Nitrogen Metabolism in *Haemonchus contortus* and *Teladorsagia circumcincta*

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Sallah Umair

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Abstract

This is the first study to characterise proline, arginine and lysine metabolism in homogenates of L3 and adult *Haemonchus contortus* and *Teladorsagia circumcincta*. The properties of glutamate dehydrogenase (GDH), glutamate synthase and the GABA shunt were also compared in the two species. The kinetic properties of 26 enzymes were determined. The gene encoding *T. circumcincta* GDH was sequenced and recombinant TcGDH expressed and biochemically characterised.

The ornithine-glutamate-proline pathway was fully functional. The mammalian α-AAA (saccharopine) and pipecolate pathways of lysine catabolism, but not the bacterial enzymes lysine dehydrogenase and decarboxylase, were present in adult worms. The pipecolate pathway was incomplete in L3 of both species, as Pip2CR activity was undetectable. Unusually, lysine ketoglutarate reductase and saccharopine dehydrogenase, Δ^1-pyrroline-5-carboxylate synthase and reductase were able to use both co-factors. The glutamine synthetase-glutamate synthase pathway of ammonia incorporation into glutamate was present, except in L3 *H. contortus*. *T. circumcincta* GDH was cloned, purified and characterised and the predicted protein sequence was very similar to *H. contortus* GDH. *T. circumcincta* recombinant and *H. contortus* homogenate GDH were both dual co-factor specific, although the latter had 50% greater activity with NAD^+/H as co-factor. GDH activity was inhibited by GTP and stimulated by ADP whereas ATP either inhibited or stimulated depending on the concentration and direction of the reaction. The GABA shunt enzymes glutamate decarboxylase and succinic semialdehyde dehydrogenase was not detected in homogenates of whole L3 or adult *H. contortus* or *T. circumcincta*.

Neither parasite had a full functional ornithine urea cycle, nor appeared to use bacterial pathways to covert arginine to ornithine. NOS

were demonstrated histochemically in nerves of adult *H. contortus*, but was undetectable in homogenates of both species. There was species variation in polyamine metabolism: *T. circumcincta* used arginase to form ornithine, followed by decarboxylation by ODC, while in *H. contortus* there was the additional pathway of first decarboxylation by ADC to form agmatine, then hydrolysis by agmatinase to putrescine. The present study helped in the better understanding of nitrogen metabolism and these enzymes can be useful targets if they differ antigenically from the host, provided the enzyme is accessible to blockage by immune effectors.

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A. aegypti	*Aedes aegypti*
A. caninum	*Ancylostoma caninum*
A. galli	*Ascaridia galli*
A. lumbricoides	*Ascaris lumbricoides*
A. suum	*Ascaris suum*
ADC	arginine decarboxylase
ADP	adenosine diphosphate
ADI	arginine deiminase
AGAT	arginine:glycine amidinotransferase
AK	arginine kinase
ASL	argininosuccinate lyase
ASS	argininosuccinate synthetase
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
B. malayi	*Brugia malayi*
B. mori	*Bombyx mori*
B. patei	*Brigia patei*
bp	base pair
cDNA	complementary deoxyribonucleic acid
C. briggsae	*Caenorhabditis briggsae*
C. elegans	*Caenorhabditis elegans*
CK	creatine kinase
cNOS	constitutive nitric oxide synthase
CP	carbamoyl phosphate
CPS	carbamoyl phosphate synthetase
D. immitis	*Dirofilaria immitis*
D. melanogaster	*Drosophila melanogaster*
DFMO	difluoromethylornithine
E. coli	*Escherichia coli*
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide

F. gigantica	*Fasciola gigantica*
F. hepatica	*Fasciola hepatica*
g	gram
g	gravitational force
GABA	gamma aminobutyric acid
GABA-T	gamma aminobutyrate transferase
GAD	glutamate decerboxylase
GDH	glutamate dehydrogenase
G. intestinalis	*Giardia intestanalis*
GOGAT	glutamate synthase
GS	glutamate synthetase
GST	glutathione S-transferases
GTP	guanosine triphosphate
h	hour
H. contortus	*Haemonchus contortus*
H. diminuta	*Hymenolepis diminuta*
H. polygyrus	*Heligmosomoides polygyrus*
H. pylori	*Helicobacter pylori*
iNOS	inducible nitric oxide synthetase
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kDa	kilodalton
LDC	lysine decarboxylase
LDH	lysine dehydrogenase
LKR	lysine ketoglutarate reductase
LO	lysine oxidase
L3	third stage larva
L4	fourth stage larva
M	molar
M. expansa	*Moniezia expansa*
mg	milligram
mGDH	mitochondrial glutamate dehydrogenase
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid

n	number
N. brasiliensis	*Nippostrongylus brasiliensis*
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NADPH-d	reduced nicotinamide adenine dinucleotide phosphate diaphroase
nmole	nanomole
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
O. volvulus	*Onchocerca volvulus*
OAA	oxaloacetic acid
OAT	ornithine aminotransferase
ODC	ornithine decarboxylase
OTC	ornithine transcarbamylase
OUC	ornithine urea cycle
PAGE	polyacrelamide gel electrophrosis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PheH	phenylalanine hydroxylase
PipO	piperideine oxidase
PLP	pyridoxal 5'-phosphate
PO	proline oxidase
P. redivivus	*Panagrellus redivivus*
P5C	pyrroline-5-carboxylate
P5CDH	pyrroline-5-carboxylate dehydrogenase
P5CR	pyrroline-5-carboxylate reductase
P5CS	pyrroline-5-carboxylate synthase
RNA	ribonucleic acid
RO	reverse osmosis
SDH	saccharopine dehydrogenase
SDS	sodium duodecyl sulphate

S. mansoni	*Schistosoma mansoni*
SOX	sarcosine oxidase
SSA	succinic semialdehyde
SSADH	succinic semialdehyde dehydrogenase
T. circumcincta	*Teladorsagia circumcincta*
TLC	thin layer chromatography
µCi	microcurie
µg	microgram
µl	microlitre
2-OG	2-oxoglutarate

INTRODUCTION

Parasitism is one of the main causes of reduced sheep meat and wool productivity and results in economic loss; severe infections can cause host mortality. Pasture-fed sheep are continuously exposed to parasite infection which is predominantly controlled by heavy reliance on anti-parasitic drenches. The heavy use of anti-parasitic drugs has led to parasites developing resistance to them (Prichard, 1994). Alternative methods are urgently needed to control worm burdens; these strategies may include breeding resistant animals, controlling the larval stages by good management practices and developing vaccines against the parasites.

Gastro-intestinal nematodes cause significant pathogenesis in sheep and co-infection with both abomasal and intestinal species in one host generally results in adverse effects. In New Zealand, the abomasal nematodes *Haemonchus contortus* (barber's pole worm) and *Teladorsagia circumcincta* (brown stomach worm) and the intestinal nematode *Trichostrongylus colubriformis* are the main nematode parasites of sheep and can result in reduced feed intake, anorexia, maldigestion, weight loss and also anemia with *H. contortus* infection. Loss of appetite is particularly associated with ill-thrift in infected animals. Adding to the production loss are the costs of treatment, veterinary care and death of infected sheep.

Developing novel effective and long lasting remedies should be based on knowledge of central aspects of parasite biology. The free-living larval stages (L1 to L3) grow rapidly and the parasitic adult worms lay large numbers of eggs each day, both requiring active nitrogen and energy metabolism. The metabolic pathways and enzymes involved would therefore be attractive targets for vaccines or new chemical anthelmintics. Relatively little is known

about nitrogen metabolism in nematodes, particularly whole pathways, and most have been limited to individual enzymes. Even fewer studies have been made in abomasal parasites. A previous student from this laboratory identified three unique *T. circumcincta* enzymes: glutamate synthase (GOGAT), aspartase and creatinase (Muhamad, 2006). GOGAT was chosen for further biochemical characterisation, as, unlike the genes for creatinase and aspartase, a partial *Caenorhabditis elegans* GOGAT gene sequence is annotated in public databases, suggesting that it may be expressed in both free-living and other parasitic nematodes.

The objective of the present study was to investigate glutamate, arginine, proline and lysine metabolism in L3 and adult *H. contortus* and *T. circumcincta*, each of which is essential for different aspects of worm growth and development. Glutamate is a central amino acid in both nitrogen and energy metabolism. Proline is a component of the collagen in the cuticle of nematode parasites. Arginine is a substrate for synthesis of the polyamines which regulate cell division and differentiation. Lysine is the site in many proteins where reversible acetylation regulates protein activity and thus many cellular functions. Chapter 1 (Literature review) will highlight the paucity of knowledge of nitrogen metabolism in the sheep parasites *H. contortus* and *T. circumcincta*. Experiments on glutamate metabolism are reported in Chapters 2-4, proline and arginine metabolism in Chapter 5 and 6 and lysine catabolism in Chapter 7.

Chapter I

Literature review

Nitrogen metabolism in abomasal nematodes of sheep

NITROGEN METABOLISM IN ABOMASAL NEMATODES OF SHEEP

Nitrogen is a component of nucleic acids, amino acids, enzymes, hormones and structural proteins of cells. Broadly, nitrogen metabolism includes catabolism and absorption of nitrogen from ingested nutrients, inter-conversion of nitrogenous compounds and excretion of excess nitrogen. Nitrogen metabolism also connects to the energy cycle at various points and provides substrates required by the cycle. Whole metabolic pathways have not been studied extensively and generally individual enzymes have been the focus of many studies, more recently using molecular biological techniques. The reports of Muhamad (2006) and Muhamad et al. (2011) on L3 and adult *T. circumcincta* are amongst the few recent studies concerned with amino acid metabolism in parasitic nematodes. Most research has been focused on a relatively small number of compounds with potential as anthelmintic targets, including neurotransmitters and their receptors, glutathione metabolism, chitin metabolism and unique enzymes such as chorismate mutase, which is expressed in several plant parasitic nematodes.

1.1. NEMATODE NEUROTRANSMITTERS

The biochemistry of the nematode neuromuscular system has been the focus of considerable research, as it is a major target of chemical anthelmintics (Köhler, 2001; Ducray et al., 2008). Many nematode neurotransmitters are nitrogen-containing molecules, including small

peptides, acetylcholine and amino acids (serotonin, histamine) or their derivatives catecholamines, dopamine, tyramine and octopamine (Blenau and Baumann, 2001). The non-mammalian transmitters, tyramine and octapamine appear to act independently as neurotransmitters in *C. elegans* (Alkema et al., 2005). The tyramine receptor Hco-LGC-55 in *H. contortus* is a chloride channel which is also gated by dopamine and octopamine and is an orthologue of a *C. elegans* receptor (Rao et al., 2010).

Synthesis of neurotransmitters from tyrosine is shown in Figure 1.1. L-Phenylalanine is catabolised to L-tyrosine in *C. elegans* by phenylalanine hydroxylase (PheH) (Calvo et al., 2008), which is expressed in the hypodermis, not the intestine (Loer et al., 1999). PheH knockout worms lacked cuticular melanin, had cuticular abnormalities, but had elevated superoxide dismutase levels (Calvo et al., 2008). Metabolism of phenylalanine to tyrosine does not appear to occur in the intestine, where the greatest tyrosine degradation takes place. Tyrosine is catabolised in all eukaryotes studied to date, including *C. elegans* (Fisher et al., 2008), by a 5 step pathway, the first of which is the removal of the amino group by tyrosine aminotransferase, then 4 steps to produce fumarate and acetoacetate.

Tetrahydrobiopterin (BH_4) is an electron donor for the three members of the family of aromatic L-amino acid decarboxylases, TrpH, TyrH and PheH which hydroxylate tryptophan, tyrosine and phenylalanine respectively, (Fitzpatrick, 2012). Synthesis of BH_4 requires GTP-cyclohydrolase, the gene encoding which has been sequenced and expression monitored during development in *T. circumcincta* and *Dictyocaulus viviparus* (Baker et al., 2011).

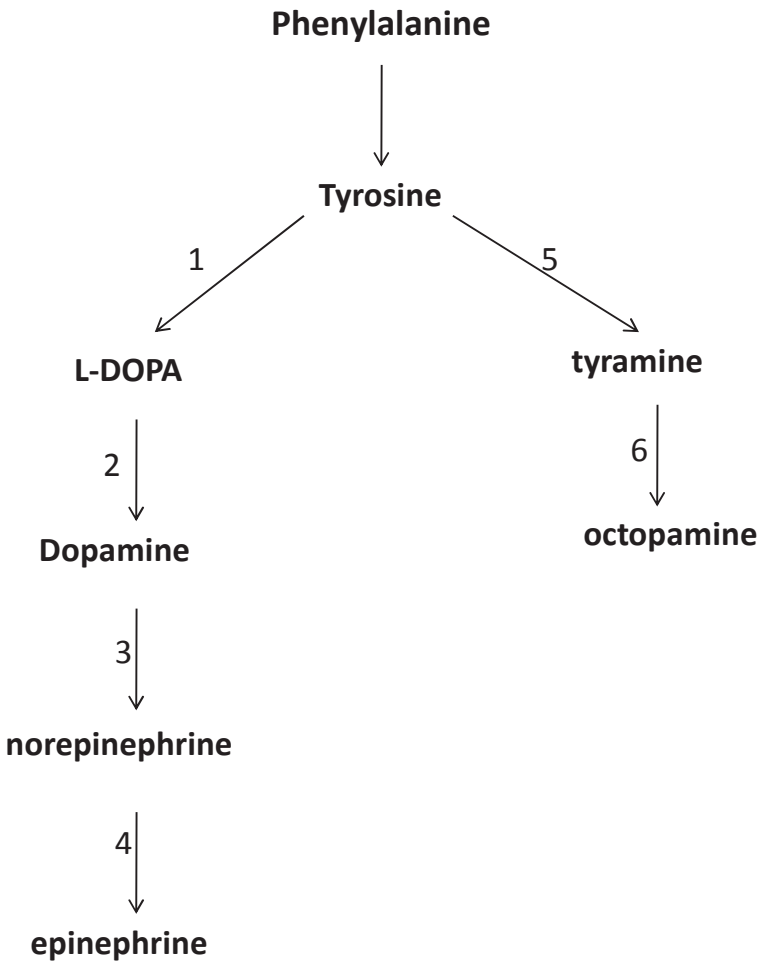

Figure 1.1. Synthesis of neurotransmitter from tyrosine. Enzymes involved are: (1) tyrosine hydroxylase (2) Amino acid decarboxylase (3) Dopamine β-hydroxylase (4) phenylethanolamineN-methyltransferase (5) Tyrosine decarboxylase and (6) Tyramine β-hydroxylase.

1.2. GLUTATHIONE METABOLISM

Glutathione is the most important low molecular weight antioxidant produced in the cell by the sequential addition of cysteine and glycine to glutamate (Forman et al., 2009). Glutathione and the cysteine sulfhydryl group (GSH) have many important cellular functions, acting as antioxidants and modulating neurotransmitters and leukotriene, steroid and prostaglandin metabolism (Forman et al., 2009). Parasite GSH and glutathione S-transferases (GSTs), enzymes which conjugate toxic compounds and xenobiotics to glutathione and protect against the host immune response, have drawn considerable interest and these compounds are considered as targets for anti-parasitic therapy and successful vaccines have been used to reduce parasite burdens (Salinas et al., 1996; Da Costa et al., 1999). Adult *H. contortus* (van Rossum et al., 2004) and *Ancylostoma caninum* (Zhan et al., 2005) have been shown to produce a novel GST, which has a binding site for hematin.

1.3. CHITIN METABOLISM

Chitin is a glucosamine polymer present in the nematode eggshell (Bird and Bird, 1991) and the feeding apparatus, for example in *Oesophagostamum dentatum* (Veronico et al., 2001). In nematodes, chitin is formed by chitin synthase, which appears to be encoded by two separate tissue specific genes, one responsible for chitin formation in the feeding apparatus and the other for egg shell and cuticle chitin synthesis (Veronico et al., 2001). Chitinase, a chitin catabolising enzyme is essential for egg hatching, larval moulting and for host tissue invasion (Adam et al., 1996; Geng et al., 2002).

1.4. CHORISMATE MUTASE

Several plant parasitic nematodes express the enzyme chorismate mutase, the last enzyme of the seven step shikimate pathway which synthesises tyrosine and phenylalanine in plants and microorganisms. There is no report of a complete shikimate pathway in nematodes. Chorismate mutase is thought to have been acquired from microorganisms through horizontal gene transfer (Yan et al., 1998) and assists the nematodes to penetrate the root nodule through the development of feeding sites. This enzyme has a probable role in the suppression of the host response (Curtis, 2007).

1.5. AMINO ACID METABOLISM IN NEMATODES

Proteins contain combinations of twenty amino acids, some of which are "essential amino acids" for mammals, as they cannot be synthesised. Earlier studies suggested that there may be no essential amino acids in nematodes, as the free living nematode *Caenorhabditis briggsae* appeared to be able to interconvert all radio-labeled amino acids (Rothstein and Tomlinson, 1962; Rothstein, 1965; Liu and Rothstein, 1976). This would require non-mammalian pathways to be present, confirmed by the demonstration in *T. circumcincta* of creatinase activity, glutamine synthetase (GOGAT), which allows excreted or added ammonia to be incorporated into glutamate and amino acids, and aspartase, which in plants and microbes reversibly converts aspartate to fumarate and ammonia (Muhamad, 2006). Several helminths appear to use the gamma amino butyric acid (GABA) bypass/shunt (Monteoliva et al., 1965; Rasero et al., 1968; Singh et al., 1983), which under anaerobic conditions could bypass part of the tricarboxylic acid cycle (TCA). The lack of a fully functional ornithine urea cycle (OUC) in many helminths (Janssens and Bryant, 1969; Grantham and Barrett, 1986a), makes

the route of synthesis of arginine uncertain. In contrast, other pathways closely resemble their mammalian counterparts, e.g. alanine and aspartate aminotransferase activity in *T. circumcincta* (Muhamad, 2006) and sulphur amino acid metabolism, including inter-conversion of methionine and cysteine (Walker and Barrett, 1997).

The sources of dietary nitrogen for gastro-intestinal nematode parasites are unknown but may include mucins, cells, leaked interstitial fluid, the digesta and bacteria. *H. contortus* is a blood sucker, but it may also have other food sources. Nematode parasites both secrete proteolytic enzymes and they are present on the intestinal brush border, which makes these parasites capable of degrading a range of proteins as a nutrient source. These proteases have been characterised in L3, L4 and adult *T. circumcincta* (Young et al., 1995), *H. contortus* (Redmond et al., 1997; Knox et al., 2003), *Ostertagia ostertagi* (Geldhof et al., 2000), *Necator americanus*, *Toxocara canis* (Loukas et al., 1998) and *A. caninum* (Williamson et al., 2003). In nematodes, the predominant excretory product ammonia could also be a source of nitrogen. In a recent study of nitrogen excretion by *T. circumcincta*, there appeared to be re-uptake of excreted ammonia during several hours of incubation (Simpson et al., 2009). It was suggested that the disappearance of ammonia from the medium was caused by its incorporation into glutamate by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway.

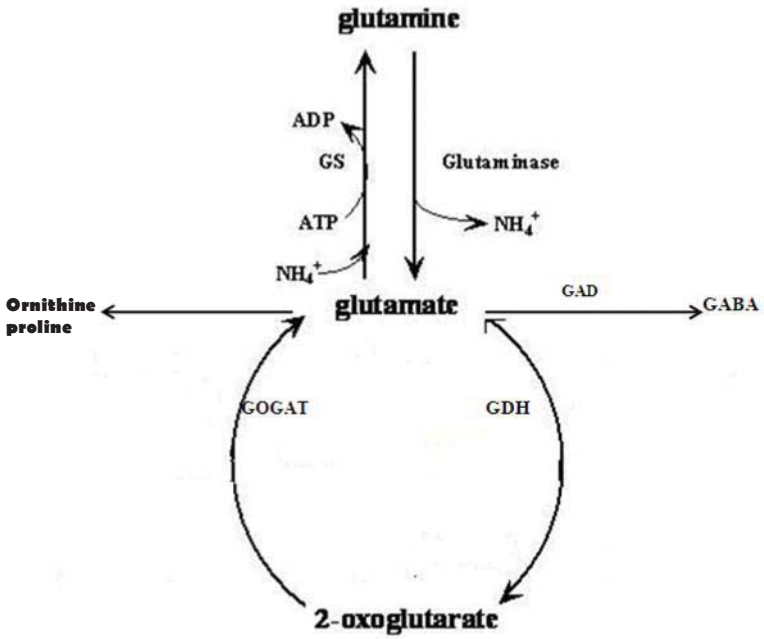

Figure 1.2. Nitrogen metabolism in which glutamate has a central position. Abbreviations: GABA, gamma amino butyric acid; GAD, glutamate decarboxylase; GS, glutamine synthetase; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; OAA, oxalo acetic acid.

1.5.1. GLUTAMATE METABOLISM

Interconversion of glutamate and the TCA cycle substrate 2-oxoglutarate (2-OG) is one link between nitrogen and energy metabolism (Hudson and Daniel, 1993; Forde and Lea, 2007). Glutamate is converted to many compounds including other amino acids by transamination, to GABA by decarboxylation and to proline and ornithine (Figure 1.2). In most animals, excreted ammonia is largely generated from glutamate by GDH. In the reverse reaction, ammonia can also be incorporated into glutamate, but is generally limited by the low affinity of GDH for ammonia. In those organisms where both GS and GOGAT are expressed, this pathway incorporates ammonia into glutamate at low cell ammonia concentrations.

1.5.1.1. Aminotransferases

Aminotransferases (previously called transaminases) are pyridoxal 5'-phosphate- (PLP) dependent enzymes which catalyse the transfer of an amino group from a donor α-amino acid to the acceptor usually 2-OG, thus forming amino acids from other carbon skeletons, for example, alanine from pyruvate and aspartate from oxaloacetic acid (OAA). During the reaction, the co-factor shifts between the PLP and pyridoxamine 5'-phosphate forms. Muhamad (2006) demonstrated high alanine and aspartate aminotransferase activities in *T. circumcincta* homogenates. Alanine is the most important amino acid involved in gluconeogenesis (Brosnan et al., 2001). Aspartate is a precursor of the essential human amino acids lysine, threonine and methionine.

1.5.1.2. Glutaminase

Glutamate is toxic to the nervous system and is stored inside cells and transported as glutamine. Glutaminase hydrolyses the conversion of glutamine back to glutamate and ammonia. High glutaminase activity was detected in *T. circumcincta* (Muhamad, 2006) whereas low activity was detected in *Heligmosomoides polygyrus* and *Panagrellus redivivus* (Grantham and Barrett, 1986) and no activity in *Litomosoides carinii* (Davies and Kohler, 1990).

1.5.1.3 Glutamine Synthetase-Glutamate Synthase (GS-GOGAT) Pathway

The GS-GOGAT pathway is important for ammonia assimilation when the ammonia concentration is low and ATP is available (Fisher and Sonenshein, 1991, Miflin and Habash, 2002). Under stress conditions and energy limitation, GDH can assimilate ammonia, if ammonia concentrations rise (Helling, 1998). Whereas GS is a universal enzyme, GOGAT is not usually present outside plants and microorganisms. In these organisms, the enzymes of glutamate metabolism and their regulatory proteins, particularly those involved in the GDH-GS-GOGAT cycle, together with the ammonium permease Amt/Rh transporter family (Tremblay and Hallenbeck, 2009), play key roles in regulating nitrogen metabolism (Forde and Lea, 2007; Arcondéguy et al., 2001; Sonoda et al., 2003; Burkovski, 2003; Moorhead and Smith, 2003).

GS catalyses the conversion of glutamate and ammonia to glutamine and the reaction is energy and co-factor dependent. There are three types of GS: GSI is present only in prokaryotes, GSII in eukaryotes while GSIII is found in some anaerobes of the rumen (Amaya et al., 2005). GS activity has been detected in the nematodes *T. circumcincta* (Muhamad, 2006), *H. polygyrus* and *P. redivivus* (Grantham and Barrett, 1988).

Depending on the co-factor requirements, there are three forms of the enzyme GOGAT, which converts glutamine and 2-OG to two molecules of L-

glutamate. NADH-GOGAT and NADPH-GOGAT are the forms in plants and bacteria, while Fd-GOGAT is present only in a few photosynthetic organisms (reviewed by Vanoni and Curti, 1999). GOGAT is generally not present in animals, but has been demonstrated in a small number of invertebrates. Hirayama et al. (1998) purified and characterised GOGAT from the silkworm *Bombyx mori*. Scaraffia et al. (2005) added the GOGAT inhibitor azaserine to the *Aedes aegypti* diet and found increased levels of glutamine, suggesting an important role for GOGAT in this mosquito. GOGAT may be expressed in nematodes, as the GOGAT gene has been identified during *C. elegans* genomic sequencing (Vanoni and Curti, 1999).

In vitro, adult and L3 *T. circumcincta* excrete small amounts of ammonia (Simpson et al., 2009) and after several hours, L3 appeared to reabsorb and metabolise the ammonia accumulating in the incubation medium. This most likely took place through the GS-GOGAT pathway. The presence of the GS-GOGAT pathway, in addition to GDH, in adult and L3 *T. circumcincta* (Muhamad, 2006; Muhamad et al., 2011) distinguishes parasite metabolism from that of the host.

1.5.1.4. Glutamate Dehydrogenase (GDH)

GDH is usually responsible for generation of much of the excreted ammonia, but may also play a role in ammonia assimilation when the energy levels are low and ammonia concentration is high. GDH catalyses the reversible oxidative deamination of glutamate to ammonium and 2-OG (Hudson and Daniel, 1993) and its activity has been demonstrated in plants (Hodges, 2002; Miflin and Habash, 2002), vertebrates (Fang et al., 2002), bacteria (Helling, 1998), as well as in helminths (Rhodes and Ferguson, 1973; Grantham and Barrett, 1986; Muhamad, 2006). Surprisingly, a developmental expression study suggested that in *H. contortus* GDH was not active in L3, whereas it was present in L4 and adult worms (Skuce et al., 1999). There are no reports of studies involving enzymatic assay of GDH in

L3 *H. contortus* which could confirm the absence of both ammonia assimilatory pathways in L3.

Based on co-factor requirements, GDH can be NAD^+-specific, $NADP^+$-specific or dual co-factor specific, (Hudson and Daniel, 1993). The dual-specificity GDH activity seen in *T. circumcincta* homogenates may be due to a single enzyme or to separate enzymes. In some organisms, separate NAD^+-GDH and $NADP^+$-GDH are responsible for the deaminating and aminating reactions; commonly dual co-factor enzymes have greater aminating activity with $NADP^+$ and greater deaminating activity with NADH (Goldin and Frieden, 1971; Hudson and Daniel, 1993). GDH activity has been demonstrated in homogenates of L3 and adult *T. circumcincta* and the enzyme(s) shown to be able to use either NAD^+ or $NADP^+$ as the co-factor (Muhamad et al., 2011). Rhodes and Ferguson (1973) reported that purified adult *H. contortus* GDH had an absolute requirement for NAD^+/H and less than 2% of that activity with $NADP^+$/H, which would classify it as NAD^+-specific and not a dual co-factor or $NADP^+$-specific enzyme. This contrasts with GDH activity in homogenates of L3 and adult *T. circumcincta* (Muhamad et al., 2011) which were active with either co-factor, consistent with a dual co-factor GDH, as are mammalian enzymes (Hudson and Daniel 1993).

GTP, ATP and ADP are short-term allosteric regulators, which are not directly involved in the reaction catalysed by GDH. Allosteric regulation of nematode GDH by single concentrations of ADP and ATP (Rhodes and Ferguson, 1973; Muhamad et al., 2011) have suggested differences from the regulation of mammalian GDH and perhaps also between nematode species. Generally, GTP is inhibitory, ADP is stimulatory and ATP can be either stimulatory or inhibitory to mammalian GDH (Fang et al., 2002; Kim et al., 2003), depending on the concentration, as ATP can bind to either the GTP or ADP binding site. A study of the concentration-dependent effects of each of the three nucleotide regulators on GDH activity in the aminating and deaminating directions of L3 and adult worm GDH should reveal any major differences in the regulation of host and nematode GDH.

1.5.1.5. Gamma-Amino Butyric Acid (GABA)

Decarboxylation of glutamate is the main route in nerves for synthesis of GABA (4-amino butyric acid), a four carbon, non-protein amino acid with an amino group on the γ-carbon atom. GABA is an inhibitory neurotransmitter in the central and peripheral nervous system of vertebrates (Ito et al., 2007) and invertebrates (Rauh et al., 1990), including nematodes (Johnson and Stretton, 1987). Plants which have high glutamate decarboxylase (GAD) activity are resistant to parasites because high levels of GABA are toxic to worms (Mclean et al., 2003). The alternative route of synthesis of GABA from putrescine (Figure 1.3) takes place in other tissues, such as the rat pancreas, where GABA controls insulin secretion (Caron et al., 1987).

The pathway that forms succinate from glutamate via GABA, the GABA shunt, bypasses part of the TCA cycle (Balazs et al., 1970); 2-OG, instead of being oxidatively decarboxylated to succinate, is converted to glutamate, which is decarboxylated irreversibly to GABA by GAD. GABA is than converted reversibly to succinic semialdehyde (SSA) by 4-aminobutyrate transaminase (GABA-T) and in the last step, SSA, by succinic semialdehyde dehydrogenase (SSADH), is irreversibly converted to succinate (Figure 1.3). The GABA shunt is present in plants (Bown and Shelp, 1997), bacteria (Metzer and Halpern, 1990), fungi and yeast (Kumar and Punekar, 1997) and in various mammalian tissues such as liver, intestines and kidneys (Erdo and Wolff, 1990; Tillakaratna et al., 1995). In plants, the GABA shunt is an alternate route in the energy cycle which provides succinate and NADH to the cell. Regulation is by the positive effect on GAD of Ca^{2+}/calmodulin and pH in the cell cytosol, thought to be involved in enzyme activation in any stress (Bouche et al., 2003). Negative regulation of SSADH is by mitochondrial NADH and ATP levels. This negative regulatory control is thought to control the shunt by mitochondrial energy charge (Bouche et al., 2003).

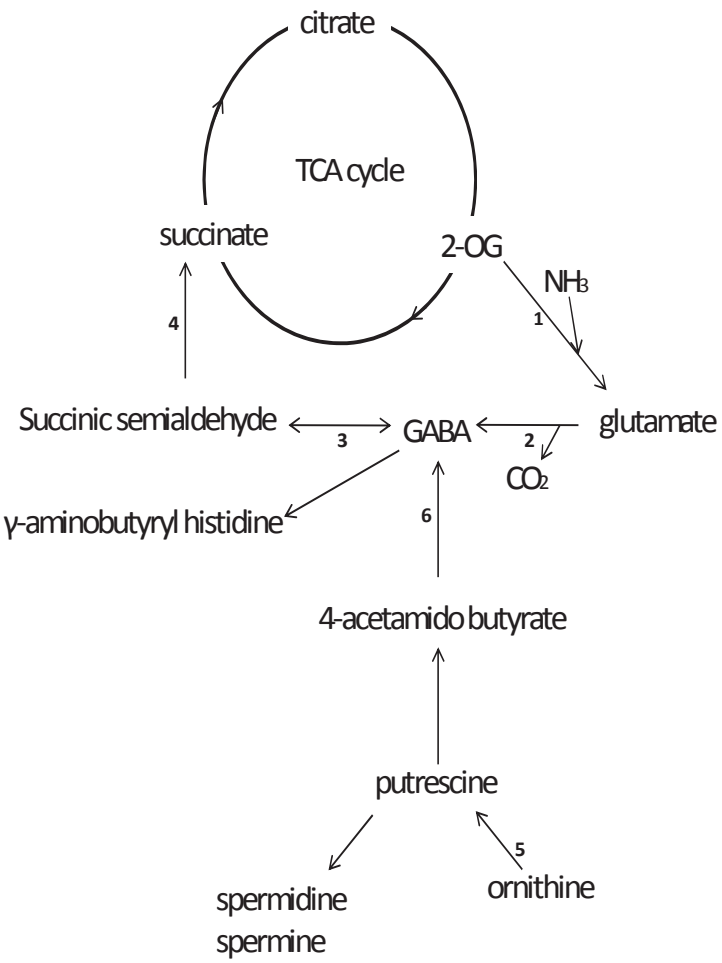

Figure 1.3. Metabolism of GABA. Enzymes involved are: (1) glutamate dehydrogenase (2) glutamate decarboxylase (3) GABA transaminase (4) succinic semialdehyde dehydrogenase (5) ornithine decarboxylase (6) 4-acetamidobutyrate deacetylase. Enzymes 2, 3 and 4 are GABA shunt enzymes.

The GABA shunt is the pathway for catabolism of GABA, the first step being catalysed by GABA-T, which has been detected in bacteria (Padmanabhan and Tchen, 1969), fungi (Yonaha et al., 1983), lower plants (Baldy, 1976), higher plants (Hulme and Arthington, 1950), vertebrates (Udenfriend, 1950), and the rat parasite *Nippostrongylus brasiliensis* (Watts and Atkins, 1983).

GABA is a well-established nematode neurotransmitter, indicating the presence of GAD and GABA-T in nervous tissue. Whether the GABA shunt is present in other tissues to bypass the TCA cycle is unclear. Invertebrates have only one form of GAD, whereas in mammals, two separate genes encode isoforms of this enzyme, GAD_{65} (molecular weight 65 kDa) and GAD_{67} (molecular weight 67 kDa) (Mackie et al., 2003).

An operative GABA shunt was detected in seven intestinal parasites (Monteoliva et al., 1965; Rasero et al., 1968). High GAD activity was found in the ovaries, uterus and intestines of *Ascaris lumbricoides, Moniezia expansa* and *Macracanthorhynchus hyrudinaceus* (Monteoliva et al., 1965). Singh et al. (1983) found some GAD activity in fractions of the mitochondrial cuticle in *Ascaridia galli*. In contrast, Cornish and Bryant (1975) found that the GABA shunt was undetectable in the intestinal cestode *M. expansa*. A high GABA-T activity was observed in the rat intestinal parasite *N. brasiliensis* (Watts and Atkins, 1983) and its kinetic properties were also studied (Watts and Atkins, 1984). Mackenzie et al. (1989) found an operative GABA shunt in *Onchocerca volvulus* and *Brugia pahangi*. GABA has been detected in tissues other than nerves in cestodes (Rasero et al., 1968), trematodes (Mendonca-Silva et al., 2004) and nematodes (Monteoliva et al., 1965).

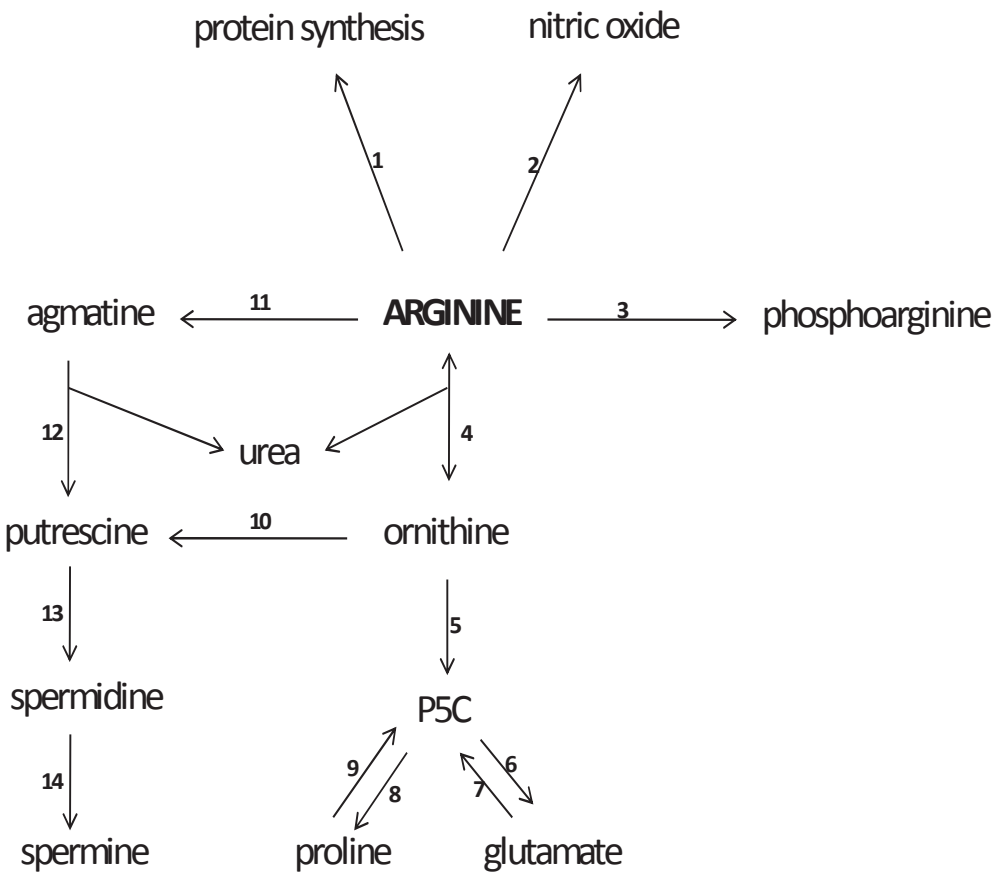

Figure 1.4. Pathways of arginine metabolism. The enzymes shown are: (1) arginyl tRNA synthetase (2) nitric oxide synthase (3) arginine kinase (4) arginase (5) ornithine aminotrahsferase (6) Δ^1-pyrroline-5-carboxylate dehydrogenase (7) Δ^1-pyrroline-5-carboxylate synthase (8) Δ^1-pyrroline-5-carboxylate reductase (9) proline oxidase (10) ornithine decarboxylase (11) arginine decarboxylase (12) agmatinase (13) spermidine synthase (14) spermine synthase.

1.5.2. ARGININE METABOLISM

Arginine is a component of proteins and is a precursor of citrulline, nitric oxide, polyamines, urea, proline, creatine and agmatine (Figure 1.4). Relatively little is known about arginine metabolism in parasitic helminths. Because of the number of competing pathways using arginine as a substrate, arginase activity is believed to be an important regulatory point, by limiting the amount of arginine available for other reactions (Vincendeau et al., 2003). A good example of this is the generation of nitric oxide (NO), a key cell-signaling molecule, neurotransmitter and toxic molecule used against some pathogens. Some parasites protect themselves from host defenses by using their arginase to deplete host arginine, thus reducing NO generation by nitric oxide synthase (NOS) (Vincendeau et al., 2003).

Enzymes of arginine catabolism are restricted to specific cells of the body and location within mitochondria or the cytosol, which determines the catabolic products of arginine e.g. arginase II and ornithine aminotransferase (OAT) in mitochondria may preferably direct arginine to the synthesis of proline and glutamate, whereas localisation of arginase I and ornithine decarboxylase (ODC) in the cell cytosol may drive synthesis of polyamines from arginine (Li et al., 2001).

Arginine is not an essential amino acid for adult mammals, as sufficient can be synthesised from citrulline in the OUC. The small intestine is a major source of citrulline by catabolism of glutamate and glutamine (Windmueller and Spaeth, 1981). Citrulline is then converted to arginine in the kidney by argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). The OUC is not generally very active or even undetectable in nematodes, so the

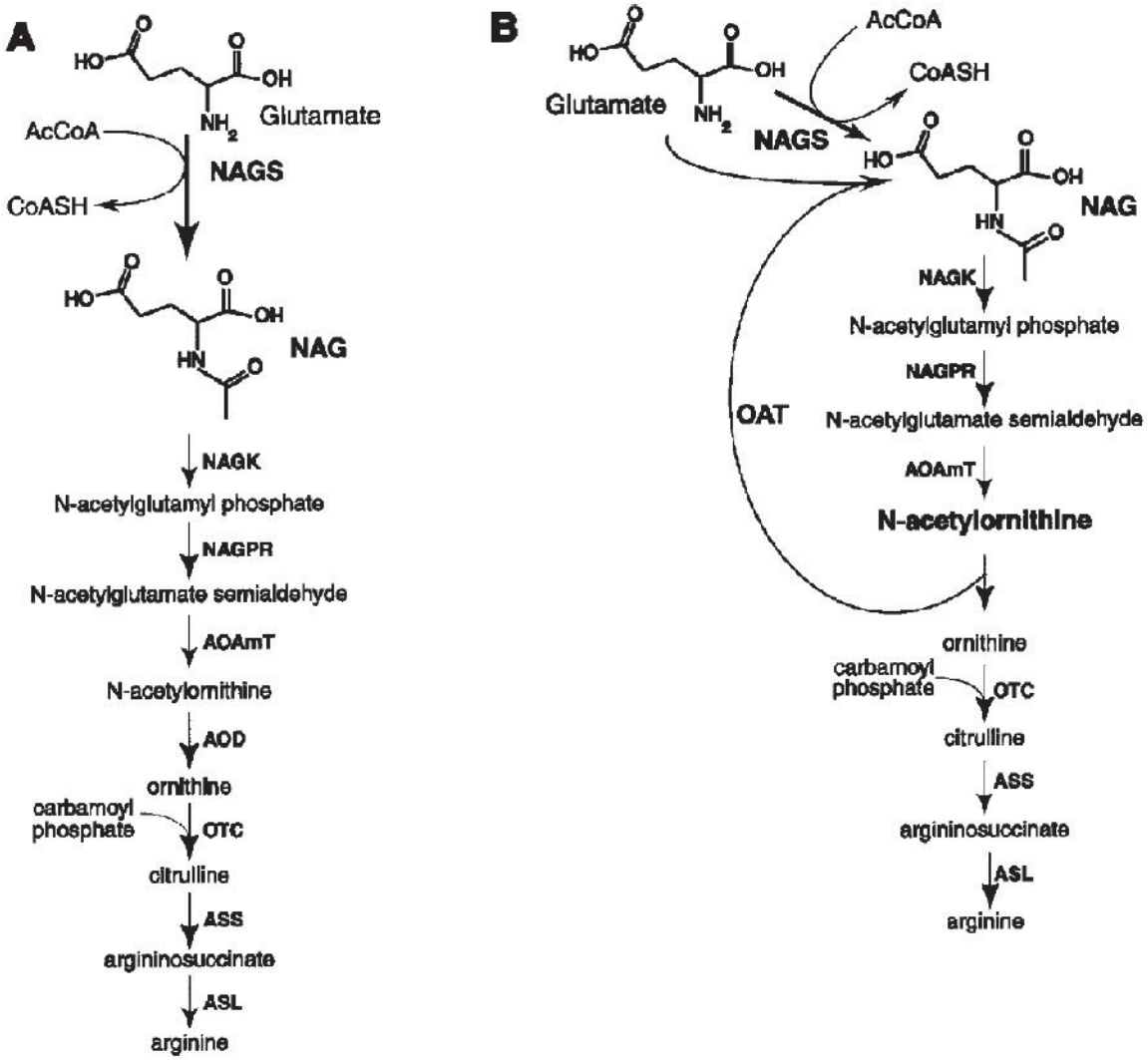

Figure 1.5. Arginine synthesis pathways in microorganisms and plants. (A) Linear (stochiometric) pathway (B) cyclic pathway. Abbreviations: NAGPR, N-acetylglutamylphosphate reductase; AOAmT, acetylornithine aminotransferase; AOD, acetylornithine deacetylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; OTC, ornithine transcarbamylase; OAT, ornithine aminotransferase; NAG, N-acetylglutamate; NAGS; NAG synthase. (From Caldovic and Tuchman, 2003)

source of arginine is not known. In lower eukaryotes, plants and microorganisms, arginine is synthesised through the intermediate N-acetylglutamate (NAG) in an eight step pathway shown in Figure 1.5 (Caldovic and Tuchman, 2003).

1.5.2.1. Ornithine Urea Cycle (OUC)

In terrestrial animals, the OUC serves two important functions: converting ammonia to less toxic urea for excretion and synthesis of arginine (Figure 1.6). The five enzymes of the urea cycle are (1) carbamoyl phosphate synthetase (CPS), (2) ornithine transcarbamylase (OTC), (3) ASS, (4) ASL and (5) arginase. The first two enzymes are mitochondrial, while the others are

cytosolic. For a fully functional OUC, mitochondrial ornithine and citrulline transporters are required as well as glutaminase to form ammonia and N-acetyl glutamate synthetase to synthesise N-acetyl glutamate, which acts as a regulator of CPS (Kobayashi et al., 1999, Watford, 1993).

Individual enzymes have other functions if there is an incomplete OUC. The isoform CPS II is located in the cytosol and is involved in pyrimidine biosynthesis (Saeed-Kothe and Powers-Lee, 2002). The activity of CPS I is low in the intestinal mucosa, where CPS along with OTC become part of citrulline synthesis (reviewed by Jackson et al., 1986). In the kidney, argininosuccinate is formed by the reversible reaction of citrulline and aspartate catalysed by ASS and ATP is converted to ADP. In lower organisms, OTC is located in the cell cytosol and converts citrulline to ornithine (Baur et al., 1987).

Arginase is the last enzyme of the OUC and is almost universally present in bacteria, yeasts, plants, invertebrates and vertebrates (Jenkinson et al., 1996). Arginase is present in many helminths, whereas other OUC enzymes have not consistently been detected. This is not surprising, as urea is a minor component of nitrogenous excretion in nematodes (Rogers, 1952; Wright, 1975). All OUC enzymes were present in *Fasciola hepatica* (Rijavec and

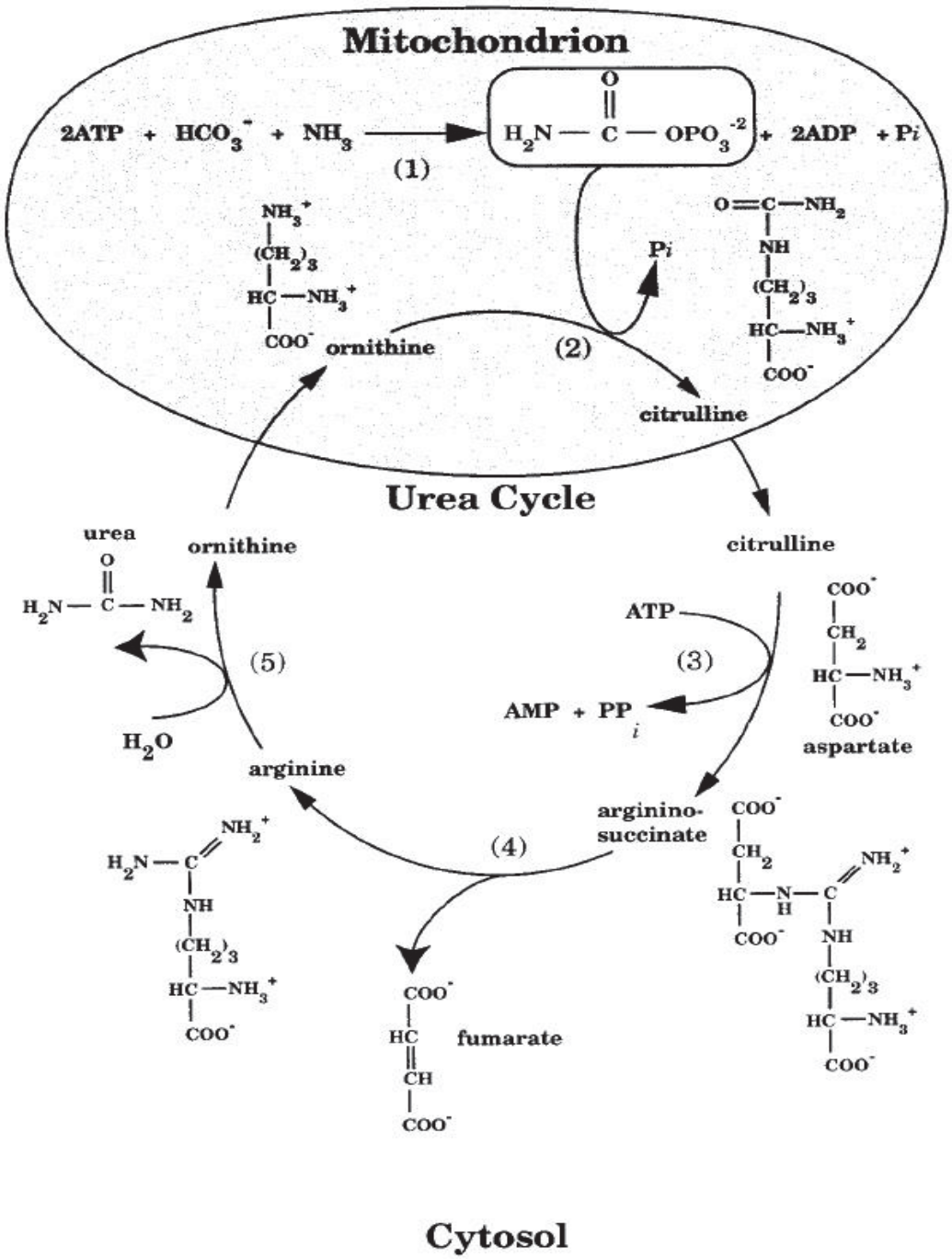

Figure 1.6. Ornithine Urea Cycle. The enzymes of OUC are (1) carbamoyl phosphate synthetase (2) ornithine transcarbamylase (3) argininosuccinate synthetase (4) argininosuccinate lyase (5) arginase. (From Holden et al., 1999).

Kuralec, 1965; Mohamed et al., 2005), *Dicrocoelium lanceatum* (Rijavec and Kuralec, 1965) and in *P. redivivus*, although ASS and ASL activities were very low (Wright, 1975). Arginase activity has been reported in parasitic trematodes (Campbell and Lee, 1963; Rijavec and Kuralec, 1965; Senft, 1966; Mohamed et al., 2005; Fitzpatrick et al., 2009), cestodes (Campbell and Lee, 1963; Janssens and Bryant, 1969) and the nematodes *T. circumcincta*, *A. lumbricoides*, *A. galli*, *Nematodirus* spp, *H. polygyrus* and *P. redivivus* (Rogers, 1952; Janssens and Bryant, 1969; Grantham and Barrett, 1986; Muhamad, 2006), although low in the latter two species.

1.5.2.2. Ornithine Synthesis

1.5.2.2.1. Arginase

Arginine is catabolised by arginase to urea and ornithine which is used as a substrate for polyamine, proline and glutamate synthesis. Conversion of arginine to ornithine is not reversible as arginase is a hydrolytic enzyme. There are two isoforms of arginase in mammals, arginase I and II, which are encoded by separate genes. Arginase l is found in the cytosol and arginase ll is located in the mitochondria.

Arginase is the only OUC enzyme definitely present in helminths (Rijavec and Kurelec, 1965; Mohamed et al., 2005). Muhamad (2006) characterised *T. circumcincta* arginase using a coupled assay and reported some unusual properties of the nematode enzyme. *T. circumcincta* arginase did not appear to require any added bivalent ion, normally Mn^{2+}. Another interesting feature was its unusual pH optimum of pH 8 (Muhamad, 2006) whereas, most arginases, including the *Schistosoma mansoni* (Fitzpatrick et al., 2009) and *Fasciola gigantica* (Mohamed et al., 2005) enzymes, are most active at pH 9-10.

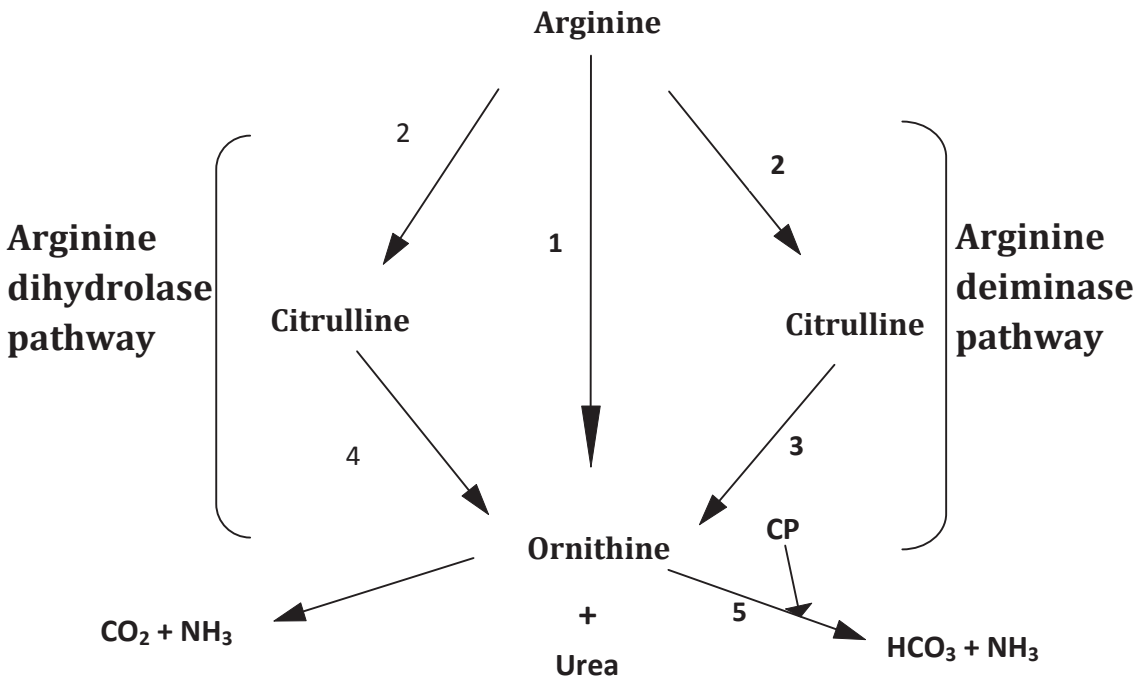

Figure 1.7. The metabolism of arginine to ornithine through the arginine deiminase and arginine dihydrolase pathways. The enzymes shown are: (1) arginase (2) arginine deiminase (3) ornithine carbamoyl transferase (4) citrullinase (5) carbamate kinase.

1.5.2.2.2. Arginine deiminase pathway

The arginine deiminase pathway generates ATP, ornithine, ammonia and CO_2 from arginine in microorganisms. The pathway consists of three steps: (1) arginine deiminase converts arginine to citrulline and ammonia; (2) ornithine carbamoyl transferase converts citrulline to ornithine and produces carbamoyl phosphate; (3) carbamate kinase converts carbamoyl phosphate to ammonia and CO_2 and ADP to ATP (Zuniga et al., 2002) (Figure 1.7). Some protozoan parasites, *Giardia intestinalis*, *Trichomonas vaginalis* and *Tritrichomonas foetus* use this pathway to catabolise arginine (Scholfield et al., 1990, Yarlett, 1988), but it is not present in the cells of higher animals (Zuniga et al., 2002). Arginine catabolism by the arginine deiminase pathway is the major ATP generating pathway for *G. intestinalis* (Scholfield et al., 1990).

1.5.2.2.3. Arginine dihydrolase pathway

There is a further pathway for converting arginine to ornithine in some prokaryotes and unicellular eukaryotes. The protozoa *Herpetomonas* and *Tetrahymena* (Beutin and Eisen, 1983) and the bacteria *Streptococcus* and *Pseudomonas* (Slade, 1952) use this pathway to metabolise arginine (Figure 1.7). This pathway is absent in mammals, plants and most microorganisms. The first enzyme of this pathway is the same as that in the arginine deiminase pathway but unlike the latter pathway, the arginine dihydrolase pathway comprises two steps: (1) arginine deiminase converts arginine to citrulline and ammonia; (2) citrulline is then metabolised to ornithine, ammonia and CO_2 at the expense of ATP in the presence of citrullinase (Slade, 1952).

1.5.2.3. Nitric Oxide Synthesis

Arginine is converted to NO and citrulline by NOS (Wu and Morris, 1998). In mammals, there are three isoforms of NOS, neuronal NOS (NOS I or nNOS), inducible NOS (NOS II or iNOS) and endothelial NOS (NOS III or eNOS). NO is a neurotransmitter thought to be involved in individual learning and memory

skills (Pitsikas, 2009). It is also a signalling molecule in the cardiovascular system, where it acts via second messenger cGMP (reviewed by Boucher et al., 1999). As in most other organisms, use of NO as a signalling molecule has been demonstrated in helminths, including *S. mansoni, Schistosoma japonicum* (Long et al., 2004), *Dirofilaria immitis, B. pahangi* (Kaiser et al., 1998) and *Ascaris suum* (Bascal et al., 2001).

Lower NO concentrations are anti-inflammatory for tissues, but overproduction of NO is thought to be a contributor in causing a number of diseases. NO is harmful to parasites (Taylor-Robinson and Looker, 1998), bacteria and cancerous cells and is a host defence strategy to a number of bacterial pathogens of vertebrates (Vincendeau et al., 2003) and invertebrates (Rivero, 2006). Possible ways of controlling NO levels are competition of arginase and NOS for their common substrate arginine (Boucher et al., 1999). Some parasites diminish host arginine (Vincendeau et al., 2003) or suppress its production as an approach to reduce NO synthesis. An example of this is infection with the nematode *Trichinella spiralis:* inducible NOS (iNOS) levels decrease significantly in the jejunum in response to the infection, when the parasitic arginase starts competing for host arginine.

1.5.2.4. Polyamine Synthesis

Arginine is a precursor for the the three principal cell growth and differentiation regulator polyamines, putrescine, spermidine and spermine (Tabor and Tabor 1985). The proportions of the three polyamines vary in different organisms: prokaryotes generally have more putrescine than spermidine and have no spermine; vertebrates have all three, with putrescine the least, while protozoa appear to be intermediate between these two groups (Pegg, 1986, Tabor and Tabor, 1985, White et al., 1983). Polyamines may be an important target in controlling protozoan and nematode infection (Henderson and Fairlamb, 1987; Walter, 1988). Difluoromethylornithine (DFMO) is an anti-cancer agent that inhibits ODC activity irreversibly and has

been successfully used as a therapeutic agent against trypanosomes (Bacchi et al., 1994). There may also be anti-parasitic targets in nematodes other than ODC: S-adenosylmethionine, the donor of an amino-propyl group to the higher polyamines spermidine and spermine (Walter, 1988) and diamine acetylase and polyamine oxidase, enzymes catabolising polyamines (Wittich and Walter, 1989).

1.5.2.4.1. Polyamine synthesis by ornithine decarboxylase (ODC)

Ornithine is converted to putrescine by ODC (Pegg, 1986). Putrescine is then catabolised to spermidine and spermine by the enzymes spermidine synthase and spermine synthase respectively (Tabor and Tabor, 1985) (Figure 1.4). Early experiments suggested that nematodes could not synthesise putrescine, but acquired it from the host (Walter, 1988). Wittich et al. (1987) could not detect any ADC or ODC activity in the filarial nematodes *D. immitis, O. volvulus, Brugia patei* and *Litomosoides carinii*, which have high spermidine and spermine levels but little putrescine. The rat parasite *N. brasiliensis* also had no detectable putrescine (Sharma et al., 1989). More recently, the need for exogenous putrescine was disproved when ODC activity was demonstrated in *C. elegans*, *H. contortus* (Schaeffer and Donatelli 1990) and *P. redivivus* (Niemann et al., 1996) and genes encoding ODC were sequenced for *P. redivivus* (Niemann et al., 1996), *C. elegans* (Macrae et al., 1995) and *H. contortus* (Klein et al., 1997). Whereas the *P. redivivus* ODC is cytosolic, as in most eukaryotes (Niemann et al., 1996), the ODC of both *C. elegans* and *H. contortus* are membrane-bound and have low K_m values for ornithine (2.7 and 3.8 μM respectively) unlike mammalian enzymes (up to 3000 μM) (Schaeffer and Donatelli, 1990). Membrane binding may explain why nematode enzyme was not detected in some studies.

1.5.2.4.2. Polyamine synthesis by arginine decarboxylase (ADC)

ADC catalyses the conversion of arginine to agmatine. Agmatine is a neurotransmitter, regulator of cell division and cell inflammation, a stimulant of insulin release (Sener et al., 1989) and an inhibitor of NOS activity by

competing for the common substrate arginine (Galea et al., 1996). Earlier, ADC was thought to be present only in plants, prokaryotes and invertebrates and not in mammals (Tabor and Tabor, 1984). More recently, its presence has been confirmed in mammals (reviewed by Zhu et al., 2004) and the human ADC gene has been cloned (Mistry et al., 2002).

Agmatine is an alternative precursor for polyamine biosynthesis to the usual arginase-ODC route. Arginine decarboxylation by ADC forms agmatine which is hydrolysed by agmatinase to putrescine and urea (Slocum et al., 1984). Agmatinase activity was not detectable in *C. elegans* (Macrae et al., 1995) or adult *N. brasiliensis* (Walker and Barrett, 1991). Arginase and agmatinase belong to the same enzyme family and are capable of using either arginine or agmatine as substrate (Ahn et al., 2004). Gilad et al. (1996) proposed that mammalian ADC and ODC are two isoforms of the same enzyme, as both can use arginine and ornithine as substrates, but their different kinetic properties and cellular location suggest they are two different enzymes (Regunathan and Reis, 2000).

1.5.2.5. Phosphagen Synthesis

The phosphagens are phosphorylated guanidine compounds formed from creatine, glycocyamine, taurocyamine, lombricine and arginine (Figure 1.8). Phosphagen kinases catalyse the reversible transfer of phosphoryl groups between ADP/ATP and the phosphagen (Uda et al., 2005). Phosphocreatine is the sole phosphagen in vertebrate cells and creatine kinase (CK) is its corresponding kinase, while invertebrates have more than six phosphagens and respective kinases (Ennor and Morrison, 1958). In vertebrates, arginine is converted to creatine by the enzyme arginine:glycine amidinotransferase (AGAT). Creatine is synthesised mainly in the kidney and liver, from where it is transported to the muscles and acts as a major source of energy. Creatine synthesis is a two step reaction catalysed by two enzymes AGAT and S-

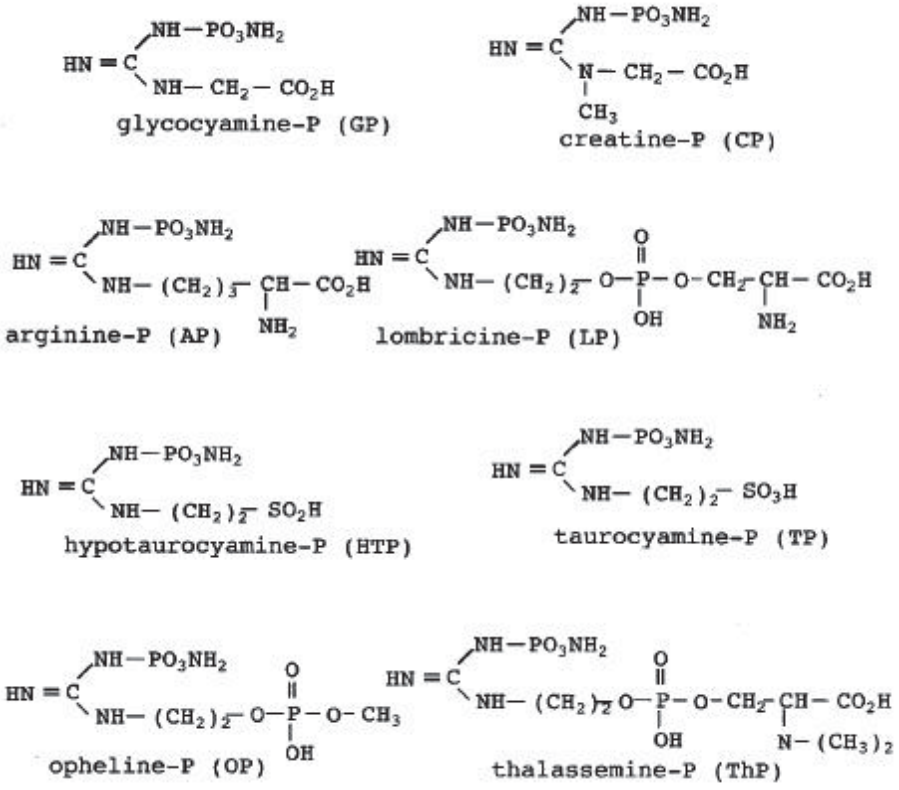

Figure 1.8. Phosphagen precursors which form the corresponding phosphagen by covalent attachment of a phosphate group to the guanidino moiety at the left of the molecule. (From Ellington, 2001).

adenosyl-L-methionine (reviewed by Ennor and Morrison, 1958; Wyss and Kaddurah-Daouk, 2000).

Phosphoarginine and arginine kinase (AK) are the most commonly found phosphagen and phosphagen kinase in invertebrates, such as arthropods, marine invertebrates (Abe et al., 2007; Platzer et al., 1995; Wallimann and Eppenberger, 1973), *H. contortus* larvae (Platzer et al., 1995), the entomopathogenic nematode *Steinernema carpocapsae* (Platzer et al., 1999) and protozoan parasite *Trypanosoma cruzi* (Pereira et al., 2000). AK is present in the monomeric as well as dimeric form; the latter form has been isolated from sea cucumber muscle (Held et al., 2007). AK activity may increase many fold in cells which have high demand for ATP, such as the flight muscles of insects (Schneider et al., 1989).

Neither phosphoarginine nor AK were detected in *A. lumbricoides* muscle (Barrett, 1973) or in *M. expansa, F. hepatica and Hymenolepis diminuta* (Barrett and Lloyd, 1981). Instead an active non-specific adenylate kinase was reported in *A. lumbricoides* and taurocyamine phosphotransferase in the other three species (Barrett and Lloyd, 1981). An ATP: phosphagen kinase gene has been cloned from the cercarial stage of *S. mansoni* by Stein et al. (1990). As they found a very low CK, but no AK activity, it was identified as CK, despite the gene having close homology to AK (Dumas and Camonis, 1993).

Invertebrate phosphagens and kinases are potential vaccine candidates because of their strong antigenicity in humans. The AK in the silkworm *B. mori* has been declared a major allergen in humans and shows cross reactivity with cockroach AK (Liu et al., 2009). Shrimp AK has shown to be the main enzyme responsible for seafood allergy in some individuals (Garcia-Orozco et al., 2007) and cross reacts with enzymes of many other invertebrates (Binder et al., 2001).

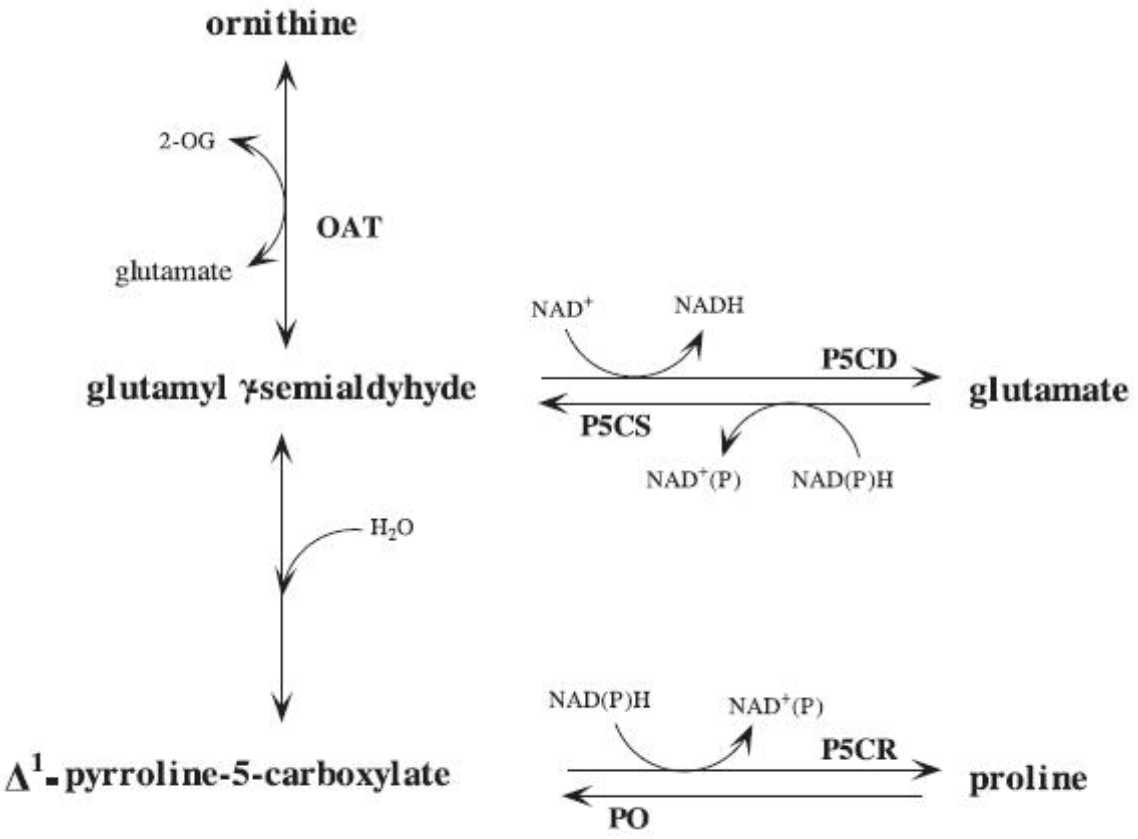

Figure 1.9. Enzymes involved in the interconversion of ornithine, glutamate and proline. Δ1-Pyrroline-5-carboxylate is spontaneously formed from glutamyl γ-semialdehyde making an equilibrium which favours P5C formation. Abbreviations used: 2-OG, 2-oxoglutarate; OAT, ornithine aminotransferase; PO, proline oxidase; P5CR, P5C reductase; P5CDH, P5C dehydrogenase; P5CS, P5C synthase

1.5.3. PROLINE METABOLISM

Proline is very important for synthesis of the cuticle of nematode parasites, as proline is a component of collagen. Proline is a non-essential amino acid for mammals, but it is not known whether nematodes are able to synthesise proline in sufficient amounts or must obtain it also from the host. In many invertebrates, proline is a source of energy (Auerswald et al., 1998; Obungu et al., 1999; Scaraffia and Wells, 2003); this may also be the case in nematodes. A fully functional ornithine-proline-glutamate pathway has been reported in the helminths *N. brasiliensis* (Walker and Barrett, 1991), *S. mansoni* (Isseroff et al., 1983), *F. gigantica* (Mohamed et al., 2008) and *F. hepatica* (Isseroff and Ertel, 1976).

Ornithine, glutamate and proline are interconvertable amino acids through an intermediate $^{1}\Delta$-pyroline-5-carboxylic acid (P5C) (Figure 1.9). Ornithine is converted reversibly by OAT to the intermediate P5C, which is then converted to proline or glutamate by pyrroline-5 carboxylate reductase (P5CR) and pyrroline-5-carboxylate dehydrogenase (P5CDH) respectively (Mohamed et al., 2008). P5C can also be synthesized from glutamate by the enzyme pyrroline-5-carboxylate synthase (P5CS) in an ATP and NADPH dependent reaction. P5C is formed from proline by proline oxidase (PO) and from glutamate by P5CS.

There are alternative routes of interconverting ornithine to proline and glutamate. In plants, instead of using P5C as an intermediate, ornithine is converted to α-keto-δ-aminovalerate, which is reduced to proline by pyrroline-2-carboxylate reductase (Aral and Kamoun, 1997). In bacteria, γ-glutamyl kinase and γ-glutamyl phosphate reductase are the enzymes responsible for converting glutamate to P5C (Smith, 1985).

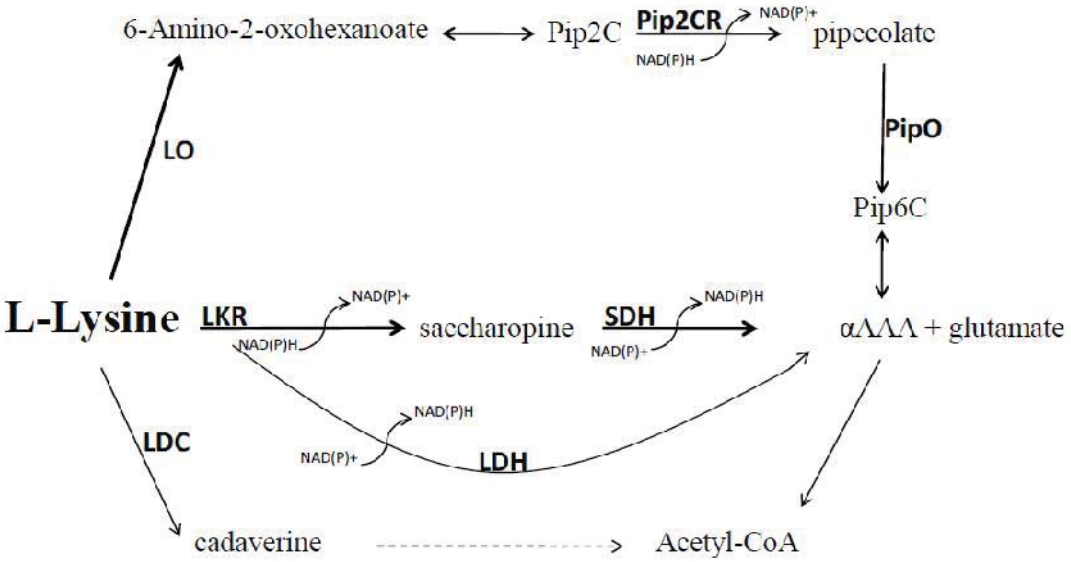

Figure 1.10. Lysine catabolism through the pipecolate, saccharopine and cadaverine pathways. Bold arrows indicate universal pathways of lysine catabolism in higher organisms. Enzymes are shown in bold. Abbreviations: α-AAA, α-amino adipic semialdehyde; LDC, lysine decarboxylase; LDH, lysine dehydrogenase; LKR, lysine ketoglutarate; LO, lysine oxidase; Pip2c, $^{1}\Delta$-piperideine-2-carboxylate; Pip2CR, $^{1}\Delta$-piperideine-2-carboxylate reductase; Pip6C, Δ^{1}-piperideine-6-carboxylate; PipO, pipecolate oxidase; SDH, saccharopine dehydrogenase.

1.5.4. LYSINE METABOLISM

The activity of proteins can be regulated by post-translational modifications of component amino acids. Lysine is the site in many nuclear and cytoplasmic proteins, including the nuclear histones, metabolic enzymes and controllers of cell differentiation and survival, where reversible acetylation plays an important role in the regulation of cellular functions in both eukaryotes (Choudary et al., 2009; Zhao et al., 2010) and prokaryotes (Zhang et al., 2009; Wang et al., 2010). Proteins can be cross-linked by transglutaminases through bridges between lysine and glutamine residues (reviewed by Greenberg et al., 1991). These enzymes are essential for nematode moulting, growth and maturation (Chandrashekar and Mehta, 2000).

Lysine is an essential amino acid in mammals (Murthy and Janardanasarma, 1999), in which only catabolic pathways are present, whereas lysine can be both synthesised from aspartate and catabolised through several different pathways in plants and microorganisms (Galili, 2002). The two universal pathways of lysine catabolism are shown in bold in Figure 1.10. Lysine metabolism has been studied in detail in plants (Azevedo and Lea, 2001) and vertebrates (Dodt et al., 2000; Ijlst et al., 2000; Goyer et al., 2004), but there appears to be no published information on lysine metabolism in nematodes.

1.5.4.1. The α-Amino Adipic Acid Pathway

The most common and well studied route of lysine catabolism in plants and animals is the saccharopine pathway, which converts lysine via saccharopine to α-amino adipic semialdehyde (α-AAA) and glutamate (Galili, 1995) (Figure 1.10). This pathway is almost always comprised of one bifunctional enzyme (Gaziola et al., 1997) with both lysine-ketoglutarate reductase (LKR) activity, that catalyses the conversion of lysine and 2-OG to

saccharopine, and saccharopine dehydrogenase (SDH) activity, which converts saccharopine to α-AAA and glutamate (Galili et al., 2001). Although there appear to be no biochemical studies on lysine metabolism in nematodes, gene sequences deposited in databases are consistent with a bifunctional LKR-SDH enzyme. In yeasts, there are separate enzymes with reversible activity, allowing lysine synthesis (Xu et al., 2007). In plants and vertebrates, LKR activity of the bifunctional enzyme strictly uses NADPH as co-factor, while the SDH activity requires either NAD^+ or $NADP^+$, although activity is usually much greater with the former (Tang et al., 1997). The gene for LKR-SDH is most highly expressed in the human liver and less so in most other organs (Sacksteder et al., 2000).

1.5.4.2. The Pipecolate Pathway

The alternative pathway of catabolism of lysine to glutamate and α-AAA is via pipecolate. In higher organisms, it is comprised of three enzymes: lysine oxidase (LO) catalyses the conversion of lysine to 6-amino-2-oxohexanoate, which spontaneously converts to Δ^1-piperideine-2-carboxylate (Pip2C); Δ^1-piperideine-2-carboxylate reductase (Pip2CR) catalyses the conversion of Pip2C to pipecolate; this is finally converted to aminoadipate 6-semialdehyde by pipecolate oxidase (PipO) (Figure 1.10). In animal tissues, L-lysine can be metabolised either through the pipecolate or saccharopine pathways (Broquist, 1991). Lysine catabolism via pipecolate is required in the brain to prevent neurological disorders (Broquist, 1991; Galili, 2002) because of low LKR-SDH activity (Chang, 1974; Chang et al., 1981). The saccharopine pathway is generally important in preventing accumulation of lysine, which is toxic in high concentrations (Epelbaum et al., 1997). In plants, lysine synthesis and catabolism via LKR-SDH are regulated to maintain homeostasis during seed formation and osmotic and other environmental stresses (Galili, 2002; Battur et al., 2009).

1.5.4.3. Non-mammalian Pathways

Microorganisms use additional and/or alternative enzymes (Figure 1.10) to those in the ubiquitous saccharopine and pipecolate pathways, often as part of pathways synthesising pharmacologically useful anti-microbial, anti-tumour and immunosuppressant chemicals (He, 2006). Some bacteria directly convert lysine to α-AAA using lysine dehydrogenase (LDH) (Dempsey et al., 1992) or lysine cyclodeaminase (Gatto et al., 2006). Lysine can be converted to Pip2C, not by LO, but by lysine-2-aminotransferase which oxidises the α-amino group to α-keto-ϵ-aminocaproic acid and Pip6C (He, 2006). D-lysine can also be converted to Pip2C by α-transamination with pyruvate (Yonaha et al., 1975). *Pseudomonas* spp. use a different Pip2CR belonging to the malate/lactate dehydrogenase family (Muramatsu et al., 2005; Revelles et al., 2007). In plants and bacteria, lysine is also catabolised by lysine decarboxylase (LDC) to cadaverine (Fecker et al., 1992), which is then converted to acetyl-CoA (Figure 1.10). LDC is involved in pH homeostasis in some bacteria when they are exposed to extremely low pH (Park et al., 1996), raising the possibility that LDC could fulfil a similar role in abomasal parasites in the acid environment of the stomach.

1.6. CONCLUSIONS

Recent studies of nitrogen metabolism in parasites have largely concentrated on neurotransmitters and their receptors, because of their well-established significance as anthelmintic targets. The second main area of interest has been parasite GSH and GSTs, enzymes which conjugate toxins and xenobiotics to protect against the host immune response. These have been used in successful vaccines to reduce parasite burdens. Other aspects of nitrogen metabolism have largely been ignored, except for the studies from this laboratory where unusual properties of some enzymes were detected in L3 and adult *T. circumcincta* and three novel enzymes, GOGAT, creatinase and

aspartase were identified as absent in the host (Muhamad, 2006). The chosen areas for the present study are glutamate, arginine, proline and lysine metabolism in L3 and adult *H. contortus* and *T. circumcincta*. Whole pathways have been examined using parasite homogenates and the next step of expressing individual enzymes has started with GDH. Studies of enzymes of glutamate metabolism are reported in Chapters 2-4, proline and arginine in Chapters 5 and 6 and lysine catabolism in Chapter 7.

Chapter II

Glutamate synthase, but not GABA shunt enzymes, contributes to nitrogen metabolism of the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair, R.J. Bland and H.V. Simpson

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Glutamate synthase, but not GABA shunt enzymes, contributes to nitrogen metabolism of the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair [a], R.J. Bland [b], H.V. Simpson [a,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand
[b] AgResearch Ltd., Private Bag 11-008, Palmerston North, New Zealand

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Succinic semialdehyde dehydrogenase (E.C. 1.2.1.24)
Ammonia assimilation

ABSTRACT

Glutamate synthase (E.C. 1.4.1.14) (GOGAT) activity was not detectable in L3 *Haemonchus contortus*, but was present in L3 *Teladorsagia circumcincta* and adult worms of both species. GOGAT activity was inhibited by 80% by azaserine. Activity (nmol min^{-1} mg^{-1} protein) was 33–59 in adult *H. contortus*, 51–91 in adult *T. circumcincta* and 24–41 in L3 *T. circumcincta*, probably depending on exposure to ammonia, as incubation with 1 mM NH_4Cl doubled GOGAT activity. The pH optimum was 7.5 in both species. Either NAD or NADP acted as co-factor. The mean apparent K_m for 2-oxoglutarate was 0.7 (0.5–0.9) mM and for glutamine was 1.0 (0.5–1.7) mM for different homogenates. There was no detectable activity in whole parasite homogenates of glutamate decarboxylase (E.C. 4.1.1.15) or succinic semialdehyde dehydrogenase (E.C. 1.2.1.24), the first and third enzymes of the GABA shunt, respectively, suggesting that the GABA shunt is not important in general metabolism in these species.

1. Introduction

Parasitic nematode larvae grow rapidly and adult worms lay large numbers of eggs, both requiring very active nitrogen and energy metabolism. Worm enzymes, particularly those not present in the host, are therefore potential targets for controlling the parasites through new therapies, either chemical or immunological. As glutamate plays a central role in nitrogen metabolism, being converted to other amino acids and in both in excretion of ammonia and its incorporation in lower organisms, some enzymes associated with glutamate metabolism are of particular interest. Two aspects which could provide opportunities for the control of parasitic nematodes are their use of the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway to synthesise amino acids from ammonium and possible use of the GABA shunt to bypass a part of the tricarboxylic acid cycle (TCA) which has low activity in anaerobic conditions.

In a recent study of nitrogen excretion by *Teladorsagia circumcincta* in vitro, there appeared to be re-uptake of excreted ammonia during several hours of incubation (Simpson et al., 2009). It was suggested that the disappearance of the ammonia from the medium was caused by its incorporation into glutamate by the GS-GOGAT pathway. Whereas GS is universally present, GOGAT is usually absent in animals, but is expressed in some nematodes and insects, particularly the silkworms *Bombyx mori* (Hirayama et al., 1998) and *Samia cynthia ricini* (Osanai et al., 2000), the mosquito *Aedes aegypti* (Scaraffia et al., 2005) and in the *Spodoptera frugiperda* Sf9 insect cell line (Doverskog et al., 2000). GOGAT activity has been demonstrated in homogenates of *T. circumcincta* by Muhamad et al. (2005) and gene sequences for GOGAT from *Caenorhabditis elegans* (Vanoni and Curti, 1999), *Caenorhabditis remanei*, *Caenorhabditis briggsae* and *Brugia malayi* have been deposited in databases.

The GS-GOGAT pathway is important for ammonia assimilation in bacteria and plants (Miflin and Habash, 2002; Magasanik, 2003) when the ammonia concentration is low and ATP is available (Helling, 1994, 2002), whereas there is a low affinity for ammonium for the alternative enzyme glutamate dehydrogenase (GDH), including the *T. circumcincta* enzyme with a K_m of 18 mM (Muhamad et al., 2004). There are three forms of GOGAT according to the co-factor requirements: NADH-GOGAT and NADPH-GOGAT are present in plants and bacteria, whilst Fd-GOGAT is the form in photosynthetic organisms (Vanoni and Curti, 1999). In the present experiments, the activity and properties of GOGAT have been investigated in *Haemonchus contortus* and *T. circumcincta*, the two important abomasal nematode parasites of small ruminants.

* Corresponding author. Fax: +64 6 350 5636.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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The GABA (gamma-amino butyric acid) shunt has been reported to play a role in nitrogen metabolism in addition to the role of GABA as neurotransmitter in nematodes (Schuske et al., 2004). The GABA shunt is a three enzyme pathway that forms succinate from glutamate via GABA, bypassing the part of the TCA cycle (Balazs et al., 1970) in which α-ketoglutarate is oxidatively decarboxylated to succinate by α-ketoglutarate dehydrogenase. First, L-glutamate is decarboxylated irreversibly to GABA by glutamate decarboxylase (GAD), then GABA is converted reversibly to succinic semialdehyde (SSA) by 4-aminobutyrate transaminase (GABA-T) and finally succinic semialdehyde dehydrogenase (SSADH) irreversibly converts SSA to succinate. The GABA shunt operates in plants (Shelp et al., 1999) and in bacteria during anaerobic conditions (Dunn, 1998) and may be important in the larger parasitic helminths, especially those in the more anaerobic environments.

GAD and GABA-T activities have been observed in *Ascaris lumbricoides*, *Ascaridia galli*, *Taenia solium, Macracanthrynchus hyrudinaceus* and *Moniezia expansa* (Monteoliva et al., 1965; Rasero et al., 1968). GAD has been located in the *A. lumbricoides* reproductive tract and intestine (Monteoliva et al., 1965) and the *A. galli* cuticle (Singh et al., 1983). GABA-T has been partially purified and characterised in *Nippostrongylus brasiliensis* (Watts and Atkins, 1983, 1984) and activity reported in this species by Walker and Barrett (1991). Evidence for a metabolic role for this pathway in helminths is conflicting, being supported by Monteoliva et al. (1965) and Rasero et al. (1968) for three species of intestinal parasites and in *Onchocerca volvulus* and *Brugia pahangi* by MacKenzie et al. (1989), but not by Cornish and Bryant (1975) for *M. expansa*. As there appear to be no studies in ruminant parasitic nematodes, the activities of GABA shunt enzymes and a possible metabolic function has been investigated in these nematodes.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (MO, USA) unless stated otherwise.

2.1. Parasites

L3 *T. circumcincta* or *H. contortus* were cultured from the faeces of sheep infected with a pure strain of parasite and stored in reverse osmosis (RO) water at 4 °C for the former and at 10 °C for the latter species. Fresh larvae were used or prior to each experiment, L3 were baermannised in RO water to remove inactive worms and re-suspended in buffer. Adult worms were recovered from the abomasa of infected sheep using the technique of Simpson et al. (1999). Briefly, abomasal contents were mixed 2:1 with 3% agar and, after solidification, the agar blocks were incubated at 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and placed in buffer.

2.2. Preparation of homogenates

About 50,000 L3 were centrifuged at 600*g* for 5 min, washed twice by re-suspending in assay buffer followed by centrifugation, then finally re-suspended in 1 ml buffer. The concentrated L3 suspension was transferred to a chilled mortar and frozen at −20 °C for at least 15 min. The frozen pellet was then disrupted using a chilled pestle and the homogenate was examined microscopically to ensure complete disruption of the parasites. Homogenates were similarly prepared using approximately 5 mg of clumped adult worms. Finely chopped sheep brain was homogenised in ice-cold 320 mM sucrose and 4.5 mM mercaptoethanol, according to the method of Hao and Schmit (1991). When homogenates were to be used for more than one assay, aliquots were kept in Eppendorff tubes on ice until required. The protein concentrations of homogenates were determined by the Bradford method (1976).

2.3. Enzyme assays

2.3.1. Glutamate synthase (GOGAT) (E.C. 1.4.1.14)

GOGAT activity was determined at 30 °C from the rate of conversion of NADH to NAD^+ during the reaction of glutamine, NADH and 2-oxoglutarate to form 2 glutamate and NAD^+. The reaction was monitored spectrophotometrically at 340 nm using an Ultraspec III (Pharmacia LKB) equipped with a temperature control unit.

(1) The presence of enzyme activity was determined in homogenates of L3 ($n = 2$) and adult ($n = 2$) *T. circumcincta* and L3 ($n = 3$) and adult ($n = 2$) *H. contortus.* The reaction mixture (total volume of 1 ml) contained 2.5 mM glutamine,10 mM 2-oxoglutarate and 50 μg homogenate protein in 100 mM phosphate buffer, pH 7.5; the reaction was initiated by the addition 0.2 mM NADH.
(2) The apparent K_m for glutamine was determined in homogenates of *T. circumcincta* L3 and adult worms and adult *H. contortus* ($n = 2$). The substrate concentrations were 10 mM 2-oxoglutarate, 0.2 mM NADH and glutamine varying from 0 to 30 mM.
(3) The apparent K_m for 2-oxoglutarate was determined in homogenates of L3 and adult *T. circumcincta* and adult *H. contortus* ($n = 2$). The substrate concentrations were 10 mM glutamine, 0.2 mM NADH and 2-oxoglutarate varying from 0 to 30 mM.
(4) Activity with NADPH as co-factor was determined in homogenates of L3 and adult *T. circumcincta* and *H. contortus* ($n = 2$). The substrate concentrations were 10 mM glutamine, 10 mM 2-oxoglutarate and 0.2 mM NADPH.
(5) The effect of azaserine was determined in homogenates of *T. circumcincta* L3 and adult worms and adult *H. contortus* ($n = 2$). Two millimolar azaserine was included in the reaction mixture with 10 mM glutamine and 10 mM 2-oxoglutarate; the reaction was initiated by the addition 0.2 mM NADH.
(6) The effect of pre-incubation of L3 with NH_4Cl was determined in L3 *T. circumcincta* and *H. contortus* ($n = 3$). Approximately 50,000 L3 were suspended in 10–15 ml 50 mM phosphate buffer, pH 7.5, containing either 0, 0.5 or 1 mM NH_4Cl. The tubes were incubated at 37 °C overnight. Homogenates were made and GOGAT activity assayed with substrate concentrations of 10 mM glutamine, 10 mM 2-oxoglutarate and 0.2 mM NADH.

2.3.2. Glutamate decarboxylase (GAD) (E.C. 4.1.1.15)

GAD activity was assayed at in homogenates of L3 ($n = 3$) and adult ($n = 2$) *T. circumcincta*, L3 ($n = 3$) and adult ($n = 2$) *H. contortus* and in sheep brain ($n = 2$) as a positive control. Enzyme activity was determined by measuring the amount of $^{14}CO_2$ released from L-[1-14C]glutamic acid (Hao and Schmit, 1991). The reaction mixture (total volume 200 μl) was prepared in 50 mM Tris–HCl, pH 5.5, containing 0.9 mM PLP, 0.9 mM EDTA, 0.9 mM 2-mercaptoethanol, 45 mM sodium piperazine-*N*,*N*′-bis (2-ethanosulphonic acid) and 50 μg homogenate, in 2 ml disposable plastic tubes with an airtight lid. A small plastic tube containing filter paper dipped in 250 μM KOH was placed in the larger tube for absorption of $^{14}CO_2$. The reaction was started by the addition of 30 mM glutamic acid and 0.055 μCi of L-[1-14C]glutamic acid (GE Healthcare Amersham, UK). After 60 min incubation in a shaking bath at 37 °C, the reaction was stopped by the addition of 200 μl of 2 N H_2SO_4. The CO_2 was allowed to be absorbed for 2 h, mixed with cocktail (Optiphase super mix, Wallac Scintillation Products, Loughborough, UK) and

the amount of $^{14}CO_2$ was determined in a scintillation counter (Wallac 1450 Microbeta Trilux, UK).

To optimise the assay, the buffer pH was varied from 3 to 7, the PLP concentration from 0 to 3 mM, glutamic acid from 5 to 50 mM, and the L-[1-14C]glutamic acid from 0 to 1 μCi. The reaction was also performed in 50 and 100 mM phosphate buffers, pH 3–7.

2.3.3. Succinic semialdehyde dehydrogenase (SSADH) (E.C. 1.2.1.24)

SSADH activity was assayed at 30 °C in homogenates of L3 (n = 3) and adult (n = 2) *T. circumcincta*, L3 (n = 3) and adult (n = 2) *H. contortus* and in sheep brain (n = 2) as a positive control. Enzyme activity was calculated from the rate of production of NADH/NADPH from $NAD^+/NADP^+$, which was monitored spectrophotometrically at 340 nm (Boer and Bruinvels, 1977). The reaction mixture (total volume 1 ml) contained 0.25 mM NAD^+, 0.3 mM succinic semialdehyde (SSA), 8.2 mM 2-mercaptoethanol and homogenate containing 50 μg protein in 50 mM Tris–HCl buffer, pH 9. The mixture was incubated for 30 min at 30 °C, then 0.1 ml ice-cold 3.4 mM SSA was added to the incubation mixture and the reaction started by placing the tubes at 30 °C.

The SSA concentration was varied from 0 to 10 mM and the pH from 6.5 to 10.5. Incubations were also carried out at 22, 25 or 37 °C. The reaction was also carried out in 50 mM phosphate buffer, pH 9. Experiments were also performed by replacing NAD^+ with $NADP^+$.

2.3.4. Data analysis

Replicate data are presented as means ± SEM. Graphpad Prisrm v4 was used to plot kinetic data and estimate apparent K_m and V_{max}. The effects of pre-incubation with NH_4Cl were analysed by one-way ANOVA and Bonferroni post-tests and of azaserine using paired t-tests.

3. Results

3.1. Glutamate synthase (GOGAT) activity

There was no detectable GOGAT activity at 30 °C in any of the six homogenates of L3 *H. contortus*, whereas enzyme activity (nmol min^{-1} mg^{-1} protein, mean ± SEM) over all experiments without inhibitors or stimulants was 48 ± 3.6 (n = 8) in adult *H. contortus*, 67 ± 4.9 (n = 8) in adult *T. circumcincta* and 31 ± 1.8 (n = 11) in L3 *T. circumcincta*. The enzyme activity and kinetic properties of GOGAT in adult *H. contortus* and *T. circumcincta* L3 and adult worms are shown in Table 1. The properties of the enzyme were generally similar in the two species. The apparent K_m for 2-oxoglutarate was in the range 0.6–0.9 mM and for glutamine 0.6 mM in L3 *T. circumcincta* but a little higher in adult worms of both species (1 mM and 1.5 mM). The pH optimum was 7.5 for the enzyme from *H. contortus* adult worms and *T. circumcincta* L3 (Fig. 1).

When NADPH was substituted for NADH, enzyme activities were all within the ranges obtained with NADH as co-factor. Activities (nmol min^{-1} mg^{-1} protein, mean ± SEM, n = 2), were 43 ± 5.0 in adult *H. contortus*, 57 ± 3.5 in adult *T. circumcincta* and 31 ± 2.5 in L3 *T. circumcincta*.

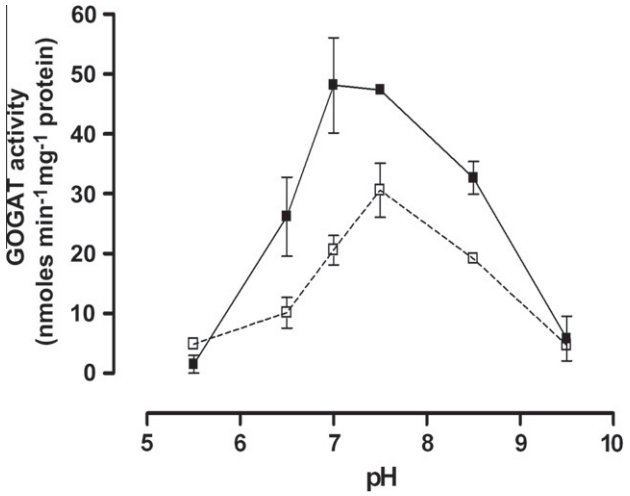

Fig. 1. Effect of pH on glutamate synthase (GOGAT) activity (nmol min^{-1} mg^{-1} protein) at 30 °C of homogenates of adult *H. contortus* (■) and L3 *T. circumcincta* (□) (mean ± SEM, n = 2).

3.2. Effect of azaserine on GOGAT activity

Addition of 2 mM azaserine inhibited GOGAT activity significantly (p = 0.001), overall by 79 ± 3.2% for two homogenates each of adult *H. contortus* and L3 and adult *T. circumcincta*. For species and life-cycle stages, the inhibitions were 89% for adult *H. contortus*, 76% for adult and 72% for L3 *T. circumcincta*.

3.3. Effect on GOGAT activity of pre-incubation of L3 T. circumcincta with NH_4Cl

Incubation of L3 *H. contortus* (n = 3) with added NH_4Cl did not result in detectable GOGAT activity, whereas 1 mM, but not

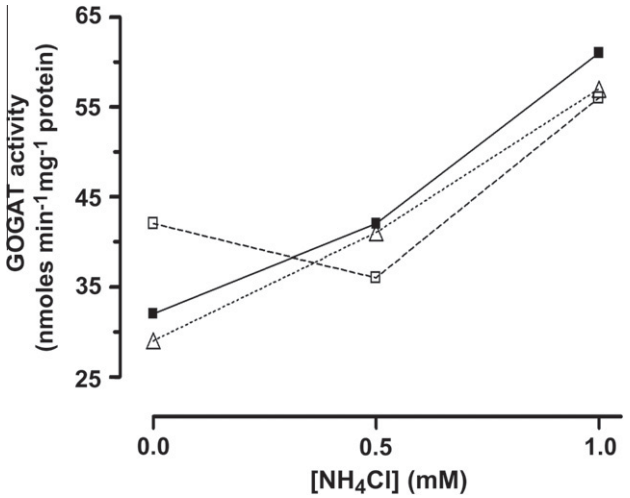

Fig. 2. Effect on glutamate synthase (GOGAT) activity (nmol min^{-1} mg^{-1} protein) at 30 °C of pre-incubation of three populations of L3 *T. circumcincta* in 100 mM phosphate buffer, pH 7.5, containing 0.5 or 1 mM NH_4Cl. Enzyme activity was significantly increased (p < 0.05) by 1 mM NH_4Cl.

Table 1
Kinetic properties of glutamate synthase (GOGAT) in adult *H. contortus* and *T. circumcincta* L3 and adult worms.

Nematode	V_{max} (nmol min^{-1} mg^{-1} protein) mean ± SEM (range)	K_m 2-OG (mM)	K_m glutamine (mM)
Adult *H. contortus* (n = 2)	42 ± 9.0 (33–59)	0.9 ± 0.05	1.0 ± 0.15
Adult *T. circumcincta* (n = 3)	65 ± 9.5 (51–91)	0.8 ± 0.05	1.5 ± 0.20
L3 *T. circumcincta* (n = 2)	37 ± 4.5 (24–41)	0.6 ± 0.05	0.6 ± 0.05

0.5 mM, NH_4Cl increased enzyme activity significantly ($p < 0.05$) in L3 *T. circumcincta* ($n = 3$) (Fig. 2). GOGAT activity after exposure to 1 mM NH_4Cl was 58 ± 1.5 nmol min^{-1} mg^{-1} protein.

3.4. GABA shunt enzyme activity

No activity of either GAD or SSADH was detected in homogenates of either adult or L3 *T. circumcincta* or *H. contortus*, whereas the activities in sheep brain, the positive control for the assays, were 185 ± 5 and 84 ± 9.1 ($n = 2$) nmol min^{-1} mg^{-1} protein, respectively.

4. Discussion

GOGAT activity has been demonstrated for the first time in *H. contortus*, but surprisingly only in adult worms, not L3. In contrast, enzyme activity was present in both the free-living and parasitic stages of *T. circumcincta*, as previously reported by Muhamad et al. (2005). The wide range of enzyme activity in different homogenates may be related to prior exposure of the parasites to ammonia, as pre-incubation of L3 *T. circumcincta* with 1 mM NH_4Cl significantly ($p < 0.05$) increased GOGAT activity to levels seen in adult worms (Fig. 2), while a lower concentration of ammonia appeared to have a proportionately lesser stimulatory effect on enzyme activity in some experiments. Exposure of L3 *H. contortus* to NH_4Cl did not induce enzyme activity. In vivo, adult worms are likely to be may be exposed to ammonia concentrations similar to those in the incubates: 1 mM in sheep abomasal fluid and 5 mM in rumen fluid (Harrop, 1974; Harrop and Phillipson, 1974), compared with 487 μM in portal blood and 200 μM in arterial blood (Parker et al., 1995).

The high degree of inhibition by azaserine (80%) supports the enzyme activity being GOGAT, as azaserine has been used to inhibit GOGAT from many organisms (Rachim and Nicholas, 1985; Mérida et al., 1991), including *B. mori* (Hirayama et al., 1998) and *A. aegypti* (Scaraffia et al., 2005). Azaserine is also a very weak inhibitor of glutamine:fructose-6-phosphate amidotransferase (Ghosh et al., 1960; Chmara et al., 1985). The nematode enzyme appears to be typical in using either NADH or NADPH as the electron donor. The pH optimum of 7.5 was the same as reported for the enzyme from *B. mori* (Hirayama et al., 1998) and *Euglena gracilis* (Miyatake and Kitaoka, 1981). The affinity for 2-oxoglutarate was greater than for glutamine, as in *B. mori* (Hirayama et al., 1998) and *Sclerotinia sclerotiorum* (Rachim and Nicholas, 1985), and both were within quite a wide range of apparent K_m values reported for different organisms, such as *Chlamydomonas reinhardii* (Cullimore and Sims, 1981) and *Lupinus angustifolicus* (Boland and Benny, 1977). The properties of the enzymes in *H. contortus* and *T. circumcincta* were very similar.

There was no detectable activity of GABA shunt enzymes in homogenates of whole L3 or adult worms of either abomasal parasitic species. Despite the positive controls for the enzymes (sheep brain), showing significant activity and assay conditions being extensively altered, no activity for either GAD or SSADH was recorded. GABA is known to be a nematode neurotransmitter, so it would be expected that at least GAD would be expressed in the neuromuscular tissues, however, the GABA shunt does not appear to operate to bypass the TCA cycle enzyme converting 2-oxoglutarate to succinate. Parasites where this appears to occur are generally larger, e.g., Ascarids (Monteoliva et al., 1965; Rasero et al., 1968) or inhabit the intestine, which may be a more anaerobic environment than the stomach.

The upregulation of GOGAT activity by exposure to ammonia is consistent with the disappearance of excreted ammonium during several hours of incubation of L3 *T. circumcincta* (Simpson et al., 2009) and probable incorporation into amino acids, as has been shown in *Samia cynthia ricini* where alanine was synthesised from labelled ammonium (Osanai et al., 2000). The importance of GOGAT in insects is shown by the increased enzyme activity associated with glutamine usage during synthesis of silk by *B. mori* (Hirayama et al., 1998). Azaserine in the diet increased glutamine and reduced proline in the haemolymph of *A. aegypti* (Scaraffia et al., 2005), interfering with metabolism of the blood meal. It would appear that nematodes also have the ability to use ammonia as a source of nitrogen as an alternative to amino acids through the GS-GOGAT pathway, which may be advantageous in the high ammonia-low amino acid environment in abomasal fluid.

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Chapter III

Molecular and biochemical characterisation of a *Teladorsagia circumcincta* glutamate dehydrogenase

S. Umair, J.S. Knight, M.L. Patchett, R.J. Bland and H.V. Simpson

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Molecular and biochemical characterisation of a *Teladorsagia circumcincta* glutamate dehydrogenase

S. Umair [a], J.S. Knight [b], M.L. Patchett [c], R.J. Bland [b], H.V. Simpson [a,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] Hopkirk Research Institute, AgResearch Ltd., Private Bag 11-008, Palmerston North, New Zealand
[c] Institute of Molecular Biosciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand

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ABSTRACT

A full length cDNA encoding glutamate dehydrogenase was cloned from *Teladorsagia circumcincta* (*Tc*GDH). The *Tc*GDH cDNA (1614 bp) encoded a 538 amino acid protein. The predicted amino acid sequence showed 96% and 93% similarity with *Haemonchus contortus* and *Caenorhabditis elegans* GDH, respectively. A soluble N-terminal 6xHis-tagged GDH protein was expressed in the recombinant *Escherichia coli* strain BL21 (DE3) pGroESL, purified and characterised. The recombinant *Tc*GDH had similar kinetic properties to those of the enzyme in homogenates of *T. circumcincta*, including greater activity in the aminating than deaminating reaction. Addition of 1 mM ADP and ATP increased activity about 3-fold in the deaminating reaction, but had no effect in the reverse direction. *Tc*GDH was a dual co-factor enzyme that operated both with NAD^+ and $NADP^+$, GDH activity was greater in the deaminating reaction with $NADP^+$ as co-factor and more with NADH in the aminating reaction.

1. Introduction

Metabolism of glutamate links nitrogen and energy metabolism through the interconversion of glutamate and 2-oxoglutarate (2-OG), a TCA cycle substrate, catalysed by glutamate dehydrogenase (GDH) and a large group of transaminases (Forde and Lea, 2007). Glutamate is also incorporated into proteins, is involved in the synthesis of other amino acids and is probably the major source of ammonia excreted by adult and L3 *Teladorsagia circumcincta* in vitro (Simpson et al., 2009). After several hours incubation, L3 appeared to reabsorb and metabolise ammonia in the incubation medium, most likely through the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway (Muhamad, 2006; Muhamad et al., 2011; Umair et al., 2011), since GOGAT activity was increased by incubation of L3 with ammonia for a similar time (Umair et al., 2011). The presence of the GS–GOGAT pathway, in addition to GDH, in adult and L3 *T. circumcincta* distinguishes parasite metabolism from that of the host and suggests potential anthelmintic targets could include these enzymes, their regulators, such as the PII receptor protein (Hsieh et al., 1998; Arcondéguy et al., 2001; Fokina et al., 2010), and the ammonium permease Amt/Rh transporter family (Tremblay and Hallenbeck, 2009).

GDH catalyses the reversible oxidative deamination of glutamate to ammonia and 2-OG and can either function in ammonia assimilation or generation of ammonia and 2-OG (Goldin and Frieden, 1971; Hudson and Daniel, 1993). Because of the low affinity for ammonia of GDH, it may assimilate ammonia under stress conditions, energy limitation or raised ammonia levels (Helling, 1998). The GS–GOGAT pathway, which requires ATP, also assimilates ammonia into glutamate in plants (Hodges, 2002; Miflin and Habash, 2002) and bacteria (Fisher and Sonenshein, 1991; Helling, 1998) when ATP levels are high and ammonia concentrations are low and GDH provides the 2-OG for the reaction (Aubert et al., 2001; Masclaux-Daubresse et al., 2006; Labboun et al., 2009).

GDH activity has been demonstrated in homogenates of L3 and adult *T. circumcincta* and the enzyme(s) shown to be able to use either NAD^+ or NADP as the co-factor (Muhamad et al., 2011). Based on co-factor requirements, GDH can be NAD^+ specific (E.C. 1.4.1.2), $NADP^+$ specific (E.C. 1.4.1.4) or dual co-factor specific (E.C. 1.4.1.3), which can use both co-factors (Hudson and Daniel, 1993). The dual-specificity GDH activity seen in *T. circumcincta* homogenates may thus be due to a single enzyme or to separate enzymes. In some organisms, separate NAD^+-GDH and NADP-GDH are responsible for the deaminating (glutamate utilisation) and aminating (glutamate formation) reactions; commonly dual co-factor enzymes have greater aminating activity with $NADP^+$ and deamination with NADH (Goldin and Frieden, 1971; Hudson and Daniel, 1993).

The enzymes in the GDH–GS–GOGAT cycle and their regulatory proteins are all possible anthelmintic targets, because of their

* Corresponding author. Address: Hopkirk Research Institute, Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand. Fax: +64 6 350 5636.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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importance in maintaining the levels of glutamate as a substrate for numerous enzymes and more widely influencing other metabolic pathways. The provision of a purified *T. circumcincta* GDH will allow kinetic studies of the purified protein and may clarify whether single or multiple enzymes are present. In this study, the gene encoding *T. circumcincta* was sequenced, the protein expressed in *Escherichia coli* and the kinetic properties of the purified enzyme determined.

2. Materials and methods

Use of experimental animals has been approved by the Massey University Animal Ethics Committee.

2.1. Parasites

Adult parasites were recovered from the abomasa of infected sheep using the technique of Simpson et al. (1999). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and stored at −80 °C.

2.2. cDNA isolation

A partial *T. circumcincta* glutamate dehydrogenase cDNA was amplified by the PCR from plasmid DNA extracted from a cDNA library prepared from an adult *T. circumcincta* (Custom cDNA Library in pCM.SPORT6.1, Invitrogen) using oligonucleotide primers based on an *Ostertagia ostertagi* putative glutamate dehydrogenase EST sequence (Genbank Accession No. BM897929). The 5′ end of the GDH cDNA was amplified from *T. circumcincta* cDNA library plasmid DNA using the PCR and *T. circumcincta* GDH-specific oligonucleotide primers, based on the partial *Tc*GDH cDNA, in conjunction with a library vector-specific oligonucleotide primers. The 3′ end of the *T. circumcincta* GDH cDNA was obtained by 3′ RACE (SMART RACE cDNA Amplification Kit, Clontech) using *T. circumcincta* adult RNA, as outlined by the manufacturer. The full length *T. circumcincta* GDH cDNA (Genbank Accession No. JF731342) was amplified by the PCR from cDNA prepared from adult *T. circumcincta* mRNA, using the oligonucleotide primers GDH expF (5′-CACCATGCTGAGCATTTTGGCACGTAC-3′) and GDH Full Rev (5′-TATCCTACGTAAAAGTGAAACCGGC-3′). *T. circumcincta* total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesised using the iScript Select cDNA Synthesis Kit (Bio-Rad). The full length product was cloned into the pET100 vector using the Champion pET Directional TOPO Expression Kit (Invitrogen) to allow the production of an N-terminal His and Xpress epitope tagged recombinant protein according to the manufacturer's instructions.

2.3. Expression and purification

E. coli strain BL21 (DE3) pGroESL (the generous gift of George Lorimer (Dupont)), transformed with pET100 *Tc*GDH, was grown in 10 ml Luria Broth (LB) containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) for 16 h at 37 °C. The culture was diluted to 3 l with LB containing antibiotics and incubated at 37 °C and 250 rpm until the OD_{600nm} reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture grown at 24 °C and 250 rpm for 6 h. Bacteria were harvested by centrifugation and the soluble extract was obtained by treating the resuspended cells with lysozyme (1 mg/ml), repeated freeze–thawing and sonication. Recombinant *Tc*GDH was purified with Ni-NTA agarose (Qiagen) using the manufacturer's standard protocol. Protein concentration was determined by the method of Bradford (1976). There was no detectable yield of soluble protein using *E. coli* strains BL21 (DE3) and Rosetta gami (DE3).

2.4. Electrophoresis

SDS–PAGE was performed using NuPAGE Novex 4–12% Bis-Tris gels according to the manufacturer's instructions (Invitrogen). Gels were stained with Coomassie Blue. A Western blot was also performed on the protein using a monoclonal anti-polyhistidine-peroxidase antibody (Sigma).

2.5. Enzyme assays

GDH activity of purified recombinant *Tc*GDH was determined both in the direction of glutamate formation and utilisation. Assays were carried out at 30 °C in 1 ml assay mixture containing 100 mM phosphate buffer pH 7.5 and 50 μg protein unless otherwise stated. Enzyme activity (V_{max}) was determined in the direction of glutamate utilisation by the rate of production of NADH/NADPH or by the rate of utilisation of NADH/NADPH in the direction of glutamate formation, measured spectrophotometrically at 340 nm.

(1) The optimum pH was determined in both directions with substrate concentrations of 0.5 mM 2-OG and 40 mM ammonia or 5 mM glutamate with pH range 5.5–9.5. Subsequent assays were carried out at pH 7.5.
(2) The optimum concentration of co-factors was determined by using $NAD(P)^+$ concentrations from 0 to 3 mM with 5 mM glutamate or 0 to 0.5 mM NAD(P)H with 0.5 mM 2-OG and 40 mM ammonia.
(3) The K_m for glutamate was determined in reaction mixtures containing 0–15 mM glutamate and 2 mM NAD^+ or $NADP^+$ and the K_m for 2-OG with 0–1 mM 2-OG, 40 mM ammonia and 0.2 mM NADH or NADPH. The K_m for ammonia was determined with 0–250 mM ammonia, 0.5 mM 2-OG and 0.2 mM NADH or NADPH.
(4) The effects of adding 1 mM $CaCl_2$, $CoCl_2$, $CuSO_4$, $MgSO_4$, $MnCl_2$, $NiCl_2$, azaserine or citrate as inhibitors/activators were determined in the direction of glutamate utilisation. The effects of adding 1 mM ATP or ADP were also determined for the purified protein in the direction of both glutamate utilisation and formation.

2.6. Data analysis

Replicate data are presented as mean ± SEM. Graph Prism v5 was used to plot kinetic data and estimate K_m and V_{max}. Student's *t*-test was used to compare K_m values.

3. Results

3.1. pET100 TcGDH gene sequence

The 1614 bp full length *T. circumcincta* glutamate dehydrogenase cDNA sequence (Supplementary Fig. 1), has been deposited in Genbank as Accession No. JF731342. The *Tc*GDH cDNA encoded a 538 amino acid protein with a predicted molecular mass of 59 kDa. Alignment of the predicted amino acid sequence showed 96%, 93% and 79% similarity to *Haemonchus contortus*, *Caenorhabditis elegans* and human GDH, respectively, using Alignmentx Vector NTI 12 (Fig. 2). Binding sites and conserved residues, deduced by comparison with the human GDH (Rosso et al., 2008), are identical.

3.2. Recombinant protein expression

Maximal production of a functional recombinant GDH was obtained in the *E. coli* strain BL21 (DE3) pGroESL when expression was induced with 0.5 mM IPTG for 6 h at 24 °C. The purified recombinant *Tc*GDH protein electrophoresed as a single band of about 60 kDa between 49 and 62 kDa on an SDS polyacrylamide gel (Fig. 1). The presence of a His-Tag was confirmed by Western blotting (data not shown).

3.3. Enzyme assays

Enzyme activities and kinetic properties of the recombinant GDH are shown in Table 1. The optimum pH for GDH activity both in the direction of glutamate utilisation and formation was 7.5 (Fig. 3). The aminating reaction had a broader pH range than the forward reaction and significant activity was detected even at pH 9.5.

Substituting $NADP^+$/H for NAD^+/H did not affect either the substrate K_m or enzyme activity (nmol min^{-1} mg^{-1} protein) in either direction ($P > 0.05$) (Table 1). The K_m for glutamate was higher than that for 2-OG and the K_m for ammonia was 37–40 mM (Table 1). The rate of the amination reaction was approximately double than that of the deamination reaction (Table 1). The affinity of the enzyme for $NAD(P)^+$ was 10-fold less than for NAD(P)H (Table 2).

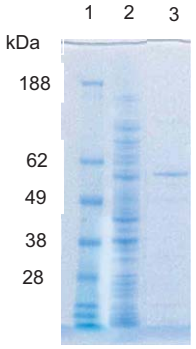

Fig. 1. SDS–PAGE of recombinant *Tc*GDH. Lane 1: standards; lane 2: non-purified glutamate dehydrogenase (cell lysate); lane 3: purified *Tc*GDH.

Table 1
Kinetic properties of recombinant *Tc*GDH in the directions of glutamate utilisation and formation with the cofactors $NAD(P)^+$ and NAD(P)H, respectively. Enzyme activity at 30 °C was monitored spectrophotometrically at 340 nm.

Substrate	Co-factor	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1} protein)
Glutamate	NAD^+	0.35 ± 0.05	380 ± 20
Glutamate	$NADP^+$	0.45 ± 0.05	410 ± 10
2-Oxoglutarate	NADH	0.1	1112 ± 70
2-Oxoglutarate	NAD^+	0.07 ± 0.01	892 ± 20
NH_4^+	NADH	37	870 ± 60
NH_4^+	NADPH	40 ± 1	810 ± 25

Table 2
The K_m (mM) of the cofactors and V_{max} (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) of recombinant *Tc*GDH at 30 °C in the directions of glutamate formation and utilisation. Enzyme activity was monitored spectrophotometrically at 340 nm.

Cofactor	K_m	V_{max}
NAD^+	0.7 ± 0.01	354 ± 21
$NADP^+$	1 ± 0.05	508 ± 17
NADH	0.05 ± 0.01	1280 ± 34
NADPH	0.03 ± 0.01	1050 ± 29

The reactions followed Michaelis–Menten characteristics with substrate (product) inhibition at high substrate concentrations, except for 2-OG (Fig. 4). In the direction of glutamate utilisation, the V_{max} was very close to being significantly greater with $NADP^+$ than with NAD^+ ($P = 0.051$) (Fig. 4A). In the reverse direction, there was no difference with the co-factors NADH and NADPH ($P = 0.9$) (Fig. 4B).

The effects of the addition of bivalent metal ions and other activators/inhibitors are presented in Supplementary Table 1. Addition of 1 mM $NiCl_2$ and $CoCl_2$ inhibited enzyme activity by 20% and Mn^{2+}, Mg^{2+} and citrate inhibited GDH activity by about 10%. Azaserine did not inhibit the enzyme activity. Addition of 1 mM ATP and ADP increased the enzyme activity about 3.5-fold in the direction of glutamate formation, whereas there was no increase in the enzyme activity in the direction of glutamate utilisation (Supplementary Table 1).

4. Discussion

In the present study, the cloning, purification and characterisation of a recombinant *Tc*GDH are reported. Recombinant *Tc*GDH and *H. contortus* GDH (*Hc*GDH) were very similar both in protein sequence (Fig. 2) and reported kinetic properties (Rhodes and Ferguson, 1973). Both enzymes contained 538 amino acids, had predicted molecular masses of approximately 60 kDa and the predicted amino acid sequences showed 91% identity and 96% similarity. *Tc*GDH was also very similar to *C. elegans* GDH (85% identity, 93% similarity). Notably, both *Tc*GDH and purified GDH from adult *H. contortus* (Rhodes and Ferguson, 1973) had a very high K_m for NH_4^+ at around 40 mM. Most nematode GDHs have pH optima between pH 7 and 8 (Rhodes and Ferguson, 1973; Turner et al., 1986). With either co-factor, the pH optima of the *Tc*GDH in both direction was 7.5 (Fig. 3), whereas in *H. contortus*, the pH optimum was pH 8 in the direction of glutamate formation and pH 8.8 in the direction of glutamate utilisation (Rhodes and Ferguson, 1973). The aminating reaction had a broader pH range than the forward reaction, with significant activity detected above the optimum pH.

The recombinant *Tc*GDH was almost certainly of nematode origin. The properties of the purified protein were virtually identical to those of the GDH in homogenates of adult or L3 *T. circumcincta* (Muhamad et al., 2011) and other nematode species (Langer, 1972; Rhodes and Ferguson, 1973). Dual co-factor specificity, in particular, distinguishes it from bacterial GDH, as the *E. coli* GDH uses only NADPH as co-factor (Hanahan, 1983; McPherson and Wootton, 1983). Recombinant *Tc*GDH had a lower specific activity than predicted from enzyme activity in homogenates (Muhamad, 2006; Muhamad et al., 2011), however, the V_{max} was similar to those reported for recombinant and purified bovine GDH (Kim et al., 2003). *Tc*GDH was relatively labile and good yields of the correctly folded protein were difficult to obtain, even in *E. coli* strain BL21 (DE3) pGroESL which expressed both soluble and insoluble protein. The initial expression vectors, *E. coli* strains BL21 (DE3) and Rosetta gami (DE3), produced only insoluble protein (inclusion bodies). Although all enzyme assays were performed soon after purification, activity began decreasing even with storage at 4 °C.

A putative mitochondrial targeting peptide (Claros and Vincens, 1996; Rosso et al., 2008) was predicted for all three nematode GDHs in Fig. 2. The mitochondrial localisation of *Tc*GDH will require experimental verification, but evidence summarised by Skuce et al. (1999) suggests a mitochondrial location for *Hc*GDH, and the putative targeting peptides of *Hc*GDH and *Tc*GDH are very similar. The 64% amino acid sequence identity between *Tc*GDH and the extensively characterised human GDH1 (Fang et al., 2002) means that the substrate and allosteric ligand binding sites can be deduced with confidence and species differences identified

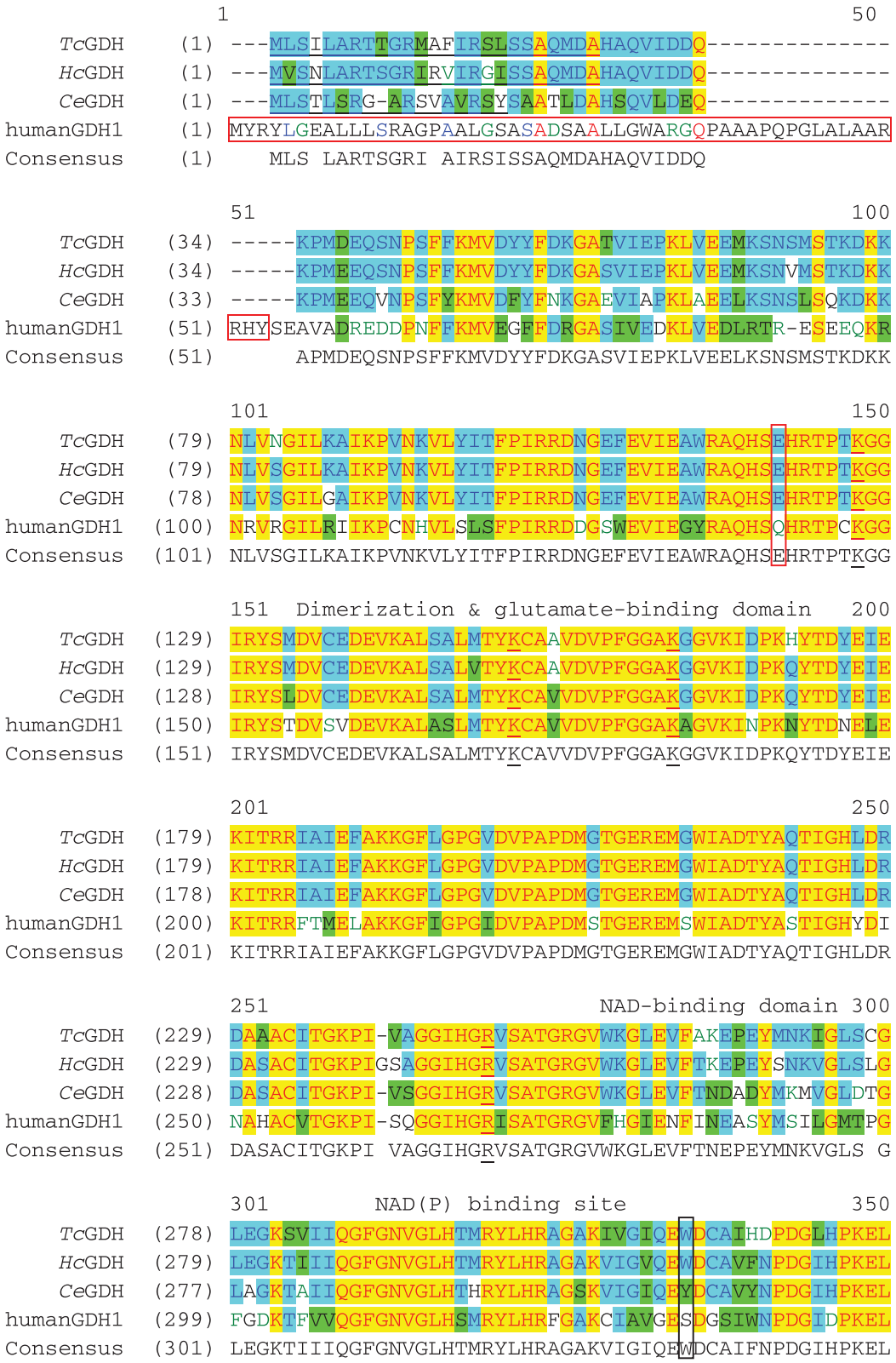

Fig. 2. Multiple sequence alignment of *T. circumcincta* GDH (*Tc*GDH) with *H. contortus* (GI:3207184), *C. elegans* (GI:17544676) and human (GI:4885281) homologues, and the consensus of the alignment. Residues coloured in yellow are completely conserved, in blue are conserved in any 3 species and in green identical to only one species. The known mitochondrial targeting peptide of human GDH1 (Rosso et al., 2008) is boxed in red. Putative nematode GDH mitochondrial targeting peptides (underlined) were based on Claros and Vincens (1996). In the N-terminal dimerization/glutamate-binding domain, conserved lysine and arginine residues involved in glutamate binding are underlined. NAD and ADP-binding residues are largely conserved; exceptions are boxed in black and red, respectively. The antenna region is shaded grey in the consensus, and pivot helices are boxed. The side chain of the pivot helix residue R516, highlighted in pink in the consensus sequence (=R463 using the processed human GDH1 numbering) binds the β-phosphate of the allosteric activator ADP and the R463A mutation abolishes ADP activation (Fang et al., 2002). Percentage identity and similarity values are from pairwise blastp alignments using default parameters. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

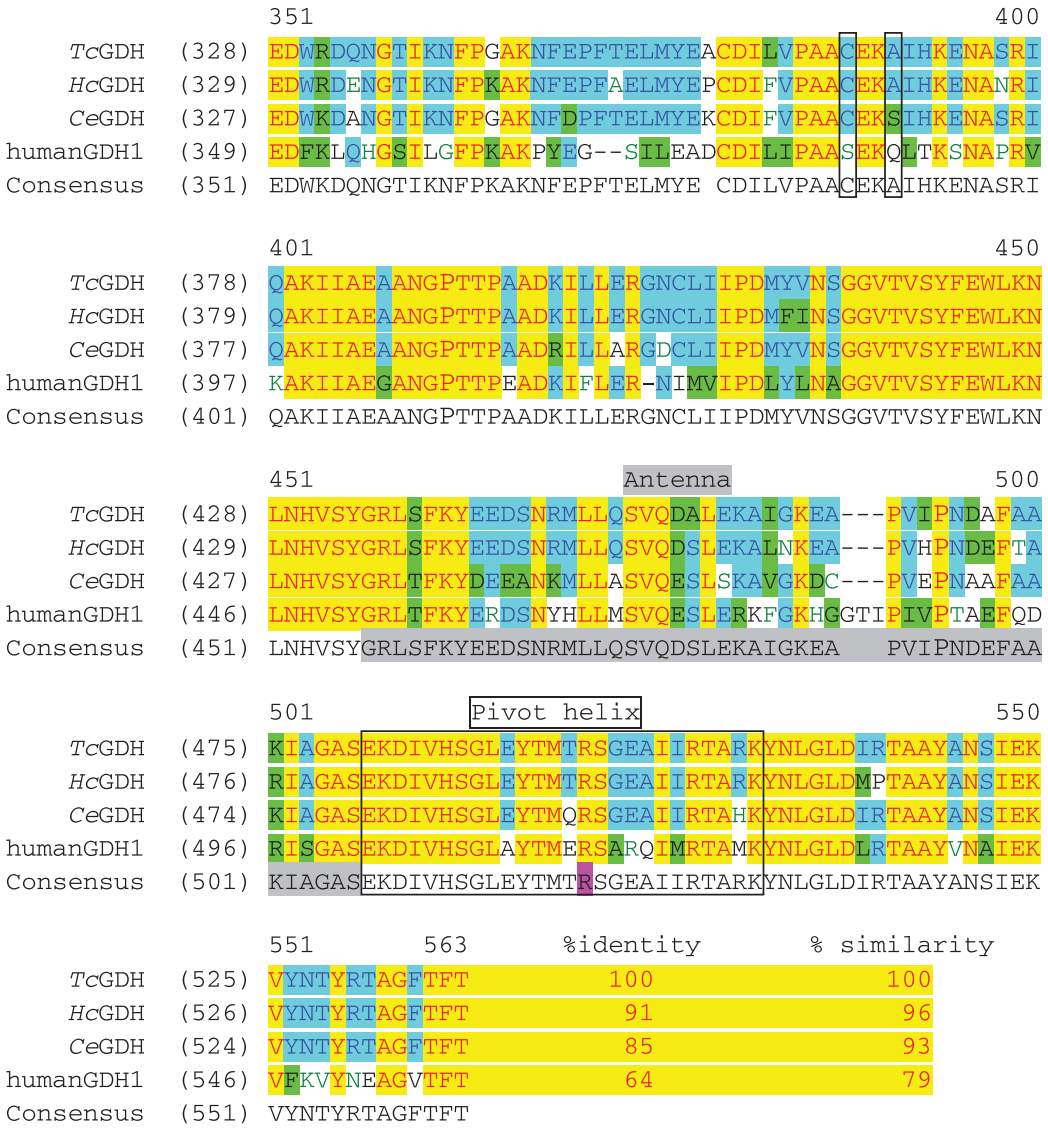

Fig. 2 (*continued*)

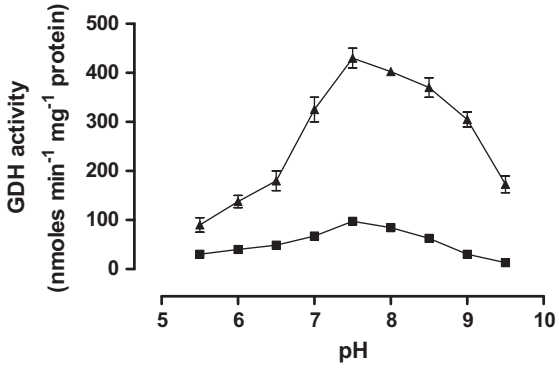

Fig. 3. Effects of pH on the activities of recombinant *Tc*GDH at 30 °C in the direction of glutamate utilisation (■) and formation (▲) (mean ± SEM, $n = 2$). Enzyme activity was calculated from the rate of oxidation or reduction of NAD^+ monitored spectrophotometrically at 340 nm.

within these conserved regions (Fig. 2). With the exception of three residues that line the NAD^+ binding site and one residue linked to ADP binding, the residues shown to be important for substrate and allosteric effector binding in human GDH1 were conserved in *Tc*GDH (Fig. 2). Given the apparent activation of *Tc*GDH by ATP, it is interesting to note that changes in residues immediately flanking a conserved arginine (in the pivot helix), that binds the β-phosphate of ADP, result in a decrease in negative charge and a decrease in side chain volume, which might enhance ATP binding at this site. The metabolic rationale for both ADP and ATP activating *Tc*GDH will be the subject of further study. Also of interest is that the greatest difference between nematode and mammalian GDHs is a three-residue deletion in the nematode enzymes that occurs in the antenna region implicated in allosteric regulation of GDH from ciliates and higher organisms (Smith and Stanley, 2008).

The recombinant *Tc*GDH was a dual co-factor enzyme (E.C. 1.4.1.3) that operated both with NAD^+ and $NADP^+$. As it was the purified product of a single gene sequence and formed a single protein on SDS–PAGE, it is unlikely that the dual co-factor specificity resulted from two isozymes (Hudson and Daniel, 1993). The properties of the purified protein were virtually identical to those of the GDH in homogenates of adult or L3 *T. circumcincta* (Muhamad et al., 2011) and dual co-factor specificity also distinguishes it from *E. coli* GDH, as the *E. coli* GDH uses only NADPH as co-factor (Hanahan, 1983; McPherson and Wootton, 1983). Enzyme activity was

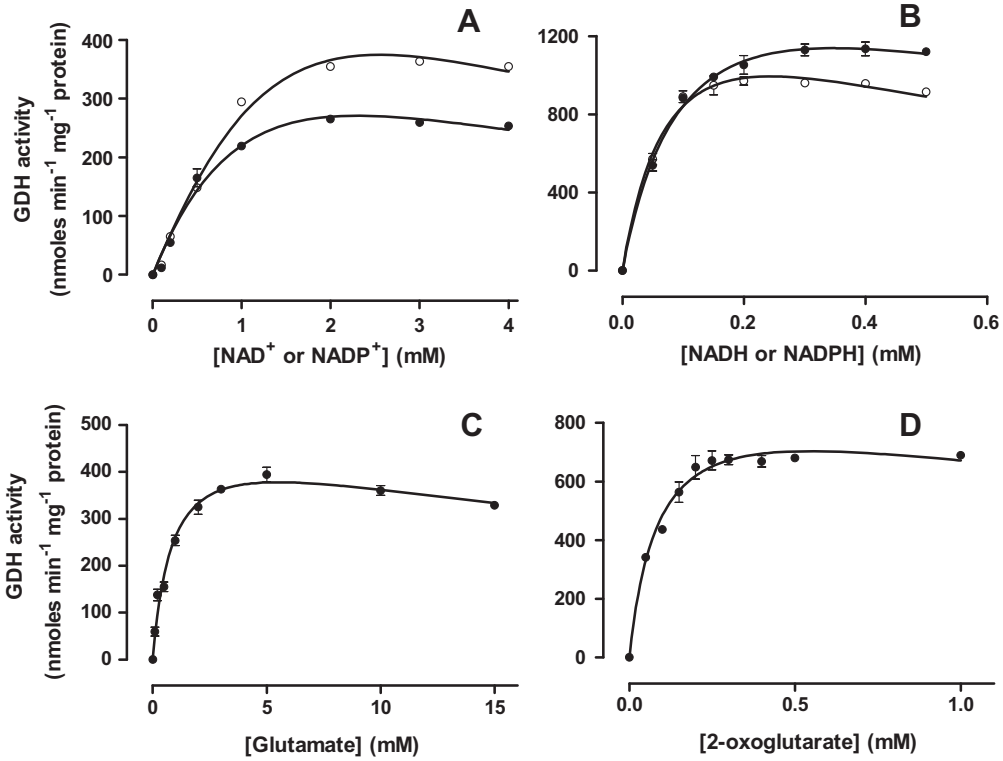

Fig. 4. Glutamate dehydrogenase (GDH) activities (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) of recombinant *Tc*GDH at 30 °C in the direction of glutamate utilisation and formation with varying concentrations of co-factors or substrates. (A) NAD^+ (●), $NADP^+$ (○); (B) NADH (●), NADPH (○); (C) glutamate and (D) 2-oxoglutarate.

more than double in the direction of glutamate formation than that for the glutamate utilisation reaction (Tables 1 and 2). Higher concentrations of co-factors inhibited enzyme activity (Fig. 4A and B). Although active with either co-factor, in the direction of glutamate formation, enzyme activity was about 40% higher with $NADP^+$ as co-factor, but 10% lower in the reverse direction. Previously, crude homogenates of adult and L3 have been used to study the properties of *T. circumcincta* GDH (Muhamad et al., 2011). The differences in activity with the two co-factors were greater in that study, because the concentration of NADP used was less than that required for maximal activity, which could not be determined accurately. In the direction of glutamate formation, generally helminth GDHs are more active with NAD^+/NADH than with $NADP^+$/NADPH (Grantham and Barrett, 1986), however, the *Hymenolepis diminuta* GDH, which is cytosolic in location, is active only with NAD^+ as co-factor (Mustafa et al., 1978).

An unusual feature of both the recombinant enzyme and GDH in homogenates was activation both with ADP and ATP in one direction and no effect in the other (Supplementary Table 1). ADP and ATP are not directly involved in the reaction catalysed by GDH (i.e. they are not substrates or co-factors), but act as allosteric effectors for short-term regulation of GDH activity. In the direction of glutamate utilisation, ATP and ADP had no effect on enzyme activity, whereas either regulator increased the enzyme activity more than threefold in the other direction. ATP can bind at the GTP binding site (Fang et al., 2002) and activate the enzyme by suppressing inhibition by GTP (Hudson and Daniel, 1993; Fang et al., 2002). As no GTP was added in the assay systems for the *Tc*GDH, this is not likely to be the key mechanism of ATP activation. ATP can either be stimulatory or inhibitory for human GDH, depending on the concentration (Fang et al., 2002), as ATP and ADP share the same binding site. As the concentration dependence appears to differ in nematode and human enzymes, a more complete study of the role of allosteric regulators of abomasal nematode GDH is being undertaken and will be reported separately.

It is still not clear whether the aminating or deaminating direction predominates *in vivo* in abomasal nematodes. The limiting factor for glutamate formation is likely to be the intracellular NH_4^+ concentration, given that the K_m for NH_4^+ was 40 mM and activity was low at predicted external concentrations in the abomasum. Nematode intracellular [NH_4^+] is unknown and may be very much lower than the 1 mM in sheep abomasal fluid, about 5 mM in ruminal fluid (Harrop, 1974; Harrop and Phillipson, 1974), 487 μM in portal blood and 200 μM in arterial blood (Parker et al., 1995). As there is a functional GS–GOGAT pathway in *T. circumcincta* (Umair et al., 2011), provided there is adequate energy and 2-OG available, ammonia incorporation is probably via this pathway, as in plants and bacteria.

In conclusion, the recombinant *Tc*GDH was similar to that of the other major abomasal parasite *H. contortus* both in protein sequence and reported kinetic properties. As was the cases for GDH activity in *T. circumcincta* homogenates, *Tc*GDH showed dual co-factor activity (both with NAD^+ and $NADP^+$), which appeared to arise from a single enzyme, not from two isozymes. Although host GDH is also a dual co-factor enzyme, there are significant differences in amino acid sequence which suggest that studies of the antigenicity of the recombinant *Tc*GDH may reveal it to be a useful anthelmintic target.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2011.08.007.

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ATGCTGAGCATTTTGGCACGTACTACGGGCCGTATGGCCTTCATACGGAGC

CTGTCGTCCGCTCAAATGGACGCTCATGCTCAAGTCATCGACGACCAGAAG

CCGATGGACGAACAAAGCAATCCTTCGTTCTTTAAGATGGTTGACTATTACT

TCGATAAGGGAGCGACCGTAATCGAGCCGAAATTGGTTGAGGAAATGAAA

TCAAACTCGATGAGCACGAAAGATAAGAAAAATCTCGTCAATGGAATTTTG

AAGGCTATCAAGCCCGTCAACAAGGTTCTATACATCACCTTCCCGATTCGTC

GTGACAATGGCGAATTCGAAGTGATCGAAGCGTGGCGTGCTCAGCATTCTG

AGCATCGAACCCCCACAAAGGGTGGTATTCGTTACTCAATGGATGTGTGCG

AGGATGAGGTGAAAGCCTTGTCAGCTCTGATGACATACAAATGTGCCGCTG

TCGATGTTCCATTCGGTGGAGCAAAAGGAGGTGTCAAGATCGATCCCAAGC

ACTATACTGATTACGAAATCGAGAAAATCACCCGTCGTATTGCTATCGAAT

TCGCCAAGAAGGGATTCCTCGGGCCTGGAGTTGACGTGCCAGCACCTGATA

TGGGAACCGGAGAACGTGAAATGGGATGGATCGCTGACACTTACGCACAG

ACAATCGGACATCTCGATAGGGATGCGGCCGCTTGTATCACTGGAAAGCCC

ATTGTTGCTGGTGGCATCCATGGACGTGTTTCTGCAACTGGTCGAGGAGTTT

GGAAGGGTCTTGAAGTATTCGCCAAAGAACCAGAGTACATGAATAAGATTG

GACTTTCATGTGGATTGGAAGGAAAGAGTGTTATCATCCAAGGTTTTGGTA

ATGTAGGACTTCACACGATGCGTTATTTGCACAGAGCTGGAGCGAAGATCG

TCGGAATTCAAGAATGGGATTGTGCTATTCACGACCCTGATGGACTTCACC

CCAAGGAACTCGAAGACTGGAGAGACCAGAATGGTACTATCAAGAACTTC

CCTGGAGCCAAGAACTTCGAGCCATTCACAGAGCTCATGTACGAGGCCTGT

GATATTCTCGTTCCTGCCGCCTGTGAAAAAGCCATCCACAAAGAAAATGCT

TCTAGAATCCAAGCTAAGATCATCGCCGAGGCAGCTAATGGTCCAACCACT

CCTGCCGCAGATAAAATCCTTCTCGAGCGCGGAAACTGCCTTATCATCCCTG

ACATGTATGTCAACTCCGGTGGTGTGACTGTCTCATACTTCGAATGGTTGAA

GAACCTTAACCACGTCTCATATGGTCGCCTTAGTTTCAAATATGAAGAGGA

CTCCAACAGAATGCTTCTGCAATCTGTACAAGACGCATTGGAGAAGGCTAT

TGGCAAGGAAGCTCCTGTGATTCCTAATGACGCTTTCGCTGCAAAGATTGCT

GGAGCAAGCGAAAAGGATATCGTACATTCCGGTCTTGAGTACACGATGACC

CGCTCAGGAGAAGCCATCATTCGCACAGCCCGAAAGTACAACTTGGGCTTA

GATATCCGCACAGCGGCTTATGCTAATTCTATAGAAAAAGTTTATAATACCT

ACAGAACTGCCGGTTTCACTTTTACGTAG

Supplementary Figure 1. Nucleotide sequence of *T. circumcincta* glutamate dehydrogenase cDNA (Genbank accession no. JF731342).

Supplementary Table 1. Glutamate dehydrogenase (GDH) activities (nmoles min^{-1} mg^{-1} protein) (mean ± SEM, *n* = 2) of recombinant *Tc*GDH at 30 ºC in the direction of glutamate utilisation in the presence of inhibitors. ATP and ADP were also studied in the reverse direction. GDH activity was calculated from the rate of reduction of NAD^+ or from the rate of oxidation of NADH, monitored spectrophotometrically at 340 nm. Enzyme activity without adding any inhibitor/bivalent metal ion is set as 100%.

Inhibitors	Concentration (mM)	Activity (%)
Control	-	100
Ca^{2+}	1	81 ± 3
Cu^{2+}	1	71 ± 4
Mg^{2+}	1	93 + 5
Mn^{2+}	1	88 ± 4
Ni^{2+}	1	79 ± 1
Azaserine	1	98 ± 3
Citrate	1	90 ± 6

ADP/ATP 1 mM	Glutamate utilisation Activity (%)	Glutamate formation Activity (%)
	100	100
ATP	90 ± 5	320 ± 19
ADP	97 ± 3	345 ± 26

Chapter IV

Nucleotide allosteric regulation of the glutamate dehydrogenases of *Teladorsagia circumcincta* and *Haemonchus contortus*

S. Umair, J.S. Knight M.L. Patchett, R.J. Bland and H.V. Simpson

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Nucleotide allosteric regulation of the glutamate dehydrogenases of *Teladorsagia circumcincta* and *Haemonchus contortus*

Saleh Umair [a], Jacqueline S. Knight [b], Mark L. Patchett [c], Ross J. Bland [b], Heather V. Simpson [a,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] Hopkirk Research Institute, AgResearch Ltd, Private Bag 11-008, Palmerston North, New Zealand
[c] Institute of Molecular Biosciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand

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ABSTRACT

The expression of glutamate dehydrogenase (GDH; EC 1.4.1.3) in L3 of the nematode *Haemonchus contortus* was confirmed by detecting GDH mRNA, contrary to earlier reports. The enzyme was active in both L3 and adult *H. contortus* homogenates either with NAD^+/H or $NADP^+$/H as co-factor. Although it was a dual co-factor GDH, activity was greater with NAD^+/H than with $NADP^+$/H. The rate of the aminating reaction (glutamate formation) was approximately three times higher than for the deaminating reaction (glutamate utilisation). GDH provides a pathway for ammonia assimilation, although the affinity for ammonia was low. Allosteric regulation by GTP, ATP and ADP of L3 and adult *H. contortus* and *Teladorsagia circumcincta* (Nematoda) GDH depended on the concentration of the regulators and the direction of the reaction. The effects of each nucleotide were qualitatively similar on the mammalian and parasite GDH, although the nematode enzymes were more responsive to activation by ADP and ATP and less inhibited by GTP under optimum assay condition. GTP inhibited deamination and low concentrations of ADP and ATP stimulated weakly. In the reverse direction, GTP was strongly inhibitory and ADP and ATP activated the enzyme.

1. Introduction

The nematodes *Teladorsagia circumcincta* and *Haemonchus contortus* are economically important gastric parasites of sheep for which new control measures, either chemical or vaccines, are urgently needed. Essential metabolic enzymes, such as those involved in glutamate metabolism, are potential targets, particularly if they are absent from the host or if there are unusual properties or significant structural differences in the protein. Glutamate dehydrogenase (GDH) is a universal enzyme which reversibly catalyses the formation of glutamate from ammonia and 2-oxoglutarate (2-OG) (Hudson and Daniel, 1993). Unlike mammals, abomasal parasites have both glutamate dehydrogenase activity (Rhodes and Ferguson, 1973; Muhamad et al., 2011; Umair et al., 2011b) and also the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway (Umair et al., 2011a), which is used for ammonia incorporation into glutamate by plants and bacteria (Masclaux-Daubresse et al., 2006; Labboun et al., 2009). Although the GS–GOGAT pathway is functional in adult *H. contortus* and L3 and adult *T. circumcincta* (Umair et al., 2011a), no GOGAT activity was detected in homogenates of sheathed L3 *H. contortus*, even after incubation with ammonia, which up-regulated GOGAT activity in L3 *T. circumcincta* (Umair et al., 2011a). Surprisingly, a developmental expression study suggested GDH was also not active in L3 *H. contortus*, whereas it was present in L4 and adult worms (Skuce et al., 1999). There are no reports of studies involving enzymatic assay of GDH in L3 *H. contortus* which could confirm the absence of both ammonia assimilatory pathways in L3.

Despite the similarity of the GDH genes in *H. contortus* and *T. circumcincta* (Umair et al., 2011b), there may be differences in the properties of *H. contortus* and *T. circumcincta* GDH activity in L3 and adult worm homogenates (Muhamad et al., 2011) and recombinant *Tc*GDH (Umair et al., 2011b). Rhodes and Ferguson (1973) reported that purified adult *H. contortus* GDH had an absolute requirement for NAD^+/H and less than 2% of that activity with $NADP^+$/H, which would classify it as NAD^+ specific (E.C. 1.4.1.2) and not a dual co-factor (E.C. 1.4.1.3) or $NADP^+$ specific enzyme (E.C. 1.4.1.4). This contrasts with GDH activity in homogenates of L3 and adult *T. circumcincta* (Muhamad et al., 2011) and recombinant *Tc*GDH (Umair et al., 2011b) which were active with either co-factor, consistent with a dual co-factor GDH, as are mammalian enzymes (Hudson and Daniel 1993). Recombinant *Tc*GDH had almost equal activity with the two co-factors, whereas activity in homogenates was greater with NAD^+ than $NADP^+$. The lack of activity of *H. contortus* GDH with $NADP^+$ is unexpected, since the predicted amino acid sequences of *H. contortus* and *T. circumcincta* (Umair et al., 2011b, Supplementary Fig. 1) had 96% similarity and 91% identity and the binding sites and

* Corresponding author at: Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand. Tel.: +64 6 356 9099; fax: +64 6 350 5636.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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conserved residues were largely identical in the proteins of the two nematode species.

Nematode GDH appeared to differ from mammalian enzymes in the response to short-term allosteric regulators, which are not directly involved in the reaction catalysed by GDH. In contrast to mammalian GDH (Fang et al., 2002; Kim et al., 2003), *T. circumcincta* GDH activity in both directions has been reported to be stimulated by 1 mM ATP (Muhamad et al., 2011; Umair et al., 2011b), while *H. contortus* GDH was slightly inhibited in the deaminating direction (Rhodes and Ferguson, 1973). In mammals, inhibition of GDH by GTP and ATP is believed to result from increased binding affinity and reduced rate of release of the product (Koberstein and Sund, 1973), whereas ADP acts as an enzyme activator by aiding product release (Smith and Stanley, 2008). ATP can be either stimulatory or inhibitory to mammalian GDH (Fang et al., 2002; Kim et al., 2003), depending on the concentration, as ATP can bind to either the GTP or ADP binding site.

In the present study, first the expression of GDH in L3 *H. contortus* was confirmed by PCR, followed by a comparison of the kinetic properties, including the role of the co-factors in both L3 and adult *H. contortus* homogenates. The concentration-dependent effects of each of the three nucleotide regulators on GDH activity in both the aminating and deaminating directions of L3 and adult worm GDH revealed differences in sensitivity to nucleotide regulators of mammalian and nematode GDH.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless stated otherwise. Use of experimental animals has been approved by the Massey University Animal Ethics Committee.

2.1. Parasites

L3 *H. contortus* or *T. circumcincta* were cultured from the faeces of sheep infected with pure strains of parasites and stored in reverse osmosis (RO) water at 10 °C for the former and 4 °C for the latter species. Sheathed larvae were either freshly collected or prior to each experiment; L3 were baermannised in RO water to remove inactive worms and re-suspended in assay buffer. Adult worms were recovered from the abomasa of infected sheep as described previously (Umair et al., 2011c). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and placed in assay buffer.

2.2. Expression of H. contortus GDH mRNA

The expression of *H. contortus* mRNA in L3 and adults was accessed by reverse transcriptase PCR. Total RNA was extracted from L3 and adult worms using Trizol (Invitrogen) according to the manufacturer's instructions and the first strand cDNA synthesised using the iScript select cDNA synthesis kit (BioRad). 20 ng cDNA was used as template in a PCR using the primers and amplification conditions described by Skuce et al. (1999). The resulting L3 amplification product was sequenced after cloning into pCR4 using the TOPO PCR TA cloning kit for sequencing (Invitrogen).

2.3. Preparation of homogenates

Approximately 50,000 L3 were centrifuged at 600 *g* for 5 min, washed twice by re-suspending in buffer followed by centrifugation, then finally re-suspended in 1 mL buffer. The concentrated L3 suspension or approximately 5 mg of clumped adult worms was transferred to a chilled mortar, frozen at −20 °C for at least 15 min and homogenised with a chilled pestle and mortar. The protein concentrations of homogenates were determined by the method of Bradford (1976).

2.4. Enzyme assays

2.4.1. Kinetic properties of H. contortus GDH

All assays were performed at 30 °C on three homogenates of sheathed L3 and two homogenates of adult worms unless stated otherwise, using 50 μg homogenate protein and 100 mM phosphate buffer in a total volume of 1 mL assay mixture.

GDH activity in L3 and adult worms was determined both for the deamination and amination reactions. Routine assays were carried out in 100 mM phosphate buffer (pH 8.5 for the deamination reaction and pH 8 for the amination reaction). Enzyme activity (V_{max}) was determined by the rate of production of NADH/NADPH in the deamination reaction or by the rate of utilisation of NADH/NADPH in the amination reaction, measured spectrophotometrically at 340 nm.

(1) In homogenates of sheathed L3, the optimum pH was determined over the pH range 5.5 to 9.5 in both direction, with substrate concentrations of 5 mM glutamate in the deaminating direction and with 1 mM 2-OG and 50 mM NH_4Cl in the opposite direction. Subsequent assays were carried out at pH 8.5 for the deamination reaction and pH 8 for the amination reaction.
(2) The apparent K_m for co-factors was determined in homogenates of sheathed L3 and adult worms with $NAD(P)^+$ concentrations from 0–3 mM with 5 mM glutamate (in the deamination reaction) or 0–0.5 mM NAD(P)H with 1 mM 2-OG and 50 mM NH_4Cl in the amination reaction.
(3) The apparent K_m for glutamate was determined in homogenates of sheathed L3 and adult worms in reaction mixtures containing 0–15 mM glutamate and 2 mM NAD^+ or $NADP^+$. The apparent K_m for 2-OG was determined with 0–3 mM 2-OG, 50 mM NH_4Cl and 0.2 mM NADH or NADPH and the apparent K_m for ammonium with 0–250 mM NH_4Cl, 1 mM 2-OG and 0.2 mM NADH or NADPH.

2.4.2. Nucleotide allosteric regulation of H. contortus and T. circumcincta GDH

The effects of the addition of increasing concentrations of GTP, ATP or ADP were determined in homogenates ($n=2$) of sheathed L3 and adult *H. contortus* and *T. circumcincta* both in the deamination and amination reactions with NAD^+/H as co-factor The reactions were carried out with concentrations of substrates and optimum buffer pH known to produce maximal enzyme activity. The buffer pH for the deamination and amination reactions were pH 8.5 and pH 8 for *H. contortus* and pH 7.5 for both reactions for *T. circumcincta* homogenates. The reaction mixtures contained 1 mM 2-OG, 50 mM NH_4Cl, 0.2 mM NADH and 0–5 mM ATP, GTP or ADP in the amination reaction and 5 mM glutamate, 2 mM NAD^+ and 0–5 mM ATP, GTP or ADP in the deamination reaction.

2.5. Data analysis

Replicate data are presented as mean ± SEM. Graph Prism v5 was used to plot kinetic data and estimate K_m and V_{max}. Student's t-test was used to compare K_m and V_{max} values.

3. Results

3.1. Expression of H. contortus GDH mRNA

H. contortus GDH cDNA was detected in both L3 and adults using reverse transcriptase PCR. The amplification products were both of

the expected size (approx. 1600 bp). The L3 sequence was identical to that obtained for adult *H. contortus* GDH by Skuce et al. (1999).

3.2. Kinetic properties of H. contortus GDH

Enzyme activities and kinetic properties of GDH in homogenates of sheathed L3 and adult *H. contortus* are shown in Table 1. The optimum pH for GDH activity for the deamination and amination reactions were 8.5 and 8 respectively (Fig. 1). The amination reaction had a broader pH range and significant activity was detected even at higher pH, whereas in the opposite reaction there was almost no activity above pH 9.

Both in L3 and adult worms, significantly less activity (V_{max}) was observed when $NADP^+$/H was substituted for NAD^+/H ($p > 0.05$), by approximately a factor of three in the deaminating reaction and by six in the aminating reaction (Table 1). The rate of glutamate formation was approximately three times higher than for glutamate utilisation (Table 1). The affinity of the enzyme for the two co-factors was similar in both forward and reverse reactions, but for NADPH in the aminating direction was approximately 10-fold higher than for $NADP^+$ in the opposite direction (Table 2). The apparent K_m for ammonia was very high: 34–36 mM in sheathed L3 and 43–44 mM in adult worms (Table 1). The apparent K_m for glutamate was about 6-fold higher than for 2-OG in sheathed L3 and 3-fold higher in adult *H. contortus*. For all 3 substrates, the K_m was lower in L3 than adult worm homogenates. The deaminating reaction with glutamate as substrate followed Michaelis-Menten characteristics with substrate (product) inhibition at high substrate concentrations (>10 mM).

3.3. Effects of ADP, ATP and GTP on H. contortus and T. circumcincta GDH activity

The effects of addition of GTP, ATP or ADP on GDH activities in both directions with NAD^+/H as co-factor are presented in Fig. 2. In both reactions, addition of 0–5 mM GTP inhibited GDH activity in the two life cycle stages of both parasites (Fig. 2A, D). GDH activity was completely inhibited by the addition of 1 mM GTP in the deamination reaction (Fig. 2A) and by 70–80% in the other direction (Fig. 2D).

The effects of ADP were concentration dependent and differed markedly in the two directions of GDH activity. In the deamination reaction, enzyme activity was generally increased by up to 20% by the addition of 0–0.5 mM ADP, except for the L3 *T. circumcincta* enzyme, which was inhibited at all concentrations. ADP at 1 mM concentration, depending on the parasite, mildly stimulated or inhibited enzyme activity by about 20% (Fig 2C inset). 1–5 mM ADP concentration-dependently inhibited deamination by 60–80% in the two life cycle stages of both parasites (Fig. 2C). In contrast, ADP was stimulatory in the aminating reaction. Addition of 0–1 mM ADP progressively increased enzyme activity in all parasites (Fig. 2F inset) to a maximum of 2–2.5-fold at 1 mM ADP, the exception being L3 *H. contortus* homogenates, in which maximum stimulation was seen at 3 mM ADP. Over the concentrations range of 2–5 mM, ADP progressively declined, except for the L3 *H. contortus* enzyme (Fig. 2F).

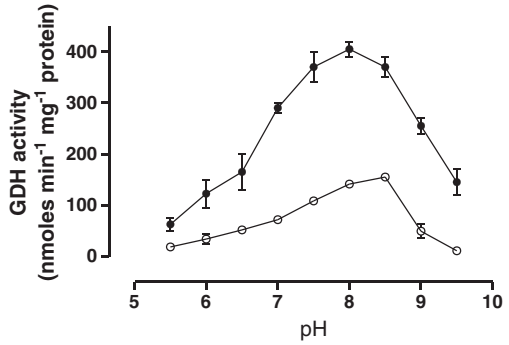

Fig. 1. Effects of pH on glutamate dehydrogenase activities at 30 °C on homogenates of sheathed L3 *H. contortus* in the direction of glutamate utilisation (○) and formation (●) (mean ± SEM, $n = 2$). Enzyme activity was calculated from the rate of oxidation or reduction of NAD^+/H calculated spectrophotometrically at 340 nm.

The effects of added ATP were generally similar to those of ADP, although slightly less stimulatory or more inhibitory at comparable concentrations (Fig. 2B, D). 1 mM ATP inhibited deamination by about 20%, except in adult *H. contortus* homogenates, in which there was no change in activity. In the range 0–1 mM, ATP only slightly stimulated activity (10-20%), while deamination was concentration-dependently inhibited by 60–70% in all homogenates by over the range 1–5 mM ATP (Fig. 2B). In the amination reaction, GDH activity was approximately doubled by the addition of 1 mM ATP to homogenates of both parasites, less so (1.5-fold) for adult *T. circumcincta*. Stimulatory activity declined rapidly at higher than 1 mM ATP, so that the addition of 5 mM ATP inhibited GDH activity to about 20% in L3 and adult *H. contortus* and about 30–40% in L3 and adult *T. circumcincta* (Fig. 2E).

4. Discussion

Allosteric regulation by nucleotides of GDH in whole parasite homogenates differed from that reported for mammalian GDH in sensitivity to nucleotides. In both cases, nucleotide regulation is not identical in the aminating and deaminating directions. ADP and ATP were more stimulatory to *H. contortus* and *T. circumcincta* GDH in the aminating reaction at high pH, especially in L3 homogenates, than for mammalian enzymes. GTP was strongly inhibitory in all cases. The present study included L3 *H. contortus* GDH, which has previously been reported not to be expressed in L3 (Skuce et al., 1999). *H. contortus* GDH, like that in *T. circumcincta*, was a dual co-factor GDH (E.C. 1.4.1.3) with considerable activity with either co-factor in both directions, significantly higher both in L3 and adult *H. contortus* with NAD^+/H in both reactions.

GDH activity in *H. contortus* L3 and adult homogenates was considerable with either co-factor in both directions, although higher with NAD^+/H as co-factor (Table 1), in contrast to purified GDH

Table 1
Substrate K_m (mM) and V_{max} (nmoles min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) of glutamate dehydrogenase in homogenates of sheathed L3 and adult *H. contortus* at 30 °C in the directions of glutamate utilisation and formation with varying co-factors.

Substrate	Co-factor	L3 *H. contortus*		Adult *H. contortus*	
		K_m	V_{max}	K_m	V_{max}
Glutamate	NAD^+	2.5 ± 0.1	101 ± 14	3.5 ± 0.1	284 ± 17
Glutamate	$NADP^+$	2.7 ± 0.2	34 ± 6	3.1 ± 0.2	61 ± 11
2-oxoglutarate	NADH	0.3 ± 0.05	375 ± 13	1 ± 0.1	665 ± 15
2-oxoglutarate	NADPH	0.5 ± 0.1	63 ± 6	1.1 ± 0.2	101 ± 8
NH_4^+	NADH	34 ± 2	364 ± 10	44 ± 4	778 ± 24
NH_4^+	NADPH	36 ± 3	51 ± 7	43 ± 4	116 ± 13

Enzyme activity was calculated from the rate of formation/utilisation of NAD^+(P)/H, monitored spectrophotometrically at 340 nm.

Table 2
The K_m (mM) of the co-factors and V_{max} (nmoles min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) of glutamate dehydrogenase in homogenates of sheathed L3 and adult *H. contortus* at 30 °C in the directions of glutamate formation and utilisation.

Co-factor	L3 *H. contortus*		Adult *H. contortus*	
	K_m	V_{max}	K_m	V_{max}
NAD^+	0.6 ± 0.05	132 ± 11	1.0 ± 0.1	296 ± 16
$NADP^+$	0.7 ± 0.1	37 ± 6	1.1 ± 0.1	88 ± 11
NADH	0.03 ± 0.01	355 ± 18	0.06 ± 0.01	748 ± 21
NADPH	0.04 ± 0.01	62 ± 9	0.05 ± 0.01	121 ± 12

Enzyme activity was monitored spectrophotometrically at 340 nm.

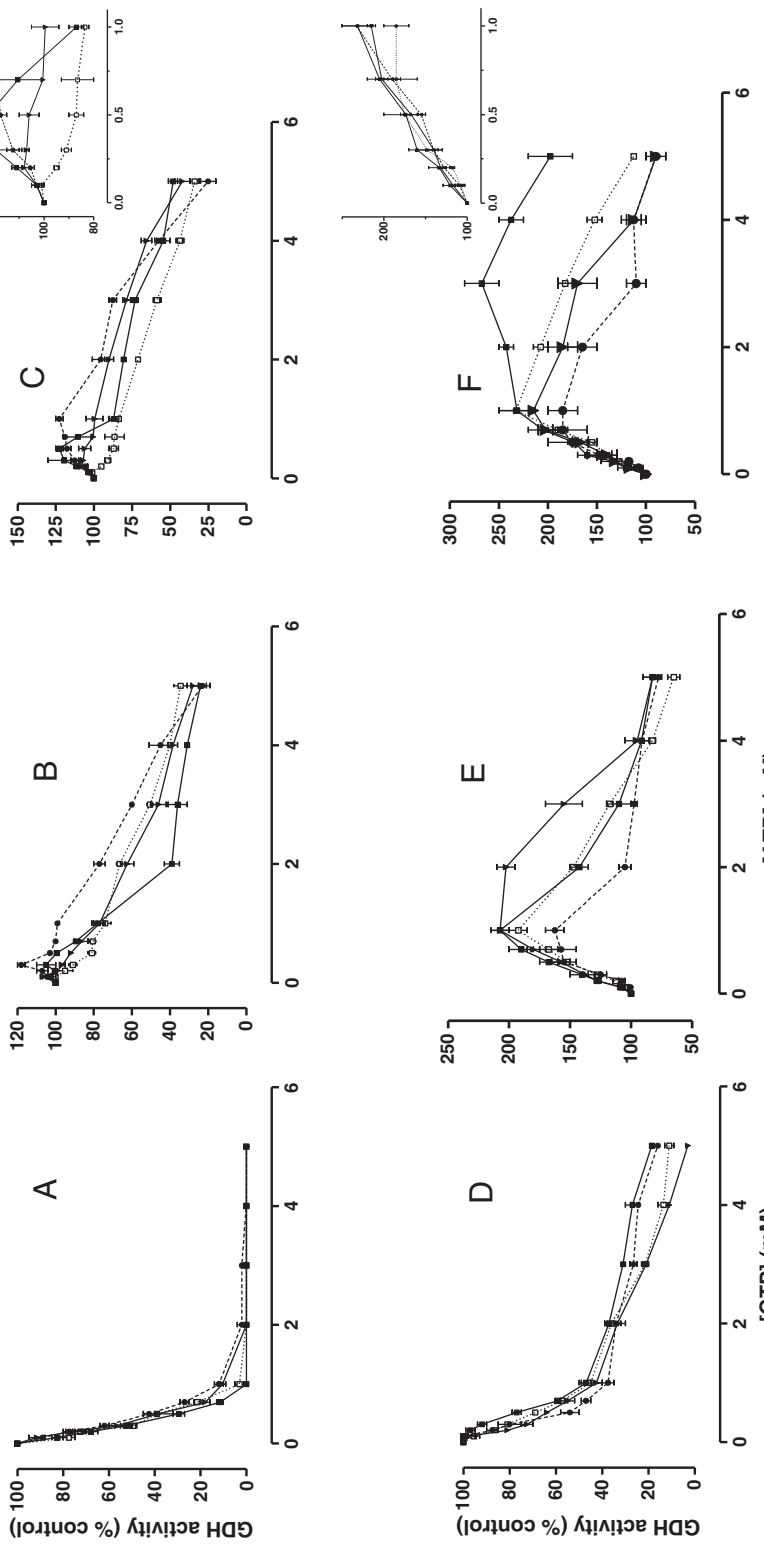

Fig. 2. Effects of the allosteric regulators GTP (A, D), ATP (B, E) and ADP (C, F) on glutamate dehydrogenase (GDH) activities (nmoles min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) with NAD^+/H as co-factor in homogenates of sheathed L3 (■) and adult (●) *H. contortus* and L3 (□) and adult *T. circumcincta* (▼) at 30 °C in the deamination (A–C) and amination (D–F) reactions; inset are responses to 0–1 mM ADP. GDH activity was calculated from the rate of reduction of NAD^+ or from the rate of oxidation of NADH, monitored spectrophotometrically at 340 nm. Enzyme activity without added regulator was set as 100%.

from adult worms, which had negligible activity with $NADP^+$ (Rhodes and Ferguson, 1973). The K_m for each co-factor was similar in the two life-cycle stages. Comparison of kinetic properties of the enzyme in homogenates (Table 2) was possible with those of purified GDH only with NAD^+/H as co-factor. There were similar pH optima and substrate K_m, notably a very high K_m for NH_4^+ (34–44 mM). Activity was present over a broader pH range in the aminating reaction than the deamination reaction (Fig. 1). The pH optima were similar with either co-factor: pH 8.5 in the deaminating direction and pH 8 in the aminating direction, as for purified adult *H. contortus* GDH (Rhodes and Ferguson, 1973).

H. contortus GDH activity and substrate affinities were generally similar to those of *T. circumcincta* GDH, but with a slightly higher pH optimum (Muhamad et al., 2011; Umair et al., 2011b). This similarity is not surprising, since the predicted amino acid sequence of *H. contortus* and *T. circumcincta* (Supplementary Fig. 1) showed 96% similarity and 91% identity and the binding sites and conserved residues are identical in the two nematode species. The most apparent differences were in enzyme activities with the different co-factors: activities were markedly less (3 and 6-fold) with $NADP^+$/H than with NAD^+/H in both L3 and adult *H. contortus* homogenates, but for *T. circumcincta* L3 homogenates, activity was 2–3-fold less in the aminating direction with $NADP^+$ than NAD^+, but similar with either co-factor in the opposite direction (Muhamad et al., 2011). In contrast, recombinant *Tc*GDH showed less than 10% difference in activity with either co-factor in both directions (Umair et al., 2011b), in marked contrast to the behaviour of purified adult *H. contortus* GDH (Rhodes and Ferguson, 1973) but very similar in recombinant (Umair et al., 2011b).

The demonstration of an active GDH in L3 *H. contortus* is contrary to the previous report of Skuce et al. (1999) that neither the GDH protein was detectable in L3 using purified antibody from the sera of immune sheep nor was mRNA. They did not carry out direct biochemical assays. In the present study, GDH mRNA was confirmed in both life cycle stages of *H. contortus* by cloning and sequencing a single PCR product of about 1600 bp. The enzyme was active in homogenates of *H. contortus* L3 in both in the deaminating and aminating directions and showed similar properties to those of the GDH in adult worm homogenates. As L3 *H. contortus* do not have an active GOGAT, unlike adult worms and both L3 and adult *T. circumcincta* (Umair et al., 2011a), GDH provides a means of ammonia assimilation, provided there are high levels of ammonia, as the alternative GS–GOGAT pathway is not active in L3 *H. contortus*.

In the present study, under optimal assay conditions for maximum enzyme activity, the use of a range of concentrations of GTP, ADP and ATP has shown that the nematode enzymes differ slightly in their sensitivities to nucleotide regulation in homogenates of larval and adult worms of the two species and from mammalian GDH, but overall showed the same qualitative effects. GTP was inhibitory to both reactions, less so for nematode GDH, whereas ADP and ATP were activators (Fig. 2), more so than for mammalian GDH. Most studies of mammalian GDH appear to be carried out at a similar high pH (Wrzeszczynski and Coleman, 1994; Fang et al., 2002; Kim et al., 2003) and more frequently in the aminating than deaminating direction. Bailey et al. (1982) observed inhibition of deamination by ADP and reduced ADP activation of amination at low pH, when basal enzyme activity was also less. At pH 6, nematode GDH was similarly much less active (20% of maximal activity) and would very likely also either be less stimulated or inhibited by low concentrations of ADP than at pH 8. Interestingly, deamination by L3 *T. circumcincta* GDH was inhibited by all concentrations of ADP (and ATP) at the optimum pH 7.5. The effects on nematode GDH of these allosteric regulators was concentration-dependent, as it is for purified human GDH in the aminating direction (Fang et al., 2002) and differed in the deaminating and aminating directions.

Previous experiments using single concentrations of ADP and ATP have suggested that nematode and mammalian GDH may respond differently to nucleotide regulators, however, over the wider range of effectors, the differences between nematode and mammalian enzymes appear to be in sensitivity, rather than qualitative differences in response. Similarly, differences between life-cycle stages and species of parasite were also seen, as well as slight differences between the present and previous studies using 1 mM ADP and ATP. The predicted co-factor binding sites on GDH appear to be identical in both nematode species and are probably not the reason for differences in kinetic properties and sensitivity to regulators. Recombinant and purified enzymes may behave differently from enzymes in homogenates, as the latter may have undergone post-translational changes, such as phosphorylation, which has been linked in a hibernating squirrel to the altered kinetic properties and allosteric nucleotide regulation of liver GDH (Bell and Storey, 2010). In addition, homogenates may contain varying amounts of regulatory nucleotides or metabolites. Although there is no evidence that different isoforms of GDH could be differentially expressed during the life-cycle, this possibility cannot be ruled out.

There are two recognised binding sites for nucleotide regulators, one for GTP and the other for ADP (Fang et al., 2002). The predicted amino acid sequences of *H. contortus* and *T. circumcincta* GDH (Supplementary Fig 1) do not appear to explain differences in sensitivity to ADP seen in homogenates of the two species and life-cycle stages, which may result instead from other components of the homogenates. The pivot helix and residues in the ADP binding pocket are identical in the two nematode species, but there are six amino changes in the antenna, which Banerjee et al. (2003) believe is not the critical part of the molecule for allosteric regulation by ADP or GTP. GTP was strongly inhibitory in the two life-cycle of both parasites (Fig. 2D), whereas ADP was stimulatory, particularly to the L3 *H. contortus* homogenates (Fig. 2F). ATP can bind either to the inhibitory GTP or stimulatory ADP site, resulting in complex effects of ATP for human GDH, in inhibition at very low concentrations (to 0.1 mM), little effect at intermediate concentrations (1–5 mM) and inhibition above 5 mM (Fang et al., 2002). These concentrations of ATP and those added in the present study were within the normal cell levels of 1–10 mM, which varies with the tissue type and whether it is obtained from vertebrates or invertebrates (Beis and Newsholme, 1975; Traut, 1994).

The present study showed that both L3 and adult *H. contortus* have an active GDH, which was a dual co-factor enzyme with more activity with NAD^+/H than $NADP^+$/H. The low affinity of GDH for ammonia may be the limiting factor for ammonia incorporation into amino acids via GDH. Allosteric nucleotide regulation was concentration- and direction-dependent and similar to that for mammalian GDH, although under optimum assay condition was more responsive to activation by ADP and ATP and less inhibited by GTP.

Supplementary materials related to this article can be found online at doi:10.1016/j.cbpb.2011.11.014.

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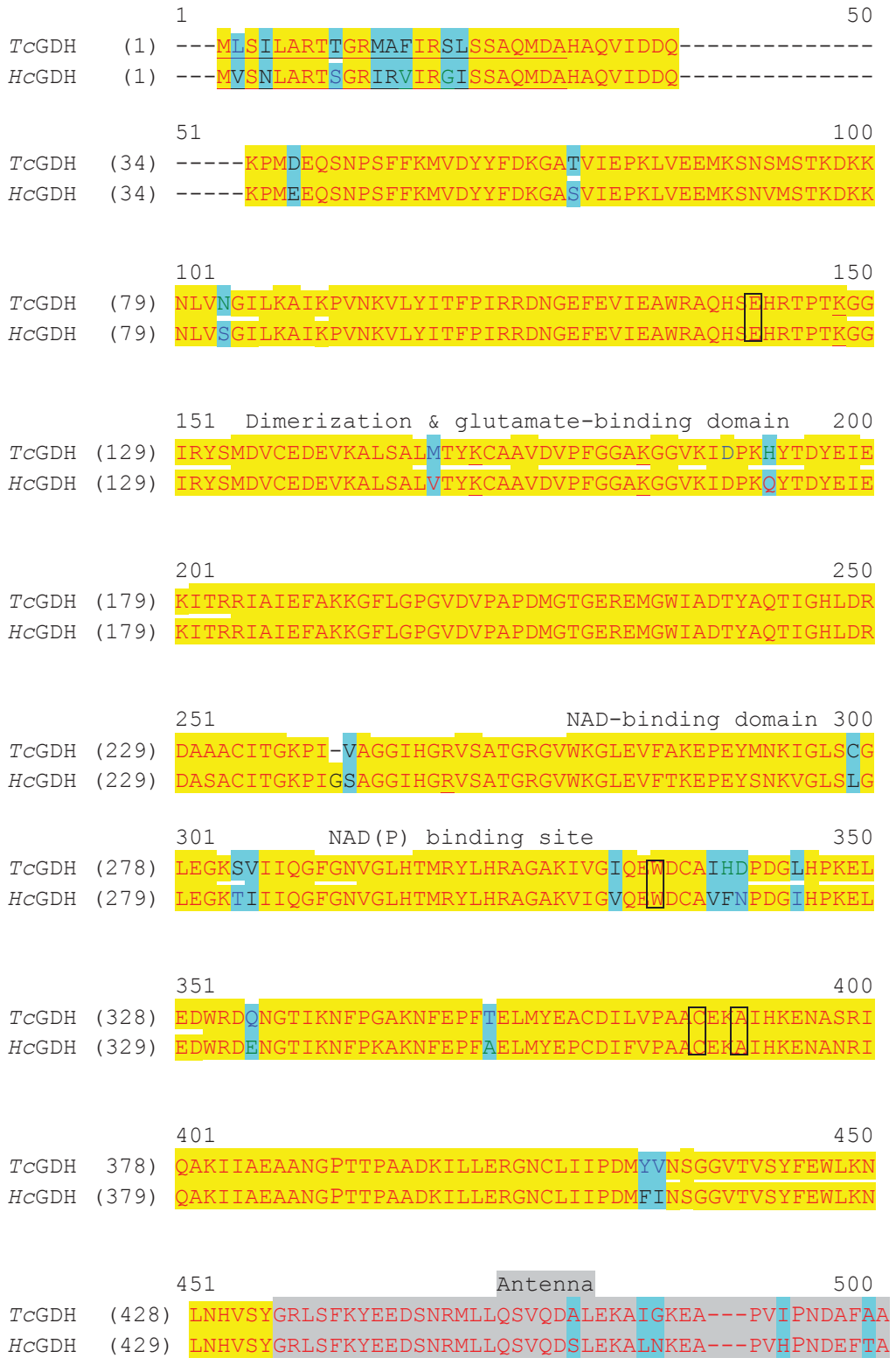
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51 100
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HcGDH (34) -----KPMEEQSNPSFFKMVDYYFDKGASVIEPKLVEEMKSNVMSTKDKK
101 150
TcGDH (79) NLVNGILKAIKPVNKVLYITFPIRRDNGEFEVIEAWRAQHSEHRTPTKGG
HcGDH (79) NLVSGILKAIKPVNKVLYITFPIRRDNGEFEVIEAWRAQHSEHRTPTKGG
151 Dimerization & glutamate-binding domain 200
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HcGDH (129) IRYSMDVCEDEVKALSALVTYKCAAVDVPFGGAKGGVKIDPKQYTDYEIE
201 250
TcGDH (179) KITRRIAIEFAKKGFLGPGVDVPAPDMGTGEREMGWIADTYAQTIGHLDR
HcGDH (179) KITRRIAIEFAKKGFLGPGVDVPAPDMGTGEREMGWIADTYAQTIGHLDR
251 NAD-binding domain 300
TcGDH (229) DAAACITGKPI-VAGGIHGRVSATGRGVWKGLEVFAKEPEYMNKIGLSCG
HcGDH (229) DASACITGKPIGSAGGIHGRVSATGRGVWKGLEVFTKEPEYSNKVGLSLG
301 NAD(P) binding site 350
TcGDH (278) LEGKSVIIQGFGNVGLHTMRYLHRAGAKIVGIQEWDCAIHDPDGLHPKEL
HcGDH (279) LEGKTIIIQGFGNVGLHTMRYLHRAGAKVIGVQEWDCAVFNPDGIHPKEL
351 400
TcGDH (328) EDWRDQNGTIKNFPGAKNFEPFTELMYEACDILVPAACEKAIHKENASRI
HcGDH (329) EDWRDENGTIKNFPKAKNFEPFAELMYEPCDIFVPAACEKAIHKENANRI
401 450
TcGDH 378) QAKIIAEAANGPTTPAADKILLERGNCLIIPDMYVNSGGVTVSYFEWLKN
HcGDH (379) QAKIIAEAANGPTTPAADKILLERGNCLIIPDMFINSGGVTVSYFEWLKN
451 Antenna 500
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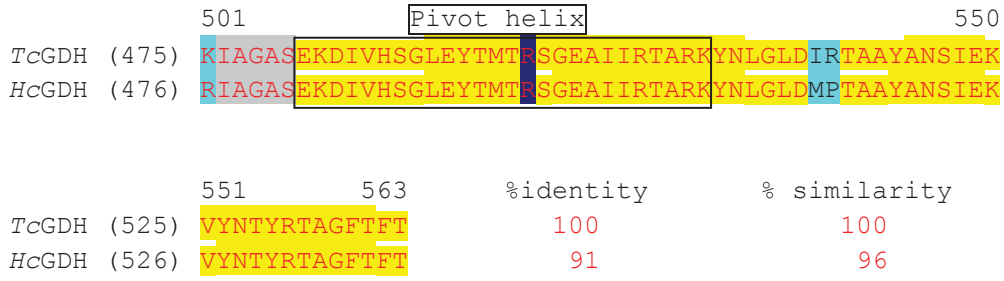

Supplementary Figure 1. Sequence alignment of *T. circumcincta* GDH (*Tc*GDH) (GI:JF731342) with *H. contortus* GDH (*Hc*GDH) (GI:3207184). Residues coloured in yellow are conserved and in blue are different. Putative nematode GDH mitochondrial targeting peptides are underlined. In the N-terminal dimerization/glutamate-binding domain, conserved lysine and arginine residues involved in glutamate binding are underlined. NAD^+- and ADP-binding residues are largely conserved. The conserved antenna region is shaded grey, and pivot helices are boxed. The side chain of the pivot helix residue R516, highlighted in purple (= R463 using the processed human GDH1 numbering) binds the ß-phosphate of the allosteric activator ADP and the R463A mutation abolishes ADP activation. Percentage identity and similarity values are from pair wise blastp alignments using default parameters.

Chapter V

Enzymes of the ornithine-proline-glutamate pathway in the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair, M-Y. Leung, R.J. Bland and H.V. Simpson

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Enzymes of the ornithine–glutamate–proline pathway in the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair [a], Y.-M. Leung [a], R.J. Bland [b], H.V. Simpson [a,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] AgResearch Ltd, Private Bag 11-008, Palmerston North, New Zealand

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Δ^1-Pyrroline-5-carboxylate synthase (E.C. 1.2.1.41)
Δ^1-Pyrroline-5-carboxylate dehydrogenase (E.C. 1.5.1.12)
Proline oxidase (E.C. 1.5.99.8)

ABSTRACT

A fully functional ornithine–glutamate–proline pathway was detected in L3 and adult *Haemonchus contortus* and *Teladorsagia circumcincta,* making the parasites capable of interconversion of these amino acids. Ornithine aminotransferase (OAT) (E.C. 2.6.1.13) was a reversible pyridoxal-5-phosphate (PLP)-dependent enzyme with an optimum pH 8.5. Hydroxylamine completely inhibited OAT activity in both parasites. For all five enzymes, substrate affinity was similar for each species and life cycle stage, the notable exceptions being the nearly 10-fold lower affinity for Δ^1-pyrroline-5-carboxylate (P5C) of P5C reductase (E.C. 1.5.1.2) in adult *T. circumcincta* and about half for P5C for L3 *H. contortus* P5C dehydrogenase (E.C. 1.5.1.12). P5C synthase (E.C. 1.2.1.41) activity was similar with either NADPH or NADH as co-factor. Proline oxidase (E.C. 1.5.99.8) was a co-factor independent enzyme with an optimal pH 8.5. Despite similarities to those in the host, enzymes of this pathway may still be useful as control targets if they differ antigenically, as a supply of proline is necessary for cuticle formation.

1. Introduction

Parasitic nematode larvae grow rapidly and adult worms lay large numbers of eggs, both requiring very active nitrogen and energy metabolism. The proline required for synthesis of collagen, an important component of the cuticle, can either be synthesised from ornithine or glutamate or obtained directly from the host. There may be other functions for proline, as in many lower organisms: in insects (Scaraffia and Wells, 2003) and trypanosomes (Obungu et al., 1999), proline acts as a source of energy; bacteria and some crustaceans use proline as an osmoprotectant (Burton, 1992; Lee et al., 2003) and in plants, bacteria and marine organisms, proline levels rise significantly during stressful conditions (Aral and Kamoun, 1997).

In animals, ornithine, glutamate and proline are interconvertable amino acids through an intermediate Δ^1-pyrroline-5-carboxylate (P5C) (Fig. 1). A reversible enzyme, ornithine aminotransferase (OAT), converts ornithine to glutamyl γ-semialdehyde, which is in equilibrium with the cyclic form P5C. P5C is then converted to proline and glutamate by Δ^1-pyrroline-5-carboxylate reductase (P5CR) and Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) respectively. In the reverse reactions, P5C is synthesised from glutamate by Δ^1-pyrroline-5-carboxylate synthase (P5CS) in an ATP- and NADPH-dependent reaction and from L-proline by proline oxidase (PO) (also called proline dehydrogenase) (Wu and Morris, 1998).

In higher plants, there is an alternative route of converting ornithine to proline. Instead of using P5C as an intermediate, ornithine is converted to α-keto-δ-aminovalerate, which is reduced to proline by Δ^1-pyrroline-2-carboxylate (P2C) reductase (Aral and Kamoun, 1997). In bacteria, γ-glutamyl kinase and γ-glutamyl phosphate reductase are the enzymes responsible for converting glutamate to P5C. The presence of one of these pathways in abomasal nematodes cannot be ruled out, as these parasites have already been shown to have differences in both arginine/ornithine and glutamate metabolism from that of the host. Both species lack a full ornithine-urea cycle, *Haemonchus contortus* can synthesise polyamines from either ornithine or agmatine (Umair et al., 2011a) and glutamate synthase (GOGAT) can incorporate ammonia into glutamate (Umair et al., 2011b).

A fully functional mammalian-type ornithine–glutamate–proline pathway has been reported in various helminths and arthropods such as *Fasciola gigantica* (Momamed et al., 2008), the mosquito *Aedes aegypti* (Scaraffia et al., 2005), the tick *Hyalomma dromedarii* (Fahmy et al., 1997), *Schistosoma mansoni* (Isseroff

* Corresponding author. Fax: +64 64 6 350 5636.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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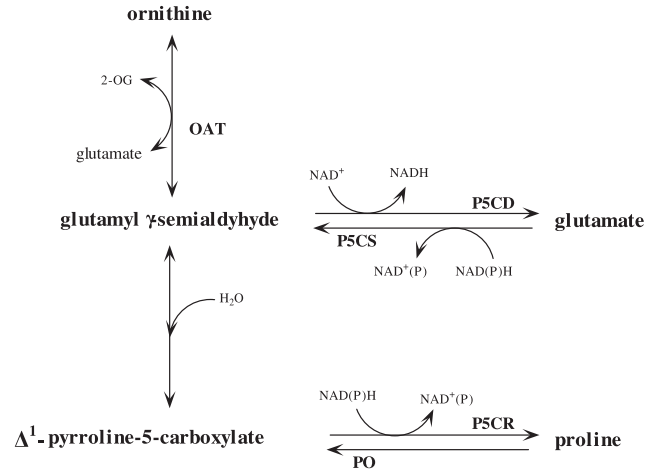

Fig. 1. Enzymes involved in the interconversion of ornithine, glutamate and proline. Δ^1-Pyrroline-5-carboxylate is spontaneously formed from glutamyl γ-semialdehyde forming an equilibrium which favours P5C formation. Abbreviations used: 2-OG: 2-oxoglutarate; OAT: ornithine aminotransferase; PO: proline oxidase; P5CR: Δ^1-pyrroline-5-carboxylate reductase; P5CDH: Δ^1-pyrroline-5-carboxylate dehydrogenase; P5CS: Δ^1-pyrroline-5-carboxylic acid synthase.

et al., 1983), *Heligmosomoides polygyris* and *Panagrellus redivivus* (Grantham and Barrett, 1986). The presence of a functional ornithine–glutamate–proline pathway in abomasal nematodes appears not to have been investigated. In the present study, the kinetic properties of the five enzymes involved in the interconversion of proline, glutamate and ornithine via P5C have been investigated in L3 and adult *H. contortus* and *T. circumcincta*.

2. Materials and methods

All chemicals were purchased from the Sigma Chemical Co. (Mo, USA), unless stated otherwise. P5C was synthesised as described by Mezl and Knox (1976). Use of experimental animals has been approved by the Massey University Animal Ethics Committee.

2.1. Parasites

L3 *T. circumcincta* and *H. contortus* were cultured from the faeces of sheep infected with pure strains of parasites and stored in reverse osmosis (RO) water at 4 °C for the former and at 10 °C for the latter species. Sheathed larvae were either freshly collected or prior to each experiment, L3 were baermannised in RO water to remove inactive worms and re-suspended in assay buffer. Adult worms were recovered from the abomasa of infected sheep using the technique of Simpson et al. (1999). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and placed in assay buffer.

2.2. Preparation of homogenates

Approximately 50,000 L3 were centrifuged at 600*g* for 5 min, washed twice by re-suspending in appropriate assay buffer followed by centrifugation, then finally re-suspended in 1 ml buffer. The concentrated L3 suspension or approximately 5 mg of clumped adult worms was transferred to a chilled mortar, frozen at −20 °C for at least 15 min and homogenised with a chilled pestle and mortar. The protein concentrations of homogenates were determined by the method of Bradford (1976).

2.3. Enzyme assays

Unless stated otherwise, all assays were performed at 30 °C on two homogenates each of sheathed L3 and adult *T. circumcincta* and *H. contortus*, using 50 μg homogenate protein and 100 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4) in a total volume of 1 ml assay mixture.

2.3.1. Ornithine aminotransferase (OAT) (E.C. 2.6.1.13)

OAT activity was determined by the rate of P5C formation (Grantham and Barrett, 1986). After incubation of the assay mixture for 30 min, the reaction was terminated by the addition of 1 ml 20% trichloroacetic acid (TCA), followed by the addition of 100 μl 0.2 M *o*-aminobenzaldehyde (in 95% alcohol) and re-incubation for 30 min. Protein was centrifuged to precipitation at 5000*g* for 5 min and OD of the supernatant was determined at 440 nm spectrophotometrically (Jenway 6505, Jenway Ltd, UK). The P5C concentration was determined from a standard curve.

(1) The optimal pH for OAT was determined in L3 homogenates with assay mixtures containing homogenate in buffer pH 6–9, 0.2 mM pyridoxal-5-phosphate (PLP), 10 mM ornithine and 10 mM 2-oxoglutarate (2-OG). Subsequent assays were performed at pH 8.5.
(2) The optimal concentration of PLP was determined with 10 mM ornithine and 2-OG and 0.01 to 2 mM PLP.
(3) The V_{max} and the apparent K_m for ornithine were determined in assay mixtures containing 10 mM 2-OG, 0.2 mM PLP and 0–50 mM ornithine and for 2-OG in mixtures containing 10 mM ornithine, 0.2 mM PLP and 0–40 mM 2-OG.
(4) The effects of addition of 1 mM hydroxylamine, isoleucine, leucine, arginine, valine or lysine were determined for L3 homogenates. The substrate concentrations were 10 mM 2-OG, 10 mM ornithine and 0.2 mM PLP.
(5) Ornithine produced by the reverse reaction of OAT, which uses P5C as substrate and generates ornithine and 2-OG, was monitored by thin layer chromatography (TLC), as performed by Zolg and Ottow (1973). Briefly, 1 μl reaction mixture and 1 mM ornithine as the standard were placed on cellulose coated TLC plates (Merck, Germany) cut to 6.5 × 9.5 cm × 0.1 mm. The plates were dipped in 20 μl of a mixture of 1-butenol, acetone, diethylamine and water (10:10:2:5). After 2 h, the plates were sprayed with ninhydrin solution (1% (w/v) in iso-propanol) and dried for 5 min. The standard and samples appeared as purple spots (Supplementary Fig. 1).

2.3.2. Δ^1-Pyrroline-5-carboxylate dehydrogenase (P5CDH) (E.C. 1.5.1.12)

P5CDH activity was determined from the rate of NADH formation monitored spectrophotometrically at 340 nm, as described by Grantham and Barrett (1986).

(1) The optimal pH was determined for L3 homogenates in reaction mixtures containing buffer pH 5.5–9, homogenate and 0.2 mM NAD^+. The reaction was started by the addition of 1 mM P5C and enzyme activity was determined from the rate of increase in absorption monitored spectrophotometrically at 340 nm. Subsequent assays were performed at pH 7.5
(2) The V_{max} and the apparent K_m for P5C were determined in assay mixtures containing 0 to 10 mM P5C and 0.2 mM NAD^+.

2.3.3. Δ^1-Pyrroline-5-carboxylate synthase (P5CS) (E.C. 1.2.1.41)

P5CS activity was determined from the rate of P5C formation (Section 2.3.1).

(1) The optimal pH for P5CS was determined for L3 homogenates with assay mixtures containing buffer pH 5.5–9, 5 mM glutamate, 0.1 mM Mg^{2+}, 0.5 mM mercaptoethanol, 0.2 mM NADH and 0.2 mM ATP. Subsequent assays were performed at pH 7.
(2) The apparent K_m and V_{max} were determined in assay mixtures containing 0–10 mM glutamate, 0.2 mM Mg^{2+}, 0.5 mM mercaptoethanol, 0.2 mM NADH and 0.2 mM ATP.
(3) Activity with NADPH as co-factor was determined in L3 homogenates. The substrate concentrations were 5 mM glutamate, 0.2 mM Mg^{2+}, 0.5 mM mercaptoethanol, 0.2 mM NADPH and 0.2 mM ATP.
(4) The effect of varying [Mg^{2+}] on P5CS activity in L3 homogenates was determined in assay mixtures containing 5 mM glutamate, 0.1–2 mM Mg^{2+}, 0.5 mM mercaptoethanol, 0.2 mM NADH and 0.2 mM ATP. The effect of varying [NADH] was determined in assay mixtures containing 5 mM glutamate, 0.2 mM Mg^{2+}, 0.5 mM mercaptoethanol, 0.1–2 mM NADH and 0.2 mM ATP.

2.3.4. Δ^1-Pyrroline-5-carboxylate reductase (P5CR) (E.C. 1.5.1.2)

P5CR was assayed by monitoring the rate of NADH utilisation spectrophotometrically at 340 nm, as described by Grantham and Barrett (1986).

(1) The optimal pH for P5CR was determined for L3 homogenates in buffer pH 6–9, 1 mM P5C and the reaction was started by the addition of 0.2 mM NADH. Subsequent assays were performed at pH 6.5 for *H. contortus* and pH 7 for *T. circumcincta*.
(2) The V_{max} and the apparent K_m for P5C were determined in assay mixtures containing 0 to 10 mM P5C and 0.2 mM NADH.
(3) Enzyme activities with NADPH as co-factor were determined in L3 homogenates in assay mixtures containing 1 mM P5C and 0.2 mM NADPH.

2.3.5. Proline oxidase (PO) (E.C. 1.5.99.8)

Proline oxidase activity was determined from the rate of P5C formation after a 30 min incubation (Section 2.3.1).

(1) The optimal pH was determined for L3 homogenates in an assay mixture consisting of 20 μg cytochrome *c* and homogenate in 50 mM Tris–HCl buffer pH 5.5–9. The reaction was started by adding 1 mM proline.
(2) The V_{max} and the apparent K_m for proline were determined in assay mixtures containing buffer pH 8, 20 μg cytochrome *c* and 0–20 mM L-proline. The reaction was terminated by the addition of 1 ml 20% TCA and [P5C] determined.
(3) Enzyme activities with NAD^+ and $NADP^+$ as co-factors were determined in L3 homogenates. The assay mixture contained 1 mM proline and 0.2 mM NAD^+(P). Enzyme activity was monitored spectrophotometrically at 340 nm from the rate of $NAD(P)^+$ utilisation.

2.4. Data analysis

Replicate data are presented as mean ± SEM. Graphpad Prism v5 was used to plot kinetic data and estimate K_m and V_{max}.

3. Results

3.1. Ornithine aminotransferase

Enzyme activities and kinetic properties of OAT in homogenates of L3 and adult *T. circumcincta* and *H. contortus* are shown in Table 1. The enzyme properties were generally similar in each of life cycle stages of the two species. Enzyme activities were higher in the adult worms than in L3. The apparent K_m for ornithine was higher than that of 2-OG in L3 and adult species of both worms. Except for L3 *H. contortus*, in which it was lower, the K_m for ornithine were very similar. The optimal pH and PLP concentration were pH 8.5 (Fig. 2A) and 0.2 mM respectively in both L3 *H. contortus* and *T. circumcincta*.

The effects of the addition of OAT inhibitors are presented in Table 2. Addition of 1 mM hydroxylamine completely inhibited OAT activity in both species, whereas, addition of 1 mM isoleucine, leucine or lysine inhibited enzyme activity by approximately 50% in L3 *H. contortus* and 40% in L3 *T. circumcincta*. Addition of 1 mM arginine did not inhibit enzyme activity in either species. Ornithine, produced by the reverse reaction of OAT, was detected on TLC plates (Supplementary Fig. 1), showing that OAT can function in either direction.

3.2. Δ^1-Pyrroline-5-carboxylate dehydrogenase

P5CDH activities were similar for two life cycle stages of the two worms, but higher in the adults than in L3 (Table 1). The apparent K_m was similar in all parasite homogenates, except those of L3 *H. contortus*, in which it was about the half. The optimal pH for P5CDH activity in both parasite species was 7.5 (Fig. 2B). In L3 *T. circumcincta*, enzyme activity appeared to have a broader pH range than that in L3 *H. contortus* and significant activity was detected even at higher pH.

3.3. Δ^1-Pyrroline-5-carboxylate synthase

P5CS activities were similar in both L3 and adult worms of the two species, but higher in adults (Table 1). The apparent K_m for L3 *T. circumcincta* P5CS was lower (0.45 mM) than that of L3 *H. contortus* (0.65 mM). The optimum pH for both L3 *H. contortus* and *T. circumcincta* was pH 7.0 (Fig. 2C).

Enzyme activities in L3 *H. contortus* and *T. circumcincta* were similar with either NADPH or NADH as co-factor. For *H. contortus*, V_{max} were 24 ± 4 and 26 ± 2 and for *T. circumcincta* 26 ± 3 and 27 ± 3 (nmoles min^{-1} mg^{-1} protein, mean ± SEM, $n = 2$) respectively. Optimal Mg^{2+} and NADH concentrations for L3 of both species were both 0.2 mM.

3.4. Δ^1-Pyrroline-5-carboxylate reductase

P5CR activities were detected in sheathed L3 and adult *H. contortus* and *T. circumcincta* (Table 1). Enzyme activities were similar with NADH or NADPH as co-factor. For *H. contortus*, V_{max} were 27 ± 2 and 24 ± 2 and for *T. circumcincta*, 30 ± 2 and 24 ± 3 (nmol min^{-1} mg^{-1} protein, mean ± SEM, $n = 2$) respectively. The apparent K_m for P5C in homogenates of L3 for both species was lower than that in adult parasites. The optimum pH for both L3 *H. contortus* and *T. circumcincta* was pH 7.0 (Fig. 2D).

3.5. Proline oxidase

Enzyme activities and kinetic properties of PO in homogenates of L3 and adult *T. circumcincta* and *H. contortus* are shown in Table 1. The enzyme properties were generally similar in both life cy-

Table 1
The apparent substrate K_m (mM) and V_{max} (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) of ornithine aminotransferase (OAT), Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH), Δ^1-pyrroline-5-carboxylate synthase (P5CS), Δ^1-pyrroline-5-carboxylate reductase (P5CR) and proline oxidase (PO) activities in L3 and adult *H. contortus* and *T. circumcincta*.

	OAT			P5CDH		P5CS		P5CR		PO	
	K_m (Orn)	K_m (2-OG)	V_{max}	K_m (P5C)	V_{max}	K_m (Glu)	V_{max}	K_m (P5C)	V_{max}	K_m (Pro)	V_{max}
L3 *H. contortus*	1.65 ± 0.05	1 ± 0.1	61 ± 5	0.04 ± 0.01	20 ± 4	0.6 ± 0.05	26 ± 2	0.06 ± 0.01	27 ± 2	0.3 ± 0.1	28 ± 3
Adult *H. contortus*	2.30 ± 0.1	1.3 ± 0.2	93 ± 15	0.10 ± .005	45 ± 4	1 ± 0.05	38 ± 2	0.15 ± 0.05	34 ± 4	0.5 ± .05	45 ± 2
L3 *T. circumcincta*	2.15 ± 0.15	1.1 ± 0.1	54 ± 12	0.09 ± 0.01	20 ± 2	0.4 ± 0.05	27 ± 3	0.07 ± 0.005	30 ± 2	0.4 ± 0.05	41 ± 4
Adult *T. circumcincta*	2.65 ± 0.05	1.3 ± 0.14	92 ± 9	0.10 ± 0.01	35 ± 6	0.7 ± 0.1	42 ± 3	0.55 ± 0.05	40 ± 2	0.5 ± 0.1	69 ± 3

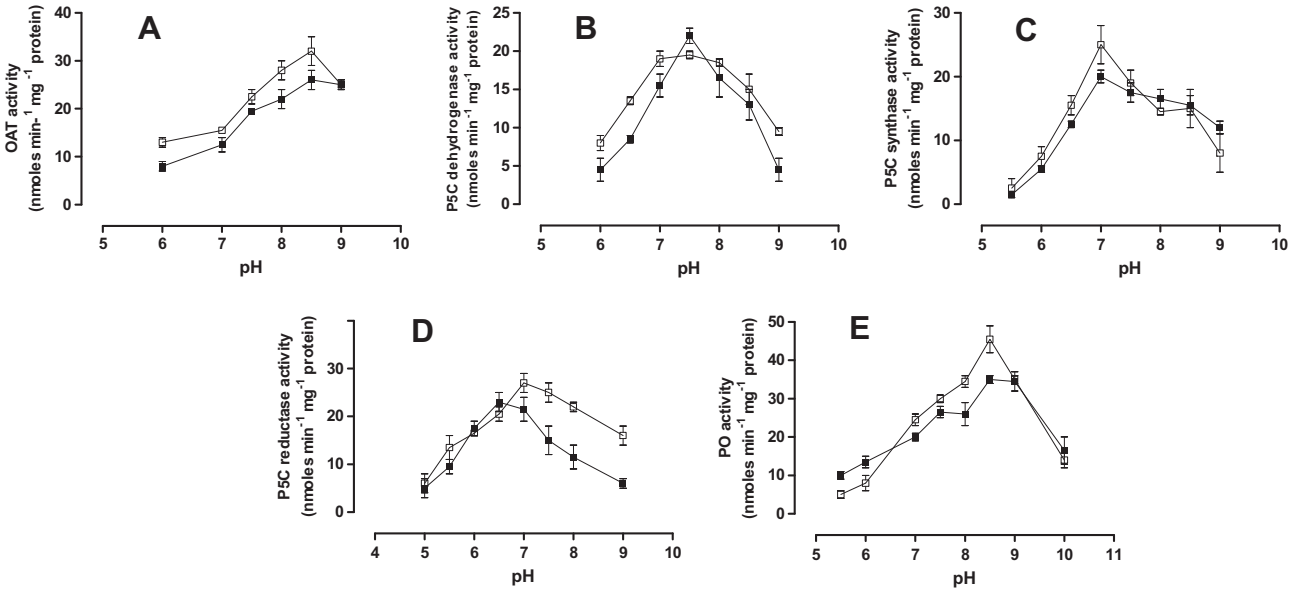

Fig. 2. Effect of pH on (A) ornithine aminotransferase, (B) Δ^1-pyrroline-5-carboxylate dehydrogenase, (C) Δ^1-pyrroline-5-carboxylate synthase, (D) Δ^1-pyrroline-5-carboxylate reductase and (E) proline oxidase activities (nmoles min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) from L3 *H. contortus* (■) or *T. circumcincta* (□).

Table 2
Ornithine aminotransferase activities (OAT) (mean ± SEM, $n = 2$) of sheathed L3 *T. circumcincta* and *H. contortus* in the presence of 1 mM OAT inhibitors. Activity was calculated from the rate of formation of Δ^1-pyrroline-5-carboxylate from 10 mM ornithine and 10 mM 2-oxoglutarate, determined spectrophotometrically at 440 nm, and expressed as a percentage of enzyme activity without any added inhibitor (100%).

Inhibitors	Activity (%) (*H. contortus*)	Activity (%) (*T. circumcincta*)
Control	100	100
Hydroxylamine	0	0
Isoleucine	50 ± 4	61 ± 3
Leucine	58 ± 2	63 + 5
Arginine	100 ± 3	98 ± 3
Lysine	56 ± 6	59 ± 1
Valine	35 ± 5	45 ± 5

cle stages of the two species. The optimum pH for both L3 *H. contortus* and *T. circumcincta* was pH 8.5 (Fig. 2E). No consumption of either NAD^+ or $NADP^+$, which were added as possible co-factors, was detected during the conversion of proline to P5C.

4. Discussion

All five enzymes of the ornithine–glutamate–proline pathway were active in both L3 and adult *H. contortus* and *T. circumcincta*, more so in adult worms. This pathway is likely to be very active in L4 and earlier larval stages which synthesise new cuticle, which has a high proline content. Although not all enzymes were assayed, there also appear to be fully functional pathways in *H. polygyrus*, *P. redivivus* (Grantham and Barrett, 1986) and *F. gigantica* (Momamed et al., 2008), whereas PO was absent in *Fasciola hepatica* (Ertel and Isseroff, 1974). In the present study, the kinetic properties of all enzymes were also investigated and revealed no major differences between the enzymes of *H. contortus* and *T. circumcincta*, nor between L3 and adult worms.

A bi-directional OAT was detected in both parasite species. The reversibility was confirmed by identifying the product ornithine on TLC plates (Supplementary Fig. 1), as ornithine could not be detected colorimetrically in the presence of the substrate P5C. Enzyme activity was higher in adults than in L3 (Table 1). The optimum pH for L3 *H. contortus* and *T. circumcincta* OAT was 8.5, similar to that in *H. polygyris* and *P. redivivus* (Grantham and Barrett, 1986), *S. mansoni* (Goldberg et al., 1979) and *F. gigantica* (Momamed et al., 2008). Of the five amino acids which partially inhibited *S. mansoni* OAT (Goldberg et al., 1979), four (not arginine) also reduced OAT activity of both L3 *H. contortus* and *T. circumcincta*. As in that study, OAT of both abomasal nematodes was completely inhibited by hydroxylamine.

Enzyme activities ranged from 20 nmol min^{-1} mg^{-1} protein for P5CDH to 93 nmol min^{-1} mg^{-1} protein for OAT (Table 1). Generally, activity was higher for each enzyme in adult worms than in L3, which may relate to the expression of activity per mg protein, rather than more active enzymes. The pH optima ranged from pH 7.0–8.5, similar to those in other helminths (Grantham and Barrett,

1986; Momamed et al., 2008). *T. circumcincta* L3 P5CR retained more activity above the optimum of pH 7 than did the *H. contortus* enzyme (Fig. 2 D). Although the optimal pH of 7.5 for both *H. contortus* and *T. circumcincta* P5CDH was a little lower than the pH 8.3 in *H. polygyris* and *P. redivivus* (Grantham and Barrett, 1986), it was similar to the pH 7 reported for *F. gigantica* (Momamed et al., 2008). There appear to be no published K_m for helminth enzymes with which the *H. contortus* and *T. circumcincta* data (Table 1) can be compared. For all five enzymes, substrate affinity was similar for enzymes from each species and life cycle stage, the notable exception being the nearly 10-fold lower affinity for P5C of P5CR in *T. circumcincta* adult worms. It is not apparent why this was case, presumably because of some interaction of the enzyme with a regulator in the homogenate, however, there was no evidence of either substrate or product inhibition.

In some plants and algae, the reaction from proline to P5C is catalysed by an NAD^+-dependent proline dehydrogenase (Mazelis and Creveling, 1974; McNamer and Stewart, 1974), whereas in animals, the corresponding enzyme is PO, which does not require this co-factor. Homogenates of both L3 and adult *H. contortus* and *T. circumcincta* were used to assay the usage of $NAD(P)^+$ during the conversion of proline to P5C to investigate whether such an enzyme were present in abomasal nematodes. As no co-factor consumption was detected, the enzyme involved appears to be the mammalian type and not an NAD^+-dependent proline dehydrogenase.

The enzymes for interconversion of proline, glutamate and ornithine are all functional in abomasal nematodes and the direction in vivo will depend on metabolic requirements and supply of nitrogen. Nematodes have a high requirement for proline as a precursor for collagen that forms the worm cuticle. Prior to moulting, large amounts of proline may be obtained directly from the host via a transporter, as gastric mucins would be a rich source of proline. Alternatively, these nematodes have a functional GS-GOGAT pathway (Umair et al., 2011b), which allows ammonia to be used as a source of nitrogen, even at low external concentrations. Ammonia incorporation into glutamate would provide a source of other amino acids, including proline, which is necessary for cuticle formation. Alternatively, the uptake of proline from mucin may be an important source of amino acids for the parasites. Despite similarities to those in the host, enzymes of this pathway may still be useful as control targets if they differ antigenically, provided the enzyme is accessible to blockage by immune effectors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2011.07.006.

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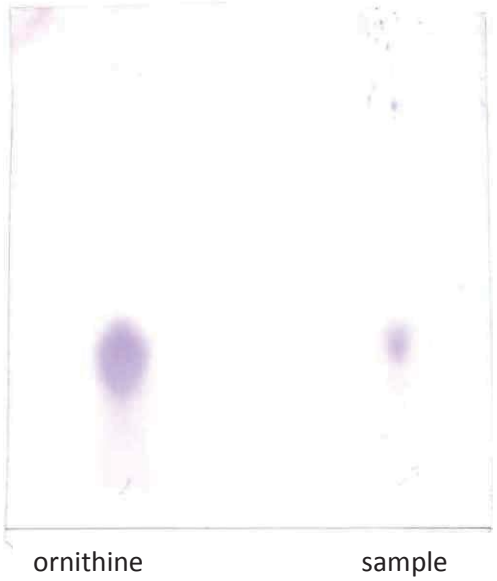

Supplementary Figure 1. Ornithine aminotransferase (OAT) activity, from P5C to ornithine, is present in homogenates of L3 abomasal nematodes. Generation of the product ornithine is confirmed by the detection of ornithine by thin layer chromatography. Ornithine (standard) and sample (assay mixture) are shown as purple spots.

Chapter VI

Arginine metabolism in the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair, M.L. Patchett, R.J. Bland and H.V. Simpson

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Arginine metabolism in the sheep abomasal nematode parasites *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair [a], M.L. Patchett [b], R.J. Bland [c], H.V. Simpson [a,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] Institute of Molecular Biosciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[c] AgResearch Ltd., Private Bag 11-008, Palmerston North, New Zealand

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Ornithine decarboxylase (E.C. 4.1.1.17)
Arginine decarboxylase (E.C. 4.1.1.19)
Polyamine

ABSTRACT

The ornithine urea cycle, polyamine synthesis, nitric oxide synthesis and metabolism of arginine to putrescine have been investigated in L3 and adult *Haemonchus contortus* and *Teladorsagia circumcincta*. Neither parasite had a detectable arginine deiminase/dihydrolase pathway nor a functional ornithine urea cycle. Nitric oxide synthase was present in central and peripheral nerves, but was not detected in whole parasite homogenates. Both arginase (E.C. 3.5.3.1) and agmatinase (E.C. 3.5.3.11) activities were present in both species. Arginase did not require added Mn^{2+} and had an optimal pH of 8.5. Polyamine metabolism differed in the two species and from that in mammals. Ornithine decarboxylase (E.C. 4.1.1.17) was present in both parasites, but no arginine decarboxylase (E.C. 4.1.1.19) activity was detected in *T. circumcincta*. The flexibility of synthesis of putrescine in *H. contortus* may make this pathway less useful as a target for parasite control than in *T. circumcincta*, in which only the ornithine decarboxylase pathway was detected.

1. Introduction

Nitrogen metabolism may present targets for novel therapies against abomasal parasitic nematodes, as it is essential for survival of all organisms and appears to differ in some aspects in nematodes from that of their host. Parasitic nematodes may not be completely dependent on ingested amino acids, but be able to incorporate ammonia into glutamate and thus into other amino acids. Excreted ammonia appeared to be reabsorbed in vitro by *Teladorsagia circumcincta* (Simpson et al., 2009) and glutamate synthase is present in both *Haemonchus contortus* and *T. circumcincta* (Umair et al., 2011). There may also be features of amino acid metabolism which differ in mammals and nematodes. Arginine is a substrate for numerous other compounds, including polyamines, agmatine, nitric oxide, citrulline, creatine, proline, glutamate and also urea via the ornithine urea cycle (OUC) (Wu and Morris, 1998). Nematodes may also use non-mammalian pathways to metabolise arginine (Fig. 1).

In most organisms, arginine is converted to ornithine and urea by arginase, which in mammals is also the final enzyme for urea synthesis by the OUC. In microorganisms and protozoan parasites, arginine is converted to ornithine via citrulline generating ATP using the arginine deiminase pathway, which is comprised of three enzymes: arginine deiminase (ADI), ornithine carbamoyl transferase and carbamate kinase (Zuniga et al., 2002). *Euglena* and some bacteria (*Streptococcus* and *Pseudomonas*) have instead the two enzyme arginine dihydrolase pathway, in which ornithine is produced from arginine by ADI and citrullinase at the expense of ATP (Slade, 1952). It is possible that some of these enzymes could be active in helminths, as Janssens and Bryant (1969) found unusual arginine metabolism in three of seven helminths studied: there was no arginase activity in *Moniliformis dubius* and citrulline, not ornithine, was the product formed from arginine in *Moniezia expansa* and *Echinococcus granulosis*.

In ureotelic animals, excreted urea is formed by the OUC (Fig. 1). As is the case in many bacteria, yeasts, plants, invertebrates and vertebrates (Jenkinson et al., 1996), arginase is present in many helminths, whereas the other OUC enzymes, carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) have not consistently been detected. This is not surprising, as urea is a minor component of nitrogenous excretion in nematodes (Rogers, 1952; Wright, 1975). All OUC enzymes were present in *Fasciola hepatica* (Rijavec and Kurelec, 1965; Mohamed et al., 2005), *Dicrocoelium lanceatum* (Rijavec and Kurelec, 1965) and in *Panagrellus redivivus*, although ASS and ASL activities were very low (Wright, 1975). Arginase activity has been reported in parasitic trematodes (Campbell and Lee, 1963; Rijavec and Kurelec, 1965; Senft, 1966;

* Corresponding author. Fax: +64 6 350 5636.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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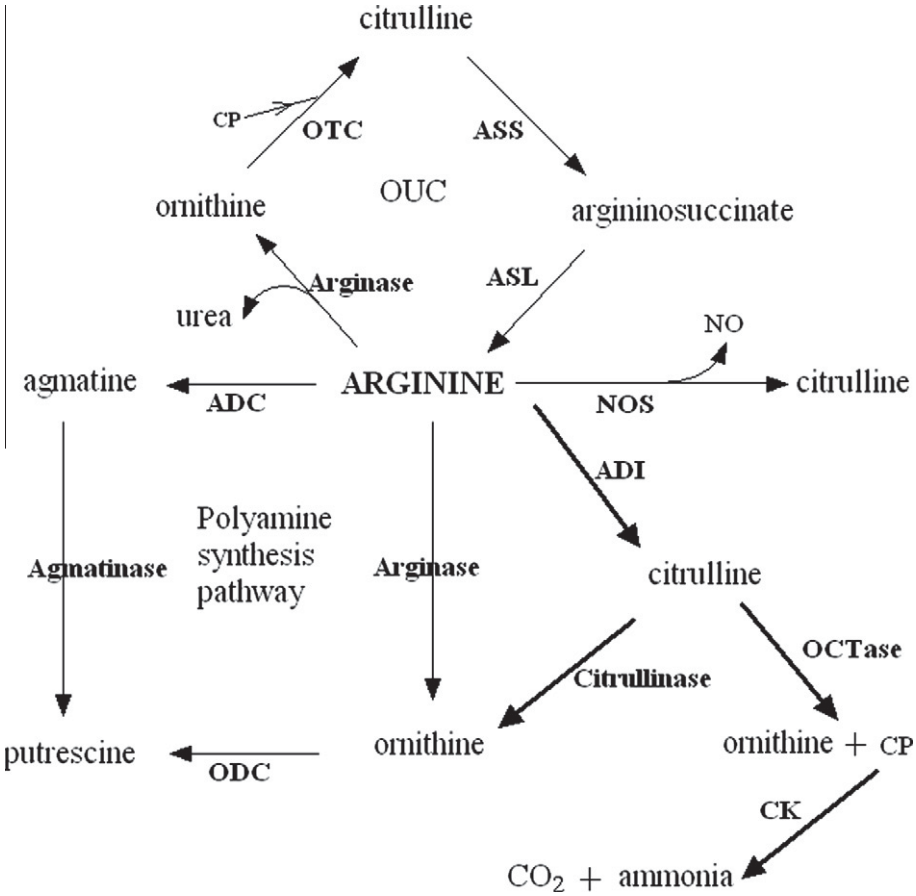

Fig. 1. Arginine metabolism by the ornithine urea cycle, nitric oxide synthase, the polyamine synthetic pathways and the non-mammalian arginine deiminase/dihydrolase pathways. Bold arrows indicate the three enzyme arginine deiminase pathway (right) and the two step arginine dihydrolase pathway (left). *Abbreviations used:* ADC, arginine decarboxylase; ADI, arginine deiminase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CK, carbamate kinase; CP, carbamoyl phosphate; NO, nitric oxide; NOS, nitric oxide synthase; OCTase, ornithine carbamoyltransferase; ODC, ornithine decarboxylase; OTC, ornithine transcarbamylase; OUC, ornithine urea cycle.

Mohamed et al., 2005; Fitzpatrick et al., 2009), cestodes (Campbell and Lee, 1963; Janssens and Bryant, 1969) and the nematodes *T. circumcincta*, *Ascaris lumbricoides*, *Ascaridia galli*, *Nematodirus* spp., *Heligmosomoides polygyrus* and *P. redivivus* (Rogers, 1952; Janssens and Bryant, 1969; Grantham and Barrett, 1986; Muhamad, 2006), although low in the latter two species.

Arginase activity is of interest because of the proposed defence strategy of parasites of using their arginase to compete with host nitric oxide synthase (NOS) for the common substrate arginine (Vincendeau et al., 2003). NO is harmful to parasites (Taylor-Robinson and Looker, 1998), bacteria and cancerous cells and is a host defence strategy to a number of bacterial pathogens of vertebrates (Vincendeau et al., 2003) and invertebrates (Rivero, 2006), although overproduction of NO is believed to contribute to host tissue damage in a number of diseases. In mammals, NO is also a neurotransmitter and signalling molecule (Wu and Morris, 1998; Boucher et al., 1999), with three isoforms of NOS, neuronal NOS, inducible NOS and endothelial NOS, located in different tissues. Using either biochemical assays or histochemical localisation with the NADPH-diaphorase reaction (Dawson et al., 1991), neuronal NOS (nNOS) has also been demonstrated in helminths, including the nematodes *Dirofilaria immitis, Brugia pahangi* (Kaiser et al., 1998) and *Ascaris suum* (Bascal et al., 2001).

Polyamines (putrescine, spermidine and spermine) are essential cell growth and differentiation regulators (Tabor and Tabor, 1984), whose synthesis can be inhibited by the irreversible inhibition of ornithine decarboxylase (ODC) by difluoromethylornithine (DFMO). DFMO has been successfully used as an anticancer agent and as a therapeutic agent against trypanosomes (Bacchi et al., 1994). Polyamine synthesis has been proposed as an important target in controlling nematode infections (Henderson and Fairlamb, 1987; Walter, 1988), although some parasites, such as filarial nematodes, may acquire host polyamines rather than synthesising these compounds themselves (Wittich et al., 1987).

In microorganisms and plants, putrescine can be synthesised from arginine either via agmatine (by arginine decarboxylase (ADC) and agmatinase) or via ornithine by the ODC pathway (Slocum et al., 1984; Bernet et al., 1999) (Fig. 1). In mammals, the ODC pathway is well established, but the existence of an ADC pathway is supported by some studies (Morrissey et al., 1995; Regunathan and Reis, 2000) but not others (Gilad et al., 1996; Coleman et al., 2004). In nematodes, ODC activity has been demonstrated in *Caenorhabditis elegans*, *H. contortus* (Schaeffer and Donatelli, 1990) and *P. redivivus* (Niemann et al., 1996) and genes encoding ODC have been sequenced for *P. redivivus* (Niemann et al., 1996), *C. elegans* (Macrae et al., 1995) and *H. contortus* (Klein et al., 1997). Neither ODC nor ADC activity was detected in the filarial nematodes *D. immitis, Onchocerca volvulus, Brugia patei or Litomosoides carinii*, the dog hookworm *Ancylostoma ceylanicum* nor in the rat parasite *Nippostrongylus brasiliensis* (Sharma et al., 1989). Whereas the *P. redivivus* ODC is cytosolic, as in most eukaryotes (Niemann et al., 1996), the ODC of both *C. elegans* and *H. contortus* are membrane-bound and have low K_m values for ornithine (Schaeffer and Donatelli, 1990). Membrane binding may explain why nematode enzymes were not detected in some studies.

In the present study, arginine metabolism has been compared in L3 and adult worms of two abomasal nematode parasites of sheep. In particular, enzymes of the OUC, NO synthesis and polyamine metabolism have been investigated, as well as non-mammalian pathways.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (Mo, USA) unless stated otherwise. Use of experimental animals has been approved by the Massey University Animal Ethics Committee.

2.1. Parasites

L3 *T. circumcincta* and *H. contortus* were cultured from the faeces of sheep infected with pure strains of parasites and stored in reverse osmosis (RO) water at 4 °C for the former and at 10 °C for the latter species. Sheathed larvae were either freshly collected or prior to each experiment were baermannised in RO water to remove inactive worms and re-suspended in assay buffer. Adult worms were recovered from the abomasa of infected sheep using the technique of Simpson et al. (1999). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and placed in assay buffer.

2.2. Preparation of homogenates

Approximately 50,000 L3 were centrifuged at 600*g* for 5 min, washed twice by re-suspending in buffer followed by centrifugation, then finally re-suspended in 1 ml buffer. The concentrated L3 suspension or approximately 5 mg of clumped adult worms was transferred to a chilled mortar, frozen at −20 °C for at least 15 min and homogenised with a chilled pestle and mortar. Finely chopped sheep liver was homogenised in 0.1% cetyltrimethylammonium bromide (1:9 w/v). When homogenates were to be used for more than one assay, aliquots were kept in Eppendorff tubes on ice until required. The protein concentrations of homogenates were determined by the method of Bradford (1976).

2.3. Enzyme assays

All assays were performed at 30 °C on two homogenates of sheathed L3 and adult *T. circumcincta* and *H. contortus* unless stated otherwise, using 50 μg homogenate protein. If no activity could be detected, the amount of homogenate was increased to 250 μg homogenate protein in an attempt to obtain enzyme activity. In no case did this produce detectable activity.

2.3.1. Ornithine transcarbamylase (OTC) (E.C. 2.1.3.3)

OTC activity was determined in homogenates of L3 and adult *T. circumcincta* and *H. contortus* and sheep liver (positive control) from the rate of formation of citrulline during the reaction of L-ornithine and carbamoyl phosphate (CP) to form citrulline and phosphate (Brown and Cohen, 1959). The citrulline concentration was measured (Boyde and Rahmatullah, 1980) after 30 min incubation of an assay mixture (total volume 1 ml) containing 50 μg homogenate protein in 50 mM glycl-glycine buffer, 1 mM ornithine and 1 mM freshly prepared CP. Assays were carried out at pH 8.3 with 0 to 10 mM ornithine and 1 mM CP or 0 to 10 mM CP and 1 mM ornithine. Assays were also carried out at pH 6–9 on homogenates of L3 *H. contortus* and *T. circumcincta*.

2.3.2. Argininosuccinate synthetase (ASS) (E.C. 6.3.4.5)

ASS activity was determined in homogenates of L3 and adult *T. circumcincta* and *H. contortus* from the decrease in citrulline concentration during the reaction of citrulline and aspartate to form argininosuccinate (Mohamed et al., 2005). The citrulline concentration was measured (Boyde and Rahmatullah, 1980) after 30 min incubation of an assay mixture (total volume 1 ml) containing 50 mM KH_2PO_4, 1 mM citrulline, 1 mM aspartate, 500 μM ATP, 1 mM $MgSO_4$ and 50 μg homogenate protein. Assays were also carried out at pH 7 with citrulline concentrations from 1 to 10 mM. Assays were also carried out at pH 6–9 on homogenates of L3 *H. contortus* and *T. circumcincta*.

2.3.3. Argininosuccinate lyase (ASL) (E.C. 4.3.2.1)

ASL activity was determined in homogenates of L3 and adult *T. circumcincta* and *H. contortus* and sheep liver (positive control) in a continuous coupled assay with malate dehydrogenase (MDH) and fumarase (Miura et al., 1987). The rate of conversion of $NADP^+$ to NADPH was monitored spectrophotometrically at 340 nm using an Ultraspec III (Pharmacia LKB) equipped with a temperature control unit. The reaction mixture (total volume of 1 ml) consisted of 50 μg homogenate protein in 50 mM Tris–HCl buffer, pH 7.3, 2 μM $MnSO_4$, 7 nM 2-mercaptoethanol, 50 nM EDTA, 1 U fumarase, 1 U MDH and 2 μM $NADP^+$. After pre-incubation for 5 min at 30 °C, 0–10 mM argininosuccinate was added to start the reaction. Assays were also carried out at pH 6–9 with 5 mM argininosuccinate on homogenates of L3 *H. contortus* and *T. circumcincta*.

2.3.4. Arginase (E.C. 3.5.3.1)

Arginase activity was measured from the rate of formation of L-ornithine from L-arginine. The assay mixture of 50 μg homogenate protein and 5 mM arginine in 50 mM HEPES buffer, pH 8.5 (total volume 1 ml) was incubated for 15 min, followed by the colorimetric determination of ornithine concentration (Mia and Koger, 1978).

(1) The optimum pH was determined for homogenates of sheathed L3 *H. contortus* and *T. circumcincta* using 50 mM HEPES buffer, pH 6.5–9.5. The optimum temperature was determined for homogenates of L3 *H. contortus* and *T. circumcincta* (pH 8.5) at 25, 30 or 37 °C. Subsequently, kinetic measurements were made at 30 °C.
(2) The apparent K_m for arginine and V_{max} were determined at pH 8.5 with 0 to 25 mM L-arginine in homogenates of L3 ($n = 3$) and adult *T. circumcincta* and *H. contortus*.
(3) The effects of the addition of 0.1 mM $MnCl_2$, $MgCl_2$, $ZnCl_2$, $CuSO_4$ or $FeSO_4$, 1 mM $MnCl_2$ or 50 mM creatine, valine or agmatine were determined for homogenates of L3 *H. contortus* and *T. circumcincta*.
(4) The effects of the addition of 20, 40 or 70 mM ornithine or putrescine were determined for homogenates of L3 *H. contortus* and *T. circumcincta*.
(5) Both products of arginase activity (ornithine and urea) were measured after assay of homogenates of L3 *T. circumcincta* and *H. contortus*. Urea was measured by the method of Geyer and Dabich (1971).

2.3.5. Arginine deiminase (ADI) (E.C. 3.5.3.6)

ADI activity was determined in homogenates of L3 and adult ($n = 1$) *T. circumcincta* and *H. contortus* from the rate of citrulline production in the reaction of L-arginine to form citrulline and NH_3. After the reaction mixture (total volume 1 ml), consisting of 50 μg homogenate protein, 1 μM L-arginine and 40 μM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6, had been incubated for 30 min, citrulline was measured by the method of Boyde and

Rahmatullah (1980). The arginine concentration was varied from 0 to 10 mM in 40 mM buffer. Assays were also carried at pH 5–8 on homogenates of L3 *H. contortus* and *T. circumcincta*.

2.3.6. Citrullinase (E.C. 3.5.1.20)

Citrullinase activity was determined in homogenates of L3 and adult *T. circumcincta* and *H. contortus* from the rate of ornithine formation from citrulline (Hill and Chambers, 1967). The assay mixture (total volume 1 ml) contained 0–10 mM L-citrulline, 20 mM potassium phosphate buffer, pH 6.8, and 50 μg homogenate protein. After 15–20 min incubation, the reaction was terminated by the addition of 0.1 ml 2 M H_2SO_4 and the ornithine concentration estimated by the method of Mia and Koger (1978). Separate reactions were conducted using 4 μM L-citrulline and added Mg^{2+}, ATP or both (0.1–1 mM). Assays were also carried on homogenates of L3 *H. contortus* and *T. circumcincta* with 4 μM citrulline in 20 mM phosphate buffer, pH 5–9.

2.3.7. Arginine decarboxylase (ADC) (E.C. 4.1.1.19)

ADC activity was determined in homogenates of L3 or adult *T. circumcincta* and *H. contortus* from the $^{14}CO_2$ released from L-[1-^{14}C] arginine (Regunathan and Reis, 2000), according to the protocol for decarboxylase assays of Umair et al. (2011). The apparent K_m for arginine and V_{max} were determined in an assay mixture containing 50 mM Tris–HCl, pH 6, 0.2 mM pyridoxal 5′-phosphate (PLP), 0.2 mM EDTA, 0.1 mM DTT, 1 mM $MgSO_4$, 0–20 mM arginine, 0.5 μCi L-[1-^{14}C] arginine and 50 μg homogenate protein was placed in the outer tube. The inner tube held a strip of filter paper dipped in 1 mM KOH to absorb the $^{14}CO_2$. After 20 min, the reaction was terminated by the addition of 0.1 ml 40% trichloroacetic acid (TCA) and the radioactivity counted in a scintillation counter (Wallac 1450 Microbeta Trilux, UK). Assays were also performed at pH 4–9 with 0.2 mM arginine using homogenates of L3 *H. contortus* and *T. circumcincta*. The optimal concentration of PLP was determined at pH 6 with 0.2 mM arginine and 0.01–0.5 mM PLP. In a preliminary experiment, the effect of 2 mM DFMO was determined on the homogenate of L3 *H. contortus* and *T. circumcincta* at pH 6 with 0.2 mM arginine and 0.2 mM PLP.

2.3.8. Ornithine decarboxylase (ODC) (E.C. 4.1.1.17)

ODC activity was determined in homogenates of L3 and adult *T. circumcincta* and *H. contortus* from the $^{14}CO_2$ released from L-[1-^{14}C] ornithine, using the protocol for ADC (Section 2.3.8) and the same reagents with ornithine instead of arginine. The apparent K_m ornithine and V_{max} were determined at pH 8 in assay mixtures containing 0.2 mM PLP with ornithine concentrations 0–20 mM. The effect of 2 or 5 mM DFMO was determined at pH 8 with 2 mM ornithine and 0.2 mM PLP. Assays were also carried at pH 4–9 with 2 mM ornithine, using homogenates of sheathed L3 *H. contortus* and *T. circumcincta*. The optimal concentration of PLP was also determined at pH 8 with 2 mM ornithine and 0.01–0.5 mM PLP.

2.3.9. Agmatinase (E.C. 3.5.3.11)

Agmatinase activity was determined from the rate of urea formation from agmatine. The optimum pH was determined in homogenates of sheathed L3 *T. circumcincta* and *H. contortus* using a reaction mixture (total volume 1 ml) containing 1 mM agmatine and 50 μg homogenate protein in 50 mM HEPES buffer, pH 6.5–9.5. After incubation for 15 min, the reaction was stopped by adding 5% TCA and the urea formed was measured according to Geyer and Dabich (1971). The apparent K_m for agmatine and V_{max} were determined in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta* at pH 8.5 for *H. contortus* and pH 9 for *T. circumcincta* with 0–20 mM agmatine. The effects of the addition of 20, 40 or 70 mM ornithine or putrescine were determined for homogenates of L3 *H. contortus* and *T. circumcincta*.

2.3.10. Nitric-oxide synthase (NOS) (E.C. 1.14.13.39)

NOS activity was determined in homogenates of sheathed L3 and adult *T. circumcincta* and *H. contortus* by monitoring spectrophotometrically at 576 nm the rate that the NO generated converted oxyhaemoglobin to methaemoglobin (Bascal et al., 2001). The assay mixture (total volume 1 ml) contained 50 mM Tris–HCl, pH 7.4, 0–2 mM arginine, 100 U catalase, 200 U superoxide dismutase, 10 μM freshly prepared oxyhaemoglobin (Murphy and Noack, 1994), 0.1 mM $CaCl_2$, 20 μg calmodulin and 50 μg homogenate protein. The reaction was started by the addition of 0.2 mM NADPH. Assays were also carried out at pH 7.4 with and 100 μM arginine, varying oxyhaemoglobin from 0 to 100 μM. In addition, 20 μM calmodulin, $CaCl_2$, FAD, FMN or BH_4 were added singly or in various combinations to the assay mixture. Assays were also carried out at pH 6–9 on homogenates of L3 *H. contortus* and *T. circumcincta*.

The generation of citrulline by NOS in the assay mixture was monitored using thin layer chromatography (TLC) as performed by Zolg and Ottow (1973). Briefly, 1 μl reaction mixture and standards (1 mM citrulline and arginine) were placed on cellulose coated TLC plates (Merck, Germany) cut to 6 × 9 cm × 0.1 mm. The plates were dipped in 20 μl of a mixture of 1-butenol, acetone, diethylamine and water (10:10:2:5). After 2 h, the plates were dried, sprayed with ninhydrin solution (1% in iso-propanol) and dried. The standards and samples appeared as purple spots.

2.4. Histochemistry

NADPH diaphorase (NADPH-d) histochemical staining (Gustafsson et al., 1998) was performed on sections of adult *H. contortus*. Adult worms were fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate buffered saline (PBS), pH 7.4, overnight at 4 °C. The worms were transferred to a solution containing 10% sucrose in PBS at 4 °C for 3–4 days, then embedded in Tissue-tek and frozen. Sections 20 μm thick were cut on a Bright cryostat, collected on gelatine-coated slides, dried at room temperature and stored at −80 °C until staining. A stock solution of incubation medium was prepared by mixing 25 mg Nitro blue tetrazolium in 6.5 ml water, 6.5 ml of 40 mM Tris–HCl, 2.5 ml $MnCl_2$ and 7.5 ml water and the pH was adjusted to 7.4. The solution was stored at −20 °C until used. The incubation mixture contained 9 ml stock solution, 1 ml water and 20 mg NADPH (pH 7.4). Incubations were performed at 37 °C for 1 h. After incubation, the slides were rinsed with 0.2% triton in 10 mM phosphate buffer thrice for 10 min and then with water for 10 min, mounted in 50% glycerol and stored at 4 °C. For the negative controls, NADPH was replaced with NAD^+.

2.5. Data analysis

Replicate data are presented as mean ± SEM. Graphpad Prism v5 was used to plot kinetic data and estimate K_m and V_{max}. One-way ANOVA with Bonferroni post-tests were used to determine the effects of inhibitors on enzyme activity.

3. Results

3.1. Ornithine urea cycle (OUC) enzymes

OTC, ASS and ASL activities were not detected in homogenates of either adult or L3 *T. circumcincta* or *H. contortus*, whereas the activities of OTC and ASL in sheep liver, the positive control, were

30 ± 6 and 24 ± 4 nmol min^{-1} mg^{-1} protein, respectively. All assays on worm homogenates were carried out over the pH range 6–9 and with substrate concentrations from 0 to 10 mM. Arginase activity is reported in Section 3.4.

3.2. Nitric oxide synthase (NOS)

NOS activity was undetectable in homogenates of adult or L3 *T. circumcincta* or *H. contortus* in assays over the pH range 6–9, with concentrations of oxyhaemoglobin from 0 to 100 μM or with the addition of calmodulin, $CaCl_2$, FAD, FMN or BH_4 singly or in combination. Citrulline, the other product of NOS, could not be detected in the reaction mixtures by TLC.

NADPH-diaphorase (nNOS) activity was detected in the nerve ring and lateral nerve cords in adult *H. contortus* (Fig. 2), but no stained nerves were seen when NADPH was replaced with NAD^+ in the negative controls.

3.3. Arginine deiminase/dihydrolase pathway

No ADI activity was detected in homogenates of either adult or L3 *T. circumcincta* or *H. contortus* in assays over the pH range 5–8 and with substrate concentrations from 0 to 10 mM, nor was citrullinase activity detected in assays over the pH range 5–9, with substrate concentrations from 0 to 10 mM or with added Mg^{2+} and ATP singly or in combination.

3.4. Arginase

Enzyme activities and kinetic properties of arginase in homogenates of L3 and adult *H. contortus* and *T. circumcincta* are shown in Table 1. The properties of the enzyme were generally similar in the two species: activities were similar for life-cycle stages of the two species, but higher in adult worms, while the apparent K_m for arginine for the *T. circumcincta* enzyme was a little higher than for *H. contortus*. The optimum pH for arginase activity in both L3 *T. circumcincta* and *H. contortus* was 8.5 (Fig. 3A) and the temperature optimum for enzymes of both species was 30 °C (Supplementary Fig. 1). Where both products of the reaction were measured, urea and ornithine were generated in nearly equimolar concentrations.

The effects of the addition of bivalent ions and amino acids are shown in Supplementary Table 1. Addition of 0.1 or 1 mM $MnCl_2$ increased arginase activity by 25 ± 5 and 20 ± 10% for L3 *H. contortus* and by 5 ± 3 and 10 ± 2% for L3 *T. circumcincta*, respectively. Whereas $MgCl_2$ was slightly stimulatory in *H. contortus*, $ZnCl_2$, $CuSO_4$ and $FeSO_4$ inhibited activity from 25% to 40% in both species. Addition of amino acids decreased the enzyme activity by approximately 10%. Added ornithine significantly reduced enzyme activity in L3 of both species by approximately 70% at 70 mM ($p < 0.001$) and also at lower concentrations (Fig. 3C), whereas putrescine reduced enzyme activity by a maximum of 15% at 70 mM ($p < 0.001$) (Fig. 3D) (Supplementary Table 2).

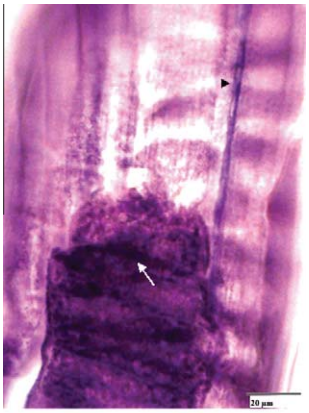

Fig. 2. Histochemical staining for NADPH-diaphorase (nitric oxide synthase) in a section of the anterior region of an adult *H. contortus*. White arrow: nerve ring; arrow head: lateral nerve cord. Scale bar: 20 μm.

3.5. Agmatinase

Agmatinase activities and apparent K_m in sheathed L3 and adult *H. contortus* and *T. circumcincta* are presented in Table 1. Activities were similar in L3 and adult worms of both species, but higher in adults than L3, particularly for *H. contortus*. The optimum pH for agmatinase activity was pH 9 in L3 *T. circumcincta* and pH 8.5 in *H. contortus* (Fig. 3B), with marked loss of activity above pH 9. The addition of ornithine inhibited the L3 enzyme activity by up to 75% ($p < 0.001$) in a concentration-dependent manner, similar to its effect on arginase (Section 3.4.), compared with only 20% by 70 mM putrescine ($p < 0.001$) (Fig. 3C and D) (Supplementary Table 2).

3.6. Arginine decarboxlyase (ADC)

ADC activity was present in both sheathed L3 and adult *H. contortus* (Table 1), but was not detected in either L3 or adult *T. circumcincta*. The optimum pH and [PLP] for L3 *H. contortus* ADC were pH 6 and 0.2 mM, at which the enzyme activities were 0.4 ± 0.01 and 0.5 ± 0.05 nmol min^{-1} mg^{-1} protein, respectively. Enzyme activity declined above 3–4 mM arginine concentration (Fig. 4). Addition of DFMO did not inhibit ADC activity in L3 *H. contortus* or *T. circumcincta*.

3.7. Ornithine decarboxylase (ODC)

ODC activities and apparent K_m in sheathed L3 and adult *H. contortus* and *T. circumcincta* are presented in Table 1. Activities were similar in L3 and adult worms of both species, but higher in adults than L3. The optimum pH and [PLP] for ODC in L3 of both species were pH 8 and 0.2 mM, respectively, with enzyme activities of 0.4–0.5 nmol min^{-1} mg^{-1} protein. Addition of DFMO significantly inhibited ODC activity by 55–65% at 2 mM ($p < 0.001$) and by 80% at 5 mM ($p < 0.001$) (Fig. 5; Supplementary Table 2).

4. Discussion

4.1. Ornithine urea cycle

No enzymes of the OUC except arginase were detected in homogenates of sheathed L3 and adult *H. contortus* or *T. circumcincta*, even when the amount of homogenate protein was increased to 250 μg. The assays had estimated sensitivities of about 1 nmol min^{-1} mg^{-1} protein (ASS and OTC) and 15 nmol min^{-1} mg^{-1} protein (ASL) and positive controls confirmed the validity of the OTC and ASL assays. Although all OUC enzymes were detected in *P. redivivus* (Wright, 1975), *D. lanceatum* (Rijavec and Kurelec, 1965) and *F. hepatica* (Rijavec and Kurelec, 1965; Mohamed et al., 2005), it is believed that helminths generally lack a fully functional urea cycle (Janssens and Bryant, 1969). The nitrogenous excretory products of free living and parasitic nematodes are approximately 80% ammonia and 20% urea (Rogers, 1952; Wright, 1975), which could be provided by arginase and/or agmatinase activities.

Table 1
Apparent K_m (mM) and V_{max} (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$ for adult worms, $n = 3$ for L3) of arginase, agmatinase, arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activities in L3 and adult *H. contortus* and *T. circumcincta*.

	Arginase		Agmatinase		ADC		ODC	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
L3 *H. contortus*	1.2 ± 0.2	33 ± 9	1.3 ± 0.1	21 ± 3	0.5 ± 0.05	0.4 ± 0.1	0.15 ± 0.05	0.6 ± 0.05
Adult *H. contortus*	1.5 ± 0.2	84 ± 5	2.1 ± 0.5	61 ± 6	0.8 ± 0.1	0.7 ± 0.06	0.35 ± 0.05	1.1 ± 0.1
L3 *T. circumcincta*	0.8 ± 0.1	30 ± 8	1.8 ± 0.1	19 ± 3	ND	ND	0.2 ± 0.05	0.6 ± 0.05
Adult *T. circumcincta*	0.8 ± 0.1	85 ± 4	2 ± 0.2	36 ± 5	ND	ND	0.25 ± 0.05	1 ± 0.5

ND, not detectable.

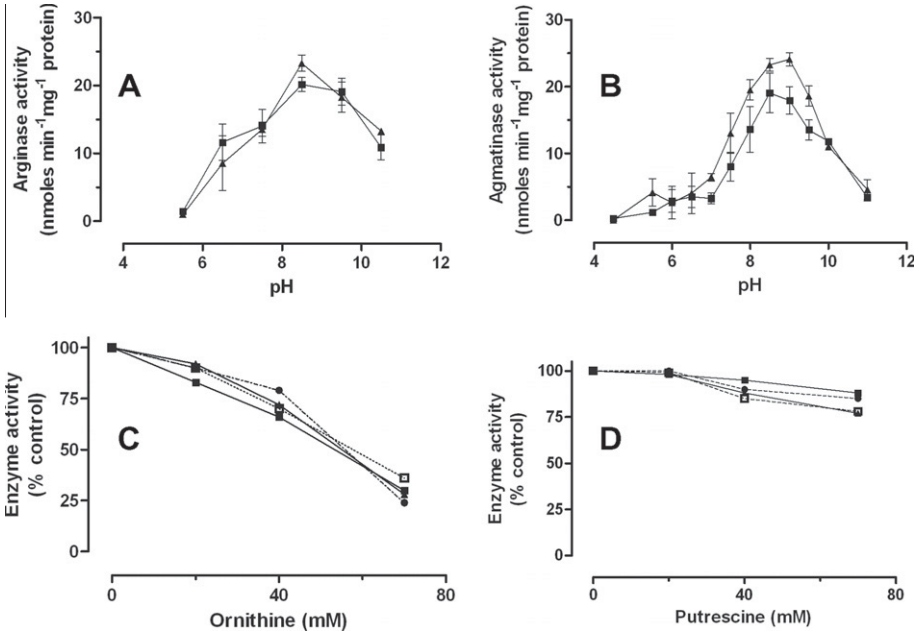

Fig. 3. Effect of pH on arginase (A) and agmatinase (B) activities and of ornithine (C) and putrescine (D) on arginase and agmatinase activities (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) at 30 °C in homogenates of sheathed L3 *H. contortus* and *T. circumcincta*. (A and B) ■: L3 *H. contortus*; ▲: L3 *T. circumcincta*. (C and D) ●: L3 *H. contortus* arginase; ■: L3 *T. circumcincta* arginase; ▲: L3 *H. contortus* agmatinase; □: L3 *T. circumcincta* agmatinase.

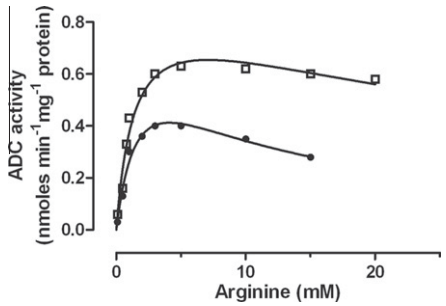

Fig. 4. Effect of arginine concentration on arginine decarboxylase (ADC) activities (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) at 30 °C in homogenates of sheathed L3 and adult *H. contortus* ●: L3 *H. contortus*; □: adult *H. contortus*.

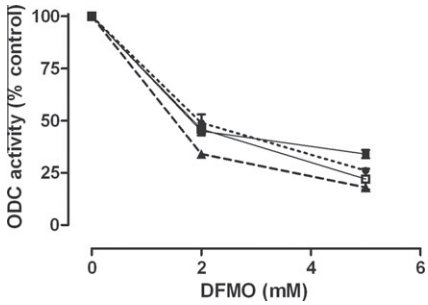

Fig. 5. Effect of Difluoromethylornithine (DFMO) on ornithine decarboxylase (ODC) activities (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) at 30 °C in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta*. ●: L3 *H. contortus*; ■: adult *H. contortus*; ▲: L3 *T. circumcincta*; □: adult *T. circumcincta*.

4.2. Nitric oxide synthase (NOS)

Although NOS activity was demonstrated in the central and peripheral nerves of adult *H. contortus* (Fig 2.), enzyme activity was undetectable in homogenates of whole adult or L3 *T. circumcincta* or *H. contortus*. Assay sensitivity was about 15 nmol min^{-1} mg^{-1} protein. A similar histochemical technique has been used to demonstrate neuronal NOS activity in cestodes and trematodes (Gustafsson et al., 1996, 1998). NOS is one of the NADPH-diaphorases (Hope et al., 1991) and is used as a marker for nNOS in the nervous system of many organisms (Dawson et al., 1991). As only some nerves stained for NOS activity, the restricted distribution of the enzyme in abomasal parasitic nematodes would account for failure to detect enzyme activity biochemically in whole worm homogenates. NOS activity is generally many-fold lower than arginase activity, with which it competes for the common substrate arginine (Iyengar et al., 1987).

4.3. Arginine deiminase/dihydrolase pathway

There was no evidence of either the arginine deiminase or dihydrolase pathways, which are used by non-mammalian organisms, including protozoan parasites, to generate ATP, ornithine, ammonia and CO_2 (Zuniga et al., 2002). The more common ADI pathway consists of three steps, whereas in the dihydrolase pathway, citrullinase replaces the last two enzymes (Fig. 1). Assays had sensitivities of about 1 nmol min^{-1} mg^{-1} protein. Neither ADI nor citrullinase activities were detected in L3 or adult parasites. Janssens and Bryant (1969) reported the generation of labelled citrulline from arginine in cestodes, possibly via the OUC, rather than from these non-mammalian pathways.

4.4. Polyamine synthesis

Generation of putrescine from arginine involved both the ODC and ADC pathways in *H. contortus*, whereas in both L3 and adult *T. circumcincta* only ODC activity was detectable. In the mammalian host, the ODC pathway predominates and even the existence of ADC has been challenged (Coleman et al., 2004). The presence of an alternative route for putrescine synthesis may make it more difficult to exploit these enzymes to control some nematode parasites.

4.4.1. Arginase

The main role for arginase activity in abomasal nematodes may be in synthesising ornithine for polyamine synthesis, rather than in generating either urea or NO. Arginase activity was present in both L3 and adult worms of both species, as in many, but not all, helminths (Rogers, 1952; Grantham and Barrett, 1986; Mohamed et al., 2005). Enzyme activity was approximately 2.5 times higher in the homogenates of adult *H. contortus* or *T. circumcincta* than in the homogenates of L3, although activities were very consistent within the life-cycle stages (Table 1). Arginase activity was confirmed by the generation of equimolar concentrations of the two products, ornithine and urea. There was no evidence of removal of urea by urease activity, consistent with biochemical assays in *T. circumcincta* homogenates (Muhamad, 2006). The affinity for arginine was similar within species, but nearly twice as great for the enzymes in *T. circumcincta* than in *H. contortus*.

Nematode arginase did not show a requirement for added Mn^{2+} or any other bivalent metal ion for its activity, in contrast to rat liver arginase (Kuhn et al., 1991). Activity was only slightly increased by the addition of 1 mM Mn^{2+}, probably because of tight binding of the cofactor Mn^{2+} to the enzyme. Other divalent cations tested were inhibitory to enzymes of both species of nematode, as in *F. hepatica* (Mohamed et al., 2005). An interesting feature of arginase activity was the unusual optimum pH. Most arginases, including the *Schistosoma mansoni* (Fitzpatrick et al., 2009) and *F. gigantica* enzymes, are most active at pH 9–10, whereas the optimal pH for the arginases of *H. contortus* and *T. circumcincta* were 8.5 (Fig. 3). There was a similar pH optimum (pH 8) for *T. circumcincta* arginase in a continuous coupled assay (Muhamad, 2006). Parasitic stages are exposed to a low pH in abomasal contents, which may explain the preference for a lower pH than most organisms. The human gastric pathogen *Helicobacter pylori*, which lives in a similar environment, has an exceptionally low optimal pH for arginase of 6.1 (McGee et al., 2004).

4.4.2. Agmatinase

Agmatinase activities and substrate affinities were lower than for arginase activities (Table 1). The two L3 enzymes had similar pH optima, pH 9 for agmatinase in L3 *T. circumcincta* and pH 8.5 in *H. contortus* (Fig. 3B); there was marked loss of activity above the optimum pH. Although agmatinase is now recognised as a neuronal enzyme in mammals (Li et al., 1994, 1995; Feng et al., 1997), it is more universally present in non-mammalian tissues (Tabor and Tabor, 1984). Arginase and agmatinase belong to the same enzyme family and are capable of using either arginine or agmatine as substrate (Ahn et al., 2004). It is unclear whether arginase is responsible for the *T. circumcincta* and *H. contortus* agmatinase activity or whether two enzymes are present. Both enzyme activities were similarly inhibited by ornithine (approximately 70%) and putrescine (15–20%) (Fig. 3C and D), suggesting a single enzyme was responsible. There may be two enzymes in *H. contortus*, but a single enzyme in *T. circumcincta*, in which there appeared to be no ADC activity (Table 1), the most likely source of the substrate agmatine. Sequencing the genes encoding these enzymes may help in resolving this issue.

4.4.3. ADC and ODC

An unusual feature of polyamine synthesis in abomasal nematodes was the demonstration of ADC activity in addition to ODC in *H. contortus* but not in *T. circumcincta* (Table 1). Polyamines are universally synthesised from ornithine by ODC (Pegg, 1986), although in microorganisms and plants, in addition to the ODC pathway, putrescine is formed from agmatine (Slocum et al., 1984) (Fig. 1). The situation in mammals is still unclear. ADC has been proposed to be a separate enzyme from ODC with distinct properties (Morrissey et al., 1995; Regunathan and Reis, 2000), but this is not considered adequate evidence by others (Gilad et al., 1996; Coleman et al., 2004).

A surprising finding was the lack of ADC in both L3 and adult *T. circumcincta*, yet the presence of agmatinase activity, since the only likely source of agmatine is arginine, requiring ADC activity. As discussed previously, this may reflect the ability of *T. circumcincta* arginase to use either arginine or agmatine as substrate, rather than a specific agmatinase being present. There may, however, be two separate enzymes in *H. contortus* and a single enzyme in *T. circumcincta*.

The current study supports the presence of separate ADC and ODC enzymes in L3 and adult *H. contortus*. First, ODC activities and substrate affinity for ornithine were similar in homogenates of L3 of both worms and similar in both adult preparations (Table 1), yet arginine was metabolised to agmatine by only one parasite species. Second, DFMO, an established inhibitor of nematode ODC (Schaeffer and Donatelli, 1990; Klein et al., 1997), inhibited ODC activity by up to 80% (Fig. 5); in contrast, a preliminary experiment showed no effect of DFMO on ADC activity in either L3 *H. contortus* or *T. circumcincta*. Third, the pH optima for the two enzymes differed markedly: pH 6 for ADC and pH 8 for ODC. Fourth, L3 *H. contortus* ADC showed declining activity at high substrate concentrations (Fig. 4), but this was not the case for ODC at high ornithine concentrations. Considered together, the most likely conclusion is that there are separate ODC and ADC enzymes in *H. contortus*.

5. Conclusions

L3 and adult *H. contortus* and *T. circumcincta* showed both similarities and significant differences in arginine metabolism. Neither parasite had a full functional OUC, nor appeared to use bacterial pathways to covert arginine to ornithine. There was no evidence of gene sequences or ESTs for genes encoding the undetectable enzymes in public databases. Both nematode species contained nNOS activity in central and peripheral nerves. Polyamine metabolism showed the greatest species variation and also demonstrated differences from that in mammals. The flexibility in synthesis of putrescine in *H. contortus* may make it less useful as a target for parasite control than in *T. circumcincta*, in which only the ODC pathway was detected.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2010.10.021.

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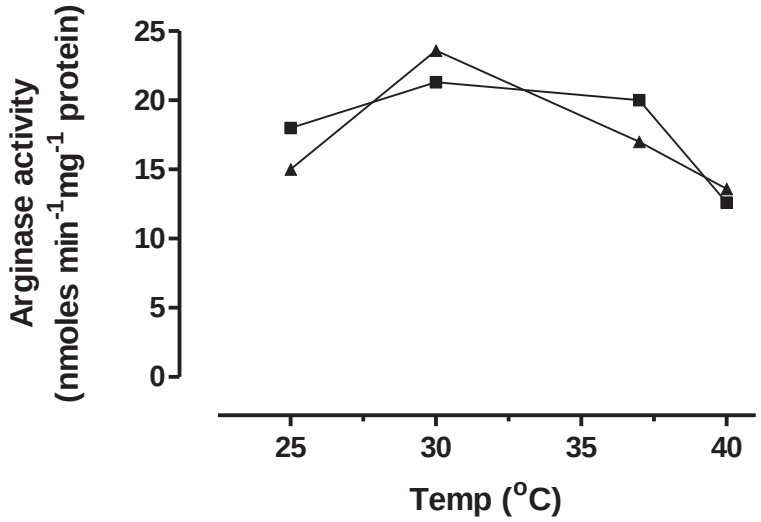

Supplementary Figure 1. Effect of temperature on arginase activity in homogenates of L3 *H. contortus* and *T. circumcincta*. Enzyme activity was calculated from the increase in the concentration of ornithine. ■: L3 *T. circumcincta:* ▲: L3 *H. contortus.*

Supplementary Table 1. The effects of addition of bivalent ions and amino acids on arginase activity at 30 °C and pH 8.5 in homogenates (*n* = 2) of L3 *H. contortus* and *T. circumcincta*. Activities are expressed as percentages of the control assay with no additives, taken as 100%.

Effectors and Inhibitors	Concentration (mM)	Activity (%) (*H. contortus*)	Activity (%) (*T. circumcincta*)
Control	-	100	100
Mn^{2+}	0.1	125 ± 5	105 ± 3
Mn^{2+}	1.0	120 ± 10	110 ± 2
Mg^{2+}	0.1	110 ± 2	101+ 5
Cu^{2+}	0.1	88 ± 3	90 ± 3
Fe^{2+}	0.1	66 ± 12	59 ± 11
Zn^{2+}	0.1	75 ± 9	74 ± 3
Creatine	50	89 ± 5	80 ± 3
Valine	50	98 ± 2	90 ± 9
Agmatine	50	92 ± 1	85 ± 1

Supplementary Table 2. Effects of added ornithine and putrescine on arginase and agmatinase activities in homogenates of sheathed L3 and effects of DFMO on ornithine decarboxylase activity of sheathed L3 and adult *H. contortus* and *T. circumcincta* (mean ± SEM, *n*= 2). All values are expressed as % control (100%).

	Ornithine			
	0 mM	20 mM	40 mM	70 mM
H. contortus arginase	100	93 ± 3	83 ± 2*	27 ± 3***
T. circumcincta arginase	100	81 ± 1.5***	65 ± 1***	31 ± 1***
H. contortus agmatinase	100	87 ± 4*	74 ± 2**	29 ±1***
T. circumcincta agmatinase	100	88 ± 3	67 ± 3**	33 ± 3***
	Putrescine			
	0 mM	20 mM	40 mM	70 mM
H. contortus arginase	100	99 ± 1	93 ± 2*	85**
T. circumcincta arginase	100	99 ± 1	95	87 ± 1.5**
H. contortus agmatinase	100	100 ± 1	91 ± 3	79 ± 1**
T. circumcincta agmatinase	100	99 ± 1	86 ± 1**	79 ± 1**
		DFMO		
		0 mM	2 mM	5 mM
L3 *H. contortus*		100	49 ± 4**	26 ± 1***
Adult *H. contortus*		100	45 ± 2***	35 ± 2.5***
L3 *T. circumcincta*		100	34 ± 1.5***	18 ± 2***
Adult *T. circumcincta*		100	46 ± 1**	22 ± 5***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Chapter VII

Lysine catabolism in *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair, R.J. Bland and H.V. Simpson

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Lysine catabolism in *Haemonchus contortus* and *Teladorsagia circumcincta*

S. Umair [a], R.J. Bland [b], H.V. Simpson [a,*]

[a] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] Hopkirk Research Institute, AgResearch Ltd., Private Bag 11-008, Palmerston North, New Zealand

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ABSTRACT

Catabolism of lysine through the pipecolate, saccharopine and cadaverine pathways has been investigated in L3 and adult *Haemonchus contortus* and *Teladorsagia circumcincta*. Both enzymes of the saccharopine pathway (lysine ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH)) were active in L3 and adult worms of both species. All three enzymes which catabolise lysine to α-amino adipic semialdehyde via pipecolate (lysine oxidase (LO), Δ^1-piperideine-2-carboxylate reductase (Pip2CR) and pipecolate oxidase (PipO)) were present in adult worms, whereas the pathway was incomplete in L3 of both species; Pip2CR activity was not detected in the L3 of either parasite species. In adult worms, the saccharopine pathway would probably be favoured over the pipecolate pathway as the K_m for lysine was lower for LKR than for LO. Neither lysine dehydrogenase nor lysine decarboxylase activity was detected in the two parasite species. Enzyme activities and substrate affinities were higher for all five enzymes in adult worms than in L3. An unexpected finding was that both LKR and SDH were dual co-factor enzymes and not specific for either NAD^+ or $NADP^+$, as is the case in other organisms. This novel property of LKR/SDH suggests it could be a good candidate for anthelmintic targeting.

1. Introduction

Metabolic enzymes which are essential for worm survival are potential anthelmintic targets for parasitic nematodes, such as the sheep abomasal parasites *Teladorsagia circumcincta* and *Haemonchus contortus*, especially if these enzymes are absent from the host or have unusual properties or significant structural differences in the protein. Lysine is the site in many nuclear and cytoplasmic proteins where reversible acetylation plays an important role in the regulation of cellular functions in both eukaryotes (Choudhary et al., 2009; Zhao et al., 2010) and prokaryotes (Zhang et al., 2009; Wang et al., 2010). This posttranslational modification regulates the activity of many proteins, including the nuclear histones, metabolic enzymes and cell differentiation and survival. Lysine is an essential amino acid in mammals (Murthy and Janardanasarma, 1999), with only catabolic pathways present, whereas in plants and microorganisms, lysine can be both synthesised from aspartate and catabolised through several pathways depending on the species (Galili, 2002). Lysine metabolism has been studied in detail in plants (Azevedo and Lea, 2001) and vertebrates (Dodt et al., 2000; Ijlst et al., 2000; Goyer et al., 2004), but there appears to be no published information on lysine metabolism in nematodes.

The two universal pathways of lysine catabolism are shown in bold in Fig. 1. The most common and well studied route of lysine catabolism in plants and animals is the saccharopine pathway, which converts lysine via saccharopine to α-amino adipic semialdehyde (α-AAA) and glutamate (Galili, 1995) (Fig. 1). This pathway is almost always comprised of one bifunctional enzyme (Gaziola et al., 1997) with both lysine-ketoglutarate reductase (LKR) activity, that catalyses the conversion of lysine and 2-oxoglutarate (2-OG) to saccharopine, and saccharopine dehydrogenase (SDH) activity, which converts saccharopine to α-AAA and glutamate (Galili et al., 2001). Nematode gene sequences deposited in databases are consistent with a bifunctional LKR–SDH enzyme. In yeasts, there are separate enzymes with reversible activity, allowing lysine synthesis (Xu et al., 2007). In plants and vertebrates, LKR activity of the bifunctional enzyme (E.C. 1.5.1.8) strictly uses NADPH as co-factor, while the SDH activity (E.C. 1.5.1.9) requires NAD^+ or $NADP^+$, but activity is usually much greater with the former (Tang et al., 1997). The gene for LKR–SDH is most highly expressed in the human liver and less so in most other organs (Sacksteder et al., 2000).

The alternative pathway of catabolism of lysine to glutamate and α-AAA is via pipecolate. In higher organisms, it is comprised of three enzymes: lysine oxidase (LO) (E.C. 1.4.3.14) catalyses the conversion of lysine to 6-amino-2-oxohexanoate, which spontaneously converts to Δ^1-piperideine-2-carboxylate (Pip2C);

* Corresponding author. Address: Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand. Fax: +64 6 350 5636.
E-mail address: H.V.Simpson@massey.ac.nz (H.V. Simpson).

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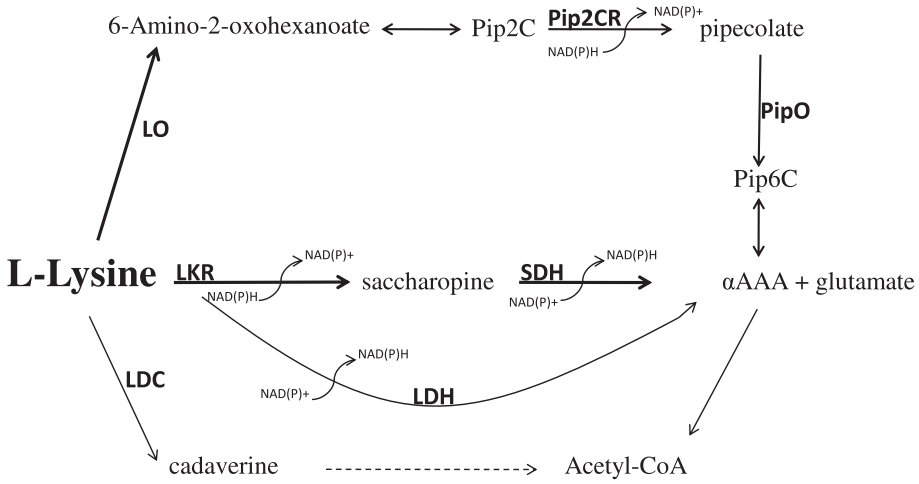

Fig. 1. Lysine catabolism through the pipecolate, saccharopine and cadaverine pathways. Bold arrows indicate universal pathways of lysine catabolism in higher organisms. *Abbreviations used:* α-AAA, α-amino adipic semialdehyde; LDC, lysine decarboxylase; LDH, lysine dehydrogenase; LKR, lysine ketoglutarate reductase; LO, lysine oxidase; Pip2C, Δ^1-piperideine-2-carboxylate; Pip2CR, Δ^1-piperideine-2-carboxylate reductase; Pip6C, Δ^1-piperideine-6-carboxylate; PipO, pipecolate oxidase; SDH, saccharopine dehydrogenase.

Δ^1-piperideine-2-carboxylate reductase (Pip2CR) (E.C. 1.5.1.1) catalyses the conversion of Pip2C to pipecolate; this is finally converted to aminoadipate 6-semialdehyde by pipecolate oxidase (PipO) (E.C. 1.5.3.7) (Fig. 1). In animal tissues, L-lysine can be metabolised either through the pipecolate or saccharopine pathways (Broquist, 1991). Lysine catabolism via pipecolate is required in the brain to prevent neurological disorders (Broquist, 1991; Galili, 2002) because of low LKR–SDH activity (Chang and Adams, 1976; Chang et al., 1981). The saccharopine pathway is generally important in preventing accumulation of lysine, which is toxic in high concentrations (Epelbaum et al., 1997). In plants, lysine synthesis and catabolism via LKR–SDH are regulated to maintain homeostasis during seed formation and osmotic and other environmental stresses (Galili, 2002; Battur et al., 2009).

Microorganisms use additional and/or alternative enzymes to those in the ubiquitous saccharopine and pipecolate pathways, often as part of pathways synthesising pharmacologically useful antimicrobial, anti-tumour and immunosuppressant chemicals (He, 2006). Some bacteria directly convert lysine to α-AAA using lysine dehydrogenase (LDH) (E.C. 1.4.1.15) (Dempsey et al., 1992) or L-lysine cyclodeaminase (Gatto et al., 2006). L-Lysine can be converted to Pip2C, not by LO, but by lysine-2-aminotransferase (L2AT) which oxidises the α-amino group to α-keto-ε-aminocaproic acid and Pip6C (He, 2006). D-Lysine can also be converted to Pip2C by α-transamination with pyruvate (Yonaha et al., 1975). *Pseudomonas* spp. use a different Pip2CR belonging to the malate/lactate dehydrogenase family (Revelles et al., 2007; Muramatsu et al., 2005). In plants and bacteria, lysine is also catabolised by lysine decarboxylase (LDC) to cadaverine (Fecker et al., 1992) which is then converted to acetyl-CoA (Fig. 1). LDC is involved in pH homeostasis in some bacteria when they are exposed to extremely low pH (Park et al., 1996), raising the possibility that LDC could fulfil a similar role in abomasal parasites in the acid environment of the stomach.

In the present study, the kinetic properties of the five enzymes involved in the two universal pathways of L-lysine catabolism via pipecolate and saccharopine have been investigated in homogenates of L3 and adult *H. contortus* and *T. circumcincta*. In addition, the possible presence of the plant and microbial enzymes LDC, which degrades lysine via cadaverine and LDH, which converts lysine to α-AAA has been investigated.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (Mo, USA) unless stated otherwise. Use of experimental animals has been approved by the Massey University Animal Ethics Committee.

2.1. Parasites

L3 *T. circumcincta* and *H. contortus* were cultured from the faeces of sheep infected with pure strains of parasites and stored in reverse osmosis (RO) water at 4 °C for the former and at 10 °C for the latter species. Sheathed larvae were either freshly collected or prior to each experiment were baermannised in RO water to remove inactive worms and re-suspended in assay buffer. Adult worms were recovered from the abomasa of infected sheep as described previously (Simpson et al., 1999). Briefly, abomasal contents were mixed 2:1 with 3% agar and the solidified agar blocks incubated at prior to each experiment L3 37 °C in a saline bath. Clumps of parasites were removed from the saline soon after emergence and placed in assay buffer.

2.2. Preparation of homogenates

Approximately 50,000 L3 of each species were centrifuged at 600*g* for 5 min, washed twice by re-suspending in assay buffer followed by centrifugation, then finally re-suspended in 1 ml buffer. The concentrated L3 suspension or approximately 5 mg of clumped adult worms was transferred to a chilled mortar, frozen at −20 °C for at least 15 min and homogenised with a chilled pestle. When homogenates were to be used for more than one assay, aliquots were kept in Eppendorff tubes on ice until required. The protein concentrations of homogenates were determined by the method of Bradford (1976).

2.3. Enzyme assays

All assays were performed at 30 °C on two homogenates of sheathed L3 and adult *T. circumcincta* and *H. contortus* unless stated otherwise, using 50 μg homogenate protein and 100 mM phosphate buffer in a total volume of 1 ml assay mixture. If no activity

were detected, the amount of homogenate was increased to 250 μg homogenate protein in an attempt to obtain enzyme activity. In no case did this produce detectable activity. All pH studies were performed on homogenates of sheathed L3 *H. contortus* and *T. circumcincta* and assays to determine the K_m and V_{max} on both L3 and adult *H. contortus* and *T. circumcincta* unless stated otherwise. All enzyme activities were expressed as nmol min^{-1} mg^{-1} protein, mean ± SEM). The lysine concentration in the reaction mixture is considered to be the added lysine and does not include any greatly diluted endogenous lysine.

2.3.1. Lysine oxidase (LO) (E.C. 1.4.3.14)

LO activity was determined from the rate of hydrogen peroxide (H_2O_2) formation during the oxidation of lysine. H_2O_2 was reacted with phenol and aminoantipyrine in the presence of peroxidase to form a quinoneimine dye, which was measured spectrophotometrically (Uwajima et al., 1984). After incubation of the assay mixture for 30 min on a shaker, the reaction was terminated by the addition of 1 ml 20% trichloroacetic acid (TCA) and centrifuged. The OD was measured spectrophotometrically at 500 nm (Jenway 6505, Jenway Ltd., U.K.) and the H_2O_2 concentration read off a standard curve.

(1) To determine the optimal pH for LO, the assay mixture contained freshly prepared homogenate in 100 mM phosphate buffer pH 5–9, 50 mM phenol, 0.5 mM aminoantipyrine, 5 mM lysine and 10U horse radish peroxidise (HRP). Subsequent assays were performed at pH 6.
(2) The V_{max} and apparent K_m for lysine were determined in assay mixtures containing 50 mM phenol, 0.5 mM aminoantipyrine, 0–25 mM lysine and 10U HRP.

2.3.2. Δ^1-Piperideine-2-carboxylate reductase (Pip2CR) (E.C. 1.5.1.1)

Pip2CR activity was determined in a continuous assay by monitoring the rate of NADH utilisation spectrophotometrically at 340 nm.

(1) To determine the optimal pH for Pip2CR, the assay mixture contained homogenates of sheathed L3 or adult *H. contortus* or *T. circumcincta* in buffer pH 5–9, 1 mM Pip2C and the reaction was started by the addition of 0.2 mM NADH. Subsequent assays were performed at pH 6.5.
(2) The V_{max} and the apparent km for Pip2C were determined in assay mixtures containing 0–20 mM Pip2C and 0.2 mM NADH.
(3) Enzyme activities with NADPH as co-factor were also determined in assay mixtures containing 1 mM Pip2C and 0.2 mM NADPH.

2.3.3. Pipecolate oxidase (PipO) (E.C. 1.5.3.7)

PipO activity was determined from the rate of H_2O_2 formation (Uwajima et al., 1984) as described in Section 2.3.1.

(1) To determine the optimal pH for PipO, the assay mixture contained homogenate in 100 mM phosphate buffer pH 5–9, 50 mM phenol, 0.5 mM aminoantipyrine, 5 mM pipecolate and 10U HRP. Subsequent assays were performed at pH 7.5.
(2) The V_{max} and the apparent K_m for pipecolate were determined in assay mixtures containing 50 mM phenol, 0.5 mM aminoantipyrine, 0–25 mM pipecolate and 10U HRP.
(3) Enzyme activities with NAD^+ or $NADP^+$ as co-factors were determined in homogenates of L3 *H. contortus* or *T. circumcincta* in assay mixtures containing 5 mM pipecolate and also 0–1 mM NAD^+ or $NADP^+$. NAD(P)H formation was monitored spectrophotometrically at 340 nm.

2.3.4. Lysine-ketoglutarate reductase (LKR) (E.C. 1.5.1.8)

LKR activity was determined from the rate of NADH utilisation monitored spectrophotometrically at 340 nm.

(1) Enzyme activities with NADH and NADPH as co-factor were compared in homogenates of L3 *H. contortus* and *T. circumcincta*. The assay mixture contained 0–20 mM lysine, 1 mM 2-OG, 0.5 mM Ca^{2+} and either 0.2 mM NADH or NADPH.
(2) To determine the optimal pH for LKR, the reaction mixtures contained 100 mM phosphate buffer pH 5.5–9, homogenate, 1 mM 2-OG, 0.5 mM Ca^{2+} and 0.2 mM NADH. The reaction was started by the addition of 1 mM lysine and enzyme activity was determined spectrophotometrically at 340 nm. Subsequent assays were performed at pH 7.
(3) The V_{max} and the apparent K_m for lysine were determined in assay mixtures containing 0–25 mM lysine, 1 mM 2-OG, 0.5 mM Ca^{2+} and 0.2 mM NADH.

2.3.5. Saccharopine dehydrogenase (SDH) (E.C. 1.5.1.9)

SDH activity was determined from the rate of NADH formation monitored spectrophotometrically at 340 nm.

(1) To determine the optimal pH for SDH, the reaction mixtures contained 100 mM phosphate buffer pH 5.5–9, homogenate and 0.2 mM NAD^+. The reaction was started by the addition of 1 mM saccharopine and enzyme activity was determined spectrophotometrically at 340 nm. Subsequent assays were performed at pH 8.
(2) The V_{max} and the apparent K_m for saccharopine were determined in assay mixtures containing 0–25 mM saccharopine and 0.2 mM NAD^+.
(3) Enzyme activities with $NADP^+$ as co-factor were also determined in homogenates of L3 *H. contortus* and *T. circumcincta*. The assay mixture contained 0–20 mM saccharopine and 0.2 mM $NADP^+$.

2.3.6. Lysine dehydrogenase (LDH) (E.C.1.4.1.15)

LDH activity was determined from the rate of NADH formation monitored spectrophotometrically at 340 nm.

(1) To determine the optimal pH for LDH, the reaction mixtures contained 100 mM phosphate buffer pH 5–9, homogenate and 0.5 mM NAD^+. The reaction was started by the addition of 1 mM lysine and enzyme activity was determined spectrophotometrically at 340 nm. Subsequent assays were performed at pH 8.
(2) The V_{max} and the apparent K_m for lysine were determined in assay mixtures containing 0–25 mM lysine and 0.5 mM NAD^+.
(3) Enzyme activities with $NADP^+$ as co-factor were determined in homogenates of L3 *H. contortus* and *T. circumcincta*. The assay mixture contained 5 mM lysine and 0.5 mM $NADP^+$.
(4) Enzyme activities with proline or sarcosine as substrate were determined in homogenates of L3 *H. contortus* and *T. circumcincta*. The V_{max} and the apparent K_m for proline or sarcosine were determined in assay mixtures containing 0–25 mM proline or sarcosine and 0.5 mM NAD^+ or 0–25 mM proline and 0.5 mM $NADP^+$.

2.3.7. Lysine decarboxylase (LDC) (E.C. 4.1.1.18)

LDC activity was determined from the $^{14}CO_2$ released from [1-^{14}C] lysine, according to the protocol for decarboxylase assays

of Umair et al. (2011a). The apparent K_m for lysine and V_{max} were determined in homogenates of L3 or adult *H. contortus* and *T. circumcincta* in assay mixtures containing 50 mM Tris HCl pH 6, 0.2 mM pyridoxal 5′-phosphate (PLP), 0.2 mM EDTA, 0.1 mM DTT, 1 mM $MgSO_4$, 0–20 mM lysine, 0.5 μCi L-[1-14C] lysine (GE Healthcare Amersham, UK) and 50 μg homogenate protein. The assay mixture was placed in the outer tube, while the inner tube held a strip of filter paper dipped in 1 mM KOH to absorb the $^{14}CO_2$. After 20 min, the reaction was terminated by the addition of 0.1 ml 40% TCA and after mixing with 5 ml cocktail (Optiphase Super Mix, Wallac Scintillation Products, Loughborough, UK) the radioactivity was counted in a scintillation counter (Wallac 1450 Microbeta Trilux, UK).

Assays were also performed over the pH range pH 5–9 with 1 mM lysine. The concentration of PLP was varied from 0.01–1 mM at pH 6 with assay mixtures containing 1 mM lysine.

2.4. Data analysis

Replicate data are presented as mean ± SEM. Graph Prism v5 was used to plot kinetic data, estimate K_m and V_{max} and compare the kinetic properties of enzymes in homogenates of L3 and adult worms of the two species using two-way ANOVA.

3. Results

3.1. Lysine oxidase

Enzyme activities and kinetic properties of LO in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta* are shown in Table 1. The optimum pH for LO activity was pH 6 (Fig. 2A). In adult worms, the V_{max} ($p < 0.05$) and apparent K_m for lysine were higher ($p < 0.05$) than in L3. The apparent K_m for lysine was higher ($p < 0.01$) in *H. contortus* than in *T. circumcincta*.

3.2. Δ^1-Piperideine-2-carboxylate reductase

Pip2CR activity was not detected in homogenates of L3 *H. contortus* or *T. circumcincta* in assays over the pH range 5–9, with NADH or NADPH and with Pip2C concentrations from 0 to 20 mM. Pip2CR activity was detected in adult worms of both species and kinetic properties were similar with NADH as co-factor (Table 1). The pH optimum was 6.5 (Fig. 2B), the apparent K_m for Pip2C were 1.4 and 1.6 mM and V_{max} were 32 and 40 nmol min^{-1} mg^{-1} protein for adult *H. contortus* and *T. circumcincta* respectively. No enzyme activity was detected when NADH was replaced with NADPH.

3.3. Pipecolate oxidase

Enzyme activities and kinetic properties of PipO in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta* are shown in Table 1. The pH optimum for PipO was 7.5 (Fig. 2C). In adult worms, the V_{max} ($p < 0.05$) and apparent K_m for pipecolate were higher ($p < 0.001$) than in L3. When either NAD^+ or $NADP^+$ was added as a possible co-factor, no consumption of the co-factor was seen during the conversion of pipecolate to Pip6C.

3.4. Lysine-ketoglutarate reductase

An initial comparison of the activities and kinetic properties with NADH and NADPH showed that the LKR in homogenates of L3 of both species were equally active with either co-factor. The V_{max} in L3 *H. contortus* and *T. circumcincta* respectively with NADH were 39 ± 2 and 33 ± 4 (nmol min^{-1} mg^{-1} protein, mean ± SEM, $n = 2$), whereas the respective activities with NADPH were 33 ± 3 and 35 ± 3 (nmol min^{-1} mg^{-1} protein, mean ± SEM, $n = 2$). The respective K_m for lysine were with NADH 0.4 ± 0.1 and 0.5 ± 0.05 mM (mean ± SEM, $n = 2$) compared with NADPH 0.5 mM for both species ($n = 1$).

Enzyme activities and kinetic properties of LKR in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta* with NADH are shown in Table 1. The pH optimum for LKR was 7 in both species (Fig. 2D). In adult worms, the V_{max} ($p < 0.01$) and apparent K_m for lysine were higher ($p < 0.05$) than in L3.

3.5. Saccharopine dehydrogenase

Enzyme activities and kinetic properties of LKR in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta* are shown in Table 1. The pH optimum for SDH was 8 (Fig. 2E). In adult worms, the V_{max} ($p < 0.01$) and apparent K_m for lysine were higher ($p < 0.01$) than in L3. When NAD^+ was replaced by $NADP^+$, the enzyme activities were all within the ranges obtained with NAD^+ as co-factor: 43 ± 2 and 37 ± 3 (nmol min^{-1} mg^{-1} protein, mean ± SEM, $n = 2$) in L3 *H. contortus* and *T. circumcincta*, respectively.

3.6. Lysine dehydrogenase

No LDH activity was detected in homogenates of either sheathed L3 or adult *H. contortus* or *T. circumcincta*, despite increasing the amount of homogenate protein up to 250 μg, the lysine concentration from 0–25 mM, and the pH from pH 5–9 and with $NADP^+$ instead of NAD^+. No dehydrogenase activity was detected with either NAD^+ or $NADP^+$ as co-factor and replacement of lysine with 0–25 mM proline.

3.7. Lysine decarboxylase

No LDC activity was detected in homogenates of either sheathed L3 or adult *H. contortus* or *T. circumcincta*, despite increasing the amount of homogenate protein up to 250 μg, the PLP concentration up to 1 mM, the lysine concentration from 0–20 mM and the pH from pH 5–9.

Table 1
The apparent substrate K_m (mM) and V_{max} (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) of lysine oxidase (LO), Δ^1-piperideine-2-carboxylate reductase (Pip2CR), pipecolate oxidase (PipO), lysine ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) in L3 and adult *H. contortus* and *T. circumcincta*.

	LO		Pip2CR		PipO		LKR		SDH	
	K_m (Lys)	V_{max}	K_m (Pip2C)	V_{max}	K_m (Pip)	V_{max}	K_m (Lys)	V_{max}	K_m (Sac)	V_{max}
L3 *H. contortus*	2.3 ± 0.1[a,c]	38 ± 4[a]	ND	ND	1 ± 0.05[a]	32 ± 5[a]	0.4 ± 0.1	39 ± 2[a]	0.5 ± 0.1[a]	40 ± 3[a]
L3 *T. circumcincta*	1.8 ± 0.1[a,d]	29 ± 4[a]	ND	ND	1.2 ± 0.1[a]	38 ± 4[a]	0.5 ± 0.05	33 ± 4[a]	0.4 ± 0.05[a]	39 ± 3[a]
Adult *H. contortus*	3 ± 0.2[b,c]	53 ± 5[b]	1.4 ± 0.2	33 ± 4	2.1 ± 0.2[b]	49 ± 3[b]	0.7 ± 0.05	65 ± 5[b]	1 ± 0.1[b]	68 ± 6[b]
Adult *T. circumcincta*	2.2 ± 0.1[b,d]	43 ± 5[b]	1.6 ± 0.1	41 ± 5	2.3 ± 0.1[b]	52 ± 4[b]	0.9 ± 0.1	69 ± 4[b]	1 ± 0.05[b]	71 ± 5[b]

ND: not detected; mean values with different superscripts different, at least $p < 0.05$.

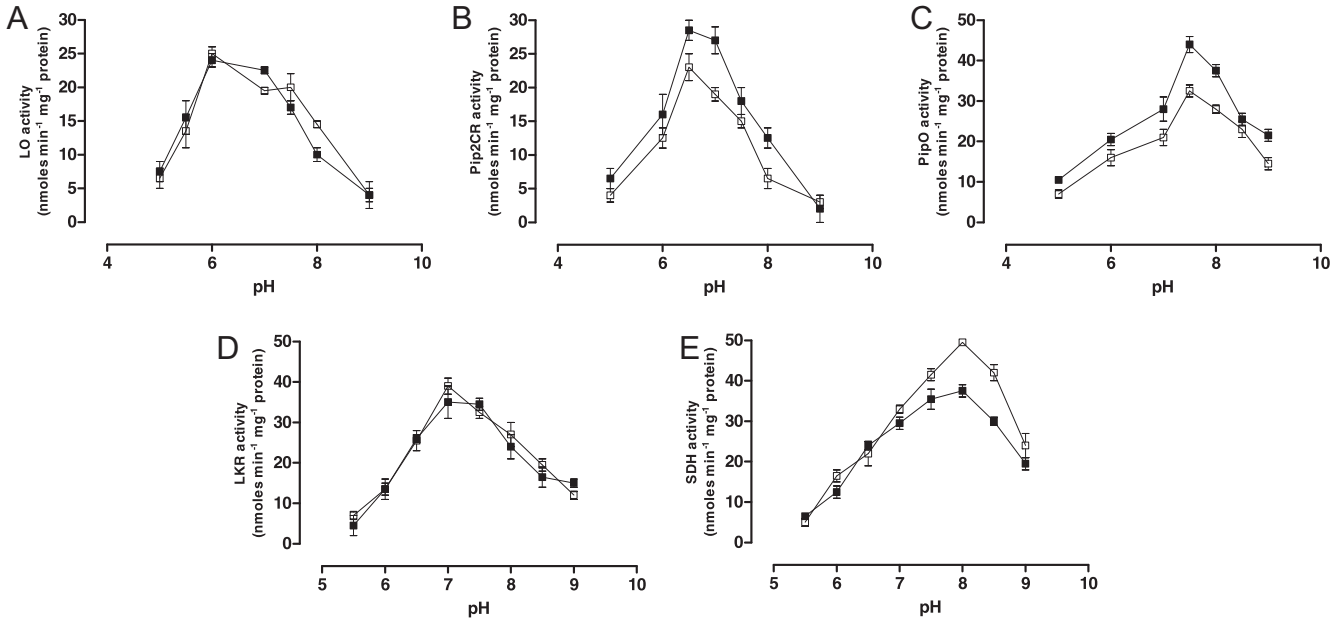

Fig. 2. Effects of pH on (A) lysine oxidase (LO), (B) Δ^1-piperideine-2-carboxylate reductase (Pip2CR) (C) pipecolate oxidase (PipO), (D) lysine ketoglutarate reductase (LKR) and (E) saccharopine dehydrogenase (SDH) activities (nmol min^{-1} mg^{-1} protein) (mean ± SEM, $n = 2$) in L3 *H. contortus* (■) and *T. circumcincta* (□), except for Pip2CR for which adult worm homogenates were used.

4. Discussion

The current study appears to be the first to investigate the kinetic properties of the enzymes of lysine catabolism in helminths. All enzymes of both the saccharopine and pipecolate pathways were active in adult *H. contortus* and *T. circumcincta*, whereas in L3 the pipecolate pathway was incomplete, as Pip2CR activity was not detected. Not surprisingly, neither LDC nor LDH activity was detected in any parasite homogenate, despite decarboxylase assays in particular being very sensitive (Umair et al., 2011a). These enzymes are not present in animals, whereas LDC is present in some bacteria (Takatsuka and Kamio, 2004) and in higher plants (Ohe et al., 2009) and LDH is a microbial enzyme (Dempsey et al., 1992). A novel finding for enzymes of the saccharopine pathway was that both LKR and SDH were dual co-factor enzymes and not specific for either NAD^+ or $NADP^+$. In contrast, reported gene sequences for *Caenorhabditis elegans* SDH and LKR are consistent with the typical eukaryote single co-factor specific enzymes.

The saccharopine pathway appears to be the sole route for lysine catabolism in L3 abomasal nematodes, whereas the pipecolate pathway was also complete in adult worms. The LKR–SDH pathway is also the major route for lysine regulation and catabolism in plants (Arruda et al., 2000) and animals (Tang et al., 1997). In adult worms, the saccharopine pathway would be predicted to be favoured over the pipecolate pathway as the K_m for lysine for LKR was 0.7 mM compared with 3 mM for LO in *H. contortus* and 0.9 mM and 2.2 mM in *T. circumcincta*. In addition, the V_{max} was higher for LKR than LO in both species. This enzyme may have other roles in invertebrate development. Using RNAi, LKR/SDH has been shown to play an important role in the reproduction and development of the tick *Haemaphysalis longicornis* (Battur et al., 2009). In *Drosophila*, the protein is involved in ecdysone-mediated transcription and the timing of developmentally regulated gene expression (Cakouros et al., 2008).

The nematode LKR/SDH does not show the reported co-factor specificity of enzymes from a wide range of organisms. Other kinetic properties, such as substrate affinity and pH optima, as well as the *C. elegans* gene sequence (KEGG R02D3.1), are consistent with a bifunctional enzyme being present in nematodes. The tick *H. longicornis* has a bifunctional LKR/SDH with very similar pH optima (Battur et al., 2009) to those of enzyme in the parasitic nematodes. The co-factor requirements of the *H. contortus* and *T. circumcincta* proteins are very different from mammalian and plant bifunctional LKR/SDH, which has NADPH-specific LKR activity and NAD^+-specific SDH activity (Tang et al., 1997). Both LKR and SDH had similar activities with either co-factor in both nematodes.

The contribution of the pipecolate pathway to lysine catabolism is uncertain, as all three enzymes, LO, Pip2CR and PipO, were present only in adult worm homogenates and there was no detectable Pip2CR activity in L3 of either species, even with five times more homogenate protein was included. Despite the lack of detectable Pip2CR activity in L3, the first and third enzyme activities were high. In mammals, the pipecolate pathway is an essential route of lysine catabolism in the brain, while lysine is catabolised via saccharopine in the rest of the body tissues (Garweg et al., 1980). The most likely reason for the absence of detectable Pip2CR is that the enzyme is limited in its distribution to specific organs, possibly the reproductive tract. Although Pip2CR and proline5CR (P5CR) are very closely related and can recognise multiple substrates (Fujii et al., 2002), it is unlikely that the adult Pip2CR activity is due to P5CR, as this is present in homogenates of both L3 and adult *H. contortus* and *T. circumcincta* (Umair et al., 2011b).

LO and PipO are likely to be separate enzymes, although L-amino oxidases, including purified mammalian PipO (Dodt et al., 2000; Goyer et al., 2004), are known to be capable of oxidising multiple substrates. Parasite LO was very labile, even on ice, and enzyme activity was detected only in freshly prepared homogenates, whereas PipO was much more stable. Furthermore, the kinetic properties and the pH optima of the two enzymes were not identical: pH 6 for LO and 7.5 for PipO (Fig. 2). There is a possibility that, in addition to PipO, there could be a pipecolate dehydrogenase in abomasal nematodes, as has been reported in *Pseudomonas putida* (Baginsky and Rodwell, 1967). This would seem to be discounted by the lack of activity with pipecolate or proline as substrate in assays under conditions to detect dehydrogenases.

The present study has shown that there are complete saccharopine and pipecolate pathways for lysine catabolism in adult *H. contortus* and *T. circumcincta*, but not in L3 of both species, in which

Pip2CR activity was not detectable. Enzyme activities and substrate affinities were higher for all five enzymes in adult worms than in L3. As enzyme activity is based on total homogenate protein, the stage-specific differences may merely reflect the tissue composition and not enzyme activity in those tissues expressing the enzyme. The substrate affinity of the enzymes of the saccharopine pathway were higher than for enzymes in the pipecolate pathway, suggesting that the former is the main pathway for lysine catabolism in adult worms and the only one in L3. A notable property of LKR/SDH was the lack of preference for NAD^+ or $NADP^+$ as co-factor, in marked contrast to the enzymes of other organisms, making it a potential candidate for anthelmintic targeting.

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Chapter VIII

General Discussion

GENERAL DISCUSSION

The aim of the project was to learn more about the metabolism of abomasal nematode parasites of sheep to identify potential targets for the development of novel, effective and long lasting remedies. As growth and reproduction of nematodes are dependent on active nitrogen and energy metabolism, these metabolic pathways are obvious targets for vaccines or new chemical anthelmintics. The current work has added significantly to knowledge of nitrogen metabolism in nematodes and differences from host metabolism. Unlike most previous research, which was on single enzymes, the present study comprehensively covered pathways of four aspects of nitrogen metabolism and identified differences between two closely related parasites.

Glutamate, proline, arginine and lysine metabolism, were chosen for study in both larval and adult stages of *H. contortus* and *T. circumcincta*. Each has a key role in worm metabolism: glutamate has a central role in amino acid inter-conversion; proline is a component of the cuticle; arginine is a precursor of polyamines and lysine is a site of protein regulation. Biochemical assays were performed on whole worm homogenates of *H. contortus* and *T. circumcincta* to establish whether typical mammalian or prokaryotic pathways were functional in either life-cycle stage. In total, the activities and kinetic properties of 26 enzymes involved in the metabolism of the four chosen amino acids were determined. In a number of cases, significant differences in the properties of the host and parasite enzymes identified them as worthy of further study of purified or recombinant enzymes. GDH was selected for cloning and expression and the properties of the recombinant enzyme were compared with GDH activity in homogenates.

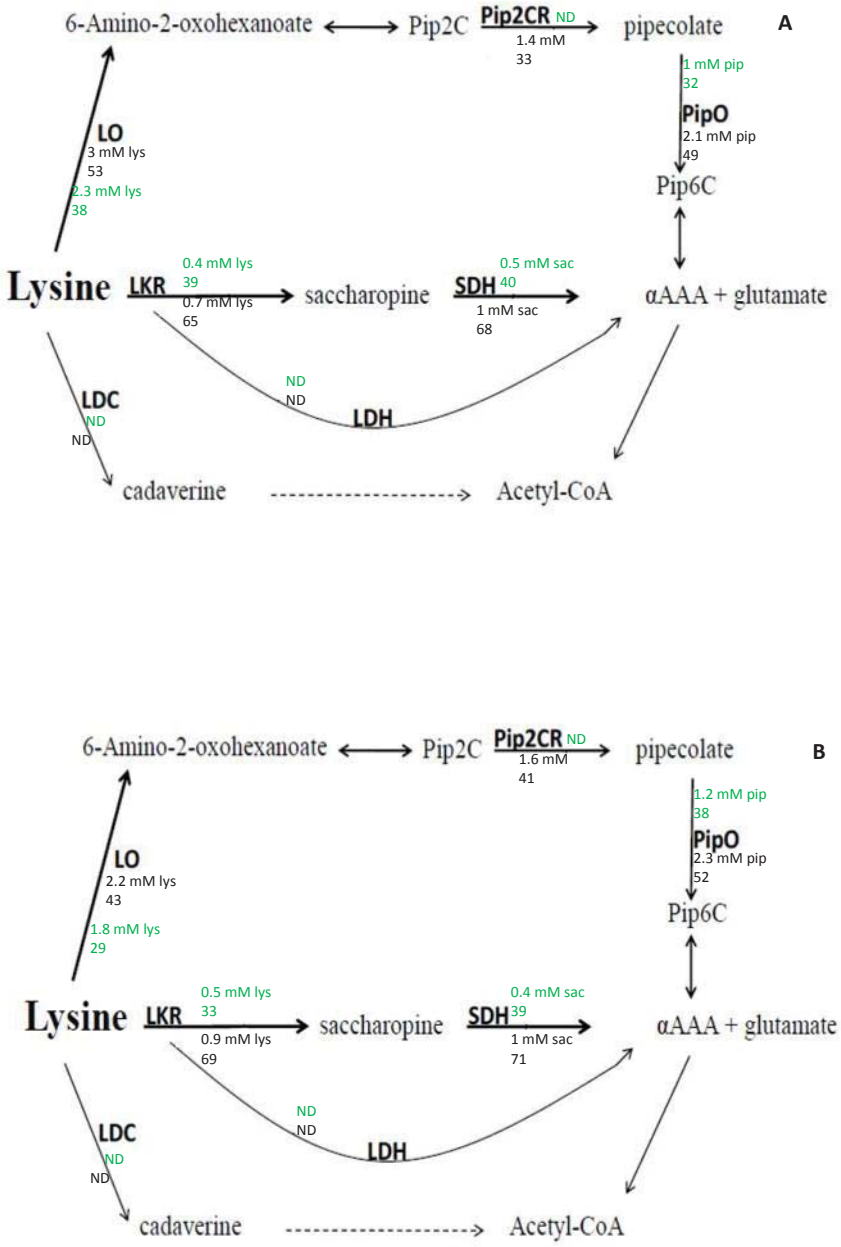

Figure 8.1. Metabolic map of enzymes of lysine catabolism identified in L3 and adult *H. contortus* (A) and *T. circumcincta* (B). Abbreviated enzyme names are shown in bold. K_m values (mM) for substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reaction are shown in green for L3 and black for adult worms.

8.1. LYSINE CATABOLISM

Reversible acetylation of lysine residues is a control mechanism of many proteins in both eukaryotes (Choudary et al., 2009; Zhao et al., 2010) and prokaryotes (Zhang et al., 2009; Wang et al., 2010). Lysine is an essential amino acid in mammals (Murthy and Janardanasarma, 1999), but can be synthesised from aspartate in plants and microorganisms (Galili, 2002). Impaired lysine catabolism causes neurological symptoms in humans (Gregory et al., 1989), highlighting the importance of catabolic pathways. As this appears to be the first study on enzymes of lysine metabolism in helminths, it is not known whether lysine can be synthesised, but catabolic pathways would be expected. The mammalian α-AAA (saccharopine) and pipecolate pathways pathways were identified in homogenates of abomasal nematodes, but not the bacterial enzymes LDH and LDC.

All enzymes of both the α-AAA and pipecolate pathways were active in adult *H. contortus* and *T. circumcincta*, whereas in L3 the pipecolate pathway was incomplete, as Pip2CR was not detected (Chapter 7). The two enzyme activities which were detected in L3 were both oxidases. These enzymes are known to accept multiple substrates (Goyer et al., 2004), so may have a function other than lysine catabolism, for example in proline metabolism. The substrate K_m and V_{max} of the reactions catalysed by enzymes in L3 and adult *H. contortus* and *T. circumcincta* are shown in Figure 8.1. The saccharopine pathway may be the main one for lysine catabolism in adult worms, as the K_m for lysine was three-fold lower and V_{max} double for LKR than for LO, the initial enzyme in the pipecolate pathway. The substrate affinities of two enzymes in the saccharopine pathway (LKR and SDH) were lower in L3 than in adult worm homogenates. Enzyme activity cannot be compared directly in L3 and adult worms, as it is based on total homogenate protein; the stage-specific differences may merely reflect the tissue composition and not enzyme activity in those tissues expressing the enzyme.

In abomasal nematodes, both LKR and SDH activities were dual co-factor enzymes, with equal activities with either NAD^+ or $NADP^+$. This is

different from the situation in plants and vertebrates, in which LKR activity of the bifunctional enzyme strictly uses NADPH as co-factor, while the SDH activity requires either NAD^+ or $NADP^+$, although activity is usually much greater with the former (Tang et al., 1997). Dual co-factor specificity of LKR/SDH is also reported from the marine sulphur degrading bacterium *Silicibacter pomeroyi*, although in this case there was greater activity with NAD^+/H than with $NADP^+$/H for both enzymes. Dual co-factor specificity of both LKR and SDH activity is in marked contrast to the enzymes of other organisms, suggesting this enzyme should be considered as a potential candidate for anthelmintic targeting.

The alternative pipecolate pathway, consisting of three enzymes, LO, Pip2CR and PipO, was incomplete in L3 of both species. One explanation is that in adult worms, Pip2CR may be expressed in specific organs, such as the reproductive tract, which are not developed in L3. Pipecolate and proline metabolism are closely related, as the substrates have similar molecular structure and the enzymes use multiple substrates (Fujii et al., 2002). Pip2CR converts Pip2C to pipecolate and P5CR converts P5C to proline, but both enzymes can recognise either substrate (Fujii et al., 2002). Pip2CR activity could be due to P5CR. If this were the case, P5C would not be expected to be metabolised to proline in homogenates of both L3 and adult *H. contortus* and *T. circumcincta* (Chapter 5).

Another feature common to pipecolate and proline metabolism is the overlapping substrate specificity of the oxidases LO, PipO and PO. L-amino oxidases, including purified mammalian PipO (Dodt et al., 2000; Goyer et al., 2004), are known to be capable of oxidising multiple substrates. Grantham and Barrett (1986b) showed that 12 amino acids (including proline and lysine) were oxidised in *H. polygyrus* and 14 amino acids in *P. redivivus* homogenates, although enzyme activities were higher with some amino acids than others. Because oxidases accept multiple substrates, the number of oxidases involved could have been less than 14. There appear to be three

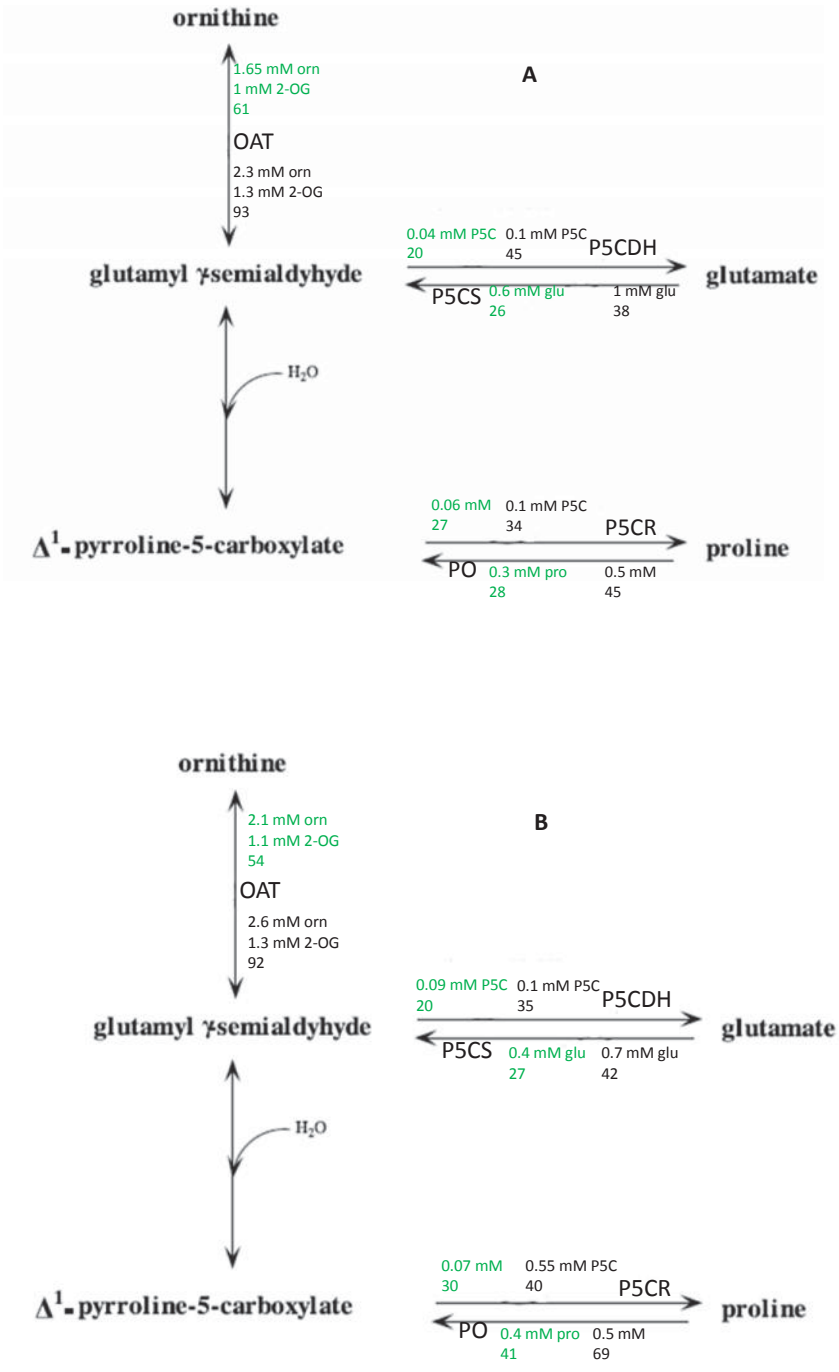

Figure 8.2. Metabolic map of enzymes of proline metabolism identified in L3 and adult *H. contortus* (A) and *T. circumcincta* (B). Enzyme names are shown in abbreviations. K_m values (mM) for substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reactions are shown in green for L3 and black for adult worms.

separate enzymes (LO, PipO and PO) in *H. contortus* and *T. circumcincta* homogenates. Parasite LO activity was very labile and enzyme activity was detected only in freshly prepared homogenates, whereas PipO was much more stable. Furthermore, the kinetic properties and the pH optima were not identical: pH 6 for LO, 7.5 for PipO and 8.5 for PO. The final conclusions about the number of separate enzymes could be made by isolating the genes for particular enzymes.

8.2. PROLINE METABOLISM

Proline is a non-essential amino acid for mammals. Proline is important for the growth and development of invertebrates, particularly for synthesis of collagen, an important component of the nematode cuticle. Insects (Scaraffia and Wells, 2003) and trypanosomes (Obungu et al., 1999) use proline as a source of energy. Bacteria and some crustaceans use proline as an osmoprotectant (Burton, 1992; Lee at al., 2003) and in plants, bacteria and marine invertebrates, proline levels rise significantly during stressful conditions (Aral and Kamoun, 1997).

Proline can readily be synthesised from either glutamate or ornithine; all five enzymes were fully functional in L3 and adult *H. contortus* and *T. circumcincta* and apparently no different from the pathway in mammals (Brunner and Neupert, 1969). The substrate K_m and V_{max} of the reactions in the inter-conversion of proline, glutamate and ornithine are shown in Figure 8.2. There are very few reported studies in helminths, the most extensive being that of Mohamed et al. (2008), who assayed four enzymes and partially purified P5CDH in *F. gigantica*. Walker and Barrett (1991) demonstrated OAT activity in *N. brasiliensis* and suggested the pathway was functional in nematodes. In the present study, the K_m and V_{max} of all five enzymes were determined and no marked species or life-cycle differences were seen.

Both P5CS and P5CR were equally active with NAD^+/H or $NADP^+$/H as co-factor, which is consistent with other nematode enzymes using these co-factors. This is not generally the case in other organisms, in which such enzymes usually prefer one or other co-factor for maximum activity, although the specific requirements either for NAD^+/H or $NADP^+$/H for enzymes in this pathway do not appear to have been examined in detail and enzyme activities have been reported for only one set of co-factors (Isseroff and Ertel, 1976; Mohamed et al., 2008)

The enzymes for inter-conversion of proline, glutamate and ornithine were all functional in abomasal nematodes, but the direction in vivo will depend on the metabolic requirements and supply of nitrogen. Nematodes have a high requirement for proline as a collagen precursor in the worm cuticle (Bird and Bird, 1991). In both species, P5CR in L3 had a high affinity for P5C compared with that in adult worms which would strongly favour proline synthesis. This was more marked in *T circumcincta* where the difference was 10-fold, compared with double in *H. contortus* L3. There is also a rich source of proline for gastro-intestinal parasites in gastric mucin, which is degraded by glycosidases and proteases. Prior to moulting, large amounts of proline could be obtained directly from the host and taken up via transporters, in addition to de novo synthesis.

8.3. ARGININE METABOLISM

Arginine is a non-essential amino acid for adult mammals, but essential for infants, piglets and felines (Morris and Rogers, 1978). In mammals, arginine is synthesised in the kidney and liver by the OUC. A fully functional OUC is generally not detectable in homogenates in parasitic nematodes, consistent with urea being a minor component of nitrogenous excretion (Wright, 1975). Arginine cannot be synthesised from ornithine, because of the irreversibility of the conversion of arginine to ornithine by the hydrolytic enzyme arginase. However, the OUC may operate sufficiently in specific

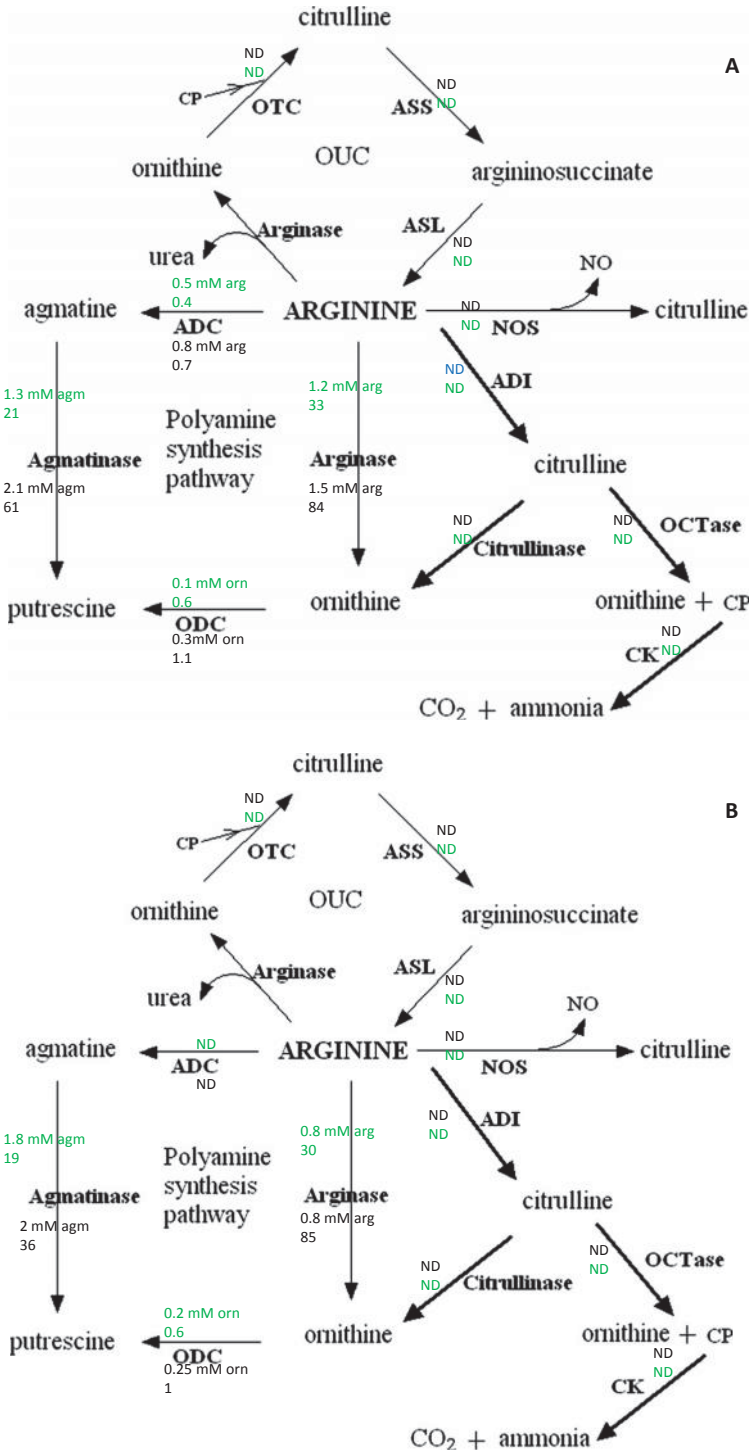

Figure 8.3. Metabolic map of enzymes of arginine metabolism identified in L3 and adult *H. contortus* (A) and *T. circumcincta* (B). Enzyme names are shown in bold. K_m values (mM) for substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reactions are shown in green for L3, black for adult worms.

tissues of the worm to generate an adequate supply of arginine. There is an alternative route of arginine synthesis in plants and microorganisms through the intermediate N-acetylglutamate (NAG) in an eight step pathway (Figure 1.5). This could operate in nematodes or arginine may be acquired from the host.

Arginine is metabolised to proline and glutamate, polyamines, NO and the phosphagens, which provide muscle energy. In the present study, the OUC, NOS activity, putrescine synthesis and non-mammalian pathways of arginine metabolism were studied in homogenates of sheathed L3 and adult *H. contortus* and *T. circumcincta*. Kinetic data of the enzymes involved are presented in Figure 8.3. The non-mammalian arginine deiminase and dihydrolase pathways were not detected in either nematode species, whereas arginine was converted to ornithine by arginase (Chapter 6).

Although the presence of NOS was demonstrated histochemically in the central and peripheral nerves of adult *H. contortus* (Chapter 6), NOS activity was undetectable in homogenates of adult or L3 *T. circumcincta* or *H. contortus*. As only some nerves stained for NOS activity, the restricted distribution of the enzyme in abomasal parasitic nematodes would account for failure to detect enzyme activity biochemically in whole worm homogenates.

8.3.1. Arginase

Arginine is metabolised by arginase to urea and ornithine, a precursor for polyamines, proline and glutamate. Arginase activity was high in L3 and adult *H. contortus* and *T. circumcincta* (Chapter 6) and may have an additional function in protecting against host NO. Other parasites and pathogens are believed to use arginase to compete with host iNOS for the common substrate arginine (Vincendeau et al., 2003). During *Trichinella spiralis* infection, jejunal iNOS was down-regulated when parasite arginase competed with the host NOS (Bian et al., 2001).

T. circumcincta arginase had an unusual pH optimum and did not require added Mn^{2+} for activity, as previously reported by Muhamad (2006). These properties were shared by *H. contortus* arginase. In contrast to other typical arginases (Kuhn et al., 1991), there was no requirement for added Mn^{2+} or any other bivalent metal ion for its activity and activity was only slightly increased by the addition of 1 mM Mn^{2+}, probably because of tight binding of the co-factor Mn^{2+} to the enzyme. Most arginases, including the *S. mansoni* (Fitzpatrick et al., 2009) and *F. gigantica* (Mohamed et al., 2005) enzymes, are most active at pH 9-10, whereas the pH optima for the arginases of *H. contortus* and *T. circumcincta* were both pH 8.5. There was a similar pH optimum for *T. circumcincta* arginase (Muhamad, 2006). There is an even lower pH optimum of pH 6.1 in the gastric pathogen *H. pylori* (McGee et al., 2004), which is also exposed to a low pH in gastric contents.

8.3.2. Polyamine synthesis

The polyamines putrescine, spermidine and spermine, regulators of cell growth and differentiation, are derived from arginine: arginine is metabolised to putrescine, which is successively metabolised to spermidine and spermine (Figure 1.6). Walter (1988) suggested that nematodes could not synthesise putrescine, but acquired it from the host, but this was subsequently disproved when ODC activity was demonstrated in *C. elegans* and *H. contortus* (Schaeffer and Donatelli, 1990).

There appeared to be two pathways of putrescine synthesis in *H. contortus* and one in *T. circumcincta* (Chapter 6). Both species have the universal pathway using arginase to form ornithine, followed by decarboxylation by ODC, while in *H. contortus* there was the additional pathway of first decarboxylation by ADC to form agmatine, then hydrolysis by agmatinase to putrescine (Figure 8.3). Putrescine synthesis via the ADC pathway in *H. contortus* is unexpected as, although this pathway is universally present in bacteria, it is controversial in mammals (Gilad et al., 1996; Regunathan and Reis, 2000). No ADC activity was detected in *N. brasiliensis* (Walker and Barrett, 1991a) and *C. elegans* (Macrae et al., 1995).

The presence of an ADC/agmatinase pathway for putrescine synthesis in *H. contortus* raises the question whether these are separate enzymes from ODC/arginase, both of which have been reported to use other substrates. In mammals, several decarboxylases are known to use multiple substrates, but with varying activities (Gilad et al., 1996). In contrast, in *E. coli*, specific decarboxylases for ornithine, arginine and lysine have been identified exhibiting strict substrate specificity (Boeker and Fisher, 1983). Nematodes are able to decarboxylate many amino acids. Singh et al. (1983) detected decarboxylase activity for serine, leucine, valine, alanine, aspartate and glutamate in the intestines, ovaries and cuticle of *A. galli* (Singh et al., 1983). Grantham and Barrett (1986b) studied the metabolism of the branched chain amino acids isoleucine, leucine and valine in *P. redivivus* and *H. polygyrus* and concluded that these pathways of amino acid catabolism were fully functional. The purified ODC from *C. elegans* was able to use arginine and lysine as substrates but the substrate affinity was low (Schaeffer and Donatelli, 1990), however, neither *H. contortus* nor *T. circumcincta* had detectable decarboxylation of lysine.

The evidence appears to support ODC and ADC being separate enzymes in *H. contortus*, as is the case in *E. coli*, in which there are separate decarboxylases with strict substrate specificity for ornithine, arginine and lysine (Boeker and Fisher, 1983). First, ODC activities and substrate affinity for ornithine were similar in homogenates of L3 of both worms and similar in both adult preparations, yet arginine was metabolised to agmatine by only one parasite species. Secondly, DFMO, an established inhibitor of nematode ODC (Schaeffer and Donatelli, 1990; Klein et al., 1997), inhibited ODC activity by up to 80%; in contrast, a preliminary experiment showed no effect of DFMO on ADC activity in either L3 *H. contortus* or *T. circumcincta*. Thirdly, the pH optima for the two enzymes differed markedly: pH 6 for ADC and pH 8 for ODC. Lastly, L3 *H. contortus* ADC showed declining activity at high substrate concentrations, but this was not the case for ODC at high ornithine concentrations (Chapter 6). Considered together, the most likely conclusion is that there are separate ODC and ADC enzymes in *H. contortus*.

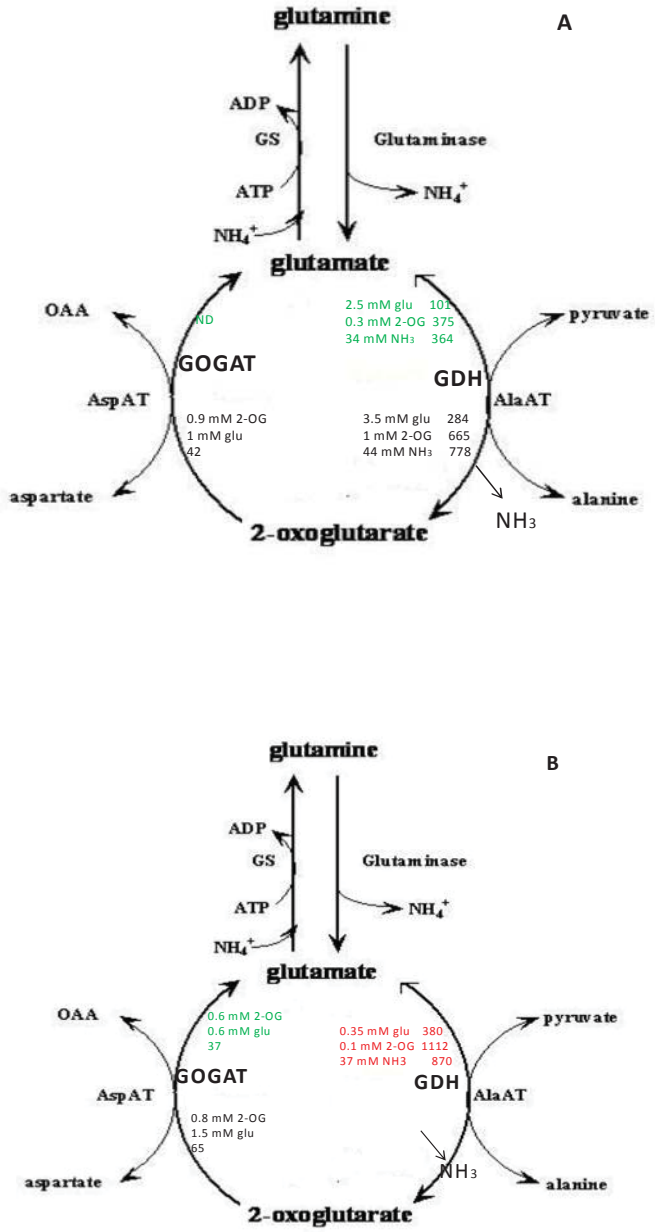

Figure 8.4. Metabolic map of enzymes of glutamate metabolism identified in L3 and adult *H. contortus* (A) and *T. circumcincta* (B). K_m values (mM) for substrates and V_{max} (nmoles min^{-1} mg^{-1} protein) for the reaction is shown in green for L3, black for adult worm and red for the purified protein with NAD^+/H as co-factor.

Agmatinase is a recognised neuronal enzyme in mammals (Li et al., 1995; Feng et al., 1997), but is universally present in non-mammalian tissues (Tabor and Tabor, 1984). As arginase and agmatinase belong to the same enzyme family and are capable of using either arginine or agmatine as substrate (Ahn et al., 2004), arginase may be responsible for the *T. circumcincta* and *H. contortus* agmatinase activity. On the other hand, there could be separate arginase and agmatinase enzymes. Both enzyme activities were similarly inhibited by ornithine (approximately 70%) and putrescine (15-20%), suggesting a single enzyme was responsible. Sequencing the genes encoding these enzymes may help in resolving this issue.

8.4. GLUTAMATE METABOLISM

Interconversion of glutamate and 2-OG provides a link between nitrogen and energy metabolism (Hudson and Daniel, 1993). Glutamate is converted to many compounds including other amino acids by transamination, to GABA by decarboxylation and to proline and ornithine through the intermediate P5C. A metabolic map linking the enzymes involved in glutamate metabolism in L3 and adult *H. contortus* and *T. circumcincta* is presented in Figure 8.4.

The universal enzymes GS, GDH and glutaminase and the non-mammalian enzyme GOGAT were characterised in L3 and adult *T. circumcincta* (Muhamad, 2006). This study and others have raised questions about the similarity of nematode and mammalian glutamate metabolism. Is the GABA shunt an important metabolic bypass of the TCA cycle in nematode parasites, as believed by Monteoliva et al. (1965) and Singh et al. (1983)? Is GS-GOGAT an important route of incorporation of ammonia into glutamate? Is GDH, a universal enzyme, not expressed in L3 *H. contortus*, as reported by Skuce et al. (1999)? Is nucleotide allosteric regulation of nematode GDH by

ADP, ATP and GTP different from that in other organisms? Are the properties of recombinant *Tc*GDH different from homogenate GDH?

8.4.1. Importance of GABA shunt as a metabolic bypass

GABA is a target of chemical anthelmintics and an inhibitory neurotransmitter (Schuske at al., 2004). In addition, previous studies showed that in the relatively anaerobic condition in the intestine, helminths use the GABA shunt to bypass the usual TCA cycle where 2-OG is converted to succinate. (Monteoliva et al., 1965; Rasero et al., 1968). There was no evidence of this in abomasal nematodes, as neither GAD nor SSADH activity was detected in homogenates of whole L3 or adult *H. contortus* or *T. circumcincta*. This was not surprising, as the small size and high surface area:volume of both *H. contortus* and *T. circumcincta* may be sufficient for oxygen delivery and the GABA shunt enzymes may be restricted to the nerves. Parasites where this appears to occur are generally larger e.g. Ascarids (Monteoliva et al., 1965; Rasero et al., 1968) or inhabit the intestine, which may be a more anaerobic environment than the stomach.

8.4.2. Ammonia incorporation by GS-GOGAT

Muhamad (2006) detected GOGAT activity for the first time in nematodes and determined substrate affinities in assays using two crude homogenates of sheathed L3 and one of adult *T. circumcincta*. In the present study, GOGAT assays were performed on L3 and adult *H. contortus* for the first time and the properties of the enzyme were compared with those in *T. circumcincta*. GOGAT activity was moderate in homogenates of sheathed L3 *T. circumcincta* and both adult *H. contortus* and *T. circumcincta*, but was undetectable in L3 *H. contortus*, even after pre-incubation overnight of L3 with NH_4Cl. In contrast, exposure to ammonia increased GOGAT activity significantly in L3 *T. circumcincta.* The properties of the enzymes in *H. contortus* and *T. circumcincta* were very similar and used either NADH or NADPH as the electron donor. The pH optimum of 7.5 was the same as

reported for the enzyme from the silkworm *B. mori* (Hirayama et al., 1998) and the protozoan *E. gracilis* (Miyatake and Kitaoka, 1981).

The high degree of inhibition by azaserine (80%) supports the enzyme activity being GOGAT, as azaserine has been used to inhibit GOGAT from many organisms (Rachim and Nicholas, 1985; Mérida et al., 1991), including the arthropods *B. mori* (Hirayama et al., 1998) and *A. aegypti* (Scaraffia et al., 2005). Azaserine is also a very weak inhibitor of glutamine:fructose-6-phosphate amidotransferase (Ghosh et al., 1960; Chmara et al., 1986).

It appears that nematodes have the ability to use ammonia through the GS-GOGAT pathway as a source of nitrogen alternative to amino acids, which may be advantageous in the high ammonia-low amino acid environment in abomasal fluid. The GS-GOGAT pathway is preferred in plants (Miflin and Habash, 2002; Magasanik, 2003) and bacteria (Helling, 1994, 2002), when there is sufficient energy and ammonia concentration is low. This pathway also can explain the disappearance of excreted ammonium during several hours of in vitro incubation of L3 *T. circumcincta* (Simpson et al., 2009). The ammonia could be incorporated into glutamate and thence into other amino acids, as has been shown in the silkworm *Samia cynthia ricini* where alanine was synthesised from labeled ammonia (Osanai et al., 2000).

8.4.3. Glutamate Dehydrogenase

The experiments on GDH were directed particularly at addressing three questions raised by earlier studies on abomasal nematodes: the absence of GDH activity in L3 *H. contortus*, possible unusual nucleotide allosteric regulation of GDH by ADP, ATP and GTP and comparison of the properties of recombinant *Tc*GDH and homogenate GDH.

8.4.3.1. *H. contortus* GDH

In contrast to previous reports (Skuce et al., 1999), GDH activity was confirmed in homogenates of *H. contortus* L3 in both the deaminating and aminating directions. The properties of the enzyme were similar to those of

the GDH in adult worm homogenates and other nematode GDHs (Langer, 1972; Muhamad et al., 2011). GDH mRNA was confirmed in both life cycle stages of *H. contortus* by cloning and sequencing a single PCR product of about 1600 bp. Previously, Skuce et al. (1999) reported that neither the GDH protein was detectable in L3 using purified antibody from the sera of immune sheep nor was mRNA detected. As L3 *H. contortus* do not have an active GOGAT (Chapter 2), the absence also of GDH would present difficulties for ammonia assimilation into glutamate. It would be expected that GDH would incorporate ammonia when there were high levels of ammonia.

Despite the similarity of the GDH genes in *H. contortus* and *T. circumcincta* (Chapter 3), some subtle differences in the co-factor affinity were observed. *H. contortus* GDH was a dual co-factor enzyme, although activity in both L3 and adult worm homogenates was about 50% higher with NAD^+/H as co-factor than with $NADP^+$/H (Chapter 4). In contrast, Rhodes and Ferguson (1973) reported that purified adult *H. contortus* GDH had an absolute requirement for NAD^+/H and less than 2% of that activity with $NADP^+$/H, which would classify it as NAD^+ specific and not a dual co-factor or $NADP^+$ specific enzyme. This difference in properties may have been caused by changes in the protein during the purification process. The K_m for each co-factor was similar in the two life-cycle stages and similar to those reported for the purified enzyme with NAD^+/H as co-factor. The predicted co-factor binding sites on GDH (Chapter 4, Supplementary Figure 1) appear to be identical in both nematode species and are probably not the reason for differences in kinetic properties and sensitivity to regulators. With either co-factor, the pH optima of the *Tc*GDH in both directions was 7.5 (Chapter 3, Figure 3), whereas in *H. contortus*, the pH optimum was pH 8 in the direction of glutamate formation and pH 8.8 in the direction of glutamate utilisation (Rhodes and Ferguson, 1973).

8.4.3.2. Nucleotide allosteric regulation of nematode GDH

Allosteric regulation of *H. contortus* and *T. circumcincta* GDH by GTP, ATP and ADP was different from that in mammals in sensitivity, rather than

showing qualitative differences in response. Previous experiments using only single concentrations of ADP and ATP, usually in only one direction, had suggested that nematode and mammalian GDH may respond differently to these regulators. The effects of these allosteric regulators was concentration-dependent, as it is for purified human GDH in the aminating direction (Fang et al., 2002) and differed in the deaminating and aminating directions. The nematode enzymes were more responsive to activation by ADP and ATP and less inhibited by GTP under optimum assay condition. GTP inhibited deamination and low concentrations of ADP and ATP stimulated weakly. In the reverse direction, GTP was strongly inhibitory and ADP and ATP activated the enzyme.

There were small differences in sensitivity to ADP and ATP between life-cycle stages and species of parasite, as well as between the present and previous studies (Muhamad et al., 2011; Rhodes and Ferguson, 1973). There are two recognised binding sites for nucleotide regulators, one for GTP and the other for ADP (Fang et al., 2002). ATP can bind either to the inhibitory GTP or stimulatory ADP site, resulting in complex effects of ATP for human GDH, in inhibition at very low concentrations (to 0.1 mM), little effect at intermediate concentrations (1–5 mM) and inhibition above 5 mM (Fang et al., 2002). Differences in sensitivity of *H. contortus* and *T. circumcincta* GDH to ATP and ADP cannot be explained by the predicted amino acid sequences and binding sites (Chapter 4, Supplementary Fig 1) and may instead be caused by other components of the homogenates.

8.4.3.3. Recombinant *T. circumcincta* GDH

GDH was the enzyme chosen to extend the study of nitrogen metabolism beyond using worm homogenates to cloning and characterisation of recombinant proteins and subsequent assessment as parasite targets. It was selected because of its established antigenicity (Skuce et al., 1999) and central position in glutamate metabolism. The gene encoding GDH was sequenced and the recombinant *Tc*GDH expressed and characterised biochemically (Chapter 3). Recombinant *Tc*GDH and *H.*

contortus GDH (*Hc*GDH) (Skuce et al., 1999) were very similar in protein sequence (Chapter 3, Figure 2). Both enzymes contained 538 amino acids, had predicted molecular masses of approximately 60 kDa and the predicted amino acid sequences showed 91% identity and 96% similarity. *Tc*GDH was also very similar to *C. elegans* GDH (85% identity, 93% similarity).

The properties of the purified protein were virtually identical to those of the GDH in homogenates of adult or L3 *T. circumcincta* (Muhamad et al., 2011). The GDH activity in homogenates of L3 and adult *T. circumcincta* (Muhamad et al., 2011) and *H. contortus* (Chapter 4) and recombinant *Tc*GDH (Chapter 3) were active with either co-factor, consistent with a dual co-factor GDH, as are mammalian enzymes (Hudson and Daniel, 1993). Differences in activity with NAD^+/H and $NADP^+$/H were small (around 10%) in homogenates and for *Tc*GDH. The K_m for individual substrates were similar in *Tc*GDH and *T. circumcincta* homogenate GDH (Muhamad et al., 2011), notably a very high K_m for NH_4^+ at around 40 mM.

Nematode GDHs have pH optima between pH 7 and 8 (Rhodes and Ferguson, 1973; Turner et al., 1986). With either co-factor, the pH optima in both direction was 7.5 in both TcGDH (Chapter 3, Figure 3)and *T. circumcincta* homogenates (Muhamad, 2006). In both cases, the aminating reaction had a broader pH range than the forward reaction, with significant activity detected above the optimum pH. Comparison of the kinetic properties of the GDH enzyme in homogenates with those of the recombinant GDH has shown that, at least for this enzyme, studies with homogenates can provide valid information.

8.5. Metabolic differences in abomasal nematodes

8.5.1. Differences between parasite and host

The major pathways of parasite and mammalian nitrogen metabolism were generally similar, although activities of some non-mammalian enzymes were detected in nematodes and the properties of some individual enzymes were significantly different. Previous studies have also identified the non-mammalian neurotransmitters octopamine and tyramine (Blenau and Baumann, 2011) and the enzymes aspartase and creatinase in abomasal nematodes (Muhamad, 2006).

A metabolic map linking all enzymes of nitrogen metabolism in this study of L3 and adult *H. contortus* and *T. circumcincta* is presented in Figure 8.5. The catabolism of lysine was very similar in abomasal nematodes and mammals and no bacterial enzymes for lysine catabolism synthesis were detected in either species (Chapter 7). The pipecolate pathway, which is particularly active in the mammalian nervous system, was complete only in adult nematodes, not L3. Both LKR and SDH were equally active with NAD^+ and $NADP^+$, which distinguishes them from the mammalian counterparts.

There was no apparent difference in L3 and adult *H. contortus* and *T. circumcincta* in the inter-conversion of ornithine, glutamate and proline from the pathway in mammals (Brunner and Neupert, 1969). Although the specific co-factor requirements of mammalian P5CS and P5CR have not been reported, co-factor specificity may be different for the nematode enzymes, which were equally active with NAD^+/H or $NADP^+$/H. This dual co-factor specificity was a common feature for other nematode enzymes, but it is not generally the case in other organisms, in which such enzymes usually prefer one or other co-factor for maximum activity

The major differences in arginine metabolism were the involvement of ADC in polyamine synthesis and the lack of a detectable OUC. Ornithine was synthesised from arginine by arginase, as in mammals, and no bacterial enzymes for ornithine synthesis were detected in either species (Chapter 6). Nematode arginase was unusual in its less alkaline pH optimum and not requiring Mn^{2+} to be added for enzyme assays in homogenates. The striking difference in glutamate metabolism was the presence of GOGAT in all but L3

H. contortus (Chapter 2), which would allow the synthesis of amino acids from ammonia.

8.5.1. Difference between life-cycle stages

The general pattern of the L3 and adult enzymes was high substrate affinity and less enzyme activity of in L3. Extreme cases of lower activity were undetectable levels of Pip2CR in both species and GOGAT in L3 *H. contortus*, even after pre-incubation overnight with NH_4Cl, which increased GOGAT activity significantly in L3 *T. circumcincta.*

Most enzymes were apparently less active in L3 by about 40%, however, PO, P5CDH and OAT activities were almost equal in L3 and adult worms. As is the usual practice, enzyme activity is expressed as activity per milligram protein in whole worm homogenates of L3 or adult worms, which is not a good basis for comparing life-cycle stages of differing tissue composition. An alternative unit of activity, based on wet weight, is unlikely to provide a better basis for comparison of different parasite populations, as when removed from the abomasum adult worms make 'worm balls', which hold water and debris. Adult parasite populations from different sheep are not identical in male:female ratios, stunting caused by host immunity and number of eggs per female worm, all of which reflect differences in parasite metabolism. When comparing L3 and adult worm enzyme activities, it is apparent when particular enzymes deviate from the usual (about 40%) greater activity in the adults.

Therefore, enzyme activity may not actually be lower in those tissues in L3 where the enzyme is expressed; the calculated activity based on total homogenate protein may lead to false conclusions of relative activity. If the lower metabolic activity is real, it may reflect L3 being a dormant phase or that there was limited tissue expression. Alternatively, enzymes may have undergone post-translational changes, such as phosphorylation,

homogenates may contain varying amounts of regulatory nucleotides or metabolites or there may be stage-specific isoforms of the enzymes.

8.5.2. Difference between parasite species

Metabolism of lysine, proline, arginine and glutamate was generally similar in the two species. The most striking difference was the failure to detect ADC activity in both L3 and adult *T. circumcincta*, suggesting only one pathway for putrescine synthesis from arginine, compared to two in *H. contortus*. In the case of GOGAT, enzyme activity was not detectable only in L3 *H. contortus*, suggesting that ammonia cannot be assimilated into glutamate as readily in larvae of this species.

There were examples of species differences in the kinetic properties of enzymes. *H. contortus* GDH was more active with NAD^+/H than with $NADP^+/H$, whereas *T. circumcincta* GDH (homogenate and recombinant) used both co-factors with equal activity. Proline synthesis by PC5R would be more strongly favoured over proline utilisation by PO in L3 *T. circumcincta* than in *H. contortus*. The 10-fold lower K_m for P5C of P5CR in L3 *T. circumcincta* contrasts with the usual less than 2-fold difference in *H. contortus*.

8.6. Future work

This study in L3 and adult *H. contortus* and *T. circumcincta* has extended the biochemical characterisation of nitrogen metabolism in abomasal nematodes and identified enzymes as future vaccine candidates or targets for novel chemical inhibitors. To achieve these practical applications, further work is needed to obtain recombinant or purified enzymes, to establish that

they are essential to parasite survival and that they can be successfully targeted in vivo.

The systematic study of glutamate, arginine, proline and lysine metabolism in this study, along with the research of Muhamad (2006) has investigated major aspects of nitrogen metabolism in abomasal nematodes, but there are other amino acids largely unexplored. There are still many aspects of the metabolism of serine, threonine, alanine, aspartate and the phosphagens to elucidate. Research needs to extend from identifiying pathways to sequencing the genes encoding key enzymes and expressing the proteins for further study, as has been done for *Tc*GDH. Obvious candidate enzymes are ODC, arginase, arginine kinase and the unique enzymes GOGAT, creatinase and aspartase identified by Muhamad (2006), as they are not expressed in mammals. Muhamad (2006) could find no asparatase or creatinase sequences in public databases, so obtaining recombinant enzymes may prove more difficult. Species differences in polyamine synthesis can be clarified from the substrate specificities of recombinant ODC and arginase and establishing whether one or two enzymes involved in ODC/ADC and arginase/agmatinase activities.

Before in vivo testing of the potential of enzymes as antigens, there are in vitro experiments which are useful for initial screening of protective antigens. An important step it is to determine which enzymes are essential for worm survival. RNA interference (RNAi) is being successfully used to knockout potential target genes, but this technique is not as straight-forward in parasitic nematodes as it is in *C. elegans* (Knox et al., 2007). Alternatively specific chemical inhibitors can be tested in short-term in vitro worm culture. As enzymes on the surface of the parasite or on its brush border are believed to be more accessible to vaccines (Skuce et al., 1999), the location of enzymes in specific tissues can be established through in situ hybridization or immunocytochemistry. Screening with host serum and mucosal antibodies can establish in vitro that the enzyme is antigenically distinct, e.g. *H. contortus* GDH is strongly recognised by the host (Skuce et al., 1999).

The ultimate assessment of enzymes as protective antigens must be carried out in vivo after preliminary in vitro screening. Vaccine development against parasitic helminths which have limited invasion of host tissues is particularly challenging. Species and life-cycle differences, such as those seen in nitrogen metabolism in *H. contortus* and *T. circumcincta* add to the difficulty in selecting suitable antigens. A protective antigen should be essential for worm growth and survival, antigenically distinct from those in host and the gut microbiota, but shared by several parasite species, accessible to host antibodies and provoke an antibody response, which is protective, not merely recognition of a foreign protein.

Vaccine development has revolutionised disease treatment and despite the challenges of producing vaccines against luminal gastro-intestinal nematodes, the current commercial antiparasite vaccines (Vercruysse et al., 2004) give cause for optimism that ruminant parasites will ultimately be controlled in this way. A second application may be the development of novel chemical therapies based on screening drug libraries against the purified protein.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Sallah Umair

Name/Title of Principal Supervisor: Prof. Heather Simpson

Name of Published Research Output and full reference:

Glutamate synthase, but not GABA shunt enzymes, contributes to nitrogen metabolism of the sheep abomasal nematode parasites Haemonchus contortus and Teladorsagia circumcincta

In which Chapter is the Published Work: 2

Please indicate either:

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  and / or

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  All experiments were performed by S Umair. Supervisors helped with the experimental design and writing.

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Sallah Umair

Name/Title of Principal Supervisor: Prof. Heather Simpson

Name of Published Research Output and full reference:

Molecular and biochemical characterisation of a Teladorsagia circumcincta glutamate dehydrogenase

In which Chapter is the Published Work: 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  All experiments were performed by S Umair. Supervisors helped with the experimental design and writing. ML Patchett assisted with the protein sequence alignment and J Knight with the protein expression.

Digitally signed by Salle
DN: cn=Salle, o=Massey University, ou=Umair, email=s.umair@massey.ac.nz, c=NZ
Date: 2012.03.27 17:06:32 +13'00'

Candidate's Signature

27.03.2012
Date

Heather Simpson

Digitally signed by Heather Simpson
DN: cn=Heather Simpson, o=Massey University, ou=IVABS, email=H.V.Simpson@masey.ac.nz, c=NZ
Date: 2012.03.27 15:10:24 +13'00'

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Name of Candidate: Sallah Umair

Name/Title of Principal Supervisor: Prof. Heather Simpson

Name of Published Research Output and full reference:

Nucleotide allosteric regulation of the glutamate dehydrogenases of Teladorsagia circumcincta and Haemonchus contortus

In which Chapter is the Published Work: 4

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Date: 2012.03.27 17:07:09 +13'00'

Candidate's Signature

27.03.2012
Date

Heather Simpson
Digitally signed by Heather Simpson
DN: cn=Heather Simpson, o=Massey University, ou=IVABS, email=H.V.Simpson@masey.ac.nz, c=NZ
Date: 2012.03.27 15:11:37 +13'00'

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Name of Candidate: Sallah Umair

Name/Title of Principal Supervisor: Prof. Heather Simpson

Name of Published Research Output and full reference:

Enzymes of the ornithine-proline-glutamate pathway in the sheep abomasal nematode parasites Haemonchus contortus and Teladorsagia circumcincta

In which Chapter is the Published Work: 5

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  All experiments were performed by S Umair. Supervisors helped with the experimental design and writing. Y-M Leung helped with OAT and P5CS assays.

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DN: cn=Salle, o=Massey University, ou=Umair, email=s.umair@massey.ac.nz, c=NZ
Date: 2012.03.27 17:08:03 +13'00'

Candidate's Signature

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Date

Heather Simpson
Digitally signed by Heather Simpson
DN: cn=Heather Simpson, o=Massey University, ou=IVABS, email=H.V.Simpson@masey.ac.nz, c=NZ
Date: 2012.03.27 15:45:53 +13'00'

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Name of Candidate: Sallah Umair

Name/Title of Principal Supervisor: Prof. Heather Simpson

Name of Published Research Output and full reference:

Arginine metabolism in the sheep abomasal nematode parasites Haemonchus contortus and Teladorsagia circumcincta

In which Chapter is the Published Work: 6

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Date: 2012.03.27 17:08:22 +13'00'

Candidate's Signature

27.03.2012
Date

Heather Simpson

Digitally signed by Heather Simpson
DN: cn=Heather Simpson, o=Massey University, ou=IVABS, email=H.V.Simpson@masey.ac.nz, c=NZ
Date: 2012.03.27 15:47:15 +13'00'

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Name of Candidate: Sallah Umair

Name/Title of Principal Supervisor: Prof. Heather Simpson

Name of Published Research Output and full reference:

Lysine catabolism in Haemonchus contortus and Teladorsagia circumcincta

In which Chapter is the Published Work: 7

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  All experiments were performed by S Umair. Supervisors helped with the experimental design and writing.

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Date: 2012.03.27 17:08:45 +13'00'

Candidate's Signature

27.03.2012
Date

Heather Simpson

Digitally signed by Heather Simpson
DN: cn=Heather Simpson, o=Massey University, ou=IVABS, email=H.V.Simpson@masey.ac.nz, c=NZ
Date: 2012.03.27 15:47:53 +13'00'

Principal Supervisor's signature

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APPENDIX 2: PARASITOLOGY

2.1. ANIMALS

Two to four months old male sheep (Romney and Romney-cross) were housed indoors in pens and fed with *ad libitum* leucerne chaff and water. Sheep were dewormed with a double dose of Matrix (1 ml/2.5 kg, body weight) and a single dose (1 ml/5kg) the next day. The parasite-stage free stage was controlled via faecal egg count (FEC). Parasite free animals were infected with either 10,000 *H. contortus* L3 or 50,000 *T. circumcincta* L3.

2.2. LARVAL CULTURE

Fresh larval culture were maintained by infecting sheep every 3 months for fresh larvae cultures of *H. contortus* and *T. circumcincta.* Sheep were cleaned from worms by drenching them with the matrix @ 1 ml/5 kg. Three weeks after drenching, sheep were checked (by microscopic faecal egg count) for worms and the sheep with zero FEC were infected with 10,000 L3 for *H. contortus* or 50,000 L3 for *T. circumcincta.* Faeces were collected in faecal bags after 3 weeks on a daily basis for about 10 days, mixed with vermiculate (Grade Fine IVL2, Nuplex Industries Ltd), moistened and mixed daily for 10-12 days at 25-27 °C.

L3 were recovered by Baermann technique. The faeces were placed in sieves with a tissue paper lining and placed in bowls filled with RO water for 24h. Water from the bowl was then passed through a 20 µm sieve. The larvae were again placed in a sieve placed over a funnel with single layer of tissue paper for 24h. Larvae were then collected and stored in RO water at 10 °C for *H. contortus* and at 4 °C for *T. circumcincta.*

2.3. FAECAL EGG COUNT

The method was the modification of the McMaster method. 2g of faeces were passed through a small sieve into 30 ml 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.

2.4. 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 cylinder.

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 twice the volume of worm mixture (final concentration 1% agar) and immediately poured into trays to set. Saline warmed to 37 °C was carefully poured over the agar blocks until they were completely covered. The worms migrated

Sheep were killed and ligated abomasa were immediately brought to the laboratory. It was cut open with scissors and washed with 0.9% NaCl and all abomasal contents were collected in a big beaker. 3% solution of agar (Bacto Agar, DIFCO Laboratories, USA) was made by heating 30 g agar in 1L of 0.9% NaCl in microwave for about 8 min, cooled to 40-50 °C and added to the double volume of worm mixture. The total mixture is mixed well and poured into the trays, allowed to be cooled at 37 °C to make agar blocks. NaCl solution is poured onto those blocks so that worms can swim out of solidified blocks and form clumps. Clumped worms were used for enzyme assays.

APPENDIX 3: BIOCHEMISTRY

3.1. PROTEIN MICROASSAY

The assay was based on the method of Bradford (1976). Test tubes were set up in duplicates. Negative control contained 0.2 ml incubation medium, standard solutions were 5-100 µg bovine serum albumin (BSA) in incubation medium while sample was 0.2 ml. 0.8 ml of Bradford reagent (Sigma) was added to each tube, mixed and left for 10 min. Absorbance of the tubes were read at 595 nm against the negative control. A standard curve was plotted to get the protein concentrations.

3.2. PREPARATION OF HOMOGENATES

(a) About 50,000 L3 *T. circumcincta* or *H. contortus* were centrifuged at 4000 rpm for 5 min in a bench centrifuge and then resuspended in assay buffer and centrifuged again. The supernatant is removed and 1 ml concentrated L3 suspension was transferred to a already chilled mortar and kept in —20 °C freezer for 15 min. The frozen L3 were then crushed by using a chilled pestle and the homogenate formed was then transferred to ependorff tube and was used soon after or stored on ice.

(b) After recovery from the sheep, about 2 clumps roughly weighing about 5 mg of adult *H. contortus* or *T*. *circumcincta* were suspended in about 1 ml of the assay buffer. The worms were then transferred to a chilled mortar and homogenised as for L3.

(c) A small portion of brain was taken soon after the sheep was killed and immediately brought to the laboratory. It was washed once with the assay buffer and then homogenized with ice-cold solutions of 0.32 M sucrose and 4.5 mM 2-mercaptoethanol in a ratio of 10% w/v. One part of the homogenate prepared is mixed with 3 parts of ice cold triton medium, which is prepared as 50mM Tris-HCl, pH 8.5, 4.5 mM 2-mercaptoethanol and 0.67% w/v triton X-100. The mixture is

kept in ice for 1h and then homogenate containing 50 µg was used for enzyme assay.

(d) A small portion of liver was brought to the laboratory soon after the sheep was killed, placed on a filter paper, weighed and homogenized with 0.1% cetyltrimethylammonium bromide in a w/v ratio of 1:9. Homogenate was placed on ice in a plastic tube and homogenate containing 50 µg was used for each assay.

3.3. PHOSPHATE BUFFER

100 mM phosphate buffer was prepared by dissolving 17.8 g $Na_2HPO_4.2H_2O$ in 900 ml RO water, adjusting the required pH with HCl and making up to 1 L.

3.4. TRIS BUFFER

To prepare 100 mM Tris buffer, dissolve 157.8 g Tris-HCl in 900 ml RO water and solution was brought to the required pH by NaOH and finally made it to 1 L.

3.5. Citrulline Estimation Assay (Boyde and Rahmatullah 1980)

The freshly prepared chromogenic solution was used in the test and was prepared as;

Reagent 1. (acid-ferric solution) was prepared by adding 250 ml of 96% concentrated sulphuric acid and 200 ml of 85% concentrated nitric acid to 550 ml water and 250 mg of $FeCl_3$ was added on cooling.

Reagent 2. (diacetyl monoxime solution) was prepared by adding 500 mg of diacetyl monoxime to 100 ml water and solution was placed in aluminium foil covered bottles.

Reagent 3. 5 mg of thiosemicarbazide.

Chromogenic reagent was made by mixing 100 ml of reagent 1 with 50 ml reagent 2 and reagent 3. After incubation for 30 min, 5% trichloroacetic acid was added to reaction tubes to stop the reaction. The tubes were then centrifuged at

4000 *g* for 5 min. 0.1 ml of supernatant was added to 3 ml chromogenic solution, mixed well and heated at 100 °C for 5 min. The tubes were then cooled to room temperature under running water and read at 530 nm spectrophotometrically against the blank.

3.6. Ornithine Estimation Assay (Mia and Kogar 1978)

3 ml ninhydrin reagent (750 mg ninhydrin reagent in 91 ml glacial acetic acid, 7.9 ml water and 1.1 ml 85% phosphoric acid) was added to all tubes containing reaction mixture and homogenate containing 50 µg protein was added to blank tube only. Tubes were placed at 95 °C for 15 min for colour development, cooled to room temperature under running tap water. Absorbance of test sample was read against blank at 515 nm spectrophotometrically.

3.7. Urea Estimation Assay (Geyer and Dabich 1971)

Tubes containing reaction mixture were centrifuged at 5000 *g* for 5 min and 0.1 ml supernatant was taken and mixed with 3 ml acid reagent (0.12M $FeCl_3$ in 56.7% H_3PO_4 and 999 ml of 20% H_2SO_4) and 2 ml colour reagent (61.7 mM diacetylmonoxime and 3.6 mM thiosemicarbazide) and mixed well. Homogenate containing 50 µg protein was added to blank only. Tubes were heated at 95 °C for 20 min, cooled to room temperature and read at 520nm spectrophotometrically against the blank.

3.8. Thin Layer Chromatography (Zolg and Ottow 1973)

Thin layer chromatography (TLC) technique was performed according to the method of Zolg and Ottow (1973). TLC plates pre-coated with cellulose, 20 by 20 cm and 0.1 mm, Merck, Germany were used. Plates were cut as required (6.5 x 9 cm) and 1µl of standards (10 mM ornithine, putrescine, arginine, agmatine, and arginine) along with samples. Plates were dip in 20 µl of solution of 1-butenol, acetone, diethylamine and water in a ratio of 10:10:2:5. After 2 h, developed plates were dried and sprayed with ninhydrin solution (1% in iso-propanol) and then dried with drier for about 1-2 min. The standards and samples appeared as purple spots at different points at TLC plates.

3.9. Conversion of Methaemoglobin to Oxyhaemoglobin (Murphy and Noack 1994)

Stock solution of ferrous haemoglobin was prepared by dissolving 25 mg haemoglobin to 1 ml assay buffer (50 mM TrisHCl pH 7.4) in a small flat-bottom flask. The flask was gently mixed for some time and then 1-2 mg sodium dithionite (sodium hydrosulfite) was added to the flask and gently mixed for several minutes.

The resulting bright, light red HbO2 solution was purified and desalted by passing it through a sephadex G-25 column. A 2 cm^2 area 15 cm long sephadex column was constructed by placing sephadex G-25 in the assay buffer for several hours and then carefully packing it in a glass tube having a glass wool plug. Sephadex column was washed with ample buffer volumes. The HbO_2 solution was carefully placed and allowed to enter the column without dilution followed by the buffer. HbO_2 was collected in a small glass tube and stored on ice in the dark. The purity of HbO_2 was checked by measuring its absorption at 415 nm. If the stock solution showed maximum absorbance at lower wavelength (413 nm), it was an indicative of methaemoglobin contamination, which was corrected by the addition of slightly higher quantity of sodium hydrosulfite in the initial step.