) and there are homologues of high affinity ammonium transporters in the *C. elegans* genome (Howitt and Udvardi, 2000; ter Schure *et al.*, 2000). In plants, bacteria and yeasts, high affinity ammonia permeases scavenge ammonia from the

environment, including recovering ammonia lost by diffusion when growing on sources of nitrogen other than ammonia (Marini *et al.*, 1997).

Ammonia is toxic in high concentrations and must either be incorporated into urea by species which have a complete OUC, or assimilated into glutamine by GS or GDH. Even in high external ammonia concentrations, plants and microorganisms maintain low cytoplasmic ammonia levels (Streeter, 1989; Roberts and Pang, 1992) primarily using the ammonia-assimilating pathway GS-GOGAT. GS has a greater affinity for ammonia than does GDH in *O. circumcincta*, as in other species, but uses large amounts of ATP, which has been estimated at 15% of ATP usage in *E. coli* (Reitzer, 2003).

The presence of GOGAT differentiates glutamate metabolism in *O. circumcincta* from that in mammals in which glutamate is formed by GDH, then aminotransferases use the glutamate-2-oxoglutarate system to generate several other amino acids from glutamate. In the silkworm, GOGAT maintains haemolymph glutamine concentrations (Hirayama *et al.*, 1997) and may have a similar role in other invertebrates. The GS-GOGAT pathway is the preferred route for assimilation of nitrogen in plants and microorganisms into glutamate, glutamine, asparagine and aspartate using the enzymes GS, GOGAT, GDH AspAT and AS (Miflin and Lea, 1976; Lam *et al.*, 1996; Miflin and Habash, 2002; Hodges *et al.*, 2003; Magasanik, 2003; Muro-Pastor and Florencio, 2003). The first four of these enzymes were established as present in *O. circumcincta*. AS is known to be present in *P. redivivus* (Grantham and Barrett, 1988) and *H. diminuta* (Zavras and Roberts, 1984) and may also be present in other nematodes.

GDH may become the main provider of the 2-oxoglutarate needed by GOGAT when there is low activity of isocitrate dehydrogenase. There is also increased GDH activity during carbohydrate starvation in plants (Dubois *et al.*, 2003). Providing 2-oxoglutarate may be a key role for GDH in *O. circumcincta*, as there is very little isocitrate dehydrogenase activity (D.C. Simcock, personal communication). In some plants, AspAT provides the 2-oxoglutarate (Hodges, 2002), but, unlike isocitrate dehydrogenase, does not result in net glutamate synthesis, however, the aspartate which is formed can be converted to asparagine or other amino acids. The enzymes involved in glutamate metabolism may have different roles in the various body organs. GDH activity

is high in the mosquito midgut but GS is low and there is no GOGAT activity, whereas in the fat body there is GOGAT, GS activity is greater and GDH is lower (Scaraffia *et al.*, 2005).

Aspartase is the second non-mammalian enzyme identified in *O. circumcincta*. It is not clear which would be the favoured direction of the reaction it catalyses *in vivo*. There was considerable aspartase activity and the K_m was favourable (10-fold lower) for competing for aspartate with AspAT, but 5-fold lower V_{max}. Alternatively, the reaction may favour aspartate formation from fumarate if the TCA cycle operates in the reverse anaerobic direction or there is only a partial cycle in the parasitic stages. The very high AspAT activity in L3 decreased by about 80% in adult worms, perhaps reflecting a shift towards more anaerobic metabolism. The TCA cycle in *O. circumcincta* is different from that in mammals, having low isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase activities and a functional glyoxylate cycle, but adult *O. circumcincta* metabolism appears to be more aerobic than in *Ascaris* (D.C. Simcock, personal communication). Aspartate may be needed as a substrate for AK to synthesis "essential amino acids" if Rothstein and Tomlinson (1961) and Kapur and Sood (1984) are correct in their interpretation that nematodes are able to synthesise these amino acids.

The third enzyme found in *O. circumcincta*, but not present in mammals is creatinase, which may be one of the sources of the excreted urea. The most obvious advantage of being able to catabolise creatine to urea and sarcosine would be as a source of nitrogen, as there may be an abundant supply of creatine in fluid exudates or intact or sloughed gut cells. The lack of urease activity in *O. circumcincta* would preclude generating ammonia through this enzyme, however, sarcosine can be degraded in mammals by sarcosine oxidase or dehydrogenase (Wittwer and Wagner, 1981; Reuber *et al.*, 1997) and nematodes may also have these enzymes. *H. contortus,* and probably also *O. circumcincta*, uses phosphoarginine rather than phosphocreatine as an energy buffer (Platzer *et al.*, 1995), which would allow creatinase to be present without the risk of degrading endogenous phosphagen.

As the three enzymes of interest in *O. circumcincta* are normally present in microorganisms, there is the possibility that the observed enzyme activity was caused by contaminating microbes, endosymbionts or residents of the

nematode gut. It was considered unlikely that there was sufficient external contaminating material after washing the parasites to account for quite large enzyme activities, however, this will be resolved by examining the type of RNA in the homogenates to distinguish eukaryote and prokaryote types. It is interesting to speculate whether the three nematode enzymes may have been acquired by horizontal gene transfer from bacteria in the soil or in the sheep gastrointestinal tract.

Endosymbionts are essential for some physiological processes in insects and the requirement of filarial nematodes for their *Wolbachia* endosymbionts is well established (Casiraghi *et al.*, 2001). Some insects, such as the pea aphid, use symbionts to recycle ammonia rather than excrete it (Sasaki and Ishikawa, 1995) and uric acid is recycled by yeast-like endosymbionts in the brown planthopper (Sasaki *et al.*, 1996) and by gut bacteria in termites (Potrikus and Breznak, 1981). Although urease is not usually present in animal tissues, the microorganisms in symbiotic relationships in the gut or tissues do usually contain the enzyme and the ammonia that they produce can be absorbed by the host (Whitehead *et al.*, 1992; Stevens and Hume, 1998). If *O. circumcincta* also has similar associated microorganisms contributing to its nutrition, these may be vulnerable to removal with deleterious effects on the nematode, as happens with tetracycline treatment of filaria (Hoerauf *et al.*, 1999; Casiraghi *et al.*, 2002).

The present experiments have shown that the nitrogen metabolism in *O. circumcincta* is not of the mammalian type but more like that in lower organisms. Nematodes may be very flexible in their nutrient requirements and like *S. cerevisiae* (ter Schure *et al.*, 2000) and cyanobacteria (Fisher, 1999) be able to use a variety of nitrogen sources, choosing the ones that best support growth and repressing other metabolic pathways. A range of forms of nitrogen, from ammonia to proteins, may be used by the free-living and adult stages of *O. circumcincta*. Although their environments appear to be very different, both larvae and adult worms are closely associated with microorganisms which inhabit the ruminant gut. These organisms may be very important in the nutrition of all stages, but additional sources of nutrients for the parasitic forms may include mucins, cells, leaked interstitial fluid or abomasal digesta.

The protein excreted by adult worms (Figure 2.10) is expected to contain the proteases identified in the ES products of L3, L4 and adult *O. circumcincta* by Young *et al.* (1995). These enzymes break down external protein to peptides and/or amino acids for which transporters have been identified in helminths (Isseroff *et al.*, 1976; Jeffs and Arme, 1985, 1987; Fei *et al.*, 1998; Meissner *et al.*, 2004) and amino acids are also taken in though the γ-glutamyl cycle (Dass and Donahue, 1986; Abidi and Nizami, 1995). The preliminary experiments reported in Chapter 2, in which there was evidence of transport of radiolabeled amino acids in adult *O. circumcincta*, indicates that similar amino acid permeases are likely to be present. Blocking the absorptive processes, which are present in the nematode cuticle and intestine (Weatherly *et al.*, 1963; Chen and Howells, 1979), could potentially starve the parasite.

Many individual enzymes involved in nitrogen metabolism have been identified in nematodes (Rogers, 1952; Rothstein and Mayoh, 1964a,b; Rasero *et al.*, 1968; Langer and Jiamperpoon, 1970; Paltridge and Janssens, 1971; Langer, 1972; Barrett, 1973; Rhodes and Ferguson, 1973; Hutchinson and Fernando, 1975; Barrett and Lloyd, 1981; Barrett, 1983; Watts and Atkins, 1983, 1984; Kawalek *et al.*, 1984; Dubinský *et al.*, 1985; Grantham and Barrett, 1986a,b, 1988; Jaffe and Lambert, 1986; Turner *et al.*, 1986; Wittich *et al.*, 1987; Walter, 1988; Sharma *et al.*, 1989; Wittich and Walter, 1989, 1990; Davies and Köhler, 1990; Schaeffer and Donatelli, 1990; Walker and Barrett, 1991a,b; Müller and Walter, 1992; Macrae *et al.*, 1995; Platzer *et al.*, 1995, 1999; von Besser *et al.*, 1995; Niemann *et al.*, 1996; Klein *et al.*, 1997; Liebau *et al.*, 1997; Walker and Barrett, 1997; Da'dara *et al.*, 1998; Lambert *et al.*, 1999; Skuce *et al.*, 1999b; Tsuji *et al.*, 1999; Veronico *et al.*, 2001; Jones *et al.*, 2003; Dufe *et al.*, 2005; Long *et al.*, 2006). There appears, however, to be insufficient information to create a model of metabolic regulation as has been done for *S. cerevisiae* (ter Schure *et al.*, 2000; Magasanik and Kaiser, 2002), *E. coli* (Reitzer, 2003) and higher plants (Lam *et al.*, 1996). ter Schure *et al.*, (2000) have searched for homologues in the *C. elegans* genome of *S. cerevisiae* genes involved in the control of glutamate metabolism, to assess whether yeast control systems were an appropriate model model for those in higher organisms. They found 35 worm sequences for homologues of *S. cerevisiae* genes encoding GS, GOGAT, the ammonium permeases MEP1,

MEP2 and MEP3, NAD-GDH, NADP-GDH, four transcription factors and a regulatory protein, but no homologues of the genes *GDH2* or *URE2*.

This study in *O. circumcincta* has revealed differences between nematode and mammalian nitrogen metabolism and three enzymes worthy of further examination as potential anthelmintic targets. Several other enzymes have been identified from reports in free-living and plant and animal parasitic nematodes as potentially also being in this category. In particular, nematodes have a very high requirement for collagen and chitin, their phosphagens are generally different from the mammalian phosphocreatine and polyamine metabolism appears to be different in some species. The shikimate pathway is currently only known in a few plant parasites, but appears not to have been investigated in animal parasites. If present in parasites of domestic ruminants, this pathway may be amenable to inhibition in both the free-living and parasitic stages. The successful exploitation of any of these enzymes will be dependent on their being essential to parasite survival.

References

Abidi, S. M. A., and Nizami, W. A. (1995). [^{3}H]-Amino acid uptake and metabolic studies on *Gigantocotyle explanatum* and *Gastrothylax crumenifer* (Digenea: Paramphistomidae). *International Journal for Parasitology* **25**, 541-549.

Acaster, M. A., and Weitzman, P. D. J. (1985). Kinetic analysis of glutamine synthetases from various plants. *FEBS Letters* **189**, 241-244.

Adam, R., Kaltmann, B., Rudin, W., Friedrich, T., Marti, T., and Lucius, R. (1996). Identification of chitinase as the immunodominant filarial antigen recognized by sera of vaccinated rodents. *Journal of Biological Chemistry* **271**, 1441-1447.

Adcock, H. J., Gaskin, P., Shaw, P., Teesdale-Spittle, P. H., and Buckberry, L. D. (1996). Novel sources of mammalian C-S lyase activity. *Journal of Pharmacy and Pharmacology* **48**, 150-153.

Addis, C. J., and Chandler, A. C. (1944). Studies on the vitamin requirement of tapeworms. *Journal of Parasitology* **30**, 229-236.

Adelsberger, H., Scheuer, T., and Dudel, J. (1997). A patch clamp study of a glutamatergic chloride channel on pharyngeal muscle of the nematode *Ascaris suum*. *Neuroscience Letters* **230**, 183-186.

Agrawal, A., Tripathi, L. M., Puri, S. K., and Pandey, V. C. (1996). Studies on ammonia-metabolizing enzymes during *Plasmodium yoelii* infection and pyrimethamine treatment in mice. *International Journal for Parasitology* **26**, 451-455.

Alagarsamy, S., Sorensen, S. D., and Conn, P. J. (2001). Coordinate regulation of metabotropic glutamate receptors. *Current Opinion in Neurobiology* **11**, 357-362.

Alekhova, T., Sof'in, A., Kobelkova, T., Marco, R., and Dournon, C. (2001). Sex-linked differences in activity of enzymes in the blood of the urodele amphibian *Pleurodeles waltl*. *Comparative Biochemistry and Physiology* **130A**, 819-825.

Alexander, F. W., Sandmeier, E., Mehta, P. K., and Christen, P. (1994). Evolutionary relationships among pyridoxal-5'-phosphate-dependent enzymes. Regio-specific α, β, and γ families. *European Journal of Biochemistry* **219**, 953-960.

Alfano, J. R., and Kahn, M. L. (1993). Isolation and characterization of a gene coding for a novel aspartate aminotransferase from *Rhizobium meliloti*. *Journal of Bacteriology* **175**, 4186-4196.

Alkema, M. J., Hunter-Ensor, M., Ringstad, N., and Horvitz, H. R. (2005). Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. *Neuron* **46**, 247-260.

Allaway, D., Lodwig, E. M., Crompton, L. A., Wood, M., Parsons, R., Wheeler, T. R., and Poole, P. S. (2000). Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. *Molecular Microbiology* **36**, 508-515.

Amaya, K. R., Kocherginskaya, S. A., Mackie, R. I., and Cann, I. K. O. (2005). Biochemical and mutational analysis of glutamine synthetase type III from the rumen anaerobe *Ruminococcus albus* 8. *Journal of Bacteriology* **187**, 7481-7491.

Anderson, C. M., and Swanson, R. A. (2000). Astrocyte glutamate transport: Review of properties, regulation, and physiological functions. *Glia* **32**, 1-14.

Anderson, M. P., Vance, C. P., Heichel, G. H., and Miller, S. S. (1989). Purification and characterization of NADH-glutamate synthase from alfalfa root nodules. *Plant Physiology* **90**, 351-358.

Anderson, P. M., Broderius, M. A., Fong, K. C., Tsui, K. N. T., Chew, S. F., and Ip, Y. K. (2002). Glutamine synthetase expression in liver, muscle, stomach and intestine of *Bostrichthys sinensis* in response to exposure to a high exogenous ammonia concentration. *Journal of Experimental Biology* **205**, 2053-2065.

Anderson, P. M., and Walsh, P. J. (1995). Subcellular-localization and biochemical properties of the enzymes of carbamoyl-phosphate and urea synthesis in the Batrachoidid fishes *Opsanus beta*, *Opsanus tau* and *Porichthys notatus*. *Journal of Experimental Biology* **198**, 755-766.

Andrews, S. J., Hole, N. J. K., Munn, E. A., and Rolph, T. P. (1995). Vaccination of sheep against haemonchosis with H11, a gut membrane-derived protective antigen from the adult parasite: prevention of the periparturient rise and colostral transfer of protective immunity. *International Journal for Parasitology* **25**, 839-846.

Antonyuk, L. P., Smirnova, V. E., Kamnev, A. A., Serebrennikova, O. B., Vanoni, M. A., Zanetti, G., Kudelina, I. A., Sokolov, O. I., and Ignatov, V. V. (2001). Influence of divalent cations on the catalytic properties and secondary structure of unadenylylated glutamine synthetase from *Azospirillum brasilense*. *BioMetals* **14**, 13-22.

Armour, J., Jarrett, W. F. H., and Jennings, F. W. (1966). Experimental *Ostertagia circumcincta* infections in sheep: development and pathogenesis of a single infection. *American Journal of Veterinary Research* **27**, 1267-1278.

Asch, H. L., and Read, C. P. (1975). Membrane transport in *Schistosoma mansoni*: transport of amino acids by adult males. *Experimental Parasitology* **38**, 123-135.

Ash, D. E. (2004). Structure and function of arginases. *Journal of Nutrition* **134**, 2760S-2764S.

Astrin, K. H., Arredondo-Vega, F. X., Desnick, R. J., and Smith, M. (1982). Assignment of the gene for cytosolic alanine aminotransferase (AAT1) to human chromosome 8. *Annals of Human Genetics* **46**, 125-133.

Audia, J. P., Webb, C. C., and Foster, J. W. (2001). Breaking the acid barrier: An orchestrated response to proton stress by enteric bacteria. *International Journal of Medical Microbiology* **291**, 97-106.

Auerswald, L., and Gäde, G. (1999). The fate of proline in the African fruit beetle *Pachnoda sinuata*. *Insect Biochemistry and Molecular Biology* **29**, 687-700.

Auerswald, L., Schneider, P., and Gäde, G. (1998). Proline powers pre-flight warm-up in the african fruit beetle *Pachnoda sinuata* (Cetoniinae). *Journal of Experimental Biology* **201**, 1651-1657.

Avery, L. (1993). Motor neuron M3 controls pharyngeal muscle relaxation timing in *Caenorhabditis elegans*. *Journal of Experimental Biology* **175**, 283-297.

Azevedo, R. A., Arruda, P., Turner, W. L., and Lea, P. J. (1997). The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. *Phytochemistry* **46**, 395-419.

Bacchi, C. J., Nathan, H. C., Yarlett, N., Goldberg, B., McCann, P. P., Sjoerdsma, A., Saric, M., and Clarkson, A. B. (1994). Combination chemotherapy of drug-resistant *Trypanosoma brucei rhodesiense* infections in mice using DL-α-difluoromethylornithine and standard trypanocides. *Antimicrobial Agents and Chemotherapy* **38**, 563-569.

Badet, B., and Walsh, C. (1985). Purification of an alanine racemase from *Streptococcus faecalis* and analysis of its inactivation by (1-aminoethyl)phosphonic acid enantiomers. *Biochemistry* **24**, 1333-1341.

Ballou, J.-M., Grzych, J.-M., Pierce, R. M., and Capron, A. (1987). A purified 28,000 dalton protein from *Schistosoma mansoni* adult worms protects rats and mice against experimental schistosomiasis. *Journal of Immunology* **138**, 3448-3453.

Barbosa-Tessmann, I. P., Chen, C., Zhong, C., Schuster, S. M., Nick, H. S., and Kilberg, M. S. (1999). Activation of the unfolded protein response pathway induces human asparagine synthetase gene expression. *Journal of Biological Chemistry* **274**, 31139-31144.

Barimo, J. F., Steele, S. L., Wright, P. A., and Walsh, P. J. (2004). Dogmas and controversies in the handling of nitrogenous wastes: Ureotely and ammonia tolerance in early life stages of the gulf toadfish, *Opsanus beta*. *Journal of Experimental Biology* **207**, 2011-2020.

Barrett, J. (1973). Nucleoside triphosphate metabolism in the muscle tissue of *Ascaris lumbricoides* (Nematoda). *International Journal for Parasitology* **3**, 393-400.

Barrett, J. (1983). The biochemistry of filarial worms. *Helminthological Abstracts* **52A**, 1-18.

Barrett, J., and Beis, I. (1973). Studies on glycolysis in the muscle tissue of *Ascaris lumbricoides* (Nematoda). *Comparative Biochemistry and Physiology* **44B**, 751-761.

Barrett, J., and Butterworth, P. E. (1981). Novel amino acid linked dehydrogenase in the sponge *Halichondria panicea* (Pallas). *Comparative Biochemistry and Physiology* **70B**, 141-146.

Barrett, J., and LLoyd, G. M. (1981). A novel phosphotransferase in the plerocercoids of *Shistocephalus solidus* (Cestoda: pseudophyllidea). *Parasitology* **82**, 11-16.

Barrett, J., Ward, C. W., and Fairbairn, D. (1970). The glyoxylate cycle and the conversion of triglycerides to carbohydrates in developing eggs of *Ascaris lumbricoides*. *Comparative Biochemistry and Physiology* **35**, 577-586.

Bascal, Z. A., Cunningham, J. M., Holden-Dye, L., O'Shea, M. G., and Walker, R. J. (2001). Characterization of a putative nitric oxide synthase in the neuromuscular system of the parasitic nematode, *Ascaris suum*. *Parasitology* **122**, 219-231.

Basch, J. J., Wickham, E. D., and Farrell, H. M. (1997). Arginase in lactating bovine mammary glands: Implications in proline synthesis. *Journal of Dairy Science* **80**, 3241-3248.

Batrel, Y., and Le Gal, Y. (1984). Nitrogen metabolism in *Arenicola marina* characterization of a NAD dependent glutamate dehydrogenase. *Comparative Biochemistry and Physiology* **78B**, 119-124.

Bearson, S., Bearson, B., and Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiology Letters* **147**, 173-180.

Beg, M. A., Fisten, J. L., Ingram, G. A., and Storey, D. M. (1995). Activities of glycogen phophorylase, alanine aminotransferase and aspartate aminotransferase in adult worms of *Litomosoides carinii* recovered from pyridoxine deficient cotton rats (*Sigmodon hispidus*). *Parasitology* **112**, 227-232.

Bertland, L. H., and Kaplan, N. O. (1968). Chicken heart soluble aspartate aminotransferase. Purification and properties. *Biochemistry* **7**, 134-142.

Bertland, L. H., and Kaplan, N. O. (1970). Studies on the conformations of mutiple forms of chicken heart soluble aspartate aminotransferase. *Biochemistry* **9**, 2653-2665.

Bhargava, K. K., Le Trang, N., Cerami, A., and Eaton, J. W. (1983). Effect of arsenical drugs on glutathione metabolism of *Litomosoides carinii*. *Molecular and Biochemical Parasitology* **9**, 29-35.

Bian, K., Harari, Y., Zhong, M., Lai, M., Castro, G., Weisbrodt, N., and Murad, F. (2001). Down-regulation of inducible nitric-oxide synthase (NOS-2) during parasite-induced gut inflammation: a path to identify a selective NOS-2 inhibitor. *Molecular Pharmacology* **59**, 939-947.

Bidigare, R. R., and King, F. D. (1981). The measurement of glutamate dehydrogenase activity in *Praunus flexuosus* and its role in the regulation of ammonium excretion. *Comparative Biochemistry and Physiology* **70B**, 409-413.

Binder, M., Mahler, V., Hayek, B., Sperr, W. R., Schöller, M., Prozell, S., Wiedermann, G., Valent, P., Valenta, R., and Duchêne, M. (2001). Molecular and immunological characterization of arginine kinase from the Indianmeal Moth, *Plodia interpunctella*, a novel cross-reactive invertebrate pan-allergen. *Journal of Immunology* **167**, 5470-5477.

Bird, A. F., and Bird, J. (1991). "The structure of nematodes.," 2nd Ed. Academic Press, San Diego.

Bird, M. I., and Nunn, P. B. (1983). Metabolic homoeostasis of L-threonine in the normally-fed rat. Importance of liver threonine dehydrogenase activity. *Biochemical Journal* **214**, 687-694.

Bird, M. I., Nunn, P. B., and Lord, L. A. J. (1984). Formation of glycine and aminoacetone from L-threonine by rat liver mitochondria. *Biochimica et Biophysica Acta* **802**, 229-236.

Bishop, J. S., and Burton, R. S. (1993). Amino acid synthesis during hyperosmotic stress in *Penaeus aztecus* postlarvae. *Comparative Biochemistry and Physiology* **106A**, 49-56.

Bishop, S. H., and Barnes, L. B. (1971). Ammonia forming mechanisms: deamination of 5'-adenylic acid (AMP) by some polychaete annelids. *Comparative Biochemistry and Physiology* **40B**, 407-422.

Bishop, S. H., Klotz, A., Drolet, L. L., Smullin, D. H., and Hoffman, R. J. (1978). NADP-Specific glutamate dehydrogenase in *Metridium senile* (L.). *Comparative Biochemistry and Physiology* **61B**, 185-187.

Blackhall, W. J., Prichard, R. K., and Beech, R. N. (2003). Selection at a γ-aminobutyric acid receptor gene in *Haemonchus contortus* resistant to avermectins/milbemycins. *Molecular and Biochemical Parasitology* **131**, 137-145.

Blanco, F., Alana, A., Llama, M. J., and Serra, J. L. (1989). Purification and properties of glutamine synthetase from the non-N_2-fixing cyanobacterium *Phormidium laminosum*. *Journal of Bacteriology* **171**, 1158-1165.

Blenau, W., and Baumann, A. (2001). Molecular and pharmacological properties of insect biogenic amine receptors: Lessons from *Drosophila melanogaster* and *Apis mellifera*. *Archives of Insect Biochemistry and Physiology* **48**, 13-38.

Bodnaryk, R. P., Bronskill, J. F., and Fetterly, J. R. (1974). Membrane-bound γ-glutamyl transpeptidase and its rôle in phenylalanine absorption-reabsorption in the larva of *Musca domestica*. *Journal of Insect Physiology* **20**, 167-171.

Boelens, P. G., van Leeuwen, P. A. M., Dejong, C. H. C., and Deutz, N. E. P. (2005). Intestinal renal metabolism of L-citrulline and L-arginine following enteral or parenteral infusion of L-alanyl-L-[2,^{15}N]glutamine or L-[2,^{15}N]glutamine in mice. *American Journal of Physiology* **289**, G679-G685.

Boldyrev, A. A., and Severin, S. E. (1990). The histidine-containing dipeptides, carnosine and anserine: Distribution, properties and biological significance. *Advances in Enzyme Regulation* **30**, 175-194.

Bond, P. A., and Sang, J. H. (1968). Glutamate dehydrogenase of *Drosophila* larvae. *Journal of Insect Physiology* **14**, 341-359.

Bonete, M.-J., Pérez-Pomares, F., Díaz, S., Ferrer, J., and Oren, A. (2003). Occurrence of two different glutamate dehydrogenase activities in the halophilic bacterium *Salinibacter ruber*. *FEMS Microbiology Letters* **226**, 181-186.

Borkovich, K., and Weiss, R. (1987). Purification and characterization of arginase from *Neurospora crassa*. *Journal of Biological Chemistry* **262**, 7081-7086.

Borza, T., Iancu, C. V., Pike, E., Honzatko, R. B., and Fromm, H. J. (2003). Variations in the response of mouse isozymes of adenylosuccinate synthetase to inhibitors of physiological relevance. *Journal of Biological Chemistry* **278**, 6673-6679.

Boucher, J. L., Moali, C., and Tenu, J. P. (1999). Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. *Cellular and Molecular Life Sciences* **55**, 1015-1028.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brophy, P. M., Ben-Smith, A., Brown, A., Behnke, J. M., and Pritchard, D. I. (1994). Glutathione *S*-transferases from the gastrointestinal nematode *Heligmosomoides polygyrus* and mammalian liver compared. *Comparative Biochemistry and Physiology* **109B**, 585-592.

Brophy, P. M., Southan, C., and Barrett, J. (1989). Glutathione transferases in the tapeworm *Moniezia expansa*. *Biochemical Journal* **262**, 939-946.

Brosnan, J. T. (2000). Glutamate, at the interface between amino acid and carbohydrate metabolism. *Journal of Nutrition* **130**, 988S-990S.

Brosnan, J. T. (2003). Interorgan amino acid transport and its regulation. *Journal of Nutrition* **133**, 2068S-2072S.

Brosnan, J. T., Brosnan, M. E., Yudkoff, M., Nissim, I., Daikhin, Y., Lazarow, A., Horyn, O., and Nissim, I. (2001). Alanine metabolism in the perfused rat liver. Studies with ^{15}N. *Journal of Biological Chemistry* **276**, 31876-31882.

Brownlee, D. J. A., and Walker, R. J. (1999). Actions of nematode FMRFamide-related peptides on the pharyngeal muscle of the parasitic nematode, *Ascaris suum*. *Annals of the New York Academy of Sciences* **897**, 228-238.

Bruce, J. I., Ruff, M. D., Belusko, R. J., and Werner, J. K. (1972). *Schistosoma mansoni* and *Schistosoma japonicum*: Utilization of amino acids. *International Journal for Parasitology* **2**, 425-430.

Brun, A., Chalot, M., Botton, B., and Martin, F. (1992). Purification and characterization of glutamine synthetase and NADP-glutamate dehydrogenase from the ectomycorrhizal fungus *Laccaria laccata*. *Plant Physiology* **99**, 938-944.

Buckner, J. S., Caldwell, J. M., and Reinecke, J. P. (1980). Uric acid excretion in larval *Manduca sexta*. *Journal of Insect Physiology* **26**, 7-12.

Buechner, M. (2002). Tubes and the single *C. elegans* excretory cell. *Trends in Cell Biology* **12**, 479-484.

Buechner, M., Hall, D. H., Bhatt, H., and Hedgecock, E. M. (1999). Cystic canal mutants in *Caenorhabditis elegans* are defective in the apical membrane domain of the renal (excretory) cell. *Developmental Biology* **214**, 227-241.

Burton, R. S. (1992). Proline synthesis during osmotic stress in megalopa stage larvae of the blue crab, *Callinectes sapidus*. *Biological Bulletin* **182**, 409-415.

Bussolati, O., Belletti, S., Uggeri, J., Gatti, R., Orlandini, G., Dall'Asta, V., and Gazzola, G. C. (1995). Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells. *Experimental Cell Research* **220**, 283-291.

Caballero, F. J., Cárdenas, J., and Castillo, F. (1989). Purification and properties of L-alanine dehydrogenase of the phototrophic bacterium *Rhodobacter capsulatus* E1F1. *Journal of Bacteriology* **171**, 3205-3210.

Caldovic, L., and Tuchman, M. (2003). N-acetylglutamate and its changing role through evolution. *Biochemical Journal* **372**, 279-290.

Campbell, A. M., Teesdale-Spittle, P. H., Barrett, J., Liebau, E., Jefferies, J. R., and Brophy, P. M. (2001). A common class of nematode glutathione *S*-transferase (GST) revealed by the theoretical proteome of the model organism *Caenorhabditis elegans*. *Comparative Biochemistry and Physiology* **128B**, 701-708.

Campbell, J. W. (1963). Urea formation and urea cycle enzymes in the cestode, *Hymenolepsis diminuta*. *Comparative Biochemistry and Physiology* **8**, 13-27.

Campbell, J. W., and Lee, T. W. (1963). Ornithine transcarbamylase and arginase activity in flatworms. *Comparative Biochemistry and Physiology* **8**, 29-38.

Campbell, L. L. (1957). Reductive degradation of pyrimidines II. Mechanism of uracil degradation by *Clostridium uracilicum*. *Journal of Bacteriology* **73**, 225-229.

Campbell, S. A., Richards, T. A., Mui, E. J., Samuel, B. U., Coggins, J. R., McLeod, R., and Roberts, C. W. (2004). A complete shikimate pathway in *Toxoplasma gondii*: an ancient eukaryotic innovation. *International Journal for Parasitology* **34**, 5-13.

Carvajal, N., Bustamante, M., Hinrichsen, P., and Torres, A. (1984). Properties of arginase from the sea mollusc *Concholepas concholepas*. *Comparative Biochemistry and Physiology* **78B**, 591-594.

Carvajal, N., Kessi, E., and Ainol, L. (1987). Subcellular localization and kinetic properties of arginase from the liver of *Genypterus maculatus*. *Comparative Biochemistry and Physiology* **88B**, 229-231.

Carvajal, N., Olave, N., Salas, M., Uribe, E., and Enriquez, S. (1996). Properties of an arginase from the cotyledons of *Phaseolus vulgaris*. *Phytochemistry* **41**, 373-376.

Carvajal, N., Uribe, E., and Torres, C. (1994). Subcellular localization, metal ion requirement and kinetic properties of arginase from the gill tissue of the bivalve *Semele solida*. *Comparative Biochemistry and Physiology* **109B**, 683-689.

Cary, S. P. L., Winger, J. A., Derbyshire, E. R., and Marletta, M. A. (2006). Nitric oxide signaling: no longer simply on or off. *Trends in Biochemical Sciences* **31**, 231-239.

Casartelli, M., Corti, P., Giovanna Leonardi, M., Fiandra, L., Burlini, N., Pennacchio, F., and Giordana, B. (2005). Absorption of albumin by the midgut of a lepidopteran larva. *Journal of Insect Physiology* **51**, 933-940.

Casiraghi, M., Anderson, T. J., Bandi, C., Bazzocchi, C., and Genchi, C. (2001). A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of Wolbachia endosymbionts. *Parasitology* **122**, 93-103.

Casiraghi, M., McCall, J. W., Simoncini, L., Kramer, L. H., Sacchi, L., Genchi, C., Werren, J. H., and Bandi, C. (2002). Tetracycline treatment and sex-ratio distortion: a role for Wolbachia in the moulting of filarial nematodes? *International Journal for Parasitology* **32**, 1457-1468.

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V., Harvey, W., and Hediger, M. (1997). Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis. *Journal of Experimental Biology* **200**, 269-286.

Cazzulo, J. J., de Cazzulo, B. M. F., Higa, A. I., and Segura, E. L. (1979). NAD-linked glutamate dehydrogenase in *Trypanosoma* cruzi. *Comparative Biochemistry and Physiology* **64B**, 129-131.

Cazzulo, J. J., Juan, S. M., and Segura, E. L. (1977). Glutamate dehydrogenase and aspartate aminotransferase in *Trypanosoma cruzi*. *Comparative Biochemistry and Physiology* **56B**, 301-303.

Cederbaum, S. D., Yu, H., Grody, W. W., Kern, R. M., Yoo, P., and Iyer, R. K. (2004). Arginases I and II: do their functions overlap? *Molecular Genetics and Metabolism* **81**, S38-S44.

Chadwick, T., and Wright, P. (1999). Nitrogen excretion and expression of urea cycle enzymes in the atlantic cod (*Gadus morhua* L.): a comparison of early life stages with adults. *Journal of Experimental Biology* **202**, 2653-2662.

Chandrashekar, R., and Mehta, K. (2000). Transglutaminase-catalyzed reactions in the growth, maturation and development of parasitic nematodes. *Parasitology Today* **16**, 11-17.

Chang, M. C., Chang, C. C., and Chang, J. C. (1992). Cloning of a creatinase gene from *Pseudomonas putida* in *Escherichia coli* by using an indicator plate. *Applied and Environmental Microbiology* **58**, 3437-3440.

Chappell, L. (1974). Methionine uptake by larval and adult *Schistosoma mansoni*. *International Journal for Parasitology* **4**, 361-369.

Chen, S. N., and Howells, R. E. (1979). The uptake *in vitro* of dyes, monosaccharides and amino acids by the filarial worm *Brugia pahangi*. *Parasitology* **78**, 343-354.

Chew, S. F., Ong, T. F., Ho, L., Tam, W. L., Loong, A. M., Hiong, K. C., Wong, W. P., and Ip, Y. K. (2003a). Urea synthesis in the African lungfish *Protopterus dolloi* - hepatic carbamoyl phosphate synthetase III and glutamine synthetase are upregulated by 6 days of aerial exposure. *Journal of Experimental Biology* **206**, 3615-3624.

Chew, S. F., Wong, M. Y., Tam, W. L., and Ip, Y. K. (2003b). The snakehead *Channa asiatica* accumulates alanine during aerial exposure, but is incapable of sustaining locomotory activities on land through partial amino acid catabolism. *Journal of Experimental Biology* **206**, 693-704.

Chmara, H., Andruszkiewicz, R., and Borowski, E. (1986). Inactivation of glucosamine-6-phosphate synthetase from *Salmonella typhimurium* LT2 by fumaroyl diaminopropanoic acid derivatives, a novel group of glutamine analogs. *Biochimica et Biophysica Acta* **870**, 357-366.

Chopra, S., Pai, H., and Ranganathan, A. (2002). Expression, purification, and biochemical characterization of *Mycobacterium tuberculosis* aspartate decarboxylase, PanD. *Protein Expression and Purification* **25**, 533-540.

Christensen, H. N. (1990). Role of amino acid transport and countertransport in nutrition and metabolism. *Physiological Reviews* **70**, 43-77.

Ciardiello, M. A., Camardella, L., Carratore, V., and di Prisco, G. (1997). Enzymes in Antarctic fish: Glucose-6-phosphate dehydrogenase and glutamate dehydrogenase,. *Comparative Biochemistry and Physiology* **118A**, 1031-1036.

Ciustea, M., Gutierrez, J. A., Abbatiello, S. E., Eyler, J. R., and Richards, N. G. J. (2005). Efficient expression, purification, and characterization of C-terminally tagged, recombinant human asparagine synthetase. *Archives of Biochemistry and Biophysics* **440**, 18-27.

Clark, A. G. (1989). The comparative enzymology of the glutathione *S*-transferases from non-vertebrate organisms. *Comparative Biochemistry and Physiology* **92B**, 419-446.

Cochran, D. G. (1981). Comparative excreta analysis on various neotropical cockroaches and a leaf mantid. *Comparative Biochemistry and Physiology* **70A**, 205-209.

Cogoni, C., Valenzuela, L., González-Halphen, D., Olivera, H., Macino, G., Ballario, P., and González, A. (1995). *Saccharomyces cerevisiae* has a single glutamate synthase gene coding for a plant-like high-molecular-weight polypeptide. *Journal of Bacteriology* **177**, 792-798.

Colasanti, M., and Venturini, G. (1998). Nitric oxide in invertebrates. *Molecular Neurobiology* **17**, 157-174.

Coll, M., Knof, S. H., Ohga, Y., Messerschmidt, A., Huber, R., Moellering, H., Russmann, L., and Schumacher, G. (1990). Enzymatic mechanism of creatine amidinohydrolase as deduced from crystal structures. *Journal of Molecular Biology* **214**, 597-610.

Colleluori, D. M., Morris, S. M., and Ash, D. E. (2001). Expression, purification, and characterization of human type II arginase. *Archives of Biochemistry and Biophysics* **389**, 135-143.

Cook, A., Aptel, N., Portillo, V., Siney, E., Sihota, R., Holden-Dye, L., and Wolstenholme, A. (2006). *Caenorhabditis elegans* ivermectin receptors regulate locomotor behaviour and are functional orthologues of *Haemonchus contortus* receptors. *Molecular and Biochemical Parasitology* **147**, 118-125.

Coombs, G. H., and Mottram, J. C. (1997). Parasite proteinases and amino acid metabolism: possibilities for chemotherapeutic exploitation. *Parasitology* **114**, S61-S80.

Cooper, A. J. L. (1977). Asparagine transaminase from rat liver. *Journal of Biological Chemistry* **252**, 2032-2038.

Cooper, A. L., Bruschi, S. A., Iriarte, A., and Carrion, M. M. (2002). Mitochondrial aspartate aminotransferase catalyses cysteine *S*-conjugate β-lyase reactions. *Biochemical Journal* **368**, 253-261.

Corrigan, J. J. (1969). D-amino acids in animals. *Science* **164**, 142-149.

Cotter, P. D., and Hill, C. (2003). Surviving the acid test: Responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews* **67**, 429-453.

Couriaud, C., Leroy, C., Simon, M., Silberstein, C., Bailly, P., Ripoche, P., and Rousselet, G. (1999). Molecular and functional characterization of an amphibian urea transporter. *Biochimica et Biophysica Acta* **1421**, 347-352.

Cox, G. N., Kusch, M., and Edgar, R. S. (1981). Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. *Journal of Cell Biology* **90**, 7-17.

Cox, G. N., Pratt, D., Hageman, R., and Boisvenue, R. J. (1990). Molecular cloning and primary sequence of a cysteine protease expressed by *Haemonchus contortus* adult worms. *Molecular and Biochemical Parasitology* **41**, 25-34.

Crow, V. L. (1987). Properties of alanine dehydrogenase and aspartase from *Propionibacterium freudenreichii* subsp. *shermanii*. *Applied and Environmental Microbiology* **53**, 1885-1892.

Curthoys, N. P. (2001). Role of mitochondrial glutaminase in rat renal glutamine metabolism. *Journal of Nutrition* **131**, 2491S-2495S.

Curthoys, N. P., Kuhlenschmidt, T., and Godfrey, S. S. (1976). Regulation of renal ammoniagenesis: Purification and characterization of phosphate-dependent glutaminase from rat kidney. *Archives of Biochemistry and Biophysics* **174**, 82-89.

Curthoys, N. P., Kuhlenschmidt, T., Godfrey, S. S., and Weiss, R. F. (1976). Phosphate-dependent glutaminase from rat kidney. Cause of increased activity in response to acidosis and identity with glutaminase from other tissues. *Archives of Biochemistry and Biophysics* **172**, 162-167.

Curthoys, N. P., and Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. *Annual Review of Nutrition* **15**, 133-159.

Cutinelli, L., Pietropaolo, C., Venuta, S., Zappia, V., and Salvatore, F. (1972). Studies on the identification and characterization of an aspartase activity in liver of elasmobranch fishes. *Comparative Biochemistry and Physiology* **41B**, 905-919.

Da Costa, A. V., Gaubert, S., Lafitte, S., Fontaine, J., Capron, A., and Gryzych, J.-M. (1999). Egg-hatching inhibition in mice immunized with recombinant *Schistosoma bovis* 28 kDa glutathione *S*-transferase. *Parasite Immunology* **21**, 341-350.

Dabir, S., Dabir, P., and Somvanshi, B. (2005). Purification, properties and alternate substrate specificities of arginase from two different sources: *Vigna catjang* cotyledon and buffalo liver. *International Journal of Biological Sciences* **1**, 114-122.

Da'dara, A. A., Mett, H., and Walter, R. D. (1998). MGBG analogues as potent inhibitors of *S*-adenosylmethionine decarboxylase of *Onchocerca volvulus*. *Molecular and Biochemical Parasitology* **97**, 13-19.

D'Aniello, A., Vetere, A., and Petrucelli, L. (1993). Further study on the specificity of D-amino acid oxidase and of D-aspartate oxidase and time course for complete oxidation of D-amino acids. *Comparative Biochemistry and Physiology* **105B**, 731-734.

Dass, P. D., and Donahue, M. J. (1986). γ-Glutamyl transpeptidase activity in *Ascaris suum*. *Molecular and Biochemical Parasitology* **20**, 233-236.

Davies, K. P., and Köhler, P. (1990). The role of amino acids in the energy generating pathways of *Litomosoides carinii*. *Molecular and Biochemical Parasitology* **41**, 115-124.

De Cian, M.-C., Regnault, M., and Lallier, F. (2000). Nitrogen metabolites and related enzymatic activities in the body fluids and tissues of the hydrothermal vent tubeworm *Riftia pachyptila*. *Journal of Experimental Biology* **203**, 2907-2920.

de Sousa, C. A. F., and Sodek, L. (2003a). Alanine metabolism and alanine aminotransferase activity in soybean (*Glycine max*) during hypoxia of the root system and subsequent return to normoxia. . *Environmental and Experimental Botany* **50**, 1-8.

de Sousa, C. A. F., and Sodek, L. (2003b). The metabolic response of plants to oxygen deficiency. *Brazilian Journal of Plant Physiology* **14**, 83-94.

de Zwaan, A., and Putzer, V. (1985). Metabolic adaptations of intertidal invertebrates to environmental hypoxia (a comparison of environmental anoxia to exercise anoxia). *In* "Symposia of the Society of Experimental Biology Physiological Adaptations of Marine Animals" (M. S. Laverack, ed.), Vol. 39, pp. 33-62. University of Cambridge.

de Zwaan, A., and Wijsman, T. C. M. (1976). Anaerobic metabolism in bivalvia (Mollusca) Characteristics of anaerobic metabolism. *Comparative Biochemistry and Physiology* **54B**, 313-323.

Depue, R. H., and Moat, A. G. (1961). Factors affecting aspartase activity. *Journal of Bacteriology* **82**, 383-386.

DeRosa, G., and Swick, R. W. (1975). Metabolic implications of the distribution of the alanine aminotransferase isoenzymes. *Journal of Biological Chemistry* **250**, 7961-7967.

Dessaux, Y., Petit, A., and Tempe, J. (1993). Chemistry and biochemistry of opines, chemical mediators of parasitism. *Phytochemistry* **34**, 31-38.

Doverskog, M., Jacobsson, U., Chapman, B. E., Kuchel, P. W., and Häggström, L. (2000). Determination of NADH-dependent glutamate synthase (GOGAT) in *Spodoptera frugiperda* (Sf9) insect cells by a selective $^{1}H/^{15}N$ NMR in vitro assay. *Journal of Biotechnology* **79**, 87-97.

Dovey, H. F., McKerrow, J. H., and Wang, C. C. (1984). Purine salvage in *Schistosoma mansoni* schistosomules. *Molecular and Biochemical Parasitology* **11**, 157-167.

Dowton, M., and Kennedy, I. R. (1986). Glutamine metabolism in fleshfly (*Parasarcophaga crassipalpis*) tissues. *Comparative Biochemistry and Physiology* **85B**, 593-600.

Drews, M., Doverskog, M., Ohman, L., Chapman, B. E., Jacobsson, U., Kuchel, P. W., and Haggstrom, L. (2000). Pathways of glutamine metabolism in *Spodoptera frugiperda* (Sf9) insect cells: evidence for the presence of the nitrogen assimilation system, and a metabolic switch by $^{1}H/^{15}N$ NMR. *Journal of Biotechnology* **78**, 23-37.

Dubinský, P., Ryboš, M., and Turceková, L. (1985). Enzymes regulating glucosamine 6-phosphate synthesis in the zygote of *Ascaris suum*. *International Journal for Parasitology* **15**, 415-419.

Dubois, F., Tercé-Laforgue, T., Gonzalez-Moro, M.-B., Estavillo, J.-M., Sangwan, R., Gallais, A., and Hirel, B. (2003). Glutamate dehydrogenase in plants: is there a new story for an old enzyme? *Plant Physiology and Biochemistry* **41**, 565-576.

Dufe, V. T., Lüersen, K., Eschbach, M.-L., Haider, N., Karlberg, T., Walter, R. D., and Al-Karadaghi, S. (2005). Cloning, expression, characterisation and three-dimensional structure determination of *Caenorhabditis elegans* spermidine synthase. *FEBS Letters* **579**, 6037-6043.

Dumas, C., and Camonis, J. (1993). Cloning and sequence analysis of the cDNA for arginine kinase of lobster muscle. *Journal of Biological Chemistry* **268**, 21599-21605.

Duncan, P. A., White, B. A., and Mackie, R. I. (1992). Purification and properties of NADP-dependent glutamate dehydrogenase from *Ruminococcus flavefaciens* FD-1. *Applied and Environmental Microbiology* **58**, 4032-4037.

Dunn, M. A., Maragoudakis, M. E., and Hait, P. K. (1978). Liver collagen hydroxylation in murine schistosomiasis. *Biochimica et Biophysica Acta* **538**, 328-333.

Dunn, M. A., Rojkind, M., Warren, K. S., Hait, P. K., Rifas, L., and Seifter, S. (1977). Liver collagen synthesis in murine schistosomiasis. *Journal Of Clinical Investigation* **59**, 666-674.

Dunn, M. F. (1998). Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS Microbiology Reviews* **22**, 105-123.

Durá, M. A., Flores, M., and Toldra, F. (2002). Purification and characterisation of a glutaminase from *Debaryomyces* spp. *International Journal of Food Microbiology* **76**, 117-126.

Edwards, M. R., Gilroy, F. V., Jimenez, B. M., and O'Sullivan, W. J. (1989). Alanine is a major end product of metabolism by *Giardia lamblia*: a proton nucelar magnetic resonance study. *Molecular and Biochemical Parasitology* **37**, 19-26.

Edwards, M. R., Schofield, P. J., O'Sullivan, W. J., and Costello, M. (1992). Arginine metabolism during culture of *Giardia intestinalis*. *Molecular and Biochemical Parasitology* **53**, 97-103.

Eisenberg, D., Gill, H. S., Pfluegl, G. M., and Rotstein, S. H. (2000). Structure-function relationships of glutamine synthetases. *Biochimica Biophysica Acta* **1477**, 122-145.

el Kouni, M. H. (2003). Potential chemotherapeutic targets in the purine metabolism of parasites. *Pharmacology and Therapeutics* **99**, 283-309.

Ellington, W. R. (1979). Octopine dehydrogenase in the basilar muscle of the sea anemone, *Metridium senile*. *Comparative Biochemistry and Physiology* **63B**, 349-354.

El-Shora, H. M., and Abo-Kassem, E. M. (2001). Kinetic characterization of glutamate dehydrogenase of marrow cotyledons. *Plant Science* **161**, 1047-1057.

Ennor, A. H., and Morrison, J. F. (1958). Biochemistry of the phosphagens and related guanidines. *Physiological Reviews* **38**, 631-674.

Ertel, J., and Isseroff, H. (1974). Proline in fascioliasis: 1. Comparative activities of ornithine-δ-transaminase and proline oxidase in *Fasciola* and in mammalian livers. *Journal of Parasitology* **60**, 574-577.

Essex-Fraser, P. A., Steele, S. L., Bernier, N. J., Murray, B. W., Stevens, E. D., and Wright, P. A. (2005). Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. *Journal of Biological Chemistry* **280**, 20268-20273.

Evans, W. R., and Axelrod, B. (1961). Pyrimidine metabolism in germinating seedlings *Plant Physiol.* **36**, 9-13.

Fahien, L., Kmiotek, E., Woldegiorgis, G., Evenson, M., Shrago, E., and Marshall, M. (1985). Regulation of aminotransferase-glutamate dehydrogenase interactions by carbamyl phosphate synthase-I, Mg^{2+} plus leucine *versus* citrate and malate. *Journal of Biological Chemistry* **260**, 6069-6079.

Fahien, L., MacDonald, M., Teller, J., Fibich, B., and Fahien, C. (1989). Kinetic advantages of hetero-enzyme complexes with glutamate dehydrogenase and the α-ketoglutarate dehydrogenase complex. *Journal of Biological Chemistry* **264**, 12303-12312.

Fahien, L. A., and Smith, S. E. (1974). The enzyme-enzyme complex of transaminase and glutamate dehydrogenase. *Journal of Biological Chemistry* **249**, 2696-2703.

Fei, Y.-J., Fujita, T., Lapp, D. F., Ganapathy, V., and Leibach, F. H. (1998). Two oligopeptide transporters from *Caenorhabditis elegans*: molecular cloning and functional expression. *Biochemical Journal* **332**, 565-572.

Felbeck, H., and Grieshaber, M. K. (1980). Investigations on some enzymes involved in the anaerobic metabolism of amino acids of *Arenicola marina* L. *Comparative Biochemistry and Physiology* **66B**, 205-213.

Fellows, F. C. I., and Hird, F. J. R. (1979). Nitrogen metabolism and excretion in the freshwater crayfish *Cherax destructor*. *Comparative Biochemistry and Physiology* **64B**, 235-238.

Feng, X.-P., Hayashi, J., Beech, R. N., and Prichard, R. K. (2002a). Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. *Journal of Neurochemistry* **83**, 870-878.

Feng, Z., Caceres, N. E., Sarath, G., and Barletta, R. G. (2002b). *Mycobacterium smegmatis* L-alanine dehydrogenase (ald) is required for proficient utilization of alanine as a sole nitrogen source and sustained anaerobic growth. *Journal of Bacteriology* **184**, 5001-5010.

Fields, J. H. A., Eng, A. K., Ramsden, W. D., Hochachka, P. W., and Weinstein, B. (1980). Alanopine and strombine are novel imino acids produced by a dehydrogenase found in the adductor muscle of the oyster, *Crassostrea gigas*. *Archives of Biochemistry and Biophysics* **201**, 110-114.

Fink, R. M., Fink, K., and Henderson, R. B. (1953). β-Amino acid formation by tissue slices incubated with pyrimidines. *Journal of Biological Chemistry* **201**, 349-355.

Fisher, S. H. (1999). Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la différence! *Molecular Microbiology* **32**, 223-232.

Fisher, S. H., and Sonenshein, A. L. (1991). Control of carbon and nitrogen metabolism in *Bacillus subtilis*. *Annual Review of Microbiology* **45**, 107-135.

Forde, B. G. (2000). Nitrate transporters in plants: structure, function and regulation. *Biochimica et Biophysica Acta* **1465**, 219-235.

Forte-McRobbie, C., and Pietruszko, R. (1986). Purification and characterization of human liver "high K_m" aldehyde dehydrogenase and its identification as glutamic γ-semialdehyde dehydrogenase. *Journal of Biological Chemistry* **261**, 2154-2163.

Fritzson, P., and Pihl, A. (1957). The catabolism of C^{14}-labelled uracil, dihydrouracil, and b-ureidopropionic acid in the intact rat. *Journal of Biological Chemistry* **226**, 229-235.

Fu, B. Q., Liu, M. Y., Kapel, C. M. O., Meng, X. P., Lu, Q., Wu, X. P., Chen, Q. J., and Boireau, P. (2005). Molecular cloning of a cDNA encoding a putative cuticle collagen of *Trichinella spiralis*. *Veterinary Parasitology* **132**, 31-35.

Fuhrman, J., Lane, W., Smith, R., Piessens, W., and Perler, F. (1992). Transmission-blocking antibodies recognize microfilarial chitinase in Brugian lymphatic filariasis. *Proceedings of the National Academy of Science of the USA* **89**, 1548-1552.

Fujii, T., Sakai, H., Kawata, Y., and Hata, Y. (2003). Crystal structure of thermostable aspartase from *Bacillus* sp. YM55-1: structure-based exploration of functional sites in the aspartase family. *Journal of Molecular Biology* **328**, 635-654.

Fujimori, T., and Abe, H. (2002). Physiological roles of free D- and L-alanine in the crayfish *Procambarus clarkii* with special reference to osmotic and anoxic stress responses. *Comparative Biochemistry and Physiology* **131A**, 893-900.

Fujimoto, S., Mizutani, N., Mizota, C., and Tamaki, N. (1986). The level of β-alanine aminotransferase activity in regenerating and differentiating rat liver. *Biochimica et Biophysica Acta* **882**, 106-112.

Gäde, G., and Grieshaber, M. K. (1986). Pyruvate reductases catalyze the formation of lactate and opines in anaerobic invertebrates. *Comparative Biochemistry and Physiology* **83B**, 255-272.

Gamble, H. R., and Mansfield, L. S. (1996). Characterization of excretory-secretory products from larval stages of *Haemonchus contortus* cultured in vitro. *Veterinary Parasitology* **62**, 291-305.

Gamble, H. R., and Pappas, P. W. (1981). Adenosine deaminase (E.C. 3.5.4.4) from *Hymenolepis diminuta*. *Journal of Parasitology* **67**, 759-760.

Garber, A. J., Karl, I. E., and Kipnis, D. M. (1976). Alanine and glutamine synthesis and release from skeletal muscle. I. Glycolysis and amino acid release. *Journal of Biological Chemistry* **251**, 826-835.

García-Domínguez, M., Reyes, J. C., and Florencio, F. J. (1997). Purification and characterization of a new type of glutamine synthetase from cyanobacteria. *European Journal of Biochemistry* **244**, 258-264.

Geldhof, P., Claerebout, E., Knox, D. P., Agneessens, J., and Vercruysse, J. (2000). Proteinases released *in vitro* by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. *Parasitology* **121**, 639-647.

Geng, J., Plenefisch, J., Komuniecki, P. R., and Komuniecki, R. (2002). Secretion of a novel developmentally regulated chitinase (family 19 glycosyl hydrolase) into the perivitelline fluid of the parasitic nematode, *Ascaris suum*. *Molecular and Biochemical Parasitology* **124**, 11-21.

Genschel, U. (2004). Coenzyme A biosynthesis: reconstruction of the pathway in Archaea and an evolutionary scenario based on comparative genomics. *Molecular Biology and Evolution* **21**, 1242-1251.

Geraghty, M., Vaughn, D., Nicholson, A., Lin, W., Jimenez-Sanchez, G., Obie, C., Flynn, M., Valle, D., and Hu, C. (1998). Mutations in the Δ^1-pyrroline 5-carboxylate dehydrogenase gene cause type II hyperprolinemia. *Human Molecular Genetics* **7**, 1411-1415.

Gerard, J. F., and Gilles, R. (1972). The free amino-acid pool in *Callinectes sapidus* (Rathbun) tissues and its rôle in the osmotic intracellular regulation. *Journal of Marine Biology and Ecology* **10**, 125-136.

Ghosh, S., Blumenthal, H. J., Davidson, E., and Roseman, S. (1960). Glucosamine Metabolism. V. Enzymatic synthesis of glucosamine-6-phosphate. *Journal of Biological Chemistry* **235**, 1265-1273.

Gilad, G. M., Gilad, V. H., and Rabey, J. M. (1996). Arginine and ornithine decarboxylation in rodent brain: coincidental changes during development and after ischemia. *Neuroscience Letters* **216**, 33-36.

Gilles, R. (1973). Oxygen consumption as related to the amino-acid metabolism during osmoregulation in the blue crab *Callinectes sapidus*. *Netherlands Journal of Sea Research* **7**, 280-289.

Giordana, B., Leonardi, M. G., Casartelli, M., Consonni, P., and Parenti, P. (1998). K^+-neutral amino acid symport of *Bombyx mori* larval midgut: a system operative in extreme conditions. *American Journal of Physiology* **274**, R1361-R1371.

Giordanengo, L., Guiñazú, N., Stempin, C., Fretes, R., Cerbán, F., and Gea, S. (2002). Cruzipain, a major *Trypanosoma cruzi* antigen, conditions the host immune response in favor of parasite. *European Journal of Immunology* **32**, 1003-1011.

Giorgianni, F., Beranova, S., Wesdemiotis, C., and Viola, R. E. (1997). Mapping the mechanism-based modification sites in L-aspartase from *Escherichia coli*. *Archives of Biochemistry and Biophysics* **341**, 329-336.

Gojković, Z., Sandrini, M. P. B., and Piškur, J. (2001). Eukaryotic β-alanine synthases are functionally related but have a high degree of structural diversity. *Genetics* **158**, 999-1011.

Goldberg, M., Flescher, E., Gold, D., and Lengy, J. (1980). Ornithine-δ-transaminase from the liver fluke *Fasciola hepatica* and the blood fluke *Schistosoma mansoni*: a comparative study. *Comparative Biochemistry and Physiology* **65B**, 605-613.

Goldberg, M., Flescher, E., and Lengy, J. (1979). *Schistosoma mansoni*: Partial purification and properties of ornithine-δ-transaminase. *Experimental Parasitology* **47**, 333-341.

Goldin, B. R., and Frieden, C. (1971). L-Glutamate dehydrogenases. *Current Topics in Cellular Regulation* **4**, 77-117.

Gooday, G. W., Brydon, L. J., and Chappell, L. H. (1988). Chitinase in female *Onchocerca gibsoni* and its inhibition by allosamidin. *Molecular and Biochemical Parasitology* **29**, 223-225.

Goodman, M. N., and Lowenstein, J. M. (1977). The purine nucleotide cycle. Studies of ammonia production by skeletal muscle *in situ* and in perfused preparations. *Journal of Biological Chemistry* **252**, 5054-5060.

Grantham, B. D., and Barrett, J. (1986a). Amino acid catabolism in the nematodes *Heligmosomoides polygyrus* and *Panagrellus redivivus* 1. Removal of the amino group. *Parasitology* **93**, 481-493.

Grantham, B. D., and Barrett, J. (1986b). Amino acid catabolism in the nematodes *Heligmosomoides polygyrus* and *Panagrellus redivivus* 2. Metabolism of the carbon skeleton. *Parasitology* **93**, 495-504.

Grantham, B. D., and Barrett, J. (1988). Glutamine and asparagine synthesis in the nematodes *Heligmosomoides polygyrus* and *Panagrellus redivivus*. *Journal of Parasitology* **74**, 1052-1053.

Green, S., Eisenstein, E., McPhie, P., and Hensley, P. (1990). The purification and characterization of arginase from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **265**, 1601-1607.

Greenberg, C. S., Birckbichler, P., and Rice, R. (1991). Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB Journal* **5**, 3071-3077.

Halpern, Y. S., and Umbarger, H. E. (1960). Conversion of ammonia to amino groups in *Escherichia coli*. *Journal of Bacteriology* **80**, 285-288.

Hamilton, P. B. (1945). Glutamine: a major constituent of free α-amino acids in animal tissues and blood plasma. *Journal of Biological Chemistry* **158**, 397-409.

Hanigan, M. H., and Frierson, H. F. (1996). Immunohistochemical detection of γ-glutamyl transpeptidase in normal human tissue. *Journal of Histochemistry and Cytochemistry* **44**, 1101-1108.

Hanlon, D. P. (1975). The distribution of arginase and urease in marine invertebrates. *Comparative Biochemistry and Physiology* **52B**, 261-264.

Hanna, R. E. B. (1980). *Fasciola hepatica*: autoradiography of protein synthesis, transport, and secretion by the tegument. *Experimental Parasitology* **50**, 297-304.

Harris, M. T., Lai, K., Arnold, K., Martinez, H. F., Specht, C. A., and Fuhrman, J. A. (2000). Chitin synthase in the filarial parasite, *Brugia malayi*. *Molecular and Biochemical Parasitology* **111**, 351-362.

Harrop, C. J. F. (1974). Nitrogen metabolism in the ovine stomach 4. Nitrogenous components of the abomasal secretions. *Journal of Agricultural Science* **83**, 249-257.

Harrop, C. J. F., and Phillipson, A. T. (1974). Nitrogen metabolism in the ovine stomach 3. Urea in the abomasal secretions. *Journal of Agricultural Science* **83**, 237-247.

Harth, G., and Horwitz, M. A. (2003). Inhibition of *Mycobacterium tuberculosis* glutamine synthetase as a novel antibiotic strategy against tuberculosis: Demonstration of efficacy in vivo. *Infection and Immunity* **71**, 456-464.

Hartman, S. C., and Stochaj, E. M. (1973). Glutaminase A of *Escherichia coli*. Subunit structure and cooperative behavior. *Journal of Biological Chemistry* **248**, 8511-8517.

Haskins, W. T., and Weinstein, P. P. (1957). Nitrogenous excretory products of *Trichinella spiralis* larvae. *Journal of Parasitology* **43**, 19-24.

Hatch, M. D. (1973). Separation and properties of leaf aspartate aminotransferase and alanine aminotransferase isoenzymes operative in the C_4 pathway of photosynthesis. *Archives of Biochemistry and Biophysics* **156**, 207-214.

Hayashi, Y. S. (1993). Alanine aminotransferase from gill tissue of the brackish-water bivalve *Corbicula japonica* (Prime): Subcellular localization and some enzymatic properties. *Journal of Experimental Marine Biology and Ecology* **170**, 45-54.

Heath, R. L. (1970). Biosynthesis de novo of purines and pyrimidines in *Mesocestoides* (Cestoda). I. *Journal of Parasitology* **56**, 98-102.

Helling, R. (1994). Why does *Escherichia coli* have two primary pathways for synthesis of glutamate? *Journal of Bacteriology* **176**, 4664-4668.

Helling, R. B. (1998). Pathway choice in glutamate synthesis in *Escherichia coli*. *Journal of Bacteriology* **180**, 4571-4575.

Helling, R. B. (2002). Speed versus efficiency in microbial growth and the role of parallel pathways. *Journal of Bacteriology* **184**, 1041-1045.

Hemmilä, I. A., and Mäntsälä, P. I. (1978). Purification and properties of glutamate synthase and glutamate dehydrogenase from *Bacillus megaterium*. *Biochemical Journal* **173**, 45-52.

Henderson, G. B., and Fairlamb, A. H. (1987). Trypanothione metabolism: a chemotherapeutic target in trypanosomatids. *Parasitology Today* **3**, 312-315.

Henry, H. A. L., and Jefferies, R. L. (2002). Free amino acid, ammonium and nitrate concentrations in soil solutions of a grazed coastal marsh in relation to plant growth. *Plant, Cell and Environment* **25**, 665-675.

Hill, D. E., Fetterer, R. H., and Urban, J. F. (1991). *Ascaris suum* stage-specific differences in lectin binding to the larval cuticle. *Experimental Parasitology* **73**, 376-383.

Hirayama, C., and Nakamura, M. (2002). Regulation of glutamine metabolism during the development of *Bombyx mori* larvae. *Biochimica et Biophysica Acta* **1571**, 131-137.

Hirayama, C., Saito, H., Konno, K., and Shinbo, H. (1998). Purification and characterization of NADH-dependent glutamate synthase from the silkworm fat body (*Bombyx mori*). *Insect Biochemistry and Molecular Biology* **28**, 473-482.

Hirayama, C., Sugimura, M., Saito, H., and Nakamura, M. (2000). Host plant urease in the hemolymph of the silkworm, *Bombyx mori*. *Journal of Insect Physiology* **46**, 1415-1421.

Hirayama, C., Sugimura, M., and Shinbo, H. (1999). Recycling of urea associated with the host plant urease in the silkworm larvae, Bombyx mori. *Journal of Insect Physiology* **45**, 15-20.

Hird, F. J. R., Cianciosi, S. C., and McLean, R. M. (1986). Investigations on the origin and metabolism of the carbon skeleton of ornithine, arginine and proline in selected animals. *Comparative Biochemistry and Physiology* **83B**, 179-184.

Hirsch-Kolb, H., Kolb, H. J., and Greenberg, D. M. (1971). Nuclear magnetic resonance studies of manganese binding of rat liver arginase. *Journal of Biological Chemistry* **246**, 395-401.

Hodges, M. (2002). Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. *Journal of Experimental Botany* **53**, 905-916.

Hodges, M., Flesch, V., Gálvez, S., and Bismuth, E. (2003). Higher plant $NADP^+$-dependent isocitrate dehydrogenases, ammonium assimilation and NADPH production. *Plant Physiology and Biochemistry* **41**, 577-585.

Hodgetts, R. B. (1972). Biochemical characterization of mutants affecting the metabolism of β-alanine in *Drosophila*. *Journal of Insect Physiology* **18**, 937-947.

Hoerauf, A., Nissen-Pahle, K., Schmetz, C., Henkle-Dührsen, K., Blaxter, M. L., Buttner, D. W., Gallin, M. Y., Al-Qaoud, K. M., Lucius, R., and Fleischer, B. (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *Journal of Clinical Investigation* **103**, 11-18.

Hoffmann, K., Schneider-Scherzer, E., Kleinkauf, H., and Zocher, R. (1994). Purification and characterization of eucaryotic alanine racemase acting as key enzyme in cyclosporin biosynthesis. *Journal of Biological Chemistry* **269**, 12710-12714.

Hoffmann, R. J., Bishop, S. H., and Sassaman, C. (1978). Glutamate dehydrogenase from Coelenterates NADP specific. *Journal of Experimental Zoology* **203**, 165-170.

Holden, H. M., Thoden, J. B., and Raushel, F. M. (1999). Carbamoyl phosphate synthetase: an amazing biochemical odyssey from substrate to product. *Cellular and Molecular Life Sciences* **56**, 507-522.

Hong, S.-J., Kang, S.-Y., Chung, Y.-B., Chung, M.-H., Oh, Y.-J., Kang, I., Bahk, Y. Y., Kong, Y., and Cho, S.-Y. (2000). *Paragonimus westermani*: a cytosolic glutathione *S*-transferase of a σ-class in adult stage. *Expermental Parasitology* **94**, 180-189.

Hong, S.-J., Lee, J.-L., Lee, D.-H., Sohn, W.-M., and Cho, S.-Y. (2001). Molecular cloning and characterization of a mu-class glutathione *S*-transferase from *Clonorchis sinensis*. *Molecular and Biochemical Parasitology* **115**, 69-75.

Hopkins, T. L., and Lofgren, P. A. (1968). Adenine metabolism and urate storage in the cockroach, *Leucophaea maderae*. *Journal of Insect Physiology* **14**, 1803-1814.

Horák, J. (1986). Amino acid transport in eucaryotic microorganisms. *Biochimica et Biophysica Acta* **864**, 223-256.

Horio, Y., Fukui, H., Taketoshi, M., Tanaka, T., and Wada, H. (1988). Induction of cytosolic aspartate aminotransferase by glucagon in primary cultured rat hepatocytes. *Biochemical and Biophysical Research Communications* **153**, 410-416.

Horvitz, H. R., Chalfie, M., Trent, C., Sulston, J. E., and Evans, P. D. (1982). Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science* **216**, 1012-1014.

Howitt, S. M., and Udvardi, M. K. (2000). Structure, function and regulation of ammonium transporters in plants. *Biochimica et Biophysica Acta* **1465**, 152-170.

Huang, G., Dong, R., Allen, R., Davis, E. L., Baum, T. J., and Hussey, R. S. (2005). Two chorismate mutase genes from the root-knot nematode *Meloidogyne incognita*. *Molecular Plant Pathology* **6**, 23-30.

Huby, F., Hoste, H., Mallet, S., Fournel, S., and Nano, J. L. (1995). Effects of the excretory/secretory products of six nematode species, parasites of the digestive tract, on the proliferation of HT29-D4 and HGT-1 cell lines. *Epithelial Cell Biology* **4**, 156-162.

Huby, F., Nano, J.-L., Mallet, S., and Hoste, H. (1999). Effects of the excretory/secretory products of *Trichostrongylus colubriformis* on the growth of different cell lines. *International Journal for Parasitology* **29**, 697-702.

Hudson, R. C., and Daniel, R. M. (1993). L-Glutamate dehydrogenases: distribution, properties and mechanism. *Comparative Biochemistry and Physiology* **106B**, 767-792.

Huerta-Saquero, A., Calderón-Flores, A., Díaz-Villasenor, A., Du Pont, G., and Durán, S. (2004). Regulation of transcription and activity of *Rhizobium etli* glutaminase A. *Biochimica et Biophysica Acta* **1673**, 201-207.

Hutchinson, G. W., and Fernando, M. A. (1975). Enzymes of the tricarboxylic acid cycle in *Obeliscoides cuniculi* (Nematoda; Trichostrongylidae) during parasitic development. *International Journal for Parasitology* **5**, 77-82.

Hutson, S. (2001). Structure and function of branched chain aminotransferases. *Progress in Nucleic Acid Research and Molecular Biology* **70**, 175-205.

Iino, T., and Shimadate, T. (1986). Purification and properties of gut arginase from earthworm *Pheretima communissima*. *Comparative Biochemistry and Physiology* **83B**, 79-84.

Irwin, J. A., Morrissey, P. E. W., Ryan, J. P., Walshe, A., O'Neill, S. M., Carrington, S. D., Matthews, E., Fitzpatrick, E., Mulcahy, G., Corfield, A. P., and Dalton, J. P. (2004). Glycosidase activity in the excretory-secretory products of the liver fluke, *Fasciola hepatica*. *Parasitology* **129**, 465-472.

Ishiyama, K., Inoue, E., Watanabe-Takahashi, A., Obara, M., Yamaya, T., and Takahashi, H. (2004). Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in *Arabidopsis*. *Journal of Biological Chemistry* **279**, 16598-16605.

Isseroff, H., Bock, K., Owczarek, A., and Smith, K. R. (1983). Schistosomiasis: proline production and release by ova. *Journal of Parasitology* **69**, 285-289.

Isseroff, H., and Ertel, J. C. (1976). Proline in fascioliasis: III. Activities of pyrroline-5-carboxylic acid reductase and pyrroline-5-carboxylic acid dehydrogenase in *Fasciola*. *International Journal for Parasitology* **6**, 183-188.

Isseroff, H., Ertel, J. C., and Levy, M. G. (1976). Absorption of amino acids by *Schistosoma mansoni*. *Comparative Biochemistry and Physiology* **54B**, 125-133.

Itagaki, T., Dry, I. B., and Wiskich, J. T. (1988). Purification and properties of NAD-glutamate dehydrogenase from turnip mitochondria. *Phytochemistry* **27**, 3373-3378.

Iyer, R., Jenkinson, C. P., Vockley, J. G., Kern, R. M., Grody, W. W., and Cederbaum, S. (1998). The human arginases and arginine deficiency. *Journal of Inherited Metabolic Diseases* **21**, S86-S100.

Jabbar, A., Dow, E. A., and Strang, R. H. C. (1987). Aerobic amino acid metabolism in the locust nervous system. *Insect Biochemistry* **17**, 207-211.

Jaffe, J. J., and Lambert, R. A. (1986). Glutathione *S*-transferase in adult *Dirofilaria immitis* and *Brugia pahangi*. *Molecular and Biochemical Parasitology* **20**, 199-206.

Janáky, R., Ogita, K., Pasqualotto, B. A., Bains, J. S., Oja, S. S., Yoneda, Y., and Shaw, C. A. (1999). Glutathione and signal transduction in the mammalian CNS. *Journal of Neurochemistry* **73**, 889-902.

Jansonius, J. N. (1998). Structure, evolution and action of vitamin B_6-dependent enzymes. *Current Opinion in Structural Biology* **8**, 759-769.

Janssens, P. A., and Bryant, C. (1969). The ornithine-urea cycle in some parasitic helminths. *Comparative Biochemistry and Physiology* **30**, 261-272.

Jao, S.-C., Chen, J., Yang, K., and Li, W.-S. (2006). Design of potent inhibitors for *Schistosoma japonica* glutathione *S*-transferase. *Bioorganic and Medicinal Chemistry* **14**, 304-318.

Javelle, A., Andre, B., Marini, A.-M., and Chalot, M. (2003). High-affinity ammonium transporters and nitrogen sensing in mycorrhizas. *Trends in Microbiology* **11**, 53-55.

Jayasekera, M. M. K., Saribas, A. S., and Viola, R. E. (1997). Enhancement of catalytic activity by gene truncation: activation of L-aspartase from *Escherichia coli*. *Biochemical and Biophysical Research Communications* **238**, 411-414.

Jeffs, S. A., and Arme, C. (1985). *Hymenolepis diminuta*: characterization of the neutral amino acid transport loci of the metacestode. *Comparative Biochemistry and Physiology* **81A**, 387-390.

Jeffs, S. A., and Arme, C. (1987). *Echinococcus granulosus*: specificity of amino acid transport systems in protoscoleces. *Parasitology* **95**, 71-78.

Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D. (1996). Comparative properties of arginases. *Comparative Biochemistry and Physiology* **114B**, 107-132.

Jensen, R. A., and Gu, W. (1996). Evolutionary recruitment of biochemically specialized subdivisions of Family I within the protein superfamily of aminotransferases. *Journal of Bacteriolology* **178**, 2161-2171.

Jiao, Y., Okumiya, T., Saibara, T., Park, K., and Sasaki, M. (1998). Abnormally decreased HbA_{1c} can be assessed with erythrocyte creatine in patients with a shortened erythrocyte age. *Diabetes Care* **21**, 1732-1735.

John, R. A. (1995). Pyridoxal phosphate-dependent enzymes. *Biochimica et Biophysica Acta* ***1248***, 81-96.

Johnstone, I. L., Shafi, Y., Majeed, A., and Barry, J. D. (1996). Cuticular collagen genes from the parasitic nematode *Ostertagia circumcincta*. *Molecular and Biochemical Parasitology* **80**, 103-112.

Jones, J. D., and Burnett, P. C. (1974). Creatinine metabolism in humans with decreased renal function: creatinine deficit. *Clinical Chemistry* **20**, 1204-1212.

Jones, J. T., Furlanetto, C., Bakker, E., Banks, B., Blok, V., Chen, Q., Phillips, M., and Prior, A. (2003). Characterization of a chorismate mutase from the potato cyst nematode *Globodera pallida*. *Molecular Plant Pathology* **4**, 43-50.

Jørgensen, N. O. G. (1987). Free amino acids in lakes: Concentrations and assimilation rates in relation to phytoplankton and bacterial production. *Limnology and Oceanography* **32**, 97-111.

Jørgensen, N. O. G., Kroer, N., Coffin, R. B., and Hoch, M. P. (1999). Relations between bacterial nitrogen metabolism and growth efficiency in an esturine and an open-water ecosystem. *Aquatic Microbial Ecology* **18**, 247-261.

Kaiser, L., Geary, T. G., and Williams, J. F. (1998). *Dirofilaria immitis* and *Brugia pahangi*: filarial parasites make nitric oxide. *Experimental Parasitology* **90**, 131-134.

Kakimoto, T., Kato, J., Shibatani, T., Nishimura, N., and Chibata, I. (1969). Crystalline L-aspartate β-decarboxylase of *Pseudomonas dacunhae*. I. Crystallization and some physiochemical properties. *Journal of Biological Chemistry* **244**, 353-358.

Kamoda, N., Minatogawa, Y., Nakamura, M., Nakanishi, J., Okuno, E., and Kido, R. (1980). The organ distribution of human alanine-2-oxoglutarate aminotransferase and alanine-glyoxylate aminotransferase. *Biochemical Medicine* **23**, 25-34.

Kang, S.-Y., Ahn, I.-Y., Park, C.-Y., Chung, Y.-B., Hong, S.-T., Kong, Y., Cho, S.-Y., and Hong, S.-J. (2001). *Clonorchis sinensis*: molecular cloning and characterization of 28-kDa glutathione *S*-transferase. *Experimental Parasitology* **97**, 186-195.

Kan-no, N., Matsu-ura, H., Jikihara, S., Yamamoto, T., Endo, N., Moriyama, S., Nagahisa, E., and Sato, M. (2005). Tauropine dehydrogenase from the marine sponge *Halichondria japonica* is a homolog of ornithine cyclodeaminase/mu-crystallin. *Comparative Biochemistry and Physiology* **141B**, 331-339.

Kapur, J., and Sood, M. L. (1984). Amino acid biosynthesis in *Haemonchus contortus* from C^{14}-labelled precursors, in vitro. *Veterinary Parasitology* **15**, 293-299.

Kashii, T., Gomi, T., Oya, T., Ishii, Y., Oda, H., Maruyama, M., Kobayashi, M., Masuda, T., Yamazaki, M., Nagata, T., Tsukada, K., Nakajima, A., Tatsu, K., Mori, H., Takusagawa, F., Ogawa, H., and Pitot, H. C. (2005). Some biochemical and histochemical properties of human liver serine dehydratase. *International Journal of Biochemistry and Cell Biology* **37**, 574-589.

Kawalek, J. C., Rew, R. S., and Heavner, J. (1984). Glutathione-*S*-transferase, a possible drug-metabolizing enzyme, in *Haemonchus contortus*: comparative activity of a cambendazole-resistant and a susceptible strain. *International Journal for Parasitology* **14**, 173-175.

Kawata, Y., Tamura, K., Yano, S., Mizobata, T., Nagai, J., Esaki, N., Soda, K., Tokushige, M., and Yumoto, N. (1999). Purification and characterization of thermostable aspartase from *Bacillus* sp. YM55-1. *Archives of Biochemistry and Biophysics* **366**, 40-46.

Khademi, S., O'Connell, J., Remis, J., Robles-Colmenares, Y., Miercke, L. J. W., and Stroud, R. M. (2004). Mechanism of ammonia transport by Amt/MEP/Rh: Structure of AmtB at 1.35 Å. *Science* **305**, 1587-1594.

Khan, F. R., and McFadden, B. A. (1980). Embryogenesis and the glyoxylate cycle. *FEBS Letters* **115**, 312-314.

Kharbuli, Z. Y., Datta, S., Biswas, K., Sarma, D., and Saha, N. (2006). Expression of ornithine-urea cycle enzymes in early life stages of air-breathing walking catfish *Clarias batrachus* and induction of ureogenesis under hyper-ammonia stress. *Comparative Biochemistry and Physiology* **143B**, 44-53.

Kielty, C. M., Povey, S., and Hopkinson, D. A. (1982). Regulation of expression of liver-specific enzymes. II. Activation and chromosomal localization of soluble glutamate-pyruvate transaminase. *Annals of Human Genetics* **46**, 135-143.

Kim, H., Ikegami, K., Nakaoka, M., Yagi, M., Shibata, H., and Sawa, Y. (2003). Characterization of aspartate aminotransferase from the cyanobacterium *Phormidium lapideum*. *Bioscience, Biotechnology and Biochemistry* **67**, 490-498.

Kimber, M. J., and Fleming, C. C. (2005). Neuromuscular function in plant parasitic nematodes: a target for novel control strategies? *Parasitology* **131**, S129-S142.

Kita, K. (1992). Electron-transfer in complexes of mitochondria in *Ascaris suum*. Parasitology Today **8**, 155-159.

Klein, M., Kaltwasser, H., and Jahns, T. (2002). Isolation of a novel, phosphate-activated glutaminase from *Bacillus pasteurii*. *FEMS Microbiology Letters* **206**, 63-67.

Klein, R. D., Favreau, M. A., Alexander-Bowman, S. J., Nulf, S. C., Vanover, L., Winterrowd, C. A., Yarlett, N., Martinez, M., Keithly, J. S., Zantello, M. R., Thomas, E. M., and Geary, T. G. (1997). *Haemonchus contortus*: cloning and functional expression of a cDNA encoding ornithine decarboxylase and development of a screen for inhibitors. *Experimental Parasitology* **87**, 171-184.

Kleiner, D. (1981). The transport of NH_3 and NH_4^+ across biological membranes. *Biochimica et Biophysica Acta* **639**, 41-52.

Kleiner, D., Traglauer, A., and Domm, S. (1998). Does ammonia production by *Klebsiella* contribute to pathogenesis? *Bulletin de l'Institut Pasteur* **96**, 257-265.

Knox, D. P., and Jones, D. G. (1990). Studies on the presence and release of proteolytic enzymes (proteinases) in gastro-intestinal nematodes of ruminants. *International Journal for Parasitology* **20**, 243-249.

Knox, D. P., Redmond, D. L., Newlands, G. F., Skuce, P. J., Pettit, D., and Smith, W. D. (2003). The nature and prospects for gut membrane proteins as vaccine candidates for *Haemonchus contortus* and other ruminant trichostrongyloids. *International Journal for Parasitology* **33**, 1129-1137.

Köhler, P. (1985). The strategies of energy conservation in helminths. *Molecular and Biochemical Parasitology* **17**, 1-18.

Köhler, P. (1991). The pathways of energy generation in filarial parasites. *Parasitology Today* **7**, 21-25.

Komuniecki, P. R., and Vanover, L. (1987). Biochemical changes during the aerobic-anaerobic transition in *Ascaris suum* larvae. *Molecular and Biochemical Parasitology* **22**, 241-248.

Konji, V. N., Olembo, N. K., and Pearson, D. J. (1984). Enzyme activities in the fat body of the tsetse fly *Glossina morsitans* and the fleshfly *Sarcophaga tibialis* in relation to proline metabolism. *Insect Biochemistry* **14**, 685-690.

Kotlyar, S., Weihrauch, D., Paulsen, R. S., and Towle, D. W. (2000). Expression of arginine kinase enzymatic activity and mRNA in gills of the euryhaline crabs *Carcinus maenas* and *Callinectes sapidus*. *Journal of Experimental Biology* **203**, 2395-2404.

Krishnan, I. S., Singhal, R. K., and Dua, R. D. (1986). Purification and characterization of glutamine synthetase from *Clostridium pasteurianum*. *Biochemistry* **25**, 1589-1599.

Krishnan, N., and Becker, D. F. (2005). Characterization of a bifunctional PutA homologue from *Bradyrhizobium japonicum* and identification of an active site residue that modulates proline reduction of the flavin adenine dinucleotide cofactor. *Biochemistry* **44**, 9130-9139.

Kuhn, N. J., Talbot, J., and Ward, S. (1991). pH-sensitive control of arginase by Mn(II) ions at submicromolar concentrations. *Archives of Biochemistry and Biophysics* **286**, 217-221.

Kujo, C., and Ohshima, T. (1998). Enzymological characteristics of the hyperthermostable NAD-dependent glutamate dehydrogenase from the Archaeon *Pyrobaculum islandicum* and effects of denaturants and organic solvents. *Applied and Environmental Microbiology* **64**, 2152-2157.

Kumada, Y., Benson, D., Hillemann, D., Hosted, T., Rochefort, D., Thompson, C., Wohlleben, W., and Tateno, Y. (1993). Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes. *Proceedings of the National Academy of Science of the USA* **90**, 3009-3013.

Kurahashi, H., Atiwetin, P., Nagaoka, S., Miyata, S., Kitajima, S., and Sugimura, Y. (2005). Absorption of mulberry root urease to the hemolymph of the silkworm, *Bombyx mori*. *Journal of Insect Physiology* **51**, 1055-1061.

Kurata, Y. (1962). On the appearance of hydrogenase, nitrate reductase and aspartase during the ontogeny of the frog. *Experimental Cell Research* **28**, 424-429.

Kurelec, B. (1975a). Molecular biology of helminth parasites. *International Journal of Biochemistry* **6**, 375-386.

Kurelec, B. (1975b). Catabolic path of arginine and NAD regeneration in the parasite *Fasciola hepatica*. *Comparative Biochemistry and Physiology* **51B**, 151-159.

Kvamme, E., Roberg, B., and Torgner, I. A. (2000). Phosphate-activated glutaminase and mitochondrial glutamine transport in the brain. *Neurochemical Research* **25**, 1407-1419.

Lain-Guelbenzu, B., Cárdenas, J., and Muñoz-Blanco, J. (1991). Purification and properties of L-alanine aminotransferase from *Chlamydomonas reinhardtii*. *European Journal of Biochemistry* **202**, 881-887.

Lam, H.-M., Coschigano, K. T., Oliveira, I. C., Melo-Oliveira, R., and Coruzzi, G. M. (1996). The molecular genetics of nitrogen assimilation into amino acids in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 569-593.

Lambert, K. N., Allen, K. D., and Sussex, I. M. (1999). Cloning and characterization of an esophageal-gland-specific chorismate mutase from the phytoparasitic nematode *Meloidogyne javanica*. *Molecular Plant-Microbe Interactions* **12**, 328-336.

Langenbuch, M., and Pörtner, H. O. (2002). Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid-base variables. *Journal of Experimental Biology* **205**, 1153-1160.

Langer, B. W. (1972). The glutamic acid dehydrogenase of adult female *Ascaris suum*. *Journal of Parasitology* **58**, 539-540.

Langer, B. W., and Jiampermpoon, D. (1970). Some metabolic processes in the microfilarial form of *Dirofilaria immitis*. *Journal of Parasitology* **56**, 144-145.

LaRossa, R. A., and Schloss, J. V. (1984). The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. *Journal of Biological Chemistry* **259**, 8753-8757.

Laue, H., and Cook, A. M. (2000). Purification, properties and primary structure of alanine dehydrogenase involved in taurine metabolism in the anaerobe *Bilophila wadsworthia*. *Archives of Microbiology* **174**, 162-167.

Lawton, D. E. B., Reynolds, G. W., Hodgkinson, S. M., Pomroy, W. E., and Simpson, H. V. (1996). Infection of sheep with adult and larval *Ostertagia circumcincta*: Effects on abomasal pH and serum gastrin and pepsinogen. *International Journal for Parasitology* **26**, 1063-1074.

Lee, D. L. (1996). Why do some nematode parasites of the alimentary tract secrete acetylcholinesterase? *International Journal for Parasitology* **26**, 499-508.

Lee, J. H., Lee, D. W., and Lee, H. S. (1983). Purification and properties of branched chain amino acid aminotransferase from *Fasciola hepatica*. *Korean Journal of Parasitology* **21**, 49-57.

Lee, J. H., Park, N. Y., Lee, M. H., and Choi, S. H. (2003). Characterzation of the *Vibrio vulnificus putAP* operon, encoding proline dehydrogenase and proline permease, and its differential expression in reponse to osmotic stress. *Journal of Bacteriology* **185**, 3842-3852.

Lee, R. M., and Hodsden, M. R. (1963). Cholinesterase activity in *Haemonchus contortus* and its inhibition by organophosphorus anthelmintics. *Biochemical Pharmacology* **12**, 1241-1252.

Lehninger, A. L. (1951). Phosphorylation coupled to oxidation of dihydrodiphosphopyridine nucleotide. *Journal of Biological Chemistry* **190**, 345-359.

LéJohn, H. B. (1968). Unidirectional inhibition of glutamate dehydrogenase by metabolites. A Possible regulatory mechanism. *Journal of Biological Chemistry* **243**, 5126-5131.

Levy, M. G., and Read, C. P. (1975). Purine and pyrimidine transport in *Schistosoma mansoni*. *Journal of Parasitology* **61**, 627-632.

Li, H., Meininger, C. J., Hawker, J. R., Jr., Haynes, T. E., Kepka-Lenhart, D., Mistry, S. K., Morris, S. M., and Wu, G. (2001). Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. *American Journal of Physiology* **280**, E75-E82.

Liebau, E., Eckelt, V. H. O., Wildenburg, G., Teesdale-Spittle, P., Brophy, P. M., Walter, R. D., and Henkle-Dührsen, K. (1997). Structural and functional analysis of a glutathione *S*-transferase from *Ascaris suum*. *Biochemical Journal* **324**, 659–666.

Liebau, E., Müller, V., Lucius, R., Walter, R. D., and Henkle-Dührsen, K. (1996). Molecular cloning, expression and characterization of a recombinant glutathione *S*-transferase from *Echinococcus multilocularis*. *Molecular and Biochemical Parasitology* **77**, 49-56.

Liebau, E., Walter, R. D., and Henkle-Dührsen, K. (1994). Isolation, sequence and expression of an *Onchocerca volvulus* glutathione *S*-transferase cDNA. *Molecular and Biochemical Parasitology* **63**, 305-309.

Lieberman, M. W., Barrios, R., Carter, B. Z., Habib, G. M., Lebovitz, R. M., Rajagopalan, S., Sepulveda, A. R., Shi, Z.-Z., and Wan, D.-F. (1995). γ-Glutamyl transpeptidase. What does the organization and expression of a multipromoter gene tell us about its functions. *American Journal of Pathology* **147**, 1175-1185.

Linstead, D., and Cranshaw, M. A. (1983). The pathway of arginine catabolism in the parasitic flagellate *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **8**, 241-252.

Linton, S. N., and Campbell, J. W. (1962). Studies on urea cycle enzymes in the terrestrial snail, *Otala lactea*. *Archives of Biochemistry and Biophysics* **97**, 360-369.

Listrom, C. D., Morizono, H., Rajagopal, B. S., McCann, M. T., Tuchman, M., and Allewell, N. M. (1997). Expression, purification, and characterization of recombinant human glutamine synthetase. *Biochemical Journal* **328**, 159-163.

Liu, A., and Rothstein, M. (1976). Nematode biochemistry - XV. Enzyme changes related to glycerol excretion in *Caenorhabditis briggsae*. *Comparative Biochemistry and Physiology* **54B**, 233-238.

Livingstone, D. R., de Zwaan, A., and Thompson, R. J. (1981). Aerobic metabolism, octopine production and phosphoarginine as sources of energy in the phasic and catch adductor muscles of the giant scallop *Placopecten magellanicus* during swimming and the subsequent recovery period. *Comparative Biochemistry and Physiology* **70B**, 35-44.

Lodwig, E., Kumar, S., Allaway, D., Bourdes, A., Prell, J., Priefer, U., and Poole, P. (2004). Regulation of L-alanine dehydrogenase in *Rhizobium leguminosarum* bv. *viciae* and its role in pea nodules. *Journal of Bacteriology* **186**, 842-849.

Long, H., Wang, X., Xu, J. H., and Hu, Y. J. (2006). Isolation and characterization of another cDNA encoding a chorismate mutase from the phytoparasitic nematode *Meloidogyne arenaria*. *Experimental Parasitology* **113**, 106-111.

Long, X.-C., Bahgat, M., Chlichlia, K., Ruppel, A., and Li, Y.-L. (2004). Detection of inducible nitric oxide synthase in *Schistosoma japonicum* and *S. mansoni*. *Journal of Helminthology* **78**, 47-50.

Lopez De Mendoza, M. E., Curtis, R. H. C., and Gowen, S. (1999). Indentification and characterization of excreted-secreted products and surface coat antigens of animal and plant-parasitic nematodes. *Parasitology* **118**, 397-405.

Louis, C. A., Mody, V., Henry, W. L., Reichner, J. S., and Albina, J. E. (1999). Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. *American Journal of Physiology* **276**, R237-R242.

Loukas, A., Selzer, P. M., and Maizels, R. M. (1998). Characterisation of *Tc-cpl-1*, a cathepsin L-like cysteine protease from *Toxocara canis* infective larvae. *Molecular and Biochemical Parasitology* **92**, 275-289.

Low, W. P., Ong, W. T., and Ip, Y. K. (1996). Different physiological functions of free D- and L-alanine in three body parts of the intertidal sipunculid *Phascolosoma arcuatum*. *Journal of Comparative Physiology B* **165**, 558-564.

Maas, W. K. (1952). Pantothenate studies. III. Description of the extracted pantothenate-synthesizing enzyme of *Escherichia coli*. *Journal of Biological Chemistry* **198**, 23-32.

MacInnis, A. J., Fisher, F. M., and Read, C. P. (1965). membrane transport of purines and pyrimidines in a cestode. *Journal of Parasitology* **51**, 260-267.

Mackenzie, N. E., Ven de Waa, E. A., Gooley, P. R., Williams, J. F., Bennett, J. L., Bjorge, S. M., Baille, T. A., and Geary, T. G. (1989). Comparison of glycolysis and glutaminolysis in *Onchocerca volvulus* and *Brugia pahang*i by ^{13}C nuclear magnetic resonance spectroscopy. *Parasitology* **99**, 427-435.

Macrae, M., Plasterk, R. H. A., and Coffino, P. (1995). The ornithine decarboxylase gene of *Caenorhabditis elegans:* cloning, mapping and mutagenesis. *Genetics* **140**, 517-525.

Mádi, A., Hoffrogge, R., Blaskó, B., Glocker, M. *O.*, and Fésus, L. (2004). Amine donor protein substrates for transglutaminase activity in *Caenorhabditis elegans*. *Biochemical and Biophysical Research Communications* **315**, 1064-1069.

Magasanik, B. (2003). Ammonia assimilation by *Saccharomyces cerevisiae*. *Eukaryotic Cell* **2**, 827-829.

Magasanik, B., and Karibian, D. (1960). Purine nucleotide cycles and their metabolic role. *Journal of Biological Chemistry* **235**, 2672-2681.

Maggini, S., Stoecklin-Tschan, F. B., Mörikofer-Zwez, S., and Walter, P. (1992). New kinetic parameters for rat liver arginase measured at near-physiological steady-state concentrations of arginine and Mn^{2+}. *Biochemical Journal* **283**, 653–660.

Magne, C., and Larher, F. (1992). High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry* **200**, 115-118.

Mahajan, S., and Atkins, W. M. (2005). The chemistry and biology of inhibitors and pro-drugs targeted to glutathione S-transferases. *Cellular and Molecular Life Sciences* **62**, 1221-1233.

Maître, M., Ciesielski, L., Cash, C., and Mandel, P. (1975). Purification and studies on some properties of the 4-aminobutyrate: 2-oxoglutarate transaminase from rat brain. *European Journal of Biochemistry* **52**, 157-169.

Male, K. B., and Storey, K. B. (1983). Kinetic characterization of NADP-specific glutamate dehydrogenase from the sea anemone, *Anthopleura xanthogrammica*: control of amino acid biosynthesis during osmotic stress. *Comparative Biochemistry and Physiology* **76B**, 823-829.

Marathe, S., Yu, Y. G., Turner, G. E., Palmier, C., and Weiss, R. L. (1998). Multiple forms of arginase are differentially expressed from a single locus in *Neurospora crassa*. *Journal of Biological Chemistry* **273**, 29776-29785.

Margolis, B., and Lifschitz, M. (1985). Ammonia production and amino acid metabolism by rat renal papillary epithelial cells in culture. *Journal of Biological Chemistry* **260**, 501-507.

Marini, A. M., Soussi-Boudekou, S., Vissers, S., and Andre, B. (1997). A family of ammonium transporters in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **17**, 4282–4293.

Márquez, J., López de la Oliva, A. R., Matés, J. M., Segura, J. A., and Alonso, F. J. (2006). Glutaminase: A multifaceted protein not only involved in generating glutamate. *Neurochemistry International* **48**, 465-471.

Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F., and Fasella, P. (1967). Isolation and characterization of multiple forms of glutamate-aspartate aminotransferase from pig heart. *Journal of Biological Chemistry* **242**, 2397-2409.

Mastorodemos, V., Zaganas, I., Spanaki, C., Bessa, M., and Plaitakis, A. (2005). Molecular basis of human glutamate dehydrogenase regulation under changing energy demands. *Journal of Neuroscience Research* **79**, 65-73.

Matsushima, O., and Hayashi, Y. S. (1992). Metabolism of D- and L-alanine and regulation of intracellular free amino acid levels during salinity stress in a brackish-water bivalve *Corbicula japonica*. *Comparative Biochemistry and Physiology* **102A**, 465-471.

Mattevi, A., Tedeschi, G., Bacchella, L., Coda, A., Negri, A., and Ronchi, S. (1999). Structure of L-aspartate oxidase: implications for the succinate dehydrogenase/fumarate reductase oxidoreductase family. *Structure* **7**, 745-756.

Mattingly, J. R., Iriarte, A., and Martinez-Carrion, M. (1995). Homologous proteins with different affinities for groEL. *Journal of Biological Chemistry* **270**, 1138-1148.

Maurizi, M., and Ginsburg, A. (1982). Active site ligand stabilization of quaternary structures of glutamine synthetase from *Escherichia coli*. *Journal of Biological Chemistry* **257**, 7246-7251.

Mayer, M. L., and Armstrong, N. (2004). Structure and function of glutamate receptor ion channels. *Annual Review of Physiology* **66**, 161-181.

McCauley, R., Kong, S.-E., Heel, K., and Hall, J. C. (1999). The role of *glutaminase* in the small intestine. *The International Journal of Biochemistryand Cell Biology* **31**, 405-413

McCracken, R. O., and Lumsden, R. D. (1975). Structure and function of parasite surface membranes - II. Concavalin A adsorption by the cestode *Hymenolepis diminuta* and its effect on transport. *Comparative Biochemistry and Physiology* **52B**, 331-337.

McGee, D. J., Zabaleta, J., Viator, R. J., Testerman, T. L., Ochoa, A. C., and Mendz, G. L. (2004). Purification and characterization of *Helicobacter pylori* arginase, RocF: unique features among the arginase superfamily. *European Journal of Biochemistry* **271**, 1952-1962.

McGivan, J. D., and Bradford, N. M. (1983). Characteristics of the activation of glutaminase by ammonia in sonicated rat liver mitochondria. *Biochimica et Biophysica Acta* **759**, 296-302.

McGivan, J. D., Lacey, J. H., and Joseph, S. K. (1980). Localization and some properties of phosphate-dependent glutaminase in disrupted liver mitochondria. *Biochemical Journal* **192**, 537-542.

McKerrow, J. H., Caffrey, C., Kelly, B., Loke, P., and Sajid, M. (2006). Proteases in parasitic diseases. *Annual Review of Pathology: Mechanisms of Disease* **1**, 497-536.

McLean, M. D., Yevtushenko, D. P., Deschene, A., Van Cauwenberghe, O. R., Makhmoudova, A., Potter, J. W., W, B. A., and Shelp, B. J. (2003). Overexpression of glutamate decarboxylase in transgenic tobacco plants confers resistance to the northern root-knot nematode. *Molecular Breeding* **11**, 277-285.

McManus, D. P., and James, B. L. (1975). Tricarboxylic acid cycle enzymes in the digestive gland of *Littorina saxatilis rudis* (Maton) and in the daughter sporocysts of *Microphallus similis* (Jäg.) (Digenea: microphallidae). *Comparative Biochemistry and Physiology* **50B**, 491-495.

McVeigh, P., Geary, T. G., Marks, N. J., and Maule, A. G. (2006). The FLP-side of nematodes. *Trends in Parasitology* **22**, 385-396.

Mégraud, F., Nemam-Simha, V., and Brugmann, D. (1992). Further evidence of the toxic effect of ammonia produced by *Helicobacter pylori* urease on human epithelial cells. *Infection and Immunity* **60**, 1858-1863.

Mehta, K., Chandrashekar, R., and Rao, U. R. (1996). Transglutaminase-catalyzed incorporation of host proteins in *Brugia malayi* microfilariae. *Molecular and Biochemical Parasitology* **76**, 105-114.

Mehta, K., Rao, U. R., Vickery, A. C., and Fesus, L. (1992). Identification of a novel transglutaminase from the filarial parasite *Brugia malayi* and its role in growth and development. *Molecular and Biochemical Parasitology* **53**, 1-15.

Mehta, P. K., Hale, T. I., and Christen, P. (1989). Evolutionary relationships among aminotransferases. Tyrosine aminotransferase, histidinol-phosphate aminotransferase, and aspartate aminotransferase are homologous proteins. *European Journal of Biochemistry* **186**, 249-253.

Mei, B., and Jiao, R. (1988). Purification and properties of glutamate synthase from *Nocardia mediterranei*. *Journal of Bacteriology* **170**, 1940-1944.

Mei, H., and LoVerde, P. T. (1997). *Schistosoma mansoni*: The developmental regulation and immunolocalization of antioxidant enzymes. *Experimental Parasitology* **86**, 69-78.

Meijer, A. J., Lamers, W. H., and Chamuleau, R. A. F. M. (1990). Nitrogen metabolism and ornithine cycle function. *Physiological Reviews* **70**, 701-748.

Meissner, B., Boll, M., Daniel, H., and Baumeister, R. (2004). Deletion of the intestinal peptide transporter affects insulin and TOR signaling in *Caenorhabditis elegans*. *Journal of Biological Chemistry* **279**, 36739-36745.

Meister, A. (1981). Metabolism and functions of glutathione. *Trends in Biochemical Sciences* **6**, 231-234.

Meister, A. (1988). Glutathione metabolism and its selective modification. *Journal of Biological Chemistry* **263**, 17205-17208.

Meister, A., Sober, H. A., and Tice, S. V. (1951). Enzymatic decarboxylation of aspartic acid to α-alanine. *Journal of Biological Chemistry* **189**, 577-590.

Menzel, R., and Roth, J. (1981). Enzymatic properties of the purified *putA* protein from *Salmonella typhimurium*. *Journal of Biological Chemistry* **256**, 9762-9766.

Mercer, J. G., and Chappell, L. (1985). *Schistosoma mansoni*: Effect of maintenance in vitro on the uptake and incorporation of leucine by adult worms. *Molecular and Biochemical Parasitology* **15**, 327-339.

Meredith, D., and Boyd, C. A. R. (2000). Structure and function of eukaryotic peptide transporters. *Cellular and Molecular Life Sciences* **57**, 754-778.

Mestichelli, L. J. J., Gupta, R. N., and Spenser, I. D. (1979). The biosynthetic route from ornithine to proline. *Journal of Biological Chemistry* **254**, 640-647.

Michalik, M., Nelson, J., and Erecinska, M. (1992). Glutamate production in islets of Langerhans: Properties of phosphate-activated glutaminase. *Metabolism* **41**, 1319-1326.

Michuda, C. M., and Martinez-Carrion, M. (1969). Distinctions in the equilibrium kinetic constants of the mitochondrial and supernatant isozymes of aspartate transaminase. *Journal of Biological Chemistry* **244**, 5920-5927.

Miflin, B. J., and Habash, D. Z. (2002). The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *Journal of Experimental Botany* **53**, 979-987.

Miflin, B. J., and Lea, P. J. (1976). The pathway of nitrogen assimilation in plants. *Phytochemistry* **15**, 873-885.

Milhon, J. L., Thiboldeaux, R. L., Glowac, K., and Tracy, J. W. (1997). *Schistosoma japonicum* GSH *S*-transferase Sj26 is not the molecular target of praziquantel action. *Experimental Parasitology* **87**, 268-274.

Miller, E., and Brenchley, J. (1981). L-Methionine *SR*-sulfoximine-resistant glutamine synthetase from mutants of *Salmonella typhimurium*. *Journal of Biological Chemistry* **256**, 11307-11312.

Milner, J., Grothe, S., and Wood, J. (1988). Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. *Journal of Biological Chemistry* **263**, 14900-14905.

Minic, Z., and Hervé, G. (2003). Arginine metabolism in the deep sea tube worm *Riftia pachyptila* and Its bacterial endosymbiont. *Journal of Biological Chemistry* **278**, 40527-40533.

Mircheff, A. K., van Os, C. H., and Wright, E. M. (1980). Pathways for alanine transport in intestinal basal lateral membrane vesicles. *Journal of Membrane Biology* **52**, 83-92.

Mistry, S. K., Burwell, T. J., Chambers, R. M., Rudolph-Owen, L., Spaltmann, F., Cook, W. J., and Morris, S. M. (2002). Cloning of human agmatinase. An alternate path for polyamine synthesis induced in liver by hepatitis B virus. *American Journal of Physiology* **282**, G375-G381.

Mitchell, A., and Magasanik, B. (1983). Purification and properties of glutamine synthetase from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **258**, 119-124.

Mixson, A. J., and Phang, J. M. (1988). The uptake of pyrroline 5-carboxylate. Group translocation mediating the transfer of reducing-oxidizing potential. *Journal of Biological Chemistry* **263**, 10720-10724.

Miyoshi, K., Taira, A., Yoshida, K., Tamura, K., and Uga, S. (1980a). Presence of creatinase and sarcosine dehydrogenase in human skeletal muscle. Proposal for creatine-urea pathway. *Proceedings of the Japanese Academy* **56**, 95-98. (cited by Wyss and Kaddurah-Daouk, 2000).

Miyoshi, K., Taira, A., Yoshida, K., Tamura, K., and Uga, S. (1980b). Abnormalities of creatinase in skeletal muscle of patients with Duchenne muscular dystrophy. *Proceedings of the Japanese Academy* **56**, 99-101. (cited by Wyss and Kaddurah-Daouk, 2000).

Mobley, H. L. T., and Hausinger, R. P. (1989). Microbial ureases: significance, regulation, and molecular characterization. *Microbiological Reviews* **53**, 85-108.

Modavi, S., and Isseroff, H. (1984). *Fasciola hepatica*: Collagen deposition and other histopathology in the rat host's bile duct caused by the parasite and by proline infusion. *Experimental Parasitology* **58**, 239-244.

Mody, I., De Koninck, Y., Otis, T. S., and Soltesz, I. (1994). Bridging the cleft at GABA synapses in the brain. *Trends in Neurosciences* **17**, 517-525.

Mohamed, S. A., Fahmy, A. S., Mohamed, T. M., and Hamdy, S. M. (2005). Urea cycle of *Fasciola gigantica*: Purification and characterization of arginase. *Comparative Biochemistry and Physiology* **142B**, 308-316.

Monastirioti, M. (1999). Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microscopy Research and Technique* **45**, 106-121.

Montanini, B., Betti, M., Márquez, A. J., Balestrini, R., Bonfante, P., and Ottonello, S. (2003). Distinctive properties and expression profiles of glutamine synthetase from a plant symbiotic fungus. *Biochemical Journal* **373**, 357-368.

Mordue, W., and Goldsworthy, G. J. (1973). Transaminase levels and uric acid production in adult locusts. *Insect Biochemistry* **3**, 419-427.

Morello, A., Repetto, Y., and Atias, A. (1982). Characterization of glutathione S-transferase activity in *Echinococcus granulosus*. *Comparative Biochemistry and Physiology* **72B**, 449-452.

Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y., and Wakayama, M. (1994). Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. *Journal of Fermentation and Bioengineering* **77**, 621-625.

Morin, P. J., Subramanian, G. S., and Gilmore, T. D. (1992). *AAT1*, a gene encoding a mitochondrial aspartate aminotransferase in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **1171**, 211-214.

Morris, S. M. (2002). Regulation of enzymes of the urea cycle and arginine metabolism. *Annual Review of Nutrition* **22**, 87-105.

Morris, S. M. (2004). Enzymes of arginine metabolism. *Journal of Nutrition* **134**, 2743S-2747S.

Morrison, C. A., Colin, T., Sexton, J. L., Bowen, F., Wicker, J., Friedel, T., and Spithill, T. W. (1996). Protection of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase. *Vaccine* **14**, 1603-1612.

Müller, I. B., Walter, R. D., and Wrenger, C. (2005). Structural metal dependency of the arginase from the human malaria parasite *Plasmodium falciparum*. *Biological Chemistry* **386**, 117-126.

Müller, S., and Walter, R. D. (1992). Purification and characterization of polyamine oxidase from *Ascaris suum*. *Biochemical Journal* **283**, 75-80.

Murase, S., Takagi, J. S., Higashi, Y., Imaishi, H., Yumoto, N., and Tokushige, M. (1991). Activation of aspartase by site-directed mutagenesis. *Biochemical and Biophysical Research Communications* **177**, 414-419.

Muro-Pastor, A. M., and Maloy, S. (1995). Proline dehydrogenase activity of the transcriptional repressor PutA is required for induction of the *put* operon by proline. *Journal of Biological Chemistry* **270**, 9819-9827.

Muro-Pastor, M. I., and Florencio, F. J. (2003). Regulation of ammonium assimilation in cyanobacteria. *Plant Physiology and Biochemistry* **41**, 595-603.

Mustafa, T., Komuniecki, R., and Mettrick, D. F. (1978). Cytosolic glutamate dehydrogenase in adult *Hymenolepis diminuta* (Cestoda). *Comparative Biochemistry and Physiology* **61B**, 219-222.

Nandakumar, R., Yoshimune, K., Wakayama, M., and Moriguchi, M. (2003). Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic* **23**, 87-100.

Nelson, D., Rumsey, W. L., and Erecińska, M. (1992). Glutamine catabolism by heart muscle. Properties of phosphate-activated glutaminase. *Biochemical Journal* **282**, 559-564.

Neuhaus, B., Bresciani, J., and Peters, W. (1997). Ultrastructure of the pharyngeal cuticle and lectin labelling with wheat germ agglutinin-gold conjugate indicating chitin in the pharyngeal cuticle of *Oesophagostomum dentatum* (Strongylida, Nematoda). *Acta Zoologica* **78**, 205-213.

Niemann, G., von Besser, G., and Walter, R. D. (1996). *Panagrellus redivivus* ornithine decarboxylase: structure of the gene, expression in *Escherichia coli* and characterization of the recombinant protein. *Biochemical Journal* **317**, 135–140.

Nimmo, G. A., and Tipton, K. F. (1981). Kinetic comparisons between soluble and membrane-bound glutaminase preparations from pig brain. *European Journal of Biochemistry* **117**, 57-64.

Nioche, P., Berka, V., Vipond, J., Minton, N., Tsai, A.-L., and Raman, C. S. (2004). Femtomolar sensitivity of a NO sensor from *Clostridium botulinum*. *Science* **306**, 1550-1553.

Nishiya, Y., Toda, A., and Imanaka, T. (1998). Gene cluster for creatinine degradation in *Arthrobacter* sp. TE1826. *Molecular and General Genetics* **257**, 581-586.

Njålsson, R., and Norgren, S. (2005). Physiological and pathological aspects of GSH metabolism. *Acta Paediatrica* **94**, 132-137.

Nna Mvondo, D., Navarro-Gonzalez, R., McKay, C. P., Coll, P., and Raulin, F. (2001). Production of nitrogen oxides by lightning and coronae discharges in simulated early Earth, Venus and Mars environments. *Advances in Space Research* **27**, 217-223.

Nomura, T., Yamamoto, I., Morishita, F., Furukawa, K., and Matsushima, O. (2001). Purification and some properties of alanine racemase from a bivalve mollusc *Corbicula japonica*. *Journal of Experimental Zoology* **289**, 1-9.

Nordlie, R. C., Varricchio, F. E., and Holten, D. D. (1965). Effects of altered hormonal states and fasting on rat-liver mitochondrial phosphoenolpyruvate carboxykinase levels. *Biochimica et Biophysica Acta* **97**, 214-221.

Nuiry, I. I., Hermes, J. D., Weiss, P. M., Chen, C. Y., and Cook, P. F. (1984). Kinetic mechanism and location of rate-determining steps for aspartase from *Hafnia alvei*. *Biochemistry* **23**, 5168-5175.

Nygaard, T., Bennett, C. C., Grossman, G., Edwards, M. R., and Schofield, P. J. (1994). Efflux of alanine by *Giardia intestinalis*. *Molecular and Biochemical Parasitology* **64**, 145-152.

Obungu, V. H., Kiaira, J. K., Njogu, R. M., and Olembo, N. K. (1999). Catabolism of proline by procyclic culture forms of *Trypanosoma congolense*. *Comparative Biochemistry and Physiology* **123B**, 59-65.

Oelze, J. (2000). Respiratory protection of nitrogenase in *Azotobacter* species: is a widely held hypothesis unequivocally supported by experimental evidence? *FEMS Microbiology Reviews* **24**, 321-333.

Ogilvie, B. M., Rothwell, T. L. W., Bremner, K. C., Schnitzerling, H. J., and Nolan, J. K. R. K. (1973). Acetylcholinesterase secretion by parasitic nematodes - I. Evidence for secretion of the enzyme by a number of species. *International Journal for Parasitology* **3**, 589-597.

Ohyama, T., Matsuda, K., Tachibana, H., Fujimoto Sakata, S., Mori, M., Horiuchi, M., and Tamaki, N. (2004). Purification and expression of a processing protease on β-alanine-oxoglutarate aminotransferase from rat liver mitochondria. *FEBS Letters* **572**, 251-255.

Okumiya, T., Ishikawa-Nishi, M., Doi, T., Kamioka, M., Takeuchi, H., Doi, Y., and Sugiura, T. (2004). Evaluation of intravascular hemolysis with erythrocyte creatine in patients with cardiac valve prostheses. *Chest* **125**, 2115-2120.

Olds, G., Ellner, J., Kearse, L., Kazura, J., and Mahmoud, A. (1980). Role of arginase in killing of schistosomula of *Schistosoma mansoni*. *Journal of Experimental Medicine* **151**, 1557-1562.

O'Malley, K. L., and Terwilliger, R. C. (1974). Structure and properties of arginase from the polychaete annelid *Pista pacifica* Berkeley. *Biochemical Journal* **143**, 591-597.

Orlacchio, A., Campos-Cavieres, M., Pashev, I., and Munn, E. A. (1979). Some kinetic and other properties of the isoenzymes of aspartate aminotransferase isolated from sheep liver. *Biochemical Journal* **177**, 583-593.

Orlowski, M., and Meister, A. (1970). The γ-glutamyl cycle: a possible transport system for amino acids. *Proceedings of the National Academy of Sciences* **67**, 1248-1255.

Orr, J., and Haselkorn, R. (1981). Kinetic and inhibition studies of glutamine synthetase from the cyanobacterium *Anabaena* 7120. *Journal of Biological Chemistry* **256**, 13099-13104.

Osanai, M., Okudaira, M., Naito, J., Demura, M., and Asakura, T. (2000). Biosynthesis of L-alanine, a major amino acid of fibroin in *Samia cynthia ricini*. *Insect Biochemistry and Molecular Biology* **30**, 225-232.

Osuji, G. O., and Madu, W. C. (1996). Ammonium ion salvage by glutamate dehydrogenase during defence response in maize. *Phytochemistry* **42**, 1491-1498.

Owen, T. G., and Hochachka, P. W. (1974). Purification and properties of dolphin muscle aspartate and alanine transaminases and their possible roles in the energy metabolism of diving mammals. *Biochemical Journal* **143**, 541-553.

Özer, N. (1985). A new enzyme-coupled spectrophotometric method for the determination of arginase activity. *Biochemical Medicine* **33**, 367-371.

Padmanabhan, B., Paehler, A., and Horikoshi, M. (2002). Structure of creatine amidinohydrolase from *Actinobacillus*. *Acta Crystallographica* **D58**, 1322-1328.

Page, A. P. (2001). The nematode cuticle: synthesis, modification and mutants. *In* "Parasitic Nematodes" (M. W. Kennedy and W. Harnett, eds.), pp. 167-193. CABI Publishing, Oxon and New York.

Paget, T. A., Raynor, M. H., Shipp, D. W. E., and Lloyd, D. (1990). *Giardia lamblia* produces alanine anaerobically but not in the presence of oxygen. *Molecular and Biochemical Parasitology* **42**, 63-67.

Paltridge, R. W., and Janssens, P. A. (1971). A reinvestigation of the status of the ornithine-urea cycle in adult *Ascaris lumbricoides*. *Comparative Biochemistry and Physiology* **40B**, 503-513.

Pan, C.-H., Chien, Y.-H., and Hunter, B. (2003). The resistance to ammonia stress of *Penaeus monodon* Fabricius juvenile fed diets supplemented with astaxanthin. *Journal of Experimental Marine Biology and Ecology* **297**, 107-118.

Panizzutti, R., Souza Leite, M., Pinheiro, C. M., and Meyer-Fernandes, J. R. (2006). The occurrence of free D-alanine and an alanine racemase activity in *Leishmania amazonensis*. *FEMS Microbiology Letters* **256**, 16-21.

Pant, R., and Kumar, S. (1978). Is a urea cycle present in insects? *Biochemical Journal* **174**, 341-344.

Panteghini, M. (1990). Aspartate aminotransferase isoenzymes. *Clinical Biochemistry* **23**, 311-319.

Pao, S. S., Paulsen, I. T., and Saier, M. H. (1998). Major Facilitator Superfamily. *Microbiology and Molecular Biology Reviews* **62**, 1-34.

Pappas, P. W. (1971). *Haematoloechus medioplexus*: uptake, localization, and fate of tritiated arginine. *Experimental Parasitology* **30**, 102-119.

Pappas, P. W. (1988). The relative role of the intestines and external surfaces in the nutrition of monogeneans and nematodes. *Parasitology* **96**, S105-S121.

Pappas, P. W., and Read, C. P. (1975). Membrane transport in helminth parasites: a review. *Experimental Parasitology* **37**, 469-530.

Pappas, P. W., Uglem, G. L., and Read, C. P. (1973). Mechanisms and specificity of amino acid transport in *Taenia crassiceps* larvae (Cestoda). *International Journal for Parasitology* **3**, 641-651.

Paris, S., Wessel, P. M., and Dumas, R. (2002). Overproduction, purification, and characterization of recombinant aspartate semialdehyde dehydrogenase from *Arabidopsis thaliana*. *Protein Expression and Purification* **24**, 99-104.

Parker, D. S., Lomax M.A., Seal C.J., Wilton J.C., Lomax, M. A., Seal, C. J., and Wilton, J. C. (1995). Metabolic implications of ammonia production in the ruminant. *Proceedings of the Nutrition Society* **54**, 549-563.

Patchett, M. L., Daniel, R. M., and Morgan, H. W. (1991). Characterisation of arginase from the extreme thermophile `*Bacillus caldovelox*'. *Biochimica et Biophysica Acta* **1077**, 291-298.

Pate, J. S. (1973). Uptake, assimilation and transport of nitrogen compounds by plants. *Soil Biology and Biochemistry* **5**, 109-119.

Pegg, A. E. (1986). Recent advances in the biochemistry of polyamines in eukaryotes. *Biochemical Journal* **234**, 249-262.

Pemberton, K. D., and Barrett, J. (1989). The detoxification of xenobiotic compounds by *Onchocerca gutturosa* (Nematoda: Filarioidea). *International Journal for Parasitology* **19**, 875-878.

Pereira, C. A., Alonso, G. D., Paveto, M. C., Iribarren, A., Cabanas, M. L., Torres, H. N., and Flawiá, M. M. (2000). *Trypanosoma cruzi* arginine kinase characterization and cloning. A novel energetic pathway in protozoan parasites. *Journal of Biological Chemistry* **275**, 1495-1501.

Pesole, G., Gissi, C., Lanave, C., and Saccone, C. (1995). Glutamine synthetase gene evolution in bacteria. *Molecular Biology and Evolution* **12**, 189-197.

Pieters, R., Klumper, E., Kaspers, G. J. L., and Veerman, A. J. P. (1997). Everything you always wanted to know about cellular drug resistance in childhood acute lymphoblastic leukemia. *Critical Reviews in Oncology/Hematology* **25**, 11-26.

Platzer, E. G., and Roberts, L. S. (1970). Developmental physiology of cestodes - Part VII Vitamin B_6 and *Hymenolepis diminuta*: vitamin levels in the cestode and effects of deficiency of phosphorylase and transaminase activities. *Comparative Biochemistry and Physiology* **35**, 535-552.

Platzer, E. G., Thompson, S. N., Borchardt, D. B., and Gamble, H. R. (1995). High energy phosphate metabolites observed by NMR in infective larvae of *Haemonchus contortus*. *Journal of Parasitology* **81**, 434-438.

Platzer, E. G., Wang, W., Thompson, S. N., and Borchardt, D. B. (1999). Arginine kinase and phosphoarginine, a functional phosphagen, in the Rhabditoid nematode *Steinernema carpocapsae*. *Journal of Parasitology* **85**, 603-607.

Potrikus, C. J., and Breznak, J. A. (1981). Gut bacteria recycle uric acid nitrogen in termites: A strategy for nutrient conservation. *Proceedings of the National Academy of Science of the USA* **78**, 4601-4605.

Prezioso, G., Indiveri, C., and Bonvino, V. (1985). Kinetic characterization of L-glutamate dehydrogenase isolated from *Drosophila melanogaster* larvae. *Comparative Biochemistry and Physiology* **80B**, 1-4.

Prichard, R. K., and Schofield, P. J. (1968). A comparative study of the tricarboxylic acid cycle enzymes in *Fasciola hepatica* and rat liver. *Comparative Biochemistry and Physiology* **25**, 1005-1019.

Prusiner, S., and Stadtman, E. R. (1971). On the regulation of glutaminase in *E. coli*: metabolite control. *Biochemical and Biophysical Research Communications* **45**, 1474-1481.

Prusiner, S., and Stadtman, E. R. (1976). Regulation of glutaminase B in *Escherichia coli*. III. Control by nucleotides and divalent cations. *Journal of Biological Chemistry* **251**, 3463-3469.

Quiroga, C., Busquets, M., Cortés, A., and Bozal, J. (1985). Separation and kinetic properties of the molecular forms of chicken liver cytoplasmic aspartate aminotransferase. *International Journal of Biochemistry* **17**, 1185-1190.

Rachim, M. A., and Nicholas, D. J. D. (1985). Glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*. *Phytochemistry* **24**, 2541-2548.

Rajamohan, F., Nelms, L., Joslin, D. L., Lu, B., Reagan, W. J., and Lawton, M. (2006). cDNA cloning, expression, purification, distribution, and characterization of biologically active canine alanine aminotransferase-1. *Protein Expression and Purification* **48**, 81-89.

Rasero, F. S., Monteoliva, M., and Mayor, F. (1968). Enzymes related to 4-aminobutyrate metabolism in intestinal parasites. *Comparative Biochemistry and Physiology* **25**, 693-696.

Rathod, P. K., and Fellman, J. H. (1985a). Identification of mammalian aspartate-4-decarboxylase. *Archives of Biochemistry and Biophysics* **238**, 435-446.

Rathod, P. K., and Fellman, J. H. (1985b). Regulation of mammalian aspartate-4-decarboxylase: Its possible role in oxaloacetate and energy metabolism. *Archives of Biochemistry and Biophysics* **238**, 447-451.

Ratti, S., Curti, B., Zanetti, G., and Galli, E. (1985). Purification and characterization of glutamate synthase from *Azospirillum brasilense*. *Journal of Bacteriology* **163**, 724-729.

Raushel, F. M., Thoden, J. B., Reinhart, G. D., and Holden, H. M. (1998). Carbamoyl phosphate synthetase: a crooked path from substrates to products. *Current Opinion in Chemical Biology* **2**, 624-632.

Rawls, J. M. (2006). Analysis of pyrimidine catabolism in *Drosophila melanogaster* using epistatic interactions with mutations of pyrimidine biosynthesis and β-alanine metabolism. *Genetics* **172**, 1665-1674.

Recasens, M., Benezra, R., Basset, P., and Mandel, P. (2003). Cysteine sulfinate aminotransferase and aspartate aminotransferase isoenzymes of rat brain. Purification, characterization, and further evidence for identity. *Biochemistry* **19**, 4583-4589.

Reczkowski, R. S., and Ash, D. E. (1994). Rat liver arginase: kinetic mechanism, alternate substrates, and Inhibitors. *Archives of Biochemistry and Biophysics* **312**, 31-37.

Reddy, S. R. R., and Campbell, J. W. (1969). Arginine metabolism in insects: Properties of insect fat body arginase. *Comparative Biochemistry and Physiology* **28**, 515-534.

Redmond, D. L., Knox, D. P., Newlands, G. F., and Smith, W. D. (1997). Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **85**, 77-87.

Reed, R. E., and Hess, J. L. (1975). Partial purification and characterization of aspartate aminotransferases from seedling oat leaves. *Journal of Biological Chemistry* **250**, 4456-4461.

Rees, T. A. V., Larson, T. R., Heldens, J. W. G., and Huning, F. G. J. (1995). In situ glutamine synthetase activity in a marine unicellular alga. *Plant Physiology* **109**, 1405-1410.

Regunathan, S., and Reis, D. J. (2000). Characterization of arginine decarboxylase in rat brain and liver: distinction from ornithine decarboxylase. *Journal of Neurochemistry* **74**, 2201-2208.

Reiss, P. M., Pierce, S. K., and Bishop, S. H. (1977). Glutamate dehydrogenases from tissues of the ribbed mussel *Modiolus demissus*: ADP activation and possible physiological significance. *Journal of Experimental Zoology* **202**, 253-258.

Reitze, M., Schöttler, U., and Luftmann, H. (1989). Alanine metabolism of the lugworm *Arenicola marina* L. (Annelida, Polychaeta) during adaptation to reduced salinity. *Comparative Biochemistry and Physiology* **93B**, 689-696.

Reitzer, L. (2003). Nitrogen assimilation and global regulation in *Escherichia coli*. *Annual Review of Microbiology* **57**, 155-176.

Rej, R. (1981). Multiple molecular forms of human cytoplasmic aspartate aminotransferase. *Clinica Chimica Acta* **112**, 1-11.

Repetto, Y., Letelier, M. E., Aldunate, J., and Morello, A. (1987). The γ-glutamyltranspeptidase of *Trypanosoma cruzi*. *Comparative Biochemistry and Physiology* **87B**, 73-78.

Reuber, B. E., Karl, C., Reimann, S. A., Mihalik, S. J., and Dodt, G. (1997). Cloning and functional expression of a mammalian gene for a peroxisomal sarcosine oxidase. *Journal of Biological Chemistry* **272**, 6766-6776.

Rhoads, M. L., Fetterer, R. H., and J.F., U. (2001). Cuticular collagen synthesis by *Ascaris suum* during development from third to fourth larval stage: identification of a potential chemotherapeutic agent with a novel mechanism of action. *Journal of Parasitology* **87**, 1144-1149.

Rhodes, M. B., and Ferguson, D. L. (1973). *Haemonchus contortus*: Enzymes III Glutamate dehydrogenase. *Experimental Parasitology* **34**, 100-110.

Ricard, B., Couée, I., Raymond, P., Saglio, P. H., Saint-Ges, V., and Pradet, A. (1994). Plant metabolism under hypoxia and anoxia. *Plant Physiology and Biochemistry* **32**, 1-10.

Richards, N. G. J., and Schuster, S. M. (1998). Mechanistic issues in asparagine synthetase catalysis. *Advances in Enzymology and Related Areas of Molecular Biology* **72**, 145-198.

Richardson, D. J., and Watmough, N. J. (1999). Inorganic nitrogen metabolism in bacteria. *Current Opinion in Chemical Biology* **3**, 207-219.

Riding, I. L. (1970). Microvilli on the outside of a nematode. *Nature* **226**, 179-180.

Rivero, A. (2006). Nitric oxide: an antiparasitic molecule of invertebrates. *Trends in Parasitology* **22**, 219-225.

Roberts, C. W. R., Fiona; Lyons, Russell E.; Kirisits, Michael J.; Mui, Ernest J.; Finnerty, John. (2002). The shikimate pathway and its branches in apicomplexan parasites. *Journal of Infectious Diseases* **185**, S25-S37.

Roberts, F., Roberts, C. W., Johnson, J. J., Kyle, D. E., Krell, T., Coggins, J. R., Coombs, G. H., Milhous, W. K., Tzipori, S., Ferguson, D. J. P., Chakrabarti, D., and McLeod, R. (1998). Evidence for the shikimate pathway in apicomplexan parasites. *Nature* **393**, 801-805.

Roberts, J. K. M., and Pang, M. K. L. (1992). Estimation of ammonium ion distribution between cytoplasm and vacuole using nuclear magnetic resonance spectroscopy. *Plant Physiology* **100**, 1571-1574.

Roberts, L. S., and Platzer, E. G. (1967). Developmental physiology of cestodes. II. Effects of changes in host dietary carbohydrate and roughage on previously established *Hymenolepis diminuta*. *Journal of Parasitology* **53**, 85-93.

Roeder, T. (1999). Octopamine in invertebrates. *Progress in Neurobiology* **59**, 533-561.

Roeder, T., Seifert, M., Kähler, C., and Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behavior and metabolism. *Archives of Insect Biochemistry and Physiology* **54**, 1-13.

Rogers, W. P. (1952). Nitrogen catabolism in nematode parasites. *Australian Journal of Scientific Research B* **5**, 210-222.

Romero, R. M., Roberts, M. F., and Phillipson, J. D. (1995). Chorismate mutase in microorganisms and plants. *Phytochemistry* **40**, 1015-1025.

Rosenthal, G. A., Hughes, C. G., and Janzen, D. H. (1982). L-Canavanine, a dietary nitrogen source for the seed predator *Caryedes brasiliensis* (Bruchidae). *Science* **217**, 353-355.

Rossjohn, J., Feil, S. C., Wilce, M. C. J., Sexton, J. L., Spithill, T. W., and Parker, M. W. (1997). Crystallization, structural determination and analysis of a novel parasite vaccine candidate: *Fasciola hepatica* glutathione *S*-transferase. *Journal of Molecular Biology* **273**, 857-872.

Rosso, M.-N., Favery, B., Piotte, C., Arthaud, L., De Boer, J. M., Hussey, R. S., Bakker, J., Baum, T. J., and Abad, P. (1999). Isolation of a cDNA encoding a β-1,4-endoglucanase in the root-knot nematode *Meloidogyne incognita* and expression analysis during plant parasitism. *Molecular Plant-Microbe Interactions* **12**, 585-591.

Rothman, A. H., and Lee, D. L. (1963). Histochemical demonstration of dehydrogenase activity in the cuticle of cestodes. *Experimental Parasitology* **14**, 333-336.

Rothstein, M. (1963). Nematode biochemistry - III. Excretion products. *Comparative Biochemistry and Physiology* **9**, 51-59.

Rothstein, M. (1965). Nematode biochemistry - V. Intermediary metabolism and amino acid interconversions in *Caenorhabditis briggsae*. *Comparative Biochemistry and Physiology* **14**, 541-552.

Rothstein, M. (1970). Nitrogen metabolism in the aschelminthes. *In* "Comparative Biochemistry Of Nitrogen Metabolism" (J. W. Campbell, ed.), pp. 91-102. Academic Press, New York.

Rothstein, M., and Mayoh, H. (1964a). Glycine synthesis and isocitrate lyase in the nematode, *Caenorhabditis briggsae*. *Biochemical and Biophysical Research Communications* **14**, 43-47.

Rothstein, M., and Mayoh, H. (1964b). Nematode biochemistry IV. On isocitrate lyase in *Caenorhabditis briggsae*. *Archives of Biochemistry and Biophysics* **108**, 134-142.

Rothstein, M., and Mayoh, H. (1966). Nematode biochemistry - VIII Malate synthase. *Comparative Biochemistry and Physiology* **17**, 1181-1188.

Rothstein, M., and Tomlinson, G. (1962). Nematode biochemistry: II. Biosynthesis of amino acids. *Biochimica et Biophysica Acta* **63**, 471-480.

Rothstein, M., and Tomlinson, G. A. (1961). Biosynthesis of amino acids by the nematode *Caenorhabditis briggsae*. *Biochimica et Biophysica Acta* **49**, 625-627.

Rubino, S. D., Nyunoya, H., and Lusty, C. J. (1986). Catalytic domains of carbamyl phosphate synthetase. Glutamine-hydrolyzing site of *Escherichia coli* carbamyl phosphate synthetase. *Journal of Biological Chemistry* **261**, 11320-11327.

Rudolph, F. B., and Fromm, H. J. (1971). The purification and properties of aspartase from *Escherichia coli*. *Archives of Biochemistry and Biophysics* **147**, 92-98.

Ruiz Ruano, A., Allende Riaño, J. L., Ruiz Amil, M., and Herranz Santos, M. J. (1985). Some enzymatic properties of NAD^+-dependent glutamate dehydrogenase of mussel hepatopancreas (*Mytilus edulis* L.) - requirement of ADP. *Comparative Biochemistry and Physiology* **82B**, 197-202.

Ruščák, M., Orlický, J., and Žúbor, V. (1982). Isoelectric focusing of the alanine aminotransferase isoenzymes from the brain, liver and kidney. *Comparative Biochemistry and Physiology* **71B**, 141-144.

Saeed-Kothe, A., and Powers-Lee, S. G. (2002). Specificity determining residues in ammonia- and glutamine-dependent carbamoyl phosphate synthetases. *Journal of Biological Chemistry* **277**, 7231-7238.

Saier, M. H., and Jenkins, W. T. (1967). Alanine aminotransferase. I. Purification and properties. *Journal of Biological Chemistry* **242**, 91-100.

Sajid, M., and McKerrow, J. H. (2002). Cysteine proteases of parasitic organisms. *Molecular and Biochemical Parasitology* **120**, 1-21.

Salama, A., Morgan, I., and Wood, C. (1999). The linkage between Na^+ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of $(NH_4)_2SO_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. *Journal of Experimental Biology* **202**, 697-709.

Salinas, G., Braun, G., and Taylor, D. W. (1994). Molecular characterisation and localisation of an *Onchocerca volvulus* π-class glutathione *S*-transferase. *Molecular and Biochemical Parasitology* **66**, 1-9.

Salinas, G., Sinha, K., Cooper, J. P., Whitworth, J. A. G., and Taylor, D. W. (1996). Human isotope antibody responses to an *Onchocerca volvulus* glutathione *S*-transferase. *Parasite Immunology* **18**, 377-386.

Salvatore, F., Zappia, V., and Costa, C. (1965). Comparative biochemistry of deamination of L-amino acids in elasmobranch and teleost fish. *Comparative Biochemistry and Physiology* **16**, 303-309.

Salvatore, L., Wijffels, G., Sexton, J. L., Panaccio, M., Mailer, S., McCauley, I., and Spithill, T. W. (1995). Biochemical analysis of recombinant glutathione *S*-transferase of *Fasciola hepatica*. *Molecular and Biochemical Parasitology* **69**, 281-288.

Samson, M.-L. (2000). *Drosophila* arginase is produced from a nonvital gene that contains the *elav* locus within its third intron. *Journal of Biological Chemistry* **275**, 31107-31114.

Sasaki, T., and Ishikawa, H. (1995). Production of essential amino acids from glutamate by mycetocyte symbionts of the pea aphid, *Acyrthosiphon pisum*. *Journal of Insect Physiology* **41**, 41-46.

Sasaki, T., Kawamura, M., and Ishikawa, H. (1996). Nitrogen recycling in the brown planthopper, *Nilaparvata lugens*: Involvement of yeast-like endosymbionts in uric acid metabolism. *Journal of Insect Physiology* **42**, 125-129.

Saz, H. J. (1981). Energy metabolism in parasitic helminths: Adaptations to parasitism. *Annual Review of Physiology* **43**, 323-341.

Scaraffia, P. Y., Isoe, J., Murillo, A., and Wells, M. A. (2005). Ammonia metabolism in *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* **35**, 491-503.

Scaraffia, P. Y., and Wells, M. A. (2003). Proline can be utilized as an energy substrate during flight of *Aedes aegypti* females. *Journal of Insect Physiology* **49**, 591-601.

Schaeffer, J. M., and Donatelli, M. R. (1990). Characterization of a high-affinity membrane-associated ornithine decarboxylase from the free-living nematode *Caenorhabditis elegans*. *Biochemical Journal* **270**, 599-604.

Scholfield, P. J., Costello, M., Edwards, M. R., and O'Sullivan, W. J. (1990). The arginine dihydrolase pathway is present in *Giardia intestinalis*. *International Journal for Parasitology* **20**, 697 - 699.

Schumann, J., Böhm, G., Schumacher, G., Rudolph, R., and Jaenicke, R. (1993). Stabilization of creatinase from *Pseudomonas putida* by random mutagenesis. *Protein Science* **2**, 1612-1620.

Schuske, K., Beg, A. A., and Jorgensen, E. M. (2004). The GABA nervous system in *C. elegans*. *Trends in Neurosciences* **27**, 407-414.

Schwartz, J. H., Reeves, J. Y., and Broome, J. D. (1966). Two L-asparaginases from *E. coli* and their action against tumors. *Proceedings of the National Academy of Sciences* **56**, 1516-1519.

Seaman, G. R. (1960). Amino acid decarboxlases in a Pseudomonad. *Journal of Bacteriology* **80**, 830-836.

Senft, A. W. (1966). Studies in arginine metabolism by schistosomes - I. Arginine uptake and lysis by *Schistosoma mansoni*. *Comparative Biochemistry and Physiology* **18**, 209-216.

Senft, A. W., Miech, R. P., Brown, P. R., and Senft, D. G. (1972). Purine metabolism in *Schistosoma mansoni*. *International Journal for Parasitology* **2**, 249-260.

Senwo, Z. N., and Tabatabai, M. A. (1999). Aspartase activity in soils: effects of trace elements and relationships to other amidohydrolases. *Soil Biology and Biochemistry* **31**, 213-219.

Setoyama, C., Ding, S., Choudhury, B., Joh, T., Takeshima, H., Tsuzuki, T., and Shimada, K. (1990). Regulatory regions of the mitochondrial and cytosolic isoenzyme genes participating in the malate-aspartate shuttle. *Journal of Biological Chemistry* **265**, 1293-1299.

Sexton, J. L., Wilce, M. C. J., Colin, T., Wijffels, G. L., Salvatore, L., Feil, S. C., Parker, M. W., Spithill, T. W., and Morrison, C. A. (1994). Vaccination of sheep against *Fasciola hepatica* with glutathione S-transferase. *Journal of Immunology* **152**, 1861-1872.

Shallig, H. D. F., Van Leewen, M. A. W., and Hendrix, W. M. L. (1994). Immune responses of Texel sheep to excretory-secretory products of adult *Haemonchus contortus*. *Parasitology* **108**, 351-357.

Shamansky, L. M., Pratt, D., Boisvenue, R. J., and Cox, G. N. (1989). Cuticle collagen genes of *Haemonchus contortus* and *Caenorhabditis elegans* are highly conserved. *Molecular and Biochemical Parasitology* **37**, 73-85.

Sharma, V., Visen, P. K. S., Katiyar, J. C., Wittich, R.-M., Walter, R. D., Ghatak, S., and Shukla, O. P. (1989). Polyamine metabolism in *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis*. *International Journal for Parasitology* **19**, 191-198.

Shaw, J. P., Petsko, G. A., and Ringe, D. (1997). Determination of the structure of alanine racemase from *Bacillus stearothermophilus* at 1.9-Å resolution. *Biochemistry* **36**, 1329-1342.

Shayakul, C., and Hediger, M. A. (2004). The SLC14 gene family of urea transporters. *Pflügers Archives - European Journal of Physiology* **447**, 603-609.

Sheers, M., Campbell, A. J., Beames, D. J., Edwards, S. R., Moore, R. J., and Montague, P. E. (1982). Fasciolicidal potential of proline analogues and proline biosynthesis inhibitors. *International Journal for Parasitology* **12**, 47-52.

Shelp, B. J., Bown, A. W., and McLean, M. D. (1999). Metabolism and function of gamma-aminobutyric acid. *Trends in Plant Science* **4**, 446-452.

Shibata, K., Shirasuna, K., Motegi, K., Kera, Y., Abe, H., and Yamada, R.-h. (2000). Purification and properties of alanine racemase from crayfish *Procambarus clarkii*. *Comparative Biochemistry and Physiology* **126B**, 599-608.

Shin, D., and Park, C. (2004). N-terminal extension of canine glutamine synthetase created by splicing alters Its enzymatic property. *Journal of Biological Chemistry* **279**, 1184-1190.

Shoemaker, C. B. (1994). The *Schistosoma mansoni* phosphagen kinase gene contains two closely apposed transcription initiation sites and arose from a fused gene duplication. *Molecular and Biochemical Parasitology* **68**, 319-322.

Shrawder, E. J., and Martinez-Carrion, M. (1973). Simultaneous isolation and characterization of chicken supernatant and mitochondrial isoenzymes of aspartate transaminase. *Journal of Biological Chemistry* **248**, 2140-2146.

Siddiqui, Z. A., and Mahmood, I. (1995). Role of plant symbionts in nematode management: A review. *Bioresource Technology* **54**, 217-226.

Sieciechowicz, K. A., Joy, K. W., and Ireland, R. J. (1988). The metabolism of asparagine in plants. *Phytochemistry* **27**, 663-671.

Silver, W. S., and Postgate, J. R. (1973). Evolution of asymbiotic nitrogen fixation. *Journal of Theoretical Biology* **40**, 1-10.

Simmons, J. E. (1960). Urease activity in Trypanorhynch cestodes. *Biological Bulletin* **121**, 535-546.

Singer, M. A. (2003). Do mammals, birds, reptiles and fish have similar nitrogen conserving systems? *Comparative Biochemistry and Physiology* **134B**, 543-558.

Singh, G., Pampori, N. A., and Srivastava, V. M. L. (1983). Metabolism of amino acids in *Ascaridia galli*: decarboxylation reactions. *International Journal for Parasitology* **13**, 305-307.

Singh, J., Joshi, M. C., and Bhatnagar, R. (2004). Cloning and expression of mycobacterial glutamine synthetase gene in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **317**, 634-638.

Singh, R. N., Chandrashekar, R., and Mehta, K. (1995). Purification and partial characterization of a transglutaminase from dog filarial parasite, *Dirofilaria immitis*. *International Journal of Biochemistry and Cell Biology* **27**, 1285-1291.

Singh, R. N., and Mehta, K. (1994). Purification and characterization of a novel transglutaminase from filarial nematode *Brugia malayi*. *European Journal of Biochemistry* **225**, 625-634.

Siranosian, K. J., Ireton, K., and Grossman, A. D. (1993). Alanine dehydrogenase (*ald*) is required for normal sporulation in *Bacillus subtilis*. *Journal of Bacteriology* **175**, 6789-6796.

Skuce, P. J., Redmond, D. L., Liddell, S., Stewart, E. M., Newlands, G. F. J., Smith, W. D., and Knox, D. P. (1999a). Molecular cloning and characterization of gut-derived cysteine proteinases associated with a host protective extract from *Haemonchus contortus*. *Parasitology* **119**, 405-412.

Skuce, P. J., Stewart, E. M., Smith, W. D., and Knox, D. P. (1999b). Cloning and characterization of glutamate dehydrogenase (GDH) from the gut of *Haemonchus contortus*. *Parasitology* **118**, 297-304.

Slocum, R. D., Kaur-Sawhney, R., and Galston, A. W. (1984). The physiology and biochemistry of polyamines in plants. *Archives of Biochemistry and Biophysics* **235**, 283-303.

Small, W., and Jones, M. (1990). Pyrroline 5-carboxylate dehydrogenase of the mitochondrial matrix of rat liver. Purification, physical and kinetic characteristics. *Journal of Biological Chemistry* **265**, 18668-18672.

Smith, C. P., and Wright, P. A. (1999). Molecular characterization of an elasmobranch urea transporter. *American Journal of Physiology* **276**, R622-R626.

Smith, E. M., and Watford, M. (1988). Rat hepatic glutaminase: Purification and immunochemical characterization. *Archives of Biochemistry and Biophysics* **260**, 740-751.

Sohocki, M. M., Sullivan, L. S., Harrison, W. R., Sodergren, E. J., Elder, F. F. B., Weinstock, G., Tanase, S., and Daiger, S. P. (1997). Human glutamate pyruvate transaminase (GPT): Localization to 8q24.3, cDNA and genomic sequences, and polymorphic sites. *Genomics* **40**, 247-252.

Sommerville, R. I. (1954). The histotrophic phase of the nematode parasite, *Ostertagia circumcincta*. *Australian Journal of Agricultural Research* **5**, 130-140.

Sommerville, R. I. (1963). Differential growth of *Ostertagia spp* in the sheep's abomasum. *Journal of Parasitology* **49**, 698-699.

Son, D., Jo, J., and Sugiyama, T. (1991). Purification and characterization of alanine aminotransferase from *Panicum miliaceum* leaves. *Archives of Biochemistry and Biophysics* **289**, 262-266.

Song, S., Lei, Y., and Tian, X. (2005). Proline metabolism and cross-tolerance to salinity and heat stress in germinating wheat seeds. *Russian Journal of Plant Physiology* **52**, 793-800.

Srivastava, A. S., I., O., T., S., and S.N., S. (1999). Activity and expression of aspartate aminotransferase during the reproductive cycle of a fresh water fish, *Clarias batrachus*. *Fish Physiology and Biochemistry* **20**, 243-250.

Stadtman, E. R. (2001). The story of glutamine synthetase regulation. *Journal of Biological Chemistry* **276**, 44357-44364.

Stafford, K. J., West, D. M., and Pomroy, W. E. (1994). Nematode worm egg output by ewes. *New Zealand Veterinary Journal* **42**, 30-32.

Stein, L. D., Harn, D. A., and David, J. R. (1990). A cloned ATP:guanidino kinase in the trematode *Schistosoma mansoni* has a novel duplicated structure. *Journal of Biological Chemistry* **265**, 6582-6588.

Stevens, C. E., and Hume, I. D. (1998). Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews* **78**, 393-427.

Strange, K. (2003). From genes to integrative physiology: ion channel and transporter biology in *Caenorhabditis elegans*. *Physiological Reviews* **83**, 377-415.

Strecker, H. J. (1953). Glutamic dehydrogenase. *Archives of Biochemistry and Biophysics* **46**, 128-140.

Streeter, J. G. (1989). Estimation of ammonium concentration in the cytosol of soybean nodules. *Plant Physiology* **90**, 779-782.

Streeter, J. G., and Thompson, J. F. (1972). Anaerobic accumulation of λ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). *Plant Physiology* **49**, 572-578.

Suárez, I., Bodega, G., and Fernández, B. (2002). Glutamine synthetase in brain: effect of ammonia. *Neurochemistry International* **41**, 123-142.

Sugimura, M., Hirayama, C., and Nakamura, M. (2001). Selective transport of the mulberry leaf urease from the midgut into the larval hemolymph of the silkworm, *Bombyx mori*. *Journal of Insect Physiology* **47**, 1133-1138.

Sumida, M., Yoshio, H., Tanaka, Y., and Matsubara, F. (1995). Developmental changes in urea concentrations in the haemolymph of Daizo (T), an original strain of the silkworm, *Bombyx mori* reared on an artificial diet and on fresh mulberry leaves. *Comparative Biochemistry and Physiology* **110A**, 33-38.

Sun, L., Bartlam, M., Liu, Y., Pang, H., and Rao, Z. (2005). Crystal structure of the pyridoxal-5'-phosphate-dependent serine dehydratase from human liver. *Protein Science* **14**, 791-798.

Surber, M. W., and Maloy, S. (1998). The PutA protein of *Salmonella typhimurium* catalyzes the two steps of proline degradation via a leaky channel. *Archives of Biochemistry and Biophysics* **354**, 281-287.

Suzuki, A., and Knaff, D. B. (2005). Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. *Photosynthesis Research* **83**, 191-217.

Suzuki, K., Sagai, H., Sugiyama, M., and Imamura, S. (1993). Molecular cloning and high expression of the *Bacillus* creatinase gene in *Escherichia coli*. *Journal of Fermentation and Bioengineering* **76**, 77-81.

Suzuki, S., Yamaguchi, J., and Tokushige, M. (1973). Studies on aspartase. I. Purification and molecular properties of aspartase from *Escherichia coli*. *Biochimica et Biophysica Acta* **321**, 369-381.

Suzuki, T., Kawasaki, Y., and Furukohri, T. (1997). Evolution of phosphagen kinase. *Biochemical Journal* **328**, 301-306.

Swick, R. W., Barnstein, P. L., and Stange, J. L. (1965). The metabolism of mitochondrial proteins. I. Distribution and characterization of the isozymes of alanine aminotransferase in rat liver. *Journal of Biological Chemistry* **240**, 3334-3340.

Szweda, L. I., and Atkinson, D. E. (1990). Response of rat liver glutaminase to magnesium ion. *Biochimica et Biophysica Acta* **1041**, 201-206.

Tabor, C. W., and Tabor, H. (1985). Polyamines in microorganisms. *Microbiological Reviews* **49**, 81-99.

Tapiero, H., Mathé, G., Couvreur, P., and Tew, K. D. (2002). Dossier: Free amino acids in human health and pathologies II. Glutamine and glutamate. *Biomedicine and Pharmacotherapy* **56**, 446-457.

Tarcsa, E., Eckerstofer, M., Breitenbach, M., Hintz, M., Schott, H.-H., Zahner, H., and Stirm, S. (1992). ξ-(λGlutamyl)lysine cross-links in *Litomosoides carinii* microfilarial sheaths. *Parasitology Research* **78**, 623-624.

Taylor, P. H., Wallace, J. C., and Keech, D. B. (1971). Gluconeogenic enzymes in sheep liver: Intracellular localization of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in normal, fasted and diabetic sheep. *Biochimica et Biophysica Acta* **237**, 179-191.

Taylor-Robinson, A., and Looker, M. (1998). Sensitivity of malaria parasites to nitric oxide at low oxygen tensions. *Lancet* **351**, 1630.

Teller, J. K. (1988a). Purification and some properties of glutamate dehydrogenase from the mealworm fat body. *Insect Biochemistry* **18**, 101-106.

Teller, J. K. (1988b). Kinetic properties of glutamate dehydrogenase purified from the mealworm fat body. The glutamate synthesizing direction. *Comparative Biochemistry and Physiology* **90B**, 329-333.

Tempest, D. W., Meers, J. L., and Brown, C. M. (1970). Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. *Biochemical Journal* **117**, 405-407.

ter Schure, E. G., van Riel, N. A. W., and Verrips, C. T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **24**, 67-83.

Teranishi, H., Kagamiyama, H., Teranishi, K., Wada, H., Yamano, T., and Morino, Y. (1978). Cytosolic and mitochondrial isoenzymes of glutamic-oxalacetic transaminase from human heart. Structural comparison with the isoenzymes from pig heart. *Journal of Biological Chemistry* **253**, 8842-8847.

Thomas, E. L., Grisham, M. B., and Jefferson, M. M. (1983). Myeloperoxidase-dependent effect of amines on functions of isolated neutrophils. *Journal of Clinical Investigation* **72**, 441-454.

Thornton, B., and Robinson, D. (2005). Uptake and assimilation of nitrogen from solutions containing multiple N sources. *Plant, Cell and Environment* **28**, 813-821.

Thorpe, E. (1968). Comparative enzyme histochemistry of immature and mature stages of *Fasciola hepatica*. *Experimental Parasitology* **22**, 150-159.

Tiwari, S., and Singh, A. (2003). Control of common freshwater predatory fish, *Channa punctatus*, through *Nerium indicum* leaf extracts. *Chemosphere* **53**, 865-875.

Toney, M. D. (2005). Reaction specificity in pyridoxal phosphate enzymes. *Archives of Biochemistry and Biophysics* **433**, 279-287.

Tormanen, C. D. (1997). The effect of metal ions on arginase from the zebra mussel *Dreissena polymorpha*. *Journal of Inorganic Biochemistry* **66**, 111-118.

Tormanen, C. D. (2001). Allosteric inhibition of rat liver and kidney arginase by copper and mercury ions. *Journal of Enzyme Inhibition and Medicinal Chemistry* **16**, 443-449.

Tormanen, C. D. (2006). Inhibition of rat liver and kidney arginase by cadmium ion. *Journal of Enzyme Inhibition and Medicinal Chemistry* **21**, 119-123.

Tornheim, K., and Lowenstein, J. M. (1972). The purine nucleotide cycle. The production of ammonia from aspartate by extracts of rat skeletal muscle. *Journal of Biological Chemistry* **247**, 162-169.

Tsahar, E., Martinez del Rio, C., Izhaki, I., and Arad, Z. (2005). Can birds be ammonotelic? Nitrogen balance and excretion in two frugivores. *Journal of Experimental Biology* **208**, 1025-1034.

Tsuji, N., Morales, T. H., V. Ozols, V., Carmody, A. B., and Chandrashekar, R. (1999). Identification of an asparagine amidohydrolase from the filarial parasite *Dirofilaria immitis*. *International Journal for Parasitology* **29**, 1451-1455.

Tuma, P. L., and Hubbard, A. L. (2003). Transcytosis: crossing cellular barriers. *Physiological Reviews* **83**, 871-932.

Turano, F. J., Wilson, B. J., and Matthews, B. F. (1990). Purification and characterization of aspartate aminotransferase isoenzymes from carrot suspension cultures. *Plant Physiology* **92**, 587-594.

Turner, A. C., Lushbaugh, W. B., and Hutchison, W. F. (1986). *Dirofilaria immitis*: Comparison of cytosolic and mitochondrial glutamate dehydrogenases. *Experimental Parasitology* **61**, 176-183.

Uglem, G. L., Pappas, P. W., and Read, C. P. (1973). Surface aminopeptidase in *Moniliformis dubius* and its relation to amino acid uptake. *Parasitology* **67**, 185-195.

Urbina, J. A., and Azavache, V. (1984). Regulation of energy metabolism in *Trypanosoma (Schizotrypanum) cruzi* epimastigotes. II. NAD^+-dependent glutamate dehydrogenase. *Molecular and Biochemical Parasitology* **11**, 241-255.

Ureta, A., and Nordlund, S. (2001). Glutamine synthetase from *Acetobacter diazotrophicus*: properties and regulation. *FEMS Microbiology Letters* **202**, 177-180.

van den Heuvel, R. H. H., Curti, B., Vanoni, M. A., and Mattevi, A. (2004). Glutamate synthase: a fascinating pathway from L-glutamine to L-glutamate. *Cellular and Molecular Life Sciences* **61**, 669-681.

Van Leuven, F. (1976). Glutamine transaminase from brain tissue. Further studies on kinetic properties and specificity of the enzyme. *European Journal of Biochemistry* **65**, 271-274.

Van Nieuwenhove, S., Schechter, P. J., Declercq, J., Bone, G., Burke, J., and Sjoerdsma, A. (1985). Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-α-difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 692-698.

Van Pilsum, J. F., Stephens, G. C., and Taylor, D. (1972). Distribution of creatine, guanidinoacetate and the enzymes for their biosynthesis in the animal kingdom. *Biochemical Journal* **126**, 325-345.

Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A., and Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. *Gene* **332**, 13-27.

Vanoni, M., A., Dossena, L., van den Heuvel, R. H. H., and Curti, B. (2005). Structure-function studies on the complex iron-sulfur flavoprotein glutamate synthase: the key enzyme of ammonia assimilation. *Photosynthesis Research* **83**, 219-238.

Vanoni, M. A., and Curti, B. (1999). Glutamate synthase: a complex iron-sulfur flavoprotein. *Cellular and Molecular Life Sciences* **55**, 617-638.

Varela-Echavarría, A., Montes de Oca-Luna, R., and Barrera-Saldaña, H. A. (1988). Uricase protein sequences: conserved during vertebrate evolution but absent in humans. *FASEB Journal* **2**, 3092-3096.

Vedavathi, M., Girish, K. S., and Karuna Kumar, M. (2004). Isolation and characterization of cytosolic alanine aminotransferase isoforms from starved rat liver. *Molecular and Cellular Biochemistry* **267**, 13-23.

Veljkovic, E., Bacconi, A., Stetak, A., Hajnal, A., Stasiuk, S., Skelly, P. J., Forster, I., Shoemaker, C. B., and Verrey, F. (2004a). Aromatic amino acid transporter AAT-9 of *Caenorhabditis elegans* localizes to neurons and muscle cells. *Journal of Biological Chemistry* **279**, 49268-49273.

Veljkovic, E., Stasiuk, S., Skelly, P. J., Shoemaker, C. B., and Verrey, F. (2004b). Functional characterization of *Caenorhabditis elegans* heteromeric amino acid transporters. *Journal of Biological Chemistry* **279**, 7655-7662.

Vender, J., and Rickenberg, H. V. (1964). Ammonia metabolism in a mutant of *Escherichia coli* lacking glutamate dehydrogenase. *Biochimica et Biophysica Acta* **90**, 218-220.

Vengušt, G., Klinkon, M., Bidovec, A., and Vengušt, A. (2003). *Fasciola hepatica*: effects on blood constituents and liver minerals in fallow deer (*Dama dama*). *Veterinary Parasitology* **112**, 51-61.

Verleur, N., Elgersma, Y., Roermund, C. W. T., Tabak, H. F., and Wanders, R. J. A. (1997). Cytosolic aspartate aminotransferase encoded by the *AAT2* gene is targeted to the peroxisomes in oleate-grown *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **247**, 972-980.

Veronico, P., Gray, L. J., Jones, J. T., Bazzicalupo, P., Arbucci, S., Cortese, M. R., Di Vito, M., and De Giorgi, C. (2001). Nematode chitin synthases: gene structure, expression and function in *Caenorhabditis elegans* and the plant parasitic nematode *Meloidogyne artiellia*. *Molecular Genetics and Genomics* **266**, 28-34.

Verrey, F., Closs, E. I., Wagner, C. A., Palacin, M., Endou, H., and Kanai, Y. (2004). CATs and HATs: the SLC7 family of amino acid transporters. *Pflügers Archive - European Journal of Physiology* **447**, 532-542.

Vílchez, S., Molina, L., Ramos, C., and Ramos, J. L. (2000). Proline catabolism by *Pseudomonas putida*: cloning, characterization, and expression of the *put* genes in the presence of root exudates. *Journal of Bacteriology* **182**, 91-99.

Vincendeau, P., Gobert, A. P., Daulouede, S., Moynet, D., and Djavad Mossalayi, M. (2003). Arginases in parasitic diseases. *Trends in Parasitology* **19**, 9-12.

Viola, R. E. (2000). L-aspartase: new tricks from an old enzyme. *In "Adances in Enzymology and Related Areas of Molecular Biology: Mechanism of Enzyme Action, Part B"* (D. L. Purich, ed.), Vol. 74, pp. 295-341. John Wiley and Sons, Inc.

Visek, W. J. (1984). Ammonia: its effects on biological systems, metabolic hormones, and reproduction. *Journal of Dairy Science* **67**, 481-498.

von Besser, H., Niemann, G., Domdey, B., and Walter, R. D. (1995). Molecular cloning and characterization of ornithine decarboxylase cDNA of the nematode *Panagrellus redivivus*. *Biochemical Journal* **308**, 635-640.

Vorhaben, J. E., Wong, L., and Campbell, J. W. (1973). Assay for glutamine synthetase activity. *Biochemical Journal* **135**, 893-896.

Waagepetersen, H. S., Qu, H., Sonnewald, U., Shimamoto, K., and Schousboe, A. (2005). Role of glutamine and neuronal glutamate uptake in glutamate homeostasis and synthesis during vesicular release in cultured glutamatergic neurons. *Neurochemistry International* **47**, 92-102.

Wagner, C. A., Lang, F., and Broer, S. (2001). Function and structure of heterodimeric amino acid transporters. *American Journal of Physiology* **281**, C1077-C1093.

Walker, J., and Barrett, J. (1991a). Pyridoxal 5'-phosphate dependent enzymes in the nematode *Nippostrongylus brasiliensis*. *International Journal for Parasitology* **21**, 641-649.

Walker, J., and Barrett, J. (1991b). Studies on alanine aminotransferase in nematodes. *International Journal for Parasitology* **21**, 377-380.

Walker, J., and Barrett, J. (1992). Biochemical characterisation of the enzyme responsible for activated L-serine sulphydrase activity in nematodes. *Experimental Parasitology* **74**, 205-215.

Walker, J., and Barrett, J. (1997). Parasite sulphur amino acid metabolism. *International Journal for Parasitology* **27**, 883-897.

Walker, J., Crowley, P., Moreman, A. D., and Barrett, J. (1993). Biochemical properties of cloned glutathione *S*-transferases from *Schistostoma mansoni* and *Schistostoma japonicum*. *Molecular and Biochemical Parasitology* **61**, 255-264.

Wallimann, T., and Eppenberger, H. M. (1973). Properties of arginine kinase from *Drosophila melanogaster*. *European Journal of Biochemistry* **38**, 180-184.

Walsh, P. J. (1997). Evolution and regulation of urea synthesis and ureotely in (Batrachoidid) fishes. *Annual Review of Physiology* **59**, 299-323.

Walsh, P. J., Grosell, M., Goss, G. G., Bergman, H. L., Bergman, A. N., Wilson, P. R., Laurent, P., Alper, S. L., Smith, C. P., Kamunde, C., and Wood, C. M. (2001). Physiological and molecular characterization of urea transport by the gills of the Lake Magadi tilapia (*Alcolapia grahami*). *Journal of Experimental Biology* **204**, 509-520.

Walter, R. D. (1988). Polyamine metabolism of filaria and allied parasites. *Parasitology Today* **4**, 18-20.

Walton, M. J., and Cowey, C. B. (1982). Aspects of intermediary metabolism in salmonid fish. *Comparative Biochemistry and Physiology* **73B**, 59-79.

Wang, L.-J., Kong, X.-D., Zhang, H.-Y., Wang, X.-P., and Zhang, J. (2000). Enhancement of the ativity of L-aspartase from *Escherichia coli* W by directed evolution. *Biochemical and Biophysical Research Communications* **276**, 346-349.

Wang, Y., Ma, X., Zhao, W., Jia, X., Kai, L., and Xu, X. (2006). Study on the creatinase from *Paracoccus* sp strain WB1. *Process Biochemistry* **41**, 2072-2077.

Ward, D. E., Kengen, S. W. M., van der Oost, J., and de Vos, W. M. (2000). Purification and characterization of the alanine aminotransferase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its role in alanine production. *Journal of Bacteriology* **182**, 2559-2566.

Watanabe, M., Hoshino, T., Kikuchi, A., and Watanabe, Y. (1999). Purification and characterization of two glutamate dehydrogenase isoenzymes from *Brassica napus*. *Plant Physiology and Biochemistry* **37**, 731-739.

Watts, S. D. M. (1970). Transamination in homogenates of rediae of *Cyptocotyle lingua* and of sporocysts of *Cercaria emasculans* Pelseneer, 1900. *Parasitology* **61**, 499-504.

Watts, S. D. M., and Atkins, A. M. (1983). Application of a quick, simple and direct radiometric assay for 4-aminobutyrate: 2-oxoglutarate aminotransferase to studies of the parasitic nematode *Nippostrongylus brasiliensis*. *Comparative Biochemistry and Physiology* **76B**, 899-906.

Watts, S. D. M., and Atkins, A. M. (1984). Kinetics of 4-aminobutyrate:2-oxoglutarate aminotransferase from *Nippostrongylus brasiliensis*. *Molecular and Biochemical Parasitology* **12**, 207-216.

Weatherly, N. F., Hansen, M. F., and Moser, H. C. (1963). *In vitro* uptake of C^{14}-labeled alanine and glucose by *Ascaridia galli* (Nematoda) of chickens. *Experimental Parasitology* **14**, 37-48.

Webb, R. A. (1986). The uptake and metabolism of L-glutamate by tissue slices of the cestode *Hymenolepis diminuta*. *Comparative Biochemistry and Physiology* **85C**, 151-162.

Weihrauch, D., Morris, S. C., and Towle, D. W. (2004). Ammonia excretion in aquatic and terrestrial crabs. *Journal of Experimental Biology* **207**, 4491-4504.

Weingand-Ziade, A., Gerber-Decombaz, C., and Affolter, M. (2003). Functional characterization of a salt- and thermotolerant glutaminase from Lactobacillus rhamnosus. *Enzyme and Microbial Technology* **32**, 862-867.

Wertheim, G., Zeledon, R., and Read, C. P. (1960). Transaminases of tapeworms. *Journal of Parasitology* **46**, 497-499.

Whitcomb, C. E. (1999). An introduction to ALS-inhibiting herbicides. *Tocicology and Industrial Health* **15**, 232-240.

White, E., Hart, D., and Sanderson, B. E. (1983). Polyamines in *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **9**, 309-318.

White, M. F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochimica et Biophysica Acta* **822**, 355-374.

White, W. H., Gunyuzlu, P. L., and Toyn, J. H. (2001). *Saccharomyces cerevisiae* is capable of *de novo* pantothenic acid biosynthesis involving a novel pathway of β-alanine production from spermine. *Journal of Biological Chemistry* **276**, 10794-10800.

Whitehead , L., Wilkinson , T., and Douglas , A. (1992). Nitrogen recycling in the pea aphid (Acyrthosiphon pisum) symbiosis. *Proceedings of the Royal Society of London* **B250**, 115-117.

Wickes, M. A., and Morgan, R. P. (1976). Effects of salinity on three enzymes involved in amino acid metabolism from the American oyster, *Crassostrea virginica*. *Comparative Biochemistry and Physiology* **53B**, 339-343.

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *Journal of Experimental Zoology* **293**, 284-301.

Willett, C. S., and Burton, R. S. (2002). Proline biosynthesis genes and their regulation under salinity stress in the euryhaline copepod *Tigriopus californicus*. *Comparative Biochemistry and Physiology* **132B**, 739-750.

Williams, C. L., Preston, T., Hossack, M., Slater, C., and McColl, K. E. L. (1996). *Helicobacter pylori* utilises urea for amino acid synthesis. *FEMS Immunology and Medical Microbiology* **13**, 87-94.

Williams, L. E., and Miller, A. J. (2001). Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 659-688.

Williams, V. R., and McIntyre, R. T. (1955). Preparation and partial purification of the aspartase of *Bacterium cadaveris*. *Journal of Biological Chemistry* **217**, 467-478.

Williamson, A. L., Brindley, P. J., Knox, D. P., Hotez, P. J., and Loukas, A. (2003a). Digestive proteases of blood-feeding nematodes. *Trends in Parasitology* **19**, 417-423.

Williamson, A. L., Brindley, P. J., and Loukas, A. (2003b). Hookworm cathepsin D aspartic proteases: contributing roles in the host-specific degradation of serum proteins and skin macromolecules. *Parasitology* **126**, 179-185.

Williamson, J. M., and Brown, G. M. (1979). Purification and properties of L-aspartate-α-decarboxylase, an enzyme that catalyzes the formation of β-alanine in *Escherichia coli*. *Journal of Biological Chemistry* **254**, 8074-8082.

Windmueller, H., and Spaeth, A. (1980). Respiratory fuels and nitrogen metabolism *in vivo* in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate. *Journal of Biological Chemistry* **255**, 107-112.

Wipf, D., Ludewig, U., Tegeder, M., Rentsch, D., Koch, W., and Frommer, W. B. (2002). Conservation of amino acid transporters in fungi, plants and animals. *Trends in Biochemical Sciences* **27**, 139-147.

Wittich, R.-M., Kilian, H.-D., and Walter, R. D. (1987). Polyamine metabolism in filarial worms. *Molecular and Biochemical Parasitology* **24**, 155-162.

Wittich, R.-M., and Walter, R. D. (1989). A novel type of putrescine (diamine)-acetylating enzyme from the nematode *Ascaris suum*. *Biochemical Journal* **260**, 265-269.

Wittich, R.-M., and Walter, R. D. (1990). Putrescine *N*-acetyltransferase in *Onchocerca volvulus* and *Ascaris suum*, an enzyme which is involved in polyamine degradation and release of *N*-acetylputrescine. *Molecular and Biochemical Parasitology* **38**, 13-17.

Wittwer, A., and Wagner, C. (1981). Identification of the folate-binding proteins of rat liver mitochondria as dimethylglycine dehydrogenase and sarcosine dehydrogenase. Purification and folate-binding characteristics. *Journal of Biological Chemistry* **256**, 4102-4108.

Wolfersberger, M. G. (2000). Amino acid transport in insects. *Annual Review of Entomology* **45**, 111-120.

Wolf-Spengler, M. L., and Isseroff, H. (1983). Fascioliasis: bile duct collagen induced by proline from the worm. *Journal of Parasitology* **69**, 290-294.

Wong, P. C. L., and Yeung, S.-B. (1981). Pathways of purine ribonucleotide biosynthesis in the adult worm *Metastrongylus apri* (nematoda: Metastronglyloidea) from pig lung. *Molecular and Biochemical Parasitology* **2**, 285-293.

Wong, P. T.-H. (1985). Rat brain aspartate β-decarboxylase. A comparative study with the liver enzyme. *Neurochemistry International* **7**, 351-355.

Wood, C. M., McDonald, M. D., Sundin, L., Laurent, P., and Walsh, P. J. (2003). Pulsatile urea excretion in the gulf toadfish: mechanisms and controls. *Comparative Biochemistry and Physiology* **136B**, 667-684.

Woods, S. A., Miles, J. S., and Guest, J. R. (1988). Sequence homologies between argininosuccinase, aspartase and fumarase: A family of structurally-related enzymes. *FEMS Microbiology Letters* **51**, 181-186.

Woolfolk, C. A., and Stadtman, E. R. (1967). Regulation of glutamine synthetase III. Cumulative feedback inhibition of glutamine synthetase from *Escherichia coli*. *Archives of Biochemistry and Biophysics* **118**, 736-755.

Wright, D. J. (1975a). Studies on nitrogen catabolism in *Panagrellus redivivus*, Goodey, 1945 (Nematoda: Cephalobidae). *Comparative Biochemistry and Physiology* **52B**, 255-260.

Wright, D. J. (1975b). Elimination of nitrogenous compounds by *Panagrellus redivivus*, Goodey, 1945 (Nematoda: Cephalobidae). *Comparative Biochemistry and Physiology* **52B**, 247-253.

Wright, D. J., and Newall, D. R. (1976). Nitrogen excretion, osmotic and ionic regulation in nematodes. *In* "The Organization of Nematodes" (N. A. Croll, ed.), pp. 163-210. Academic Press Inc., London.

Wright, P. (1995). Nitrogen excretion: three end products, many physiological roles. *Journal of Experimental Biology* **198**, 273-281.

Wright, P. A., Felskie, A., and Anderson, P. M. (1995). Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. *Journal of Experimental Biology* **198**, 127-135.

Wu, G., and Morris, S. M. (1998). Arginine metabolism: nitric oxide and beyond. *Biochemical Journal* **336**, 1-17.

Wu, Y., Egerton, G., Underwood, A. P., Sakuda, S., and Bianco, A. E. (2001). Expression and secretion of a larval-specific chitinase (family 18 glycosyl hydrolase) by the infective stages of the parasitic nematode, *Onchocerca volvulus*. *Journal of Biological Chemistry* **276**, 42557-42564.

Wyss, M., and Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiological Reviews* **80**, 1107-1213.

Yamada, A., and Matsushima, O. (1992). The relation of D-alanine and alanine racemase activity in molluscs. *Comparative Biochemistry and Physiology* **103B**, 617-621.

Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi-Shinozaki, K., Shinozaki, K., and Yoshiba, Y. (2005). Effects of free proline accumulation in petunias under drought stress. *Journal of Experimental Botany* **56**, 1975-1981.

Yan, Y., Smant, G., Stokkermans, J., Qin, L., Helder, J., Baum, T., Schots, A., and Davis, E. (1998). Genomic organization of four β-1,4-endoglucanase genes in plant-parasitic cyst nematodes and its evolutionary implications. *Gene* **220**, 61-70.

Yang, R.-Z., Blaileanu, G., Hansen, B. C., Shuldiner, A. R., and Gong, D.-W. (2002). cDNA Cloning, genomic structure, chromosomal mapping, and functional expression of a novel human alanine aminotransferase. *Genomics* **79**, 445-450.

Yarlett, N. (1988). Polyamine biosynthesis and inhibition in *Trichomonas vaginalis*. *Parasitology Today* **4**, 357-360.

Yarlett, N., Lindmark, D. G., Goldberg, B., Moharrami, M. A., and Bacchi, C. J. (1994). Subcellular localization of the enzymes of the arginine dihydrolase pathway in *Trichomonas vaginalis* and *Tritrichomonas foetus*. *Journal of Eukaryotic Microbiology* **41**, 554-559.

Yatsuda, A. P., Krijgsveld, J., Cornelissen, A. W. C. A., Heck, A. J. R., and de Vries, E. (2003). Comprehensive analysis of the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition. *Journal of Biological Chemistry* **278**, 16941-16951.

Yoon, M.-Y., Park, J.-H., Choi, K.-J., Kim, J., Kim, Y.-O., Jun-Bum, P., and Kyungand, J.-B. (1998). Purification and characterization of aspartase from *Hafnia alvei*. *Journal of Biochemistry and Molecular Biology* **31**, 345-348. (abstract).

Yoshida, N., Jankevicius, J. V., Roitman, I., and Camargo, E. P. (1978). Enzymes of the ornithine-arginine metabolism of trypanosomatids of the genus *Herpetomonas*. *Journal of Protozoology* **25**, 550-555.

Yoshikawa, N., Dhomae, N., Takio, K., and Abe, H. (2002). Purification, properties, and partial amino acid sequences of alanine racemase from the muscle of the black tiger prawn *Penaeus monodon*. *Comparative Biochemistry and Physiology* **133B**, 445-453.

Yoshimoto, T., Oka, I., and Tsuru, D. (1976). Creatine amidinohydrolase of *Pseudomonas putida*: crystallization and some properties. *Archives of Biochemistry and Biophysics* **177**, 508-515.

Young, A. R., Mancuso, N., and Bowles, V. M. (1999). Biochemical aspects of egg hatch in endo- and ectoparasites: potential for rational drug design. *International Journal for Parasitology* **29**, 861-867.

Young, C. J., McKeand, J. B., and Knox, D. P. (1995). Proteinases released *in vitro* by the parasitic stages of *Teladorsagia circumcincta*, an ovine abomasal nematode. *Parasitology* **110**, 465-471.

Yu, C.-J., Lin, Y.-F., Chiang, B.-L., and Chow, L.-P. (2003a). Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *Journal of Immunology* **170**, 445-453.

Yu, H., Yoo, P. K., Aguirre, C. C., Tsoa, R. W., Kern, R. M., Grody, W. W., Cederbaum, S. D., and Iyer, R. K. (2003b). Widespread expression of arginase I in mouse tissues: Biochemical and physiological implications. *Journal of Histochemistry and Cytochemistry.* **51**, 1151-1160.

Yuan, H.-F., Wang, C.-M., and Kung, H.-W. (2001). Purification and characterization of glutamine synthetase from the unicellular cyanobacterium *Synechoccus* RF-1. *Botanical Bulletin of Academia Sinica* **42**, 23-33.

Zavras, E. T., and Roberts, L. S. (1984). Developmental physiology of cestodes: Characterization of putative crowding factors in *Hymenolepis diminuta*. *Journal of Parasitology* **70**, 937-944.

Zhang, Y. (1987). Malaria: an intra-erythrocytic neoplasm? *Parasitology Today* **3**, 190-192.

Zhu, M.-Y., Iyo, A., Piletz, J. E., and Regunathan, S. (2004). Expression of human arginine decarboxylase, the biosynthetic enzyme for agmatine. *Biochimica et Biophysica Acta* **1670**, 156-164.

Ziv, E., and Bendayan, M. (2000). Intestinal absorption of peptides through enterocytes. *Microscopy Research and Technique* **49**, 346-352.

Zúñiga, M., Pérez, G., and González-Candelas, F. (2002). Evolution of arginine deiminase (ADI) pathway genes. *Molecular Phylogenetics and Evolution* **25**, 429-444.

APPENDIX 1: PARASITOLOGY

1.1 LARVAL CULTURE

Infective L3 were regularly passaged through parasite-free sheep to maintain cultures of pure strains of *O. circumcincta*. Existing worm burdens were removed from donor sheep by drenching with a double dose of Leviben (8mg/kg ricobendazole + 15mg/kg levamisole; Young's Animal Health Ltd., N.Z.) and ivermectin (Ivomec, Merial, Auckland, NZ; 7ml). Dexamethazone (Dex 5, Virbac NZ, 0.15mg/kg) was injected intramuscularly twice weekly. After faecal floats were negative, the sheep were infected intraruminally via stomach tube with L3. Faeces collected in faecal bags were mixed with vermiculite (Grade Fine IVL2, Nuplex Industries Ltd), moistened and incubated in a 25-27°C room for 10-14 days. The mixture was moistened and mixed daily.

L3 were recovered by a Baermann technique. The faeces mixture was placed in sieves lined with tissue paper in bowls of RO water and left for 24h. The water containing the larvae was then passed through a 20-µm filter, the larvae resuspended and placed in a funnel lined with a single layer of tissue for a further 24h. L3 were stored at 4°C in RO water.

1.2 FAECAL EGG COUNTS

The method was the modified McMaster method of Stafford *et al.* (1994). 2g of faeces were passed through a small sieve into 30ml of saturated NaCl solution. Aliquots of the resulting suspension were transferred with a Pasteur pipette into the chambers of a McMaster slide. The suspension was continuously stirred while aliquots were being removed to assure an even distribution of eggs. After 2 min, eggs were counted under a microscope. Each egg represented 50 e.p.g.

1.3 EXSHEATHING L3

Larvae were counted, transferred to a 50 ml tube and centrifuged at 1800 g for 5 min. L3 were exsheathed at 37°C in 0.2% sodium hypochlorite. Exsheathing was monitored microscopically and when complete, the suspension was centrifuged, the L3 washed several times by resuspension

and centrifugation with water and finally Baermannized to remove inactive worms, as described above in Appendix 1.1.

1.4 RECOVERY OF ADULT WORMS

Ligated abomasa were removed from donor animals immediately after death, opened and the contents collected. The luminal surface of the mucosa was washed with warm 0.9% NaCl. The washings were mixed with the abomasal contents and the worms were allowed to settle in a large measuring cylinder in a waterbath at 37°C. The volume in the cylinder was reduced by aspirating fluid at the surface fluid from which worms had sedimented.

A solution of 3% agar (Bacto Agar, DIFCO Laboratories, USA) was heated in a microwave oven and allowed to cool. When it had cooled to 40-50°C, it was rapidly mixed with twice its volume of worm mixture (final concentration 1% agar) and immediately poured into trays to set. Saline (0.9%) warmed to 37°C was carefully poured over the agar blocks until they were completely covered. The agar blocks were placed in large trays of saline at 37°C. The worms migrated out of the agar blocks into the saline and formed clumps. About 90% of those worms that migrated from the agar had done so in the first two hours. Clumps of worms were picked out of the saline and suspended in the appropriate medium.

1.5 BAERMANNISATION AND COUNTING OF LARVAE

Before use in experiments, L3 were resuspended in RO water and placed in a funnel lined with a single layer of tissue for 2 hours to allow active larvae to migrate through the tissue into RO water. The worm suspension was well mixed and an aliqot of 20-100 μl placed on the graduated counting slide. A drop of iodine was added to inhibit worm motility and larvae were counted under 10x magnification. Counts were made in triplicate and worm density calculated before use in experiments.

APPENDIX 2: ASSAYS

2.1 AMMONIA ASSAY

The assay was based on the reaction of ammonia with hypochlorite and phenol to produce indophenols, which were monitored spectrophotometrically at 635nm.

Assay solutions:

0.1 mM NH_4Cl (positive control)

Milli Q H_2O (negative control)

Reagent A (kept at 4°C) 0.5 M phenol and 0.4 mM sodium nitroferricyanide

Reagent B (kept at 4°C) 0.67 M NaOH and 0.45 mM sodium hypochlorite

Method:

Set up in duplicate in 5ml plastic test tubes:

Negative control	0.5 ml Milli Q H_2O
Positive control	0.5 ml 0.1mM NH_4Cl
Standard solutions	0.5 ml of 0.001-1 mM NH_4Cl in Milli Q H_2O
Sample	0.5 ml sample

To each tube, add 100 µl of reagent A and briefly vortex the tubes.

Then add 100 µl of reagent B and again briefly vortex the tubes.

Place the tubes on a 50°C heating block for at least 30 min.

Remove the tubes and cool to room temperature.

Read the absorbance at 635 nm against the negative control.

Construct a standard curve from which ammonia concentrations can be read.

2.2 UREA ASSAY

The assay was based on the conversion of urea to ammonia and carbon dioxide by urease followed by the estimation of the ammonia concentration as in Appendix 2.1.

Set up in duplicate in 5ml plastic test tubes:

Ammonia blank	0.5 ml sample
Urea	0.5 ml sample plus 1 unit of urease (Sigma)

The tubes were incubated at 37°C for 30 min.
Ammonia was estimated in both sample and blank tubes and the urea concentration calculated after the ammonia blank had been subtracted.

2.3 PROTEIN MICROASSAY

The assay was based on the method of Bradford (1976).

Set up in duplicate in 5 ml plastic test tubes:

Negative control	0.2 ml incubation medium
Standard solutions	5 -100 µg BSA in incubation medium
Sample	0.2 ml sample

Add 0.8 ml of Bradford reagent (Sigma) to each tube, mix and leave for 10 min
Read the absorbance at 595 nm against the negative control.
Construct a standard curve to obtain protein concentrations.

2.4 TOTAL AMINO ACID ASSAY

The assay was a modification of the method of Magne and Larher (1992).

0.5 ml sodium citrate buffer (0.2M, pH 4.6) was pipetted into a test tube, followed by 1 ml of sample or amino acid standards and 1 ml of 1% ninhydrin solution (Sigma). The tubes were shaken well and heated to 100°C in a heating block for 15 min. After cooling to room temperature, 3 ml of 60% ethyl alcohol (diluted in distilled water) was added to each tube and the absorbance read at 570 nm within 1 hour. Amino acid concentrations were read from standard curves constructed with either glutamate or aspartate in concentrations from 0-1 mM.

2.5 URIC ACID ASSAY

The method was a modification of that of Sasaki *et al.* (1996). The wavelength of 290 nm at which absorbances were read was selected from the high absorbance detemined in a preliminary spectral study.

Method:
Set up in duplicate in 1ml Eppendorf tubes:

Negative control	0.5 ml 100 mM phosphate buffer, pH 7.5
Standard solutions	0 to 0.1 mM uric acid (Sigma) in phosphate buffer
Sample	0.5 ml sample

Absorbances were read at 290 nm against the negative control. Uric acid concentrations of the samples were read from the standard curve constructed with the uric acid standards.

2.6 PREPARATION OF HOMOGENATES

About 50,000 L3 *O. circumcinta* were centrifuged at 600 *g* for 5 min in a bench centrifuge and then resuspended in assay buffer. The larvae were washed by repeating this treatment twice and finally resuspended in about 1 ml of buffer. The concentrated L3 suspension was transferred to a chilled mortar and frozen at -20°C for at least 15 minutes. The frozen pellet was then disrupted using a chilled pestle and the homogenate was examined under the microscope to ensure the complete disruption of the parasites.

About 5 mg of clumped adult *O. circumcinta* were suspended in about 1 ml of buffer. The suspension was transferred to a chilled mortar, frozen and homogenised as for L3.

About 5 mg of sheep muscle was cut into small pieces using a sharp razor blade and suspended in about 1 ml of buffer. The suspension was transferred to a chilled mortar, frozen and homogenised as for L3.

When homogenates were to be used for more than one assay, aliquots were frozen and kept in Eppendorff tubes in liquid nitrogen until required.

2.7 CONTINUOUS ENZYME ASSAYS

All enzyme assays were carried out using an Ultraspec III (Pharmacia LKB) equipped with a temperature control unit (Pharmacia LKB – autofill III) which was controlled, and the data were collected, by a computer using software and hardware developed in-house (Brown, unpublished). The software also allowed the estimation of the slope (change in the absorbance over time) by least squares regression. An example of using this method is

shown below in Figure A2.1 for an assay in which the rate of NADH utilisation was followed at 340 nm.

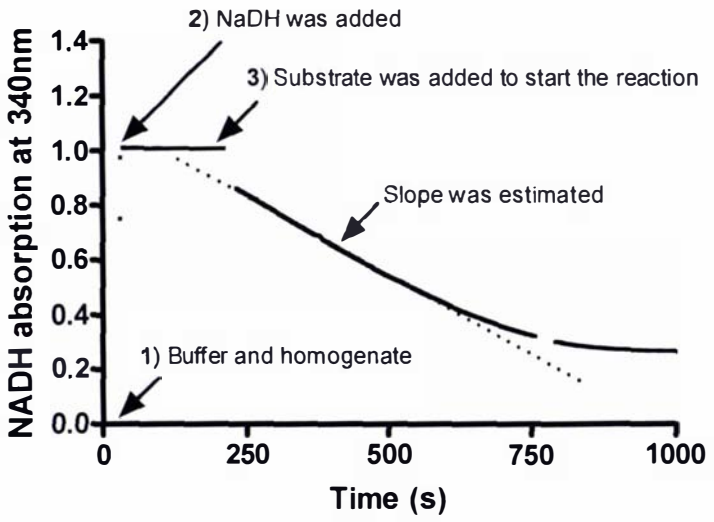


Figure A2.1. Example of a continuous assay in which the rate of NADH utilisation was monitored spectrophotometrically at 340 nm.

(1) Buffer, homogenate and specific assay components were mixed in the cuvette, which was placed in the cuvette housing, and data collection was initiated. This first stage was useful to (a) check whether there was interference in the assay at the wavelength selected due to any of the components present and (b) monitor the stability of the homogenate in the suspension.

(2) Cofactor was added (frequently this was NADH or NAD^+), usually after a fixed period of time, and the absorbance was monitored. This was useful to establish whether there was any other reaction arising from the components present in the cuvette that might have been mistakenly attributed to the enzyme of interest.

(3) Substrate was added to the cuvette to initiate the reaction and the absorbance was monitored continuously for an appropriate time.

(4) Frequently other compounds were added to the cuvette after this point to assist in quality control. Various controls were carried out as appropriate to ensure that the reported activity was dependent on the

added protein, on the substrates added and on any coupling enzymes present, and was not affected unexpectedly by any assay

2.8 DETERMINATION OF EXTINCTION COEFFICIENT

Solutions of the analyte of interest were prepared at a range of concentrations in the relevant medium. Spectra were obtained for each concentration at 30°C, at 1 nm intervals over the appropriate wavelength range (see figure 2.1 for an example using phenylalanine) using the Ultrospec III (LKB Pharmacia). In each case the reference was the medium in which the analyte was dissolved. Having identified the relevant peak position (λ_{max}) for this analyte in these conditions, the absorbance at λ_{max} was plotted as a function of analyte concentration and the slope of the regression line was taken as the extinction coefficient (see Figure A2.2 inset for an example).

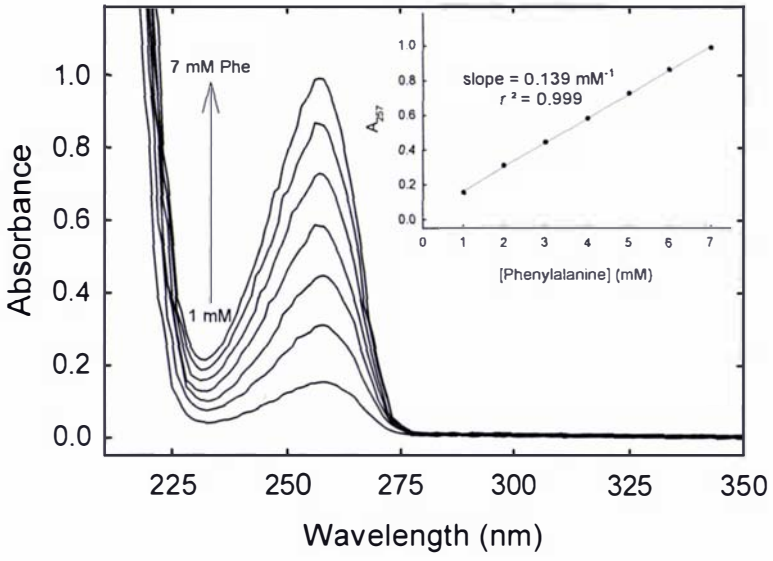


Figure A2.2. The spectra of 1-7 mM phenylalanine in phosphate medium at 30°C. The apparent λ_{max} was 257 nm and the inset shows A_{257} as a function of the concentration of phenylalanine. The slope of the regression line was 0.139 mM^{-1}, corresponding to ε_{257}^{Phe} = 139 M^{-1} cm^{-1}. Neither the apparent λ_{max} nor the ε_{257}^{Phe} differed significantly from that reported by Minke *et al.* (2000) for phenylalanine, although they reported that λ_{max} = 255 nm.

As standard cuvettes were used at all times, it was assumed that the optical path length was 1 cm. In some instances (fumarate, for example), the extinction coefficient was estimated at a wavelength other than λ_{max}.

The advantages of this approach were that (1) it facilitated identification of the relevant λ_{max} rather than simply assuming a given value, (2) it provided some indication as to the region over which the absorbance depended linearly on analyte concentration and (3) it provided independent samples from which to determine $\varepsilon_{\lambda}^{analyte}$.

2.9 CALCULATION OF ENZYME ACTIVITY

The rate of enzyme activity based on NADH oxidation was calculated based on the equation 2:

$$V = \frac{(\textit{Slope estimation\_} / \varepsilon_{340}^{NADH}) \times t \times Vt}{\textit{Protein}} \qquad \text{(Equation 2)}$$

Where :

V = rate of activity; ε_{340}^{NADH} = *extinction coefficient of 340*nm i.e. 6220M^{-1};

t = timeframe of assay in minutes;

Vt = final volume of assay in Litre;

Protein = amount of homogenate used in the assay in mg.

Data were plotted using Graphpad Prism version 4. V_{max} and K_m were obtained by fitting the data to a hyperbola using the with non-linear regression function to obtain B_{max} and K_d values.

2.10 GLUTAMINE SYNTHETASE ASSAY

GS activity was determined by the rate of conversion of hydroxylamine to γ-glutamyl hydroxamate, which was determined by a colorimetric method (Vorhaben *et al.*, 1973).

Set up in duplicate in 5 ml plastic tubes:

Negative control 0.2ml incubation medium

Standard solutions	0 to 0.6 mM γ-glutamyl hydroxamate in incubation medium
Sample	0.2 ml sample

Add 1.5 ml of a solution containing 3.5% $FeCl_3$, 2% trichloroacetic acid and 0.25 N HCl. A brown colour developed. Read the absorbance at 500 nm in a Pharmacia LKB Novaspec II against the negative control.

Construct a standard curve to obtain γ-glutamyl hydroxamate concentrations GS activities were calculated from the equation in Appendix 2.9, using the extinction coefficent for γ-glutamyl hydroxamate at 500nm i.e 842 M^{-1}.

APPENDIX 3: SOLUTIONS

3.1 PHOSPHATE BUFFER

100 mM phosphate buffer was made by dissolving 17.8 g $Na_2HPO_4.2H_2O$ (MW 178) in 950 ml RO water, adjusting the pH with HCl and making up to 1 litre.

0.8 mM phosphate buffer was made up similarly with 0.142 g per litre.

3.2 TRIS BUFFER

100 mm Tris buffer was made up by was made by dissolving 157.8 g Tris-HCl in 950 ml RO water, adjusting the pH with NaOH and making up to 1 litre.

3.3 PHOSPHATE BUFFERED SALINE

9 g NaCl was dissolved in 1 litre of 100 mM phosphate buffer pH 7.0.