Studies on the energy metabolism of *Eimeria tenella* and other coccidia

Helen Denton

University of Glasgow

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This thesis is dedicated to Geoff Ballinger,
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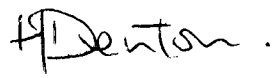
DECLARATION

I hereby declare that this thesis has been composed by myself, that the work it describes is my own, except where stated in the text, and that it has not been accepted in any previous application for a degree.



Helen Denton, 27th June 1996.

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ABBREVIATIONS

DTT	Dithiothreitol
E64	Trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane
F16P2	Fructose 1,6-bisphosphate
F26P2	Fructose 2,6-bisphosphate
F6P	Fructose 6-phosphate
fplc	Fast protein liquid chromatography
G6P	Glucose 6-phosphate
Hepes	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
Hexo.	Hexokinase
hplc	High-performance liquid chromatography
ICDH	Isocitrate dehydrogenase
K_m	Michaelis constant
n	Number of determinations
LDH	Lactate dehydrogenase
NMR	Nuclear magnetic resonance
NTP	Nucleotide triphosphate
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid-Schiff staining
PBS	Phosphate buffered saline (0.1 M)
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PK	Pyruvate kinase
PMSF	Phenylmethanesulphonyl fluoride
PPDK	Pyruvate phosphate dikinase
PP_i	Inorganic pyrophosphate
P_i	Inorganic phosphate
SD	Standard deviation
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
TCA	Tricarboxylic acid
V_{max}	Maximal (limiting) velocity

SUMMARY

The protozoan parasite *Eimeria tenella* has a complex life cycle, including phases in aerobic and essentially anaerobic environments. Previous studies had indicated that carbohydrates serve as the major energy substrate throughout the life-cycle of the parasite, but little was known about the pathways by which these are catabolised. In particular the relative dependence on aerobic versus anaerobic modes of energy production was far from clear. In this project I have undertaken an analysis of the enzymes and pathways of energy metabolism in *Eimeria*, with a particular view to determining how it is affected by, and adapted towards, the different environmental conditions encountered during its life cycle. The study has been dominated by two main approaches: characterisation of glycolytic enzymes, and analysis of end-products. In some areas it has been possible to carry out comparative studies using the related coccidial species *Cryptosporidium parvum* and *Toxoplasma gondii*.

The activities of selected enzymes of energy metabolism were measured in an attempt to determine which pathways of energy metabolism were present in the parasites, and to assess their relative significance in different species and stages. *Eimeria*, *Toxoplasma* and *Cryptosporidium* all contained high levels of the glycolytic enzymes phosphofructokinase and pyruvate kinase, as well as lactate dehydrogenase. The TCA-specific enzyme succinate dehydrogenase could not be detected in *Eimeria* or *Cryptosporidium* but was present in tachyzoites of *T. gondii*. *T. gondii* and *E. tenella* contained an $NADP^+$-specific isocitrate dehydrogenase but no NAD^+-dependent enzyme; *C. parvum* contained neither isocitrate dehydrogenase variety. The activities of pyruvate kinase and lactate dehydrogenase were significantly higher in bradyzoites than in tachyzoites of *T. gondii*, suggesting that the former may be more reliant on fermentative modes of energy generation. In *E. tenella*, the enzyme activities investigated were generally much lower in sporulated than in unsporulated oocysts, probably reflecting the dormant nature of the latter. Sporozoites contained high levels of most enzymes indicating that significant activation or synthesis of enzymes occurs during, or shortly after, excystation.

All three coccidian species were found to be unusual in containing a phosphofructokinase specific for PP_i rather than ATP. The equivalent ATP-dependent enzyme was not present. Pyrophosphate-dependent phosphofructokinases have been found in a number of micro-organisms, most of which are fermentative in their energy metabolism, and are believed to represent adaptations towards anaerobiosis. Analysis of the phosphofructokinases of *E. tenella* and *C. parvum* in crude parasite extracts revealed properties similar to those associated with pyrophosphate-phosphofructokinases from other micro-organisms. Most significantly both enzymes displayed simple Michaelis-Menton kinetics with respect to both of their substrates and were not affected by fructose-2,6-bisphosphate, the major allosteric regulator of ATP-phosphofructokinases. The eimerian enzyme appeared to catalyse a readily reversible reaction and there was no evidence for a separate gluconeogenic activity. Pyrophosphatase activity could not be detected in any of the coccidial lysates which is similar to the situation in other organisms using pyrophosphate-dependent enzymes and consistent with the use of this molecule as an energy donor.

Unlike some other organisms that have a pyrophosphate-phosphofructokinases, the coccidia were found to possess an ADP-linked pyruvate kinase activity rather than one utilising pyrophosphate. The pyruvate kinase of all three parasites required one divalent and one monovalent metal ion for full activity, with Mg^{2+} and K^+ representing the optimal combination. The *Cryptosporidium* pyruvate kinase differed from those in the other two parasites in requiring the presence of DTT for full activity; this feature may reflect a fundamental difference in the catalytic mechanism of the enzyme. Each of the coccidian pyruvate kinases presented simple hyperbolic saturation kinetics with respect to ADP, but the eimerian and *Toxoplasma* enzymes presented strongly sigmoidal saturation curves with phosphoenolpyruvate as the variable substrate. A number of compounds were found to have some ability to activate the enzymes when tested at 1 mM concentration. The most effective effectors identified were: for the *Eimeria* enzyme (in order of potency), glucose 6-phosphate, fructose 6-phosphate and AMP; and for the *Toxoplasma* enzyme, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate. Glucose 6-phosphate, the most potent activator in each case, had a K_{ac} of 20-120 μM. These are unusual sets of effectors: in most eukaryotes fructose 1,6-bisphosphate is the main regulator of pyruvate kinase activity, being effective at μM

concentrations. It appears likely that the relative lack of effect of this compound on the coccidian pyruvate kinases relates to the lack of regulation at phosphofructokinase, which renders fructose 1,6-bisphosphate (as its product) a less useful feed-forward indicator of glycolytic load. Unlike the enzymes in *Eimeria* and *Toxoplasma*, the pyruvate kinase from *C. parvum* exhibited no evidence of allosteric properties and presented a simple hyperbolic saturation curve with respect to PEP. No allosteric modulators of this enzyme could be identified although its product, ATP, appeared to be a competitive inhibitor of the reaction. This is one of only very few eukaryotic pyruvate kinases known not to be subject to allosteric regulation.

Hexokinase was also investigated as a potential regulatory enzyme of glycolysis in *Eimeria*. This enzyme is of particular interest because it plays a role not only in glycolysis, but also in the recently identified mannitol cycle in the coccidia. The enzyme had to be partially purified prior to kinetic analysis to remove apparent ATPase activity, which interfered with the coupled assay system. During fractionation, both gluco- and fructo- kinase activities were found to co-purify, implying that the presence of only one hexokinase activity that was capable of phosphorylating either glucose (as in glycolysis), or fructose (as in the mannitol cycle). Results from experiments using mixed substrates were consistent with this conclusion, however the enzyme had a significantly greater affinity for glucose than for fructose. Analyses suggested that, unlike most mammalian hexokinases, the eimerian enzyme is not subject to allosteric inhibition by its phosphorylated products glucose 6-phosphate and fructose 6-phosphate; hence entry of glucose into glycolysis is probably not controlled at this level.

Studies using ^{13}C-NMR showed that *E. tenella* sporozoites catabolised glucose to lactate, acetate and glycerol. Acetate had not previously been identified as a product of eimerian energy metabolism. Experiments using selectively labelled (C1 or C6) glucose indicated that the carbons were completely randomised into the detected products - probably reflecting high triose phosphate isomerase activity. Mannitol was the only detectable derivative of glucose detected within the sporozoites following incubations. The apparent ability of the cells to convert glucose to mannitol further confirms the existence of the mannitol cycle, and also the direction of its operation in this stage of the parasite. Quantitative analysis of the products of glucose catabolism by sporozoites was

carried out using a combination of enzymatic assays and hplc techniques. The results showed that the parasites maintain a relatively high rate of lactate production even under aerobic conditions. This implies a fairly low potential for full aerobic respiration and indicates that the parasites might indeed be geared towards an anaerobic mode of life. This would accord with the specific adaptation represented by the pyrophosphate-phosphofructokinase and may reflect the low oxygen tensions encountered in the host gut. The parasites did not consume amino acids from medium either in the presence or absence of glucose, implying that these are not major substrates for either energy production or other functions. Some amino acids were found to be released during sporozoite incubations. Exogenous mannitol was not consumed.

Metabolite concentrations in sporulated and unsporulated oocysts were compared in an attempt to gain insight into the metabolic processes fuelling sporulation. Both stages contained comparable levels of lactate, glycerol and acetate, while sporulated oocysts also contained apparently high levels of an another organic acid whose identity could not be ascertained - (the compound presented unusual physical properties which have hampered its identification by physico-chemical techniques). The profile of free amino acids in the oocysts underwent defined changes during sporulation, with an overall decrease in the concentration of all amino acids. Several non-standard amino compounds were also detected in oocyst extracts, some of these were also released during sporozoite incubations. One of these compounds was tentatively identified as γ-aminobutyrate, the rest remain unidentified.

Investigations were also made into proteinase activities in *Eimeria tenella*. These studies were complicated by the fact that high concentrations of trypsin are used in the procedures for oocyst isolation and excystation. This trypsin could not be effectively removed from the cells by washing and interfered with many types of proteinase analysis. That parasite-specific proteinases do exist was indicated by the rapid changes in protein profile of crude extracts upon incubation at 41°C. This protein degradation was only marginally affected by the presence of trypsin inhibitor, but could be prevented by the inclusion of other specific proteinase inhibitors in the incubation medium. The most effective inhibition was achieved by a combination of PMSF and E64, which are inhibitors of serine and cysteine-type proteinases. Despite these results,

no proteinase activity (apart from trypsin) could be detected in *Eimeria* lysates using the standard procedures of substrate SDS-PAGE (attempted under a comprehensive range of conditions). This suggests that the proteinases of *Eimeria* must have either an unusually narrow substrate-specificity, or be of very low activity (or both). Investigations were therefore continued using alternative techniques which do not depend on the direct detection of proteolytic activity. Biotinylated inhibitors with specificity for cysteine-type proteinases reacted with a single protein of approximately 46 kDa present in unsporulated oocyst extract. The presence of E64 considerably reduced the intensity of the binding signal, thus confirming the specificity of the reaction. A band of similar molecular weight was also detected in unsporulated oocyst extracts by Western blotting with antisera against a leishmanial cysteine proteinase with homology to cathepsin L. The protein was not detected in sporozoite extracts suggesting that *Eimeria* might show stage-specificity of proteinase activity.

1. INTRODUCTION

1.1 THE COCCIDIA:

1.1.1 Taxonomy and phylogeny

The term 'coccidia', which is often used imprecisely, refers most correctly to members of the sub-order Eimeriina within the phylum Apicomplexa (Levine, 1973). The group contains, in current classifications, 10 families, 37 genera and some 1,500 named species (Levine, 1982). The taxonomic classification of the group is illustrated in Figure 1.1. Like all members of the Apicomplexa, the coccidia are parasitic and include within their numbers several pathogens of medical and veterinary importance. These are contained within three main families: Eimeriidae (which includes the genera *Eimeria* and *Isospora*); Sarcocystidae (containing *Sarcocystis* and *Toxoplasma*); and Cryptosporidiidae (containing *Cryptosporidium* alone). Together these genera constitute the much studied 'true-coccidia'. Other families within the sub-order contain no notable pathogens and as a consequence receive little scientific attention.

The coccidia are grouped among the Apicomplexa by virtue of their possession of ultrastructural components comprising the apical complex. Beyond this their detailed classification has been based upon a variety of morphological and biological features including host and tissue specificities. Although their current classification is the product of many years revision and refinement it remains, in the words of Levine *et al.* (1980) "one of convenience [which] does not necessarily indicate evolutionary relationships". Recently there have been a number of attempts to assess the evolutionary

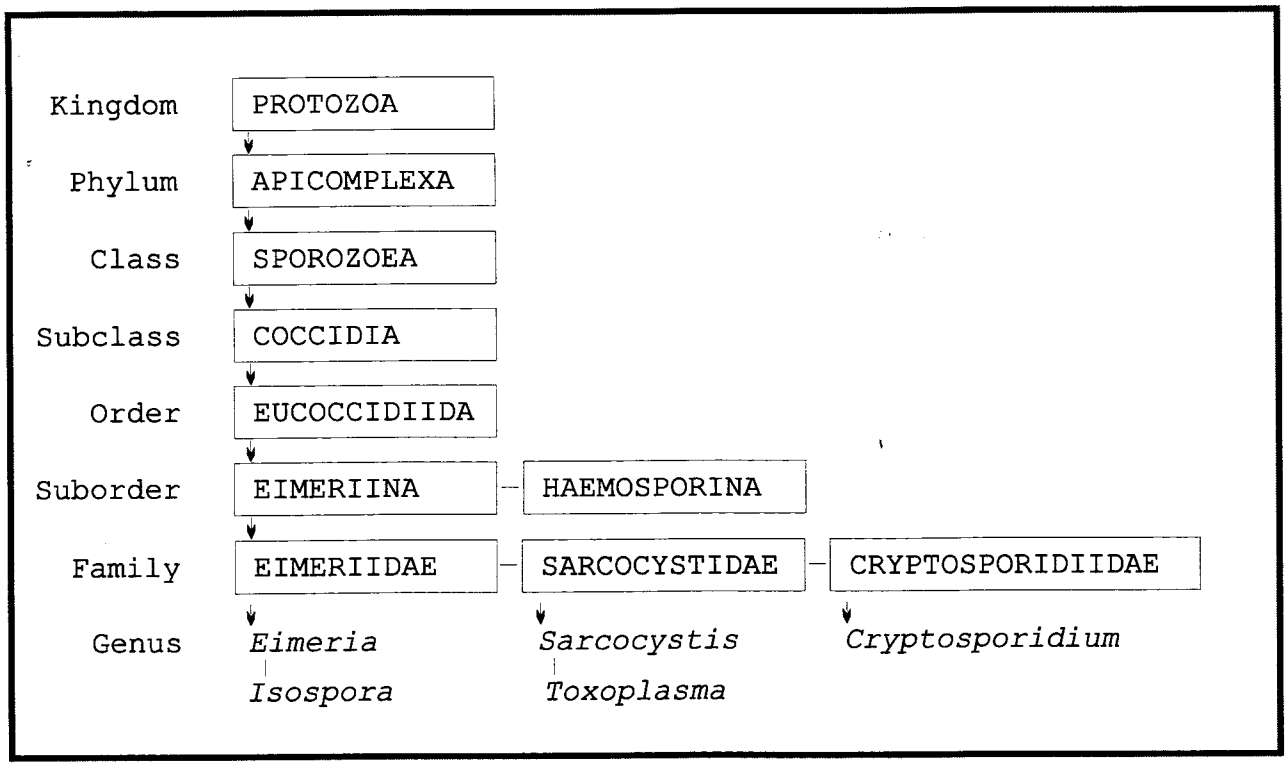

Figure 1.1. Taxonomy of the coccidia.

relationships of the Apicomplexa and coccidia using small ribosomal RNA (srRNA) sequences (Fenger *et al.*, 1994; Gagnon *et al.*, 1993; Johnson and Baverstock, 1989; Johnson *et al.*, 1990; Barta *et al.*, 1991; Wolters, 1991; Escalante and Ayala, 1995). In general these analyses agree with the pre-existing taxonomic classifications, supporting both the monophyly of the Apicomplexa and the coherence of the coccidia as a group within the phylum. The precise relationships within the group, however, remain controversial, and it is probably beyond the scope of molecular analyses to resolve them completely. There is one point on which the molecular analyses show significant and interesting deviation from the previously accepted taxonomic status. This is on the placing of *Cryptosporidium* species. On the basis of srRNA data, members of this genus are consistently placed outside the main coccidia group (Barta *et al.*, 1991; Escalante and Ayala, 1995) and even, on one analysis, outside the Apicomplexa altogether (Johnson *et al.*, 1990). This is interesting in view of the fact that *Cryptosporidium* species show marked morphological differences from other species of coccidia (see Section 1.1.3) and suggests that the classification of this group should be reconsidered.

While many of the branches within the Apicomplexa are very deep, the phylum itself represents a relatively recent branch of the Eucarya. In modern phylogenetic trees (see for example Figure 1.2) it is shown, juxtaposed between the plants and the fungi, among the recently diverged 'crown groups' (Embley *et al.*, 1994). As such the coccidia are probably more closely related to man than they are to some of the more 'primitive' parasitic protozoa such as *Giardia* or the trichomonads (Johnson and Baverstock, 1989). This fact, and the other evolutionary relationships discussed above, may be worth bearing in mind when considering the morphological and biochemical characteristics of the coccidia discussed later.

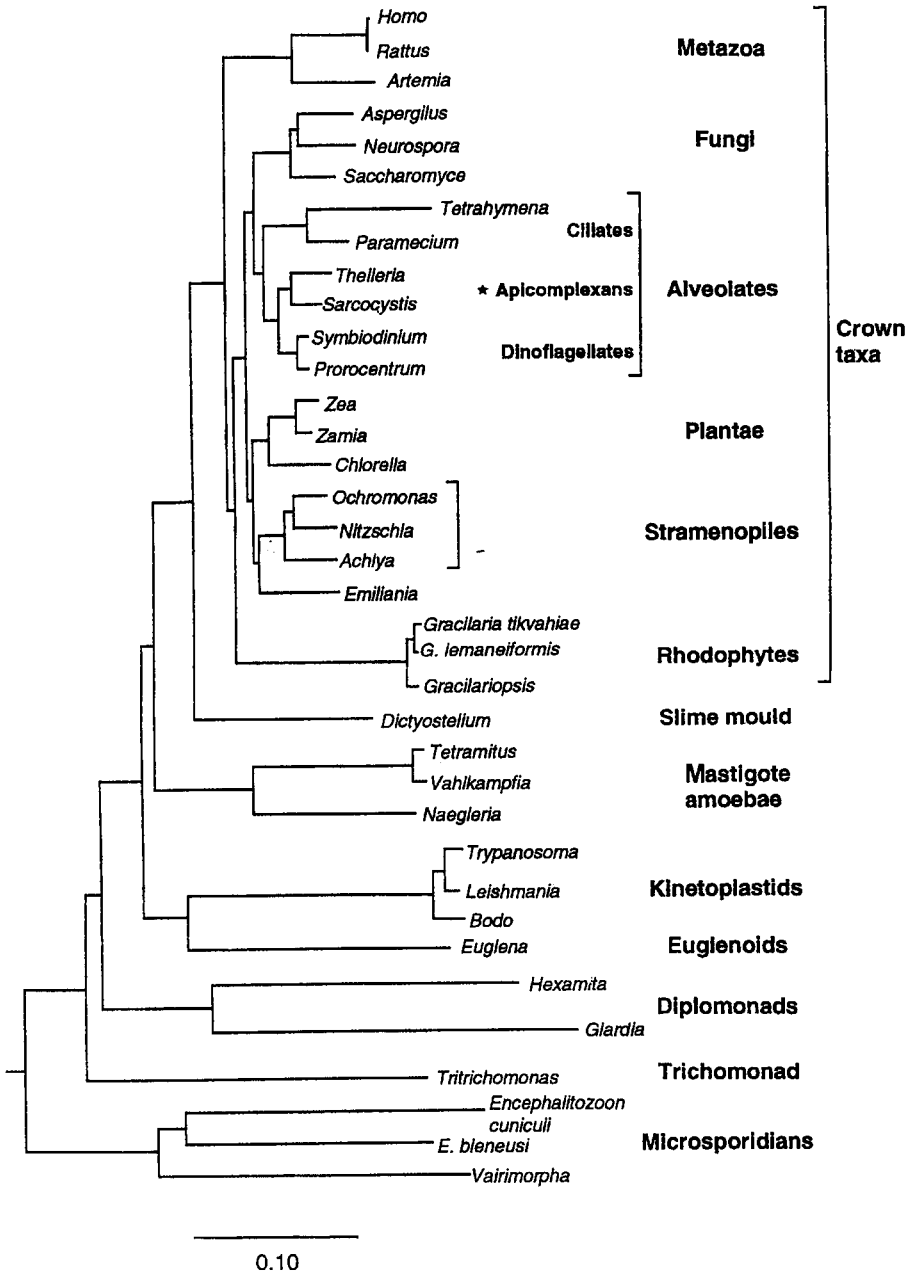

Figure 1.2. Relationships among a representative sample of the Eucarya based upon small subunit ribosomal RNA sequence comparisons. Adapted from Embley *et al.* (1994).

1.1.2 Life cycles

The life cycles of all the coccidia contain three distinct multiplicative phases: schizogony, gametogony and sporogony. These are exemplified in the life cycle of the chicken parasite *Eimeria tenella* shown in Figure 1.3; the numbers in the following text refer to this figure.

Infection by *E. tenella* is initiated when a bird ingests sporulated oocysts [24] present in food or drink. The infective stages of the parasite, the sporozoites, are released from the oocyst in the host gut in a two stage process called excystation. Firstly mechanical grinding in the host gizzard disrupts the oocyst wall freeing the sporocysts, then proteolytic enzymes and bile in the small intestine combine to remove the Steidae body and stimulate the sporozoites to escape from the sporocyst. Once free, the motile banana-shaped sporozoites [1] make their way through the intestine to the caecum, where they pass through the basement membrane and lamina propria to invade epithelial cells within the glands of Leiberkuhn [2]. Here, lying below the cell nucleus and within a parasitophorous vacuole, they initiate a form of asexual reproduction (merogony or schizogony) [3] which gives rise to 900 or so first-generation merozoites [4]. When mature, the merozoites break out of the host cell [5] and move off to invade surrounding epithelial cells [6] where they become second generation schizonts [7 and 8]. These produce some 200-350 larger, second-generation merozoites [9 and 10] which in turn break out invade new host intestinal epithelial cells. Some of the merozoites initiate a third round of schizogony [12 and 13], producing third generation merozoites [14 and 15] which are in turn released and invade cells. These third generation merozoites, along

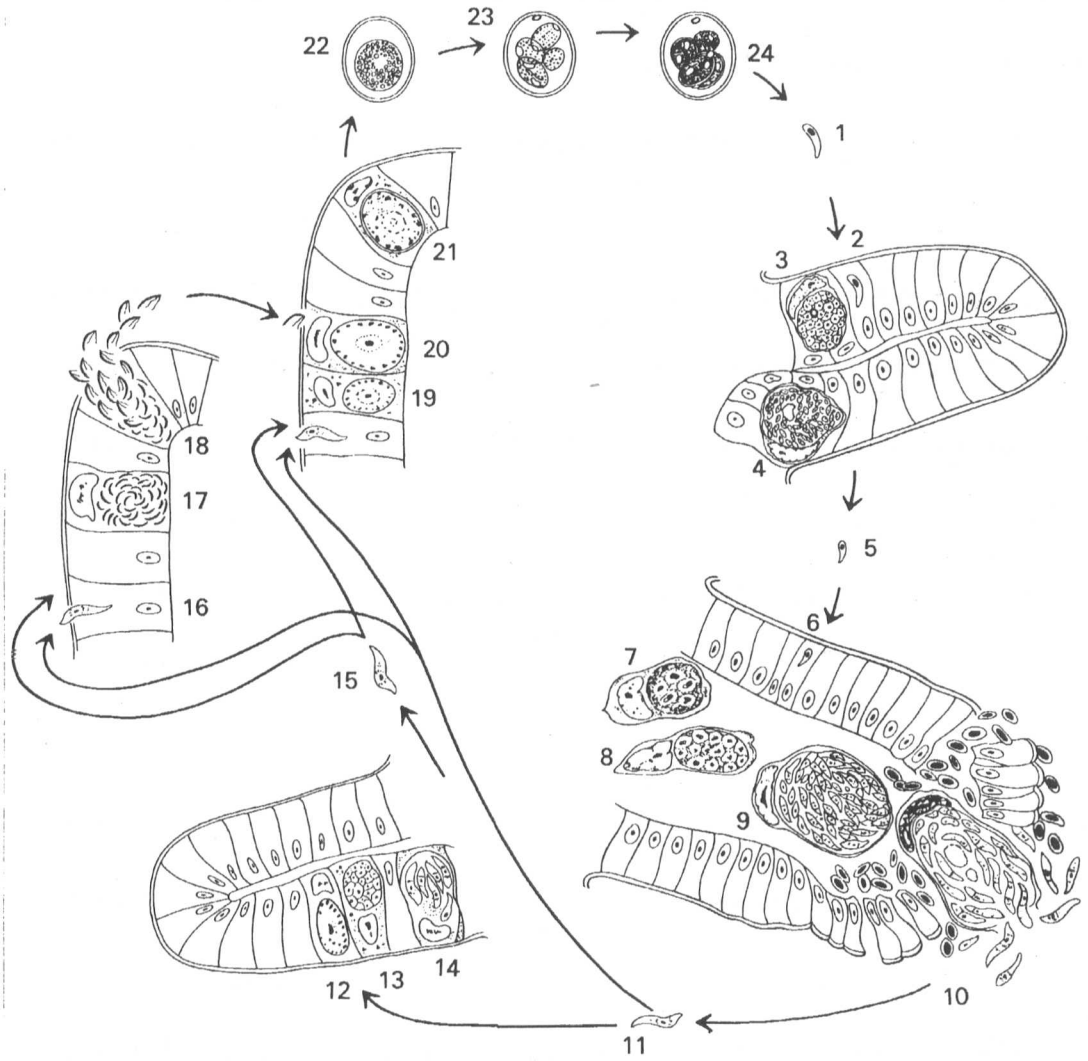

Figure 1.3. Life cycle of the chicken coccidian *Eimeria tenella*. Details are given in the text. Adapted from Schmidt and Roberts (1989).

with the great majority of the second generation merozoites [11], now initiate the sexual stage of the life cycle (gametogony). Some become micro (male) gametocytes [16 and 17] which produce a large number of biflagellate micro gametocytes [18]. The rest develop into macro (female) gametes [19 and 20]. The macrogametes are fertilised by the microgametes within the host cells to form zygotes [21] which lay down a heavy wall around themselves and become young oocysts. The oocysts develop and are eventually released from the host cells and pass out of the gut in the faeces [22].

At this point the oocysts contain only a single cell. In response to oxygen and the lower temperatures within the environment the sporont throws off a polar body and forms four sporoblasts [23] each of which forms a sporocyst containing two sporozoites. This process is known as sporulation, and the resultant sporulated oocyst [24] is ready to initiate another life cycle. Sporulated oocysts are highly resistant to desiccation and may remain viable for many months in the environment.

The life cycles of the coccidia differ primarily in terms of host and site specificity. While all species of *Eimeria* are extremely specific, both in terms of hosts and sites of infection, most coccidian species are more indiscriminate. In *Cryptosporidium,* for example, only five distinct species are recognised, however these are all able to cross host-boundaries with 20 or more host species known to be infected (Levine, 1984; Current, 1989; Sterling and Arrowood, 1993).

Other coccidia, including *Toxoplasma gondii* have a heteroxenous life cycle with different stages of the life cycle being played out in different hosts. The definitive host of *T. gondii* is the domestic cat, while a wide range of mammals (including humans) and

some birds may act as intermediate hosts. Two forms of asexual reproduction may occur in the intermediate hosts: a rapid form, involving the production of tachyzoites (fast growing zoites); or a much slower form leading to production of bradyzoites (slow growing zoites) (Schmidt and Roberts, 1989). The latter are contained within a cyst-like structure and are considered to be a latent phase.

The life cycles of these, and other coccidia, are described in detail elsewhere (see for example, Levine; 1982; Ball *et al.*, 1989; Schmidt and Roberts, 1989).

1.1.3 Ultrastructure

The morphology of a typical *Eimeria* sporozoite is shown in Figure 1.4.; the ultrastructural features are fairly representative of equivalent developmental forms in other coccidia. In addition to the conventional eukaryotic apparatus the parasite possesses a number of unusual ultrastructural features (Chobotar and Scholtyseck, 1982). The most distinctive of these, and the defining characteristic of the Apicomplexa, is the apical complex, comprising the rhoptries, conoid, micronemes and polar ring. This assembly is commonly considered to be involved in the process of cell invasion although this function is not yet proven (Dubremetz and Schwartzman, 1993; Jensen and Edgar, 1976; Perkins, 1992). Also typical are the minute invaginations in the pellicle known as micropores. These are found in other most other members of the Apicomplexa as well as among the Dinoflagellates and are thought to involved in ingestion of food during the intracellular stage of the parasites (Scholtyseck and Mehlhorn, 1970).

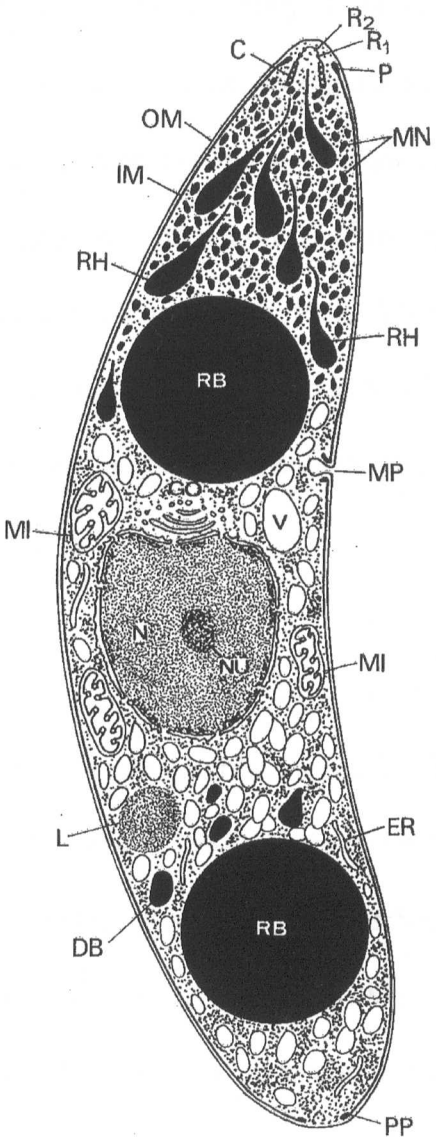

Figure 1.4. ***Eimeria ninakohlyakimovae*: Diagram of a sporozoite to show the principal fine structures.** C, conoid; DB, dark bodies; ER, endoplasmic reticulum; IM, inner membranous complex; L, lipid inclusion; MI, mitochondrion; MN, micronemes; MP, micropore; N, nucleus; NU, nucleolus; OM, outer membrane; P, polar ring; PP, posterior polar ring; $R_{1,2}$, preconoidal rings; RB, refractile bodies; RH, rhoptries; V, vesicle. From Scholtyseck (1979).

The most prominent and distinctive structures in *Eimeria* sporozoites are the refractile bodies, positioned on either side of the nucleus. These are electron-dense, homogeneous organelles with no apparent sub-structure. They disappear in later stages of the life cycle (Augustine *et al.*; 1988; Abrahamsen *et al.*, 1994) and are not present in all members of the coccidia. In some species, such as *Cryptosporidium* and *Sarcocystis*, they appear to be replaced by smaller organelles with a lattice-like internal structure and are known as crystalloid bodies (Chobotar and Scholtyseck, 1982). There is no evidence regarding the function of either of these organelles, although it has been suggested that they may constitute energy reserves for the parasite (Roberts and Hammond, 1970; Hammond, 1973; Trefiak and Desser, 1973).

Mitochondria appear to be a normal feature of the coccidia. In *Eimeria* they are present in all developmental forms and are described as having a normal, cristate, appearance consistent with functionality (Wang, 1982). *Cryptosporidium* appears to be exceptional within the group in that structurally identifiable mitochondria are absent from most, if not all, species (Vetterling *et al.*, 1971; Current, 1989; Tetley, L., Brown, S.M.A. and Coombs, G.H., unpublished data). The genus is also unusual in lacking micropores and the subpellicular tubules common to other coccidia (Vetterling *et al.*, 1971).

Polysaccharide granules and lipid droplets have been described in most members of the coccidia and generally appear to be differentially built up and consumed throughout their life cycles. Their function is discussed in Section 1.2.1.1.1.

For detailed reviews of the ultrastructure of the coccidia see: Roberts and Hammond, 1970; Scholtyseck, 1973; Scholtyseck, 1979; Chobotar and Scholtyseck, 1982.

1.1.4 Pathology and Pathogenesis

Until recently the coccidia were considered of importance primarily as the aetological agents of veterinary diseases. The genus *Eimeria*, which contains the economically important poultry parasites such as *E. tenella,* attracted the most attention and was quite extensively studied. More recently other coccidian parasites have been recognised as important opportunistic parasites in immunocompromised hosts, including humans. Considerable attention is now focused on *Toxoplasma gondii* and *Cryptosporidium parvum* which cause life-threatening complications in many patients with acquired immunodeficiency syndrome (AIDS) (Ambroise-Thomas and Pelloux, 1993; Cook, 1990; Sterling and Arrowood, 1993). The increased interest in these parasites has also led to their further recognition as a source of disease in many immunocompetent hosts.

The symptoms of the coccidioses differ, being largely dependent on the tissue specificity of the infecting species (Long, 1973; Fernando, 1982). Intestinal infections, such as those caused by *Eimeria tenella* and *Cryptosporidium*, are frequently characterised by diarrhoea which, with its associated plasma electrolyte changes and dehydration, is the final cause of death in many infections. Other symptoms associated with *Eimeria* include, anorexia, anaemia and intestinal haemorrhage: even if infection does not kill a bird, it leads to general thriftiness and consequent loss of productivity for the farmer. Symptoms of species such as *T. gondii*, which infect multiple tissues are, not surprisingly, diverse.

The pathogenic mechanisms underlying the symptoms of coccidiosis are poorly understood (Fernando, 1982). The lesions and haemorrhage which characterise many

infections are probably the result of direct physical damage by the parasite, thought to be inflicted particularly during the late growth phases of gametogony and schizogony. In contrast, the more general villous atrophy associated with intestinal disease appears to be the consequence of host-mediated hypersensitivity reactions (type I). In *Eimeria* infections it has been reported that host intestinal absorption is impaired and this probably accounts, at least partly, for the weight loss associated with the disease. The cause of the diarrhoea is unknown although there is speculation that it may be mediated by specific parasite toxins.

Since most coccidia possess a self-limiting life cycle, terminating in oocyst production, the severity of a coccidial infection is partly dictated by the number of sporulated oocysts originally ingested. This explains the severity of the coccidia problems in modern farming conditions, where intensive rearing permits the build-up of high doses of infective oocysts in the environment. The outcome of the disease is also heavily influenced by the immune status and genetic makeup of the infected individual (Fernando, 1982).

Although methods exist for controlling most coccidial diseases, none have yet proved fully reliable. *Eimeria* infections have been successfully treated (or prophylactically prevented) with a number of compounds over the years (see Section 1.2.3 for details), however the intensive use of many of these has led to the rapid rise of drug-resistant strains within the parasite population (Chapman, 1982 and 1993). The resistance problem is such that the development of new chemo- or immunotherapeutic strategies remains a constant priority. Attempts to develop such therapies have so far been hampered by our poor understanding of the detailed biology and biochemistry of the

coccidia. More impetus in these areas is the most likely way of providing answers to the current problems. Furthermore, elucidation of the mechanisms of action and resistance of current drugs (see Section 1.2.3) may lead to novel chemical strategies for obtaining the new anticoccidial drugs that are urgently required.

1.2 BIOCHEMICAL ASPECTS OF THE COCCIDIA

The following section constitutes a review of some biochemical aspects of the coccidia relevant to my project, and is mainly based on the literature available at the start of my project. The information in all areas is fragmentary and, sometimes, contradictory. For the time being I have attempted just to summarise the available facts, as published, rather than giving much discussion as to their merits, meanings, or inadequacies. The emphasis of the discussion reflects that of my own work, in that *Eimeria* is given most prominence, while *Toxoplasma* and *Cryptosporidium* are dealt with in less detail. In general, however, this emphasis gives a true reflection of the relative amounts of attention that have been focused on these organisms in the past and very little information has actually been excluded.

1.2.1 Energy metabolism

The following section refers almost exclusively to eimerian species, what little is known about other coccidial parasites is detailed separately at the end of the section.

1.2.1.1 Energy Substrates

Current evidence suggests that carbohydrates serve as major energy substrates throughout the life cycle of *Eimeria*, however the possibility that other compounds may be catabolised for energy generation has been little explored. Lipid droplets are present to varying extents in all stages of the parasite and there has been a suggestion (Wilson and Fairbairn, 1961), as yet unconfirmed, that these may provide the energy for the final stages of sporulation as well as for metabolism during dormancy of *E. acervulina* cysts (see Section 1.2.1.4.1). The possibility that *Eimeria*, like some other parasitic protozoa, may catabolise amino acids or proteins for energy production has not been investigated (but see Section 1.2.2).

1.2.1.1.1 Endogenous polysaccharide energy reserves

Dense granules, identifiable as polysaccharide by iodine- and periodic acid-Schiff-staining, are present to varying degrees in almost all developmental forms of *E. tenella*. The localisation of these granules is variable, although in sporozoites they are predominantly found around the nucleus and refractile bodies. Decreases in the number and size of the polysaccharide granules are associated with the processes of sporulation (Patillo and Becket, 1955) and extracellular survival (Vetterling and Doran, 1969), while resynthesis has been observed during late schizogony and gametogony (Edgar *et al.*, 1944; Ferguson *et al.*, 1977; Ferguson and Hutchinson, 1987). Several studies have found a strong relationship between polysaccharide content and sporozoite or oocyst viability, as assessed by both dye exclusion and *in vivo* infectivity assays (Augustine, 1980; Nakai and Ogimoto, 1983 *a*, *b* and *c*). These observations are clearly consistent with the use of polysaccharide as an energy reserve.

Originally, the polysaccharide granules were thought to be composed of glycogen (Wilson and Fairbairn, 1961), but a more thorough chemical and enzymatic analysis of the isolated granules showed them to be amylopectin (Ryley *et al.*, 1969), a storage polysaccharide normally found in plants and fungi. Only a few other protozoa (including the rumen ciliate *Entodinium caudatum* and *Gregarina blaberae)* have been shown to use amylopectin as an energy reserve. Ryley *et al.* (1969) measured the average chain lengths of amylopectin from *Eimeria* species and found them to be between 18 to 23 units, depending on the species. This is slightly shorter than typical plant amylopectins but similar to the chain profile of amylopectin from *Gregarina blaberae.*

Amylopectin phosphorylase has been identified as the major enzyme involved in amylopectin mobilisation during sporogony of *Eimeria* (Wang *et al.*, 1975). This enzyme cleaves and phosphorylates glucose residues from non-reducing ends of amylopectin chains (presumably, but yet to be confirmed, a debranching enzyme is also involved in mobilisation). Unsporulated oocysts contain high levels of amylopectin phosphorylase but the activity decreases almost linearly during the sporulation process such that sporulated oocysts contain less than 8% of the original activity. In most eukaryotes, glycogen phosphorylase is regulated by phosphorylation and dephosphorylation, the end result of a cAMP cascade. Wang *et al.* (1975) reported, however, that the eimerian enzyme seemed unsusceptible to activation by phosphorylation and there was no change in cAMP concentrations during sporulation. Thus it was concluded that the changes in the phosphorylase activity must be mediated by a different mechanism. It seems probable, given the extended time-scale of

sporulation, that the activity of this enzyme might simply be modulated by the relative rates of gene expression and protein turnover; such a control strategy would, however, seem inadequate for the apparently more volatile requirements of the intra-host stages of the parasite.

Karkhanis *et al.* (1993) identified an amylopectin synthase in extracts of unsporulated oocysts of *E. tenella.* This soluble enzyme, optimally active at pH 7.5, catalysed the transfer of uridine 5'-diphosphate-glucose to a primer of either glycogen or, at a higher rate, amylopectin. This specificity is unusual in that ADP-glucose is the normal substrate for amylopectin synthases from plants. UDP-glucose, however, is the activated intermediate used by most eukaryotic glycogen synthases. The enzyme presented typical Michaelis-Menten kinetics for its substrates and no regulatory features were noted. If, as seems likely, amylopectin synthesis in *Eimeria* is analogous to glycogen synthesis in other eukaryotes, then a self-glycosylating protein and a branching enzyme will also be involved in the biosynthesis.

Polysaccharide granules have been observed in many other species of coccidia, being variously identified as either amylopectin or glycogen (Sheffield *et al.*, 1977; Chaudry *et al.*, 1985; Ferguson and Hutchinson, 1987; Current, 1989).

One of the most interesting recent discoveries of coccidian biochemistry is that mannitol is accumulated in large amounts in some stages of *Eimeria* and may serve as an energy reserve during parts of the life cycle. This is dealt with in detail in Section 1.2.1.2.2.

1.2.1.1.2 Use of exogenous carbohydrates

The accumulation of carbohydrate during the intracellular stage of the parasite must be dependent on the uptake of energy substrates from the environment. Although uptake of substrates has been observed in several stages of the *Eimeria*, the biochemical basis of the process is poorly characterised.

Sporozoites of *E. tenella* have been observed to take up exogenous monosaccharides, and either catabolise them or convert them to mannitol or amylopectin. Nakai and Ogimoto (1983*c*) incubated sporozoites of *E. tenella* with C^{14}-glucose and showed that radioactivity could be recovered both in amylopectin granules and released CO_2. They also investigated the influence of exogenous carbohydrates on sporozoite viability (as assessed by dye exclusion) and amylopectin reserves (as assessed by PAS staining). In aerobic incubations, glucose, fructose, mannose and maltose were effective in sparing the utilisation of amylopectin; galactose, glycogen, sucrose, lactose, pyruvate and glycerol were not. The presence of glucose in the incubation medium also increased the length of time that sporozoites survived at 41°C and in addition the PAS content of 'starved' sporozoites increased when they were placed in media containing glucose.

Similarly Smith and Lee (1986) showed that exogenous glucose greatly improved the *in vitro* survival of *E.tenella* sporozoites over a twenty-four hour period. They went on to investigate the mechanism of monosaccharide accumulation using radiolabelled glucose and some of its supposedly non-metabolisable analogues. The uptake mechanism proved to be saturable (V_{max} = 312 μg glucose (10^6 sporozoites) min^{-1} and K_T = 20 mM

approximately) and relatively specific (in that glucose was accumulated faster than some of its analogues), suggesting that the transport was carrier-mediated.

Glucose uptake did not appear to be influenced by exogenous sodium levels or by ouabain (an inhibitor of Na^+-K^+ pumps) but was inhibited by phloretin, an inhibitor of facilitated-diffusion systems in many cell types. These properties are suggestive of a passive carrier-mediated sugar transport system. Similar transport mechanisms have been described in other protozoan parasites and are typical of glucose transport systems in many other cell types. The high capacity but unusually low affinity of the carrier in sporozoites suggest that it is adapted to function in conditions of high glucose concentration.

Growing cells of *E. tenella* have also been shown to take up ^{3}H-glucose (administered to chickens). When assessed by electron microscopic autoradiography, first generation schizonts were found to become labelled around the nucleus, nucleolus, mitochondria and endoplasmic reticulum, while macrogametocytes showed less extensive overall labelling (Matsuzawa, 1979).

1.2.1.2 Pathways of carbohydrate metabolism

There has been no comprehensive study of the pathways of carbohydrate catabolism in any coccidian including *Eimeria*. Much of our knowledge of the enzyme systems operating in the parasites therefore comes from surveys of the isoenzyme activities which have been used to discriminate between species and intraspecific strains. Fortunately enzymes involved in carbohydrate catabolism are generally highly

expressed and are particularly suited for such analyses. Table 1.1 (parts *a*, *b* and *c*) summarises the enzyme activities which have been detected in *Eimeria* and some other coccidia species.

The following sections details mainly enzymatic evidence for the existence of key pathways of energy metabolism, and the properties of some of the enzymes mediating them. Evidence for the functionality of the pathways is dealt with in Section 1.2.1.4.

1.2.1.2.1 Glycolysis

All enzymes of the Embden-Meyerhoff glycolytic pathway have been identified in extracts of sporozoites, merozoites and oocysts of *Eimeria* species, and most have also been reported in other coccidial species (see Table 1.1.*a*). *T. gondii* has been shown to be unusual in possessing a phosphofructokinase (PFK) specific for pyrophosphate (PP_i) rather than ATP (Peng and Mansour, 1992). This is interesting in that it suggests the parasite might be adapted towards anaerobiosis (see Section 3.1.2). The enzyme has been purified and found to be typical of the type I PP_i-PFKs associated with anaerobic micro-organisms (Peng and Mansour, 1992). Unlike most ATP-PFKs, type I PP_i-PFKs are unregulated which means that species which have them must employ unusual mechanisms of glycolytic control. Such mechanisms had not been explored in the coccidia.

Fructose 1,6-diphosphate aldolase of *Eimeria steidae* has also been purified and identified as a type 1 enzyme typical of those found in mammalian cells (Mitchell and Daron, 1982; Wang, 1982).

All coccidial species which have been investigated contain high levels of lactate dehydrogenase (LDH), an enzyme responsible for mediating NADH oxidation under anaerobic conditions. It has been suggested that this might reflect a high dependence on anaerobic energy production. The enzyme has been purified and characterised from *Eimeria steidae* by Fransden and Cooper (1972). A single enzyme was identified with an electrophoretic mobility corresponding to the LD4 of vertebrates. The enzyme was specific for L(+) lactate but was capable of catalysing the reduction of both β- and α-NAD (the rate with the latter was only 3% that with the former). The enzyme subunits

	Eimeria							*Toxo-plasma*	*Sarco-cystis*	*Crpto-sporidium*
	Unsporulated oocyts	Sporulated oocysts	Sporozoites	Merozoites	Schizonts	Micro-gametocytes	Macro-gametocytes			
Glycolysis:										
Hexokinase	✓ 7	✓ 6						✓ 29,30,31	✓ 38	
PGI	✓ 7,8	✓ 6,8,25						✓ 27,30,31		✓ 32,33
PFK		✓ 6								✓ 1
Aldolase	✓ 3,7	✓ 6						✓ 30	✓ 38	
TPI	✓ 7	✘ 6								
GPDH		✓ 6								
PGK	✓ 7									
PGM	✓ 7,9	✓ 6,25						✓ 30,31	✓ 34	✓ 32,33
Enolase	✓ 7	✓ 6								
PK	✓ 7	✓ 6							✓ 38	✓ 1
LDH	✓ 2,7,8	✓ 6,8,25	✓ 19					✓ 30,31	✓ 38	✓ 33
TCA:										
Citrate synthetase		✘ 6								
Aconitase								✓ 30		
Isocitrate DH		✘ 6						✓ 30,31		
α-Ketoglutarate DH		✘ 6								
Succinyl CoA synth.		✘ 6								
Succinate DH	✓ 4,15	✘ 6 ✓ 4		✓ 4	✓ 4	✘ 4	✘ 4,15			
Fumarase		✘ 6						✓ 29,30		✓ 33
Malate DH		✓ 6								

Table 1.1 *a*. Enzyme activities in the coccidia.

	Eimeria							*Toxo-plasma*	*Sarco-cystis*	*Crypto-sporidium*
	Unsporulated oocysts	Sporulated oocysts	Sporozoites.	Merozoites	Schizonts	Micro-gametocytes	Macro-gametocytes			
Pentose Phosphate:										
G6PDH	✓ 5	✓ 6,8	✓ 19					✓ 29,30,31	✓ 38	
6-PGDH	✓ 8	✓ 8						✓ 30	✓ 34,38	
Gluconeogenesis:										
G6Pase	✓ 4	✓ 4,25		✓ 4	✓ 4	? 4	✓ 4			
F16BPase	✓ 7									
Pyruvate carboxylase								✓ 29		
Mannitol Cycle:										
M1PDH	✓ 20,21	✓ 21	✓ 21							
M1Pase	✓ 20,21	✓ 21	✓ 21	✓ 21						
MDH	✓ 20,21	✓ 21	✓ 21	✓ 21						
Hexokinase	✓ 20,21	✓ 21	✓ 21							
Misc. CHO metab.:										
Phosphoglucomutase	✓ 7	✓ 6						✓ 30,31	✓ 34	✓ 32,33
Amylopectin phosphorylase	✓ 16	✓ 16								
Amylopectin synthase	✓ 17									
α-Glycerophospate DH	✓ 15						✓ 15			
PEPCK		✓ 6								
Malic enzyme		✓ 6								
Glycerol 3P DH								✓ 30		
Amylase								✓ 4		

Table 1.1 *b*. Enzyme activities in the coccidia, continued.

	Eimeria							*Toxo-plasma*	*Sarco-cystis*	*Crypto-sporidium*
	Unsporulated oocyts	Sporulated oocysts	Sporozoites	Merozoites	Schizonts	Micro-gametocytes	Macro gametocytes			
'General':										
Glutamate DH	✓ 14	✓ 14								
Aspartate aminotrans.	✓ 14	✓ 14						✓ 27,30	✓ 39	
IMP DH	✓ 24									
Adenosine kinase		✓ 22								
ATPase	✓ 4	✓ 4		✓ 4	✓ 4, 26	✓ 4	✓ 4	✓ 1		
Alkaline phosphatase	✓ 4	✓ 4	✗ 19	✓ 4	✓ 4	✓ 4	✓ 4	✓ 28,30,31	✓ 35,37	
Acid phosphatase	✓ 4,10	✓ 4,10,11	✓ 10,19	✓ 4,18	✓ 4,18,26	✓ 4,26	✓ 4,18,26	✓ 27,28,31	✓ 35,37	
Non-specific esterase	✓ 4	✓ 4		✓ 4	✓ 4	✓ 4	✓ 4	✓ 30,31		
β-Glucuronidase	✗ 4	✗ 4		✗ 4	✗ 4	✗ 4	✗ 4	✓28		
Leucine napthylamidase	✗ 4	? 4		✗ 4	✗ 4	✗ 4	✗ 4			
Aconitate hydratase	✓ 7									
Glyoxalase I	✓ 7									
Leucine aminopeptidase	✓ 7,19		✗ 19	✓ 19	✓ 19		✓ 19	✓ 30		
Purine nucleoside phosphorylase	✓ 7									
Adenosylmethionine decarboxylase		✓12								
Arylsulphatase			✓ 13							
β-Galactosidase			✗ 19	✓ 19		✓ 19	✓ 19	✓ 28	✓ 35	
β-Glucosidase			✗ 19	✗ 19		✗ 19	✗ 19			
Carboxylic ester hydrolases			✗ 19							
GTPase	✓ 23									
5-nucleotidase					✓ 26	✓ 26	✓ 26			

Table 1.1 *c*. Enzyme activities in the coccidia, continued.

Key to Tables 1.1. *a*, *b* and *c*.

References

1 Takeuchi *et al.*. (1980)
2 Fransden and Cooper (1972)
3 Mitchell and Daron (1982)
4 Michael and Hodges (1973)
5 Fransden (1976)
6 Smith *et al.* (1994)
7 Andrews *et al.* (1990)
8 Shirley (1975)
9 Rollinson *et al.* (1979)
10 Hosek *et al.* (1988)
11 Farooqui and Hanson (1988)
12 San-Martin Nunez *et al.* (1987)
13 Farooqui and Hanson (1983)
14 Wang *et al.* (1979)
15 Beyer (1970)
16 Wang *et al.* (1975)
17 Karkhanis *et al.* (1993)
18 Heller and Scholtyseck (1970)
19 Fransden (1970)
20 Schmatz *et al.* (1989)
21 Michalski *et al.* (1992*b*)
22 Miller *et al.* (1982)
23 Maga *et al.* (1994)
24 Hupe *et al.* (1986)
25 Shirley *et al.* (1977)
26 Vetterling and Waldrop (1976)
27 Darde *et al.* (1992)
28 Manafi *et al.* (1993)
29 Takeuchi *et al.* (1980)
30 Darde *et al.* (1988)
31 Barnet *et al.* (1988)
32 Awad-El-Kariem *et al.* (1993)
33 Ogunkolade *et al.* (1993)
34 Atkinson and Collins (1981)
35 Farooqui *et al.* (1987)
36 Chaudry *et al.* (1985)
37 Chaudry *et al.* (1986*a*)
38 Gupta *et al.* (1992)

Abbreviations

aminotrans., aminotransferase
CHO, carbohydrate
DH, dehydrogenase
F16BPase, fructose 1,6-bisphosphatase
G6Pase, glucose 6-phosphatase
G6PDH, glucose 6-phosphate dehydrogenase
GPDH, glyceraldehyde 3-phosphate dehydrogenase
LDH, lactate dehydrogenase
M1Pase, mannitol 1-phosphatase
M1PDH, mannitol 1-phosphate dehydrogenase
MDH, mannitol dehydrogenase
P, phosphate
PEPCK, phosphoenolpyruvate carboxykinase
PFK, phosphofructokinase
6-PGDH, 6-phosphogluconate dehydrogenase
PGI, phosphoglucoisomerase
PGK, phosphoglycerate kinase
PGM, phosphoglyceromutase
PK, pyruvate kinase
synth., synthetase
TPI, triose phosphate isomerase

✓ = detected
✗ = apparently absent
gap means 'no report'

proved highly resistant to dissociation and showed no obvious hybridisation with H subunits from chick heart, implying a fundamentally different subunit construction to the isoenzymes characterised from vertebrates.

1.2.1.2.2 Mannitol metabolism

When Wilson and Fairbairn (1961) measured the total masses of lipid, protein and anthrone-sensitive carbohydrate in unsporulated oocysts of *E. acervulina*, 25% of the oocyst dry mass was left unaccounted for. Recently this missing component has been identified as predominately mannitol, a carbohydrate previously identified only in fungi. Further investigation revealed the presence of enzymes associated with a mannitol cycle (see Figure 1.5). With the exception of hexokinase (which can accept either glucose or fructose as substrates), the enzymes are all very specific in their reactions; their k_ms are such to imply that the pathway acts only in one direction (as shown in Figure 1.5).

Schmatz *et al.* (1989) reported that mannitol was present at very high levels (up to 300 mM) in unsporulated oocysts of *E. tenella* but fell during sporulation to about 10 mM. Michalski *et al.* (1992*b*) however obtained slightly different results, finding only small amounts of mannitol (50-80 nmol per 10^6 oocysts) in unsporulated oocysts of the same species. These concentrations increased rapidly, however, during the early stages of sporulation, concomitant with a decrease in amylopectin levels, and then decreased during the later stages of sporulation, reaching a basal level after 40 hours. These changes were correlated with changing activities of the mannitol cycle enzymes. The discrepancy between the results of the two groups may be due to the fact that Michalski *et al.* used oocysts obtained directly from the caecum rather than from faeces (as were

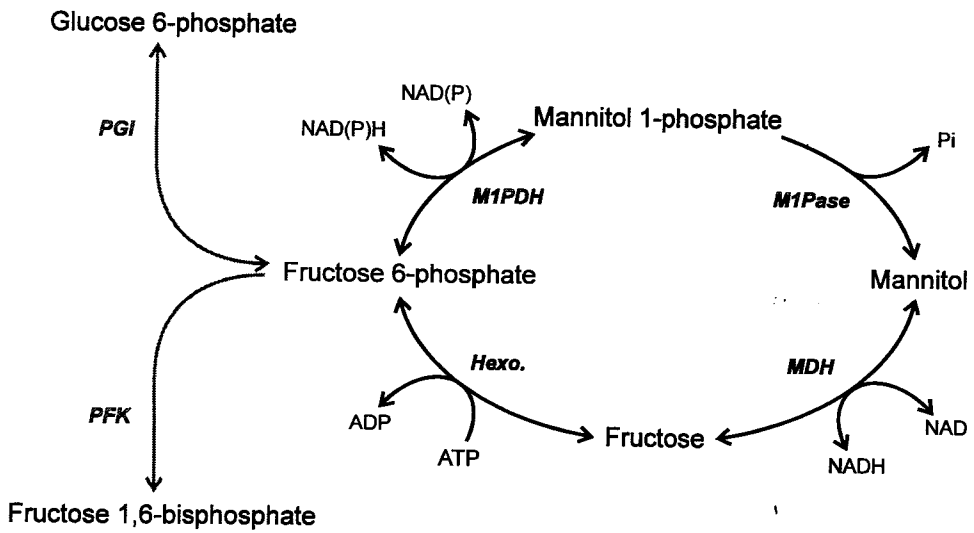

Figure 1.5. The mannitol cycle in *Eimeria tenella*. The enzymes involved are: M1PDH, mannitol 1-phosphate dehydrogenase; M1Pase, mannitol 1-phosphatase; MDH, mannitol dehydrogenase; and Hexo., hexokinase. Fructose 6-phosphate enters the cycle via M1PDH and is reduced to mannitol 1-phosphate. Mannitol 1-phosphate is dephosphorylated by M1Pase to yield mannitol which is then oxidised to fructose by MDH. Fructose is rephosphorylated by hexokinase producing fructose 6-phosphate; this may either re-enter glycolysis via phosphofructokinase (PFK), or enter the pentose phosphate pathway via phosphoglucoisomerase (PGI).

Schmatz's) and that these may not have been fully mature. As such the initial changes that occurred in the study may have been due to the final maturation of the oocysts which normally occurs within the host . If we assume this to be true, then the two sets of results are, in fact, very comparable.

During sporulation Michalski *et al.* (1992) found the mannitol to accumulate within the developing sporocysts and sporozoites; mannitol cycle enzymes were also detected in sporozoites and merozoites; and sporozoites were shown to be capable of converting ^{14}C-glucose into mannitol. Together these observations constitute strong evidence that the pathway is important in stages beside the oocysts. Recent evidence, however, would seem to suggest that the synthetic part of the cycle is fully functional only during the sexual phase of the life cycle (Schmatz, personal communication) and that this leads to the large concentration of mannitol in the oocyst. Most interestingly, this part of the cycle appears to be mainly controlled through the binding of a protein inhibitor of the first enzyme, mannitol 1-phosphate dehydrogenase. It is currently not known how the degradation of mannitol is regulated but it is clear that there must be co-ordinated regulation of the fluxes to and from mannitol and amylopectin, and through glycolysis.

The function of the mannitol cycle in either fungi or the coccidia is unclear. One possibility is that mannitol is functioning as an energy reserve, although the following additional functions have all been suggested for mannitol or its cycle; as yet there is no direct evidence for, or against, any of these:

1) NADH generated during the breakdown of mannitol may be used directly for oxidative phosphorylation;

2) the first part of the pathway may acts as an electron sink for replenishment of NAD^+ under anaerobic conditions;
3) mannitol may act as an osmoregulator, keeping the oocyst wall rigid during maturation;
4) mannitol may have a protective effect against superoxide ions;
5) mannitol phosphate might be polymerised to act as a structural component in the oocyst or sporocyst wall .

These functions are considered in more detail in Schmatz (1989).

The mannitol cycle appears to be present in both *Toxoplasma* and *Cryptosporidium* and so may be a common feature of all coccidia. Both mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase have been detected in *C. parvum* (Schmatz, 1989) and specific antibodies have been used to show that mannitol-1-phosphate, but not its inhibitor, is present in the sexual stages of both *Toxoplasma* and *Cryptosporidium,* whereas both proteins occur in other stages of the life cycle (Schmatz, personal communication).

1.2.1.2.3 Tricarboxylic acid (TCA) cycle

From the available enzymatic evidence, it is unclear whether *Eimeria* (or any of the other coccidia) contain a functional TCA cycle at any stage of their life cycle. Of the TCA enzymes, Smith *et al.* (1994) was able to detect only malate dehydrogenase in sporulated oocysts of *E. tenella.* Both phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme were present, however, and he concluded that *Eimeria* lacks a

conventional TCA cycle but contains a PEPCK by-pass similar to those found in anaerobic protozoa such as *Giardia lamblia* and *Trichomonas vaginalis* (see Figure 1.6, below)

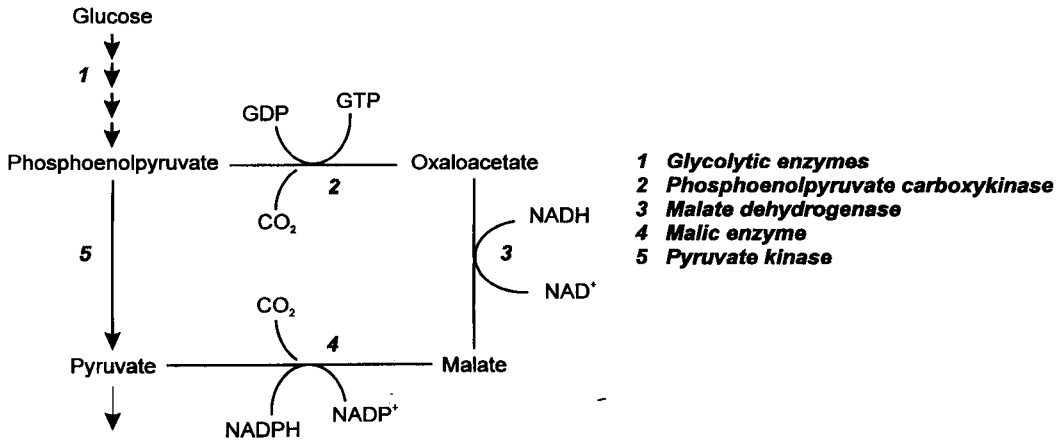

Figure 1.6 The PEPCK by-pass, as found in *G. lamblia* and *T. vaginalis*.

These results, however, conflict with Wang's assertion (1982) that: "There is ample evidence indicating a functional tricarboxylic acid cycle in coccidia in general". The only evidence quoted as justification for this statement, however, was the detection of isocitrate dehydrogense (ICDH) and malate dehydrogenase in unsporulated oocysts, and the demonstration by cytochemical analysis of succinate dehydrogenase (SDH) and ICDH in proliferative *Toxoplasma*. SDH has also been cytochemically detected in several stages of *Eimeria* (Beyer, 1970; Michael and Hodges, 1973).

The evidence for or against the TCA cycle is therefore ambiguous. It remains a possibility that the pathway is operative in just some stages of the parasite(s).

1.2.1.2.4 Respiratory chain

All developmental stages of *Eimeria* species possess distinctive elongate, cristate mitochondria (Wang, 1982). The processes of sporulation and excystation are associated with vigorous respiratory activity (consumption of O_2 and production of CO_2) (see section 2.4 for details). This is reversibly inhibited by cyanide and other inhibitors of electron transport (Wang, 1982), implying that it is mediated, at least partially, by a cytochrome-containing respiratory chain. The detailed composition of the cytochrome chain is yet to be elucidated but there is evidence that it differs from those found in mammalian mitochondria.

Mitochondria isolated from unsporulated oocysts of *E. tenella* consumed oxygen in the presence of conventional respiratory substrates, including NADH and succinate (Wang, 1975; Fry and Williams, 1984). The isolated mitochondria were uncoupled with respect to oxidative phosphorylation: oxygen consumption was not dependent on ADP, and there was no effect by the uncoupler carbonyl *m*-chlorophenylhydrazone (Fry and Williams, 1984). It remains unclear whether this uncoupling is an inherent property of the mitochondria or a result of damage inflicted during the isolation procedure. Spectrophotometric analysis of the mitochondria revealed absorbance maxima characteristic of a- and b-type cytochromes but no clear indication of a c-type cytochrome. Interaction with CO suggested that there might be two a-type cytochromes present, cytochrome a_3 of cytochrome oxidase and an *o* cytochrome. The mitochondrial respiration was inhibited by cyanide, azide, carbon monoxide (inhibitors of cytochrome

oxidase) and also by antimycin A, which blocks CoQ-cytochrome reductase. However rotenone and amytal were largely without effect, suggesting that NADH-Q reductase was either absent or presented unusual properties. Similar findings have been reported for *Plasmodium* which is thought to lack a NADH-Q reductase, with succinate being the major feed-in point to the respiratory chain.

The respiratory pathway in *Eimeria* has attracted particular interest as the apparent site of action of several anti-coccidial agents. The quinolone and pyridine coccidiostats and the 2-hydroxynapthoquinones appear to act by blocking different sections of the respiratory chain (Wang, 1975; Wang, 1976;-Fry and Williams, 1984). Resistance to quinolones and pyridines also appears to be manifest at the level of electron transport as much higher concentrations of drug are required to inhibit respiration in mitochondria from drug-resistant than from drug-sensitive parasite strains (Wang, 1975; Wang, 1976; Fry and Williams, 1984). The resistance and sensitivity relationships between quinolone anti-coccidials (in particular decoquinate) and the pyridine clopidol show some peculiar features which may provide insights into the structure of the eimerian respiratory chain. These two drugs show a marked synergistic effect both *in vivo* and *in vitro*; however acquisition of resistance to one of the two drugs consistently leads to a greater sensitivity to the other (Fry and Williams, 1984). These observations have led to the suggestion that *E. tenella* has a branched or parallel electron transport chain: one part of which is more sensitive to 4-hydroxyquinolones, while the other is blocked by clopidol. Resistance to one drug may therefore result from electron transport being diverted towards the less sensitive of the pathways. This model would explain both the collateral sensitivities and the apparent synergism between the two drugs. The pathway that is most sensitive to clopidol also appears to be more readily blocked by cyanide and azide

as resistance to the drug is also associated with a decreased sensitivity to these agents (the reverse being true for the decoquinate-sensitive route). It is interesting to view these results in the light of the detection of two a-type cytochromes in the mitochondria: perhaps these represent two terminal oxidases?

1.2.1.2.5 Pentose phosphate pathway

Glucose 6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway, has been purified and characterised from unsporulated oocysts of *Eimeria steidae* (Fransden, 1976). Like mammalian enzymes, the enzyme was specific for $NADP^+$ and could accept glucose, at a low rate, as well as glucose-6-phosphate. A large range of compounds were tested a potential regulators of the enzyme. Of these, the purine triphosphates (ATP, ITP and GTP) and the pyrimidine triphosphates (CTP and UTP) were effective inhibitors of the enzyme at mM concentrations, while phosphoenolpyruvate (a powerful inhibitor of some bacterial enzymes) had no effect. There was, however, significant inhibition by oleic and linoleic fatty acids. 6-phosphogluconate dehydrogenase activity has also been detected in starch gels of sporulated and unsporulated oocysts (Shirley, 1975). From the presence of these enzymes, it would seem likely that a functioning pentose phosphate shunt exists in *Eimeria* species. It could be envisaged that this pathway would be particularly important during the rapid growth phases of schizogony and gametogony where NADPH and ribose requirements would be high. Indeed, James (1980) has presented evidence that the pathway is very active in isolated schizonts (see Section 1.2.1.5.).

1.2.1.2.6 Gluconeogenesis

Fructose 1,6-bisphosphatase and glucose 6-phosphatase have both been detected in extracts of *Eimeria* and so it would seem that the parasites have gluconeogenic capabilities. This may relate to the importance of amylopectin and mannitol as energy reserves, although at present there is no evidence that exogenous substrates other than carbohydrates are used in their synthesis. Perhaps the endogenous reserves of lipid can be converted to the carbohydrate stores?

1.2.1.2.7 Glyoxylate cycle

No isocitrate lyase or malate synthetase activity could be detected in crude extracts of *Eimeria tenella* unsporulated oocysts (Wang, 1982) implying that a glyoxylate pathway is not present.

1.2.1.3 Other catabolic pathways

The possible use of amino acids as energy substrates by the coccidia has not been investigated in any detail. Anaerobic parasitic protozoa such as *Trichomonas vaginalis* and *Giardia lamblia*, however, do use some, especially arginine, in this way (Coombs and Muller, 1995) and so it will be interesting to see if this is an anaerobic characteristic also shared by coccidia.

High activities of glutamate dehydrogenase (Wang *et al.*, 1979) and aspartate aminotransferase (Shirley and Rollinson, 1979) have been detected in oocyst extracts, but there are no reports on other enzymes possibly involved in amino acid breakdown. It

has been shown that most, if not all, of the 'intra-host' stages of the parasite are capable of taking up amino acids from the environment, although these appear to be mainly incorporated into parasite proteins (Krylov and Svanbaev, 1980).

1.2.1.4 ***Variation with phase of development***

1.2.1.4.1 Oocysts and Sporulation

The oocyst wall is a complex, highly resistant structure (Stotish *et al.*, 1978) which is impermeable to all but the smallest molecules and respiratory gases (El-Moukdad, 1976). Since it cannot take in nutrients from the environment, the oocyst must be dependent on its endogenous energy reserves to fuel the processes of sporulation and to maintain viability during dormancy.

Early studies suggested that the initial phase of sporulation is fuelled by carbohydrate catabolism (Wilson and Fairbairn, 1961), but the later stages by lipid oxidation. Wilson and Fairbairn (1961) noted that the concentration of alkali-stable carbohydrate decreased from 83 to 46 μg $(10^6$ oocysts$)^{-1}$ during the first 10 hours of sporulation of *E. acervulina* oocysts. During the later phases of the process there was some resynthesis, which appeared to be driven by lipid oxidation. The respiratory quotient was maintained at values considerably less than one during these later stages of sporulation, which is consistent with lipid being the major substrate used. More recently, Michalski *et al.* (1992*b*) observed similar decreases in amylopectin content during sporulation of *E. tenella* oocysts and also showed an overall decrease in mannitol concentration during

the process, implying that both components might be being used as energy sources (but see section 1.2.2.2).

The process of sporulation seems to be strictly aerobic and will not proceed either at low oxygen tension or in the presence of respiratory inhibitors such as cyanide (Wang, 1976). Several researchers have observed vigorous respiratory activity during the process, with O_2 being consumed and CO_2 released into the environment. Wilson and Fairbairn (1961) found respiratory activity to be maximal at the start of sporulation; thereafter it fell off steadily with a constant, very low rate being achieved after 2-3 days. The respiratory profiles reported by Wagenbach and Burns (1969) for the sporulation of *E. tenella* and *E. steidai* are more complex, showing variable rates of respiration throughout the process. They found the rate to peak around the '2-nucleus' stage (between 10 and 18 hours), this maximal activity was followed by a marked depression, correlating with the appearance of the early spindle stage. Once sporulation is complete respiration falls to a barely detectable level until either the fuel reserves are exhausted or ingestion by a host causes excystation to occur.

Wagenbach and Burns (1969) noted that sporulated oocysts kept under anaerobic conditions remained viable for only a quarter of the time of those kept under aerobic conditions. This presumably reflects less efficient usage of energy substrate under anaerobic conditions; however the polysaccharide content of the oocysts was not monitored.

1.2.1.4.2 Sporozoites and excystation

Excystation occurs efficiently under either aerobic or anaerobic conditions, or in the presence of respiratory chain inhibitors such as cyanide (Wagenbach and Burns, 1969; Wang, 1976). The process is associated with the disappearance of amylopectin granules from the cytoplasm, suggesting that these are the source of the energy required.

Excystation under aerobic conditions was reported to be accompanied by vigorous respiratory activity (Vetterling, 1968). This was initiated upon breakage of the oocyst wall and reached a maximal level 2-10 minutes into excystation, before declining to a constant low level which was maintained until the sporozoites were stimulated to invade host cells. Ryley (1973) compared the rates of respiration reported by Vetterling (1968) with the amount of carbohydrate consumed during the equivalent period (Vetterling and Doran, 1969) and found that approximately 6 moles of oxygen were consumed per mole of glucose. This ratio suggests that glucose is being completely oxidised to CO_2 and H_2O and implies that both a TCA cycle and respiratory chain are operative in this developmental form of the parasite.

The fact that *E. tenella* sporozoites can excyst and remain motile in the absence of oxygen, however, suggests that they are facultative anaerobes. It is to be remembered that they exist in the gut lumen, where oxygen concentrations are low[1]. Ryley (1973) monitored the anaerobic endogenous metabolism of *Eimeria tenella* sporozoites (Table 1.2) and identified lactate as the major end product with some CO_2 and glycerol also

[1] See Bryant and Behm (1989) or Coombs and Muller (1995) for a discussion of conditions in the digestive tract.

being produced. Together these products accounted for 97% of the amylopectin consumed over the experimental period.

Thus it seems that under conditions of limited oxygen *Eimeria* sporozoites obtain energy through glycolytic substrate level phosphorylation and depend largely on lactic acid production for regeneration of the intracellular pool of NAD^+. The origin of the CO_2 is unclear although some is probably produced via the hexose monophosphate shunt.

Table 1.2 Anaerobic endogenous metabolism of *Eimeria tenella* sporozoites.

(from Ryley, 1973)

	μmoles	Moles/mole glucose
Amylopectin (as glucose)	- 30.05	- 1.00
Acid (from bicarbonate)	+ 50.25	+ 1.67
CO_2	+ 14.95	+ 0.50
Lactic acid	+ 45.50	+ 1.49
Pyruvic acid	0.00	0.00
Succinic acid	0.00	0.00
Glycerol	+ 8.60	+ 0.28

A recent study showed that the invasion rate of *E. tenella* sporozoites into host cells in an *in vitro* system was significantly greater under anaerobic than under aerobic

conditions (Wrede *et al.*, 1993) (a similar situation has also been reported for *C. parvum*: Upton *et al.*, 1991). This observation suggests that the sporozoites function better at low oxygen tensions, perhaps because they are specifically adapted towards anaerobiosis.

1.2.1.4.3 Intracellular stages

The metabolic activities of the intracellular stages of *Eimeria* are difficult to monitor directly and interpretation of data is complicated since it is hard to know whether observed changes are caused by activity of the parasite or changes in host cell metabolism. Several groups have attempted to compare the rates of respiration or glycolysis in normal and parasitised caecal tissue but these experiments have yielded unreproducible and contradictory results (see Ryley, 1973). A common feature of chicken coccidiosis, however, is a markedly lowered intestinal pH (van der Horst and Kouwenhoven, 1973). This might be due to high levels of lactic acid production by parasitised cells, which would imply that there are significant periods of anaerobic metabolism during this phase of the life cycle.

James (1980) showed that isolated schizonts of *E. tenella* were able to take up and catabolise radiolabelled glucose to CO_2 (other products were not investigated). The rate of CO_2 production was approximately 3 times higher when 1-^{14}C-glucose was used as opposed to 6-^{14}C-glucose. This would suggest high hexose monophosphate shunt activity. (Tissues with very rapid hexose monophosphate shunt activity have previously been reported to produce as much as 20 times more CO_2 from 1-^{14}C-glucose than from 6-^{14}C-glucose (Rognstad and Katz, 1969)).

Beyer (1970) used cytochemical techniques to investigate the levels of succinate dehydrogenase and α-glycerophosphate dehydrogenase in the intracellular stages of *E. magna*. Growing macrogametes had high levels α-glycerophosphate dehydrogenase but no detectable succinate dehydrogenase, while the zygote immediately after fertilisation showed high levels of both enzymes. Beyer interpreted this as reflecting changes in mitochondrial activity during the life cycle of the organism and considered that under the ample nutritional means provided by the host cells, glycolysis can easily cover the energy requirements for the developing parasites. Later, as the host substrate becomes exhausted and the merozoites or zygote prepares for an extracellular phase of life, there is a switch to more efficient aerobic metabolism using the TCA cycle. An interesting alternative hypothesis is that oxygen becomes limited from fairly early on in the intracellular phase (the parasite grows and multiplies very rapidly and thus would soon consume the available oxygen) and that the switch to aerobic potential represents a pre-adaption to an extracellular existence in an aerobic environment.

1.2.1.5 Toxoplasma

Until recently the biochemistry of *Toxoplasma* species had attracted very little attention, The situation is now beginning to change with the recognition of the parasites as major causes of mortality in immunosupressed individuals but there has so-far been relatively little work on their energy metabolism.

Like *Eimeria*, *Toxoplasma gondii* possesses cristate mitochondria throughout its life cycle. Amylopectin is present in abundance in bradyzoites, but appears to be absent or

present at only low levels in tachyzoites (Ferguson and Hutchison, 1987; McLeod *et al.*, 1991).

The enzymes which have been detected in the species are summarised in Table 1.1. and include several enzymes of glycolysis and the TCA cycle. The glycolytic pathway, as has already been described (Section 1.2.1.2.1), contains a pyrophosphate-dependent PFK of the type normally associated with anaerobic micro-organisms. However there is evidence for a respiratory chain containing cytochrome oxidase as well as cytochromes b and c (Fulton and Spooner, 1960)

In the most detailed analysis of *T. gondii* energy metabolism so-far undertaken, Fulton and Spooner (1960) demonstrated the ability of a range of substrates to stimulate oxygen consumption by intact tachyzoites of *T. gondii*. Glucose was the most effective substrate identified, with a respiratory quotient of 0.83-1.14; glutamine was almost as good, while lactate and various intermediates of the TCA cycle were ineffective in supporting respiration. Interestingly, low levels of respiration were observed in the presence of glucose-free glycogen - a substance which is likely to be present at high concentrations in host cells.

Using paper chromatography, lactate and acetate were identified as the two main soluble products of aerobic glucose catabolism by tachyzoites, traces of propionic, butyric and valeric acid were also detected by the more sensitive technique of gas chromatography. Under aerobic conditions, about 80% of the glucose consumed was accounted for by the products CO_2 and lactate (in the approximate ratio 3:1). Under anaerobic conditions, levels of lactate production were 2-3 times greater.

The aerobic catabolism of glucose to lactate and acetate by *T. gondii* tachyzoites has been confirmed by further studies using ^{1}H-NMR (Ohsaka *et al.*, 1982). These showed the overall production of lactate to be much greater than that of acetate, with there being an apparent delay in the onset of production of the latter. No end-products were detected in the absence of added substrate - in accord with the apparent lack of endogenous energy reserves in this stage of the species.

Apparent evidence that the respiratory chain is indeed functional in energy production comes from experiments by Werk and Bommer (1980), working on host cell invasion. Using an *in vitro* system, they found that preincubating *T. gondii* tachyzoites with cyanide decreased their efficiency of invasion by more than 80%. The invasion rate was, however, restored to normality when glucose was present during the invasion process. Excluding non-specific effects, the obvious interpretation is that energy production is far less efficient under the enforced fermentative metabolism induced by cyanide, and that the cells consequently consume their finite energy reserves too quickly for cell invasion to occur.

1.2.1.6 Cryptosporidium

Very little is known about energy metabolism in *Cryptosporidium* species. The apparent lack of mitochondria in *C. parvum* suggests that oxygen is unimportant to this species and that glycolysis is the main source of energy generation. Such a conclusion is consistent with the observation that development of the parasite was enhanced under reduced oxygen tensions (Upton *et al.*, 1991). Mitochondria appear to be present in *C.*

muris (Uni *et al.*, 1987), suggesting that the metabolism of this species may differ very greatly from that of *C. parvum.* Further studies are required to verify that two supposedly closely related species do indeed differ so markedly.

1.2.2 Protein and amino acid metabolism

1.2.2.1 Protein synthesis

The mechanisms by which coccidia synthesise proteins have been investigated little in recent years. The few studies reported have been confined to determinations of the sensitivity of the parasites to some standard protein synthesis inhibitors (as quoted in Edlind, 1991, and those in Table 1.3).

1.2.2.2 Amino acid interconversions

Some enzymes likely to be involved in the interconversions of amino acids in coccidia have been detected, mainly during isoenzyme studies aimed at distinguishing species/lines, but there have been no detailed analyses of the enzymes themselves or of the ability of the parasites to interconvert amino acids. The enzymes detected to date are detailed in Table 1.1.

1.2.2.3 Protein catabolism

Parasite proteinases have attracted considerable attention in recent years (North, 1982; McKerrow, 1989; North *et al.*, 1990) and are considered to be good targets for

chemotherapeutic attack. Coccidia undoubtedly possess many proteinases, as is thought to be the case for all eukaryotic cells, but there have been relatively few reports on them.

Wang and Stotish (1978) looked for proteinases which might be involved in protein turnover during sporulation or in the release of sporozoites during excystation. Very low proteinase activities were found using C^{14}-glycinated haemoglobin as substrate at pH 4 and extracts of sporulated or unsporulated oocysts of *E. tenella*, whereas none was apparent at neutral or basic pH. The activity was completely inhibited by PMSF, implicating a serine proteinase.

Three leucine aminopeptidase activities were detected when extracts of unsporulated oocysts of *E. tenella* were subjected to electrophoretic analysis (Wang & Stotish, 1978). These activities gradually diminished during sporulation while a different isoenzyme appeared, such that it was the only activity that could be detected sporulated oocysts. This was shown to be located primarily in the cytoplasm surrounding the sporocysts; it was not found in either in sporozoites or merozoites. All four aminopeptidase activities had similar substrate specificities and were unaffected by inhibitors of serine proteinases. The enzymes present in unsporulated oocysts had pH optima in the range 8.0 to 8.5, while that in sporulated oocysts was found to be more active at pH 8.5-9.0. The latter activity differed form the others in that it was greatly reduced in the absence of metal ions (Mg^{2+} or Mn^{2+}) and was inhibited by chelating agents, thus it had characteristics of a metallo-proteinase. As there is limited protein synthesis occurring at the late stages of sporulation, it was suggested that the leucine aminopeptidase activity appearing at that stage may result from the processing of the pre-existing activities. The rather different properties of the isoenzymes, however, make this unlikely and their

presence at different stages during sporulation suggests that the isoenzymes may perform specific roles in this process and excystation.

Using a different substrate (azocollogen), Farooqui and Hanson (1983) detected high proteinase activities in sporulated and unsporulated oocysts and sporozoites of *E. tenella*. The activities detected were in the order sporozoites > sporulated oocysts > unsporulated oocysts. Fuller and McDougald (1990) investigated azocasein digestion by intact sporozoites and merozoites and detected extremely low levels of activity sensitive to inhibition by PMSF and to a lesser degree by TPCK and TLCK. The activities associated with the sporozoites and merozoites had different pH optima and inhibitor sensitivities, implying stage-specific proteinases.

Michalski *et al.* (1992*a*) succeeded in purifying a 20 kDa proteinase from *E.tenella* oocysts, even though it was not readily detected in crude homogenates because of some 'interfering factor'. The purified enzyme had a pH optimum of 8.0, using either azocasein or gelatin as substrate, and was sensitive to inhibition by PMSF, aprotonin and TLCK. It was unaffected by inhibitors specific for other proteinase types and by divalent cations and so appeared to be a serine proteinase. Antisera raised against the enzyme recognised proteins in both oocyst and sporozoite homogenates on Western blots, but did not react with trypsin thus confirming that it was indeed of parasite origin.

This group also noted that the proteins in crude oocyst homogenates are hydrolysed if proteinase inhibitors are not added, thus confirming that proteinases are present (Michalski *et al.* 1992*a*). The degradation can be partially prevented by inclusion of PMSF in the lysis buffer, but a combination of PMSF and E64 is required to achieve

full protection. This provides convincing evidence that oocysts contain proteinases of both the serine and cysteine types.

Adams and Bushell (1988) and Fuller and McDougald (1990) used proteinase inhibitors to investigate the possibility that proteinases might be involved in the process of host cell invasion by eimerian sporozoites. Using an *in vitro* system and preincubation of the sporozoites with various proteinase inhibitors, it was reported that invasion was significantly inhibited by PMSF and, in the second study, a range of other inhibitors specific for serine proteinases and cysteine proteinases.

It has been reported that *E. tenella* has a gene with high homology to those encoding apartic proteinases in other organisms (Laurent *et al.*, 1993). Immunolocalisation studies suggested that the enzyme was associated with the refractile bodies of the sporozoite, however the activity of the enzyme encoded by the gene has not been described to date. It is yet to be determined if the expression of the gene is developmentally regulated.

The proteinases of *Toxoplasma* and *Cryptosporidium* have been studied even less extensively than those of *Eimeria.* Two proteinase activities were separated from tachyzoites of *T. gondii* (Choi *et al.*, 1989). One had characteristics of a cysteine proteinase and was optimally active at pH 6.0, the other was an ATP-dependent serine proteinase optimally active at pH 8.5. This parasite was also reported to contain a cystatin-like inhibitor of cysteine proteinase. This is likely to play a role in protecting the parasite from its own enzymes. An aminopeptidase activity has been detected in *C. parvum* sporozoite lysates (Okhuysen *et al.*, 1994). A role in excystation was suggested

for this enzyme. Several other aminopeptidases were also detected in *C. parvum* using a range of chromogenic substrates.

The role of proteases in host cell invasion has been studied with *S. muris* (Strobel *et al.* 1992). The results suggested that a cysteine proteinase is involved and may possibly enhance parasite invasion by modifying the parasitophorous vacuole (Strobel *et al.*, 1992).

1.2.3 Biochemical Action of Anticoccidial Agents

In general, agents which are effective against the genus *Eimeria* are also effective for *Toxoplasma*, *Sarcocystis* and *Isospora* (Stuart and Lindsay, 1986; Kirkpatrick and Dubey, 1987; Cawthorn and Speer, 1990; Johnson, 1990; MacDougald, 1982) but until recent years the vast majority of effort was devoted to the economically important avian parasites. The realisation that toxoplasmosis and cryptosporidiosis are important AIDS-related infections resulted in increased efforts to obtain better chemotherapy against these infections, but with limited success so far. Although many different compounds, including those active against *Eimeria*, have been tested against *Cryptosporidium,* none except halofuginone (Current and Blagburn, 1990; Naciri *et al.*, 1993) were found to be effective and it has yet to be shown to be useful clinically. There is little information on the chemotherapy of *Caryospora* or *Hammondia* (Kirkpatrick and Dubey, 1987; Upton and Sundermann, 1990; Dubey, 1993) and only one report for *Besnoitia* (Shkap *et al.*, 1987). Therefore, the main focus of this section is *Eimeria.*

There has been no commercial launch of any novel class of synthetic or semisynthetic avian anticoccidial agents since the early 1980s. This is a major concern because of the resistance problems with current agents (Chapman, 1982 and 1993; Edgar, 1993;

Vertommen and Peek, 1993). Recombinant vaccines are now becoming available and perhaps they will set the trend for cheap coccidiosis control in the future (Wallach, 1993; Barriga, 1994).

There have been two reviews covering the biochemical action of avian anticoccidial drugs (Wang, 1982; Looker *et al.*, 1986). MacDougald (1982, 1990) also commented on the mechanism of action of these drugs in his reviews of the anticoccidial chemotherapy literature. There have been few advances in this area in recent years and so the current understanding is simply summarised in Table 1.3. It needs to be stressed that in most cases the precise mechanism of action is not known. When compared to the detailed information available on, for instance, many antimalarial drugs (for example, Foote and Cowman, 1994), current knowledge of the biochemical action of avian anticoccidial drugs is superficial. There have been just a few recent papers on the effects of drugs on parasite morphology and site of action of anticoccidial drugs (Smith and Strout, 1980; Maes *et al.*, 1988; Verheyen *et al.*, 1988; Guyonnet *et al.*, 1990; Daszak *et al.*, 1991; Raether *et al.*, 1991; Zhu and MacDougald, 1992; Conway *et al.*, 1993; Ferguson *et al.*, 1994). In addition, studies on the mechanism of resistance to ionophores in *Eimeria* have revealed differences in drug uptake (Augustine *et al.*, 1986) and protein content (Zhu *et al.*, 1994) of parasites. Despite the major impact that drug resistance has had on anticoccidial chemotherapy, the mechanisms of drug resistance are have not been elucidated in any case. More basic but focused research in coccidia is needed to provide better understanding of drug-parasite interactions.

There have been several recent reports of novel experimental compounds with anticoccidial activities. Some of these interesting anticoccidial leads and their putative biochemical actions are given in Tables 1.3.*a* and 1.3.*b*. It is apparent that macrolides are well represented (see Table 1.3.*b*), suggesting that protein synthesis provides an important coccidial target. On the other hand, nothing is known about the identity of the molecular targets of many of the compounds (see Table 1.3.*a*). It remains to be seen

whether any of these compounds or their analogues will become viable commercial products

COMPOUND	ORGANISM	ACTIVITY	PUTATIVE MECHANISM	REFERENCE
Azithromycin	*Cryptosporidium/ Toxoplasma*	*in vitro/in vivo*	Protein synthesis	Araujo *et al.*, 1991 Rehg, 1991 Araujo and Remington, 1992 Blais *et al.*, 1993 (*a* & *b*) Vargas *et al.*, 1993
Clarithromycin	*Toxoplasma*	*in vivo*	Protein synthesis	Edlind, 1991 Georgiev, 1994
Roxithromycin	*Toxoplasma*	*in vivo*	Protein synthesis	Araujo *et al.*, 1991
Paromomycin	*Cryptosporidium*	*in vivo*	Protein synthesis	Edlind, 1991 Fayer and Ellis, 1993
Rifabutin	*Toxoplasma*	*in vivo*	DNA-dependent RNA polymerase	Araujo *et al.*, 1994
Sinefugin	*Cryptosporidium*	*in vivo*	Methylation reactions?	Brasseur *et al.*, 1994
Artemesinin	*Toxoplasma*	*in vitro*	Free-radical ? Haem polymerisation?	Holfels *et al.*, 1994 Hong *et al.*, 1994
Hydroxynapthoquinone (Atovoquone)	*Toxoplasma* *Eimeria*	*in vivo/in vitro*	Mitochondrial electron transport Cytochrome bc 1 complex	Fry and Williams, 1984 Fry *et al.*, 1984 Edlind, 1991 Fry and Pudney, 1992 Romand *et al.*, 1993
Epiroprim	*Toxoplasma*	*in vitro/in vivo*	Dihydrofolate reductase	Chang *et al.*, 1994

Table 1.3.a. Putative mechanisms of action of potential anticoccidial agents.

COMPOUND	ORGANISM	ACTIVITY	PUTATIVE MECHANISM	REFERENCE
Difluoromethylornithine	*Eimeria*	*in vivo*	Ornithine decarboxylase	McCann *et al.*, 1981
Carboxyemimycin	*Eimeria*	*in vivo*	Orotic acid analogue Pyrimidine metabolism?	Matsuno *et al.*, 1984
Frenolicin B	*Eimeria*	*in vivo*	Not known	Omura *et al.*, 1985
Cyclosporin A	*Eimeria*	*in vitro/in vivo*	Cyclophilin?	McCabe *et al.*, 1986*b* Rose and Hesketh, 1989 Harrison and Stein, 1990
Primaquine	*Eimeria* *Sarcocystis*	*in vivo*	Via free-radical? Oxidative phosphorylation?	Matsuno *et al.*, 1991 Bisby, 1990 Baker *et al.*, 1986
Hydroxycoumarin 1992	*Eimeria*	*in vivo*	Not known	Int. Pub. No. WO 92/06083
Xanthoquinodin	*Eimeria*	*in vitro/in vivo*	Not known	Tabata *et al.*, 1993*a*
WS-5995	*Eimeria*	*in vitro/in vivo*	Not known	Devi *et al.*, 1994
Benzimidazole 1993	*Eimeria*	*in vitro*	Not known	Eur. Pat, No. 931172243.1
Diolmycin	*Eimeria*	*in vitro*	Not known	Tabata *et al.*, 1993*c*
Cytosaminomycin	*Eimeria*	*in vitro*	Not known	Hanenda *et al.*, 1994
Hynapene	*Eimeria*	*in vitro*	Not known	Tabata *et al.*, 1993*b*

Table 1.3.b. Putative mechanisms of action of potential anticoccidial agents

COMPOUND CLASS	EXAMPLES	MECHANISM OF ACTION
Polyether ionophore	Salinomycin Lasalcoid Maduramicin	Perturb ion gradients
Carbanilide/ pyrimidine	Nicarbazin	Oxidative phosphorylation uncoupler?
Febrifugine	Halofuginone	Not known
Triazine	Diclazuril Toltrazuril	Pyrimidine metabolism? Mitochondrial respiration? Chlorophyll *a* D1 complex?
Quinolone	Decoquinate Buquinolate	Mitochondrial respiration/ electron transport
Pyridinol	Clopidol	Mitochondrial respiration/ electron transport
Thiamine anologue	Amprolium	Thiamine uptake and utilisation
Nitrobenzamide	Zoalene Nitromide	Nicotinamide antagonist?
Guanidine	Robenidine	Oxidative phosphorylation uncoupler?
Benzylpurine	Arprinocid	Purine salvage? Ion chelation (N-oxide active metabolite?)
Organic arsenical	Roxarsone	Binds protein sulphdryl groups?
Polyketide	Oxytetracycline Chlorotetracycline	Protein synthesis?
Sulphonamide	Sulphadimethoxine Sulphaquinoxaline	Dihydropteroate synthetase
Aminopyrimidine and Sulphonamide	Ormethoprim and Sulphadimethoxine	Dihydrofolate reductase and dihydrofoltae synthetase

Table 1.3.c. Mechanism of action of commercial avian anticoccidial drugs

1.3 AIMS OF THE PROJECT

The previous section highlights some of the deficiencies in our knowledge of energy metabolism in the coccidia. This project was aimed mainly at enhancing our understanding of this area. The overall aims could be stated as follows:

- To investigate and identify the pathways of energy metabolism operating in coccidia.
- To establish the physiological significance of the pathways for the survival, growth, and development of the parasites within the host.

A secondary aim was to identify parasite-specific targets for chemotherapeutic exploitation.

In the pursuit of these aims, two main approaches were adopted:

- Detection and characterisation of enzymes of energy metabolism (Chapter 3).
- Elucidation of substrates and end products of energy metabolism (Chapter 4).

A particular concern of the project was to elucidate how the coccidia are adapted towards the differing gaseous conditions encountered during their life cycle. A working hypothesis was that the invasive stages of the parasites might be adapted towards a fermentative energy metabolism due to the low oxygen tensions encountered in the host gut. In view of this, particular emphasis was placed on assessing the relative importance of aerobic and anaerobic pathways of energy metabolism in coccidia, and on investigating specific adaptations towards anaerobiosis.

In addition to the work on energy metabolism, limited investigations were carried out into proteinase activity in the parasites (Chapter 5). These were prompted by the

availability of local expertise in proteinase analysis; the deficit of previous analyses; the possibility that these enzymes may play a role in energy metabolism and the perceived potential of these enzymes as drug targets.

Most studies were restricted to sporozoites and oocysts of *E. tenella*, the parasite material most readily available, however some comparative analyses were possible using tachyzoites and bradyzoites of *T. gondii*, and cysts of *C. parvum*.

2. PARASITE METHODOLOGY

2.1 INTRODUCTION

The parasitic stages of the coccidia are predominately intracellular and as a consequence most are difficult to obtain in sufficient numbers, or at a suitable level of purity, for biochemical analyses. Current biochemical information is therefore derived primarily from the oocysts, which can be obtained in large numbers from the tissue or faeces of infected animals, and from the invasive stages of the parasites. Being highly resistant, the oocysts are very amenable to purification and will remain viable for many months under appropriate storage conditions. Unsporulated oocysts may be sporulated by aerobic incubation to yield sporulated oocysts which may then be excysted *in vitro* to release sporozoites. The conditions for *in vitro* excystation are well defined, usually involving exposure to bile salts and proteolytic enzymes - thought to be the key effectors of the process in the host gut.

Tachyzoites of *T. gondii* are currently the only intracellular stage of a coccidian that can be obtained with relative ease. These can be harvested in large numbers and relatively high purity from the peritoneal fluid of infected laboratory animals (Roberts and Alexander, 1992). While it is possible to grow the intracellular phases of other coccidia, including *Eimeria*, in vertebrate cell lines *in vitro*, these approaches generally yield only limited amounts material at low purity and as yet have not provided material satisfactory for many biochemical studies.

This project mainly concerns *Eimeria tenella*. Like most studies of this parasite, it was restricted largely to the oocyst and sporozoite stages, although crude preparations of merozoites and schizonts were also available for a few analyses. In part of the project, comparative studies were carried out using oocysts of *C. parvum*, and both tachyzoites and bradyzoites of *T. gondii*. All of these parasites were provided by other workers. While most were produced using well-established and reported techniques, the method for growing and purifying bradyzoites of *T. gondii* is novel and so is presented here in some detail. The following section briefly summarises the techniques used to obtain the parasites used in this project and describes, in more detail, the methods used in the experimental work.

2.2 CHEMICALS AND REAGENTS

Unless otherwise stated, the reagents used in this project were of analytical grade and were purchased from Sigma (Poole, UK). In cases where the precise source or grade of a reagent might affect the reproducibility of experiments, details (including catalogue numbers) are given in the text.

2.3 *EIMERIA*:

2.3.1 Oocyst supply

Oocysts of *Eimeria tenella* were supplied by Pfizer Central Research, Kent. Two strains were used during the course of this project: an ionophore-sensitive strain, Et2, which formed the basis for the enzymatic analyses described in Chapter 3; and an ionophore-resistant strain, Ret5, which was used throughout the end-product analyses (excepting NMR studies) reported in Chapter 4. Both strains were used during the proteinase analyses of Chapter 5.

The oocysts were obtained from the caeca of infected birds using standard methods of homogenisation and trypsin digestion (Davis, 1973) and were partially purified by sieving and salt flotation. They were supplied, either sporulated or unsporulated, in 2.5% (w/v) potassium dichromate solution and stored at 4°C until use.

2.3.2 Bleach treatment of oocysts

The oocyst preparations received in the laboratory generally contained high levels of contaminating bacteria and gut debris. After investigation of a variety of techniques the following process of bleach treatment was chosen as the most efficient and reliable method of removing the contaminating material. The bleach acts both to sterilise the oocysts and break down tissue material (Davis, 1973) and, by virtue of its high viscosity, facilitates separation of debris by differential centrifugation.

Oocysts were washed out of the dichromate by centrifugation (5 min at 1200 g, 4°C) and resuspended in a minimal volume (approximately 15 ml per $5x10^7$ oocysts) of 20% (v/v) bleach (Tesco). The suspension was incubated for 20 min at 0°C, then diluted 5 fold with distilled water and centrifuged for 2 min at 1000 g. The supernatant (containing bacteria and small debris) was carefully removed and the loose pellet resuspended in 50-100 ml distilled water and centrifuged for a further two min at 1000 g. The resultant pellet, containing cleaned oocysts, was washed by centrifugation (5 min at 1500 g, 4°C) and resuspension in distilled water until free of the smell of hypochlorite (a minimum of five washes). The cleaned oocysts were used directly or resuspended in 2.5% (w/v) potassium dichromate and stored at 4°C.

2.3.3 Oocyst sporulation

When required, oocyst sporulation was effected by incubation at 29°C in a rapidly shaking water bath (Davis, 1973). The incubation vessel was left open to the atmosphere and chosen to ensure good aeration of the suspension. The oocysts were normally resuspended in 2.5% (w/v) potassium dichromate solution to inhibit bacterial growth,

however 0.1 M phosphate buffered saline (PBS), pH 7.4, and distilled water were also used on occasions. When fresh oocysts were used, sporulation efficiencies of 75-85% were normally achieved (as assessed by microscopic examination), however efficiency rapidly declined as the oocysts aged and was negligible in preparations that had been stored for more than three weeks at 4°C.

2.3.4 *In vitro* excystation and purification of sporozoites

The excystation protocol used throughout this project is an adaptation of the method currently employed at Pfizer Central Research. The main modification is the inclusion of Mg^{2+} and chymotrypsin in the sporozoite excystation mix. These components have previously been reported to enhance excystation (Dulski, 1990; Guyonnet *et al.*, 1991), a fact which my own investigations seemed to confirm (data not shown).

[2]Sporulated oocysts were washed out of dichromate by centrifugation (5 min at 1200 g, 4°C) and resuspended in 10 ml PBS, pH 7.4, in a 50 ml Falcon tube. An equal volume of 3 mm diameter glass beads was added and the tube was vortexed at maximum speed for 3 - 3.5 min, or until microscopic examination showed the majority of oocysts to be ruptured. The sporocyst suspension was transferred to another Falcon tube, centrifuged for 5 min at 1500 g at 4°C, and the resultant pellet resuspended in 15 ml PBS, pH 7.4, containing 2% (w/v) taurodeoxycholic acid (Calbiochem), 0.1% trypsin (porcine, Sigma

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[2] The volumes and containers specified in the protocol are appropriate for dealing with approximately $1x10^8$ oocysts. To maintain the efficiency of excystation when using quantities other than this, the reagent volumes etc. would have to be scaled appropriately.

#T-4674), 0.01% α-chymotrypsin (from bovine pancreas, Sigma #C-4129) and 10 mM $MgCl_2$. The suspension was transferred to a 75 ml culture flask and incubated at 44°C in a shaking water bath for 1 hr, or until microscopic examination showed the majority of sporozoites to be free. The sporozoite suspension was then transferred to another Falcon tube and combined with 25 ml RPMI containing 10% (v/v) heat inactivated foetal calf serum. This suspension was centrifuged up to 1000 g in a bench centrifuge (the centrifuge being switched off when it attained this speed) and the supernatant (containing mainly sporozoites) removed. The loose pellet, containing oocyst shells and large debris, was resuspended in 45 ml PBS, pH 7.4, and recentrifuged up to 1000 g, with the supernatant being again retained. This process was repeated if significant numbers of sporozoites remained in the pellet. The final supernatant fractions were centrifuged for 5 min at 2800 g (4°C) and the pelleted sporozoites resuspended to approximately $5x10^6$ ml^{-1} in PBS, pH 7.4. The suspension was filtered through 10 ml (packed volume) of pre-wet cotton wool packed in a 50 ml syringe barrel and the 'column' was washed with a further 50-100 ml of PBS, pH 7.4. The purified sporozoites were collected from the combined column eluent by centrifugation (5 min at 2800 g, 4°C) and either used immediately or stored as pellets at -70°C.

2.3.4.1 Efficiency of excystation

The average yields associated with the different stages of the excystation process are shown in Table 2.1; an average of 1.56 sporozoites were recovered per oocyst.

The final sporozoite preparation was typically more than 90% pure, in terms of the proportion of sporozoites to other particles. Empty sporocyst shells were the main

Table 2.1 Excystation of *E. tenella* oocysts.

Excystation step	Efficiency of step [a] (%)	Overall yield [b] (%)
Sporocyst release	39.6 ± 7.3	39.6
Sporozoite release	62.1 ± 20.4	24.6
Filtration	79.2 ± 12.2	19.5

Means ± SD, $n = 20$

[a] Actual yield as a percentage of theoretical yield

[b] Calculated from data in previous column

contaminant. The most important factor in determining the final purity of the sporozoite preparation proved to be the cleanliness of the initial oocyst suspension, while the overall yield was largely dictated by the age of the sporulated oocysts used. The speed and efficiency of excystation fell off gradually as the oocysts aged, with very low or negligible excystation being achieved after 9-12 months in storage. In some instances it was observed that while sporozoites from aged oocysts were capable of leaving the sporocyst, they lysed shortly after release into the excystation mix. Preliminary investigations indicated that this lysis could be partially prevented by including 10 mM glucose in the excystation mix - suggesting that the sporozoites might be running out of energy reserves - however this aspect was not investigated in detail.

2.3.5 Motility and viability of sporozoites

The viability of *Eimeria* sporozoites is commonly assessed by infectivity assays either *in vivo*, or using cell lines or chick embryos. While decisive, these techniques are time-consuming and were considered both impractical and unnecessary for the purposes of this project. Instead, viability was usually assessed by using phase contrast microscopy to assess whether sporozoites were intact and motile. This approach was not wholly satisfactory since sporozoites from different batches of oocysts frequently exhibited very different levels of motility. Indeed, despite trying a variety of conditions (ranging from media composition, pH, temperature, gaseous composition (see Glover, 1992)) motility of excysted sporozoites of the Et2 strain was hardly ever observed, although the parasites remained intact and consumed substrate during incubation experiments. In contrast, high levels of motility were usually observed with the Ret5 strain of the parasite. While this observation might reflect a true difference between the two strains of parasite, it is also possible that the transition from use of one strain to the other coincided with a change in the batch of one of the chemical components used for excystation which was adversely affecting motility. Nevertheless, significant variations in motility levels were sometimes observed between batches of the Ret5 strain which did not correlate with any obvious factor (including age). It was noted that motility was usually higher when sporozoites were removed from excystation mix into PBS, pH 7.4, and that motility levels were largely proportional to temperature. Hence, observations were normally carried out in PBS, pH 7.4, at a temperature of 37-41°C. It was found that freshly excysted sporozoites could be stored at 4°C for up to 48 hours in PBS, pH 7.4, and still regain motility upon warming to 37°C. In incubation experiments motility could be maintained throughout 8 hours at 41°C in PBS, pH 7.4, containing glucose

under both aerobic and anaerobic conditions (see Section 4.3.2.2). Recently Brown *et al.* (1996) developed a novel dye-staining technique which appears to have general applicability to the diagnosis of viability of both *C. parvum* and *E. tenella* sporozoites. This technique is more rapid and less subjective than making observations of motility. In comparative tests, higher levels of sporozoites were usually judged to be viable using the dye-staining technique rather than by motility observations of the same sample. The technique will no doubt prove valuable in future biochemical studies using whole cells, and may prove ideal for mass screening of anticoccidial agents..

2.3.6 Production of merozoites and schizonts

Merozoites were grown in primary chick kidney cell cultures by Dr Debbie Smith of Pfizer Central Research, Kent. The cultures were infected with sporozoites on day 2 following initiation, and first and second generation merozoites were recovered from the medium on days 5 and 7, respectively. The post-infected cells were used as a source of schizonts. All preparations contained high levels of host cell material.

2.4 TOXOPLASMA

Tachyzoites and bradyzoites of *Toxoplasma gondii* were produced by Dr Craig Roberts of the Department of Immunology, University of Strathclyde. The method of bradyzoite purification is novel and as a consequence is described here in some detail.

2.4.1 Production and purification of tachyzoites.

Tachyzoites of *T. gondii* (RH strain) were grown in the peritoneum of cotton rats as described previously (Roberts and Alexander, 1992). Cotton rats were inoculated intraperitoneally with $5x10^5$ tachyzoites suspended in PBS, pH 7.2. 48 hours later, tachyzoites were harvested from the peritoneum through a 21 gauge needle into PBS, pH 7.2, with heparin (20 U ml^{-1}). The parasites were sedimented (200 g for 10 min, 4°C), washed twice in PBS, pH 7.2, and either lysed immediately or stored as pellets at -70°C.

2.4.2 Production and purification of bradyzoites

Production in mice. Bradyzoites were obtained from tissue cysts harvested from the brains of B10 mice. This strain of mice has been demonstrated to develop large tissue cyst burdens in their brains during *T. gondii* infections (Hunter *et al.*, 1992; Blackwell *et al.*, 1993). B10 mice were infected using cysts obtained from the brains of Strathclyde Albino mice infected with *T. gondii* 17-21 weeks previously. The brains were homogenised in 2 ml PBS, pH 7.2, by six passes through a 21 gauge needle, the cyst density was determined microscopically and each B10 mouse was inoculated intraperitoneally with 10 cysts.

Purification. Cysts were harvested from the brains of B10 mice which had been infected 28-30 days previously, and purified by a modification of the method previously described (Blewett *et al.*, 1993). The brain of each mouse was removed and homogenised in PBS, pH 7.2, by repeated passage through a 21 gauge needle. Aliquots

containing the equivalent of 3-4 homogenised brains were diluted to 13 ml with PBS. 6.5 ml 90% (v/v) Percoll produced by addition of 10× concentrated PBS, pH 7.2, to Percoll, were added and the mixture left to settle for 30 min at 18°C. 2 ml 90% (v/v) Percoll was then added as a bottom layer and the mixture centrifuged for 30 min at 2500 g and 18°C, which sedimented most of the cysts into the bottom layer. This and the bottom 1 ml of the upper layer, which also contained tissue cysts and most of the erythrocytes, were removed, diluted 10-fold in PBS, pH 7.2, and centrifuged at 2500 g for 15 min (18°C) to remove the Percoll. The supernatant was discarded, 1.8 ml of distilled water added to the pellet to lyse the erythrocytes, and 0.2 ml of 10 × concentrated PBS, pH 7.2, added immediately. The cysts were sedimented by centrifugation at 1000 g for 10 min, resuspended in 1 ml of PBS, pH 7.2, for counting, resedimented (1000 g for 10 min) and resuspended in 5 ml of 1% (w/v) pepsin (Sigma) in HCl (pH 1.5) to release the bradyzoites. Following a 5 min incubation at 37°C, the pepsin solution was deactivated by addition of 15 ml PBS and the bradyzoites sedimented by centrifugation at 1000 g for 10 min. The bradyzoites were washed once, resuspended in PBS, and either lysed immediately or stored as pellets at -70°C.

2.4.2.1 Efficiency of bradyzoite production

The method used for growing and purifying bradyzoites was successful in terms of both parasite numbers and the low level of contaminating material. In a typical purification, which used the brains of 15 mice, 6.9×10^4 cysts were found to be present in unpurified brain homogenate. 6.4×10^4 cysts were purified (92% recovery) yielding 7.5×10^6 bradyzoites (approx. 20 μg protein). There was less than 1% cell/cell contamination, as

determined microscopically. The amount of contaminating host material was also assessed by analysing enzyme activities apparently absent from the parasite but present in the host cells. The results are described in Section 3.3.1 Tachyzoite preparations were essentially free from host contamination.

2.5 *CRYPTOSPORIDIUM*

Sporulated oocysts of *Cryptosporidium parvum* where supplied by Dr Vincent McDonald of The London School of Hygiene and Tropical Medicine.

Oocysts were passaged in C57 mice (OLAC) which had previously been immunosuppressed by subcutaneous administration of dexamethasone (1 mg on alternate days for one week). 10^5-10^6 purified *C. parvum* oocysts were administered orally at the same time as the last dexamethasone injection and faeces collected on days 3-7 following infection. Oocysts were isolated and purified from the faeces using methods described previously (Iseki, 1986; McDonald *et al.*, 1990; Ogunkolade *et al.*, 1993) and stored at 4°C in 2.5% (w/v) potassium dichromate until use.

2.6 PREPARATION OF CELL HOMOGENATES

The precise conditions used for cell lysis were dictated by the intended use of the resultant extract, however the same basic methods were used throughout.

Bradyzoites and tachyzoites of *T. gondii*, and sporozoites of *E. tenella* were generally lysed directly by resuspension in buffer containing 0.25% Triton X-100. The suspension was aspirated thoroughly and left on ice for 5 min prior to further processing. The cells could also be lysed by sonication (3×10 sec at 18 μm) and this procedure was used where indicated in Chapters 3-5.

Oocysts of *E. tenella* and *C. parvum* were resuspended to the required density in buffer (generally containing 0.25% Triton X-100) and broken by vortexing with an equal volume of 3 mm glass beads. Vortexing was continued until microscopic examination revealed the majority of oocysts and their contents to be disrupted. This typically took 3-4 min for unsporulated oocysts of *E. tenella*; 10-12 min for sporulated oocysts of the species; and 15-20 min for cysts of *C. parvum*[3].

The following proteinase inhibitors were normally included in the lysis buffer: 0.5 mM PMSF, 5 μM E64, 1 μM pepstatin and 0.2 mM 1,10-phenanthroline; all lyses and subsequent processes were carried out at 0-4°C.

Soluble extracts were obtained by centrifugation for 10 min at 14000 g and 4°C. The amount of protein associated with these soluble extracts is shown in Table 2.2.

[3] Investigation showed that much more rapid disruption of the C. *parvum* cysts could be achieved if smaller (e.g. 0.3 mm) beads were used, however these were not generally available during the project.

Table 2.2 Protein in soluble extracts of coccidial parasites.

	Protein (μg per 10^7 cells)
Eimeria tenella	
unsporulated oocysts	1500 ± 318 (19)
sporulated oocysts	1131 ± 146 (15)
sporozoites	170 ± 81 (12)
Toxoplasma gondii	
tachyzoites	65 ± 29 (19)
bradyzoites	27 ± 12 (5)
Cryptosporidium parvum	
cysts	33 ± 18 (8)

Means ± SD, *n* in parentheses.

3. ENZYMES OF ENERGY METABOLISM IN THE COCCIDIA

3.1 INTRODUCTION

3.1.1 Why look at enzymes?

The pathways of energy metabolism operating in an organism are reflected in its repertoire of enzyme activities. Since the conditions under which most common enzymes operate are well defined, it is theoretically straightforward to assay extracts of an organism and say whether a particular pathway, such as glycolysis or the TCA cycle, appears to be present. Inspection of the specific activities of enzymes may give an indication of the relative flux through particular metabolic pathways, or of their relative importance in different species, or stages of a species life cycle. In parasites, there are often very extreme variations in enzyme expression at different stages of the life cycle (Bryant and Behm, 1989). This 'developmental regulation' usually constitutes an adaptive response to the different environments encountered during the life cycle and is particularly driven by changes in nutrient availability and gaseous composition.

Some parasites possess unusual pathways (or steps) of energy metabolism which are catalysed by enzymes which have no direct counterpart in their host species. Two examples are the acetate thiokinase of *Giardia lamblia* and the hydrogenase of *Trichomonas vaginalis* (Muller, 1988). Other parasite enzymes have been found which

catalyse apparently conventional steps, but on examination present very different properties to their host counterparts. These differences may be at the level of substrate-specificity and regulatory properties, or in the fine details of structure and catalytic mechanism. Unusual enzymes within parasites are of inherent interest as potential drug targets and may also provide particular insight into the evolutionary origins of, or adaptive pressures acting on, an organism.

3.1.2 Pyrophosphate-dependent glycolytic enzymes

Glycolysis is an ancient metabolic pathway which is present, at least in part, in all organisms. It is central to both aerobic and anaerobic modes of carbohydrate catabolism. While the reactions of glycolysis (shown in Figure 3.1) and the enzymes mediating them are generally highly conserved (Fothergill-Gilmore and Michels, 1993), it has become increasingly evident that some variations do exist. One departure is the utilisation of inorganic pyrophosphate (PP_i) rather than nucleotide phosphate (NTP) as a phosphoryl donor in some reactions. The two most common pyrophosphate-dependent reactions are shown in Figure 3.2.

Pyrophosphate-dependent phosphofructokinase (PP_i-PFK) is the best-studied PP_i-dependent enzyme. This enzyme has been identified in a variety of organisms including the following parasitic protozoa: *Entamoeba histolytica* (Reeves *et al.*, 1974), *Giardia lamblia* (Mertens, 1990), *Trichomonas vaginalis* and *Tritrichomans foetus* (Mertens and Muller, 1990), *Naegleria fowleri* (Mertens *et al.*, 1993) and *Toxoplasma gondii* (Peng and Mansour, 1992). The first two organisms on the list also contain a pyruvate

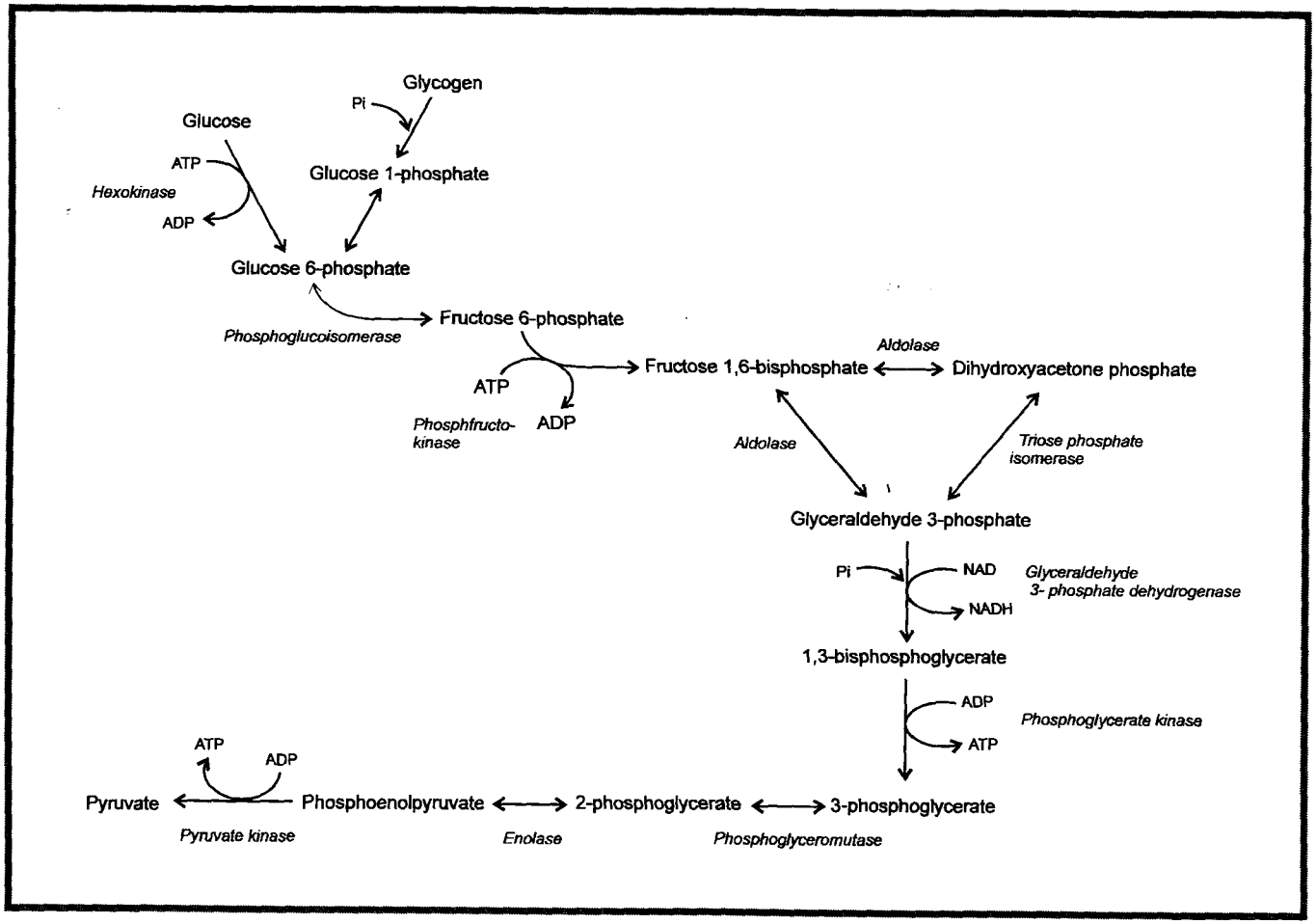

Figure 3.1. The glycolytic pathway.

Classical NTP-linked reactions	Pyrophosphate-linked reactions
$ATP + F6P \rightarrow F16P_2 + ADP$ ATP-dependent phosphofructokinase	$PP_i + F6P \leftrightarrow F16P_2 + P_i$ PP_i-dependent phosphofructokinase
$PEP + ADP \rightarrow pyruvate + ATP$ pyruvate kinase	$PEP + AMP + PP_i \leftrightarrow pyruvate + P_i + ATP$ pyruvate phosphate dikinase

Figure 3.2. Pyrophosphate-dependent glycolytic reactions and their nucleotide-dependent equivalents.

phosphate dikinase (PPDK) which apparently replaces the conventional ADP-specific pyruvate kinase (PK) (Reeves, 1968; Mertens, 1993).

The PP_i-PFKs in parasitic protozoa present essentially similar properties and have been termed type I PP_i-PFKs (Mertens, 1991). This distinguishes them from the type II variety which are found in plants. The most distinctive feature of the type I enzymes is an apparent lack of regulatory properties. Most specifically, they show no evidence of allosteric properties and are unaffected by fructose 2,6-bisphosphate (F26P2), the major activator of most ATP-PFKs. In addition, organisms which use these enzymes are unusual in possessing no separate fructose 1,6-bisphosphatase activity. It is thought that these features both relate to the ready reversibility of the pyrophosphate-dependent reaction which, theoretically, renders it less useful as metabolic control and also means that the enzyme can perform both a glycolytic and the gluconeogenic function. The properties are in marked contrast to those of ATP-dependent enzymes which catalyse an essentially irreversible reaction and, in most organisms, are regarded as the key regulatory enzymes of glycolysis.

The organisms containing type I PP_i-PFKs present no close phylogenetic relationship, but are apparently united by a heavy reliance on fermentative modes of energy production. Most lack mitochondria and are therefore obligate anaerobes - this usually correlates with an existence in largely anaerobic environments. The use of a PP_i rather than an ATP-PFK increases the energy yield of glycolysis from 2 to 3 molecules of ATP per molecule of glucose (simply because one less ATP is hydrolysed during the process). While this increase in energetic efficiency would only be of only slight benefit to aerobes, it would represent a significant advantage to organisms which are solely

dependent on glycolysis for their energy production. This observation, along with the unusual phylogenetic distribution of the type I PP_i-PFKs enzymes, has led to the suggestion that PP_i-dependent enzymes may represent evolutionary adaptations towards anaerobiosis (see Mertens, 1991 and 1993); this hypothesis is widely accepted.

In most organisms PP_i is a product of biosynthetic reactions and is rapidly cleared from the cytosol by inorganic pyrophosphatases (Baltscheffsky and Baltscheffsky, 1992). This is thought to be necessary to prevent inhibition of biosynthetic reactions. Organisms which use PP_i-dependent enzymes have been found to be unusual in lacking cytosolic pyrophosphatase activity (McLaughlin *et al.*, 1978; Searle and Muller, 1991). This is clearly consistent with the use of PP_i compound as an energy donor but it is so-far unclear how pyrophosphate levels are regulated to favour both glycolysis and biosynthetic reactions.

The type II PP_i-PFKs, found in plants and other photosynthetic organisms, differ from the type I variety in co-existing with both an ATP-PFK and a fructose 1,6-bisphosphatase, and also in being allosterically activated by $F26P_2$. The relationship between these and the type I PP_i-PFKs of bacteria and protozoa is unclear, however Mertens (1991) considers (and cites data to indicate) that the former are also associated with anaerobiosis, or with other conditions where ATP/ADP ratios are low.

3.1.3 Aims of this study

A number of enzymes of energy metabolism have previously been detected in the coccidia, detailed in Table 1.1 (*a*). The enzymatic evidence implies that a glycolytic

pathway is a common feature of the coccidia but it is unclear whether or not any species or stage contains a functional TCA cycle (discussed in Section 1.2.1.2). There have been very few quantitative analyses of enzyme activities involved in energy metabolism in the coccidia and hardly any enzymes have been characterised.

In the following study, an attempt was made to assess the relative significance of aerobic and anaerobic modes of energy metabolism in the coccidia, by comparing the activities of selected enzymes of glycolysis and the TCA cycle in various stages of *E. tenella*, *T. gondii* and *C. parvum*. PP_i-dependent activities were specifically included in the study, both because of the recent finding of PP_i-PFK in tachyzoites of *T. gondii,* and the idea that these enzymes might be a common adaptation of the coccidia towards the largely anaerobic conditions encountered by the invasive stages in the host gut. The discovery of PP_i-PFKs in *C. parvum* and *E. tenella* led to a detailed analysis of their properties and, in turn, to an investigation of how glycolysis is regulated in the coccidia. This was investigated by studying the kinetic and regulatory properties of hexokinase and pyruvate kinase, two enzymes which are classically involved in glycolytic control.

The results from this section are presented in Denton *et al.* (1994 and 1996 (*a* and *b*)), (attached).

3.2 MATERIALS AND METHODS

3.2.1 Preparation of extracts for use in enzyme assays

Parasite extracts were prepared as described in Section 2.6 using the following lysis buffer: 50 mM HEPES, pH 7.3 containing 20% (v/v) glycerol, 0.25% Triton X-100 and proteinase inhibitors (0.5 mM PMSF, 5 μM E64, 1 μM pepstatin, 0.2 mM 1,10-phenanthroline). DTT (1 mM) was also included if the enzyme under investigation was enhanced by reducing conditions.

Mouse brain homogenates were prepared by addition of the suspension buffer used with the parasites and subjecting the brains to 5 passes through a 21 gauge needle and then three passes through a 6 gauge needle.

The extracts were either used immediately, or stored at -70°C, as specified below.

3.2.2 Concentration of extracts

When required, soluble extracts were concentrated by ultrafiltration using an Amicon Centricon (10 kDa molecular weight cut-off).

3.2.3 Buffer exchange

In specified experiments the soluble parasite extracts were buffer exchanged prior to assay to remove low molecular weight components. This was achieved either by dialysis against 100 volumes of appropriate assay buffer (20 h, 4°C, one change); by desalting

on a Pharmacia Sephadex G-25M PD-10 column; or by repeated concentration and dilution using a Centricon10 concentrator (see above). The methods used are specified in the text.

3.2.4 Enzyme assays

All assays were carried out at 37°C in a final volume of 1 ml and, with the exception of the succinate dehydrogenase assay (for which crude homogenates were used), contained soluble cell extract. Unless indicated otherwise, reactions were monitored spectrophotometrically at 340 nm and initiated by the addition of substrate at saturating concentrations. To confirm the specificity of the reaction, control assays were carried out lacking one or more of the assay components. Experiments were also conducted to ensure a linear relationship between reaction rate and sample size over the normal working range. Kinetic calculations were performed using the computer program 'Grafit'(Leatherbarrow, 1992). All pyruvate kinase assays and kinetic analyses were carried out using freshly prepared cell and tissue extracts; other enzyme assays were performed on extracts which had been stored at -70°C.

Source and type of coupling enzymes:

Aldolase, type IV from rabbit muscle, Sigma #A-1893; glucose 6-phosphate dehydrogenase, type VII from bakers yeast, Sigma #G-7877; glutamic-pyruvic transaminase (L-alanine aminotransferase, from porcine heart, Sigma #G-9880; glycerokinase, from *Cellulomanas* spp., Sigma #G-6142; α-glycerophosphate dehydrogenase, type I from rabbit muscle, Sigma #G-6751; α-glycerophosphate dehydrogenase/ triose phosphate isomerase mix, Sigma #G-1881; hexokinase, type III

from bakers yeast, Sigma #H-5000; inorganic pyrophosphatase, from yeast, Boehringer Mannheim #108 987; L-lactic dehydrogenase, type II from rabbit muscle, Sigma #L-2500; phosphoglucose isomerase, type II from bakers yeast, Sigma #P-5381; pyruvate kinase, type II from rabbit muscle, Sigma #P-1506; triose phosphate isomerase, type I from bakers yeast, Sigma #T-2507.

3.2.4.1 Phosphofructokinase

Forward reaction: the standard assay contained: 50 mM HEPES, pH 7.0; 1 mM $MgCl_2$; 1 mM DTT; 0.28 mM NADH; 1 mM fructose 6-phosphate; 1.5 U aldolase; 2 U α-glycerophosphate dehydrogenase; 20 U triose phosphate isomerase; 1 mM PP_i or 1 mM ATP and crude enzyme. 5U of yeast pyrophosphatase was included in assays for the ATP-dependent activity.

Reverse reaction: the standard assay contained: 50 mM HEPES, pH 7.0; 1 mM $MgCl_2$; 1 mM DTT; 1 mM $NADP^+$; 1 mM fructose 1,6-bisphosphate; 1 mM P_i; 2 U glucose 6-phosphate dehydrogenase; 5 U phosphoglucose isomerase and crude enzyme.

3.2.4.2 Pyruvate kinase (and pyruvate phosphate dikinase)

The standard assay contained: 50 mM HEPES, pH 7.5; 1 mM DTT; 7.5 mM $MgCl_2$; 75 mM KCl; 0.28 mM NADH; 10 mM phosphoenolpyruvate; 2 U lactate dehydrogenase; 5 mM ADP (or 5 mM PP_i and 5 mM AMP) and crude enzyme.

3.2.4.3 Hexokinase

Hexokinase activity was assayed according to two methods (Jenkins and Thompson, 1989). The glucose 6-phosphate dehydrogenase method was used to determine specific activities and to assess the substrate-specificity of the enzyme, while the pyruvate kinase-coupled method was used to investigate product inhibition. This second assay could not be applied to crude extracts because of interference by apparent ATPase activity - this could be effectively removed by MonoQ fractionation (see Sections 3.2.3 and 3.3.4.1.).

Glucose 6-phosphate dehydrogenase-coupled assay (Method A)

The standard assay contained: 50 mM HEPES, pH 7.6; 1 mM DTT; 90 mM KCl; 3 mM $MgCl_2$; 2 mM glucose (or fructose); 1 mM ATP; 0.2 mM $NADP^+$; 1 U glucose 6-phosphate dehydrogenase and crude enzyme. 2U phosphoglucoisomerase was also included when fructose was used as a substrate.

Pyruvate kinase-coupled assay (Method B)

The standard assay contained: 50 mM HEPES, pH 7.6; 1 mM DTT; 90 mM KCl; 3 mM $MgCl_2$; 2 mM glucose (or fructose); 1 mM ATP; 0.28 mM NADH; 5 U pyruvate kinase; 5 U lactate dehydrogenase and crude enzyme. 2U phosphoglucose isomerase was also included when fructose was used as a substrate.

3.2.4.4 Lactate dehydrogenase

The standard assay contained: 100 mM Tris, pH 7.4; 200 mM NaCl; 0.28 mM NADH; 1.6 mM pyruvate and crude enzyme.

3.2.4.5 Isocitrate dehydrogenase (NAD^+- and $NADP^+$- dependent)

The standard assay contained: 50 mM HEPES, pH 7.5; 2 mM $MnCl_2$; 6.7 mM isocitrate; 0.4 mM $NADP^+$ or NAD^+ and crude enzyme. 2 mM ADP was included in assays for the NAD^+-dependent enzyme.

3.2.4.6 Succinate dehydrogenase

Succinate dehydrogenase activity was assayed in whole cell homogenates by following the oxidation of potassium ferricyanide. The standard assay contained in a final volume of 1 ml: 100 mM sodium phosphate buffer, pH 7.3; 10 mM KCN; 2 mM potassium ferricyanide; 15 mM sodium succinate and crude cell homogenate. The reaction was followed continuously at 420 nm (for 1-2 h). Control assays were carried out in the absence of succinate.

3.2.4.7 Pyrophosphatase

Samples of parasite extract were incubated at 37°C in 50 mM HEPES, pH 7.5, containing 2.5 mM $MgCl_2$ and 2 mM PP_i. The reaction was terminated after 30-90 min by addition of SDS to 6% (w/v), and the amount of released phosphate determined according to the method of Chifflet *et al.* (1988). Assays were routinely carried out in a final volume of 250 µl in microtitre plates (50 µl sample volume).

3.2.4.8 Other enzymes

Phosphoglyceromutase, phosphoglucose isomerase, glutamate dehydrogenase, malate dehydrogenase, fumarase, glucose 6-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase and glycerol dehydrogenase were assayed by standard spectrophotometric techniques as described in Bergmeyer *et al.* (1983). Serine sulphydrase was assayed as described in Thong and Coombs (1985).

3.2.4.9 Microtitre plate protocols

When it was more important to obtain a qualitative rather than a quantitative detection of enzymes (for example, when analysing fractions from column chromatography), the above assays were adapted to be carried out in 250 µl volumes in microtitre wells. Assays typically contained 10 µl of sample and the same concentrations of reagents as specified for the standard assay. Plates were incubated at 37°C, and absorbances measured at intervals until an appropriate end-point was achieved.

3.2.5 MonoQ fractionation

Unsporulated oocysts of *E. tenella* were resuspended to a concentration of 2-5x10^7 ml^{-1} in 20 mM Tris-HCl, pH 7.3, containing proteinase inhibitors and lysed by vortexing with glass beads (Section 2.6). The crude homogenate was sonicated (3×10 sec at 18 µm), then centrifuged at 30,000 g for 1 h at 4°C. The non-sedimentable fractionation (shown to contain 100% of the original hexokinase activity) was clarified by filtration through a 0.22 µm pore GS membrane (Millipore) and samples (2 ml) were injected onto a MonoQ HR 5/5 (Pharmacia) anion-exchange column pre-equilibrated with 20

mM Tris-HCl, pH 7.3, (Buffer A). The column was washed with 5 ml of Buffer A at a rate of 1 ml min^{-1} and the protein eluted with 0-350 mM gradient of KCl in the same buffer (Buffer B). Fractions (0.5 ml) were analysed for enzyme activities using microtitre plate protocols and pooled and concentrated as required.

3.2.6 SDS-PAGE

Discontinuous SDS-PAGE was carried out as described by Hames (1990).

3.2.7 Protein determinations

Protein concentrations were determined using the Pierce BCA assay kit with bovine serum albumin as a standard.

3.3 RESULTS

3.3.1 Enzyme activities in coccidial extracts

Tables 3.1 and 3.2 show the activities of selected enzymes of energy metabolism in different stages of *E. tenella*, *T. gondii* and *C. parvum*. The number of activities investigated was largely limited by the availability of parasite material and in particular by the availability of *T. gondii* bradyzoites. Since it was considered possible that the bradyzoite samples used in this study might be contaminated with host cell material, the activity of each enzyme was also measured in mouse brain extracts. By reference to the sensitivity of the assay for NAD^+-ICDH (an enzyme found to be present in mouse brain but not in bradyzoites) it was possible to calculate that the bradyzoite preparation could comprise, at most, 4.3% host protein. This figure was used to calculate the possible contribution by host enzymes to the enzyme activities detected in the parasite preparation. The results (detailed in the legend to Table 3.1) provide assurance that the data presented for bradyzoite activities are valid. The remaining parasite preparations were essentially free from host cell contamination.

The presence of high activities of PK and PP_i-PFK in all the parasite extracts investigated is consistent with the presence of a high activity glycolytic pathway - as suggested by previous results (see Section 1.2.1.2). The fact that hexokinase could not be detected in cysts of *C. parvum*, and was present only at very low levels in sporulated oocysts of *E. tenella* is somewhat surprising but it may simply reflect the reliance of these parasite stages on polysaccharide reserves rather than on free sugars.

Table 3.1. Activities of enzymes of energy metabolism in *Toxoplasma*, *Cryptosporidium* and mouse brain extracts.

Sample:	Enzyme activity (nmol min^{-1}(mg protein)$^{-1}$)						
	HEXO.	PP_i - PFK	PK	LDH	$NADP^+$-ICDH	NAD^+-ICDH	SDH
T. gondii, tachyzoites	1.9 ± 1.0 (3)	634 ± 203 (3)	4658 ± 1275 (7)	655 ± 196 (6)	130 ± 33 (3)	n.d. < 0.2 (3)	13 ± 6 (3)
T. gondii, bradyzoites	n.a.	265 ± 15 (3)	11338 ± 2201 (3)	2259 ± 827 (6)	130 ± 38 (3)	n.d. < 1.0 (4)	n.d. < 26 (3)
Mouse brain	n.a.	n.d. < 1.0 (3)	5219 ± 398 (6)	n.d. < 0.3 (4)	64 ± 5 (3)	23 ± 5 (3)	25 ± 3 (3)
C. parvum, cysts	n.d. < 1.0 (3)	331 ± 33 (3)	2947 ± 775 (5)	82 ± 44 (6)	n.d. < 0.9 (3)	n.d. < 0.9 (3)	n.d. < 1.0 (3)

Means ± SD, *n* in parentheses. n.d., not detectable, n.a., not assayed

Maximum contribution of host cell enzymes to activities measured in bradyzoite extracts (nmols min^{-1} (mg protein)$^{-1}$):
PFK, 0; PK, 227; LDH, 0; $NADP^+$-ICDH, 2.8; SDH, 1.1.

Table 3.2. Activities of enzymes of energy metabolism in different developmental stages of *Eimeria tenella*.

Sample:	Enzyme activity (nmol min^{-1}(mg protein)$^{-1}$)						
	HEXO.	PP_i - PFK	PK	LDH	$NADP^+$-ICDH	NAD^+-ICDH	SDH
E.tenella, unsporulated oocysts	36 ± 4 (3)	191 ± 46 (3)	2105 ± 572 (3)	2617 ± 244 (3)	8 ± 3 (5)	n.d. < 0.4 (4)	n.d. < 0.4 (3)
E.tenella, sporulated oocysts	3 ± 2 (3)	56 ± 24 (4)	124 ± 60 (4)	114 ± 77 (4)	20 ± 8 (5)	n.d. < 0.6 (4)	n.d. < 0.6 (3)
E.tenella, sporozoites	53 ± 8 (4)	105 ± 15 (5)	1349 ± 167 (4)	1254 ± 74 (3)	122 ± 74 (7)	n.d. < 0.4 (4)	n.d. < 0.4 (3)

Means ± SD, *n* in parentheses. n.d., not detectable

The absence of both ICDH activity and SDH from cysts of *C. parvum* suggests that these parasites do not possess a TCA cycle and is consistent with their apparent lack of mitochondria (Current, 1989; Tetley, L., Brown S.M.A. and Coombs, G.H., unpublished data) , however, the evidence regarding the presence or absence of a TCA cycle in the other species investigated is more ambiguous. This is partly because of uncertainty regarding the function of $NADP^+$-ICDH. This enzyme was detected in both *T. gondii* and *E. tenella*, while the NAD^+-dependent version was found only in mouse brain extracts. Most eukaryotic cells contain both an NAD^+ and $NADP^+$-specific ICDH. The current view is that TCA cycle activity is mediated largely by the NAD^+-dependent variety, while $NADP^+$-ICDH, which is present in both the cytosol and mitochondria, fulfils a separate, but as yet undetermined, function (Sazanov and Jackson, 1994). This scenario would seem to argue against the presence of a TCA cycle in the coccidia. To complicate things, however, it has been observed that many parasites, both protozoa and helminths, contain an $NADP^+$- but not a NAD^+-dependent ICDH. Some of these parasites have been reported to mediate full aerobic energy metabolism, suggesting that in these organisms $NADP^+$-ICDH participates in the TCA cycle (Barrett, 1981). In light of this, the presence of an $NADP^+$-, but not an NAD^+-ICDH in the coccidia is difficult to interpret. SDH is less problematic in that it presents no ambiguities of function. However SDH activities are generally low and may be difficult to detect by conventional assay systems. The apparent absence of this activity in *Eimeria* and in *Toxoplasma* bradyzoites, for which material was particularly limited, should not therefore be regarded as definitive.

All the parasite extracts contained high activities of lactate dehydrogenase, the enzyme responsible for mediating NADH reoxidation under anaerobic conditions. This would be

consistent with a heavy reliance on fermentative modes of energy generation (see Chapter 6 for further discussion).

3.3.1.1 Phosphoryl substrate specificites of PK and PFK

A significant finding of this study was, that like *T. gondii* (Peng and Mansour, 1992), both *E. tenella* and *C.parvum* contain a PP_i-specific PFK. A low level of apparent ATP-PFK was also detectable in extracts of these parasites, however this was abolished by addition of 5U of yeast pyrophosphatase to the assay mix, indicating that it was probably due to the conversion of some ATP to PP_i in the reaction mix - a similar effect has been observed in other PP_i-PFK harbouring protozoa (Peng and Mansour, 1992; Mertens, 1990). It was concluded that ATP-PFK activity is not present in these parasites. PP_i-PFK activity was also detected in preparations of first and second generation merozoites of *E. tenella* as well as in extracts of host cells infected with schizonts. Since these samples were heavily contaminated with host material it was not possible to obtain a meaningful specific activity for the enzyme, or to determine whether there was any parasite specific ATP-PFK activity. PP_i-PFK, however, was not detected in extracts of uninfected host cells.

Unlike some PP_i-PFK utilising protozoa (Reeves *et al.*, 1974; Mertens, 1990), the parasites contained high levels of ADP-specific pyruvate kinase but no PPDK (the limit of detection for the latter was, 1 nmol min^{-1} mg $protein^{-1}$).

3.3.1.2 Pyrophosphatase activity

Inorganic pyrophosphatase activity could not be detected under the standard assay conditions in any of the coccidial lysates (the limit of detection was 2 nmol min^{-1} mg $protein^{-1}$) - however high levels were measured in mouse brain and leishmanial extracts used as positive controls.

3.3.1.3 Differences between developmental stages

While the different developmental stages of *Eimeria* and *Toxoplasma* contained the same basic repertoire of enzymes, the activities of the enzymes differed between stages. Bradyzoites of *T. gondii* contained 2-3 times more PK and LDH activity than did tachyzoites, but only half the activity of PP_i-PFK. This may imply that the former place a greater reliance on anaerobic modes of energy metabolism (see Section 6.6). A more general trend was discernible between the different developmental stages of *Eimeria*. Here, lower levels of each enzyme (apart from $NADP^+$-ICDH) were found in sporulated than in unsporulated oocysts, while higher levels were found again in sporozoites. Since sporozoites account for more than half the soluble protein in sporulated oocysts (see Table 2.2), and the enzyme activities in sporozoites were more than double (in fact, up to 20-fold) those in oocysts, it appears that synthesis or activation of enzymes must be occurring rapidly during, or shortly after, the excystation process.

3.3.1.4 Detection of other enzyme activities

Phosphoglyceromutase (2,3-bisphosphoglycerate-dependent variety (Fothergill-Gilmore and Watson, 1989)), phosphoglucoisomerase, glutamate dehydrogenase, malate

dehydrogenase and glucose 6-phosphate dehydrogenase were also detected in extracts of *E. tenella* on one or more occasion. Although specific activities were not determined, these enzymes were all clearly present at high levels. Fumarase, serine sulphydrase, phosphoenolpyruvate carboxykinase and glycerol dehydrogenase were similarly assayed for on one or more occasion, but not detected. Since these assays were performed under a single set of conditions and with no positive controls, the negative results should be treated with caution.

3.3.2 Characterisation of PP_i-PFKs from *E. tenella* and *C. parvum*

Previous analyses of purified PP_i-PFK from *T. gondii* (Peng and Mansour, 1992) have shown that its properties conform to those of the type I enzymes found in other parasitic protozoa. The following section describes some properties of the PP_i-PFKs of *C. parvum* and *E. tenella*. These were investigated in soluble extracts of cysts and unsporulated oocysts, respectively. Analysis of the *C. parvum* enzyme was limited by the low availability of parasite material.

3.3.2.1 Stability

The PP_i-PFKs from both organisms proved extremely stable under standard conditions. No diminution of activity was observed in extracts stored either at 4°C for up to six hours, or at -70°C for several months. The omission of proteinase inhibitors from the lysis buffer did not noticeably affect the stability of either enzyme.

3.3.2.2 Kinetic properties

The kinetic properties of both enzymes were similar to those of PP_i-PFKs from other protists (Mertens, 1993; Wood *et al.*, 1985). Most significantly, both displayed hyperbolic kinetics with respect to the substrate fructose 6-phosphate (F6P) and were not affected by concentrations of F26P2 up to 10 μM (see Figure 3.3). In contrast, ATP-PFK from rabbit muscle, displayed strong positive cooperativity with respect to F6P and was allosterically activated by F26P2 (see Figure 3.4) - this is typical of most eukaryotic ATP-PFKs. A number of other substances (ATP, ADP, AMP, succinate and citrate, all at 1 mM) were also investigated as potential modulators of the eimerian enzyme but

were without effect at either saturating or sub-saturating concentrations of F6P. The enzyme from *Eimeria* was also found to display hyperbolic kinetics with respect to its second substrate, PP_i (not shown). The kinetic response of this enzyme remained the same following buffer exchange of the extract to remove potential low molecular weight effectors.

3.3.2.3 Cation dependence

Like all PFKs, both PP_i- and ATP- dependent, the coccidian enzymes had an absolute requirement for divalent cations. Highest activity was attained in the presence of Mg^{2+} (1 mM), however this could be partially replaced by Mn^{2+}, at certain concentrations. (Data not shown).

3.3.2.4 The reverse reaction

Activity catalysing the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate was also detectable in *Eimeria* lysates. This activity co-eluted with the forward PP_i-PFK activity in anion exchange (Mono Q) chromatography (albeit in the non-binding fraction, see Figure 3.14. *a*) indicating that both activities might be due to the same enzyme.

3.3.2.5 pH optima

The reverse reaction and a pH optimum of approximately 7.5 while that of the forward reaction was around pH 6.5 (Figure 3.5). These optima are similar to those observed for other protozoon PP_i-PFKs (Mertens, 1993; Peng and Mansour, 1992)

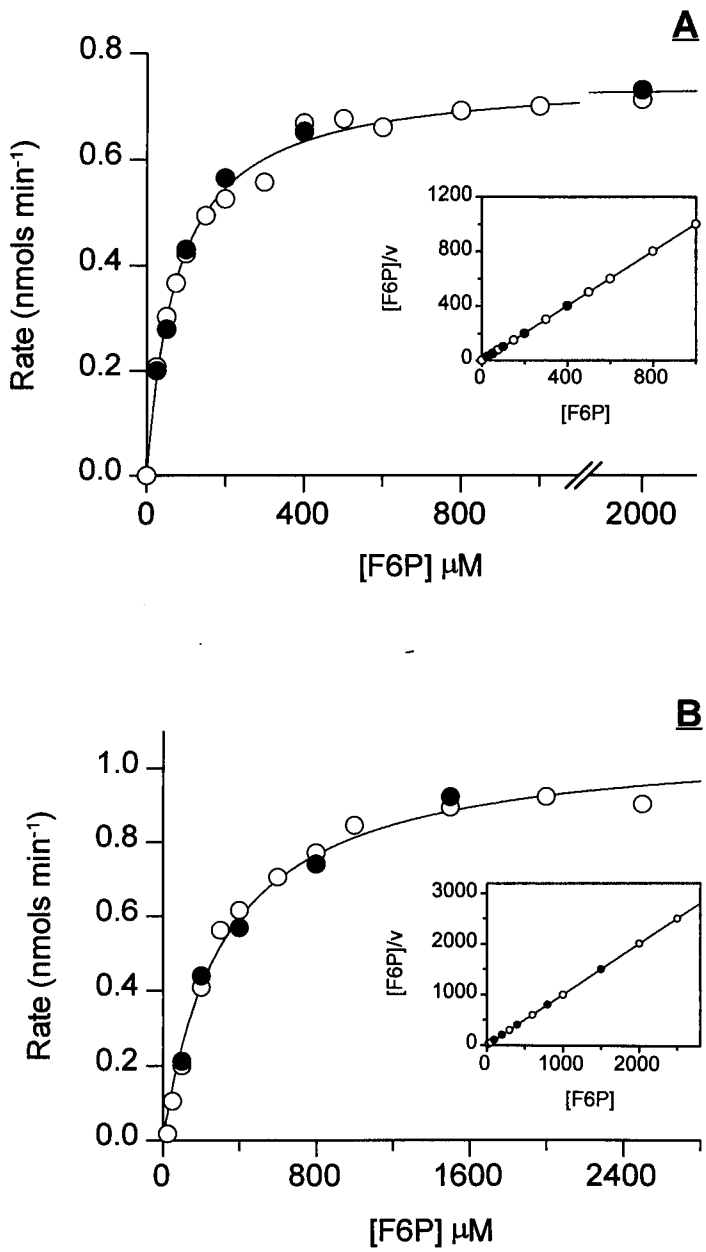

Figure 3.3. Representative fructose 6-phosphate saturation curves of PP_i-PFK from (A) *E. tenella* and (B) *C. parvum*. The activity associated with a fixed quantity of lysate was assayed at different concentrations of F6P, with (●) and without (○) fructose 2,6-bisphosphate (10µM). Other conditions were as described in Materials and Methods. The inserts show Hanes' plots of the data. The kinetic parameters for these experiments were: for the *E. tenella* enzyme - K_m, 77 µM and V_{max}, 0.76 nmol min^{-1}; for the *C. parvum* enzyme - K_m, 320 µM and V_{max}, 1.07 nmol min^{-1}.

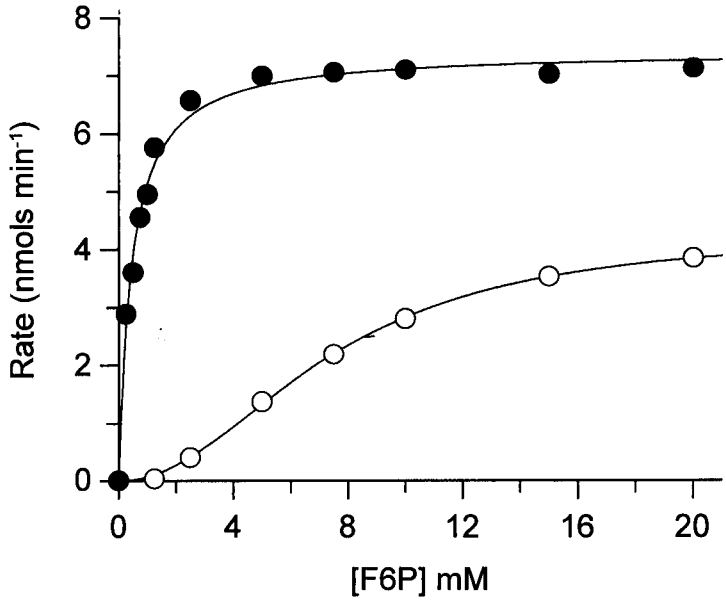

Figure 3.4. Fructose 6-phosphate saturation curve of ATP-PFK from rabbit muscle - illustrating allosteric activation by fructose 2,6-bisphosphate. The enzyme, a contaminating activity in a commercial preparation of rabbit muscle pyruvate kinase (Sigma, type I), was assayed at different concentrations of F6P, with (•) and without (o) fructose 2,6-bisphosphate (10μM). Other conditions were as described in Materials and Methods. The kinetic parameters for this experiment were: in the absence of glucose 6-phosphate - $K_{0.5}$, 4.3 mM, V_{max}, 6.5 nmol min^{-1} and Hill coefficient, 2.07; in the presence of glucose 6-phosphate - K_m, 0.45 mM, V_{max}, 7.44.

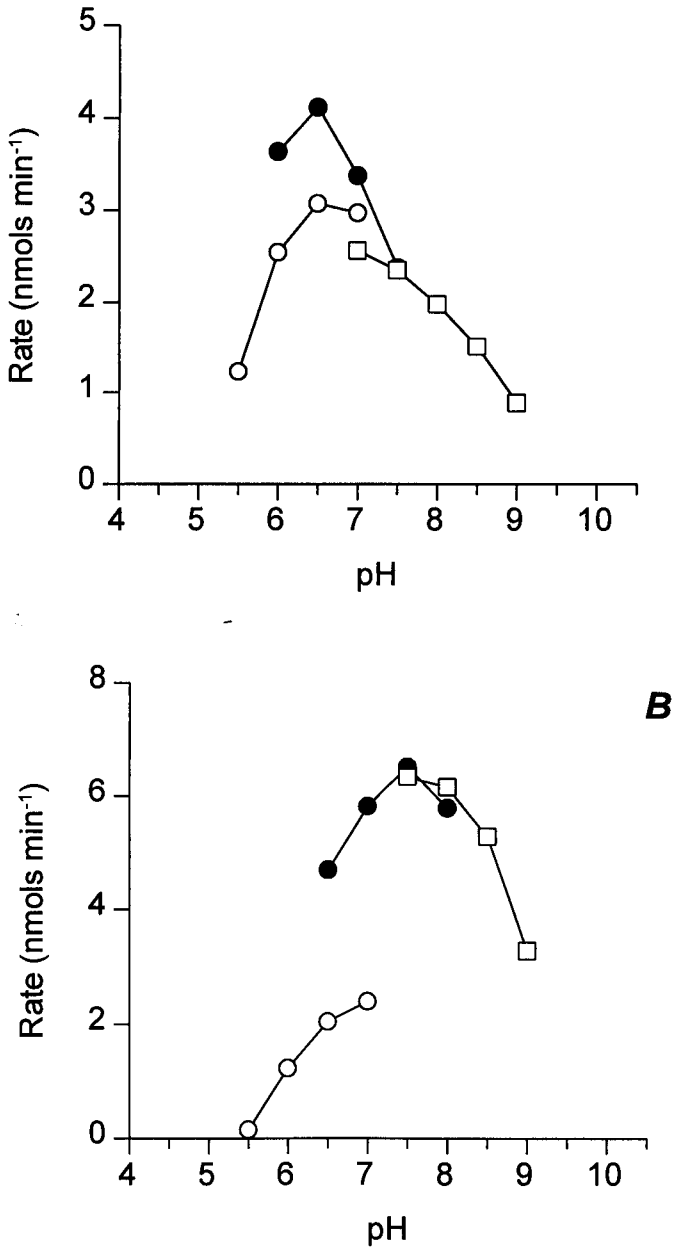

Figure 3.5. Effect of pH on the forward (A) and reverse (B) reactions of PP_i-PFK from *E. tenella*. The activity of a fixed quantity of crude extract was measured at 0.5 unit intervals using the following buffers: 100 mM Bis-Tris (○); 100 mM Hepes (●); 100 mM Tris (□). Other conditions were as described in Materials and Methods. Experiments were carried out to ensure that the activity of the coupling enzymes was not limiting under any of the pH conditions used. All points were assayed in duplicate.

3.3.3 Characterisation of the PKs of *E. tenella*, *T. gondii* and *C. parvum*

The fact that type I PP_i-PFKs are unregulated means that organisms which use them must use fundamentally unusual mechanisms of glycolytic control. In most organisms hexokinase and pyruvate kinase are believed to play secondary roles in glycolytic regulation. These are usually allosteric enzymes whose activities are modulated by various metabolic intermediates. A wide range of PK effectors are known, the most common and potent of which is fructose 1,6-bisphosphate (F16P2) - an activator of virtually all eukaryotic PKs (Fothergill-Gilmore and Michels, 1993). Interestingly, the parasitic protozoon *Trichomonas vaginalis*, which contains a PP_i-dependent PFK, has recently been shown to contain an ADP-specific PK that is regulated by an unusual set of effectors which does not include F16P2 (Mertens *et al.*, 1992). PKs with unusual allosteric regulators have also been found in PP_i-PFK utilising bacteria related to the class *Mollicutes* (Petzel *et al.*, 1989 *a* and *b*) It has been suggested that the unusual regulation of PK in these organisms might be a consequence of the lack of regulation at PFK, which renders F16P2 a less useful feed-forward indicator of glycolytic load (see Mertens *et al.*, 1992).

To see how the coccidial PKs compared with other eukaryotic enzymes, a general characterisation of pyruvate kinase activities in *Eimeria*, *Toxoplasma* and *Cryptosporidium* was undertaken. The study was particularly aimed at identifying regulatory features.

3.3.3.1 Stability

The PKs of all three coccidia were unstable in crude lysates. In typical extracts (not containing glycerol), PK activity exhibited a half life of 1-2 hours at 0°C. Investigations using the eimerian enzyme showed that this half life could be significantly extended by including 20% (v/v) glycerol in the lysis buffer. Glycerol was therefore made a standard component of the lysis mix, both in investigations of PK and other enzymes. Tests showed that it did not interfere in assays. The coccidian PKs showed very different degrees of cold lability. The *Eimeria* PK was almost completely inactivated by freezing, while those of *T. gondii* and *C. parvum* could be stored at -70°C for several months without major loss of activity.

3.3.3.2 Effect of DTT

The reducing agent DTT had a marked stimulatory effect on the PKs of *E.tenella* and *C. parvum*. Addition of 1 mM DTT typically resulted in activation of these enzymes by 50-70%. In contrast, the *Toxoplasma* PK was largely unaffected by this compound.

3.3.3.3 pH optima

The PKs from *Eimeria* and *Toxoplasma* had approximate pH optima of 7.5 and 6.5 respectively (Figure 3.6). The pH optimum of the *C. parvum* PK was not investigated.

3.3.3.4 Cation dependence

Like PKs from many other sources (Wood *et al.*, 1985), the coccidian enzymes required the presence of one monovalent and one divalent cation for full activity. Mg^{2+} and K^{+}

appeared to be the most effective combination in all cases, but could be partially substituted by Mn^{2+} and NH^{4+}. The response of the eimerian PK to these ions is shown in Figures 3.7. Similar results were obtained with the enzymes from *C. parvum* and *T. gondii* (data not shown).

3.3.3.5 Kinetic properties

The PKs of *T. gondii* and *E. tenella* presented simple Michaelis-Menten kinetics with respect to their substrate ADP, but produced a strongly sigmoidal saturation curve with phosphoenolpyruvate as the variable substrate (Figures 3.8). In contrast, the *C. parvum* PK presented Michaelis-Menten kinetics with respect to both substrates (Figure 3.9). The PEP saturation curve of this enzyme remained hyperbolic both at sub-saturating concentrations of ADP (0.3 mM) and following buffer-exchange of the crude enzyme to remove potential low molecular weight effectors.

A number of compounds, all effectors of PKs from other sources, were tested for their ability to modulate the activity of the coccidial PKs. The effects of a number of these, at sub-saturating concentrations of PEP are shown in Table 3.3. The following compounds were also tested (all at 1 mM) on one or more occasion but appeared without effect: 2-phosphoglycerate, fructose 2-phosphate, succinyl CoA, malate, citrate, acetate, succinate, GTP, ITP. No significant effect was noted for any compound at saturating concentrations of PEP.

Several of the test compounds had a stimulatory effect on the PKs of *T. gondii* and *E. tenella*. The most potent activators in each case were glucose 6-phosphate (G6P) and

F6P (Table 3.3). In the presence of either of these, the PEP saturation curve was converted to hyperbolic form (Figure 3.8). The activation kinetics and approximate K_{ac}s for these compounds are shown in Figures 3.10 and 3.11.

None of the compounds tested were found to modulate the *C. parvum* enzyme (Table 3.3). ATP had a small inhibitory effect, as it also did on the enzymes of *T. gondii* and *E. tenella*, however analyses showed that this was through purely competitive inhibition (data not shown).

This apparent lack of allosteric properties makes the *C. parvum* PK exceptional: to date the only other PK reported not to present cooperative binding of PEP is the mammalian Type I enzyme from muscle (Fothergill-Gilmore and Michels, 1993). The use in this study of fresh parasite lysates and the inclusion of proteinase inhibitors argue against the enzyme having been degraded with loss of allosteric properties.

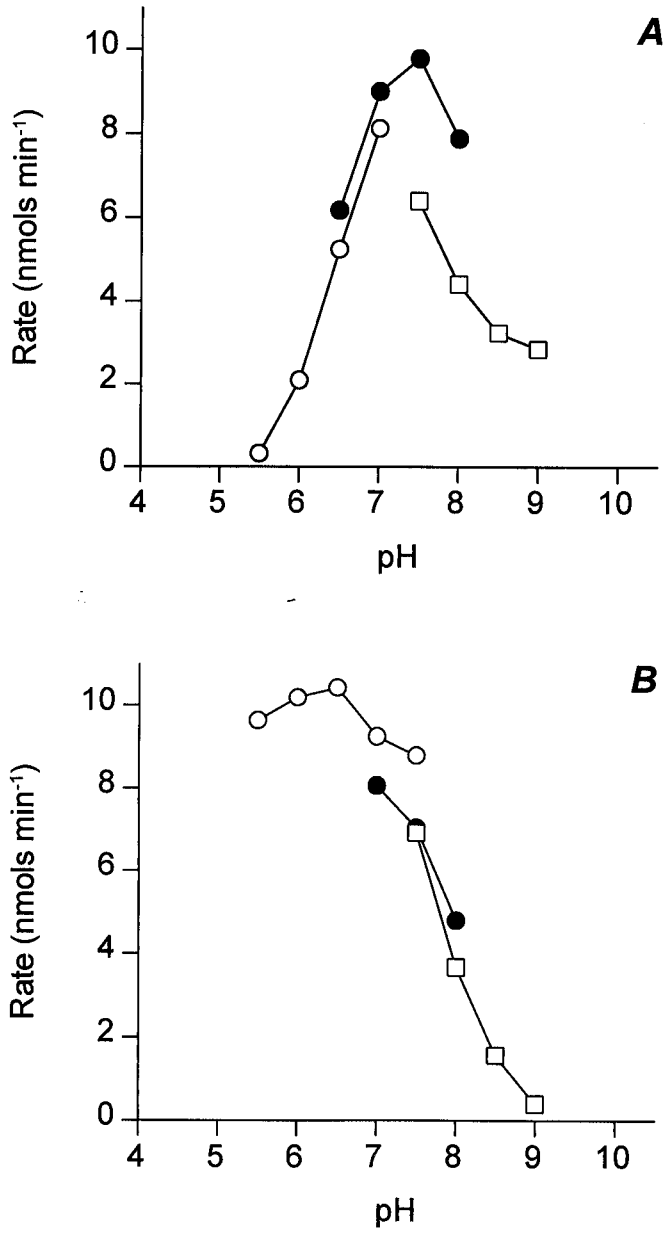

Figure 3.6. Effect of pH on the PKs from *E. tenella* (A) and *T. gondii* (B). The activity of a fixed quantity of crude extract was measured at 0.5 unit intervals using the following buffers: 100 mM Bis-Tris (○); 100 mM Hepes (●); 100 mM Tris (□). Other conditions were as described in Materials and Methods. Experiments were carried out to ensure that the activity of the coupling enzymes was not limiting under any of the pH conditions used. All points were assayed in duplicate.

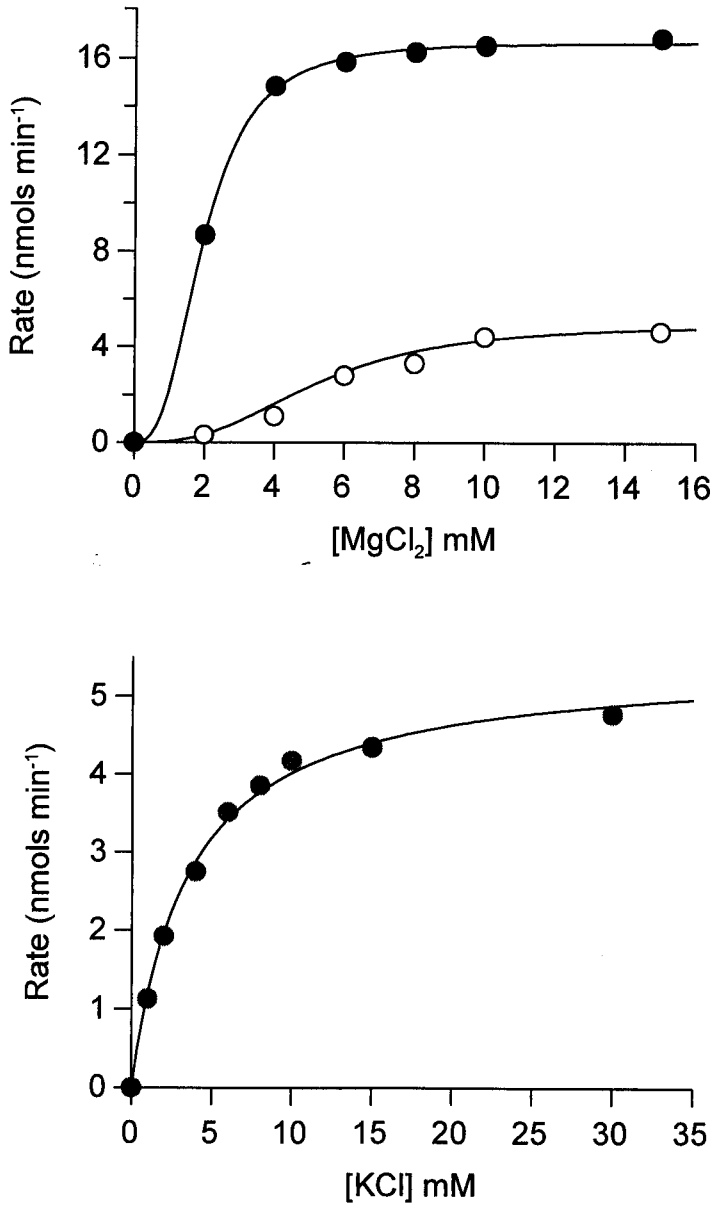

Figure 3.7. Effect of cations on the activity of pyruvate kinase from *T. gondii*. *Upper graph*: Activity as a function of Mg^{2+} concentration. Activity was measured in the presence (●) or absence (○) of 75 mM KCl. *Lower graph:* Activity as a function of K^+ concentration; assays contained 15 mM $MgCl_2$. Other conditions were as described in Materials and Methods. These are the results of a single, but representative experiment.

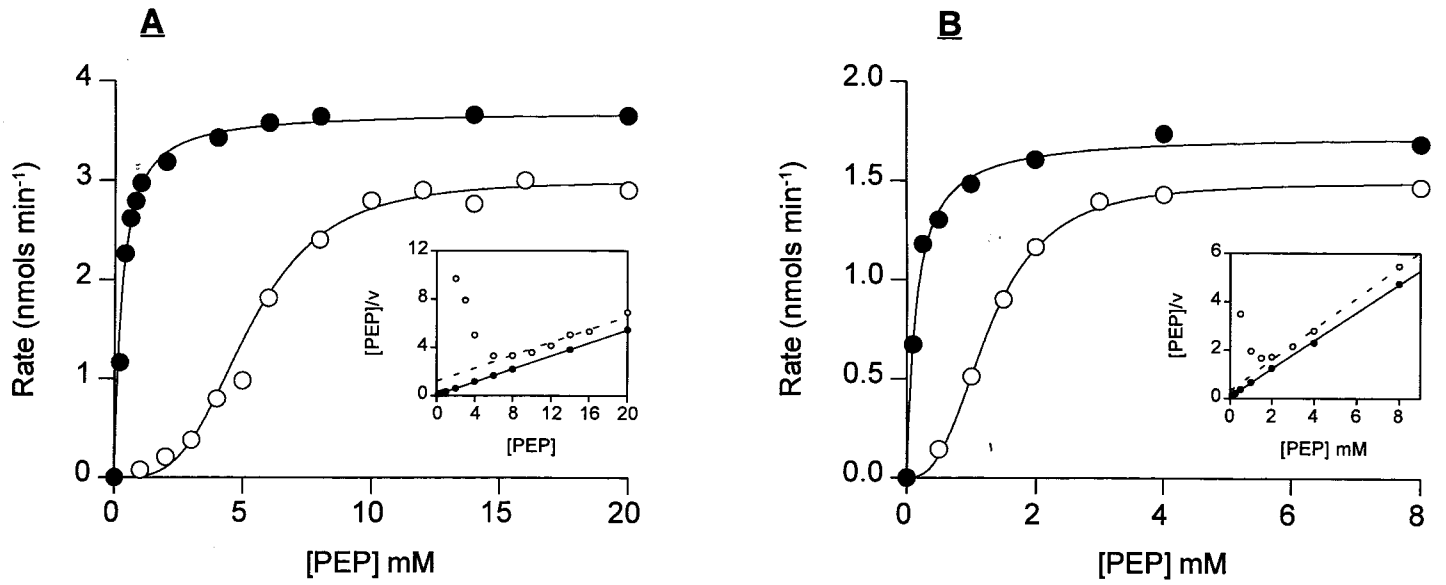

Figure 3.8. Representative phosphoenolpyruvate saturation curves of PKs from *E. tenella* (A) and *T. gondii* (B). The activity was assayed in the presence (●) or absence (○) of 1 mM glucose 6-phosphate. Other conditions were as described in Materials and Methods. The inserts show Hanes' plots of the data. The kinetic parameters for the *E. tenella* PK were: in the absence of glucose 6-phosphate - $K_{0.5}$, 5.2 mM, V_{max}; 3.0 nmol min^{-1} and Hill coefficient, 3.5; in the presence of glucose 6-phosphate - K_m, 0.26 mM; V_{max}, 3.7. The parameters for the *T. gondii* PK were: in the absence of glucose 6-phosphate - $K_{0.5}$, 1.89 mM, V_{max}; 1.5 nmol min^{-1} and Hill coefficient, 2.7; in the presence of glucose 6-phosphate - K_m, 0.14 mM; V_{max}, 1.74.

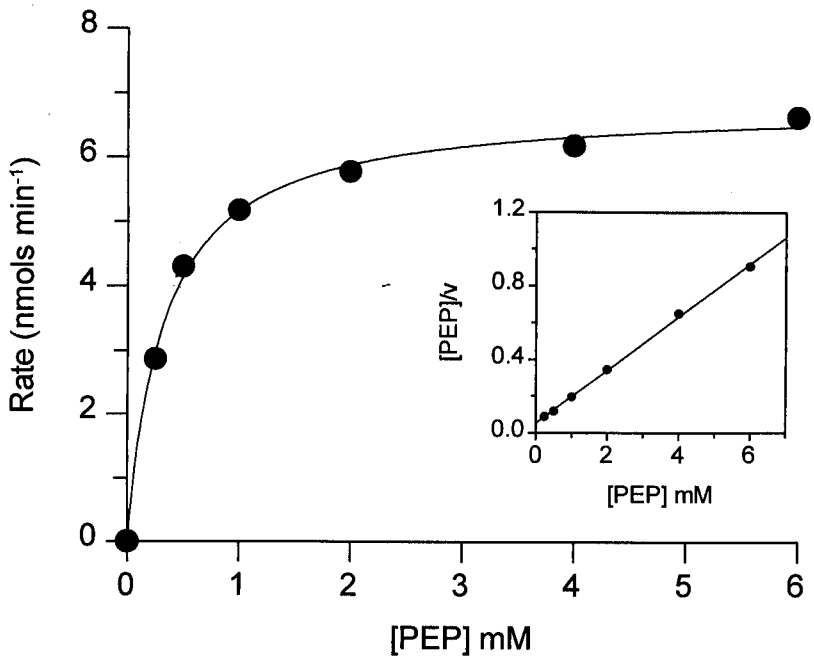

Figure 3.9 A representative PEP saturation curve of *C. parvum* PK. The activity associated with a fixed quantity of lysate was assayed at varying concentrations of PEP. Other conditions were as described in Materials and Methods. The insert shows a Hanes' plot of the data. The kinetic parameters for this experiment were: K_m, 0.32 mM; V_{max}, 6.82 nmols min^{-1}.

Table 3.3. Effects of metabolites on the activity of *E. tenella*, *T. gondii* and *C. parvum* PKs.

	Percent of control activity (%)		
Compound	*E. tenella*	*T. gondii*	*C. parvum*
None	100	100	100
Fructose 6-phosphate	239	728 ± 83	103 ± 3
Glucose 6-phosphate	218	746 ± 22	111 ± 3
Fructose 1,6-bisphosphate	125	397 ± 54	96 ± 2
ATP	91	86 ± 5	89 ± 6
AMP	204	163 ± 8	101 ± 1
Fructose 2,6-bisphosphate	99	162 ± 35	108 ± 6
Ribose 5-phosphate	94	298 ± 28	105 ± 1
3-Phosphoglycerate	87	98 ± 4	99 ± 5
Glucose 1-phosphate	111	248 ± 43	102 ± 4

The activity associated with a fixed quantity of lysate was assayed at sub-saturating concentrations of PEP (0.15 mM and 0.5 mM for the *C. parvum* and *T. gondii* PKs, respectively, and both 2.0 and 2.5 mM for the *E. tenella* enzyme) in the presence of the test compound (all at 1 mM except for fructose 2,6-bisphosphate which was at 10 μM). Other conditions were as described in Materials and Methods. Activities are expressed as a percentage of the control activity (100%) measured in the absence of effector. Figures are means ± S.D., n = 3. The results for the *E. tenella* PK are the means of activities measured at *different* sub-saturating concentrations of PEP hence standard deviations are not quoted.

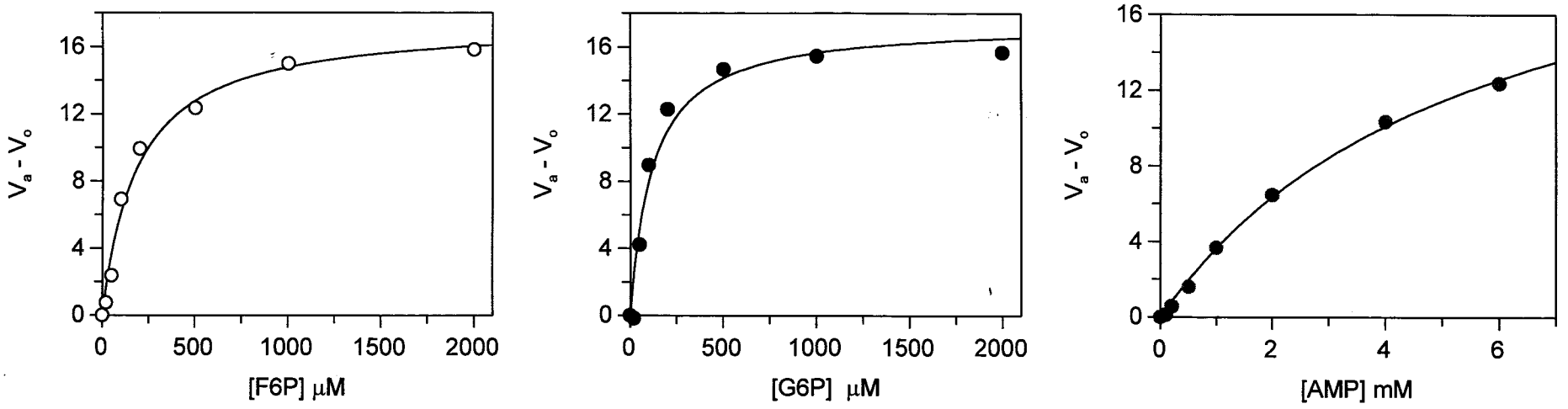

Figure 3.10. Kinetics of activation of *E. tenella* PK by fructose 6-phosphate, glucose 6-phosphate and AMP. Assays were carried out under standard conditions in the presence of varying concentrations of activator. V_a is the velocity in the presence, and V_o in the absence of the activator. The $K_{activation}$ values were: for fructose 6-phosphate, 185 μM; for glucose 6-phosphate, 117 μM; for AMP, 5.7 mM.

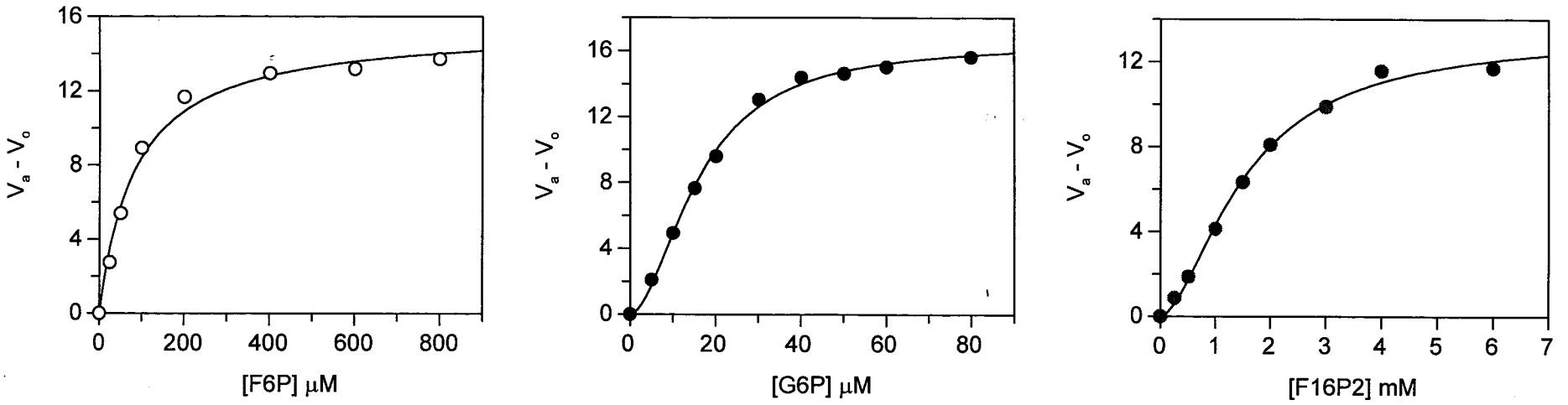

Figure 3.11. Kinetics of activation of *T. gondii* PK by fructose 6-phosphate, glucose 6-phosphate and fructose 1,6-bisphosphate. Assays were carried out under standard conditions in the presence of varying concentrations of activator. V_a is the velocity in the presence, and V_0 in the absence of the activator. The $K_{activation}$ values were: for fructose 6-phosphate, 84 μM; for glucose 6-phosphate, 16 μM; for fructose 1,6-bisphosphate, 2.07 mM.

3.3.4 Characterisation of hexokinase from *Eimeria*

Hexokinase is believed to be a regulatory enzyme of glycolysis in most eukaryotes. The most common form of regulation, as displayed by the mammalian enzymes types I, II and III, is allosteric inhibition by its product glucose 6-phosphate. Several other low molecular weight compounds are known to modulate hexokinases from other sources (Kosow and Rose, 1971; Fothergill-Gilmore and Michels, 1993). Unusually, the few protozoan hexokinases that have been investigated present no evidence of allosteric properties and appear to be unregulated (Berens *et al.*, 1980; Urbina and Crepso, 1984; Mertens and Muller, 1990).

In *Eimeria,* hexokinase is particularly interesting in that it plays a role not only in glycolysis but in the recently discovered mannitol cycle. The following study was aimed at determining whether these two roles are performed by one or by separate enzymes, and whether the reactions were subject to metabolic regulation. All analyses were performed using extracts of unsporulated oocysts.

3.3.4.1 Assays and MonoQ fractionation

The substrate specificity of the *Eimeria* hexokinase was investigated using a glucose 6-phosphate dehydrogenase-coupled enzyme system. This assay could not be used to investigate end-product inhibition, so another assay, using pyruvate kinase in an ADP regenerating system, was also employed (Jenkins and Thompson, 1989). Crude extracts of unsporulated oocysts contained high levels of apparent ATPase activity, which drove the pyruvate kinase-coupled assay in the absence of a hexose substrate, and had to be

removed before it could be applied effectively. This removal was effected by anion exchange (MonoQ) chromatography as described in Materials and Methods (Section 3.2.3). The fractionation achieved good separation of parasite proteins, including the main glycolytic activities investigated (see Figures 3.12, 3.13 and 3.14). The hexokinase-containing fractions were essentially free from interfering activity and could be used in regulatory analyses.

3.3.4.2 Glucokinase versus fructokinase activities

Crude extracts of *E. tenella* catalysed the phosphorylation of both glucose and fructose. Under standard assay conditions the rate of reaction with fructose was approximately 70% that with glucose. During MonoQ fractionation, gluco- and fructo- kinase activities were found to co-purify (see Figure 3.14.*B*) - implying the presence of only one enzyme that is capable of phosphorylating either glucose (as in glycolysis) or fructose (as in the mannitol cycle). Experiments using mixed substrates seemed to confirm this: when both glucose and fructose were present in the assay mix, the observed rates were intermediate between those observed with the individual substrates - rather than additive as might have been expected if separate enzymes were present.

3.3.4.3 Regulatory studies

The hexokinase from unsporulated oocysts presented simple Michaelis-Menten kinetics with respect to fructose, glucose and ATP. While similar maximal velocities were achieved for both substrates, the apparent K_m for fructose was approximately 6× greater than that for glucose (see Figure 3.15);. Glucose 6-phosphate and fructose 6-phosphate

showed some ability to inhibit the partially purified enzyme at both saturating and sub-saturating concentrations of hexose (See Figure 3.16.*A*). However, this inhibition was only significant at high product concentrations, and proved to be purely competitive with respect to both glucose and ATP (Figure 3.16.*B*). No other compounds were assessed as regulators of the enzyme.

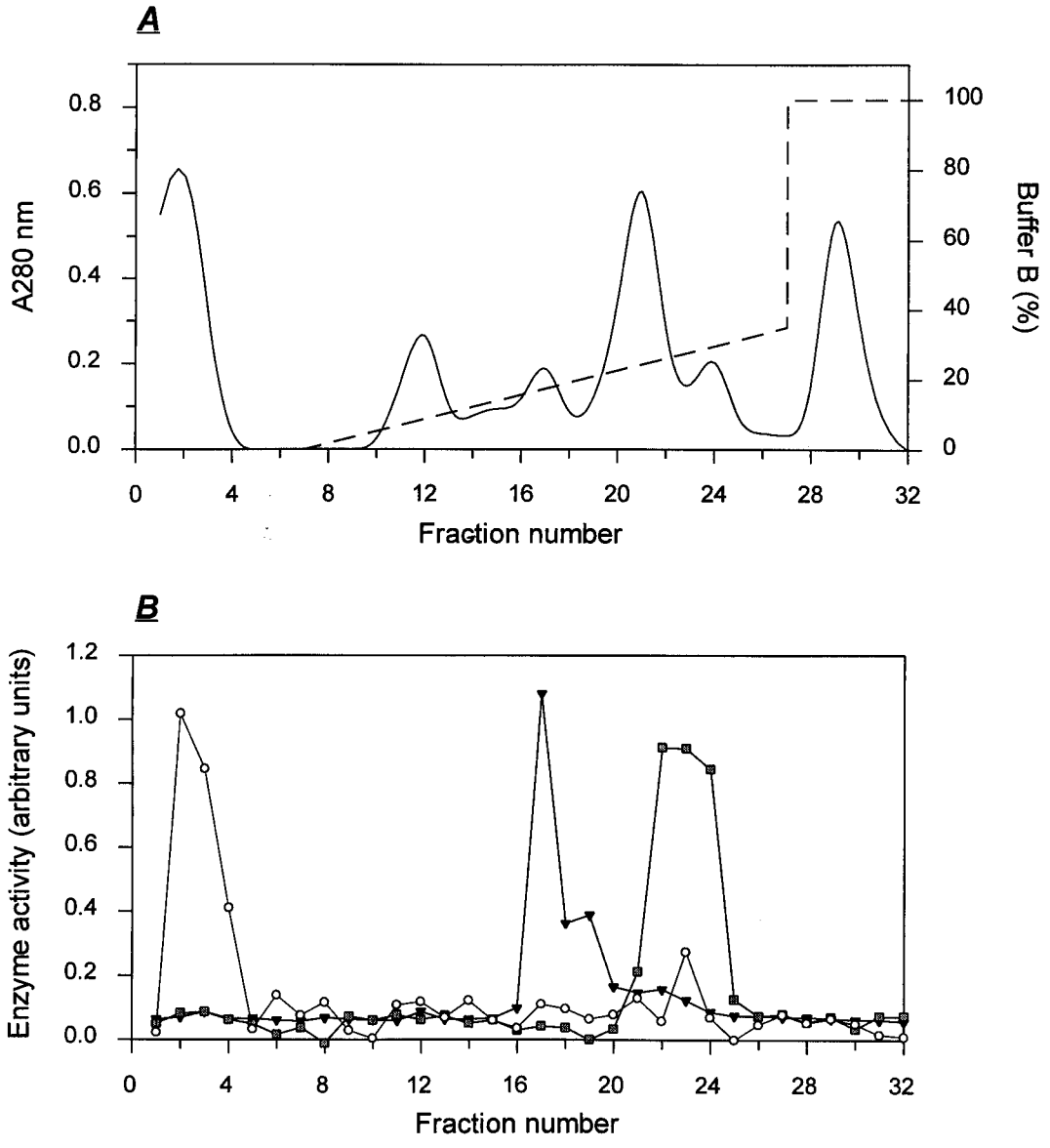

Figure 3.12. MonoQ fractionation of *E. tenella* unsporulated oocyst extract. Extracts of unsporulated oocysts were subjected to MonoQ fractionation as described in Materials and Methods. ***A:*** Elution of protein (——), and salt gradient (- - - -). ***B:*** Elution of glycolytic enzymes: PP_i-PFK *(circles)*, PK *(squares)*, and hexokinase *(triangles)*.

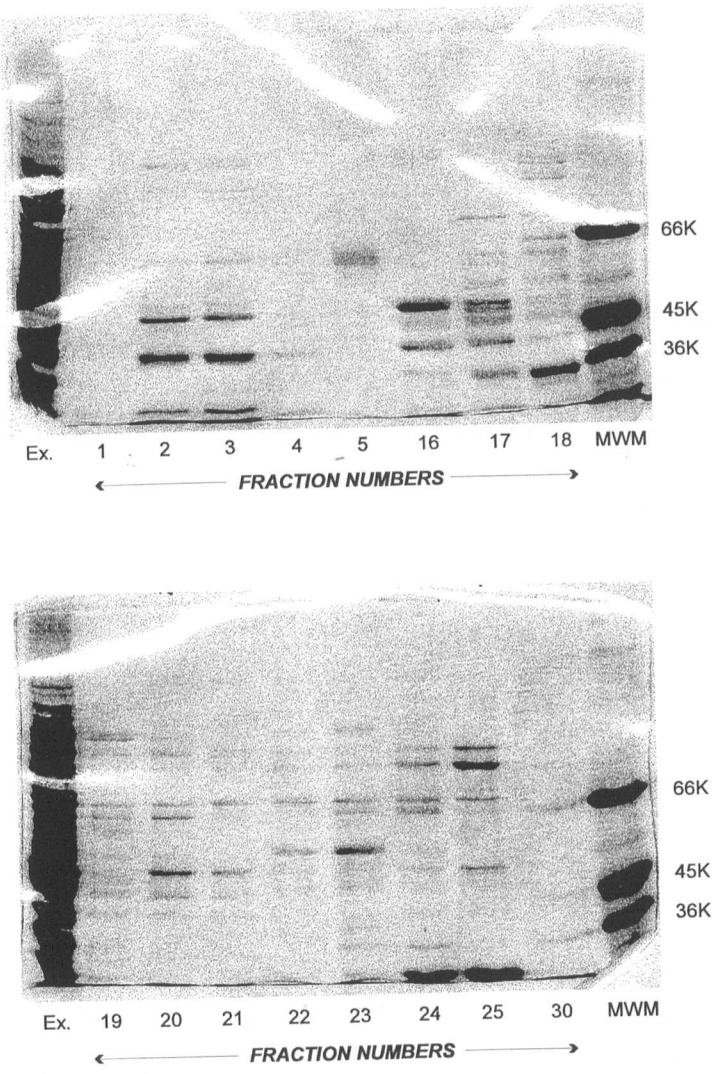

Figure 3.13. SDS-PAGE analysis of MonoQ fractions of *E. tenella* unsporulated oocyst extract. The gel was stained with Coomassie Blue. Fraction numbers correspond to those in Figures 3.12. and 3.14. Ex., unfractionated oocyst extract; MWM, molecular mass markers.

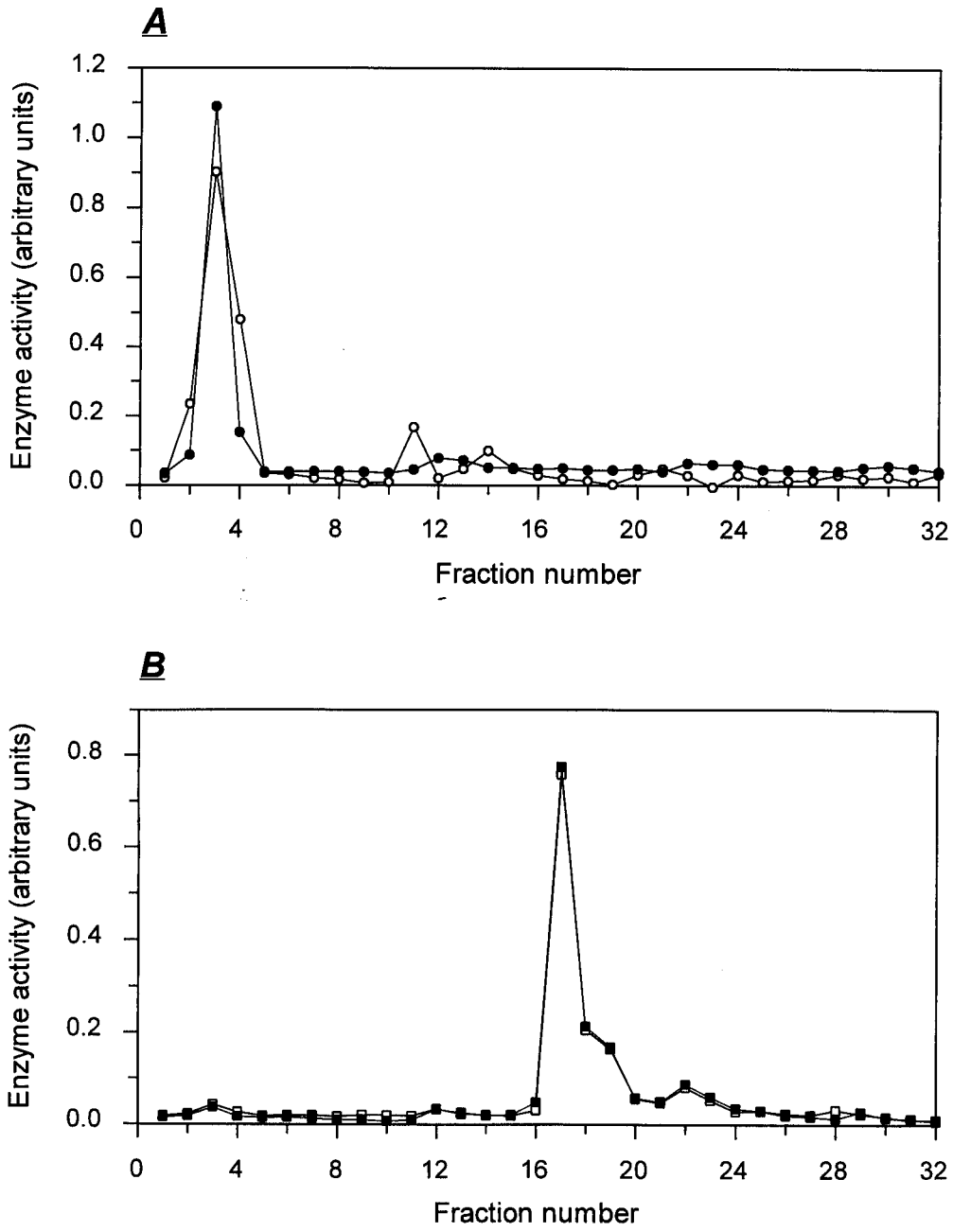

Figure 3.14. Coelution of enzyme activities during MonoQ fractionation of *E. tenella* unsporulated oocyst extract. Extracts of unsporulated oocysts were subjected to MonoQ fractionation as described in Materials and Methods. ***A:*** PP_i-PFK: forward reaction (○) and reverse reaction (●). ***B:*** glucokinase activity (□) and fructokinase activity (■).

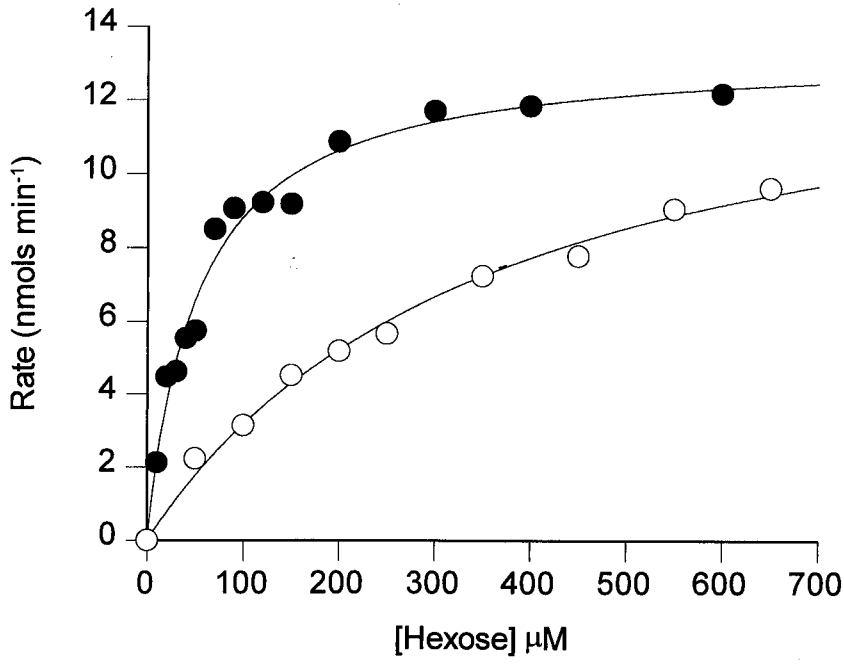

Figure 3.15. Representative glucose and fructose saturation curves of hexokinase from unsporulated oocysts of *E. tenella*. The activity associated with a fixed quantity of partially purified enzyme was assayed at different concentrations of glucose (○) or fructose (●). K_m for glucose, 51 μM; for fructose, 364 μM.

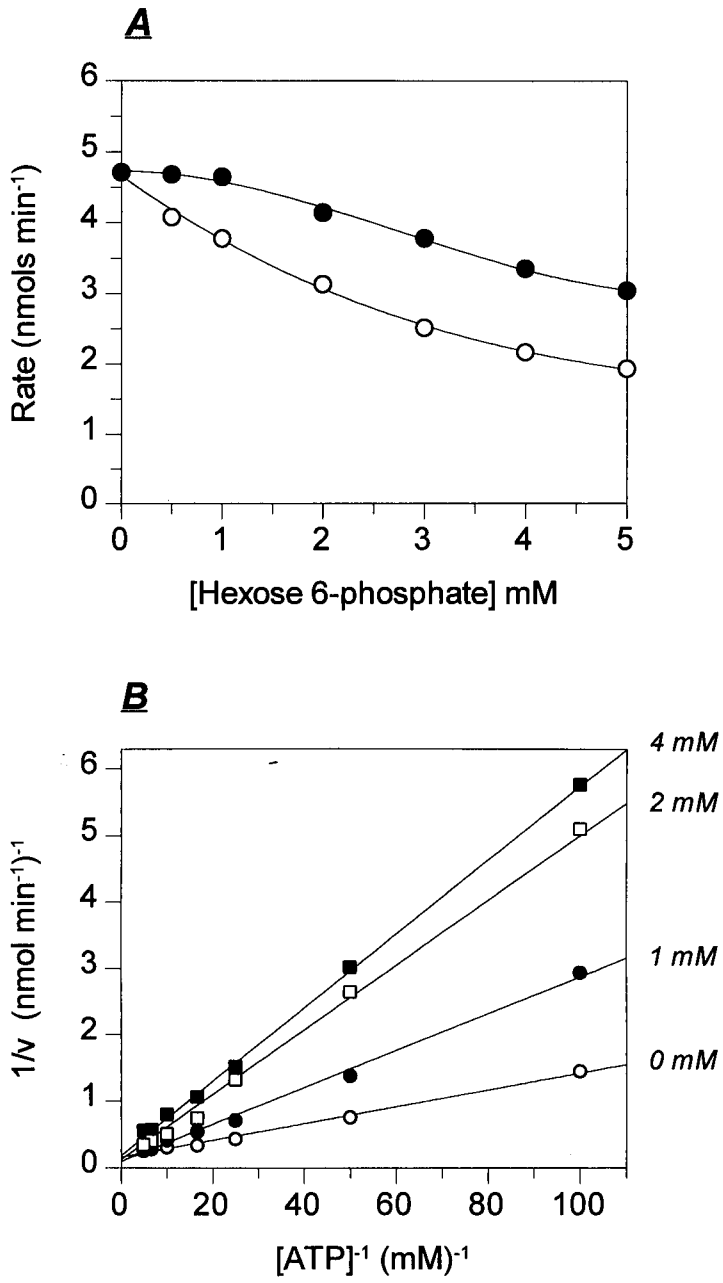

Figure 3.16. (A) Inhibition of *E. tenella* hexokinase by hexose 6-phosphates. A fixed quantity of partially purified hexokinase was assayed (method B) at a sub-saturating concentration of glucose in the presence of various concentrations of glucose 6-phosphate (o) and fructose 6-phosphate (●). **(B) Competitive inhibition of glucose phosphorylation by glucose 6-phosphate.** A fixed quantity of partially purified hexokinase was assayed (method B) at varying concentrations of ATP in the presence of the indicated concentrations of glucose 6-phosphate. This is a Lineweaver-Burke plot, the intersection of lines at the Y-axis indicates that inhibition is competitive. Similar results were obtained with glucose as the variable substrate.

3.4 RESULTS SUMMARY

- *Eimeria*, *Toxoplasma* and *Cryptosporidium* were found to contain high levels of PP_i-PFK, PK, and LDH. These activities suggest the presence of a glycolytic pathway and a high potential for fermentative energy metabolism.
- SDH and ICDH activity could not be detected in sporulated cysts of *C. parvum*. This argues against the presence of a TCA cycle and is consistent with the apparent lack of mitochondria in this species.
- All stages of *Toxoplasma* and *Eimeria* investigated contained an $NADP^+$-ICDH but no NAD^+-ICDH. Tachyzoites of *T.gondii* contained SDH activity, but the enzyme was not detected in bradyzoites of the species, nor in any stage of *E. tenella*. This is not sufficient information to deduce whether or not a functional TCA cycle is present in these species.
- Bradyzoites of *T. gondii* contained significantly higher levels of LDH and PK than did tachyzoites. This suggests that the former may rely more heavily on fermentative energy generation.
- Enzyme activities were found to be generally lower in sporulated than in unsporulated oocysts of *E. tenella*. This probably reflects the dormant nature of the latter. In contrast, high enzyme activities were found in sporozoites indicating significant and rapid activation or synthesis of enzymes during excystation.
- All stages of *E. tenella* and oocysts of *C. parvum* were shown to contain a pyrophosphate-dependent PFK, similar to that previously described in *T. gondii*. The enzymes showed no evidence of allosteric properties and were unaffected by $F26P_2$. This enzyme may represent an adaptation towards anaerobiosis.

- Cytosolic pyrophosphatase activity could not be detected in any of the three parasites. This is consistent with the use of PP_i as an energy donor.
- All the parasite stages investigated contained a PK specific for ADP rather than for PP_i/AMP. The enzymes from *T. gondii* and *E. tenella* displayed strong positive cooperativity with respect to their substrate PEP and were heterotropically activated by F6P and G6P. The PK of *C. parvum* showed no evidence of allosteric properties.
- Unsporulated oocysts of *E. tenella* were shown to contain a single hexokinase capable of phosphorylating either glucose or fructose. The enzyme was not allosterically modulated by G6P or F6P.

4. SUBSTRATES AND END-PRODUCTS OF ENERGY METABOLISM IN *EIMERIA TENELLA*

4.1 INTRODUCTION:

4.1.1 Substrates and end-products of energy metabolism in parasitic protozoa

While enzyme activities may give an indication of the pathways of energy metabolism that are present in an organism, they do not necessarily indicate that these pathways are operational, and provide no information about their relative operation under different environmental conditions. Theoretically, in order to demonstrate that a pathway is operational, it is necessary to be able to feed substrates in at one end and follow their metabolism, through all intermediates, to the final products. In practice, however, most pathways are well-defined and their operation can usually be accepted on observations of substrate depletion and end-product formation. The rate of flux through a pathway may be determined by quantitating these processes and it can often be informative to investigate how it is affected by changes in environmental conditions. Such analysis may, for example, give an indication of an organisms relative potential for aerobic and anaerobic modes of energy generation.

While most parasites use carbohydrates as their primary energy substrates, it is increasingly being recognised that some species also catabolise other compounds, e.g.

amino acids, fatty acids, for energy production (Bryant and Behm, 1989). These may be used throughout the life-cycle, or at specific stages, to accord with the major nutrients available in the environment. In parasites in general it is unusual for energy substrates to be catabolised completely to CO_2 and H_2O. More commonly, a large proportion are converted to fermentation products - organic acids and alcohols - even under aerobic conditions. The end-products of carbohydrate catabolism produced by some parasites are shown in Table 4.1.

The reasons why parasites should place such a high dependence on anaerobic modes of energy metabolism, and why such a diversity of end-products are produced, have been much discussed (see for example, Barrett, 1981; Bryant and Behm, 1989; Cox, 1982; Fairlamb, 1989; Bryant, 1993). While there are no definitive answers, it is generally agreed that there are two major explanations which apply to different groups of organisms.

Explanation 1 applies to protozoa such as *Giardia lamblia*, *Trichomonas vaginalis* and *Entamoaeba histolytica*, which live in lumenal habitats. The oxygen tensions in these environments are thought to be low and restrictive to full aerobic respiration. As a consequence the parasites have either lost, or never evolved, the capacity for full aerobic respiration and rely largely on glycolysis for their energy production (Muller, 1988; Muller, 1992; Coombs and Muller, 1995). Nutrients may also be fairly limited in these habitats, so in order to maximise energetic efficiency some species have evolved extensions to glycolysis which generate ATP by substrate-level phosphorylation. These extensions may give rise to relatively unusual end-products, such as acetate. Other unusual products may be produced out of the requirement to regenerate NAD^+ and

Table 4.1. Major end-products of carbohydrate catabolism in some parasitic protozoa.

SPECIES	ANAEROBIC	AEROBIC
Entamoeba histolytica	Ethanol, acetate, CO_2	Ethanol, CO_2
Giardia lamblia	Ethanol, acetate, CO_2, glycerol	Ethanol, acetate, CO_2
Trichomonas vaginalis	Glycerol, lactate, acetate, H_2, CO_2	Glycerol, lactate, acetate, CO_2
Tritrichomonas foetus	Glycerol, succinate, acetate, H_2, CO_2	Glycerol, succinate, acetate, CO_2
Plasmodium falciparum	[not viable]	Lactate
Trypanosoma brucei		
blood stream stage:	Pyruvate, glycerol	Pyruvate
insect stage:	Succinate, alanine, acetate	Succinate, alanine, acetate, CO_2
Leishmania braziliensis panamensis (promastigotes)	Succinate, glycerol, D-lactate, pyruvate, alanine	Succinate, glycerol, D-lactate, pyruvate, CO_2
Toxoplasma gondii	Lactate, acetate, CO_2	Lactate, acetate, CO_2

Data taken from: Fairlamb and Opperdoes, 1986; Fulton and Spooner, 1960; Muller, 1991; Rainey and MacKenzie, 1991; Sherman, 1979.

maintain the redox balance within the organism. Parasites belonging to this group of anaerobes often lack mitochondria and may be adversely affected by the presence of oxygen.

Explanation 2 applies to the blood-stream or tissue-dwelling stages of species such as *Plasmodium*, *Trypanosoma* and *Leishmania* and in some ways is the converse of the above. The environments in which these parasites live contain essentially unlimited supplies of both nutrients (glucose) and oxygen. While there is no theoretical barrier to full aerobic energy production, the constant availability of substrate means that the parasites have no real need to mediate efficient energy metabolism. Instead they have 'stream-lined' their metabolism, losing the fully oxidative components, and depending largely on glycolysis for ATP production. This is satisfactory providing that the pathway can operate fast enough to meet ATP requirements. In the kinetoplastids, glycolysis occurs within a glycosome; this compartmentalisation may be a strategy to increase the efficiency of the pathway (Opperdoes, 1991).

4.1.2 Techniques for analysing metabolites.

Until recently identifying end-products of energy metabolism was a rather hit-or-miss affair. Researchers had to presuppose which metabolites might be produced, and then assay for them using reaction-based techniques. In this way it was easy for unusual end-products to be overlooked. In recent years the increasing availability of objective techniques such as nuclear magnetic resonance (NMR), high-performance liquid chromatography (hplc) and gas chromatography has led to more confident assessments of the full complement of metabolic end-products of parasitic protozoa. The technique

of NMR has proved particularly powerful in this respect (O'Sullivan *et al.*, 1989; Thompson, 1991).

While the technique of NMR has been available for several decades (Williams and Fleming, 1987; Silverstein and Bassler, 1987), it is only recently that its potential for exploring biological systems has begun to be realised. For the biologist, NMR has a number of inherent advantages. To begin with, the technique is both non-invasive and non-destructive. This means that analyses can be carried out not only on cell-extracts, but on whole cells; and if required, these can be monitored continuously over an extended period of time. Secondly, since absorption spectra may be interpreted directly, the technique requires no a priori assumptions to be made regarding the identity of the metabolites. All compounds, even in a complex mix, can be detected simultaneously.

The nucleus most frequently used in the study of biological pathways is ^{13}C. Although ^{13}C is present only at low levels in natural compounds, it is possible to synthesise compounds which are artificially enriched in the nucleus and can therefore be readily detected (Williams and Fleming, 1987). These compounds may be administered to cells or other biological systems, and their fate, in terms of final products, determined by NMR. The ability to synthesis compounds containing specifically-labelled atoms is particularly useful in elucidating the precise reactions of metabolic pathways.

There have been a number of reports of ^{13}C-NMR being applied to the detection of end-products of energy metabolism in parasitic protozoa (Chapman *et al.*, 1985; Chatterjee and Datta, 1973; Darling *et al.*, 1987; Edwards *et al.*, 1989; Gilroy *et al.*, 1988; MacKenzie *et al.*, 1982; Rainey and MacKenzie, 1991; Santos *et al.*, 1985) and ^{1}H-NMR has also been used occasionally. Particularly relevant to this project was the use

of ^{1}H-NMR to analyse the soluble products of aerobic glucose catabolism by tachyzoites of *T. gondii* (Ohsaka *et al.*, 1982). Two products were identified: lactate and acetate (see Section 1.2.1.5).

4.1.3 Aims of this study.

The finding that tachyzoites of *T. gondii* catabolise significant proportions of glucose to lactate and acetate even under aerobic conditions has led to the supposition that these parasites have a low capacity for full aerobic respiration (Fulton and Spooner, 1960; Ohsaka *et al.*, 1982). While several investigators have reported CO_2 to be released by various stages of *Eimeria* species. (and correlated it with glucose consumption), there has been no comprehensive analysis of soluble products of glucose catabolism by such species. Ryley (1973) reported lactate and glycerol to be products of anaerobic endogenous energy metabolism by sporozoites of *E. tenella*, and produced quantitative data on this (see Table 1.2). However these products were identified using simple quantitative assay techniques and it is possible that other compounds were released but not detected in the study. There had been no reported analyses of soluble products of aerobic energy metabolism by *E. tenella*, and the possibility that the parasites might use energy substrates other than carbohydrates had not been investigated.

In this study, ^{13}C-NMR was used to produce a more objective analysis of the end-products of both aerobic and anaerobic glucose catabolism by sporozoites of *E. tenella*. The soluble products identified by this technique were quantified by a combination of enzymatic assays and hplc to obtain a direct assessment of the relative capacity of the sporozoites for aerobic and anaerobic modes of energy metabolism. The possibility that

compounds other than glucose (amino acids and mannitol) were catabolised for energy production was also investigated.

In an extension to the study, quantitative techniques were used to compare metabolite levels in extracts of sporulated and unsporulated oocysts of *E. tenella*. It was hoped that this would provide insight into the metabolic processes occurring during sporulation.

4.2 MATERIALS AND METHODS

4.2.1 Qualitative analyses: NMR

4.2.1.1 Incubation of sporozoites

Sporozoites of *E. tenella* (ionophore-sensitive strain, Et2) were excysted and purified according to standard techniques and resuspended to a concentration of 1-2 × 10^8 ml^{-1} in PBS, pH 7.4, containing 10 mM ^{13}C-enriched glucose (either 1-^{13}C, 6-^{13}C or $^{13}C_6$). 0.75 ml aliquots of parasite suspension were placed in 5 ml glass bottles and incubated at 41°C in a shaking water bath. The tubes were either left open to the air (aerobic conditions), or fitted with a self-sealing rubber cap and flushed with nitrogen for 5-10 min prior to incubation (anaerobic conditions). At the end of the incubation period, the cells and incubation media were separated by centrifugation (5 min at 7000 g at room temperature) and the supernatant removed to another tube. The cells were washed three times by centrifugation (5 min at 7000 g at room temperature) and resuspended to a final volume of 0.75 ml in fresh PBS, pH 7.4. Cells and medium were stored at -70°C until analysis.

Cell densities were determined at the beginning and end of all incubations

4.2.1.2 Preparation of samples for NMR

Cell suspensions and incubation media were made up to 8% (w/v) with respect to perchloric acid and incubated for 1 hr at 0°C. Following incubation the samples were centrifuged for 15 min at 12000 g at 4°C whereupon the supernatant was removed and

neutralised by titration with ice cold 5M KOH. Excess salt was removed by centrifugation (2 min at 7000 g at room temperature) and the supernatant was freeze-dried and stored at -70°C. When required for NMR analysis, the freeze-dried material was resuspended in 0.75 ml deuterium oxide and clarified by filtration through a 0.22 μm pore membrane.

4.2.1.3 NMR spectroscopy

^{13}C-NMR spectra were recorded on a Varian Unity Plus 500 spectrometer operating at 125.6 MHz. (78K time domain data points; acquisition time, 1.301 seconds; flip angle, 30°). Deuterium oxide was used as an internal lock in all samples and the temperature was maintained at 30°C throughout. Acquisition times varied from 20 min to 16 hr, depending on the strength of metabolite signals.

4.2.1.4 Identification of metabolites

Metabolites were identified by reference to the chemical shifts of a set of standard metabolites: succinate, mannitol, fumarate, sodium acetate, malate, trehalose, lactate, pyruvate, glycerol, mannitol 1-phosphate, propionic acid and alanine. These were made up to 10 mg ml^{-1} in deuterium oxide, adjusted to pH 7.0 with KOH, and analysed alongside the experimental samples.

4.2.2 Quantitative analyses

4.2.2.1 Sporozoite incubations

Freshly excysted sporozoites (ionophore-resistant strain, Ret5) were resuspended to a concentration of 0.5-1×10^8 ml^{-1} in PBS, pH 7.4, and incubated as described in Section 4.2.1.1. The PBS was supplemented as required with either glucose (15 mM) or a mixture of amino acids containing 1% (v/v) 50×MEM amino acids solution without glutamine, and 1% (v/v) 100×MEM non-essential amino acids solution (Gibco). In some experiments, as indicated, the parasite suspension was continuously bubbled with the gas phase (nitrogen or air / 5% CO_2) throughout the incubation (at a rate of approximately 2 bubbles per second). This procedure was intended to minimise changes in the gaseous composition of the medium due to cell metabolism. At the end of the incubation period the cells were removed by centrifugation (5 min at 7000 g at room temperature) and the incubation medium was stored at -70°C until analysis.

Cell densities were determined at the beginning and end of all incubations and the sporozoites were monitored microscopically for motility.

4.2.2.2 Preparation of oocyst extracts for metabolite analyses

Sporulated and unsporulated oocysts were resuspended to a concentration of approximately 10^7 ml^{-1} and lysed by vortexing with glass beads (Section 2.6). The normal solvent used was H_2SO_4, pH 2.7, however PBS, pH 7.4, and Millipore water were also used on occasions. Proteinase inhibitors were always included when preparing samples for amino acid analysis. In some experiments, the oocysts were boiled for two

min immediately prior to lysis in order to inhibit metabolic processes which might change the metabolite profiles following lysis. This treatment, however, made no appreciable difference to the metabolite concentrations measured; similarly fresh lysates could be incubated for 2 hr at 37°C without any detectable change in their organic acid profiles.

4.2.2.3 Enzymatic assays

Metabolites were quantified enzymatically as described below by coupling their metabolism to NAD(P)(H) reduction/oxidation and determining the change in absorbance at 340 nm. Concentrations were extrapolated from standard curves of the test compound. All assays were carried out in a final volume of 250 µl in microtitre plates (50 µl sample volume); incubation was at 37°C. Coupling enzymes were as described in Section 3.2.2.

Glucose: The standard assay contained: 50 mM HEPES, pH 7.6; 90 mM KCl; 3 mM $MgCl_2$; 1 mM ATP; 1 mM $NADP^+$; 0.5 U hexokinase; 0.5 U glucose 6-phosphate dehydrogenase and sample.

Lactate: The standard assay contained: 120 mM glutamic acid, pH 8.9; 1 mM NAD^+; 1 U alanine aminotransferase; 1 U lactate dehydrogenase and sample.

Glycerol: The standard assay contained: 150 mM glycine containing 650 mM hydrazine, pH 9.8; 1.5 mM $MgCl_2$; 5 mM ATP; 1 mM NAD^+; 3 U glycerophosphate dehydrogenase; 3 U glycerol kinase and sample.

4.2.2.4 Amino acid hplc

Samples were prepared and analysed for amino acids using the hplc technique described by Zuo and Coombs (1995). This technique was capable of resolving 14 individual amino acids. The detection of lysine and glycine was inconsistent so they were generally excluded from studies. Two pairs of amino acids, histidine/glutamine and valine/methionine, consistently co-eluted and hence could not be quantified while neither cysteine nor proline react with OPA and hence could not be detected

4.2.2.5 Organic acid hplc:

4.2.2.5.1 Extraction procedure

Organic acids were extracted from samples using a modification of the technique described by Guerrant *et al.* (1992): 200 µl sample was vortexed for 1 min with 0.12 g NaCl, 40 µl 18N H_2SO_4 and 1 ml diethyl ether. The mixture was centrifuged for 1 min at 1400 g at room temperature and the upper ether layer removed to another tube containing 100 µl of 1 M NaOH. The mixture was vortexed and centrifuged as previously and the upper ether phase discarded. The tube was then left open at room temperature for 10-20 min to allow residual ether to evaporate and the remaining material was analysed immediately or stored at -20°C.

4.2.2.5.2 Separation procedure

Organic acids were separated using a Hamilton PRP-X300 ion exclusion column (7 µm bore size, 250 × 4.1 mm) at a flow rate of 2 ml min^{-1} with H_2SO_4 as the eluent. The sample size was 20 µl and the total run time 10 min. Detection was at 210 nm.

Unknowns were identified by comparing retention times with those of reagent grade standards (see Table 4.2)[4]. Since eluent pH was found to have a strong effect on the elution characteristics of the column, duplicate analyses were routinely performed at two or more pH values (different pH solvents were produced by titrating millipore water with 18 N H_2SO_4). This procedure proved useful for verifying the identity of unknown compounds and frequently enhanced the separation of co-eluting compounds. Organic acids were quantified according to peak area by reference to external standard curves. The detected quantities had to be adjusted to compensate for the individual extraction efficiencies of the acids. The extraction efficiencies of lactate, acetate and succinate were 27.5 ± 1.38 %, 61.5 ± 2.5 % and 46.3 ± 0.2 % respectively (as determined by experiment, n=6).

[4] Some common organic acids did not elute from the column with H_2SO_4 as the solvent but could be eluted by 50% methanol. These included: propionic acid, butyric acid, isobutyric acid, *n*-valeric acid, iso-valeric acid, methylvaleric acid, methyl-butyric acid, caproic acid, α-ketoisocaproic acid and α-hydroxyisocaproic acid. Preliminary investigations indicated that *Eimeria* did not produce any acids requiring methanol elution so subsequent analyses were restricted to the H_2SO_4 system.

Table 4.2 Retention times[1] of selected organic acids during hplc with H_2SO_4 as the eluent.

	Eluent pH:		
Acid	*2.2*	*2.7*	*3.7*
Tartaric acid		1.19	
Pyruvic acid		1.55	
Malic acid		1.67	
Malonic acid		1.74	1.51
α-Ketoglutaric acid		1.90	1.90
Lactic acid		2.64	2.12
Phenyl acetic acid		3.72	
Acetic acid		3.86	3.60
α-Ketobutyric acid	10.15	4.44	1.58
β-Hydroxybutyric acid	5.43	5.06	4.27
Succinic acid	6.62	5.63	4.72
Hydroxyphenylacetic acid		6.18	
Fumaric acid		7.40	3.03
Acetoacetic acid		7.50	
α-Hydroxybutyric acid		8.88	
'X'	7.84	5.65	2.27

[1] Time in minutes.

4.3 RESULTS

4.3.1 Glucose catabolism by sporozoites: NMR analyses

4.3.1.1 Identification of soluble products of glucose catabolism

Figure 4.1 shows spectra of media in which sporozoites have been incubated, aerobically and anaerobically, with universally labelled ^{13}C-glucose. The spectra are representative of those obtained in multiple experiments. The profile of the starting medium (PBS, pH 7.4, containing 10 mM $^{13}C_6$-glucose) is also shown (Figure 4.1.A). In all experiments, the glucose-specific peaks of the incubation media were found to diminish during the course of the incubation, giving way to a new set of spectral peaks. The products responsible for these peaks were identified, by reference to the chemical shifts of standard compounds as lactate, glycerol and acetate. While lactate and glycerol were identified as end-products of sporozoite metabolism by Ryley (1973), acetate has not previously been reported as a product of glucose catabolism by sporozoites. Although no other products were detected in these analyses, it should be remembered that ^{13}C-NMR is a relatively insensitive technique. The detection limits for different compounds depend on their precise absorption characteristics, but are generally in the mM range. Hence the possibility that there are other soluble products of glucose catabolism by sporozoites cannot be excluded. While lactate and glycerol were produced in all incubations, both aerobic and anaerobic, acetate was only ever detected in media from aerobic incubations. In a time course experiment (carried out once), metabolite production was observed to continue throughout the 12 hr incubation period (monitored at 2 hr intervals), by the end

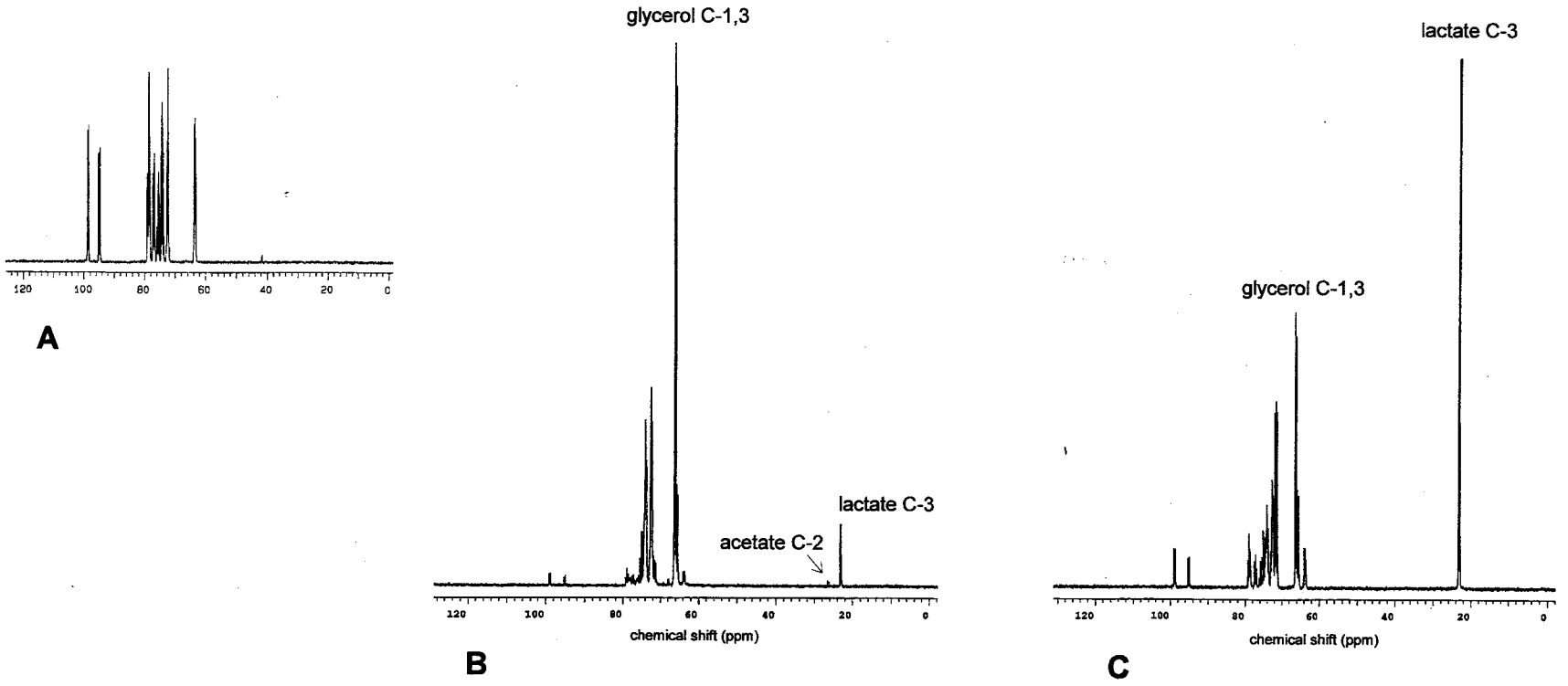

Figure 4.1. ^{13}C-NMR spectra showing catabolism of universally enriched [^{13}C]glucose by *E. tenella* sporozoites. (A) Fresh medium: all peaks are glucose-specific. (B) Medium in which 2.5×10^8 sporozoites have been incubated aerobically for 9 hr. (C) Medium in which 2.5×10^8 sporozoites have been incubated anaerobically for 9 hr. The most easily distinguishable peaks are labelled for each product. For conditions of incubation and acquisition see Materials and Methods.

of which the glucose was virtually exhausted. This indicated that the sporozoites were able to survive for extended periods in this medium. Sporozoites retained their integrity throughout this, and all other incubations with substrate, as judged by microscopy. Although no quantitative information was derived from these NMR experiments, the ratio of lactate to glycerol was consistently found to be greater under anaerobic than aerobic conditions. Glucose to glycerol ratios were similar in all samples[5].

4.3.1.2 Catabolism of selectively labelled ^{13}C-glucose

When sporozoites were incubated with selectively labelled, C-1 or C-6, ^{13}C-glucose, the labelled atoms were detected equally in all end-products, both aerobic and anaerobic (see Figure 4.2). This randomisation of carbon atoms is consistent with the presence, and near-equilibrium operation, of the glycolytic enzyme triosephosphate isomerase.

4.3.1.3 Intracellular products

Lysates of incubated sporozoites contained low intensity peaks corresponding to the chemical shifts of mannitol and lactate (see Figure 4.3). No other compounds were detected and there was no difference in the profiles of cells from aerobic and anaerobic

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[5] In one experiment, sporozoites of the ionophore-resistant strain, Ret5, were used in place of the sensitive variety. The aerobic incubation media from these cells was found to contain significantly higher proportions of acetate - relative to both lactate and glycerol - than equivalent media from the sensitive strain. Some acetate was also detected in the anaerobic incubation medium. These results hint at metabolic differences between the two strains - however the experiment was not repeated.

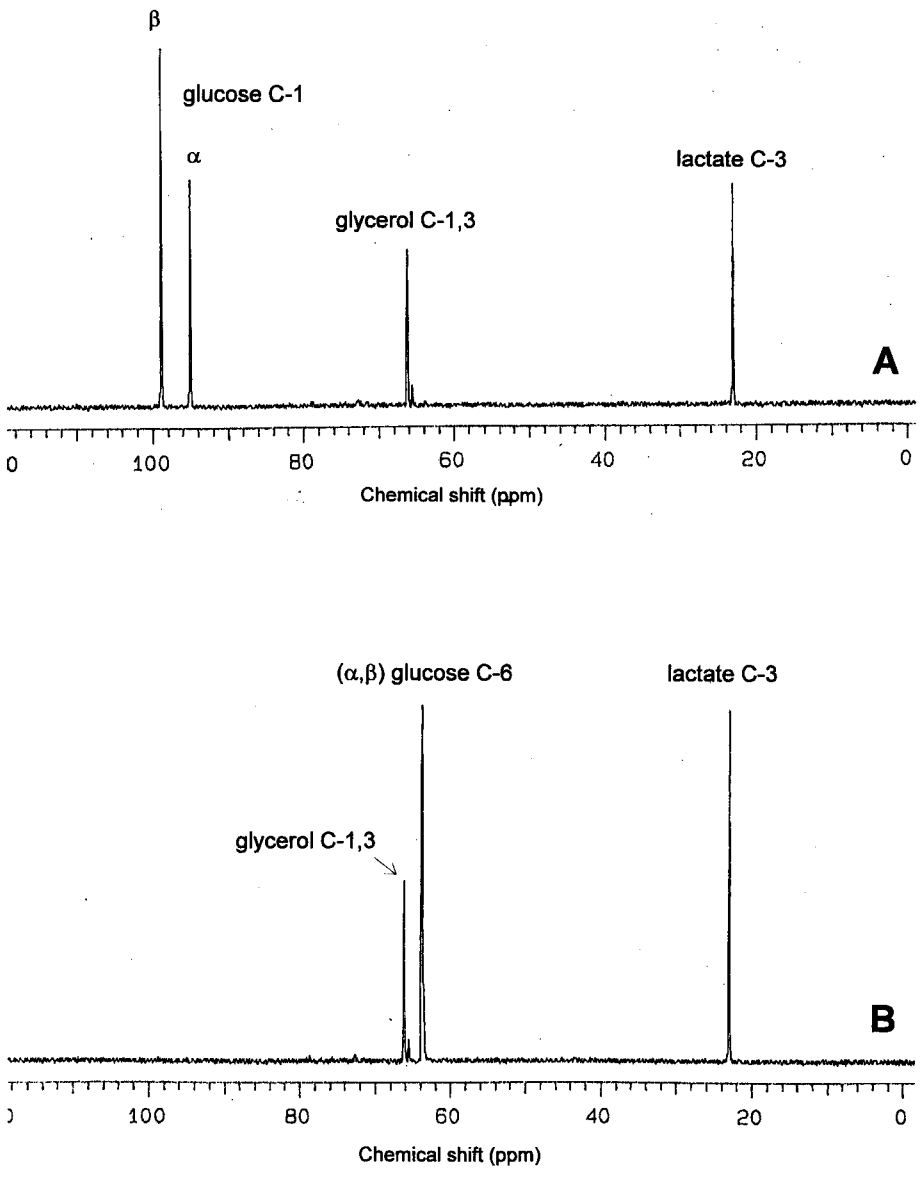

Figure 4.2. ^{13}C-NMR spectra of medium in which *E. tenella* sporozoites (1.25×10^{8} ml^{-1}) have been incubated aerobically with [1-^{13}C] (A) or [6-^{13}C] (B) glucose. The incubation time was 9 hr. For conditions of incubation and acquisition see Materials and Methods.

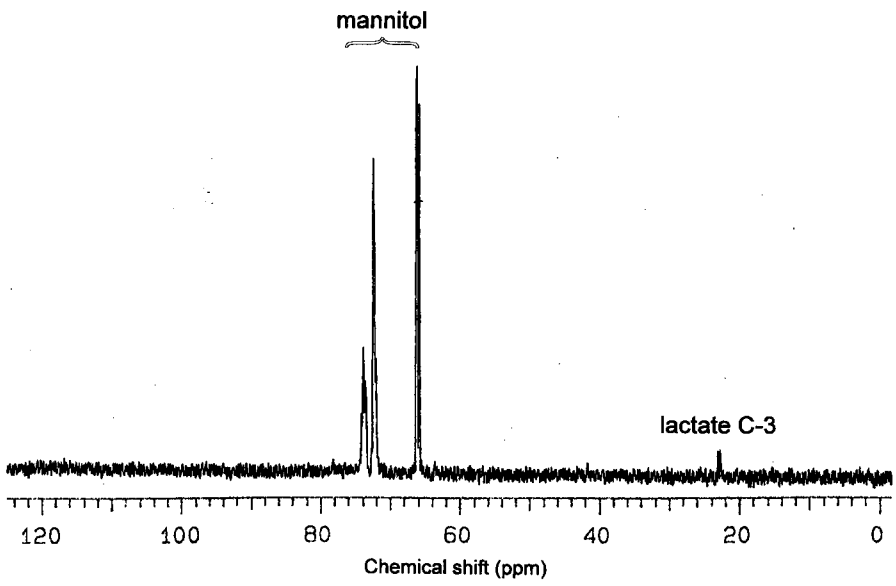

Figure 4.3. ^{13}C-NMR spectra of perchloric acid lysate of sporozoites (1.8×10^{8} ml^{-1}) previously incubated aerobically for 8 hr with [$^{13}C_6$]glucose. For conditions of incubation and acquisition see Materials and Methods.

incubations. The ability of the cells to convert glucose to mannitol further confirms the existence of a functioning mannitol cycle in this stage of the parasite.

4.3.2 Glucose catabolism by sporozoites: quantitative analyses

To obtain a clearer picture of glucose catabolism by sporozoites, quantitative techniques were used to measure the concentrations of glucose and end-products in incubation media. Glucose and glycerol were quantified by enzymatic assays while lactate and acetate were analysed using an hplc system specialised for the separation and detection of organic acids. These studies were performed using the Ret5 strain of *E. tenella* and were limited by the availability of parasite material.

4.3.2.1 Kinetics of glucose catabolism

Figure 4.4 shows the kinetics of glucose consumption and lactate and glycerol production by sporozoites incubated under aerobic and anaerobic conditions. As can be seen, the rates of glucose consumption and glycerol production were comparable under both gaseous conditions; while about twice as much lactate was produced anaerobically than aerobically. The rates of glucose consumption and product formation decreased steadily over the incubation time which was interpreted as reflecting a decrease in the viability of the parasites. This apparent decrease in viability did not correlate to cell lysis since there was no decrease in the number of intact cells over the course of the experiment. These are the results of a single experiment and acetate was not analysed.

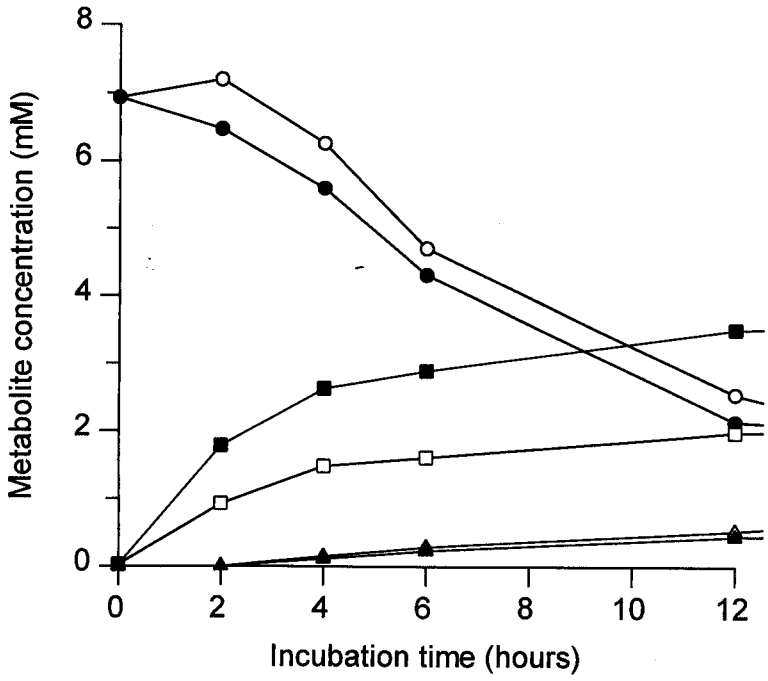

Figure 4.4. Glucose catabolism by *E. tenella* sporozoites, time course experiment. Sporozoites ($6x10^7$ ml^{-1}) were incubated, either aerobically *(open symbols)* or anaerobically *(closed symbols)*, in PBS, pH 7.4, containing glucose. Samples were removed at the times indicated and analysed for metabolites: glucose *(circles)*; lactate *(squares)*; and glycerol *(triangles)*.

4.3.2.2 Quantitative analysis of products under aerobic and anaerobic conditions: fixed length incubations

In order to obtain more precise data on aerobic and anaerobic glucose catabolism, metabolites were quantified in medium from fixed length incubations carried out in replicate. Since we were more interested in establishing the relative proportions of end-products rather than their precise rates of production, the fairly long incubation time of 8 hr were used throughout. This allowed sporozoite numbers to be kept to a minimum while the metabolite levels were sufficiently high to be accurately quantified.

The results of individual sporozoite incubations are shown in Table 4.3 (parts *a*, *b* and *c*) Different batches of parasites were used for each experiment and each experiment included concomitant incubations under aerobic and anaerobic conditions. Although the same end-products were produced in all experiments[6], the precise proportions of the individual end-products under the two gaseous conditions showed variation. Inspection of the data reveals that the experimental results fall into two discrete groups[7]. In one, (GROUP 1), rather similar levels of lactate were produced under both aerobic and anaerobic conditions, while acetate production was slightly greater in the absence of oxygen (Table 4.3.a.). In GROUP 2, significantly more lactate was produced in the absence of oxygen, while more acetate was found aerobically (Table 4.3.b). The GROUP 2 results seem to reflect more closely those obtained by NMR. The averages of the

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[6] Trace amounts of pyruvate were sometimes detected in incubation medium by hplc analysis; the levels were extremely low (< 20 nmol per 10^8 sporozoites over 8 hr) and were not quantified.

[7] While the data groups are too small to allow convincing statistical analysis, the differences noted are all statistically significant as judged by the Mann-Whitney test at a 10% confidence limit.

Table 4.3. *a.* Aerobic and anaerobic glucose catabolism by *E. tenella* sporozoites. Result GROUP 1.

	AEROBIC			ANAEROBIC		
experiment number:	*1*	*2*	*3*	*1*	*2*	*3*
Age of oocysts at excystation (months)	1	1	1	1	1	1
Bubbled?	✓	×	×	✓	×	×
	µmol per 10^8 sporozoites			µmol per 10^8 sporozoites		
GLUCOSE	- 13.20	- 9.27	- 9.60	- 10.70	- 9.21	- 7.82
LACTATE	+ 3.29	+ 4.88	+ 3.96	+ 4.08	+ 5.29	+ 3.75
ACETATE	+ 1.46	+ 2.40	+ 2.42	+ 2.44	+ 3.05	+ 4.19
GLYCEROL	+ 2.30	+ 1.16	+ 1.25	+ 2.14	+ 1.64	+ 1.56
TOTAL END-PRODUCTS	7.05	8.44	7.63	8.66	9.98	9.50

Sporozoites were incubated for 8 hr in PBS, pH 7.4, containing 15 mM glucose. Other conditions were as described in Materials and Methods. Each experiment comprised aerobic and anerobic incubations and experiments were carried out on different days and using different batches of sporozoites. Where indicated the suspensions were continuously bubbled with gas phase throughout incubation. The concentrations of glucose and glycerol in incubation media were quantitated by enzymatic assay; lactate and acetate were quantitated by hplc. Results are expressed as the amount of metabolite (µmol) produced (+) or consumed (-) by 10^8 sporozoites over the incubation period.

Table 4.3.*b*. Aerobic and anaerobic glucose catabolism by *E. tenella* sporozoites. Results GROUP 2.

	AEROBIC			ANAEROBIC		
experiment number:	*4*	*5*	*6*	*4*	*5*	*6*
Age of oocysts at excystation (months)	< 1	6	<1	< 1	6	<1
Bubbled ?	✓	×	×	✓	×	×
	µmol per 10^8 sporozoites			µmol per 10^8 sporozoites		
GLUCOSE	- 9.43	- 9.48	- 7.44	- 9.52	- 10.76	- 8.95
LACTATE	+ 2.81	+ 3.70	+ 3.16	+ 7.27	+ 9.54	+ 8.24
ACETATE	+ 4.82	+ 4.52	+ 4.99	+ 0.90	+ 1.93	+ 1.67
GLYCEROL	+ 1.28	+ 1.20	+ 1.32	+ 1.07	+ 1.08	+ 1.07
TOTAL END-PRODUCTS	8.91	9.42	9.47	9.24	12.55	10.98

Legend as for Table 4.3.*a*.

Table 4.3.*c*. Aerobic and anaerobic glucose catabolism by *E. tenella* sporozoites. MEAN DATA.

	AEROBIC			ANAEROBIC		
Data group:	*Group 1*	*Group 2*	*All experiments*	*Group 1*	*Group 2*	*All experiments*
	µmol per 10^8 sporozoites			µmol per 10^8 sporozoites		
GLUCOSE	- 10.69 ± 2.18	- 8.78 ± 1.16	- 9.74 ± 1.88	- 9.53 ± 1.21	- 9.47 ± 0.93	- 9.49 ± 1.12
LACTATE	+ 4.04 ± 0.80	+ 3.22 ± 0.45	+ 3.63 ± 0.73	+ 4.37 ± 0.81	+ 8.35 ± 1.14	+ 6.36 ± 2.35
ACETATE	+ 2.09 ± 0.55	+ 4.77 ± 0.24	+ 3.44 ± 1.52	+ 3.23 ± 0.89	+ 1.50 ± 0.54	+ 2.36 ± 1.15
GLYCEROL	+ 1.57 ± 0.63	+ 1.27 ± 0.06	+ 1.42 ± 0.44	+ 1.78 ± 0.31	± 1.07 ± 0.01	+ 1.43 ± 0.44
TOTAL END-PRODUCTS	7.71 ± 0.70	9.27 ± 0.31	8.49 ± 0.98	9.38 ± 0.67	10.92 ± 1.66	10.15 ± 1.41

individual data groups as well as the overall means are shown in Table 4.2.c. The reason for this division of results is not apparent since they do not correlate with any obvious factor, such as age of oocysts or mode of incubation (i.e. whether or not continuously bubbled). The main unambiguous finding of this study was that significant amounts of lactate, acetate and glycerol are produced under aerobic as well as under anaerobic conditions. This implies that the parasites have a fairly low potential for full aerobic respiration. In addition, only about half the glucose consumed in any experiment was detected in the measured end-products. This may indicate that there are other end-products of glucose catabolism not yet accounted for, or simply be a consequence of significant amounts of glucose being incorporated into polysaccharide reserves within the parasites (this is discussed more fully in Section 6.3).

In many instances the sporozoites used in these studies were motile on excystation and retained their motility throughout the incubations. There was no appreciable difference between the levels of motility observed under aerobic and anaerobic conditions, or between sporozoites producing different data groups .

4.3.2.3 Production of end-products from endogenous energy reserves

When sporozoites were incubated in the absence of exogenous substrates, some metabolites were still released into the medium. These were presumably derived from endogenous carbohydrate reserves. Table 4.4 summarises the quantities of metabolites produced by sporozoites during 8 hr incubations in PBS, pH 7.4. It seems probable that these quantities reflect the total amount of storage carbohydrate present in the sporozoites at the beginning of incubation. If so, this must be equivalent to at least 2

μmol of glucose per 10^8 cells. Since some carbohydrate must already have been consumed by the parasites during storage and excystation, it can be assumed that their total storage capacity is somewhat higher than this.

Some decline in cell numbers was usually observed by the end of these experiments.

Table 4.4 Metabolite release in the absence of exogenous substrates.

	μmol per 10^8 sporozoites/ 8 hr	
PRODUCTS:	*AEROBIC*	*ANAEROBIC*
LACTATE ($n = 3$) ACETATE ($n = 3$) GLYCEROL ($n = 4$)	1.14 ± 0.38 2.45 ± 1.55 0.31 ± 0.17	2.66 ± 1.01 0.92 ± 0.36 0.38 ± 0.14
TOTAL END-PRODUCTS:	3.90	3.96

Sporozoites were resuspended in PBS, pH 7.4, and incubated as described in Materials and Methods. Results are expressed as the amount (μmol) of product released by 10^8 sporozoites over 8 hr. The kinetics of production were not investigated.

4.3.3 Investigation of alternative substrates

4.3.3.1 Amino-acids

In recent years it has become increasingly evident that some parasitic protozoa, including the anaerobes *Giardia*, *Entamoeba* and *Trichomonas* (Lockwood and Coombs, 1991; Schofield and Edwards, 1994; Zuo and Coombs, 1995), catabolise

significant amounts of amino acids for energy generation. The possibility that the coccidia might utilise amino acids in this way had not been investigated.

In this study the possibility that *E. tenella* sporozoites might consume amino acids was investigated by monitoring changes in the amino acid profile of sporozoite incubation medium. Sporozoites were suspended in simple medium supplemented with either glucose or a mixture of standard amino acids, and incubated under aerobic or anaerobic conditions. Amino acids were detected and quantified by hplc.

Sporozoites did not consume any amino acids from the standard incubation mix, instead there appeared to be a general release of all amino acids analysed into the medium (see Table 4.5 (A)). This occurred in both amino acid- and glucose-supplemented media, but was greater in the former; there was no substantial difference between levels of release under aerobic and anaerobic conditions. The relative levels of release were fairly similar in all comparable experiments with no one amino acid being especially highly represented. In addition to the standard amino acids, a number of unidentified amino compounds were detected, both in cells extracts and in the incubation media (see Table 4.6); these are discussed in more detail in Section 4.3.4.4. This apparent release of amino acids is surprising and may be artifactual. For example: although there was no significant decrease in sporozoite numbers during any of the incubations, it is possible that lysis of only small numbers of sporozoites, followed by proteolytic degradation of their protein, could contribute largely to amino acid concentrations within the medium. The amino acid pools within the sporozoites were also analysed (see Table 4.5(B) and 4.6 (B)). These proved too small to account for the amino acid concentrations in the incubation media - even if all the cells had lysed!

Table 4.5. (A) Release of amino acids during incubation of *E. tenella* sporozoites (B) Amino acid content of *E. tenella* sporozoites.

A	nmols/ 10^8 sporozoites/ 8 hr[1]			
	PBS/amino acids ($n = 3$)		PBS/glucose ($n = 4$)	
amino acids	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
asp	+16.6 ± 7.4	+22.2 ± 11.5	+1.0 ± 1.2	+1.5 ± 1.8
glu	+27.3 ± 4.0	+34.3 ± 10.1	+9.3 ± 0.5	+9.7 ± 1.7
asn	+4.7 ± 3.2	+9.7 ± 9.4	+2.4 ± 1.5	+2.5 ± 0.6
ser	+39.0 ± 19.7	+42.1 ± 15.0	+14.4 ± 4.8	+19.7 ± 3.4
thr	+32.8 ± 7.9	+44.2 ± 19.1	+6.2 ± 0.9	+8.1 ± 3.6
arg	+55.2 ± 11.8	+70.4 ± 29.8	+11.3 ± 1.7	+9.0 ± 4.5
ala	+28.1 ± 5.2	+37.8 ± 10.1	+25.2 ± 4.7	+24.0 ± 3.0
tyr	+16.7 ± 3.6	+22.8 ± 11.2	+3.7 ± 0.3	+3.7 ± 0.6
trp	+3.5 ± 4.9	+5.5 ± 4.6	+8.3 ± 3.8	+6.9 ± 5.8
phe	+12.6 ± 5.3	+20.3 ± 12.1	+4.9 ± 0.6	+5.2 ± 1.1
ile	+28.5 ± 10.3	+41.9 ± 25.4	+4.3 ± 0.2	+5.0 ± 0.9
leu	+30.2 ± 6.3	+41.8 ± 19.7	+11.8 ± 0.9	+12.1 ± 1.8

B	nmols/ 10^8 sporozoites[2] ($n = 3$)
amino acids	
asp	0.4 ± 0.1
glu	6.4 ± 2.2
asn	2.0 ± 0.7
ser	1.1 ± 0.3
thr	8.5 ± 1.7
arg	5.3 ± 1.5
ala	2.7 ± 0.3
tyr	0.5 ± 0.1
trp	2.1 ± 0.4
phe	0.9 ± 0.3
ile	0.5 ± 0.1
leu	0.4 ± 0.1

Means ± SD

[1] Sporozoites were incubated at 41°C in PBS, pH 7.4, containing either 10 mM glucose (PBS/glucose) or 1% (v/v) MEM essential and non-essential amino acids (PBS/amino acids). Amino acids were quantitated by hplc at $t = 0$ and $t = 8$ hr of incubation. Results are expressed as nmols of amino acid released per 10^8 sporozoites over the incubation time.

[2] Amino acids were quantitated in sporozoite lysates prepared at a concentration of 10^8 ml^{-1}.

Table 4.6. (A) Release of unidentified amino-compounds and co-eluting amino acids during incubation of *E. tenella* sporozoites

(B) Unidentified amino-compounds and co-eluting amino acids in lysates of *E. tenella* sporozoites

A	Peak area [1] (fluorescence units)			
	PBS/amino acids ($n = 3$)		PBS/glucose ($n = 4$).	
amino acids	AEROBIC	ANAEROBIC	AEROBIC	ANAEROBIC
his/glu	+50.3 ± 10.6	+62.7 ± 22.6	+14.2 ± 0.7	+14.9 ± 6.9
val/met	+125.7 ± 24.9	+165.0 ± 67.7	+15.2 ± 1.3	+15.8 ± 1.6
a	+4.1 ± 3.4	+2.2 ± 1.2	+1.2 ± 0.6	+0.7 ± 0.3
b	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
c	+24.8 ± 11.1	+30.1 ± 11.8	+16.9 ± 0.5	+14.9 ± 6.9
d	+6.0 ± 0.7	+7.9 ± 1.0	+2.4 ± 0.5	+2.8 ± 0.4
e	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

B	peak area (10^8 sporozoites ml^{-1})[2] ($n = 3$)
amino acids	
his/glu	1.6 ± 0.4
val/met	1.0 ± 0.3
a	16.1 ± 3.3
b	0.9 ± 0.3
c	1.1 ± 1.6
d	3.5 ± 2.4
e	2.2 ± 0.5

Means ± SD

[1] Sporozoites were incubated at 41°C in PBS, pH 7.4, containing either 10 mM glucose (PBS/glucose) or 1% (v/v) MEM essential and non-essential amino acids (PBS/amino acids). Results are expressed as the change in (hplc) peak area induced by sporozoites incubated for 8 hr at a concentration of 10^8 ml^{-1}.

[2] Peak areas were determined in sporozoites lysates prepared at a concentration of 10^8 ml^{-1}.

4.3.3.2 Mannitol

It has been suggested (K-W Thong, personal communication) that the sugar mannitol might be highly represented in the chicken diet and could be taken up by sporozoites and catabolised via the mannitol cycle. To investigate this possibility, sporozoites (1×10^8 ml^{-1}) were incubated in PBS, pH 7.4, containing 20 mM mannitol and the medium analysed for the normal end-products of glucose catabolism (lactate, acetate and glycerol). The quantities of end-products detected under these conditions were the same as when no exogenous substrate was included in the medium (see Section 4.3.2.3). This suggests that either exogenous mannitol is not a useful energy substrate for this stage of the parasites, or, if it is, it is catabolised via different pathways to glucose.

4.3.4 Sporulation studies

Oocysts of *Eimeria* species sporulate only in the presence of oxygen. The process, as detailed in Section 1.2.1.5., involves consumption of amylopectin and mannitol and is accompanied by vigorous respiratory activity and release of CO_2. Wang (1976) was unable to detect any soluble products being released into medium during sporulation of *E. tenella* oocysts. Observations such as these have led to the assumption that oocyst sporulation is fuelled by fully aerobic catabolism of carbohydrates to CO_2 and H_2O. While not refuting this possibility, it seemed to us that the highly impermeable nature of the oocysts wall would mean that soluble products of oocyst metabolism may be retained within the oocysts rather than being released to the environment. This possibility does not seem to have been previously investigated. In the following study, the quantitative methods used in the previous section were used to compare levels of

metabolites - organic acids, glycerol and amino acids - in extracts of both sporulated and unsporulated oocysts of *E. tenella*. It was hoped that the results might provide further insight into the metabolic events occurring during sporulation.

4.3.4.1 Investigation of released products

None of the metabolites assayed for (glycerol, lactate and acetate) could be detected in medium in which oocysts had been incubated and successfully sporulated over a 24 hr period. However, although the oocysts were incubated at a fairly high concentration (2×10^6 ml^{-1}), the final dilution of any excreted compounds would still have been relatively large. Hence, given the sensitivity of the detection system, it can only be definitively stated that release of lactate was < 0.5 μmols/10^6 oocysts/24 hr.

4.3.4.2 Levels of organic acids and glycerol in sporulated and unsporulated oocysts

Table 4.7.a. shows the amounts of glucose, glycerol, lactate and acetate associated with extracts of sporulated and unsporulated oocysts of *E. tenella*. These figures were also used to estimate the approximate concentration of each of these metabolites within the oocysts. These were calculated using an estimated volume of 3.05×10^{-4} litres per 10^8 oocysts which is based on an average oocyst diameter of 18 μm. The concentrations of all the compounds were fairly high (notably glycerol), but the levels were effectively the same, within the limits of standard deviations, in both oocyst stages. In conjunction with the data in the previous section, this implies that there is no net synthesis of fermentative end-products during sporulation. It is interesting to note that the

Table 4.7.*a*. Metabolites in sporulated and unsporulated oocysts of *E. tenella*.

Metabolite:	μmols per 10^8 oocysts		Concentration within oocysts (mM) [1]	
	Unsporulated oocysts	Sporulated oocysts	Unsporulated oocysts	Sporulated oocysts
GLUCOSE	1.1 ± 0.4 (3)	1.7 ± 1.1 (3)	3.7	5.4
LACTATE	8.2 ± 1.2 (5)	7.2 ± 1.5 (3)	26.8	23.6
ACETATE	1.0 ± 0.5 (5)	0.3 ± 0.2 (3)	3.2	1.1
GLYCEROL	48.3 ± 17.6 (3)	58.1 ± 18.9 (3)	158.4	190.4

Means ± SD, *n* in parentheses

[1] Concentrations were calculated from the figures in the previous columns, using an estimated volume of 3.05×10^{-4} litres per 10^8 oocysts; this is based on an average oocyst diameter of 18 μm.

Table 4.7.*b*. The mysterious 'X' peak !

	Unsporulated oocysts	Sporulated oocysts
'X' (peak area $\times 10^3$) [2]	1268 ± 339 (4)	11431 ± 3654 (4)

[2] Peak areas were determined in oocysts lysates prepared at a concentration of 10^8 ml^{-1}.

proportions of the metabolites do not reflect the proportions produced by sporozoites during glucose catabolism.

4.3.4.2.1 The 'X' peak !

An interesting feature of this study was the discovery of an unknown peak in oocyst extracts applied to organic acid hplc. This peak - designated the X peak - was present in all extracts investigated, but was approximately 10 times larger in sporulated than in unsporulated oocyst lysates (see Figure 4.5 and Table 4.7.b). Accumulation of the X peak appeared to be specifically associated with the process of sporulation in that it did not build up in unsporulated oocysts incubated under anaerobic conditions which hence did not sporulate (see Figure 4.5). The elution time of the peak was extremely sensitive to pH. Although at pH 2.7 it co-eluted with the compound succinate, the peaks were convincingly separated at other pH values. It did not consistently co-elute with any of the other standard organic acids applied to the column (Table 4.2); the one that showed the most similar response to pH was α-ketobutyrate. Since the absorption characteristics of the compound are unknown it clearly cannot be quantified. If for the sake of interest, however, we assign it a fairly average absorption coefficient - that of succinate - then its concentration in sporulated oocysts would be approximately 100 mM.

In an attempt to obtain a pure sample of X for identification by physico-chemical means, X-containing fractions were collected from the hplc column. Unfortunately the relatively low capacity of the column and the large dilution experienced during elution, meant that it was impossible to obtain a sufficiently concentrated sample for analysis by NMR or mass spectrometry (analysis was attempted, but nothing was detected). The

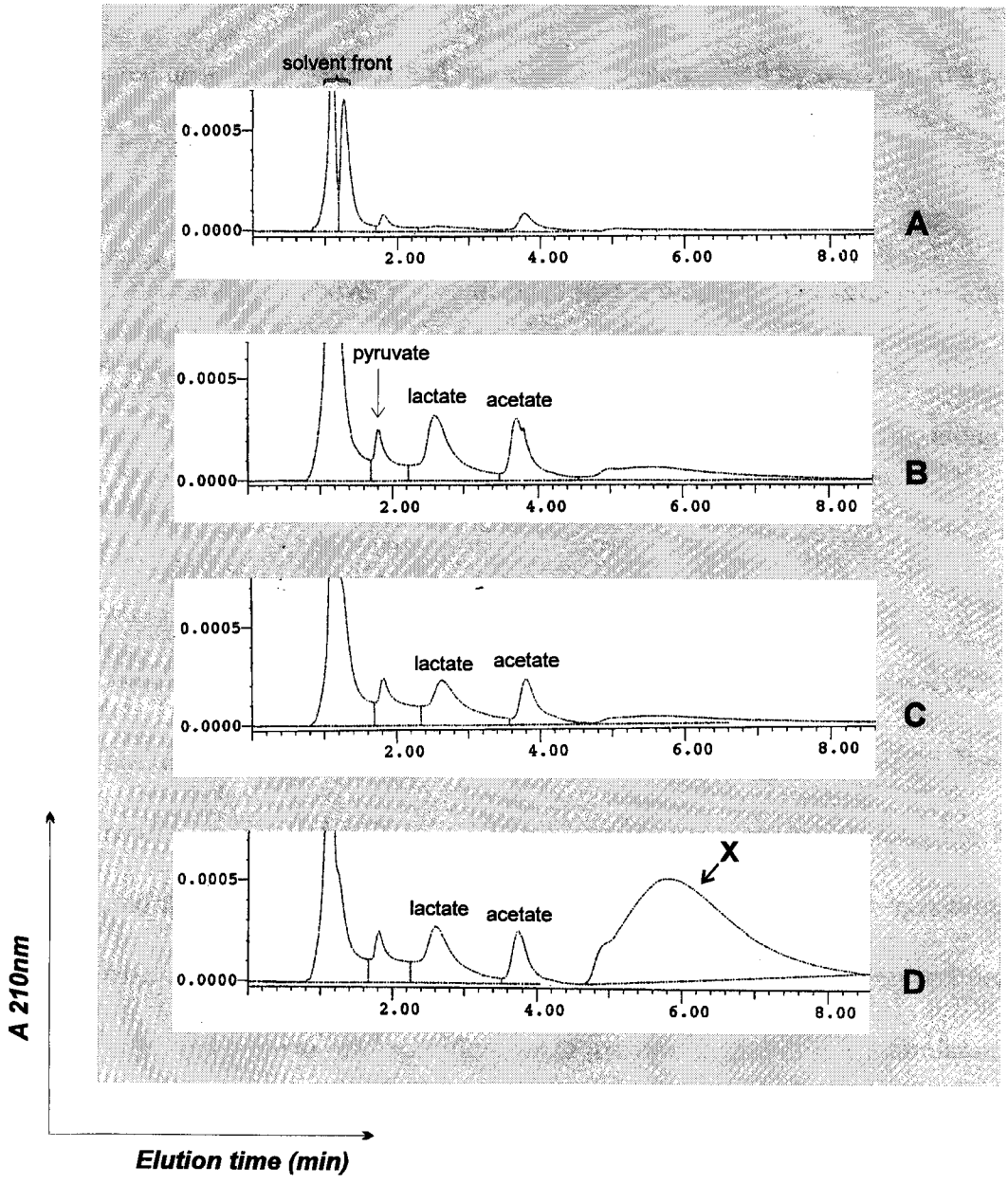


Figure 4.5. Hplc chromatograms of free acids in sporulated and unsporulated oocysts of *E. tenella*. (A) Blank run, extracted solvent only. (B) Unsporulated oocyst extract. (C) Extract of unsporulated oocysts which had been incubated at 29°C for 24 hr, anaerobically. (D) Extract of oocysts which had been incubated at 29°C for 24 hr, aerobically; 82% sporulation was achieved. Equivalent amounts of extract were applied to each analysis.

approach was further complicated by the fact that the compound did not extract efficiently into dichloromethane - the solvent of choice for physico-chemical analyses. It also appeared unstable or volatile and could not be concentrated by conventional freeze-drying.

In view of the problems above, it was decided that it would be too time consuming to pursue the identity of the compound further. Hence the 'X peak' remains an enigma; its interest must be increased by the fact that it was also found to be present, along with lactate and acetate, in extracts of sporulated oocysts of *C. parvum*.

4.3.4.3 Comparison of free amino acids in sporulated and unsporulated oocysts

The free amino acids pools of sporulated and unsporulated oocysts were analysed by hplc, the results are detailed in Tables 4.8. All amino acids were present at lower levels in sporulated than in unsporulated oocysts. Different amino acids were 'consumed' to different extents with alanine undergoing the greatest change. The combined peak of his/glu was the only amino-acid-specific peak to increase in size during sporulation (see Table 4.9).

Clearly we cannot say in what way the amino acids are being used during sporulation: whether they are being incorporated into proteins, catabolised for energy production, or entering other metabolic pathways. Certainly massive protein synthesis must occur during sporulation suggesting that at least some of the change is due to incorporation into proteins.

Table 4.8. Amino acids in sporulated and unsporulated oocysts of *E. tenella.*

amino acid	Retention time (mins) ($n = 7$)	nmol per 10^7 oocysts		
		UNSPORULATED ($n = 3$)	SPORULATED ($n = 4$)	DIFFERENCE (SPD-UNSPD)
asp	2.15 ± 0.06	61.0 ± 3.1	41.3 ± 16.4	- 19.7
glu	3.25 ± 0.14	182.7 ± 48.5	108.8 ± 35.6	- 73.9
asn	7.14 ± 0.22	35.4 ± 4.7	12.1 ± 1.3	- 23.3
ser	8.45 ± 0.25	197.4 ± 25.2	67.2 ± 18.0	- 130.2
gly	10.98 ± 0.33	167.3 ± 23.3	77.5 ± 18.0	- 89.8
thr	11.45 ± 0.32	120.7 ± 13.9	45.6 ± 10.4	- 75.1
arg	12.93 ± 0.32	84.3 ± 12.7	56.4 ± 13.1	- 27.9
ala	13.62 ± 0.34	405.7 ± 50.7	70.4 ± 25.4	- 335.3
tyr	15.38 ± 0.30	51.4 ± 6.8	27.7 ± 3.2	- 23.7
trp	21.50 ± 0.33	18.1 ± 2.6	16.4 ± 2.3	- 1.7
phe	22.14 ± 0.36	72.2 ± 7.6	38.2 ± 4.0	- 34.0
ile	22.51 ± 0.35	50.3 ± 5.3	39.2 ± 4.1	- 11.1
leu	23.24 ± 0.36	127.0 ± 15	117.0 ± 8.6	- 10.0
γ-aminobutyric acid **(d)** [1]	14.56 ± 0.35	327.7 ± 52.5	52.1 ± 19.3	- 275.6

[1] The unidentified amino-compound, d, was quantified according to a standard curve of γ-aminobutyric acid.

Table 4.9. Unidentified amino-compounds and co-eluting amino acids in sporulated and unsporulated oocysts of *E. tenella*.

Peak	Retention time (mins) ($n = 7$)	Peak area [1] (fluorescence units)		
		UNSPORULATED ($n = 3$)	SPORULATED ($n = 4$)	DIFFERENCE (SPD-UNSPD)
a	6.96 ± 0.27	0.0 ± 0.0	30.1 ± 6.9	+30.1
b	9.49 ± 0.28	66.4 ± 22.6	0.0 ± 0.0	-66.4
c	10.51 ± 0.31	0.0 ± 0.0	48.8 ± 10.4	+48.8
d (γ-aminobutyrate?)	14.56 ± 0.35	306.1 ± 47.5	56.5 ± 16.7	-249.6
e	20.00 ± 0.35	16.7 ± 4.6	18.5 ± 6.3	+1.7
his/glu	9.28 ± 0.28	44.5 ± 14.1	91.5 ± 63.3	+47.0
val/met	19.55 ± 0.34	196.1 ± 75.0	101.2 ± 7.6	-94.9

[1] Peak areas were determined for samples of oocyst lysates prepared at a concentration of 10^7 ml^{-1}.

4.3.4.3.1 Unknown amino compounds

In addition to the 16 standard amino acid peaks observed in oocyst extracts, a number of unusual peaks were detected. Five main 'unknowns' were identified. These were designated, in order of retention time, a, b, c d and e. The compounds were consistently present and underwent defined changes in concentration during sporulation. The levels of these compounds in sporulated and unsporulated oocysts are described in terms of peak area in Table 4.9. Some of these compounds were also present in extracts of sporozoites and also appeared to be released during incubation of these cells (Table 4.6).

The identity of these peaks has not been ascertained. Since the compounds react with OPA, they must certainly contain a terminal amino group. However, at least 150 non-protein amino compounds have been described in biological systems (Barrett, 1981) and in view of these numbers it did not seem reasonable to try to identify the compounds by testing all possibilities. Some of the apparently more likely possibilities were, however, applied to the column. Of these, the polyamines, spermidine, cadaverine, putrescine, spermine and their precursor, ornithine, were not elutable under the same conditions as the unknowns; neither were α-, β- or di- aminobutyric acids. γ-Aminobutyrate, however, was found to consistently co-elute with the peak known as 'd'. While co-elution of a known and unknown compound under a single set of conditions cannot be taken as proof of identity, γ-aminobutyrate has been reported to be present in a number of other parasites, and so its presence would not be unprecedented. Its function, in all cases, is unclear. The low concentrations of the amino-compounds present mean that there is little possibility of their being identifiable by physico-chemical techniques.

4.4 RESULTS SUMMARY

- Using ^{13}C-NMR, lactate, acetate and glycerol were identified as soluble end-products of ^{13}C-glucose catabolism by sporozoites of *E. tenella*. ^{13}C-mannitol was detected within sporozoites incubated with ^{13}C-glucose.
- The soluble end-products of aerobic and anaerobic glucose catabolism were quantified by a combination of hplc and enzymatic assays. While the results showed some variation, significant quantities of all end-products were produced under both gaseous conditions. This implies that the sporozoites have a limited capacity for full aerobic respiration.
- Sporozoites of *E. tenella* did not consume any amino acids from incubation medium. There was a net increase in the medium of all the amino acids analysed.
- Exogenous mannitol was not apparently catabolised by sporozoites of *E. tenella*.
- Sporulated and unsporulated oocysts of *E. tenella* contained comparable levels of lactate, acetate and glycerol. None of these metabolites were detected in sporulation medium.
- An unidentified organic acid was detected in oocyst extracts. This seemed to accumulate during sporulation, with levels being approximately 10× higher in sporulated than in unsporulated oocysts. It was also detected in sporulated oocysts of *C. parvum*.
- Levels of all free amino acids were lower in sporulated than in unsporulated oocysts of *E. tenella*.
- Five unknown amino compounds were distinguished in oocyst extracts. Some of these were also produced during sporozoite incubations. One compound was tentatively identified as γ-aminobutyrate.

5. PROTEINASE STUDIES

5.1 INTRODUCTION: PROTEINASES OF PARASITIC PROTOZOA

Proteinases are enzymes that catalyse the hydrolysis of peptide bonds within proteins or polypeptides. Such enzymes have been found in virtually all organisms that have been investigated. While proteinases can be classified in many different ways, the most satisfactory is according to catalytic mechanism (Hartley, 1960). On this basis, four distinct classes of proteinase are recognised - serine, cysteine, aspartyl and metallo - these are most readily distinguished according to their different sensitivities to various inhibitors (Beynon and Bond, 1989) (see Table 5.1).

While proteinases have long been known to play a role in nutrient digestion and protein turnover, they are increasingly being recognised as effectors of other important biological functions. In parasitic protozoa proteinases have been implicated in such diverse roles as facilitation of invasion of host cells, evasion of host immune response, retardation of blood coagulation, and regulatory modification of parasite proteins (McKerrow, 1989; Healer *et al.*, 1991). Recent interest in the function of parasite proteinases has led to detailed analyses of some of the enzymes. Such studies provide optimism that some parasite proteinases may be sufficiently different from their host

counter parts to be suitable targets for chemotherapeutic attack (North, 1982; McKerrow, 1989; Cohen *et al.*, 1991). Cysteine proteinases, which appear to be especially prevalent in protozoa, have attracted particular attention in this respect (North *et al.*, 1990).

Information on parasitic proteinases is reviewed in several chapters of Coombs and North (1991).

Table 5.1 Inhibitor sensitivities of different proteinase classes.

Inhibitors of:			
Serine proteinases	**Cysteine proteinases**	**Metallo-proteinases**	**Aspartic proteinases**
DFP, diisopropylflourophosphate 3,4-Dichloroisocoumarin	Iodoacetamide Iodoacetate E-64 Heavy metals	Chelating agents: EDTA 1,10-phenanthroline 8-Hydroxyquinoline Phosphoramidon	Pepstatin
PMSF Leupeptin TLCK (trypsin, other serine and cysteine proteinases)			

5.1.1 Aims of this study

While the general literature on parasite proteinases has expanded greatly over the last decade, there have been relatively few published investigations of proteinases in coccidial parasites. The available information on coccidial proteinases is summarised in Section 1.2.2.3. although it should be noted that several of the key references in this

section were published after the start of my project in 1992. The apparent deficit of knowledge of coccidial proteinases prompted me to undertake a summary analysis of proteinase activity in *Eimeria*. I was particularly interested in the possibility that such enzymes might be involved in the degradation of protein for nutritional means. Analysis was to commence with the detection of proteinase activity by standard techniques, such as substrate SDS-PAGE, and to proceed to more detailed characterisations if appropriate.

5.2 MATERIALS AND METHODS

5.2.1 Parasites

Analyses were initially performed using the Et2 strain of *E. tenella* and subsequently repeated using the Ret5 strain. Identical results were obtained with both strains.

5.2.2 Preparation of samples for SDS-PAGE

Parasites were lysed directly into gel sample buffer (Hames, 1990) to give the equivalent of $0.5\text{-}2\times10^6$ sporozoites or $2.5\text{-}5\times10^5$ oocysts per 25 µl well.

5.2.3 Substrate SDS-PAGE

Gelatin SDS-PAGE was performed as described in Robertson and Coombs (1990). Activities against bovine serum albumin and haemoglobin were investigated by substituting these for the gelatin in the gel mix. All gels were incubated at 37°C for a minimum of 16 hr prior to staining.

The effect of pH on substrate hydrolysis was investigated by incubating gel strips (after electrophoresis) in the following buffers: pH 3-5, sodium acetate (0.1 M); pH 6-8, sodium phosphate (0.1 M); pH 9-10, sodium bicarbonate (0.1 M). Inhibitor sensitivities were investigated by supplementing the incubation buffer with proteinase inhibitors: PMSF (0.5 mM), E64 (5 µM), pepstatin (1 µM), EDTA (1 mM), trypsin inhibitor (0.1%). Stock solutions of inhibitors were made up and stored as recommended in Beynon and Bond (1989). Metal ion dependency was investigated using the following

salts: $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$, and NaCl (all at 10 mM). DTT (1 mM) was included in some incubation buffers.

5.2.4 Investigation of proteolysis in crude homogenates

Sporulated or unsporulated oocysts were resuspended (2.5×10^7 ml^{-1}) in 100 mM Tris pH 8.0 and lysed by vortexing with glass beads (Section 2.5). Aliquots of freshly lysed extract were supplemented with proteinase inhibitors or DTT (concentrations specified above) and incubated at 41°C. Reactions were terminated after the required incubation time by addition of an equal volume of SDS-PAGE loading buffer (denaturing) and the samples were stored at -70°C. The protein composition of the homogenates was analysed by SDS-PAGE with Coomassie Blue staining

5.2.5 Western blotting

Western blotting was performed as described in Mottram *et al.* (1992). Polyclonal antisera to *Leishmania mexicana* group A and group C cysteine proteinases (Robertson and Coombs, 1990 and 1993) were used at dilution of 1: 500.

5.2.6 Use of biotinylated inhibitors

Protein extracts were incubated for 30 min at 37°C with biotin-phe-ala-CHN_2 (3 mM) (Biosyn) then separated by SDS-PAGE and transferred to nitrocellulose. Bound inhibitor was detected according to the manufacturers instructions using streptavidin/alkaline phosphatase (1:500 dilution) and 5-bromo-4-chloro-3-indolyl

phosphate/nitro blue tetrazolium (Sigma). The specificity of binding was checked by preincubating some samples with E64 (3 mM).

5.3 RESULTS

5.3.1 Presence of trypsin in parasite homogenates

One band of proteinase activity was detected in extracts of sporozoites, sporulated and unsporulated oocysts of *E. tenella* using gelatin SDS-PAGE. The proteinase had an apparent molecular weight of approximately 20 kDa and was optimally active at pH 7-8. It was inhibited by PMSF and TLCK but not affected by DTT. These properties were noted to be similar to those of the serine proteinase trypsin (Beynon and Boyd, 1989), which is used both to effect excystation of sporozoites and to facilitate release of oocysts from caecal tissue (see Section 2.2.4). Subsequent analyses indicated that, as suspected, the detected activity was almost certainly due to trypsin left over from preparative procedures. The electrophoretic mobility of the parasite-associated proteinase (as shown in Figure 5.1) was identical with that of trypsin, and both were largely inhibited by trypsin inhibitor and TLCK. Although the apparent trypsin activity associated with most extracts was quite low (typically overnight incubation was required to provide a good signal on substrate gels), it could not be effectively removed from the cells by repeated washing with low salt buffers. Indeed lysates of washed cells were found to contain more trypsin activity than medium in which they had been washed. These observations implied that the activity detected was due to trypsin which was associating with the parasites, possibly though ionic interactions with the cell surface. In one experiment it was observed that a high salt solution (0.5 M NaCl) was more effective in removing the activity from oocysts than a low salt buffer: this would seem to support the notion of ionic associations.

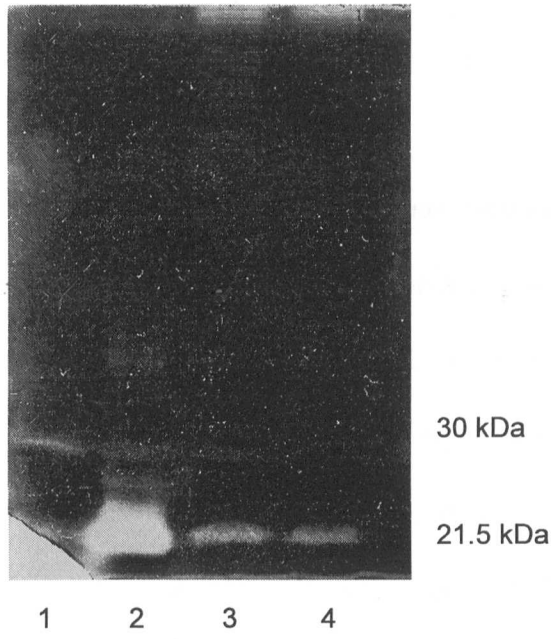


Figure 5.1. Gelatin SDS-PAGE analysis of *E. tenella* extracts showing presence of trypsin. Lane 1, chymotrypsin (from bovine pancreas, Sigma #C-4129), 0.15 μg; lane 2, trypsin (porcine, Sigma #T-4674), 0.35 μg; lane 3, sporozoite extract (equivalent to 1×10^6 cells); lane 4, unsporulated oocyst extract (equivalent to 5×10^5 cells). Note, proteins often run anomalously on gelatin gels so molecular weights should be treated with caution.

It is interesting to note that some of the proteinase activities reported in *Eimeria* by other researchers have been identified as serine proteinases with properties resembling those of trypsin (Farooqui *et al.*, 1983; Fuller and McDougald, 1990; Michalski *et al.*, 1992). Since trypsin is almost universally used in parasite production, it seems possible that some of these reported activities were actually due to residual trypsin present in the extracts. Its association with cell surfaces has not previously been noted and will clearly complicate many forms of proteinase analysis.

With the exception of the apparent trypsin activity, no proteinases were detected in *Eimeria* lysates using either gelatin, haemoglobin or BSA as substrates in SDS-PAGE. In each case, incubations were carried out over a pH range of 3-10, plus and minus various metal salts ($MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$ and NaCl, all at 10 mM), and with and without DTT (1 mM). The lack of positive results with this technique suggests that proteinases of *E. tenella* must be of relatively high specificity or of low activity (or both!).

5.3.2 Proteolysis in parasite homogenates

In a further attempt to detect proteinase activity, crude extracts of *E. tenella* oocysts were incubated at 41°C and monitored for changes in protein composition by SDS-PAGE. As shown in Figure 5.2.A., the protein profile of crude homogenates underwent rapid modification upon incubation, with most bands showing significant lessening of intensity. Degradation of certain bands was evident within 10 minutes of the start of incubation, while more general proteolysis appeared to continue for up to 24 hours. The specificity of the proteinases mediating these changes was investigated by including

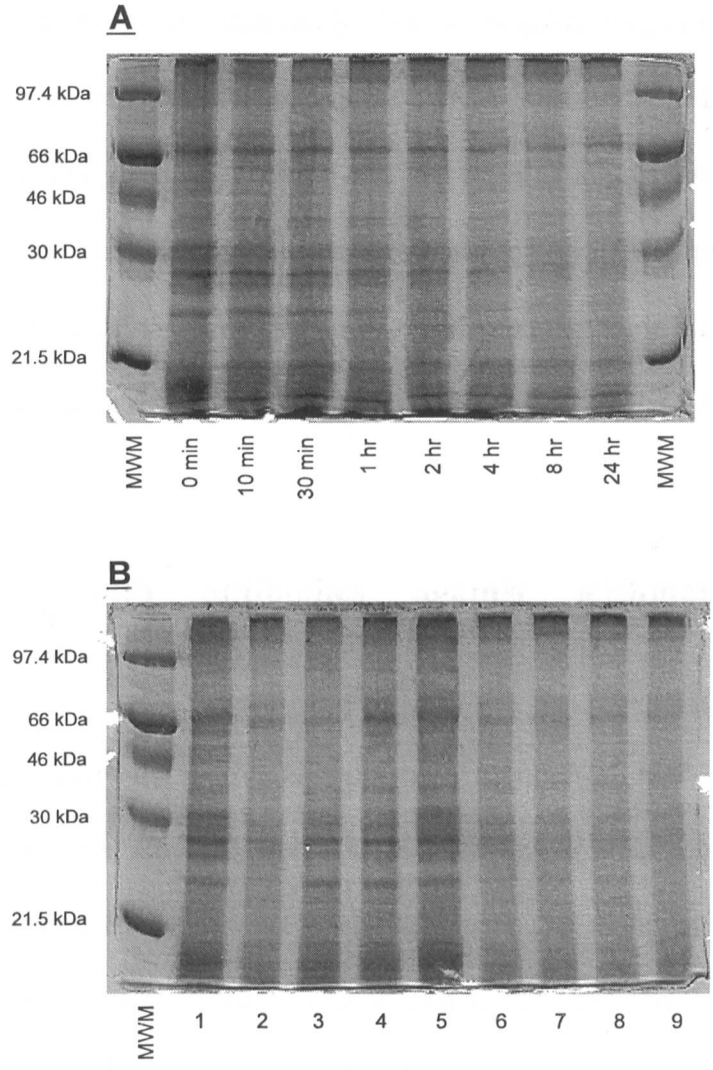


Figure 5.2. Proteolysis in homogenates of unsporulated oocysts of *E. tenella*. The protein composition of homogenates was analysed by SDS-PAGE with Coomassie blue staining (equivalent of 1.25×10^5 oocysts per lane). MWM, molecular mass markers. **(A) Time course experiment:** homogenates were incubated at 41°C for the indicated lengths of time. **(B) Effect of proteinase inhibitors:** lane 1, fresh homogenate; lane 2, homogenate incubated at 41°C for 2 hr; lane 3, homogenate incubated with E64; lane 4, homogenate incubated with PMSF; lane 5, homogenate incubated with both E64 and PMSF; lane 6, homogenate incubated with pepstatin; lane 7, homogenate incubated with 1,10-phenanthroline; lane 8, homogenate incubated with DTT; lane 8, homogenate incubated with trypsin inhibitor.

specific proteinase inhibitors in the incubation mix (Figure 5.2.B.). The serine/cysteine proteinase inhibitor, PMSF, was extremely effective in preventing proteolysis; E64 also afforded some protection; while pepstatin, EDTA and trypsin inhibitor had no appreciable effect. The presence of PMSF and E64 together almost completely inhibited proteolytic damage. This information indicates that proteolysis was probably mediated by parasite-specific proteinases rather than by trypsin and that these were of the cysteine and serine variety. These results duplicate those of Michalski *et al.* (1992).

5.3.3 Reaction with antibodies against leishmanial cysteine proteinases

Amastigotes of *Leishmania mexicana* contain a number of high activity cysteine proteinases which have attracted attention as potential drug targets and have been purified and analysed at the genetic level (Robertson and Coombs, 1990 and 1993; Mottram *et al.*, 1992). The parasite genome contains at least three types of cysteine proteinase gene. Two of these, *lmcpa* and *lmcpb*, code for a cathepsin L-like proteinases, while *lmcpc* codes for cathepsin B-type activity. Polyclonal antisera have been raised against each of these gene-products.

In a further attempt to detect proteinases in *E. tenella*, lysates were subjected to Western blotting using antisera against the *lmcpb* and *lmcpc* gene products. The anti-LmCPa antibodies did not appear to recognise any epitopes in sporozoite extracts, but reacted specifically with one band of approximately 46 kDa in unsporulated oocysts. A representative blot is shown in Figure 5.3, similar results were obtained on three

separate occasions. The sera specific for the *lmcpb* gene product did not recognise epitopes in either of the parasite extracts.

While the possibility that the antibodies are recognising a superficially homologous but functionally different protein cannot be excluded, this result gives preliminary indications that there is a developmentally-regulated cathepsin L-type proteinase in *Eimeria.*

5.3.4 Reaction with biotinylated inhibitors

The biotinylated inhibitor biotin-phe-ala-CHN_2, which is directed at cysteine-type proteinases, reacted with one band of protein on blots of *E. tenella* unsporulated oocyst extract (see Figure 5.4). The band had an approximate molecular weight of 46 kDa and may therefore have been the same as that recognised by the α-LmCPa antisera. When extracts were preincubated with the cysteine proteinase inhibitor E64 prior to addition of affinity label, the intensity of the binding signal was greatly reduced. This confirms the specificity of the reaction.

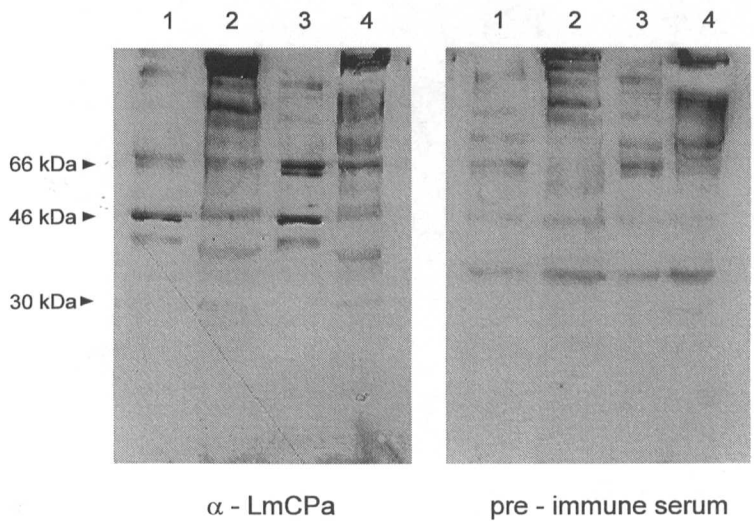


Figure 5.3. Reaction of α-LmCPa with *E. tenella* extracts. Western blot analysis: Lanes 1 and 3, unsporulated oocyst extract (equivalent of 3×10^5 cells per lane), reduced and non-reduced respectively; lanes 2 and 4, sporozoite extract (equivalent of 2.5×10^6 cells per lane), reduced and non-reduced respectively. Molecular weights are indicated on the left.

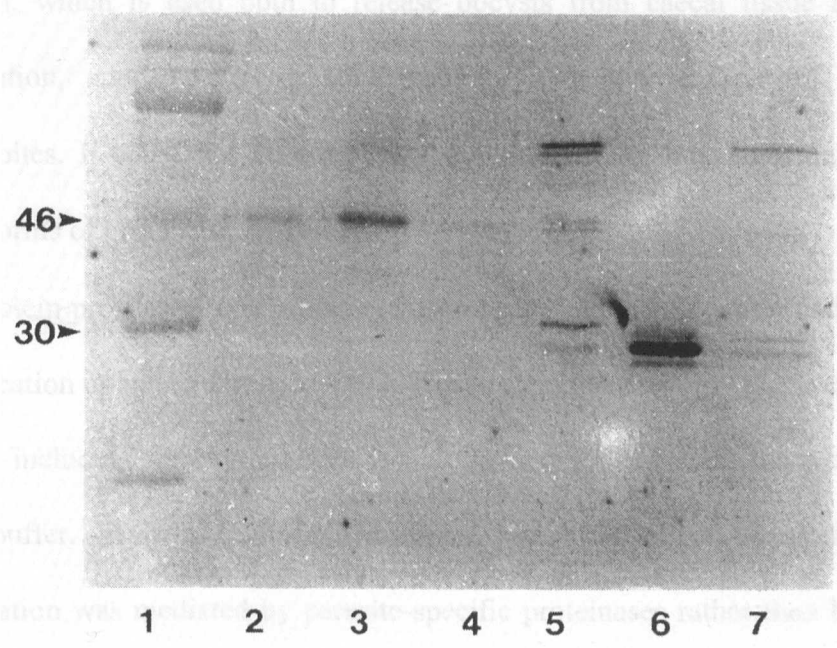


Figure 5.4. Detection of cysteine proteinases in *E. tenella* using biotinylated inhibitors. *Leishmania mexicana* was used as a positive control. Lane 1, Rainbow markers (Amersham); lane 2, *E. tenella* unsporulated oocysts (2×10^5 per well) incubated with E64 and biotinylated inhibitor; lane 3, *E. tenella* incubated with biotinylated inhibitor; lane 4, *E. tenella* without inhibitor; lane 5, *L. mexicana* amastigotes (1×10^7 per well) incubated with E64 and biotinylated inhibitor; lane 6, *L. mexicana* incubated with biotinylated inhibitor; lane 7, *L. mexicana* without inhibitor.

5.4 RESULTS SUMMARY

- Parasite-specific proteinases could not be detected in *Eimeria* lysates using substrate SDS-PAGE under a comprehensive range of conditions. Proteinases in the parasite must therefore be of relatively high specificity or low activity.
- Trypsin, which is used both to release oocysts from caecal tissue and to effect excystation, appeared to associate strongly with the surfaces of oocysts and sporozoites. It could not be effectively removed by washing and may complicate some forms of proteinase analysis.
- The protein profiles of crude homogenates of unsporulated oocysts underwent rapid modification upon incubation at 41°C. This protein degradation could be prevented in part by including serine proteinase inhibitors or cysteine proteinase inhibitors in the lysis buffer. Trypsin inhibitor, however, had little effect, indicating that the degradation was mediated by parasite-specific proteinases rather than by associated trypsin.
- Antibodies directed against the leishmanial *lmcpa* gene product recognised a 46 kDa protein in unsporulated oocysts extracts, but no equivalent in sporozoite extracts. This implies the presence of a developmentally-regulated cathepsin L-like cysteine proteinase.
- Biotinylated inhibitors directed against cysteine proteinases reacted specifically with a single protein in unsporulated oocyst extracts. The protein had a molecular weight of 46 kDa and may therefore be the same as that recognised by α-LmCPa antisera.

6. GENERAL DISCUSSION

6.1 SPOROZOITES OF *EIMERIA TENELLA*: AEROBIC VERSUS ANAEROBIC ENERGY METABOLISM.

One of the primary concerns of this project, as stated in Section 1.3, was to investigate the relative dependence of *Eimeria* sporozoites on aerobic and anaerobic pathways of carbohydrate catabolism. This was investigated by comparing the activities of selected enzymes of glycolysis and the TCA cycle in the parasites, and by analysing the soluble end-products of glucose catabolism produced under aerobic and anaerobic conditions.

The enzyme analyses, while fruitful in their demonstration of PP_i-PFK (discussed later), provide little advance to previous analyses as far as elucidating which pathways of energy metabolism operate in the organism. The detection of PP_i-PFK, PK and hexokinase, is consistent with presence of a glycolytic pathway - for which there is ample other evidence anyway. However, the apparent absence of SDH and NAD^+-ICDH but presence of $NADP^+$-ICDH activity, provide yet more ambiguous evidence both for and against the presence of a TCA cycle and do not resolve the issue (this was discussed to some extent in Sections 1.2.1.2.3 and 3.3.1). Overall, the enzymatic evidence would seem to argue against there being a complete TCA cycle in any stage of *Eimeria* that has been investigated, however, this conclusion should be treated with extreme caution. The possibility that the pathway is present but at very low activity can certainly not be

excluded. It is notable that *Eimeria*, and indeed all other coccidia investigated, contains high levels of lactate dehydrogenase, the enzyme responsible for mediating NAD^+ regeneration during fermentative metabolism. This suggests that the parasites have a high potential for anaerobic carbohydrate catabolism and would be consistent with a low aerobic capacity. It would also correlate with the presence of PP_i-dependent PFK, an apparent adaptation towards anaerobism (see later).

The NMR analyses described in Chapter 4 constitute the first objective analysis of end-products of carbohydrate catabolism by the species. Although lactate and glycerol were previously identified as end-products of carbohydrate catabolism by sporozoites (Ryley, 1973), the production of acetate has not previously been reported. In quantitative analyses, the precise proportions of these products of glucose catabolism showed some variation between apparently replicate experiments. The reason for this variation remains unclear, however while there was on average a slight difference in the relative proportions of lactate, acetate and glycerol produced under the different gaseous conditions, the total amount of fermentative end-products produced by the parasites was extremely consistent - and rather similar - under both aerobic and anaerobic conditions. On average, 44% of the consumed carbohydrate was accounted for in fermentative products under aerobic conditions, as compared with 53% under anaerobic conditions. This result suggests that the sporozoites have a low capacity for full aerobic energy metabolism and rely largely on glycolysis for energy production, regardless of gaseous conditions. It is notable that there was no evidence of malate or succinate being produced which might have indicated the presence of a partial reverse TCA cycle such as in *Leishmania* or *Trypanosoma* spp (Chatterjee and Datta, 1973; Blum, 1991; Urbina, 1994).

A possible criticism of the end-product experiments is that the gas compositions within the incubation chambers were not monitored, and that high parasite concentrations might have led to a partial anaerobiosis in the supposedly aerobic incubations. While this possibility cannot be excluded, the broad consistency of results obtained both in open incubations and when suspensions were bubbled continuously (and quite vigorously) with air/CO_2 suggests that the problem is unlikely to have been significant.

The overall sum of the enzymatic and end-product information suggests that sporozoites of *E. tenella* have, at-best, a low capacity for full aerobic energy metabolism, and rely largely on fermentative modes of carbohydrate catabolism. It should be noted, however, that the parasites appeared to remain equally viable in both aerobic and anaerobic incubations (as judged by motility and the kinetics of glucose catabolism and end-product release). This suggests that the sporozoites are not adversely affected by oxygen and hence are certainly not obligate anaerobes. The sporozoites must certainly encounter relatively high oxygen tensions during their passage to, and residence within, caecal epithelial cells, so it is only to be expected that they are able to deal with its presence. Whether or not this is involved in energy production in the intracellular stages remains a tantalising question.

6.2 PUTATIVE PATHWAYS OF END-PRODUCT FORMATION IN *EIMERIA TENELLA*.

While the lactate produced by *E. tenella* is clearly formed by the action of lactate dehydrogenase, which was present at high levels in all stages investigated, the

mechanisms of acetate and glycerol production have not been investigated. These compounds, however, are end-products of energy metabolism in many other parasites, both protozoa and helminths. Analysis has shown that they are produced by a limited number of similar pathways - described in Boxes 1 and 2. It would seem reasonable to assume that analogous pathways operate in *Eimeria*. These could easily be investigated by spectrophotometric assays.

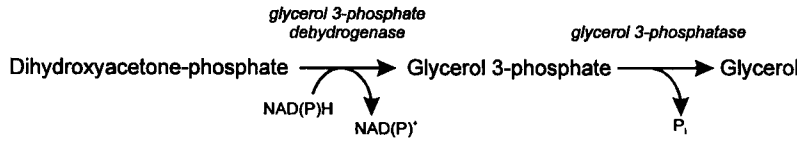
6.3 ANOMALIES OF THE END-PRODUCT ANALYSES: REDOX BALANCE AND CARBON BALANCE.

The main significance of the end-product analyses clearly relates to the potential of the parasites for aerobic and anaerobic energy metabolism. However the data (Table 4.3) present features which may provide further insights into energy metabolism in *Eimeria* sporozoites.

It is generally accepted that, in the absence of oxygen as a terminal electron acceptor, the production of fermentation products is required to maintain a supply of NAD^+ for use in glycolysis. Thus, the reduction of NAD^+ during glycolysis, and energy-yielding extensions to the pathway, must be balanced by the oxidation of NADH in reactions such as lactate formation.

Box 1. Glycerol production in parasites

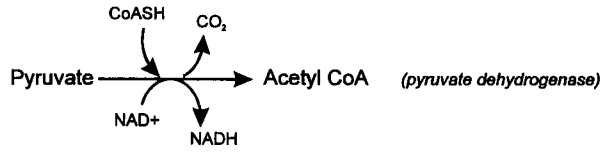
In most parasites **glycerol** is formed from the glycolytic intermediate dihydroxyacetone phosphate, by the sequential action of glycerol 3-phosphate dehydrogenase and glycerol 3-phosphatase (Bryant and Behm, 1989):



In glycosome-containing protozoa, such as *Trypanosoma brucei*, the dephosphorylation step is linked to ATP production by the enzyme glycerol kinase. This reaction is made possible by the compartmentalisation, which allows sufficient concentration of substrate to build up to drive the energetically unfavourable forward reaction.

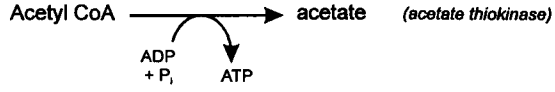
Box 2. Pathways of acetate production in parasites

Acetate production in parasites is usually linked to energy production via a substrate level phosphorylation. Two different mechanisms have been identified in protozoa (Bryant and Behm, 1989). The common starting compound is acetyl CoA, formed from pyruvate by the action of either pyruvate: ferredoxin oxidoreductase or the pyruvate dehydrogenase complex:

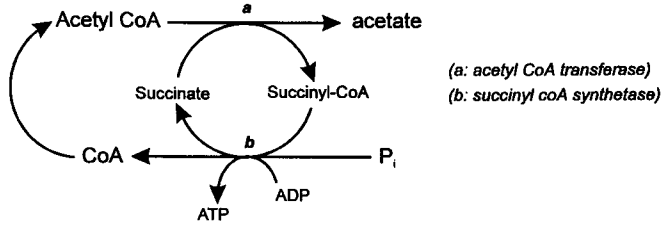


Pyruvate: ferredoxin oxidoreductase catalyses the equivalent reaction to pyruvate dehydrogenase but uses ferredoxin as an electron acceptor. It is particularly associated with fermentative protozoa.

The anaerobic protozoa, *Entamoeba histolytica* and *Giardia intestinalis*, appear able to couple ATP formation directly to the cleavage of the thioester bond of acetyl CoA:

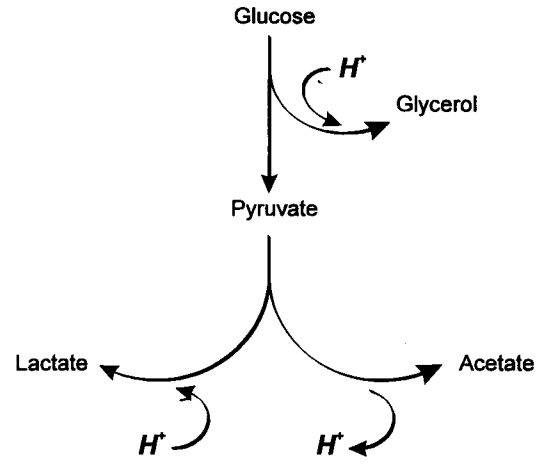


In others (e.g. *Trichomonas vaginalis*), CoA is first transferred to succinate forming succinyl CoA, then succinyl CoA is involved in a substrate-level phosphorylation:



It thought that alternative pathways of acetate production exist in some helminths (Barrett, 1981).

Although the precise pathways of end-product formation in *E. tenella* sporozoites have not been defined, the pattern of proton incorporation and release from the detected end-products must be as follows:



The production of lactate is self-balancing, since for every molecule of NADH reduced in its production, one is formed during glycolysis. If we assume that oxidation of pyruvate is NAD^+-linked, as it is in virtually all eukaryotes, then the production of acetate will result in generation of two molecules of NADH: one from glycolysis and one from acetyl CoA formation. For this to be balanced by the production of glycerol, glycerol 3-phosphate dehydrogenase must be NADH-linked and two molecules of glycerol must be produced for every molecule of acetate formed[8]. If we refer to the end-product tables of Section 4.3.2.2, however, we find that this clearly does not occur: more acetate than glycerol is produced even under anaerobic conditions. This suggests

[8] Note, however, that if glycerol production did balance acetate production, the apparent energetic advantage of acetate production would be lost: the gain of ATP through acetate production being offset by the reduced flux through pyruvate kinase due to diversion of dihydroxyacetone phosphate.

that some other terminal electron acceptor may also be used by *Eimeria* sporozoites. Clearly, under aerobic conditions this could be oxygen acting through a respiratory chain or other mechanism: but what about in the absence of oxygen? One possibility that has previously been suggested (Schmatz, 1989) is that under anaerobic conditions the mannitol cycle could act as an electron sink - with NAD^+ being generated during the incoming step. If this was the case, then mannitol would be expected to accumulate in the sporozoites under anaerobic conditions; it would be interesting to see if this is the case. Alternatively this could be the role of the PEPCK by-pass which Smith *et al.* (1994) reported to be present in sporulated oocysts (see Figure 1.6). If the cofactor specificities of these reactions were appropriate (and it isn't clear from the report if they are) then the forward cycle could be used to drive a transhydrogenation of NADH to NADPH with consequent regeneration of NAD^+. (Note, however, that this is a CO_2-fixing pathway and might not have been operative under the purely nitrogenous atmosphere used in anaerobic incubations.) A third possibility is that the result reflects the existence of additional end-products of glucose catabolism, either gaseous or soluble, which have not yet been detected. While NMR is an objective technique, theoretically capable of detecting all products of glucose catabolism, it is also relatively insensitive (as was noted in Section 4.3.1.1), hence the possibility that further soluble products exist cannot be excluded.

The second anomaly of the end-product analyses concerns carbon balance. It was observed in Section 4.3.2.2 that only about half the carbon consumed by the sporozoites, under both gaseous conditions, was accounted for in the detected end-products. Where, then, was the rest of the glucose going?

If the situation pertained only under aerobic conditions, it might be concluded that the remaining glucose was completely catabolised to CO_2, which was not analysed. However this can hardly be the case under anaerobic conditions. It also seems improbable that such high proportions could be accounted for by the pentose phosphate pathway which, although evidently active in the reproductive phases of the life-cycle (Section 1.2.1.2.5), is unlikely to be very significant in the non-dividing sporozoites. This leaves two possibilities: either, that the carbon is being converted to additional end-products which we have not yet identified; or that it is being incorporated into endogenous energy reserves such as amylopectin and possibly mannitol. This latter explanation would seem the most likely for a number of reasons. To begin with, in Section 4.3.2.3 it was shown that in the absence of exogenous glucose the equivalent of about 4 μmols of end-product were produced per 10^8 sporozoites over 8 hour incubations under both aerobic and anaerobic conditions. Since by the time of the experiment, the energy reserves must already have been depleted by storage and excystation, the capacity of the parasites for carbohydrate storage is likely to be somewhat greater than this. In other words, they almost certainly have the capacity to store the 'missing' 5 or so μmols of glucose. It is also interesting to note that in the NMR experiments, ^{13}C-mannitol was detected in lysates of sporozoites incubated with exogenous ^{13}C-glucose - hence demonstrating the ability of this stage of the parasite to 'store' glucose in this form.

Further evidence against additional end-products may be gleaned from the data of Ryley (1973) on anaerobic endogenous energy metabolism of *E. tenella* sporozoites. In these experiments, no exogenous substrate was supplied to the parasites, and glucose consumption was measured in terms of amylopectin depletion. The measured end-

products - lactate, glycerol and CO_2 - accounted for 92% of the amylopectin consumed in the experiments. If acetate had also been present, in similar proportions as in my experiments, then all the carbon would have been accounted for. It is also of note that the amount of CO_2 detected by Ryley would have been consistent with its production through pyruvate decarboxylation during acetate formation.

The apparent incorporation of excess glucose into energy reserves by sporozoites suggests that the cell's catabolism is carefully regulated to accord with its energetic needs. This suggests that sporozoites are relatively conservative and efficient in their energy metabolism which may be a reflection of low or unreliable nutrient levels in the environments in which they reside.

6.4 OOCYSTS AND SPORULATION OF *EIMERIA TENELLA*

Oocysts are often regarded as dormant stages in the life-cycle of *E. tenella*; however this is not strictly justified. The process of sporulation (sporozoite and sporocyst production) involves massive biosynthesis and, clearly, energy expenditure. This is reflected in the depletion of amylopectin granules from the cytoplasm and major use of mannitol. Since sporulation will not occur in the absence of O_2, and is reversibly inhibited by cyanide and other inhibitors of the cytochrome respiratory chain, it has been commonly assumed that the process is fuelled by fully aerobic energy production, involving a TCA cycle and a respiratory chain (Wang, 1982). Sporulated oocysts, however, are able to maintain viability under either aerobic or anaerobic conditions, suggesting that by this stage they are able to rely, if necessary, on fermentative energy metabolism.

In the course of this project I attempted to gain more insight into the processes fuelling sporulation by investigating enzyme activities within the sporulated and unsporulated oocysts, and also by analysing the concentrations of putative end-products of energy metabolism in oocyst lysates. While the results obtained provide no clear-cut picture of energy metabolism during sporulation, they do provide some interesting material for speculation.

Both sporulated and unsporulated oocysts contained the same repertoire of enzymes as did sporozoites. Glycolytic enzymes were present, as was LDH, but SDH was not detected. Interestingly the activity of the one potential TCA enzyme detected, NADP-ICDH, was some 10 × higher in unsporulated oocysts than in sporozoites; LDH activity was also twice as high in the former. These data are surprising since it suggests that prior to sporulation oocysts may have an even lower potential for full aerobic metabolism than do sporozoites. Clearly, however, specific activities give only an indication of flux through a pathway and it would be unwise to put much reliance on this interpretation. The specific activities of the other enzymes assayed were significantly reduced in sporulated relative to unsporulated oocysts. This can be readily explained in terms of down-regulation of metabolic flux during dormancy. In contrast, the enzyme activities measured in sporozoites were between 2 and 15 times higher than those in the sporulated oocysts. Since sporozoites account for more than twice the soluble protein in these oocysts (see Table 2.2), the increase in specific activity cannot be solely due to changes in protein concentration. There must be rapid synthesis or activation of enzymes occurring during or shortly after excystation which therefore seems to be an activation-like event. It is interesting to consider that the measured differences in activity will be much amplified by the differences in temperature

experienced by the two stages in the life-cycle. Perhaps some of the increased enzyme activity is due to expression of new isoforms of the enzymes which are more suited to the higher temperatures encountered in the host? Similar scenarios have been certainly been observed in other parasites (Barrett, 1981).

A limitation of the enzyme data is that they are confined to the sporulated and unsporulated oocysts: we have no knowledge of the enzyme activities during sporulation. It has been shown (Michalski *et al.*, 1992) that the mannitol cycle enzymes of *E. tenella* undergo significant changes in activity during sporulation which seem to correlate with the build up and degradation of mannitol. It is conceivable that other enzyme activities alter in this way. Perhaps, a whole TCA cycle is developed during early sporulation, but lost by the end? Only further analysis will tell.

Previous reports have stated that CO_2 is the only product to be released during sporulation. My own studies seemed to confirm that no significant amounts of organic acids (or glycerol) are released from the oocysts during sporulation. However, high levels of the end-products identified from sporozoites (lactate, acetate and glycerol) were measured in extracts of oocysts; presumably the oocyst wall is too impermeable to allow their exit. It is interesting that the levels of these metabolites were not significantly higher in sporulated than in unsporulated oocysts, suggesting that they are not produced during the sporulation (unless there is a dynamic balance of production and consumption). This, then, appears to support the notion of full aerobic respiration. It should, however, be remembered that the unidentified organic acid, 'X', was found to accumulate in oocysts during sporulation. Perhaps X is an additional, and if so, highly unusual, end-product of energy metabolism. Formation of 'X' appeared to be strictly

linked to sporulation - in that it did not appear to accumulate further in sporulated oocysts incubated anaerobically - hence it is probably not a general fermentation product.

The lactate, acetate and glycerol found within unsporulated oocysts are presumably derived from metabolism during maturation of the cyst within the host gut. Their presence suggests that this process may be at least partially fermentative. It is interesting to note the proportions of end-products detected do not reflect those produced by the sporozoite stage: there being far more glycerol relative to both lactate and acetate. This may reflect either a different pattern of metabolism in developing oocysts compared to sporozoites, or simply be a consequence of differences in the permeability of the developing cyst wall to the different metabolites.

Finally, although sporulation clearly requires O_2 and respiratory chain activity, it should be remembered that respiratory chain activity is not obligatorily linked to energy production. The assumption that it is appears to have been commonly made in previous assessments of eimerian energy metabolism (Wang, 1982). While this scenario is certainly the norm, it is being increasingly recognised that in some organisms electron transport is not linked to oxidative phosphorylation but may fulfil other purposes. *Plasmodium falciparum*, for example, consumes O_2 via a cytochrome-containing respiratory chain, but appears to be a homolactate fermentor. Respiration in this organism is thought instead to be linked to the activity of dihydroorotate dehydrogenase, a key enzyme in *de novo* pyrimidine biosynthesis and an important chemotherapeutic target (Gutteridge *et al.*, 1979; Fry, 1991; Hudson, 1992 and 1993). It is possible that this situation also pertains in *Eimeria* (and other coccidia species). If the respiratory

chain was required for biosynthetic precursors in *Eimeria*, then the complete and immediate cessation of sporulation upon addition of respiratory inhibitors might be more adequately explained. Such precursors are clearly vital to the sporulation process. If the inhibitor blocked oxidative phosphorylation only, then - given the apparent potential of the organism for fermentative energy metabolism - one might rather expect sporulation to proceed, but possibly less efficiently, until polysaccharide reserves were exhausted. Similarly, oxygen consumption by sporozoites should not be equated too decidedly with energy metabolism, or even with respiratory chain activity. The possibility that oxygen may be involved in processes other than oxidative phosphorylation does not appear to have been investigated.

6.5 THE ROLE OF PROTEINASES AND AMINO ACIDS IN *EIMERIA TENELLA*

The apparent inability of *Eimeria* sporozoites to take up amino acids from incubation medium (see Section 4.3.3.1) suggests that, at least in this stage of the parasite, amino acids are not utilised for energy production. The apparent release of significant amounts of amino acids by the parasites during these investigations was surprising and may be artifactual, as was discussed: Section 4.3.3.1, however it remains a possibility that the effect is a positive adaptive response to osmotic stress. Certainly other parasitic protozoa, e.g. *Leishmania* (Blum, 1991), have been observed to release amino acids under hypo-osmotic conditions *in vitro*, although usually a single or discrete range of amino acids are released, rather than the whole spectrum (Barrett, 1981). It is worth noting that the apparent lack of catabolism of amino acids would be consistent with the absence of a TCA cycle in *Eimeria* which is the major point of entry of amino acids into

energy metabolism in most, although not all (Lockwood and Coombs, 1991), protozoan species.

The apparent absence of high activity, broad specificity proteinases in the parasite stages investigated might also be consistent with exogenous amino acids being unimportant - such proteinases are usually associated with the digestion of proteins for nutritional means. The inability to detect parasite-specific proteinases in the stages of *Eimeria* investigated using standard activity-dependent techniques suggests that proteinases in the parasite must be of relatively high specificity or of low activity (or both). This can only increase their interest, not least as potential drug targets, since it suggests that they may be unusual and mediate very specific functions within the cells.

The apparent stage-specific cathepsin L-type proteinase identified in this study has not previously been described and merits further investigation.

6.6 *TOXOPLASMA GONDII*: APPARENT DIFFERENCES IN THE ENERGY METABOLISM IN TACHYZOITES AND BRADYZOITES

While bradyzoites and tachyzoites of *T. gondii* present clear biological and structural differences, little is known of how they compare biochemically. This is largely due to the difficulty of obtaining bradyzoites in a suitable form for biochemical analyses. During the course of this project C. Roberts of Strathclyde University developed a new technique for purifying bradyzoites from the brains of infected mice and kindly provided us with a limited supply of pure bradyzoites. These were analysed, along with

tachyzoites, for selected enzymes of energy metabolism. The results, detailed in Table 3.1, constitute the first comparative analysis of enzymes on the two stages and indicate that the two stages may present certain differences in energy metabolism.

Like *Eimeria*, both stages of *T. gondii* contained high levels of PP_i-dependent PFK, ADP-dependent PK and LDH. Tachyzoites however contained readily detectable levels of both $NADP^+$- (but not NAD^+-) dependent ICDH and SDH. Since two other TCA cycle enzymes, aconitase and fumarase, have previously been reported in tachyzoites, the parasites present far more complete and unambiguous evidence for a TCA cycle than does *Eimeria*. Further evidence in favour of a capacity for aerobic energy metabolism comes from the work of Fulton and Spooner (1960) who analysed the end-products of glucose catabolism by tachyzoites of *T. gondii*. Under aerobic conditions about 40% of the consumed glucose was accounted for by released CO_2, while 20% was lactate; anaerobically lactate production increased by 2-3 fold, at the expense of CO_2. This represents a much more significant shift to fermentative end-products dependent on gaseous conditions than was seen with *Eimeria*, and hence implies some potential for full aerobic energy metabolism. The continued production of lactate, and also acetate, under aerobic conditions implies, however, that this capacity is relatively low.

While bradyzoites contained similar level of $NADP^+$-ICDH as did tachyzoites, it could not be ascertained (given the limited material and relative insensitivity of the assay) whether they also contained SDH. Most interestingly however, the bradyzoites were found to contain significantly higher levels of both PK and LDH than did tachyzoites. This suggests, very tentatively, that the former may place a higher reliance on fermentative energy metabolism. This is particularly interesting in light of recent reports

that bradyzoites are more resistant to atovaquone - a potent inhibitor of the cytochrome respiratory chain - than are tachyzoites (Araujo *et al.*, 1991; Durand *et al.*, 1995; Kovacs, 1992; Tomavo and Boothroyd, 1995). While this drug is thought to act against the respiratory chain-linked dihydroorotate dehydrogenase in *Plasmodium* (Gutteridge *et al.*, 1979; Hudson, 1993), there is evidence that this is not the case in *T. gondii* (Pfefferkorn *et al.*, 1993). An alternative mechanism of action would be inhibition of oxidative phosphorylation. This would be compatible with the observed resistance pattern if bradyzoites were less dependent on a respiratory chain for ATP-production than are tachyzoites.

These results and their implications are discussed more fully in Denton *et al.*, 1996 (attached).

6.7 *CRYPTOSPORIDIUM PARVUM*: AN OBLIGATE ANAEROBE?

Analysis of *C. parvum* was restricted in this project to the determination of enzyme activities in the cyst stage of the parasite. Results are shown in Table 3.1.

Like both *Eimeria* and *Toxoplasma*, *C. parvum* contained a PP_i-linked PFK and an ADP-PK. The fact that *C. parvum* shares this unusual enzyme combination with the other coccidia favours its current taxonomic grouping which has recently been thrown into question by ssRNA analyses (see Section 1.1). A significant feature that distinguishes *C. parvum* from other coccidia is its reported lack of mitochondria (Current, 1990; Tetley, L., Brown, S.M.A. and Coombs, G.H., unpublished data) which suggests that the parasite may be an obligate anaerobe in terms of energy metabolism. This feature appeared to be underlined in my results by the absence of both the TCA

enzymes investigated, ICDH and SDH. The fact that this species, in which there is ultrastructural evidence against a TCA cycle, contained neither the $NADP^+$- nor NAD^+-dependent ICDHs suggests, albeit indirectly, that the $NADP^+$-ICDH present in *Eimeria* and *Toxoplasma* may be participating in a TCA cycle. The presence of LDH suggests that glycolysis linked to lactate production may be a major pathway of energy production in *C. parvum*. It is interesting to note that hexokinase activity could not be detected in oocysts of *C. parvum* and was also at low activity in sporulated oocysts of *E. tenella*. This may simply reflect the dormant nature of these stages of the parasites, and a dependence on endogenous energy reserves rather than free glucose. However it is interesting to note that some anaerobic organisms have recently been found to use polyphosphate, GTP-linked and even ADP-linked hexokinases in place of the conventional ATP-dependent variety (Kengen *et al.*, 1994; Takahashi *et al.*, 1995). It would be interesting to investigate excysted sporozoites of *C. parvum* to see if hexokinase is up-regulated here, as in *Eimeria*; or whether there was any evidence for activity dependent on an alternative phosphoryl donor.

6.8 PP_i-PFK: AN ADAPTATION TOWARDS ANAEROBIOSIS?

When PP_i-utilising enzymes were first discovered in the apparently 'primitive' protozoa, *E. histolytica* and *G. lamblia*, it was suggested that they might represent remnants of an ancestral energy metabolism which existed before the advent of mitochondria and the development of ATP as an energy currency. The subsequent finding of similar enzymes in organisms of apparently much higher evolutionary status, often with close relationship to organisms using ATP-PFKs, shattered this hypothesis. However it was noted that organisms using unregulated PP_i-PFKs are, in the main, dependent on

anaerobic means of energy generation, usually by virtue of lacking mitochondria. This observation led to the hypothesis, discussed in Section 3.1.2, that PP_i-PFKs represent adaptations towards anaerobiosis and have arisen in several independent events from ATP-PFKs (an example of convergent evolution). The theory is well-supported by the theoretical increase in energetic efficiency effected by the use of PP_i as an energy donor.

While the circumstantial evidence in support of convergent evolution is compelling, the relationship between ATP- and PP_i-dependent PFKs can only be established by systematic comparisons of their sequences. In recent years a number of PP_i-PFKs from plant, bacterial and protozoan sources have been sequenced. The sequences show a loose gradation of homology (see Table 6.1) with the protozoan enzymes appearing to stand between the plant and the bacterial enzymes in terms of similarity. The greatest homology in all cases is between the residues thought to be involved in substrate binding in the *P. freudenreichii* (Phillips and Li, 1995; Xu *et al.*, 1994) enzyme. These residues are also conserved in all known ATP-PFKs providing strong evidence that the ATP- and PP_i-PFKs are members of a structurally and functionally homologous superfamily of common ancestry. The low level of overall similarity observed between the ATP- and PP_i- dependent enzymes compared so far has, however, led some to argue that the enzymes cannot have diverged recently, but instead have branched from an ancient common ancestor which probably used PP_i as a substrate (Todd *et al.*, 1995; Alves *et al.*, 1996). While the reasoning leading to this conclusion is valid, the scenario is hardly compatible with modern phylogenetic trees based in ssRNA, which, it should be noted, are strongly supported by taxonomic classifications. It should also be noted that most of the ATP-PFK sequences available for comparison are from sources which are clearly phylogenetically distant from any PP_i-PFK. As the differences observed

between these ATP- and PP_i- dependent PFKs frequently fall within the range observed for phylogenetically distant ATP-PFKs (and also PP_i-PFKs) (Fothergill-Gilmore and Michels, 1993), the comparison is not ideal. A more satisfactory assessment of the situation might be obtained by comparing ATP- and PP_i-PFK sequence from apparently closely related organisms. As yet such sequences are not available, however the Apicomplexa and the Ciliates (both of which contain ATP- and PP_i- PFK utilising organisms) may well provide candidates highly suited to such analyses. It is always possible that a combination of both divergent and convergent evolution has taken place: with the PP_i-PFKs in primitive protozoa representing an ancestral form, while those in the more derived organism are a regression from an ATP-dependent derivative.

Table 6.1 Pairwise comparisons of phosphofructokinase amino acid sequences.

		PERCENTAGE IDENTITY							
		potato	*G.l* [a]	*N.f*	*E.h*	*P.f*	*E.c*	*B.s*	rabbit
PP_i	potato (β-subunit)	100	59	28	25	27	22	-	-
↓	*G. lamblia* [a]		100	-	-	25	27	-	-
↓	*N. fowleri*			100	28	23	25	-	-
↓	*E. histolytica*				100	27	-	-	-
↓	*P. freundenreichii*					100	23	15	-
ATP	*E. coli*						100	26	29-39 [b]
↓	*B. stearothermophilus*							100	43
↓	rabbit muscle								100

[a] Comparisons are with the amino-terminal region of the *G. lamblia* sequence.
[b] Comparisons are with carboxyl- and amino- halves of the rabbit muscle gene respectively.
Dashes indicate sequence comparisons which have not been reported.
Percentage identities are derived from analyses reported in the following references: Carlisle *et al.*, 1990; Fothergill-Gilmore and Michels, 1993; Li and Phillips, 1996; Montavon and Kruger, 1993; Xu *et al.*, 1994; Huang *et al.*, 1995; Rozario *et al.*, 1995; Todd *et al.*, 1995; Wessberg *et al.*, 1995.

While most organisms in which PP_i-PFK has been identified are strict fermentors - usually by virtue of lacking mitochondria - the coccidia, and also *Naegleria* in which a PP_i-PFK has been identified, have not classically been regarded in this light. Their use of PP_i could therefore be regarded as evidence against the adaptive theory for PP_i-PFKs. However, it has been shown in this study, and also in others, that the coccidia, during at least some stages of their life-cycle, are heavily dependent on fermentative modes of energy generation. Clearly, given the phylogenetic position of these organisms, and their general possession of mitochondria, this tendency towards anaerobism must be a secondary adaptive feature, and hence in some way a response to the habitats in which they reside.

It was stated in Section 4.1.1 that there are basically two types of fermentative organism: those which have anaerobism imposed on them by the low oxygen tensions in which they reside, and those which live in the midst of plenty and hence have no need for efficient energy metabolism. The sporozoites of *E. tenella*, which pass the most active part of their existence in the low oxygen tensions of the host gut, are most likely to correspond to the former definition. The limiting conditions with which of this organism must contend are further indicated by its possession of polysaccharide reserves which would not be required if the parasite lived in the midst of plenty. Hence, adaptation towards anaerobism in this, and other invasive stages of the coccidia, may well be driven by the transient anaerobic phase in the host gut. It would seem likely that these stages represent the weakest link in the life cycle: both because they exist under the most limiting environmental conditions, and because perpetuation is often dependent

on low numbers of ingested parasites. Hence one would expect them to be particularly powerful in driving adaptive evolution.

While we have little direct knowledge regarding metabolism during the intracellular stages of coccidial parasites, the persistence of PP_i-PFK in merozoites and schizonts, and their apparent production of lactate (Section 1.2.1.4.3), suggest that fermentative metabolism may also occur intracellularly. While oxygen tensions within cells are normally high, it is possible that they may become limiting during the rapid growth phases of the parasite, hence imposing anaerobism. Alternatively, it is possible that nutrient levels remain high enough that the intracellular parasites have no need to mediate full aerobic energy metabolism.

If we accept that PP_i-PFKs are adaptations towards anaerobism, then an alternative explanation of their presence in the coccidia might be that they are the legacy of a common ancestral form of the coccidia, which was an anaerobe - perhaps by virtue of being confined to the host gut. In this respect it is interesting to note that it is commonly considered that the Apicomplexa probably originated as gut parasites of free-living marine invertebrates (Barta, 1989).

Finally, the fact that the coccidia species investigated apparently exhibit different levels of anaerobism (with *C. parvum* representing the extreme with its lack of mitochondria) is interesting and raises questions concerning matters such as, the difference between aerobes and anaerobes, the factors driving evolution, and the ease with which metabolic changes can take place. The apparent proximity of mitochondrial and amitochondriate

organisms within both the Apicomplexa and the Ciliates suggests that the aerobic to anaerobic transition is not too arduous to make!

6.9 PP_i-PFK AS A DRUG TARGET

Any disruption of the pathways of energy metabolism is likely to have a profound and possibly catastrophic effect on any organism. The coccidia, being apparently heavily dependent on fermentative carbohydrate catabolism, are likely to be particularly vulnerable to any disruption of the glycolytic pathway. In this respect, PP_i-PFK, an enzyme that is present in the parasites but not in the host species, would seem an ideal candidate for chemotherapeutic attack. While the coccidial PFKs have not been sequenced, the sequences available from other PP_i-PFKs suggest that the enzymes may be sufficiently different from the host ATP-PFKs to be susceptible to selective inhibition. In support of this, it was recently reported that certain phosphonic acid derivatives are capable selectively inhibiting PP_i-PFK from *T. gondii* (Peng *et al.*, 1995). Some of the compounds also showed some ability to selectively inhibit replication of the intracellular parasites in an *in vitro* system, and also protected the mammalian cells from damage by the parasite. The main limiting factor in the efficacy of the compounds appeared to be their ability to cross cell membranes and reach the intracellular parasites. If the compounds can be chemically manipulated to increase their

lipophilicity then they have the potential to form a new class of broad-spectrum anticoccidial drugs[9].

6.10 HOW IS ENERGY METABOLISM REGULATED IN THE COCCIDIA?

The changes in enzyme activity between different stages of the coccidia constitute one form of metabolic regulation; this is a relatively long-term method of control and is ultimately controlled at the genetic level. More immediate mechanisms of adjusting metabolic flux also exist in most organisms; these usually involve direct modulation of enzyme activities - either by allosteric effectors or by covalent modification. In general the most important regulatory enzymes appear to be those which catalyse non-equilibrium reactions which, in standard glycolysis, are PFK, PK and hexokinase. In most organisms these are allosteric enzymes whose activities are modulated by a variety of low molecular weight effectors (Fothergill-Gilmore and Michels, 1993). ATP-PFK is considered to be the most important enzyme of glycolytic control in most eukaryotes.

The PP_i-PFKs of the coccidia resemble those in most other micro-organisms in apparently not being subject to allosteric control. This is consistent with the ready reversibility of the reaction, as discussed in Section 3.1.2 and indicates that these species must employ fundamentally unusual mechanisms of glycolytic control.

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[9] Since this section was written it has been reported that certain synthetic pyrophosphate analogues are also able to kill *Entamoeba histolytica* (Bruchhaus *et al.*, 1996), a protozoan which contains a number a PP_i-dependent enzymes. This further underscores the potential of PP_i-dependent enzymes as drug targets.

In *Eimeria* and *Toxoplasma*, the ADP-specific PKs were identified as potential sites of glycolytic regulation. The enzymes from both organisms showed homotropic cooperativity with respect to PEP but could be activated to hyperbolic kinetics by a number of low molecular weight effectors. The most potent effectors identified in each case were F6P and G6P. F16P2, the major activator of most mammalian enzymes, had relatively little effect. Unusual regulation of PK, such as this, has been found in other PP_i-utilising micro-organisms (see Section 3.3.3; Petzel *et al.*, 1989 *a* and *b*; Mertens *et al.*, 1992). It has been suggested that the feature may relate to the lack of regulation at PFK, which renders F16P2 - as its product - a less useful feed-forward indicator of glycolytic load (Mertens *et al.*, 1992). The finding of an unusually regulated PK now in two more PP_i-utilising organisms, *Eimeria* and *Toxoplasma*, lends further weight to this theory. In general, important regulatory enzymes are expressed only at very low activity in cells. However the activities of the coccidial PKs are relatively high; a fact which may argue against the physiological significance of their regulation - (Although PK activities also appear anomalously high in mammalian tissues (Bergmeyer *et al.* 1983) where they are considered to play a role in regulation, so maybe this enzyme is an exception to the rule?)

The PK of *C. parvum* appeared to lack allosteric properties. This makes it exceptional: to date the only other PK known not to present cooperative binding of PEP is the mammalian type 1 enzyme from muscle. The physiological activity of some eukaryotic PKs, however, is regulated by protein kinase-effected phosphorylation (Fothergill-Gilmore and Michels, 1993) and the possibility that such a mechanism exists for the *C. parvum*, or indeed for either of the other coccidial PKs, cannot be excluded. It should be

noted that allosteric properties do not necessarily indicate that an enzyme plays a major regulatory role: only direct analyses of flux kinetics can confirm this and it is increasingly being recognised that metabolic control may be shared in practical terms between all enzymes in a pathway (Westerhoff *et al.*, 1991). Overall however, *C. parvum* certainly appears to be an unusual protist in terms of glycolytic regulation.

While the PKs of *Eimeria* and *Toxoplasma* show potential for activation, it is clear that mechanisms must also exist for damping down glycolytic flux when the ATP/ADP ratio is high. Such mechanisms have not yet been identified in the coccidia. In many cells, hexokinase is important in controlling the entry of glucose into glycolysis. Most eukaryotic hexokinases are allosterically inhibited by their phosphorylated product, glucose 6-phosphate (Fothergill-Gilmore and Michels, 1993). The enzyme from *Eimeria*, however, showed no evidence of such regulation, at least in the oocyst stages that were investigated. In the absence of regulation at this level it seems likely that important factors controlling glycolytic flux in the coccidia are either the rate of substrate uptake from the environment, or the levels of amylopectin and/or mannitol biosynthesis and mobilisation. The analogous steps to these are tightly regulated in all mammalian cells, usually by a combination of protein kinase-dependent phosphorylation and allosterism. Such mechanisms have yet to be explored in the coccidia.

6.11 'CONCLUSIONS'

While this project has provided some new insights into energy metabolism in the coccidia, the overall picture is still far from complete. Some of the remaining gaps in

our knowledge will be easy to fill. For example, determining, the precise pathways of end-product formation in *E. tenella* sporozoites should be a simple matter of applying enzyme assays. In contrast, analysis of the energy metabolism of most intracellular stages of the coccidia will be largely dependent on the development of improved techniques for producing and handling these stages. Some very basic information remains to be elucidated, for example: what are the precise characteristics of the environments in which the parasites reside? Detailed information on these would greatly aid our attempts to understand how the parasites are adapted to their infection sites, and may also aid in the development of satisfactory culture techniques.

The data presented in this thesis suggests that the *Eimeria*, *Toxoplasma* and *Cryptosporidium* share a tendency towards anaerobism. However, it also shows that, despite their apparently close phylogenetic relationship, the species do not present identical patterns of energy metabolism; nor do the patterns so-far elucidated precisely match those described in any other parasitic protist to date. This observation underscores the complexity of energy metabolism which is increasingly being recognised among micro-organisms and among parasites in particular. As yet the evolutionary and adaptive basis of this complexity is poorly understood but may become clearer as more observational analyses, of the type carried out in this project, are performed on different organisms.

The unusual glycolytic enzymes identified in the coccidia are worthy of in-depth study in their own right. Sequence information from the coccidial PP_i-PFKs (and related ATP-PFKs) would certainly be of interest to molecular evolutionists who are currently debating the origins of such PP_i-dependent enzymes. In addition, analysis of the

coccidial PKs might well contribute to our understanding of the basis and evolution of allosteric enzyme regulation (an area which is of continued interest to biochemists). Finally, further analysis of the PP_i-PFKs of the coccidia must certainly be merited by their apparent potential as targets for chemotherapeutic attack.

7. REFERENCES

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Comparison of the phosphofructokinase and pyruvate kinase activities of *Cryptosporidium parvum*, *Eimeria tenella* and *Toxoplasma gondii*

Helen Denton[a], Samantha M.A. Brown[a], Craig W. Roberts[b], James Alexander[b], Vincent McDonald[c], Kam-Wah Thong[d], Graham H. Coombs[a,*]

[a]*Parasitology Laboratory, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK*

[b]*Department of Immunology, University of Strathclyde, Glasgow, G4 0NR, UK*

[c]*Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, London, WC1E 7HT, UK*

[d]*Animal Health Discovery, Pfizer Central Research, Sandwich, Kent, CT13 9NJ, UK*

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Abstract

Oocysts of *Cryptosporidium parvum* were shown to contain a pyrophosphate-dependent phosphofructokinase (PP_i-PFK) similar to those previously described for *Eimeria tenella* and *Toxoplasma gondii*. PP_i-PFK of *C. parvum* displayed simple hyperbolic kinetics with respect to its substrate fructose 6-phosphate and was not affected by fructose 2,6-bisphosphate, the major allosteric activator of most ATP-PFKs. Inorganic pyrophosphatase was not detectable in any of the three parasites. *T. gondii* tachyzoites and *C. parvum* cysts both contained a pyruvate kinase (PK) specific for ADP rather than PP_i/AMP. The PK of *T. gondii* was similar to that of *E. tenella* in that it displayed strong positive cooperativity with respect to its substrate phosphoenolpyruvate and was heterotropically activated by glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate. PK of *C. parvum* showed no evidence of allosteric properties. The results suggest that the three coccidia are similar in depending heavily on anaerobic energy production via glycolysis but that the mechanisms for regulating glycolysis are not common to all species.

Keywords: *Toxoplasma gondii*; *Cryptosporidium parvum*; *Eimeria tenella*; Pyrophosphate-dependent phosphofructokinase; Pyruvate kinase; Anaerobiosis

* Corresponding author, Tel.: +44 141 3304777; Fax: +44 141 3078016; E-mail: graham.coombs@udcf.gla.ac.uk.

Abbreviations: Hepes, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]; PMSF, phenylmethanesulphonyl fluoride; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; DTT, dithiothreitol; PEP, phosphoenolpyru-vate; PFK, phosphofructokinase; PK, pyruvate kinase; PP_i, inorganic pyrophosphate

1. Introduction

The coccidia are a diverse group of parasitic protozoa which include several species of medical and veterinary importance. The parasites have basically similar life cycles but varied host and tissue specificities. Infection is generally initiated

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by invasive forms released into the host gut which invade host cells and reproduce intracellularly. Like most biochemical aspects of the coccidia, the area of energy metabolism has been studied very little [1]. Carbohydrates appear to represent the main energy substrate for all species that have been investigated, but little is known about the pathways by which these are catabolised. *Cryptosporidium parvum* is unusual in that it appears to lack a mitochondrion [2] and therefore is presumably an obligate fermentor, although there have been no reports on the energy metabolism of this parasite. The situation in *Eimeria tenella* and *Toxoplasma gondii* is less clear-cut. Both possess cristate mitochondria throughout their life cycles and appear to have some capacity for full aerobic respiration. This capacity, however, appears to be relatively low, at least in the stages that have been studied, and it has been reported that they produce high concentrations of lactic acid, and possibly other organic acids, even under aerobic conditions [3,4] (Denton et al., unpublished data). Thus the data available suggest that these three coccidia rely heavily on anaerobic energy production via glycolysis and that this may be a common characteristic of members of this group.

Phosphofructokinase (PFK) has been found to be specific for inorganic pyrophosphate (PP_i) rather than ATP in both *E. tenella* and *T. gondii* [5,6]. Similar enzymes have previously been detected in a number of other microbes including some parasitic protozoa [7–10]. The pyrophosphate-specific PFKs (PP_i-PFKs) identified in the coccidial species so far conform to the definition of the Type I enzyme as proposed by Mertens [11]. The most characteristic feature of this group of enzymes is their apparent lack of allosteric regulation. In contrast, Type II PP_i-PFKs, found in plants and *Euglena*, are allosterically regulated by fructose 2,6-bisphosphate and tend to co-exist with ATP-PFKs. The occurrence of PP_i-PFKs is peculiar in that it does not map to a distinct phylogenetic lineage, instead they have been found in a disparate group of microorganisms which are fermentative in their energy metabolism [12]. This finding, coupled with the fact that the use of PP_i-PFK increases the energetic efficiency of glycolysis by 50%, has led to the suggestion that these PP_i-dependent enzymes represent evolutionary adaptations towards anaerobiosis [11,13]. Some protozoa with Type I PP_i-PFK also have another PP_i-dependent enzyme, pyruvate phosphate dikinase (PPDK). This catalyses a reaction analogous to pyruvate kinase [14,15]. *E. tenella*, however, has been shown to possess a pyruvate kinase (PK) specific for ADP [6]. Interestingly, this PK is an allosteric enzyme that is modified by an unusual group of effectors [6].

These previous data suggested that fermentative metabolism involving PP_i-PFK and an unusually-regulated ADP-PK could be a characteristic of coccidia in general, at least at some stage during their life cycle. We have investigated this possibility by studying the PKs of *C. parvum* and *T. gondii* and PFK of *C. parvum*.

2. Materials and methods

2.1. Parasites

Tachyzoites of *Toxoplasma gondii* (RH strain) were grown in the peritoneum of cotton rats as described previously [16]. Cotton rats were inoculated intraperitoneally with 5×10^5 tachyzoites suspended in 0.1 M phosphate-buffered saline, pH 7.2 (PBS). Forty-eight h later, tachyzoites were harvested from the peritoneum through a 21-gauge needle into PBS with heparin (20 U ml^{-1}). The parasites were sedimented ($200 \times g$ for 10 min), washed twice in PBS and either lysed immediately or stored as pellets at -70°C. Samples to be assayed for pyrophosphatase were washed in 0.9% (w/v) NaCl, pH 7.2, instead of PBS.

Cryptosporidium parvum oocysts were passaged in C57 mice (OLAC) which had been immunosuppressed by subcutaneous administration of dexamethasone (1 mg on alternate days for one week). Purified *C. parvum* oocysts (10^5–10^6) were administered orally at the same time as the last dexamethasone injection and faeces collected on days 3–7 following infection. Oocysts were isolated and purified from the faeces using methods described previously [17–19] and stored in 2.5% (w/v) potassium dichromate until use.

Table 1
PK and PFK activities in coccidial extracts. All activities were determined using fresh cell extracts and are expressed as means ± SD, with *n* in parentheses.

	PFK (nmol min^{-1} (mg protein)$^{-1}$)		PK (nmol min^{-1} (mg protein)$^{-1}$)	
Phosphoryl substrate:	PP_i	ATP[a]	PP_i	ADP
C. parvum (oocysts)	331 ± 33 (3)	<1 (3)	<0.5 (3)	2947 ± 775 (5)
T. gondii (tachyzoites)	634 ± 203 (3)	<1 (3)	<0.5 (3)	4658 ± 1275 (7)
E. tenella[b]				
Unsporulated oocysts	183 ± 71 (4)	<1 (4)	<0.5 (4)	2105 ± 572 (4)
Sporozoites	105 ± 15 (5)	<1 (4)	<0.5 (4)	1349 ± 167 (4)

[a]Assays contained 5U yeast pyrophosphatase.
[b]From Denton et al. [6].

2.2. Preparation of parasite extracts

T. gondii tachyzoites were lysed directly by resuspension (to 1–2 × 10^8 cells ml^{-1}) of the cell pellets in 50 mM Hepes (pH 7.4) containing 20% (v/v) glycerol, 0.25% Triton X-100 and proteinase inhibitors (0.5 mM PMSF, 5 μM E64, 1 μM pepstatin, 0.2 mM 1,10-phenanthroline). The washed cysts of *C. parvum* were resuspended (to 10^8 cells ml^{-1}) in the same buffer including proteinase inhibitors and broken by vortexing for 20–30 min with 3-mm glass beads. Insoluble material was removed by centrifugation (10 min at 14000 × *g*) and the supernatant used in the enzyme assays.

2.3. Glycolytic enzyme assays

All analyses except for the stability studies were carried out on extracts freshly prepared from living cells. Activities were measured in the forward (glycolytic) direction as described by Denton et al. [6]. pH optima were determined using the pH range 4–9 (in 0.5 unit increments) and the following buffers: Mes (pH 4.0–5.5); Bistris (pH 5.5–8.0); Hepes (pH 7.0–8.5); Tris (pH 7.5–9.0).

2.4. Pyrophosphatase assay

Samples of parasite extract were incubated at 37°C in 50 mM Hepes (pH 7.5) containing 2.5 mM $MgCl_2$ and 2 mM PP_i. The reaction was terminated after 30–90 min. by addition of SDS to 6% (w/v), and the amount of released phosphate determined according to the method of Chifflet et al. [20]. Assays were routinely carried out in a final volume of 250 μl in microtitre plates (50 μl sample volume).

2.5. Protein determinations

- Protein concentrations were determined using the Pierce BCA protein assay kit (microtitre plate protocol) with bovine serum albumin as standard.

3. Results

3.1. PP_i-PFK, ADP-PK and pyrophosphatase activities

Extracts of all three parasites were shown to contain PP_i-PFK but no ATP-PFK and an ADP-specific PK activity but no PPDK activity (Table 1). PK activities were higher than the corresponding PFK activities and the highest activites were detected in *T. gondii*. Pyrophosphatase activity was undetectable (< 1 nmol min^{-1} (mg protein)$^{-1}$) under the assay conditions used in both *C. parvum* and *T. gondii* extracts, as previously reported for *E. tenella* [6]. High activities were detected in both *Leishmania* and mouse brain homogenates, used as positive controls.

3.2. Kinetic properties of PP_i-PFK of C. parvum

PP_i-PFK of *C. parvum* resembled the equivalent enzymes of *Eimeria*, *Toxoplasma* and other para-

sitic protozoa. Most distinctively, it presented simple Michaelis-Menten kinetics with respect to its substrate fructose 6-phosphate (Fig. 1) and was not affected by fructose 2,6-bisphosphate, the major allosteric activator of most ATP-PFKs. Like all PFKs, both ATP and PP_i-specific, the enzyme of *Cryptosporidium* had a requirement for divalent cations (preferably Mg^{2+}, although Mn^{2+} could suffice).

3.3. PK of C. parvum and T. gondii

The PKs of *T. gondii* and *C. parvum* were considerably more stable than that of *Eimeria* [6]. Similar activities were detected in lysates of both fresh and frozen cells and little diminution of activity was observed upon storage of lysates for several weeks at −70°C (this being in contrast to the *Eimeria* enzyme which is largely inactivated by freezing). Like PKs from many other sources, both enzymes required the presence of one monovalent and one divalent cation for full activity. Mg^{2+} and K^+ was the most effective combination tested (Mn^{2+} and NH_4^+ were slightly less effective), with the concentrations used in the standard assay producing maximal activity for both enzymes. The enzyme of *C. parvum* resembled that of *Eimeria* in being stimulated by the reducing agent DTT, addition of 1 mM DTT resulted in activation of 50–70%. In contrast, the *Toxoplasma* PK was unaffected by this compound. The pH optimum of the *T. gondii* enzyme was shown to be 6.5.

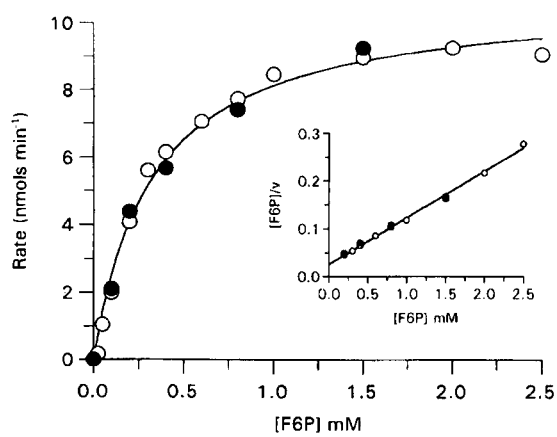

Fig. 1. A representative fructose 6-phosphate saturation curve of *C. parvum* PP_i-PFK. The activity associated with a fixed quantity of lysate was assayed at varying concentrations of fructose 6-phosphate, with (closed circles) and without (open circles) 10 μM fructose 2,6-bisphosphate. Other conditions were as described in Materials and methods. The insert shows a Hanes' plot of the data. The kinetic parameters for this experiment were: K_m, 0.32 mM; V_{max}, 10.7 nmol min^{-1}.

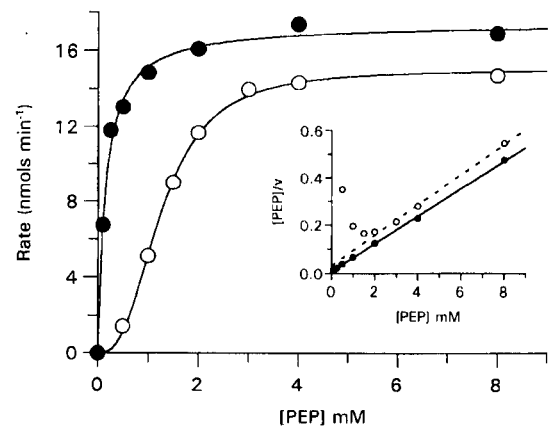

Fig. 2. Representative PEP saturation curves of *T. gondii* PK. The activity was assayed in the presence (closed circles) or absence (open circles) of 1 mM glucose 6-phosphate. Other conditions were as described in Materials and methods with ADP present at saturating concentration (5 mM). The insert shows a Hanes' plot of the data. The kinetic parameters for this experiment were: in the absence of glucose 6-phosphate, $K_{0.5}$, 1.89 mM; V_{max}, 15.0 nmol min^{-1} and Hill Coefficient, 2.70; in the presence of glucose 6-phosphate, K_m, 0.14 mM; V_{max}, 17.4 nmol min^{-1}.

PK of *Toxoplasma* presented simple Michaelis-Menten saturation kinetics with respect to its substrate ADP, but produced a strongly sigmoidal saturation curve with PEP as the variable substrate (Fig. 2). In contrast, the *C. parvum* PK presented Michaelis-Menten kinetics with respect to both its substrates (Fig. 3). The PEP saturation curve for this enzyme remained hyperbolic both at sub-saturating concentrations of ADP (0.3 mM) and following buffer-exchange of the crude enzyme to remove potential low molecular mass effectors.

A number of compounds, all effectors of PKs from other sources, were tested for their ability to modulate the activity of the coccidial PKs. The effects of a number of these, at sub-saturating

concentrations of PEP, are shown in Table 2. Several of the test compounds had a stimulatory effect on the PK of *T. gondii*. The most potent activators identified were glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate. In the presence of each of these, the PEP saturation curve was converted to hyperbolic form (see Fig. 2). The approximate K_{ac}s were as follows: fructose 6-phosphate, 84 μM; glucose 6-phosphate, 16 μM; fructose 1,6-bisphosphate, 2.07 mM. The following compounds were also similarly tested (all at 1 mM) on one or more occasions but without effect: 2-phosphoglycerate, fructose 2-phosphate, succinyl CoA, malate, citrate, acetate, succinate, GTP, ITP. No significant effect was noted for any compound at saturating concentrations of PEP. None of the compounds tested were found to modulate the *C. parvum* enzyme. ATP had a small inhibitory effect, as it did also on the enzymes of *T. gondii* (Table 2) and *E. tenella*. This was probably through competitive inhibition as it is one of the reaction products.

Table 2
Effects of metabolites on the activity of *C. parvum* and *T. gondii* PKs

Compound	Percent of control activity (%)	
	C. parvum	*T. gondii*
None	100	100
Fructose 6-phosphate	103 ± 3	728 ± 83
Glucose 6-phosphate	111 ± 3	746 ± 22
Fructose 1,6-bisphosphate	96 ± 2	397 ± 54
ATP	89 ± 6	86 ± 5
AMP	101 ± 1	163 ± 8
Fructose 2,6-bisphosphate	108 ± 6	162 ± 35
Ribose 5-phosphate	105 ± 1	298 ± 28
3-Phosphoglycerate	99 ± 5	98 ± 4
Glucose 1-phosphate	102 ± 4	248 ± 43

The activity associated with a fixed quantity of lysate was assayed at sub-saturating concentrations of PEP (0.15 and 0.5 mM for the *C. parvum* and *T. gondii* PKs, respectively) in the presence of the test compound (all at 1 mM except for fructose 2,6-bisphosphate which was at 10 μM). Other conditions were as described in Materials and methods. Activities are expressed as a percentage of the control activity (100%) measured in the absence of effector. Control activities were typically 1.5–3.0 nmol min^{-1}. Figures are means ± S.D., $n = 3$.

4. Discussion

The finding of PP_i-PFK in three members of the coccidia suggests that the presence of this enzyme along with an ADP-PK may be a general feature of this group of protozoa. The lack of readily detectable inorganic pyrophosphatase activity is typical of microorganisms with a Type I PP_i-PFK and appears to correlate with the need for a cytoplasmic concentration of PP_i at metabolite level in support of glycolysis [21]. Two such organisms, *Entamoeba histolytica* and *Trichomonas vaginalis*, have been reported to contain low activity of an acid inorganic pyrophosphatase, apparently located within organelles [21,22]. Such an enzyme is unlikely to be involved in regulating the concentration of cytoplasmic PP_i and was not investigated in this study.

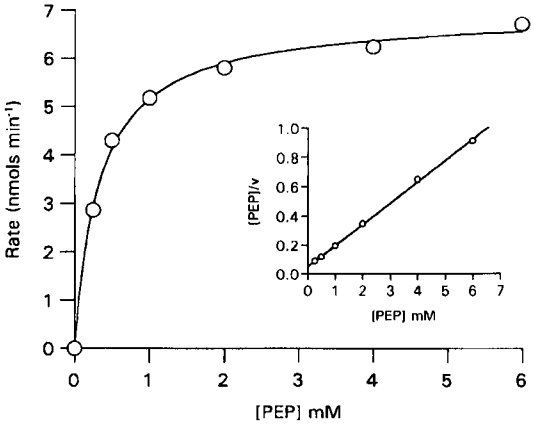

Fig. 3. A representative PEP saturation curve of *C. parvum* PK. The activity associated with a fixed quantity of lysate was assayed at varying concentrations of PEP. Other conditions were as described in Materials and methods. The insert shows a Hanes' plot of the data. The kinetic parameters for this experiment were: K_m, 0.32 mM; V_{max}, 6.82 nmol min^{-1}.

Type I PP_i-PFKs are believed to represent an adaptation towards anaerobiosis and are commonly associated with fermentative microorganisms [11,13]. The data reported to date, however, do not suggest that all coccidia are solely fermentative. Both *E. tenella* and *T. gondii* appear to have some capacity for full aerobic respiration

and both reside, at least during parts of their life cycle, in environments in which O_2 levels will be sufficient to sustain this. Why then should they have an enzyme thought to be associated with anaerobiosis? One possibility is that the feature has evolved as an adaptation for the transient anaerobic phase experienced by the invasive stage of the parasite in the host gut. It is also possible that, even in the intracellular environment, oxygen could become limited during the rapid growth phases of schizogony and gametogony. An alternative explanation is that the PP_i-PFK is the legacy of a common ancestral form of the coccidia, which was an anaerobe (perhaps by virtue of being confined to the host gut). This might also account for the parasites' low capacity for full aerobic respiration even when oxygen is present in reasonable amounts. Further analysis of the distribution of PP_i-dependent enzymes in general and in coccidia in particular (both in different species and in the different life cycle stages) should shed more light on the matter.

It was reported recently that inhibitors of PP_i-PFK of *Toxoplasma* selectively killed the intracellular parasite in an in vitro system [23]. If, as appears likely, PP_i-PFKs are a common feature of the coccidia, then selective inhibitors of these enzymes could become a new class of broad spectrum anticoccidial drugs.

The significance of the PP_i-PFK and ADP-PK combination is unclear. From the limited data known, it appears that in such a situation the regulation of PK, and glycolysis itself, is likely to be unusual. Certainly this is the case with *Eimeria* [6], *Toxoplasma* (this study) and *Trichomonas vaginalis* [24]. Most eukaryotic PKs are allosterically activated by fructose 1,6-bisphosphate, which is effective at very low concentrations. Although this compound had some effect on the PKs of *E. tenella* and *T. gondii* (Table 2), it was far less potent than glucose 6-phosphate and fructose 6-phosphate. This unusual regulation may relate to the lack of control of PFK, which renders fructose 1,6-bisphosphate, its product, a far less useful feed-forward indicator of glycolytic load (see Ref. [24]). The apparent lack of allosteric properties of the *C. parvum* PK makes it exceptional; to date the only other PKs known not to present cooperative binding of PEP are the mammalian Type I enzyme from muscle [12]. The use in the current study of fresh parasite lysates and the inclusion of proteinase inhibitors argue against the *Cryptosporidium* enzyme having been degraded with the loss of allosteric regulation. The physiological activity of some PKs is regulated through phosphorylation [12], such a regulatory mechanism for the *Cryptosporidium* enzyme cannot be excluded. Indeed, the way in which glycolysis is regulated in this organism requires and deserves further investigation.

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Eimeria tenella contains a pyrophosphate-dependent phosphofructokinase and a pyruvate kinase with unusual allosteric regulators

Helen Denton *[a], Kam-Wah Thong [b] and Graham H. Coombs [a]

[a] *Laboratory for Biochemical Parasitology, Department of Zoology, University of Glasgow, Glasgow, G12 8QQ, UK, and*
[b] *Animal Health Discovery, Pfizer Central Research, Sandwich, Kent, CT13 9NJ, UK*

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Eimeria tenella contains a pyrophosphate-dependent phosphofructokinase and a pyruvate kinase with unusual allosteric regulators

Helen Denton *[,a], Kam-Wah Thong [b] and Graham H. Coombs [a]

[a] *Laboratory for Biochemical Parasitology, Department of Zoology, University of Glasgow, Glasgow, G12 8QQ, UK, and* [b] *Animal Health Discovery, Pfizer Central Research, Sandwich, Kent, CT13 9NJ, UK*

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Abstract: Sporozoites and unsporulated oocysts of *Eimeria tenella* were shown to contain a pyrophosphate-dependent phosphofructokinase (PP_i-PFK) but apparently lack an ATP-specific activity. The PP_i-PFK resembles those that occur in a number of other protists in being reversible and not subject to metabolic control. In contrast, the ADP-utilising pyruvate kinase, present in two developmental stages of the parasite, exhibited strong positive cooperativity with respect to its substrate, phosphoenolpyruvate, and was shown to be allosterically activated by glucose 6-phosphate, fructose 6-phosphate and AMP. It is suggested that the PP_i-PFK represents an adaptation of the parasite towards life in an environment containing only low concentrations of oxygen and that the unusual allosteric regulation of pyruvate kinase evolved to compensate for glycolysis not being controlled at the PP_i-PFK step.

Key words: Eimeria tenella; Pyrophosphate-dependent phosphofructokinase; Pyruvate kinase; Anaerobiosis; Metabolic regulation

Introduction

The chicken parasite *Eimeria tenella* has a complex lifecycle including phases in aerobic and essentially anaerobic environments. The extracellular stages of the parasite are thought to be heavily dependent upon carbohydrate metabolism for energy generation and contain endogenous energy reserves of amylopectin [1] and mannitol [2]. Experimental studies have indicated that under aerobic conditions both the oocysts and the sporozoites undergo respiration mediated by a TCA cycle and respiratory chain, while under conditions of low oxygen tension these stages produce ATP via glycolysis and rely largely on lactate production for regeneration of NAD^+ [3,4].

In recent years it has become increasingly evident that significant deviations from standard glycolysis occur in some protists. One such departure is the utilisation of pyrophosphate (PP_i) rather than nucleotide phosphates as phosphoryl substrates in some reactions. Pyrophosphate-dependent enzymes have been identified in a variety of organisms, including a number of largely fermentative parasitic protists [5–9]. It has been suggested that they may represent an adaptation towards an anaerobic mode of life [10,11].

* Corresponding author.

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Pyrophosphate-dependent phosphofructokinase (PP_i-PFK) is probably the best-studied PP_i-dependent enzyme. In most organisms, ATP-PFK catalyses the first committed step of glycolysis, and as such constitutes a major control point of glycolytic activity. The utilisation of PP_i rather than ATP renders this reaction readily reversible and hence less useful as a metabolic control point. This appears to be reflected in the lack of allosteric properties associated with many PP_i-PFKs. Most protists containing PP_i-PFK also possess pyruvate phosphate dikinase (PPDK) [12], a PP_i-dependent enzyme catalysing the analogous step to pyruvate kinase in standard glycolysis, and are also unusual in lacking cytosolic pyrophosphatase activity.

Despite the reports that *E. tenella* is not an obligate fermenter, the sporozoite, merozoite and oocyst stages of the parasite exist for periods in the gut lumen and are likely to have adaptations for survival under the relatively low oxygen tensions present. This line of thought, and the recent report that a related organism, *Toxoplasma gondii*, contains PP_i-PFK [7], prompted us to look for PP_i-dependent enzymes in *E. tenella*.

Materials and Methods

Parasites

Sporulated and unsporulated oocysts of *E. tenella* (Weybridge 281) were produced and purified by standard methods and stored at 4°C in 2.5% (w/v) potassium dichromate solution until required. Sporozoites were obtained from sporulated oocysts by in vitro excystation. Briefly, oocysts resuspended in phosphate-buffered saline, pH 7.3 (PBS) were broken by vortexing with 3-mm glass beads and the freed sporocysts were treated for 1 h at 44°C in PBS containing 20 mM $MgCl_2$, 2% (w/v) taurodeoxycholic acid, 0.1% trypsin and 0.01% chymotrypsin to effect release of sporozoites. Debris was removed by differential centrifugation at intermediate steps and the sporozoites were finally purified by filtration through cotton wool. Purified sporozoites were used immediately or stored at −70°C.

Preparation of crude extracts

Unsporulated oocysts were resuspended (2–5 $\times 10^6$ oocysts ml^{-1}) in 50 mM HEPES (pH 7.0) with 1 mM DTT and broken by vortexing for 5 min with 3-mm glass beads. Sporozoites were lysed in the same buffer ($1–4 \times 10^7$ cells ml^{-1}) either by sonication (3×10 s at 17 μm) or addition of Triton X-100 (0.25%).

Insoluble material was removed by centrifugation (10 min at $14000 \times g$) and the supernatant used in enzyme assays; either directly or following gel filtration to remove low molecular mass components. Proteinase inhibitors (50 μM TLCK, 5 μM E64, 0.5 mM PMSF, 1 mM 1,10-phenanthroline) were initially included in the lysis buffer but appeared to have no beneficial effects.

Glycolytic enzyme assays

Enzyme activities were coupled to NADH oxidation and monitored spectrophotometrically at 340 nm. All assays were carried out at 41°C and initiated by the addition of substrate at saturating concentration. Control assays lacking one or more of the reaction components were also carried out.

Phosphofructokinase, forward reaction. The standard assay contained in a final volume of 1 ml: 50 mM HEPES (pH 7.0); 1 mM $MgCl_2$; 1 mM DTT; 0.2 mg NADH ml^{-1}; 1 mM fructose 6-phosphate; 1.5 U aldolase; 2 U α-glycerophosphate dehydrogenase; 20 U triose phosphate isomerase; 1 mM PP_i or 1 mM ATP; and crude enzyme.

Phosphofructokinase, reverse reaction. The standard assay contained in a final volume of 1 ml: 50 mM HEPES (pH 7.0); 1 mM $MgCl_2$; 1 mM DTT; 1 mM $NADP^+$; 1 mm fructose-1,6-bisphosphate; 1 mM P_i; 2 U glucose-6-phosphate dehydrogenase; 5 U phosphoglucose isomerase; and crude enzyme.

Pyruvate kinase. The standard assay contained in a final volume of 1 ml: 50 mM HEPES (pH 7.5); 1 mM DTT; 7.5 mM $MgCl_2$; 75 mM KCl; 0.2 mg NADH ml^{-1}; 10 mM phosphoenolpyruvate; 2 U lactate dehydrogenase; 5 mM ADP or 5 mM PP_i; and crude enzyme.

Determination of pH optima

The activity of a fixed quantity of crude enzyme was measured at 0.5-U intervals over the

pH range 4–10 using the following buffers: MES (pH 4.0–5.5); BIS-Tris (pH 5.5–7.5); HEPES (pH 7.5–8.5); Tris (pH 7.5–9.5). Experiments were carried out to ensure that the activity of the coupling enzymes was not limiting under any of the pH conditions used.

Pyrophosphatase assay

Samples of crude extract were incubated for 30–90 min at 41°C in 50 mM HEPES (pH 7.5), containing 5 mM PP_i and 20 mM $MgCl_2$, and the amount of released phosphate was determined according to the absorbance at 690 nm 20 min after the addition of SDS, ascorbic acid, ammonium molybdate, sodium citrate, sodium *meta*-arsenite and acetic acid to 1.2% (w/v), 0.6% (w/v), 0.1% (w/v), 1.2% (w/v), 1.2% (w/v) and 1.2% (v/v), respectively [13]. Sodium dihydrogen orthophosphate was used as a standard and assays were carried out in a final volume of 250 μl in microtitre plates.

Protein determinations

Protein concentrations were determined using the Pierce BCA assay kit with bovine serum albumin as a standard.

Results and Discussion

Soluble extracts of *E. tenella* sporozoites and unsporulated oocysts contained PP_i-PFK and ADP-utilising pyruvate kinase (PK) (Table 1). PP_i-PFK activity was also found in extracts of first and second generation merozoites. A low level of apparent ATP-PFK activity was detected in the extracts of sporozoites and oocysts but this was abolished by addition of 5 U yeast pyrophosphatase to the standard assay mixture, indicating that it was probably due to the conversion of some ATP to PP_i in the reaction mixture [7,8]. Pyrophosphatase activity was not detectable in lysates of *E. tenella* (< 1 nmol min^{-1} (mg protein)$^{-1}$) – this being in contrast to high levels detected in leishmanial lysates used as a positive control.

In the glycolytic direction, the properties of the *E. tenella* PP_i-PFK were shown to be similar to those of PP_i-PFKs in other protists [11,14]. Most significantly, it displayed hyperbolic kinetics with respect to both substrates and was not af-

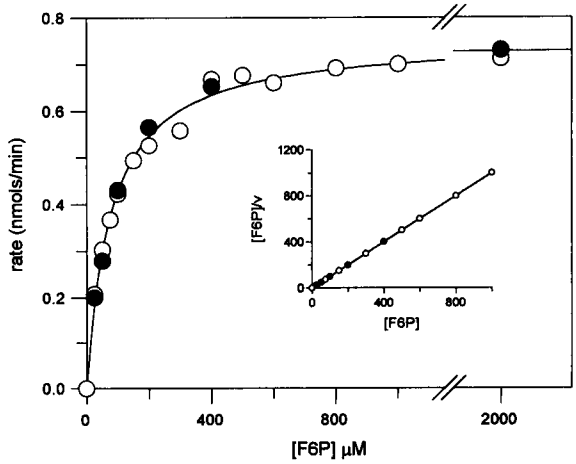

Fig. 1. A representative F6P saturation curve of PP_i-PFK and Hanes plot of data (insert). The activity associated with a fixed quantity of unsporulated oocyst lyaste was assayed at different concentrations of F6P, with (closed circles) and without (open circles) $F26P_2$. The kinetic parameters for this experiment were: K_m, 77 μM and V_{max}, 0.76 nmol min^{-1}.

Table 1

PK and PFK activities in extracts of sporozoites and unsporulated oocysts of *E. tenella* (nmol min^{-1} (mg protein)$^{-1}$)

	PFK		PK	
Phosphoryl substrate:	PP_i	ATP [a]	PP_i	ADP
Unsporulated oocysts	183 ± 71 (4)	n.d. < 1(4)	n.d. < 2 (4)	2105 ± 572 (4)
Sporozoites	105 ± 15 (5)	n.d. < 1 (4)	n.d. < 2 (4)	1349 ± 167 (4)

Means ± SD, *n* in parentheses. n.d., not detectable.

[a] Assays contained 5 U of yeast pyrophosphatase.

fected by concentrations of fructose-2,6-bisphosphate ($F26P_2$) up to 10 μM (Fig. 1). This is in contrast to most ATP-PFKs and plant PP_i-PFKs, which display strong positive cooperativity with respect to fructose-6-phosphate (F6P) and are allosterically activated by $F26P_2$ [15]. A number of other substances (ATP, ADP, AMP, succinate and citrate at 1 mM), investigated as potential modulators of the enzyme, were also without effect at either saturating or subsaturating concentrations of F6P. The enzyme was dependent on Mg^{2+} ions for activity and had a pH optimum around 6.8 in the forward direction. Activity catalysing the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate was also detectable in lysates. This had a pH optimum of approximately 7.5 and co-eluted with the forward PP_i-PFK activity in anion exchange (MonoQ) chromatography.

E. tenella differs from most other PP_i-PFK-containing protists in having an ADP-specific PK but no PPDK. The PK of *E. tenella* rapidly lost activity in lysates but was largely stabilised by addition of 20% (v/v) glycerol. Like PKs from most other sources [16], the enzyme required Mg^{2+} ions for activity and was enhanced by addition of K^+ ions (optimal concentrations of each were used in the standard assay). The pH optimum of the enzyme was shown to be around 7.5. The enzyme presented Michaelis-Menten kinetics for ADP (at saturating concentrations of phosphoenolpyruvate (PEP)), but produced a sigmoidal saturation curve with PEP as the variable substrate (Fig. 2). A range of substances, all known effectors of PKs from other sources, were tested as possible modulators of the eimerian PK. Of these, glucose 1-phosphate, fructose 1,6-bisphosphate ($F16P_2$), ribose 5-phosphate, 3-phosphoglycerate, ATP, acetyl-CoA (all at 1 mM concentration) and $F26P_2$ (at 10 μM) were largely without effect, while AMP, F6P and glucose 6-phosphate (G6P) proved to be allosteric activators, capable of converting the PEP saturation curve to hyperbolic form (see Fig. 2). This unusual combination of activators, which has not been reported for other protozoan PKs, may be related to the lack of regulation at PP_i-PFK (see [17]). It seems possible that in this organism PK has replaced PFK as the major regulatory enzyme of glycolysis.

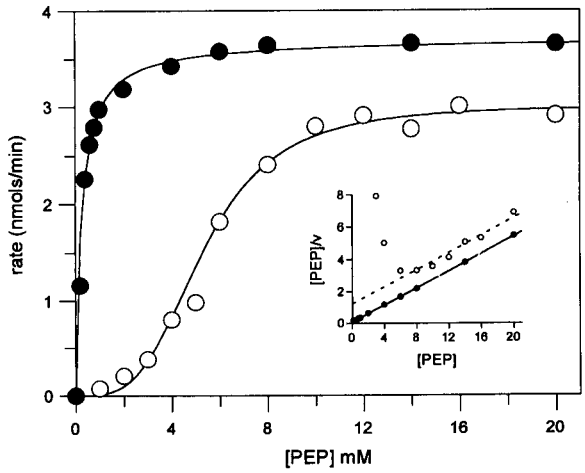

Fig. 2. A representative PEP saturation curve of PK and Hanes plot of data (insert). The activity associated with a fixed quantity of unsporulated oocyst extract was assayed at different concentrations of PEP, with (closed circles) and without (open circles) G6P. The kinetic parameters for this experiment were: K_m, 0.26 mM and V_{max}, 3.7 nmol min^{-1}, in the presence of G6P; in its absence the parameters became: $K_{0.5}$, 5.2 mM, V_{max}, 3.0 nmol min^{-1} and Hill coefficient, 3.5.

For some time, it was considered that unregulated PP_i-dependent glycolytic enzymes were solely a feature of various obligate fermentative protists. Clearly, however, *Eimeria* now joins *Naegleria* [9] and *Toxoplasma* [7] as organisms which contain PP_i-PFK but also appear to have some capacity for aerobic respiration. These findings suggest that the parasites are not obligate aerobes in all stages of their life cycles but indeed are adapted for living under conditions of reduced oxygen tension. The phylogenetic distribution of PP_i-PFK implies that the enzyme may have evolved on more than one occasion as an adaptation for anaerobiosis, another example of convergent evolution.

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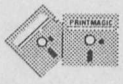

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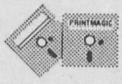

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Enzymes of energy metabolism in the bradyzoites and tachyzoites of *Toxoplasma gondii*

Helen Denton [a], Craig W. Roberts [b,1], James Alexander [b], Kam-Wah Thong [c], Graham H. Coombs [a,*]

[a] *Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK*
[b] *Department of Immunology, The Todd Centre, University of Strathclyde, Glasgow G4 0NR, UK*
[c] *Animal Health Discovery, Pfizer Central Research, Sandwich, Kent CT13 9NJ, UK*

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Abstract

The bradyzoite and tachyzoite forms of *Toxoplasma gondii*, purified from infected animals, were analysed for their activities of phosphofructokinase, pyruvate kinase, lactate dehydrogenase, NAD^+- and NADH-linked isocitrate dehydrogenases, and succinic dehydrogenase. Both developmental stages contained high activities of phosphofructokinase (specific for pyrophosphate rather than ATP), pyruvate kinase and lactate dehydrogenase, suggesting that energy metabolism in both forms may centre around a high glycolytic flux linked to lactate production. The markedly higher activity of the latter two enzymes in bradyzoites suggests that lactate production is particularly important in this developmental form. NAD^+-specific isocitrate dehydrogenase was not detectable in either stage of the parasite (and proved useful as a measure of the purity of the bradyzoite preparation), whereas both parasite forms contained low activities of $NADP^+$-linked isocitrate dehydrogenase. The results are consistent with the bradyzoites lacking a functional TCA cycle and respiratory chain and are suggestive of a lack of susceptibility of this developmental stage to atovaquone.

Keywords: *Toxoplasma gondii*; Tachyzoite; Bradyzoite; Enzymes; Glycolysis; Lactate dehydrogenase; TCA cycle

1. Introduction

Toxoplasma gondii is an important cause of congenital disease and abortion in humans and domestic animals. While foetal death and abortion have been attributed to the rapidly dividing tachyzoite stage of the parasite's life cycle, the CNS lesions and chorioretinitis observed as a result of congenital infection are caused either directly by cysts containing bradyzoites or by reactivation of the disease from this, so-called, dormant stage. In addition, toxoplasmic

Abbreviations: PFK, phosphofructokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; $NAD(P)^+$-ICDH, $NAD(P)^+$-linked isocitrate dehydrogenase; SDH, succinate dehydrogenase; PBS, phosphate buffered saline; PP_i, inorganic pyrophosphate

* Corresponding author. Tel.: +44 (141) 330 4777; Fax: +44 (141) 307 8016; E-mail: graham.coombs@udcf.gla.ac.uk

[1] Present address: Michael Reese Hospital and Medical Center, Division of Infectious Diseases, 2929 South Ellis Avenue, Chicago, IL 60616-3390, USA.

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encephalitis as a result of reactivation from latent cyst stage bradyzoites is often a life threatening condition in immunosuppressed individuals such as transplant recipients and AIDS patients where it constitutes the greatest cause of cerebral mass lesions [1]. The development of a chemotherapeutic regimen that would lead to removal of bradyzoites is therefore a high priority. Unfortunately there is an almost total lack of information on the biochemistry of bradyzoites and this is a major obstacle in identifying possible drug targets. The known structural and biological differences between the bradyzoite and tachyzoite stages of the parasite suggest, however, that they may also differ significantly in their metabolism. Such stage-specific variation would have profound implications for drug development strategies, most of which currently involve using tachyzoites for determining the efficacy of potential drugs. Our aim is to compare the two forms in order to determine the ways in which bradyzoites differ biochemically from (and are similar to) tachyzoites.

Energy metabolism was chosen as the first aspect to study for two main reasons. Firstly, previous studies have revealed unusual features of the energy metabolism of tachyzoites [2–4] and there is some evidence that the two developmental stages of the parasite may differ in this respect. The different growth characteristics of the two forms indicate that their energy requirements are not the same and also it seems likely that the two forms will have a different range of nutrients available to them as they reside in distinct environments. In particular, the bradyzoites are surrounded by a cyst wall which may reduce the availability of exogenous materials to this form of the parasite. The second reason was to probe the basis of the anti-*Toxoplasma* activity of the naphthoquinone atovaquone [5]. It is thought that atovaquone acts by inhibiting the respiratory chain and so clearly the susceptibility of *Toxoplasma* to this and similar compounds will be determined in part by the importance, or otherwise, of electron transport in energy generation and the maintenance of redox balance. One objective of this study was to provide data on the likely importance of electron transport to bradyzoites and so their innate susceptibility to this drug.

Biochemical investigations on bradyzoites have been greatly curtailed in the past by the impossibility of obtaining parasite material. We have now developed a method that enables us to obtain pure bradyzoites in sufficient quantities for some studies. This paper reports both the procedure for obtaining bradyzoites in quantity and the first information on the activities in bradyzoites of enzymes involved in energy metabolism.

2. Materials and methods

2.1. Animals

Cotton rats, Strathclyde Albino and C57BL/10ScSn (B10) mice were bred and maintained in the University of Strathclyde under conventional conditions and used at 8–14 weeks of age.

2.2. Production of bradyzoites in mice

Bradyzoites were obtained from tissue cysts harvested from the brains of B10 mice. This strain of mice has been demonstrated to develop large tissue cyst burdens in their brains during *T. gondii* infections [6]. B10 mice were infected using cysts obtained from the brains of Strathclyde Albino mice infected with *T. gondii* 17–21 weeks previously. The brains were homogenised in 2 ml 0.1 M phosphate buffered saline, pH 7.2 (PBS) by six passes through a 21 gauge needle, the cyst density was determined microscopically and each B10 mouse was inoculated intraperitoneally with 10 cysts.

2.3. Bradyzoite purification

Cysts were harvested from the brains of B10 mice which had been infected 28–30 days previously. The brain of each mouse was removed and homogenised in PBS by repeated passage through a 21 gauge needle. Aliquots containing the equivalent of 3–4 homogenised brains were diluted to 13 ml with PBS. 6.5 ml 90% (v/v) Percoll (Sigma, Poole, UK), produced by addition of 10 × concentrated PBS to Percoll, was added and the mixture left to settle for 30 min at 18°C. 2 ml 90% (v/v) Percoll was then added as a bottom layer and the mixture centrifuged for 30 min at 2500 × *g* and 18°C, which sedimented most of the cysts into the bottom layer. This and the

bottom 1 ml of the upper layer, which also contained tissue cysts and most of the erythrocytes, were removed, diluted 10-fold in PBS and centrifuged at $2500 \times g$ for 15 min to remove the Percoll. The supernatant was discarded, 1.8 ml of distilled water added to the pellet to lyse the erythrocytes, and 0.2 ml of $10 \times$ concentrated PBS added immediately. The cysts were sedimented by centrifugation at $1000 \times g$ for 10 min, resuspended in 1 ml of PBS for counting, resedimented ($1000 \times g$ for 10 min) and resuspended in 5 ml of 1% (w/v) pepsin (Sigma) in HCl (pH 1.5) to release the bradyzoites. Following a 5 min incubation at 37°C, the pepsin solution was deactivated by addition of 15 ml PBS and the bradyzoites sedimented by centrifugation at $1000 \times g$ for 10 min. The bradyzoites were washed once, resuspended in PBS and either lysed immediately or stored as pellets at −70°C.

2.4. Production and purification of tachyzoites

Tachyzoites of *T. gondii* (RH strain) were grown in the peritoneum of cotton rats as described previously [7]. Cotton rats were inoculated intraperitoneally with 5×10^5 tachyzoites suspended in PBS. 48 h later, tachyzoites were harvested from the peritoneum through a 21 gauge needle into PBS with heparin (20 U ml^{-1}). The parasites were sedimented ($200 \times g$ for 10 min), washed twice in PBS and either lysed immediately or stored as pellets at −70°C.

2.5. Preparation of cell extracts

T. gondii tachyzoites and bradyzoites were lysed directly by resuspension (to $1-2 \times 10^8$ cells ml^{-1}) in 50 mM HEPES (pH 7.4) containing 20% glycerol, 0.25% Triton X-100 and proteinase inhibitors (0.5 mM PMSF, 5 μM E64, 1 μM pepstatin, 0.2 mM 1,10-phenanthroline). Soluble extracts were obtained by centrifugation for 10 min at $14000 \times g$. Mouse brain homogenates were prepared by addition of the suspension buffer used with the parasites and subjecting the brains to five passes through a 21 gauge needle and then three passes through a 6 gauge needle. Pyruvate kinase assays were carried out using freshly prepared cell and tissue extracts; all other enzyme assays were performed on extracts which had been stored at −70°C.

2.6. Enzyme assays

All assays were carried out at 37°C in a final volume of 1 ml. Reaction was initiated by the addition of substrate to a saturating concentration. With the exception of the succinate dehydrogenase assay, all assays contained soluble cell extract and were monitored spectrophotometrically at 340 nm. To confirm the specificity of the reaction, control assays were carried out lacking one or more of the assay components. Phosphofructokinase (PFK) and pyruvate kinase (PK) were assayed in the forward (glycolytic) direction as described [8]. Lactate dehydrogenase (LDH) was assayed in a mixture containing 100 mM Tris (pH 7.4), 200 mM NaCl, 0.2 mg NADH ml^{-1}, 1.6 mM pyruvate and soluble cell extract. NAD^+- and $NADP^+$-dependent isocitrate dehydrogenase (ICDH) were assayed using a mixture containing 50 mM HEPES (pH 7.5), 2 mM $MnCl_2$, 6.7 mM isocitrate, 0.4 mM $NADP^+$ or NAD^+ and soluble cell extract. 2 mM ADP was included in assays for the NAD^+-dependent enzyme. Succinate dehydrogenase (SDH) activity was assayed in whole cell homogenates by following the oxidation of potassium ferricyanide. The standard assay contained 100 mM sodium phosphate buffer (pH 7.3), 10 mM KCN, 2 mM potassium ferricyanide, 15 mM sodium succinate and crude cell homogenate. The reaction was followed continuously at 420 nm (for 1–2 h). Control assays were carried out in the absence of succinate. Protein concentrations were determined using the Pierce BCA protein assay kit (microtitre plate protocol) with bovine serum albumin as a standard.

3. Results

The method devised for growing and purifying bradyzoites was successful in terms of both parasite numbers and the low level of contaminating material. In a typical purification which used the brains of 15 mice, 6.9×10^4 cysts were found to be present in unpurified brain homogenate. 6.4×10^4 cysts were purified (92% recovery), yielding 7.5×10^6 bradyzoites (approx. 13 μg protein). There was less than 1% cell/cell contamination, as determined microscopically. The amount of contaminating host material was also assessed by analysing enzyme activities

Table 1
Activities of enzymes of energy metabolism in *Toxoplasma* and mouse brain extracts

Sample	Enzyme activity (nmol min^{-1} (mg protein)$^{-1}$)					
	PP_i-PFK	PK	LDH	$NADP^+$-ICDH	NAD^+-ICDH	SDH
T. gondii, tachyzoites	634 ± 203 (3) [a]	4658 ± 1275 (7) [a]	655 ± 196 (6)	130 ± 33 (3)	n.d., < 0.2 (3)	13 ± 6 (3)
T. gondii, bradyzoites	265 ± 15 (3)	11338 ± 2201 (3)	2259 ± 827 (6)	130 ± 38 (3)	n.d., < 1.0 (4)	n.d., < 26 (3)
Mouse brain	n.d., < 1.0 (3)	5219 ± 398 (6)	n.d., < 0.3 (4)	64 ± 5 (3)	23 ± 5 (3)	25 ± 3 (3)

Means ± S.D., n in parentheses. n.d., not detectable.
Maximum contribution of host cell enzymes to activities in bradyzoite extracts (nmol min^{-1} (mg protein)$^{-1}$): PFK, 0; PK, 227; LDH, 0; $NADP^+$-ICDH, 2.8; SDH, 1.1.
[a] Data from [4].

apparently absent from the parasite. Both ATP-PFK and NAD^+-ICDH were used, but the latter activity proved to be more useful because of the difficulty in obtaining coupling enzymes for the PFK assay which were free from ATP-dependent activity. NAD^+-ICDH was undetectable in the bradyzoite preparation and by taking into account the sensitivity of the assay it was calculated that at most 4.3% of the protein in the bradyzoite preparation could be of host origin. This figure was used to calculate the possible contribution by host enzymes to the enzyme activities detected in the parasite preparation. The results (detailed in the legend to Table 1) provide assurance that the data presented for bradyzoite enzyme activities in Table 1 are valid. The tachyzoite preparation was essentially free from host contamination.

The enzyme activities detected in the parasite preparations and mouse brain extract are detailed in Table 1. Both parasite forms lacked ATP-PFK but contained PFK specific for inorganic pyrophosphate (PP_i-PFK), PK, LDH and $NADP^+$-ICDH. In contrast, neither PP_i-PFK nor LDH was detectable in the mouse extract whereas NAD^+-ICDH was detected only in this sample. The two glycolytic enzymes and LDH were present at high activity in the parasite samples (for comparison, the activities in similar extracts of sporulated oocysts of *Eimeria tenella* and oocysts of *Cryptosporidium parvum* were, respectively, 56 ± 24 and 331 ± 33 (PP_i-PFK), 124 ± 60 and 2947 ± 775 (PK), 114 ± 77 and 82 ± 44 (LDH), 20 ± 8 and < 0.9 ($NADP^+$-ICDH), < 0.6 and < 0.9 (NAD^+-ICDH) and < 0.6 and < 1 (SDH) nmol min^{-1} (mg protein)$^{-1}$, $n = 3$ or more [4,8] (and unpublished). Bradyzoites contained 2–3 times more PK and LDH activity than did tachyzoites, but only half the activity of PP_i-PFK. The PK activity in the parasite extracts was found to be allosterically activated by glucose 6-phosphate and fructose 6-phosphate, but the 10 mM PEP used in the assays ensured that the activities were maximal. There was more $NADP^+$-ICDH in the parasites than in the host extract, and SDH activity was moderately low in tachyzoites and not detectable in bradyzoites (although the relatively low sensitivity of the assay combined with the limiting amounts of parasite material available mean that this result should be interpreted with caution).

4. Discussion

Bradyzoites are considered to be a relatively dormant stage in the life cycle of *T. gondii* whereas the tachyzoites multiply rapidly in almost every tissue of the infected animals. Thus it is likely that the tachyzoites are adapted for more rapid and greater energy generation than the bradyzoites. Tachyzoites, however, rely heavily upon glycolysis for energy production and some 20% of the glucose catabolised by them is converted to lactate even under aerobic conditions [2]. Significant amounts of acetate are also produced. Current data suggest that oxidation through the TCA cycle and associated respiratory chain contributes in only a minor way to energy metabolism in this developmental form of the parasite [2]. This is one explanation for the presence of PP_i-PFK rather than ATP-PFK, as it results in 50% greater energy production in glycolysis. Therefore it was expected that the glycolytic flux in the relatively inactive bradyzoites would be lower than in tachy-

zoites, reflecting the down regulation of energy generating pathways. Thus it is surprising that both PK and LDH activities in bradyzoites are considerably greater than in tachyzoites (Table 1). Specific activities of enzymes give only an indication of flux through the pathways, nevertheless the results do suggest that fermentation to lactate plays the major role in energy generation in bradyzoites. Amylopectin is present in abundance in bradyzoites (in contrast to tachyzoites) and so it seems likely that this stage relies heavily on the energy generated in the catabolism of amylopectin to lactate. The differences observed in this study between the LDH activities of the two developmental stages of the parasite are also consistent with the findings that tachyzoites can express more than one isoenzyme of LDH [9] and that there is stage specificity of LDH gene expression, one gene being expressed only in bradyzoites [10]. Another way in which bradyzoites differ significantly from tachyzoites is that they are highly resistant to low pH. This is thought to be an adaptation that enables the parasite to survive passage through the acid conditions of the host's stomach, but it may also be an important adaptation of the bradyzoite for survival in the cyst; the lactate produced by the bradyzoite is likely to be retained within the cyst with the result that the fluid surrounding the bradyzoite becomes quite acid. It has been reported that low pH will stimulate differentiation of tachyzoites to bradyzoites [11] and so the lactate produced by bradyzoites in the tissue cysts is likely to help maintain the parasite in the bradyzoite form. It will be interesting to find out if there are environmental changes within the cyst, such as a consumption of lactate and consequent rise in pH, in advance of, and maybe as a trigger for, reactivation of the cysts, and if so how this is mediated.

Lactate production does not involve a respiratory chain. There is also other evidence that bradyzoites may not require a functional respiratory chain. It has been suggested that as bradyzoites mature they divide rarely if at all, and so it is probable that there is little or no pyrimidine biosynthesis in bradyzoites. Thus a role for the electron transport chain in association with dihydroorotate dehydrogenase (its main function in the asexual stages of *Plasmodium*, although it is not known if this is also the case in *T. gondii* tachyzoites [12]) seems unlikely. It has also been shown that molecules that interfere with mitochondrial function, such as oligomycin, antimycin A and nitric oxide, promote the transformation of tachyzoites to bradyzoites [13]. This occurs even in host cells that lack a mitochondrion and one possible explanation is that a functional mitochondrion is important to tachyzoites but not bradyzoites. These findings lead logically to the hypothesis that the tachyzoite to bradyzoite conversion which occurs in infected animals may be a means of escaping from compounds such as nitric oxide that are produced as part of the host's immune response to infection. In this context, it is relevant that atovaquone itself triggers transformation of tachyzoites to bradyzoites [14]. The lack of a functional respiratory chain in bradyzoites would be likely to make them innately resistant to atovaquone, irrespective of whether or not the drug is able to pass through the cyst wall and reach the parasites, such that the drug would have little efficacy against tissue cysts. There is some evidence that bradyzoites in vitro are susceptible to atovaquone [15], although they are less sensitive than tachyzoites (see [14]). There are conflicting reports on the usefulness or otherwise of the drug against tissue cysts although it has been reported recently that atovaquone was ineffective against cerebral toxoplasmosis [16]. This latter finding could be due to failure to take the drug with food as recommended, and so only low plasma levels of the drug being achieved, or acquired drug resistance, but the results presented in this paper suggest that the innate sensitivity of bradyzoites to atovaquone should be re-evaluated. One reason for some of the conflicting data on the drug's usefulness clinically may be that extremely high concentrations of the drug can be achieved during its use and that at these concentrations the drug has effects other than against dihydroorotate dehydrogenase and electron transport.

The data presented suggest that bradyzoites differ from tachyzoites in their energy metabolism. Nevertheless, bradyzoites are no doubt biochemically similar to tachyzoites in many respects and so the more freely available form can be used with confidence for many investigations. It is important, however, to confirm rather than assume that the stages are similar. This study has shown that procedures enabling key biochemical studies on bradyzoites to be carried out have now been developed. These methods will

have important applications in both validating the use of tachyzoites in some studies and the discovery of bradyzoite-specific biochemical features.

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