# STUDIES ON THE ADAPTATIONS FOR SURVIVAL AND INVASION BY *CRYPTOSPORIDIUM* AND *EIMERIA TENELLA*

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**ABSTRACT**

*Cryptosporidium* and *Eimeria* are two coccidian parasites that infect a wide range of animals including humans for *Cryptosporidium*, resulting in disease and, in some cases, death of the host. Infection involves three stages: ingestion, excystation and the subsequent invasion of the epithelial cells lining the gastrointestinal or respiratory (as can be the case for *Cryptosporidium*) tract. The mechanisms employed by the parasites to withstand the hostile environment of the host, and to penetrate to and invade a host cell are not clearly understood. The objective of this study was to provide more information on these three steps to infection, to elucidate the mechanisms involved for *Cryptosporidium* and *Eimeria tenella*, and to establish the extent to which these features are shared by the parasites.

An *in vitro* procedure for inducing *Cryptosporidium* excystation was optimised. Anaerobic reducing conditions produced the highest excystation rates, with oocysts from different species excysting most efficiently at the pH values akin to their *in vivo* locations in the gastrointestinal tract. Oocyst age affected the excystation process, with the number of sporozoites released declining with increasing age.

A novel method for determining the viability of sporozoites was developed. This employed the use of the vital stains acridine orange and bis-benzimide using fluorescence microscopy. Sporozoite survival during incubation with different pH buffers varied with the species of parasite, exemplifying that each species is adapted for survival at the pH similar to their infection site *in vivo*.

Inhibitors of mitochondrial function had little effect on either *C. parvum* or *E. tenella*. These data are consistent with *C. parvum* lacking a mitochondrion, and *E. tenella* sporozoites not requiring the function of this organelle. *C. muris* was, to an extent, affected by some of the metabolic inhibitors, suggesting that this species either relies in part on a mitochondrion, or that the inhibitors were affecting other processes in the parasite.

Biotinylated probes and antibodies raised against *Leishmania mexicana* cysteine proteinases were used to analyse the lysates of *C. parvum, E. tenella* and *Toxoplasma gondii* for the presence of proteinases. Evidence was found for the presence of both cysteine and serine proteinases in all of the coccidia, with the apparent localisation of a cysteine proteinase on the surface of *E. tenella* sporozoites. Stage-specific differences were observed when sera against *L. mexicana* cysteine proteinases were used to analyse unsporulated oocysts and sporozoites of *E. tenella*.

A survey of a range of enzyme activities showed the presence of a number of enzymes and some apparent variations between life cycle stages. However attempts to detect sialidase in *Cryptosporidium* and *Eimeria* using fluorogenic substrates were not successful.

A system was developed to study the penetration of mucus layers. The results showed that *C. parvum* sporozoites and *E. tenella* sporozoites and merozoites were able to penetrate the layers and, through the use of specific

enzyme inhibitors, that motility, cysteine and serine proteinases, polyamines and sialidase were involved in mucus penetration by the parasites.

Host cell invasion by *C. parvum* sporozoites and *E. tenella* sporozoites and merozoites was investigated using Madin Darby Bovine Kidney (MDBK) cells and specific enzyme inhibitors. The results suggest that cysteine, serine, metallo- and aspartic proteinases play a role in sporozoite invasion, as do polyamines, whereas sialidase was not important. Invasion by merozoites also involved cysteine proteinases and polyamines, but sialidase did appear to have a role.

The MDBK cell cycle was analysed using bromodeoxyuridine (BrdU) labelling and 4,6,-diamino-2-phenylindole (DAPI) staining and its influence on parasite invasion was investigated. Sporozoites of both *E. tenella* and *C. parvum* invaded the host cells at the highest rate when the host cells were four hours into S phase. It was also discovered that more MDBK cells from cultures infected with *E. tenella* were in S phase than in control cultures.

Thus the studies performed provide information on how *Cryptosporidium* and *Eimeria* survive within the hosts intestinal tract and penetrate to and invade a host cell. The two parasites appear to employ some similar mechanisms to facilitate the infection process, yet there are stage-specific features of each parasite.

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| | |
|---|---|
| **ADC** | Argenine decarboxylase |
| **ADP** | Adenosine diphosphate |
| **AIDS** | Aquired immunodeficiency syndrome |
| **ATP** | Adenosine triphosphate |
| **BCIP/NBT** | 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium |
| **BFTE** | Bovine fallopian tube epithelial |
| **BrdU** | Bromodeoxyuridine |
| **BSM** | Bovine submaxillary mucin |
| **CL** | Cardiolipin |
| **$CO_2$** | Carbon dioxide |
| **CTP** | Cytidine triphosphate |
| **DAPI** | 4,6,-diamino-2-phenylindole |
| **DFMO** | α-DL-difluoromethylornithine |
| **DIFP** | Diisopropylfluorophosphate |
| **DNA** | Deoxyribonucleic acid |
| **E64** | Trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme linked immunosorbent assay |
| **FCS** | Foetal calf serum |
| **FITC** | Fluoroscein isothiocyanate |
| **$G_1$** | Gap period before DNA synthesis |
| **$G_2$** | Gap period after DNA synthesis |
| **GPI** | Glycosylphosphatidylinositol |
| **GTP** | Guanosine triphosphate |
| **HDA** | Histone deacetylase |
| **HIV** | Human immunodeficiency virus |
| **HRP** | Horse radish peroxidase |
| **IEL** | Intraepithelial lymphocytes |

| | |
|---|---|
| **IgA** | Immunoglobulin A |
| **ITP** | Inosine triphosphate |
| **LDH** | Lactate dehydrogenase |
| **M** | Mitosis |
| **MDBK** | Madin Darby bovine kidney |
| **MDCK** | Madin Darby canine kidney |
| **4-Mu-Neu5Ac** | 2'-(4-methylumbelliferyl)-α-N-acetyl-D-neuraminic acid |
| **NADH** | Nicotinamide adenine dinucleotide (reduced form) |
| **NAD** | Nicotinamide adenine dinucleotide (oxidised form) |
| **5NeuAc2en** | 2-deoxy-2,3-dehydro-N-acetylneuraminic acid |
| **ODC** | Ornithine decarboxylase |
| **PAS** | Periodic acid-schiff |
| **PBS** | Phosphate buffered saline |
| **PC** | Phosphatidylcholine |
| **PCR** | Polymerase chain reaction |
| **PEPCK** | Phosphoenolpyruvate carboxykinase |
| **PK** | Pyruvate kinase |
| **$PLA_2$** | Phospholipase $A_2$ |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **$PP_i$-PFK** | Pyrophosphate-linked phosphofructokinase |
| **PV** | Parasitophorous vacuole |
| **RNA** | Ribonucleic acid |
| **S** | Period of DNA synthesis |
| **SAMDC** | S-adenosylmethionine decarboxylase |
| **SCID** | Severe combined immunodeficient |
| **SDS-PAGE** | Sodium dodecyl sulphate - polyacrylamide gel electrophoresis |
| **SHAM** | Salicylhydroxamic acid |
| **TBS** | Tris buffered saline |
| **TCA** | Tricarboxylic acid |
| **TDC** | Taurodeoxycholic acid |

| | |
|---|---|
| **TEMED** | Tetramethylethylenediamine |
| **TLCK** | Tosyl-lysine chloromethyl ketone |
| **TPCK** | Tosylamido-2-phenylethyl chloromethyl ketone |
| **UTP** | Uridine triphosphate |
| **VLP** | Virus like particle |
| **ZFA** | Z-phenylalanine diazomethane |

## DECLARATION

I hereby declare that all the results presented in this thesis are my own work unless stated differently.

Sections of the general and chapter introductions have already been published as:

Coombs, G.H., Denton, H., Brown, S.M.A., Thong, K-W. (1997) Biochemistry of the Coccidia (review). *Advances in Parasitology* 39, 141-226.

Some results from chapter three have been published as:

Brown, S.M.A., McDonald, V., Denton, H., Coombs, G.H. (1996) The use of a new viability assay to determine the susceptibility of *Cryptosporidium* and *Eimeria* sporozoites to respiratory inhibitors and extremes of pH. *FEMS Microbiology Letters* **142,** 203-208.

Results obtained in Appendix 1 were from Dr. Laurence Tetley's work in the electron microscope laboratory and are due to be submitted to *Microbiology.* Some of these results have already been published as:

Tetley, L., Brunton, C., Brown, S.M.A., McDonald, V. and Coombs, G.H. (1995) The sporozoite of *Cryptosporidium*: the use of cryotechniques and energy filtering electron microscopy to reveal the three-dimensional ultrastructure. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **89,** 594.

Glycolytic enzyme studies were performed by Helen Denton and are presented as a paper in the back of this thesis:

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xxxxxxxxxxxxxxxxxx

**"He who conquers himself, conquers all."**

**-Iain Hopkins**

# CHAPTER ONE

## 1.1 General review

### 1.1.1 Introduction

Cryptosporidiosis is a disease caused by the protozoan parasite *Cryptosporidium* which belongs to the subclass of Coccidia along with other parasites including *Eimeria* and *Toxoplasma*. *Cryptosporidium* infects the mucosal epithelium of the digestive and respiratory tracts and, although named almost ninety years ago, it was not until recently that much research has been performed on this organism. Found to be primarily a gastrointestinal disease, cryptosporidiosis is known to manifest as a self-limiting diarrhoeal illness in immunocompetent humans, lasting 3-20 days depending on the immune and nutritional status of the host (Current and Garcia, 1991). In contrast, life threatening cholera-like symptoms are seen in immunocompromised patients. Indeed, approximately 10-20% of AIDS (Acquired Immune Deficiency Syndrome) patients develop cryptosporidiosis (Fayer and Leek, 1984; Goodgame, 1996). Additionally *Cryptosporidium* has a major economic significance due to the high mortality in young animals infected with this parasite (Wang, 1982), with *Cryptosporidium* having been reported as the fourth most widespread diarrhoea-causing pathogen in cattle (Müller *et al.*, 1993). It is estimated that in the United States there is an annual cost of $6.2 million due to cryptosporidial diarrhoea in cattle (Sterling and Arrowood, 1993).

### 1.1.2. History

In 1907 Tyzzer named a protozoan parasite infecting the gastric epithelium of laboratory mice as *Cryptosporidium muris* to highlight the fact that this parasite has no sporocysts (*crypto*-absent or concealed). In 1910 he then proceeded to describe the life cycle of this organism although he could not verify its intracellular nature. It was not until 1976 that the first human case of cryptosporidiosis was reported (Nime *et al.*, 1976), after which further reports were rare until *Cryptosporidium* was recognised to produce a diarrhoeal illness which is life-threatening in immune deficient patients. Between 1968 and 1981 other *Cryptosporidium* species were named after every host in which they were observed, bringing the total to more than 20 species (Sterling and Arrowood, 1993). It was not until later that cross-transmission and morphological studies showed that there are possibly only six species: *C. parvum* which infects humans and mammals (Tyzzer, 1912); *C. muris* usually present in rodents but has been observed in cattle (Müller *et al*, 1993; Bukhari and Smith, 1996); *C. baileyi* and *C. meleagridis* which infect birds (however a *C. baileyi* infection was also reported in an HIV-positive patient, Ditrich *et al.*, 1991); *C. serpentis* which is found in reptiles and *C. nasorum* which occurs in fish (Levine, 1984; Webster *et al.*, 1993). Indeed further investigation has revealed that there are some differences - especially between the oocysts of the various *Cryptosporidium* species. One such study used western blotting and immunofluorescence to analyse the oocyst strains, it was observed that *C. parvum* and *C. baileyi* were much more closely related antigenically than either was to *C. muris* (Nina *et al.*, 1992). Isoenzyme studies using electrophoretic mobilities on *Cryptosporidium* (Ogunkolade *et al.*, 1993) and *Eimeria* (Johnston and Fernando, 1997)

were also able to distinguish between species, strains and clones.

The differences between human and animal isolates have also been investigated. Isoenzyme studies have shown consistent differences in electrophoretic mobilities between animal and human isolates of *C. parvum* (Awad-el-Kariem *et al.*, 1993; Ogunkolade *et al.*, 1993; Awad-el-Kariem *et al.*, 1995).

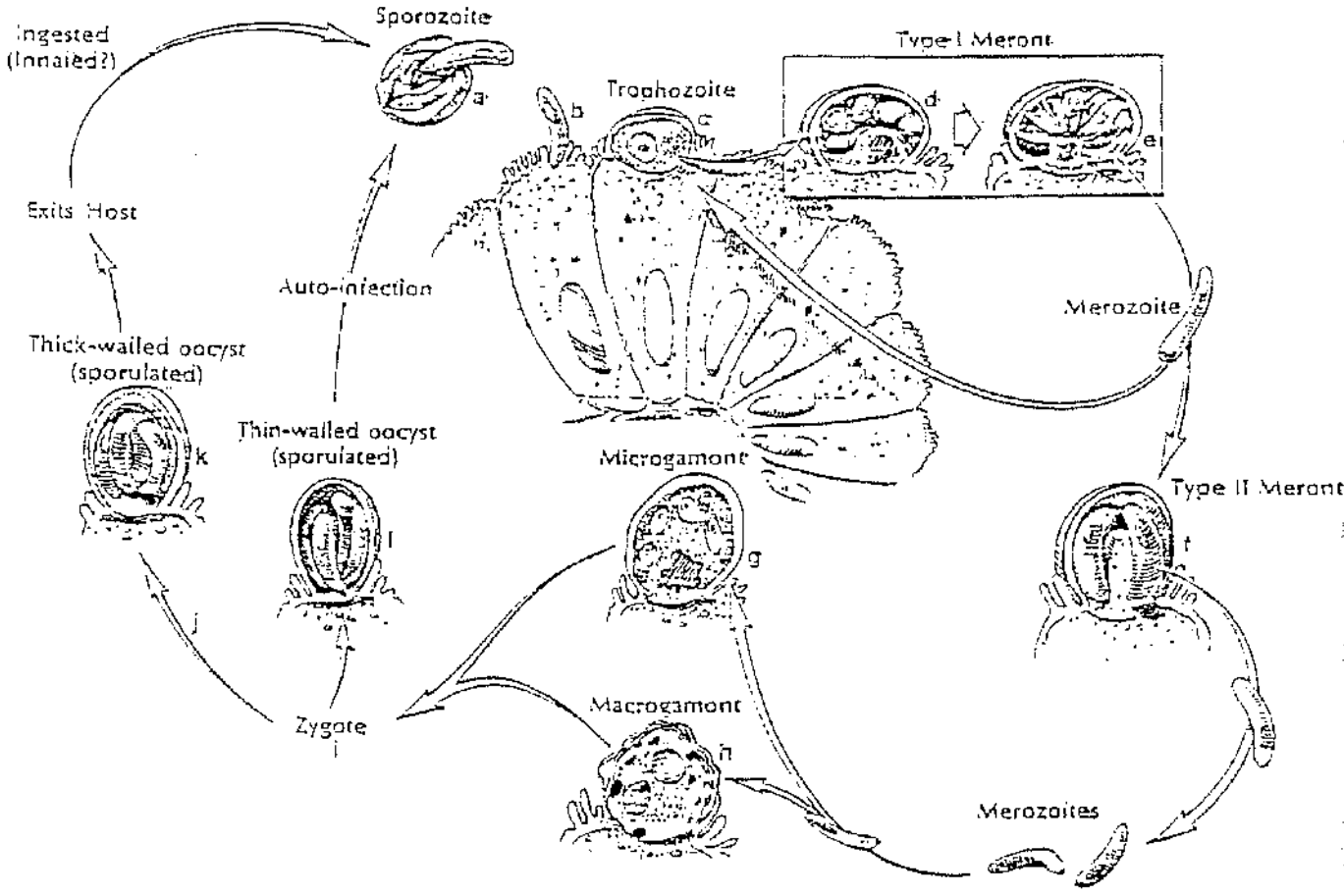
Recently more molecular techniques have enabled researchers to study the differences between isolates. Random amplified polymorphic DNA (RAPD) analysis was used successfully to distinguish *C. serpentis* and *C. parvum* isolates, with *C. parvum* being able to be divided into a further two groups: human or animal isolates (Morgan *et al.*, 1995). Similarly a PCR-restriction fragment length polymorphism method was able to distinguish between *Cryptosporidium* species and between human and animal isolates (Spano *et al.*, 1997).

In terms of phylogeny, RNA data has revealed that *Cryptosporidium* appears to be less related to other coccidia - namely *Eimeria*, *Toxoplasma* and *Sarcocystis* - and is hypothesised to be monophyletic with *Plasmodium* (Johnson *et al.*, 1990; Barta *et al.*, 1991).

### 1.1.3. Life cycle

A detailed study on the life cycle of *Cryptosporidium* was performed by Tyzzer in 1910 and 1912. The life cycle of *C. parvum* is illustrated in Figure 1.1.3.1. and for which the following summary is given. The life cycle of *Cryptosporidium* is monoxenous (completed in a single host) and can be divided into five major stages: oocysts, sporozoites, trophozoites, merozoites and gametes. The life cycle starts when

**Figure 1.1.3.1: Diagrammatic representation of *C. parvum* life cycle in the mucosal epithelium of an infected mammal**
(From Current and Garcia, 1991)




excystation of ingested oocysts (~4.0 x 3 μm, Reduker *et al.*, 1985), which occurs (a) in the gastrointestinal or respiratory tract, results in the release of four sporozoites (4.0 x 0.6 μm, Reduker *et al.*, 1985). These penetrate (b) host epithelial cells which envelop the sporozoite in a parasitophorous vacuole (PV) surrounded by the host cell membrane.

The PV remains extracytoplasmic - unlike other coccidia - and is connected to the host cell via a 'feeder organelle' from which the parasite is thought to obtain nutrients directly from the host cell (Wang, 1982; Marcial and Madara, 1986; Tzipori, 1988). The sporozoite (c) develops into a trophozoite (~3.0 x 3.0 μm, O'Donoghue, 1995) which undergoes a process called merogony (asexual division) (d) and (e) to produce merozoites. These organisms may either invade epithelial cells to produce additional Type I meronts (5.3 x 1.0 μm), or go on to form Type II meronts (f) (4.7 x 1.2 μm) (Sterling and Arrowood, 1993). Type II meronts then proceed to enter adjacent epithelial cells and produce macrogamonts (h) (4.0 x 4.0 μm, O'Donoghue, 1995) and microgamonts (g) (4.0 x 5.0 μm, Sterling and Arrowood, 1993). These produce gametes which in turn produce a zygote (i) which may go on to form a thin-walled oocyst (l) which is thought to auto-infect the host. However 80% of oocysts are thick-walled (Sterling and Arrowood, 1993) and fully sporulate to produce four infective sporozoites before being shed by the host. This is an unusual feature among coccidia as many others, for example *Eimeria*, are released as unsporulated oocysts. In fact, *Caryospora* and *Sarcocystis* are the only other coccidians known to sporulate before release from the host; interestingly *Caryospora* is also capable of autoinfection (Fayer and Ungar, 1986).

### 1.1.4. Transmission

It is now known that, as for the other coccidia, *Cryptosporidium parvum* can be transmitted via a number of routes including contaminated water and food (Petersen, 1995); and, for *Cryptosporidium*, animal to animal, animal to person and person to

person (Baxby and Taylor, 1983) with different isolates being able to infect certain hosts more readily, indicating the adaptibility and lack of host specificity of this parasite (Tzipori *et al.*, 1980; Tzipori, 1988). Hence cryptosporidiosis is more prevalent (4-20%, Smith and Rose, 1990) in areas of low standards of living and in less developed countries where livestock are in close contact with the inhabitants. Yet water-borne *Cryptosporidium* proves a problem for developed countries also (with a prevalence of 0.6-20%, Smith and Rose, 1990) since intact oocysts are extremely resistant to most disinfectants (Riggs and Perryman, 1987) including chlorine (Hoepelman, 1996), and the routine cleansing used to eliminate most waterborne organisms (Current and Garcia, 1991; Robertson *et al.*, 1992). Indeed data from laboratories that screen for *Cryptosporidium* infections in the United States show positive diagnoses in the range 0.5-0.9% of stool samples examined (Li and Stanley, 1996).

Prevalence of this parasite is also seasonal, with there being a higher rate during the warmer and wetter months (Current and Garcia, 1991). This presumably reflects increased contamination of water supplies from farm land. It has also been noticed that children are much more prone to infection than adults, with a peak incidence occurring in 1-5 year olds (Casemore, 1990). It is thought that immunity is gained through separate exposures over time, hence most individuals exhibit an immune response to *Cryptosporidium* as adults. It is known that the elimination of *Cryptosporidium* in neonatal calves occurs with the first appearance of IgA (Hoepelman, 1996).

The minimum number of oocysts required to cause 'full blown' cryptosporidiosis has not been determined unequivocally, and probably differs between patients due to immunological status etc.. However studies performed on mice indicate that this can occur with less than one thousand oocysts (Ernest *et al.*, 1986). Recent studies led to the

authors concluding that the infection dose for healthy persons without previous exposure must be approximately 100 oocysts (Martins and Guerrant, 1995; Petersen, 1995).

### 1.1.5. Diagnosis

Various methods for the diagnosis of *Cryptosporidium* have been reported and most still rely on identifying oocysts in faecal, sputum or bile specimens, employing light and transmission electron microscopy to examine stained faecal smears (Boldorini *et al.*, 1996; Casemore *et al.*, 1985). Immunofluorescence, including the use of flow cytometry, has been developed for detecting oocysts in faecal samples and water providing an easy and rapid screening method (Tzipori, 1988; Campbell *et al.*, 1992; Grimason *et al.*, 1994; Alles *et al.*, 1995; Arrowood *et al.*, 1995; Zimmerman and Needham, 1995; Garcia and Shimizu, 1997). PCR techniques for identifying *C. parvum* have been described (Laxer *et al.*, 1991; Webster *et al.*, 1993), and further development has led to faster and simplified PCR techniques to detect oocysts in both faecal debris and water supplies (Leng *et al.*, 1996).

Recently an assay combining cell culture with reverse transcriptase PCR was developed. This enables water authorities to measure whether or not the detected *Cryptosporidium* oocysts in the water supply are a threat to the public safety (Rochelle *et al.*, 1997).

### 1.1.6. Pathogenicity

Cryptosporidiosis in humans is primarily a gastrointestinal disease with the main

clinical feature being diarrhoea. Other symptoms that have been reported are nausea, vomiting - which may lead to the reported infections in the respiratory tract - fever, abdominal pain, anorexia, dehydration, and weight loss (Wang, 1982; Current and Garcia, 1991). The disease can be split into two categories. That of the immunocompetent, well nourished host where the illness manifests itself as a self limiting diarrhoea lasting on average 3 to 12 days, and that in the immunodeficient patient where cryptosporidiosis may last indefinitely with symptoms of a cholera-like illness sometimes accompanied by extra-intestinal diseases like hepatitis, pancreatitis and respiratory problems (Current and Garcia, 1991). In addition, the diarrhoeal symptoms, together with malnutrition, lead to further weight loss with prolonged illness which is often fatal (Bogaerts *et al.*, 1984). Hence the immunological status of the host determines the extent and severity of the illness.

*Cryptosporidium parvum* occurs mainly in the epithelial cells in the gastrointestinal tract - predominantly in the ileum (Tzipori *et al.*, 1980; Hill *et al.*, 1991) - and (ultrastructurally), it was observed that the majority of infected cells (85%) were at the villus tip with crypt cells rarely being infected (Moore *et al.*, 1995). Maldigestion and malabsorption due to villous atrophy are thought to result in a watery diarrhoea containing fats, carbohydrates, proteins and fluid (Wang, 1982; Tzipori, 1988; Griffiths *et al.*, 1994). However, some infections of *C. parvum* that involve these morphological changes due to atrophy do not result in diarrhoeal symptoms, leading to the suggestion that alternative mechanisms are responsible for the illness (Sears and Guerrant, 1994). It has been suggested that an enterotoxin-mediated mechanism is involved since there is no inflammation and the diarrhoea is of a secretory nature (Garza *et al.*, 1986;

Massimillo *et al.*, 1995). Indeed, one study demonstrated that nine out of eleven diarrhoea samples tested from patients with cryptosporidosis were of an enterotoxic nature. However the finding that not all samples contained enterotoxin indicated other mechanisms, such as villous atrophy, may be at least partly responsible for the diarrhoea (Guarino *et al.*, 1995). Parasites in the trachea also cause flattening of the epithelial cells in the lining, along with a thickening of the mucosa cells. This leads to a severe cough, hoarseness, wheezing, shortness of breath and croup (Current and Garcia, 1991; Sterling and Arrowood, 1993). It has been reported that HIV-positive patients have a 17% prevalence of pulmonary cryptosporidiosis (Hojlyng and Jensen, 1988) although it is still considered that respiratory cryptosporidiosis is most likely secondary to an intestinal infection (Clavel *et al.*, 1996).

Two *Cryptosporidium* infections - a human case and that of a young foal (the first case reported in horses) - were reported to be complicated by an adenovirus infection (Bird and Smith, 1980). Additionally, a picobirnavirus was recently reported in association with a human *Cryptosporidium* infection (Gallimore *et al.*, 1995). The nature of these associations remains unclear but, as suggested, it may be possible that the viruses replicate and transmit better in individuals infected with *Cryptosporidium* (Gallimore *et al.*, 1995).

### 1.1.7. *In vitro* culture

Research on *Cryptosporidium* has been impeded by the lack of an *in vitro* culture system that is able to support the good development and growth of this protozoan

parasite. Intracellular growth is possible with *T. gondii* tachyzoites and, more recently, bradyzoites (Weiss *et al.*, 1994). Additionally, for *Eimeria*, reports have stated that first and second generation schizonts can be produced (Doran and Augustine, 1978). However, both *Eimeria* and *Cryptosporidium* cannot be serially propagated under *in vitro* conditions.

For *Cryptosporidium parvum* the following cell lines have been tested as host cells for *in vitro* culture and intracellular stages: mouse fibroblast L929 cells, human enterocyte HT29.74 cells, Caco-2 cells (Petersen, 1993), human intestinal cells, human embryonic cells, MDCK cells (Tzipori, 1988), HCT-8 cells (Upton *et al.*, 1995), RL95-2 cells (Rasmussen *et al.*, 1993) and MDBK cells (Villacorta *et al.*, 1996). However none was able to support *Cryptosporidium* propagation. Yet it was observed that human foetal lung cells, primary chicken kidney, porcine kidney cells and chicken embryos all supported development from sporozoite to sporulated oocyst (Current and Long, 1983; Current and Haynes, 1984). More recently, bovine fallopian tube epithelial (BFTE) cells were assessed for *in vitro* cultivation of *C. parvum* and compared with MDCK cells. The results showed that at 24 hours post-inoculation BFTE cells had an infection rate of 39%, whereas the rate with MDCK cells was much lower (4%) (Yang *et al.*, 1996). However autoinfection was not observed with either cell line.

Various media supplements have also been tested to see if they improve the growth of *C. parvum in vitro*. These include 10% foetal bovine serum; various sugars: glucose, galactose, maltose and mannose; insulin; and the four vitamins: ascorbic acid, calcium panthothenate, folic acid and *para*-aminobenzoic acid. All were found to enhance *C. parvum in vitro* development (Upton *et al.*, 1995) and a number of them also enhanced *E. tenella* development (Strout and Schmatz, 1990). Alternatively, glucose

availability had no effect on *E. tenella* development in MDBK cells (Strout and Schmatz, 1990). In addition to the nutrients present in the media, environmental pressures also seem to play a part in parasite development (Upton *et al.*, 1994), and it was demonstrated that invasion was increased two fold by a 4% oxygen environment compared to atmospheric oxygen tensions (Strout and Schmatz, 1990).

The failure of *in vitro* culture of *Cryptosporidium* seems to be due to the fact that few sporozoites are able to develop and that there is a lack of autoinfective (thin walled) oocysts as seen in the *in vivo* situation, thus *Cryptosporidium in vitro* only undergoes one developmental cycle (Tzipori, 1988; Rasmussen *et al.*, 1993). Additionally it has been observed that most microgametes fail to leave microgametocytes, again leaving the cycle incomplete *in vitro* (Upton *et al.*, 1995) disallowing any studies to be performed on *in vitro* development after a certain point in time. Nevertheless studies are progressing with recent reports stating that *C. parvum* can be cultivated for up to 15 days with oocysts being produced, however most of these oocysts remained intracellular and therefore subculturing still remained unsuccessful (Lawton *et al.*, 1996).

### 1.2. Biochemistry of the coccidia

At present hardly anything is known about the biochemistry of *Cryptosporidium*, thus the following review on the biochemistry of the coccidia compares *Cryptosporidium* primarily with *Eimeria*.

### 1.2.1. Energy metabolism

#### 1.2.1.1. Pathways of carbohydrate metabolism

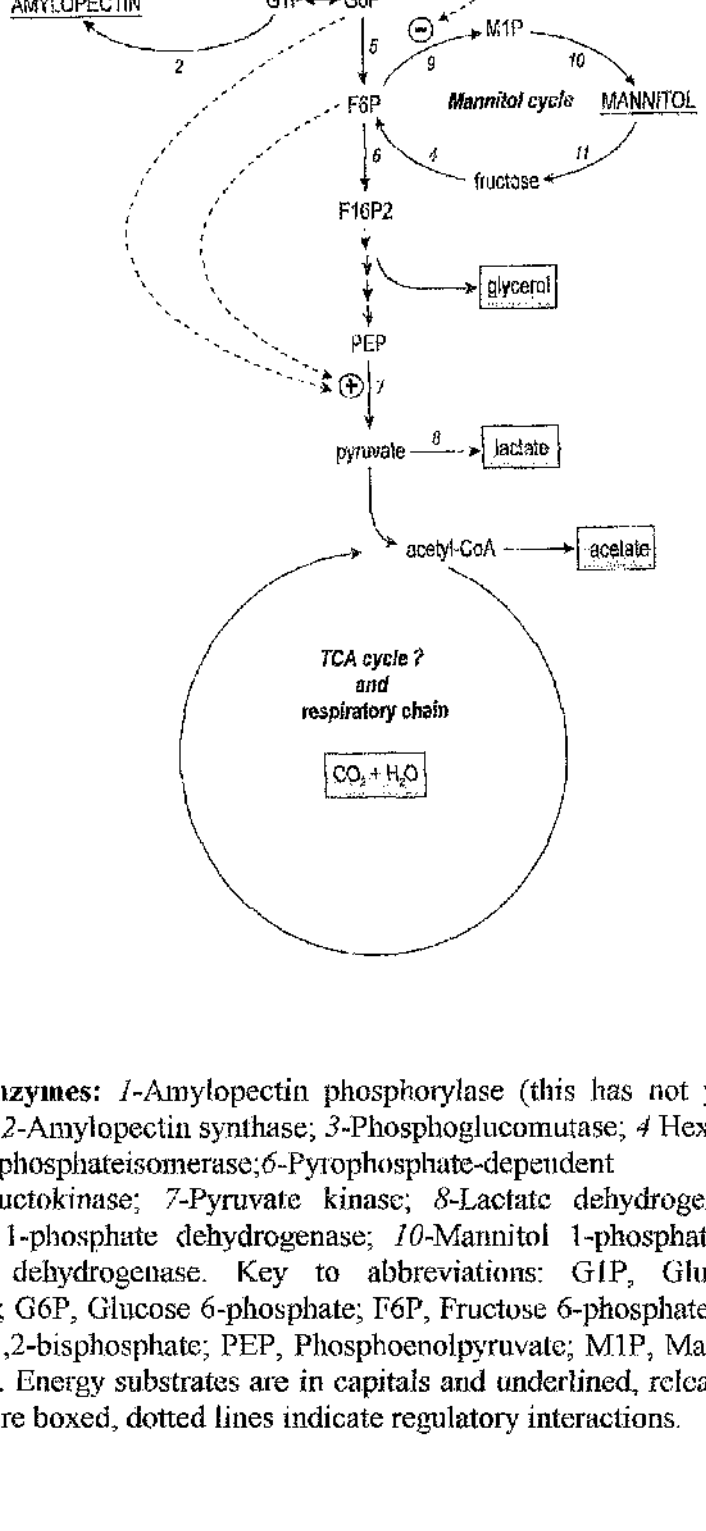
The pathways of carbohydrate metabolism in *E. tenella* are shown in Figure 1.2.1.1.

##### 1.2.1.1.1. Glycolysis

Glycolysis has been suggested to be the main source of energy for *C. parvum* due to the apparent lack of mitochondria and TCA cycle (Current, 1989) and it has also been observed that parasite development was enhanced under reduced oxygen tensions (Upton *et al.*, 1994).

The Embden-Meyerhoff glycolytic pathway (as seen in Figure 1.2.1.1. for *E. tenella)* in *C. parvum, E. tenella* and *T. gondii* is unusual in containing, at least in the stages investigated, a pyrophosphate-linked phosphofructokinase ($PP_i$-PFK) instead of the conventional ADP-linked enzyme (Peng and Mansour, 1992; Denton *et al.*, 1994, 1996a). These $PP_i$-PFKs appear to be of the type I variety which is normally associated with fermentative micro-organisms. Their distribution, and the fact that their use increases the energetic yield of glycolysis by 50%, suggest that these coccidia are adapted for anaerobic modes of energy production, at least for parts of their life cycle (Coombs and Muller, 1995). Like other micro-organisms containing $PP_i$-utilising glycolytic enzymes, the coccidia lack cytosolic pyrophosphatase activity. The key

**Figure 1.2.1.1.: Pathways of energy metabolism operating in *Eimeria tenella***




**Key to enzymes:** *1*-Amylopectin phosphorylase (this has not yet been reported); *2*-Amylopectin synthase; *3*-Phosphoglucomutase; *4* Hexokinase; *5*-Glucosephosphateisomerase;*6*-Pyrophosphate-dependent phosphofructokinase; *7*-Pyruvate kinase; *8*-Lactate dehydrogenase; *9*-Mannitol 1-phosphate dehydrogenase; *10*-Mannitol 1-phosphatase; *11*-Mannitol dehydrogenase. Key to abbreviations: G1P, Glucose 1-phosphate; G6P, Glucose 6-phosphate; F6P, Fructose 6-phosphate; F16P2, Fructose 1,2-bisphosphate; PEP, Phosphoenolpyruvate; M1P, Mannitol 1-phosphate. Energy substrates are in capitals and underlined, released end-products are boxed, dotted lines indicate regulatory interactions.

feature of type I $PP_i$-PFKs is their lack of regulatory features. Since PFK is a key enzyme in regulating glycolytic flux in most eukaryotes, organisms which possess type I $PP_i$-PFK's must utilise relatively unusual mechanisms of glycolytic control. This feature has now raised interest as a novel target for therapy in *T. gondii* (Peng *et al.*, 1995).

The PK from *C. parvum* shows no evidence of regulatory properties, and presents simple Michaelis-Menten kinetics with respect to both its substrates (Denton *et al.*, 1996a). The only other PK so far reported not to be under allosteric regulation is the type I enzyme from mammalian muscle (Fothergill-Gilmore and Michels, 1993). Yet in *E. tenella* and *T. gondii* it seems likely that glycolytic control is exerted, at least partially, through their pyruvate kinase. Unlike some other micro-organisms which have a $PP_i$-PFK, the coccidia species which have been investigated have an ADP-specific pyruvate kinase (PK) rather than a $PP_i$-specific activity (Denton *et al.*, 1994, 1996a). Hexokinase, another regulatory enzyme in most eukaryotes, appears to be unregulated, at least in *Eimeria.* Interestingly, the parasite appears to contain only one hexokinase which is capable of phosphorylating both glucose (as in glycolysis) and fructose (as in the mannitol cycle) (Schmatz *et al.*, 1989; Denton *et al.*, unpublished).

All coccidia species which have been investigated contain high levels of lactate dehydrogenase (LDH), the enzyme capable of mediating NADH oxidation under anaerobic conditions. The enzyme was purified from *E. stiedae* and characterised by Fransden and Cooper (1972). The enzyme subunits 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. Structural data have now been obtained through analyses of the genes. Interestingly, *Toxoplasma* has two LDH genes which appear to be entirely stage-specific (Yang and Parmley, 1995). These genes encode an unusual five amino acid insert around the active site. Fructose 1,6-diphosphate aldolase of *E. stiedae* has also been purified and identified as a type I enzyme typical of those found in mammalian cells (Mitchell and Daron, 1982; Wang, 1982).

**1.2.1.1.2. Mannitol metabolism**

The mannitol cycle appears to be a common feature of all coccidia. Both mannitol 1-phosphate dehydrogenase and mannitol 1-phosphatase have been detected in *C. parvum* (Schmatz, 1989) and specific antibodies have been used to show that mannitol 1-phosphate dehydrogenase, 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 *et al.*, 1997).

More research has been performed on *Eimeria*. When the total masses of lipid, protein and anthrone-sensitive carbohydrate were measured in unsporulated oocysts of *E. acervulina*, 25% of the oocyst dry mass was left unaccounted for (Wilson and Fairbairn, 1961). This missing component has now been shown to be predominantly mannitol (Schmatz, 1989; Schmatz *et al.*, 1989), a carbohydrate previously known to occur only in fungi. Further investigation revealed the presence of enzymes associated with a mannitol cycle. With the exception of hexokinase (which, as previously stated, can accept either glucose or fructose as substrates), the enzymes are all very specific in their reactions and their $K_m$s suggest that the pathway acts only in one direction (as

shown in Figure 1.2.1.1).

Schmatz and colleagues (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. In contrast, Michalski *et al.*, (1992) found only small amounts of mannitol (50-80 nmoles per $10^6$ oocysts) in unsporulated oocysts of the same species. They reported, however, that mannitol concentrations increased rapidly during the early stages of sporulation, concomitant with a decrease in amylopectin levels, and then diminished slowly and reached a basal level after 40 hours. These changes correlated with changing activities of the mannitol cycle enzymes. This discrepancy between the results of these two groups may be due to the fact that Michalski *et al.*, (1992) used oocysts obtained directly from the caecum rather than from faeces (the source used by Schmatz *et al.*, 1989) and that these may not have been fully mature such that the initial changes that occurred in their study may have been due to the final maturation of the oocysts which normally occurs within the host. Sporozoites also contain mannitol cycle enzymes and are capable of converting glucose into mannitol (Michalski *et al.*, 1992). Recent evidence suggests, however, that the synthetic part of the cycle is fully functional only during the sexual phase of the life cycle (Schmatz, 1997) 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 (Schmatz, 1997).

How the degradation of mannitol is regulated remains unknown. It seems that there is only one hexokinase isoenzyme (rather than one specific for glucose that participates in glycolysis and one specific for fructose that functions in mannitol

mobilisation) which is not tightly controlled. It is clear that there must be co-ordinated regulation of the fluxes to and from mannitol and amylopectin and through glycolysis, but the details remain to be elucidated.

However the function of the mannitol cycle in coccidia is uncertain, as indeed is the case for fungi. An obvious possibility is that it is acting as an energy reserve. However several other roles have also been proposed (see Schmatz, 1989; Schmatz, 1997) which include: (a) NADH generated during the breakdown of mannitol may be used directly for oxidative phosphorylation and so results in energy production; (b) the first part of the pathway may act as an electron sink for replenishment of $NAD^+$ under anaerobic conditions; (c) mannitol may act as an osmoregulator, keeping the oocyst wall rigid during maturation; (d) mannitol may have a protective effect against superoxide ions; (e) mannitol phosphate might be polymerised and act as a structural component in the oocyst or sporocyst wall.

**1.2.1.1.3. TCA cycle**

No TCA cycle enzymes have been detected in *C. parvum* and, of the classical TCA cycle enzymes, only malate dehydrogenase in sporulated oocysts of *E. tenella* was detected (Smith *et al.*, 1994). Both phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme were present, however, and it was concluded that *Eimeria* sporulated oocysts lack a conventional TCA cycle but contain a PEPCK by-pass similar to that in anaerobic protozoa such as *Giardia lamblia* and *Trichomonas vaginalis* (Coombs and Muller, 1995). These results, however, conflict with the following statement: "There is ample evidence indicating a functional tricarboxylic acid cycle in coccidia" (Wang,

1982). Yet the only evidence given as justification for this statement was the detection of isocitrate dehydrogense and malate dehdrogenase in unsporulated oocysts and the demonstration by cytochemical analysis of succinate dehydrogenase and isocitrate dehydrogense in *Toxoplasma*. Succinate dehydrogenase also was apparently detected cytochemically in several stages of *Eimeria* (Beyer, 1970; Michael and Hodges, 1973), however others have seen no presence of this activity (Denton, 1994; Smith *et al.*, 1994).

Thus there is evidence both for and against a functional TCA cycle. A possibility is that the TCA cycle is operative in only some life cycle stages of the parasite. Sporulation of eimerian oocysts occurs only under aerobic conditions. It can be inhibited by inhibitors of the respiratory chain (see 1.2.1.1.4), and results in carbon dioxide production (Nakai *et al.*, 1983), thus suggesting that this form of the parasite may also have a functional, but perhaps only partial, TCA cycle. Conversely, however, the end products released by eimerian sporozoites and their lack of sensitivity to respiratory inhibitors (see section 1.2.1.1.4.) are consistent with the TCA cycle playing little part in their energy metabolism. There are few data on other developmental stages.

**1.2.1.1.4. Respiratory chain**

*C. parvum* has not been reported to possess a mitochondrion which is consistent with the lack of detectable TCA cycle enzymes and antioxidant enzymes such as catalase (Entrala *et al.*, 1997). However mitochondria have been reported 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* and this is further discussed in Chapter 3.

In contrast, all developmental stages of *Eimeria* species possess distinctive elongate, cristate mitochondria. The processes of sporulation and excystation are associated with vigorous respiratory activity which is reversibly inhibited by cyanide and other inhibitors of electron transport, implying that it is mediated, at least partially, by a cytochrome-containing respiratory chain.

The detailed composition of the cytochrome chain in *Eimeria* 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 the uncoupler carbonyl *m*-chlorophenylhydrazone had no effect (Fry and Williams, 1984). However this lack of coupling may be a result of damage caused during the isolation procedure. Spectrophotometric analysis revealed absorbance maxima characteristic of a- and b-type cytochromes but no clear indication of a c-type cytochrome. Interaction with carbon monoxide suggested that there might be two a-type cytochromes present, cytochrome $a_3$ of cytochrome oxidase and an *o*-type 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. These findings are similar to those having been reported for *Plasmodium* which is thought to lack the NADH-Q reductase and use succinate dehydrogenase as the major

feed-in point (Fry, 1991).

The quinolone and pyridine coccidiostats and the 2-hydroxynapthoquinones act by blocking different sections of the respiratory chain. The use of these led to the suggestion that *E. tenella* has a branched or parallel electron transport chain. If so, resistance to one of the drugs could be mediated by electron transport being diverted towards the less sensitive of the pathways. 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.

Plastid-like organelles have been found to occur in coccidia and the DNA associated with them encodes components of an oxidative chain (Hackstein *et al.*, 1995) which may contribute to the cell's respiration (see section 1.2.2.2.)

**1.2.1.1.5. Other pathways**

No information is available on the following pathways for *Cryptosporidium* and so they focus on that which has been documented for *Eimeria.*

*Pentose phosphate pathway*

Glucose 6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway, has been purified and characterised from unsporulated oocysts of *E. stiedae* (Fransden, 1976, 1978). Like mammalian enzymes, the enzyme was specific for NADP and could accept glucose, at a low rate, as well as glucose 6-phosphate. 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 in the rapid growth situations of schizogony and gametogony where NADPH and ribose requirements would be high. Indeed, James (1980) has presented circumstantial evidence that the pathway is very active in isolated schizonts.

*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 possibly 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.

*Glyoxylate cycle*

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

### 1.2.1.2. Other catabolic pathways and enzymes

The possible use of amino acids as energy substrates has not been investigated in any great detail in any of the coccidia, although some studies have been performed on *Eimeria* and *Toxoplasma*. It was found that there was a marked decrease in the concentration of most free amino acids during sporulation of oocysts (Denton *et al.*, unpublished), however sporozoites of *E. tenella* did not consume amino acids from the incubation medium (Denton *et al.*, unpublished). 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, and 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 were mainly incorporated into parasite proteins (Krylov and Svanbaev, 1980).

ATPases are an important group of enzymes that regulate intracellular ATP and ion levels within cells (Pederson and Carafoli, 1987). ATPase activity has been detected by histochemical means in most stages of *Eimeria* (Michael and Hodges, 1973; Vetterling and Waldrop, 1976), with the activity in *Eimeria* sporozoites (Thong, unpublished) and *T. gondii* tachyzoites (Takeuchi *et al.*, 1980) having been characterised. Both parasites appear unusual in lacking (or having very low levels) of $Na^+/K^+$-ATPase, the enzyme responsible for maintaining high potassium ion concentrations within most eukaryotic cells. The apparent absence of this enzyme

within *Toxoplasma* led Takeuchi *et al.* (1980) to speculate that the parasite plasma membrane might be freely permeable to $Na^+$ and $K^+$ and that transmembrane fluxes of these ions occurring during transition between different environments (in particular during invasion of host cells) might be important effectors of the parasite's metabolism. In apparent support of this conjecture, they demonstrated that the parasite's protein synthesis was markedly stimulated by potassium ion concentrations up to 150 mM and also showed that there were significantly higher levels of $Na^+$ than $K^+$ in lysates of tachyzoites. While lacking a $Na^+/K^+$ ATPase, both *Eimeria* and *T. gondii* contain $Mg^{2+}$-ATPase activity (considered likely to be of the mitochondrial variety which participates in oxidative phosphorylation). $Ca^{2+}$-ATPase activity was also detected in membrane preparations of the parasites. The $Mg^{2+}$- and $Ca^{2+}$-ATPases of *Eimeria* presented similar kinetic parameters and pH optima to the equivalent ATPases in chick liver cells, but had significantly different inhibitor sensitivities (Thong, unpublished). In particular, the parasite enzymes were much less affected by azide than their host cell counterparts while N-ethyl maleimide proved to be a potent inhibitor of the eimerian $Ca^{2+}$-ATPase but had little effect on the host enzyme. The activities of a number of ionophores and synthetic anticoccidials were investigated but appeared not to inhibit the ATPases in *Eimeria*.

### 1.2.2. Nucleic acids

#### 1.2.2.1. Genes and proteins

Information on a number of coccidian genes and the corresponding proteins has

been gained including heat shock proteins (HSPs) in *Eimeria* (Laurent *et al.*, 1994; Dunn *et al.*, 1995; Clark *et al.*, 1996), *Cryptosporidium* (Khramtsov *et al.*, 1995) and *Toxoplasma* (Lyons and Johnson, 1995;) which have been suggested to possibly target proteins to the apical complex; tubulin in *Cryptosporidium* (Edlind *et al.*, 1994, 1997) and *Eimeria* (Zhu and Keithly, 1996); NAD transhydrogenase in *Eimeria* (Kramer *et al.*, 1993) which encodes a protein apparently associated with the refractile body (Vermeulen *et al.*, 1993); acetyl CoA synthetase and hemolysin in *C. parvum* (Khramtsov *et al.*, 1996 and Steele *et al.*, 1995 respectively). However, some of the genes show no significant homology with any already characterised, for example, the acetyl CoA synthetase and hemolysin of *Cryptosporidium*, and so the role of the gene product remains to be elucidated. Analysis of RNA levels themselves and the expression of different genes has shown that there is variation during the life cycle (Ellis and Thurlby, 1991; Herbert *et al.*, 1992; Abrahamsen *et al.*, 1994, 1995) thus providing useful new insights into the specific adaptations of individual developmental stages. Molecular techniques have progressed most rapidly with *Toxoplasma* and are now used widely (Sibley *et al.*, 1993; Donald and Roos, 1994; Sibley *et al.*, 1994b; Messina *et al.*, 1995; Soldati *et al.*, 1995; Seeber and Boothroyd, 1996).

The ways in which nucleic acids are synthesised, processed and catabolised in coccidia have not been studied to any great extent. Ribonuclease P of *T. gondii* has been partially characterised (Mack *et al.*, 1994), a type II topoisomerase gene has been identified in *C. parvum* (Christopher and Dykstra, 1994) and it has been reported that DNA polymerase activity of *T. gondii* correlated with virulence (Makioka and Ohtomo, 1995).

### 1.2.2.2. Plastids of the coccidia

More recently extranuclear circular DNA closely related to that of plastids has been found in coccidia (Egea and Lang-Unnasch, 1995; McFadden *et al.*, 1996). Similar DNA appears to be a characteristic of the Apicomplexa and is reported to be a 35 kb circular molecule (Köhler *et al.*, 1997) which has been studied extensively in *Plasmodium* (Williamson *et al.*, 1994; Wilson, 1997) and *T. gondii* (Köhler *et al.*, 1997). Köhler *et al.* (1997) reported this DNA molecule - which is similar to chloroplast genomes - to be localised to a membranous region in the tachyzoite, next to the nucleus and distinct from either the mitochondria or golgi apparatus. It appears, however, that there is high conservation between the different groups of parasites and the name 'Apicoplast' has been suggested for the organelle (Köhler *et al.*, 1997). The DNA includes genes encoding RNA polymerase, tRNA and various ribosomal proteins (Jeffries and Johnson, 1996). Evidence has also been presented for the presence of a chlorophyll *a*-D1 complex in *Toxoplasma* (Hackstein *et al.*, 1995) and, as this is a key component of the electron transport chain of plastids, this is highly suggestive of electron transport occurring in the organelle. The importance and functional significance of the plastid is still a matter of debate but it is possible that in addition to energy metabolism it provides essential proteins. The findings that some herbicides - thought to act by inhibiting the components of the electron transport chain encoded by the plastid - have anticoccidial activity, (Hackstein *et al.*, 1995) and that malaria parasites are sensitive to inhibitors of plastid metabolism (see McFadden *et al.*, 1996) has led to great interest in this area.

It has been reported that some *Eimeria* may contain viral nucleic acids (Revets *et al.*, 1989; Ellis and Revets, 1990; Roditi *et al.*, 1994; Lee *et al.*, 1996). Sporulated oocysts of *E. nieschulzi* were seen to have an RNA-dependent RNA polymerase activity which was absent in both *E. tenella* and *E. acervulina*. This activity correlated with an unknown nucleic acid species which may represent the genomic RNA of a possible virus (Sepp *et al.*, 1991; Roditi *et al.*, 1994). In another study, performed on *E. necatrix* and *E. maxima*, RNA molecules possibly of viral origin were isolated and thought to be located in the cytoplasm of the parasite (Ellis and Revets, 1990). Those of *E. necatrix* were icosahedral and 42–44 nm in diameter with no envelope (Lee *et al.*, 1996).

The implications of these virus-like-particles (VLPs) have not been determined.

### 1.2.3 Protein and amino acid metabolism

#### 1.2.3.1 Protein synthesis

Few studies have reported on coccidian protein synthesis and have mainly been limited to determine the sensitivity of the parasites to some standard protein synthesis inhibitors which have activity against coccidia *in vitro* and *in vivo* suggesting that protein synthesis is an important target for chemotherapy. Hence little detail is known of the mechanisms of protein synthesis. The gene encoding elongation factor-2, a protein essential for protein synthesis, has been cloned from *C. parvum* (Jones *et al.*, 1995), and *Toxoplasma* has been shown to contain a cyclophilin (High *et al.*, 1994; Page *et al.*, 1995) thought to be involved in protein folding.

### 1.2.3.2. Protein catabolism

Parasite proteinases have attracted considerable attention in recent years (McKerrow, 1989; North *et al.*, 1990; McKerrow *et al.*, 1993; North and Lockwood, 1995; Coombs and Mottram, 1997), largely because they are considered to be good targets for chemotherapeutic attack. Coccidia undoubtedly possess many proteinases themselves, as is thought to be the case for all eukaryotic cells, but only a few have been reported to date and these are described in the introduction of Chapter Four.

### 1.2.4 Polyamine metabolism

Effects of inhibitors of ornithine decarboxylase (ODCase) and S-adenosylmethionine decarboxylase (SAMDCase) have provided the most useful information on the significance of polyamine metabolism in the coccidia species. However a recent report indicates that *C. parvum* polyamine biosynthesis occurs via the enzyme arginine decarboxylase (ADC) rather than ODC with α-DL-difluoromethylornithine (DFMO - an enzyme-activated irreversible inhibitor of ODCase and an effective antitrypanosomal drug) having no effect on growth (Yarlett *et al.*, 1996).

The efficacy of DFMO against *E. tenella* infections in chickens was investigated by Hanson *et al.* (1981). When adminstered at 0.5% in drinking water, starting one day before infection and continuing for five days, the compound was as effective in preventing symptomatic coccidiosis as was 0.012% of amprolium. Following a 'cure' with DFMO, the birds were immune to reinfection by the parasite

when challenged one week later. If, however, putrescine was administered along with the DFMO then no cure was effected. These results are similar to those obtained with other parasites and imply that DFMO acts specifically by blocking putrescine production, that its effects are cytostatic rather than directly cytotoxic, and it is ineffective if exogenous putrescine is available and can be scavenged by the parasite.

There have been very few reports on polyamine metabolism in coccidia. Spermidine was detected in sporulated oocysts of *E. acervulina* (van der Horst and Kouwenhoven, 1973). SAMDCase has also been investigated using crude extracts of *E. stiedae* oocysts (San-Martin Nunez *et al.*, 1987). The enzyme differed from its counterparts in rat or rabbit liver in having a considerably lower $K_m$, a slightly higher pH optimum and being relatively insensitive to several potent inhibitors of the mammalian enzyme.

### 1.2.5. Lipid metabolism

Lipid distribution has been investigated using cytochemical techniques with several coccidia. Large numbers of intensely staining lipid droplets are present in the macrogametocyte stages of *E. tenella*, *T. gondii* (Ryley, 1973) and *Isospora belli* (Lindsay and Blagburn, 1994). At least in *Eimeria*, these droplets appear to persist through the process of zygote development and into the sporulated oocysts, where they accumulate within the sporocyst structures. Small lipid droplets have also been reported in the microgametocytes of the above mentioned species, as well as in the sporozoites of *Eimeria*. In the remaining stages of *Eimeria*, as well as the tachyzoites of *Toxoplasma*,

lipid staining is very diffuse and suggestive of only structural rather than storage lipids (Ryley, 1973; Fransden, 1970). Although the presence of these lipid droplets suggests a role as an energy store, the part that they play is far from clear. Using quantitative mass spectroscopy, Wilson and Fairbairn (1961) found that during sporulation the lipid content of *E. acervulina* oocysts decreased by about 50% and they concluded that the lipids were being used as an energy substrate. However a contradictory conclusion was obtained by Weppelman *et al.* (1976), working with *E. tenella* oocysts. Using gravimetric analysis they found that although the fatty acid content of the oocysts fell by around 50% during sporulation, this decrease was almost compensated for by a 30% increase in the weight of non-saponifiable lipids (in particular C24 and C26 fatty alcohols). From this they concluded that there was no net oxidation of lipids during sporulation, but rather a general incorporation of fatty acids into fatty alcohols. The newly synthesised fatty alcohols appeared to remain in the cytoplasm, although whether in the oocyst fluid, the sporocysts or the sporozoites was not determined.

Compositional studies have been carried out on tachyzoites of *T. gondii* (Foussard *et al.*, 1991), sporulated and unsporulated oocysts of *E. tenella* (Weppelman *et al.*, 1976; Stotish *et al.*, 1978), and oocysts of *C. parvum* (Mitschler *et al.*, 1994). In the last study, the host cells (Madin-Darby Bovine Kidney (MDBK)) cells, which are often used for *in vitro* growth of coccidial parasites) were also analysed with the results shown in Table 1.2.5.1. Phosphatidylcholine (PC) represents the major phospholipid in both *T. gondii* and *C. parvum*, and was also detected in the oocyst walls of *E. tenella*. The proportion of PC in both the former two parasites was found to be high in comparison with MDBK cells and some other protozoa, for example *Plasmodium falciparum* (45%)

*Trypanosoma cruzi* (44%).

Hexacosanol is the predominant lipid present (~60%) in the oocyst walls of *E. tenella* with phosphatidylcholine, ethanolamine, serine and lysophosphatidylcholine being the four predominant phospholipids (Stotish *et al.*, 1978).

The phospholipid composition of *C. parvum* cysts is unusual in that the amount of cardiolipin (CL) is extremely low compared, for example, with the levels in MDBK cells. CL in eukaryotic cells is almost exclusively a component of the inner mitochondrial membrane, so this observation is consistent with the suggestion that

**Table 1.2.5.1.: Phospholipid composition of some coccidia extracts**

| Phospholipid | *Eimeria tenella* (oocyst wall) | *Toxoplasma gondii* (tachyzoites) | *Cryptosporidi--um parvum* (oocysts) | MDBK cells |
|---|---|---|---|---|
| PC | √ | 62.0 ± 4.2 | 65.5 ± 4.1 | 40.6 ± 1.8 |
| PE | √ | 11.2 ± 4.2 | 7.3 ± 1.8 | 27.2 ± 3.6 |
| PI/PS | √ | 15.0 ± 5.6 | 1.8 ± 0.9 | 12.6 ± 2.3 |
| SM | ? | 8.0 ± 4.3 | 24.4 ± 5.2 | 14.0 ± 1.5 |
| CL | ? | ? | 0.9 ± 0.8 | 5.8 ± 0.5 |
| LPC | √ | ? | ? | ? |

Results are expressed % of total phosphate. Means ± SD. Key: PC-phosphatidylcholine; PE-phosphatidylethanolamine; PI-phosphatidylinositol; PS-phosphatidylserine; SM-sphingomyelin; CL-cardiolipin; LPC-lysophosphatidyl choline; √, detected but not quantified; ?, not investigated.

*C. parvum* either lacks a mitochondrion altogether or has only a rudimentary structure (Current, 1989). It should be remembered, however, that CL was not detected at all in *T. gondii* and this parasite contains structures that resemble mitochondria on morphological criteria along with evidence for a functional TCA cycle and respiratory

chain.

The fatty acid compositions reported for coccidia are summarised in Table 1.2.5.2. It is important to take into account that the various sets of data are not directly comparable as they refer to different lipid fractions. However, *Toxoplasma* and *Cryptosporidium* are similar in containing 16:0, 18:1 and 18:2 as their major acyl chains while *Eimeria* contains predominantly 18:1 and has only trace amounts of 18:2. Recently the effect a high n-3 fatty acid on chickens infected with *E. tenella* was

**Table 1.2.5.2.: Fatty acid composition of some coccidia extracts**

| Fatty acid | *Eimeria tenella* | | *T. gondii* | *C. parvum* (oocysts) | |
|---|---|---|---|---|---|
| | Unsporulated oocysts | Sporulated oocysts | (tachyzoites) | phospholipids | neutral lipids |
| C 14:0 | 1 | 2 | 9 | 0 | 5 |
| C 16:0 | 12 | 8 | 17 | 31 | 27 |
| C 16:1 | 1 | 3 | nd | trace | 3 |
| C 18:0 | 10 | 17 | 11 | 16 | 20 |
| C 18:1 | 75 | 68 | 31 | 22 | 27 |
| C 18:2 | trace | trace | 20 | 29 | 15 |
| C 20:0 | nd | nd | 6 | trace | 1 |

Results are expressed as % of total phospholipid in the fraction.
nd: not detected.
Data taken from: Weppelman *et al.*, 1976; Stotish *et al.*, 1978; Foussard *et al*, 1991; Mitschler *et al.*, 1994.

studied, with the hypothesis being that the fatty acids cause nutritionally oxidative stress. The effect was that degeneration of both asexual and sexual stages of the parasite was observed (Danforth *et al.*, 1997).

Mitschler *et al.* (1994) noted that the fatty acid profile of *C. parvum* was significantly different from that of MDBK cells and concluded that *C. parvum* must have the ability either to synthesize or selectively sequester specific acyl chains.

Cholesterol is the only sterol that has been detected in either *Toxoplasma* or *Cryptosporidium*, and it has also been identified in *Sarcocystis* and *Eimeria* species. In *Eimeria*, several other components are also found in the non-saponifiable lipid fraction. From their retention times in gas-liquid chromatographic analysis, two of these components in *E. acervulina* were suggested to be squalene and progesterone (Van der Horst and Kouwenhoven, 1973). However, Weppelman *et al.* (1976) used *E. tenella* extracts and found no evidence for these compounds but identified the non-saponifiable lipids as cholesterol and a range of fatty alcohols of 22-32 carbons (hexacosinol was the most abundant). In the unsporulated oocysts, the majority of these alcohols were located in the oocyst wall. It seems likely that they contribute to the impermeability and general resistance of this structure.

### 1.2.6. Treatment and control

As cryptosporidiosis is self-limiting in immunocompetent patients, oral or intravenous hydration is a sufficient therapy (Fayer and Ungar, 1986). However, in immunodeficient patients, for example AIDS patients, hydration is simply not enough since cryptosporidiosis is often severe and may be fatal. Chemotherapy against *Cryptosporidium* has proved ineffective (Tzipori, 1988) and there are few drugs active against *Cryptosporidium*, far fewer than against other coccidia. Many studies using various drugs have been carried out with some having been reported to inhibit *C.*

*parvum* development *in vitro*. In some, an enzyme linked immunosorbent assay (ELISA) was used to evaluate growth and effects of various anti-microbials (Woods *et al.*, 1995). Whilst not all studies report the same activities, the data suggest that monensin and halofuginone inhibit parasite development by more than 90% (Yvore and Naciri, 1989; McDonald *et al*, 1990).

Dinitroaniline herbicides are known to inhibit plant tubulin polymerisation, thus arresting mitosis in cells (Arrowood *et al.*, 1996). More recently, these compounds have been reported to have antiproliferative effects on a number of protozoan parasites including other apicomplexans. A study on the anticryptosporidial activity of dinitroaniline herbicides *in vitro* concluded that of the five tested all exhibited anticryptosporidial activity with no toxicity to the host cell itself (Arrowood *et al.*, 1996).

A newly discovered fungal product known as Apicidin also has interesting anticryptosporidial activity by inhibiting apicomplexan histone deacetylases (HDA) and therefore transcriptional control (Darkin-Rattray *et al.*, 1996).

However many of the drugs active *in vitro* showed little or no activity *in vivo*. Using animal models, halofuginone was seen to prevent acute cryptosporidiosis in the ileum of infected rats, yet it was less effective for chronic cryptosporidiosis of the colon - which is usually the case in immunocompromised patients (Rehg, 1995). Azithromycin and sinefungin were also reported to prevent infection in immunosuppressed rats (Rehg, 1991; Brasseur *et al.*, 1993). However, against *C. serpentis* infections halofuginone and spiramycin did not provide any therapeutic aid (Graczyk *et al.*, 1996).

In clinical trials a study performed on zidovudine claimed that a HIV-positive

patient fully recovered from *Cryptosporidium* after a four week course of treatment (Greenberg, *et al.*, 1989). This may work indirectly by restoring T cell ($CD4^+$) numbers as it kills the HIV virus. Alternately, Nousbaum *et al.* (1991) reported that zidovudine alone could not resolve cryptosporidiosis but when used with SMS 201-995 (a somatostatin analogue) the infection was controlled within 15 days of treatment. Additionally spiramycin (a macrolide antibiotic) has been reported to resolve *C. parvum* infections in immunosuppressed patients (Portnoy *et al.*, 1984) whereas diloxanide furoate, furazolidone, interleukin-2, quinine and clindamycin were all reported to ease symptoms (Sterling and Arrowood, 1993). More recently paromomycin, an antibiotic poorly absorbed by intact gut epithelial cells (Hoepelman, 1996), has been used in clinical trials. The results showed all patients responded to the drug and there was improvement in both clinical and parasitological parameters (Armitage *et al.*, 1992; White *et al.*, 1994). Paromomycin and maduramicin also exhibited anti-cryptosporidial activity as assessed using a chemiluminescence immunoassay to evaluate *in vitro* growth of *C. parvum* (You *et al.*, 1996). Additionally, paromomycin succeeded in curing respiratory cryptosporidiosis (Mohri *et al.*, 1995).

Methods other than chemotherapy that have potential for preventing cryptosporidiosis have also been researched. Bovine colostrum hyperimmune to *Cryptosporidium* was seen to eliminate the infection of an AIDS patient after as little as 48 h direct duodenal infusion (Ungar *et al.*, 1990). However, other studies have reported no success using bovine colostrum (Tzipori, *et al.*, 1986; Saxon and Weinstein, 1987) whereas others report no benefit from feeding children with immune mother's milk (Sterling *et al.*, 1991).

An alternative method of reducing the prevalence of the infection is to eradicate oocysts from the water supply. Unfortunately, *Cryptosporidium* oocysts are extremely resistant to the majority of common disinfectants (Campbell *et al.*, 1982). Recent studies have shown that ozone may be useful in controlling oocyst numbers (Owens *et al.*, 1994). Both ozone and chlorine dioxide proved to be more effective than chlorine and monochlorine (Korich *et al.*, 1990). However these studies also showed that *C. parvum* oocysts are some 40x fold more resistant to ozone and usual water treatments than are *Giardia* cysts (Korich *et al.*, 1990), indicating that these cleansing techniques cannot be relied upon to eradicate infective *C. parvum* oocysts from the water supply.

The present situation has therefore led researchers to investigate disinfectants that may be used other than those used routinely. However the quest to find a compound that is effective in destroying oocyst viability but is not harmful to both humans and animals continues.

The search for drugs effective against *Eimeria* has been more successful than that involving *Cryptosporidium*. The drugs of choice for avian *Eimeria* are polyether ionophores (Dutton *et al.*, 1995, and see Table 1.2.6.1.) which have captured approximately 70% of the current market. It is anticipated that these compounds will dominate this market for many years to come because there has been no commercial launch of any novel class of synthetic or semisynthetic avian anticoccidial drug since the early 1980s. This is a major concern as resistance problems have arisen with current agents (Chapman, 1993; Edgar, 1993; Vertommen and Peek, 1993). However experimental recombinant vaccines are now being developed and perhaps

**Table 1.2.6.1.: Mechanism of action of commercial avian anticoccidial drugs**

| Compound Class | Examples | Mechanism of action |
|---|---|---|
| Polyether ionophore | Salinomycin | Perturb ion gradients |
| | Lasalocid | |
| | Maduramicin | |
| Carbanilide/pyrimidine | Nicarbazin | Oxidative phosphorylation uncoupler? |
| Febrifugine | Halofuginone | Not known |
| Triazine | Diclazuril | Pyrimidine metabolism? |
| | Toltrazuril | Mitochondrial respiration? (see Harder and Haberkorn, 1989) |
| | | Chlorophyll *a*-D1complex? (see Hackstein *et al.*, 1995) |
| Quinolone | Decoquinate | Mitochondrial respiration/electron transport |
| | Buquinolate | Topisomerase? |
| Pyridinol | Clopidol | Mitochondrial respiration/electron transport |
| Thiamine analogue | Amprolium | Thiamine uptake and utilisation |
| Nitrobenzamide | Zoalene | Nicotinamide antagonist? |
| | Nitromide | |
| Guanidine | Robenidine | Oxidative phosphorylation uncoupler? |
| Benzylpurine | Arprinocid | Purine salvage? |
| | | Ion chelation (N-oxide active metabolite)? |
| Organic arsenical | Roxarsone | Binds protein sulphydryl groups? |
| Polyketide | Oxytetracycline | Protein synthesis? |
| | Chlortetracycline | |
| Sulphonamide | Sulphadimethoxine | Dihydropteroate synthetase |
| | Sulphaquinoxaline | |
| Aminopyrimidine and Sulphonamide | Ormethoprim and | Dihyrofolate reductase and |
| | Sulphadimethoxine | Dihydropteroate synthetase |

they will set the trend for cheap avian coccidiosis control in the future (Buxton, 1993; Wallach, 1993; Barriga, 1994; Buxton and Innes, 1995). However elucidation of the mechanisms of action and resistance of current drugs may lead to novel chemical strategies for obtaining the new anticoccidial drugs that are urgently required.

Yet in most cases the precise mechanism of action of the avian anticoccidial drugs listed in Table 1.2.6.1. is not known. When compared with the detailed information available on, for instance, many antimalarial drugs, 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 Stout, 1980; Maes *et al.*, 1988; Verheyen *et al.*, 1988; Guyonnet *et al.*, 1990; Daszak *et al.*, 1991; Rather *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). Despite the major impact that drug resistance has had on anticoccidial chemotherapy (Chapman, 1993; Edgar, 1993; Vertommen and Peek, 1993), and studies on the basis of resistance to some compounds (Pfefferkorn *et al.*, 1989; Pfefferkorn and Borotz, 1994), the mechanisms of drug resistance have not been elucidated in detail in any case. More basic but focused research on coccidia is needed to provide better understanding of drug-parasite interactions.

There have been several recent reports of novel experimental compounds with

**Table 1.2.6.2.(a):. Putative mechanisms of action of potential anticoccidial agents**

| Compound | Organism | Activity | Putative Mechanism | Reference |
|---|---|---|---|---|
| Azithromycin | *Cryptosporidium*<br>*Toxoplasma* | *in vitro/in vivo* | Protein synthesis | Araujo *et al.*, 1991<br>Rehg, 1991<br>Araujo and Remington, 1992<br>Blais *et al.*, 1993a<br>Vargas *et al.*, 1993 |
| Clarithromycin | *Toxoplasma* | *in vivo* | Protein synthesis | Edlind, 1991<br>Georgiev, 1994 |
| Roxithromycin | *Toxoplasma* | *in vivo* | Protein synthesis | Araujo *et al.*, 1991 |
| Paromomycin | *Cryptosporidium* | *in vivo* | Protein synthesis | Edlind, 1991<br>Fayer and Ellis, 1993 |
| Rifabutin | *Toxoplasma* | *in vivo* | DNA-dependent RNA polymerase | Araujo *et al.*, 1994, 1996a,b<br>Olliaro *et al.*, 1994 |
| Sinefungin | *Cryptosporidium* | *in vivo* | Methylation reactions? | Brasseur *et al.*, 1994 |
| Artemesinin | *Toxoplasma* | *in vitro* | Free-radical?<br>Haem polymerisation? | Holfels *et al.*, 1994<br>Hong *et al.*, 1994 |
| Hydroxynaphthoquinone (Atovaquone) | *Toxoplasma*<br>*Eimeria* | *in vitro/in vivo* | Mitochondrial electron transport (Complex III) | Fry and Williams, 1984<br>Fry *et al.*, 1984<br>Fry and Pudney, 1992<br>Romand *et al.*, 1993 |
| Epiroprim | *Toxoplasma* | *in vitro/in vivo* | Dihydrofolate reductase | Chang *et al.*, 1994<br>Martinez *et al.*, 1996 |

**Table 1.2.6.2.(b): 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<br>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 |
| | *Toxoplasma* | | P-glycoprotein? | Rose and Hesketh, 1989<br>Harrison and Stein, 1990<br>Page *et al.*, 1995 |
| Primaquine | *Eimeria* | *in vivo* | Via free-radical? | Matsuno *et al.*, 1991 |
| | *Sarcocystis* | | Oxidative phosphorylation? | Bisby, 1990<br>Baker *et al.*, 1986 |
| Hydroxycoumarin | *Eimeria* | *in vivo* | Not known | Int. Pub. No. WO 92/06083, 1992 |
| Xanthoquinodin | *Eimeria* | *in vitro/in vivo* | Not known | Tabata *et al.*, 1993a |
| WS-5995 | *Eimeria* | *in vitro/in vivo* | Not known | Devi *et al.*, 1994 |
| Benzimidazole | *Eimeria* | *in vivo* | Not known | Eur. Pat. No. 931172243.1, 1993 |
| Diolmycin | *Eimeria* | *in vitro* | Not known | Tabata *et al.*, 1993c |
| Cytosaminomycin | *Eimeria* | *in vitro* | Not known | Haneda *et al.*, 1994 |
| Hynnapene | *Eimeria* | *in vitro* | Not known | Tabata *et al.*, 1993b |

anticoccidial activities including those against *Cryptosporidium*. Some of these interesting anticoccidial leads and their putative biochemical actions are given in Tables 1.2.6.2.(a) and (b). It is apparent that macrolides/azalides are well represented, 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. It remains to be seen whether any of these compounds or their analogues will become viable commercial products.

### 1.2.7. The oocyst wall

The 'thick walled' oocysts of *C. parvum* have three membranes and an outer wall of two chitinous layers, and the thin walled oocysts have a single unit membrane (Sterling and Arrowood, 1993). More recently, *Cryptosporidium* oocysts were reported to be similar to those of *Eimeria* in having two walls (Petersen, 1993). The first cloned *C. parvum* oocyst wall protein (COWP) has been ultrastructurally located to the inner layer of the oocyst wall (although the resolution could not distinguish the two layers of the wall) (Spano *et al.*, 1997).

Three species of *Cryptosporidium* were analysed for oocyst wall composition using surface labelling and antibodies (Tilley *et al.*, 1990; Nina *et al.*, 1992). The oocysts of *C. baileyi, C. muris* and *C. parvum* could all be distinguished, but *C. parvum* and *C. baileyi* were shown to be more similar to each other than either was to *C. muris*. Additionally oocysts of the same species of *Cryptosporidium* have been seen to vary in antigenic profiles according to their geographic location (Nina *et al.*, 1992) and different isolates vary in virulence (Petersen, 1992).

The oocyst walls of *E. tenella* have two layers, an outer 10 nm layer and an inner 90 nm layer (Stotish *et al.*, 1978). Studies performed using gas liquid chromatography have revealed that glucose is the predominant neutral sugar with small amounts of mannose and galactose (Stotish *et al.*, 1978). Unsporulated oocyst walls were found to be 67% protein, 14% lipid and 19% carbohydrate. They contain an unusual polysaccharide, initially thought to be chitin but which on further analysis was found to differ in detail (Ryley, 1973). One of the protein components has been characterised (Eschenbacher *et al.*, 1996).

### 1.2.8 Functional surface molecules

Parasite surfaces have received particular attention from scientists because of their role in the initial interaction of a parasite with its host. However many of the studies have used limited approaches for analysing surface molecules and most of the molecules detected in this way have been characterised only with respect to their mobility in gels and the presence or absence of carbohydrates or lipids. Very little research has been focused on this area in coccidia.

### 1.2.9 Glycosylation

There have been several reports on the presence of glycoproteins in *Eimeria* and *Toxoplasma* (Schwarz *et al.*, 1993), but in most cases the subcellular location of the molecules has not been established. Dieckmann-Schuppert *et al.* (1992) reported that exogenous dolichol pyrophosphoryl-oligosaccharides were utilised by *T. gondii* for

glycosylation of its proteins. It was not until 1993 that biochemical evidence was obtained showing that *T. gondii* tachyzoites synthesise N-linked glycoproteins and the lipid-linked glycan precursor for N-linked oligosaccharides (Odenthal-Schnittler *et al.*, 1993). Additionally in *E. tenella* a protein extracted from oocysts walls indicated that O-linked carbohydrates were present (Eschenbacher *et al.*, 1996).

### 1.2.10 GPI-anchors

Many surface molecules are attached to the plasma membrane via glycosylphosphatidylinositol (GPI) anchors. However, this is an area in which coccidia have been investigated in much less detail than some other protozoan parasites. The major surface proteins on the tachyzoites of *T. gondii* do have GPI-anchors (Nagel and Boothroyd, 1989; Tomavo *et al.*, 1992a) and the parasite is capable of synthesising GPI and also N- and O-glycans (Tomavo *et al.*, 1992b; Schwarz and Tomavo, 1993). Some undefined antigens on the surface of *Eimeria* sporozoites are also attached via GPI-anchors (Gurnett *et al.*, 1990).

### 1.2.11 Lectins

Lectins are the class of carbohydrate-binding proteins present on most cell surfaces and are thought to be involved in cell-cell interactions, including host-parasite invasion. N-acetylglucosamine-specific lectins were seen to be the main ones of *C. parvum* oocysts (Llovo *et al.*, 1993). The sporozoites of *C. parvum* have also been studied for lectins with the aim of elucidating their role in

sporozoite attachment to host cells (Thea *et al.*, 1992). Of the glycoproteins tested, bovine submaxillary mucin (BSM) and the blood group antigen-related $P_1$ glycoprotein proved to be the most inhibitory on sporozoite haemagglutination activity. Galactose and N-acetylgalactosamine were the most effective monosaccharide inhibitors (Joe *et al.*, 1994). Using invasion studies with MDCK cells, *C. parvum* sporozoites pre-incubated with BSM or fetuin showed a reduction in attachment by 77% and 63%, respectively (Joe *et al.*, 1994). Invasion was also reduced by 28% and 27%, respectively, thus supporting the theory that lectins do play a major role in *C. parvum* host cell invasion. Additionally it has been reported that both *Toxoplasma* and *Cryptosporidium* have a thrombospondin-related adhesive protein that appeared to be located, at least in part, on the sporozoite surface and is likely to be involved in binding to the prospective host cell.

Three *Eimeria* species and various developmental stages of *E. tenella* were evaluated for lectin activity using haemagglutination. It was found that *Eimeria* has lectins that are developmentally regulated (Strout *et al.*, 1994), suggesting stage-specific functions. The lectin specificity of each species was also found to be different. L(-)-fucose and D(+)-arabinose inhibited invasion of *E. tenella* and *E. acervulina*, respectively, whilst none of the 30 monosaccharides tested inhibited *E. maxima* (Strout *et al.*, 1994). Additionally the pH optima of the lectins from the three species was found to differ and, to an extent, correlate with the *in vivo* conditions found in the part of the gut that each infects (Strout *et al.*, 1994). These results suggest that lectins may play a role in site-selection by these coccidial parasites (see Chapter 3 for further details). Lectins seem to play a role in host cell attachment, although the study of Baba *et*

*al.* (1996) suggested that a lectin-like receptor on the host cell recognises galactose on the sporozoite surface.

### 1.2.12 Cell signalling

Cell signalling is an area of coccidian biology, however, that is largely unexplored. Genes encoding protein kinases and a phosphatase in *T. gondii* have been reported (Russell and Dwyer, 1993; Ng *et al.*, 1995), and evidence for GTP-binding proteins has been presented (Halonen *et al.*, 1996). A $Ca^{++}$-dependent protein kinase, with some plant-like features, has been cloned from *E. tenella* (Dunn *et al.*, 1996). Interestingly it appears to be cytosolic in sporozoites but to move to the apical tip during host cell penetration.

**Aims of study**

The general aims of my project were to elucidate the mechanisms that enable the parasites *Cryptosporidium* and *Eimeria* to survive in the gastrointestinal tract of the host and to penetrate to and invade the host cells themselves.

It was intended that the following working hypotheses would be tested:

1. Sporozoites are biochemically adapted for a period of anaerobiosis after excystation.
2. Sporozoites have surface enzyme activities or release enzymes that aid the parasite's penetration through the mucus layer covering the host's epithelial cells, and the subsequent invasion of a host cell.
3. The condition of the potential host cell affects its susceptibility to infection by the parasite, and infection of the host cell modifies its growth.
4. *Eimeria* and *Cryptosporidium* have similar biochemical adaptations.

# CHAPTER 2

# PARASITE MAINTENANCE AND EXCYSTATION TO PRODUCE SPOROZOITES

## 2.1. Introduction

Excystation, the process that results in the release of invasive sporozoites from oocysts, is the initial stage of *Cryptosporidium* and *Eimeria* infections. For some coccidia, for example *Eimeria* spp., studies began on excystation in the early 1960s and standard excystation protocols have been defined for this parasite which are widely accepted. In comparison fewer studies have been performed with *Cryptosporidium* to define a method of *in vitro* excystation. Initially, many researchers presumed that the process of excystation of *Cryptosporidium* would involve the same mechanisms as with *Eimeria.* The excystation of *Eimeria* oocysts has been observed to proceed via two steps: the first, an incubation under anaerobic reducing conditions, followed by a second step involving exposing the sporocysts to the pancreatic enzyme trypsin and also bile (Fayer and Leek, 1984). This results in the release of the sporozoites through an opening in the sporocyst wall called the steida body (Woodmansee, 1987). However *Cryptosporidium* oocysts differ from those in *Eimeria* in that they sporulate before leaving the host and also lack sporocysts (Sundermann *et al.*, 1987), and the suture through which *Cryptosporidium* sporozoites are released is on the oocyst wall itself, as can be seen by electron microscopy (Reduker *et al.*, 1985). Thus it is not surprising that there are some differences between the excystation of oocysts of *Cryptosporidium* and *Eimeria.*

An early study suggested that tap water and the temperature of 37°C was sufficient for *C. parvum* excystation (Fayer and Ungar, 1984). However this is in direct contrast to later reports which state that excystation fluid is essential to the process as well as a temperature of 37°C (Speer and Reduker, 1986). It has become generally accepted that the use of bile or bile salt and a temperature elevation to 37°C are important for excystation of *C. parvum* (Fayer and Ungar, 1986). Interestingly, *C. muris* is different: bile is not necessary for excystation, reflecting that excystation for this species occurs in the stomach of the host (Nina *et al.*, 1992b). Nevertheless, a number of excystation protocols have been reported by different researchers. Various preincubation treatments have been analysed to determine whether or not they increase excystation. Commonly used ones are sodium hypochlorite and bleach, although quite differing effects have been reported by different researchers (Reduker *et al.*, 1985; Sundermann *et al.*, 1987; Woodmansee, 1987; Robertson *et al.*, 1993). Supposedly, these cause an alteration in oocyst wall permeability (Robertson *et al.*, 1992). Additionally the effect of a number of protease inhibitors on excystation was also studied. It was observed that the proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DIFP), aprotinin and $\alpha_1$-antitrypsin all inhibited excystation yet E64 did not, thus suggesting that serine proteinases are functionally associated with excystation (Forney *et al.*, 1996b). However the mechanism by which these proteinase inhibitors inhibit this process is not fully understood and, as the authors clearly state, the oocysts had been pretreated with bleach, possibly providing a means for the inhibitors to penetrate the oocyst (Forney *et al.*, 1996). Preincubation with saliva was curiously noted to reduce the excystation of *Cryptosporidium* oocysts (Robertson *et al.*, 1993).

Bile/bile salt have been used in excystation media, with concentrations ranging from 0.15% (Woodmansee, 1987) to 0.75% (Fayer and Ungar, 1986).

The lower concentrations of bile resulted in decreased excystation but better survival of the resulting sporozoites (Woodmansee, 1987). Surprisingly, the source of host bile was unimportant with *C. baileyi*. This suggests that *Cryptosporidium* differs from *Sarcocystis*, since with the latter parasite, bile is thought to play a major role in determining host specificity (Sundermann *et al.*, 1987).

There are conflicting reports on whether trypsin is necessary for the excystation of *Cryptosporidium* - as it is for *Eimeria* (Fayer and Leek, 1984; Fayer and Ungar, 1986; Woodmansee, 1987). Recent studies suggested that trypsin has no beneficial effect on the excystation process of *Cryptosporidium* (Robertson *et al.*, 1992). This is believable, as the role of trypsin is thought to be to dissolve the steida body of the *Eimeria* sporocyst and, as mentioned above, *Cryptosporidium* does not possess such a structure (Sundermann *et al.*, 1987). *Cryptosporidium* also differs from other coccidia in that it appears not to require reducing conditions for excystation to occur (Fayer and Leek, 1984; Sundermann *et al.*, 1987). Also, although excystation rates are reported to be higher at lower pH values (5.5-6.0), sporozoite survival is poor at pH ≤6.5 (Woodmansee, 1987).

For both *Cryptosporidium* and *Eimeria* the age of the oocyst sample is one factor that is often overlooked. Excystation decreases over time as oocysts are stored at 4°C, such that after several months they are unable to excyst (Fayer and Leek, 1984; Speer and Reduker, 1986) and infectivity to animals is lost by 18 months (Yang *et al.*, 1996). These observations support the hypothesis that, based on the findings, excystation is a process dependent on temperature, pH, age of oocysts and enzymes within the oocyst (Fayer and Leek, 1984; Woodmansee, 1987). Different oocyst isolates also have different excystation efficiencies although this may reflect the different purification techniques used (Campbell *et al.*, 1992; Robertson *et al.*, 1993); for example centrifugation with percoll

gradients, saturated sodium chloride or discontinuous sucrose gradients have been employed to isolate oocysts (Arrowood and Sterling, 1987).

Most researchers use different lengths of incubation period for excystation. A sixty minute incubation has been reported to be sufficient for maximal excystation (Woodmansee, 1987), whereas other researchers stated that maximal excystation occurs after four hours (Campbell *et al.*, 1992; Robertson *et al.*, 1993). However it has also been reported that excystation should not be stopped earlier than 30 minutes if high sporozoite numbers are required (Robertson *et al.*, 1993).

The following experiments were performed to study and consequently optimise the *in vitro* excystation of *Cryptosporidium* and to provide more information on this initial step in parasite infection.

## 2.2. Materials and Methods

### 2.2.1. Maintenance of *Cryptosporidium* species

#### 2.2.1.1. Infection of animals

Both the MD isolate of *C. parvum* (originally isolated from a deer and obtained from the Moredun Institute for Animal Research) and the RN 66 strain of *C. muris* (originally isolated from rats) were routinely passaged in mice. Before infection with *C. parvum* female C57 mice (OLAC) were immunosuppressed using dexamethasone (10 mg $ml^{-1}$, SIGMA D-1756), administered subcutaneously (0.1 ml) on alternate days for one week. At the same time as the last dexamethasone injection, $10^5$-$10^6$ purified *C. parvum* oocysts were administered orally. Faeces were collected on days 3-7 after the initial infection (day 0) and each day's collection was stored in 50 ml water at 4°C.

*C. muris* was passaged in CB-17 SCID mice infected with 2 x $10^5$ oocysts. Faeces were isolated on each day after day 14 of infection and stored as described above.

This animal work was entirely carried out at Dr. V. McDonald's laboratory at the London School of Hygiene and Tropical Medicine.

#### 2.2.1.2. Monitoring infections

A smear was made from the faeces of an infected mouse and, after fixing, was stained using the Ziehl-Neelsen method. The smear was air-dried and fixed by covering in methanol and allowing to dry. The smear was then immersed in carbol fuschin for 30 min, rinsed with water and then covered with a 1% acid

alcohol solution (500 ml methanol, 5 ml concentrated hydrochloric acid) for a few seconds. The slide was rinsed with water and the acid alcohol step was repeated. The smear was then immersed in malachite green for a few seconds and was subsequently washed well with water. After drying, a number of fields (approximately ten) of the slide were observed under oil immersion microscopy to determine whether oocysts were present in sufficient numbers to isolate from the faeces.

**2.2.1.3. Isolating oocysts**

The collected faeces and water mixture was shaken to produce a homogenate and passed through a 200 μm metal sieve into a beaker to remove large particles. More water was washed through the large particles in the sieve to obtain as many oocysts as possible. The sieved material was left to settle for 15 min in the beaker whereupon the liquid above the sediment was poured off and centrifuged at 1000 x g for 10 min at 4°C. The supernatant was discarded and the pellets containing the oocysts were resuspended in 45 ml saturated salt solution (32% w/v sodium chloride). Each suspension was then transferred to a 50 ml sterile plastic conical tube and approximately 5ml of water was run down the side of each tube to form an upper layer. The tubes were then centrifuged at 1000 x g for 10 min at 4°C and the oocysts, having risen to the upper water layer, were pipetted off and pooled together in a sterile tube. Having pelleted the oocysts by centrifugation (1000 x g for 10 min), the pellet was resuspended in the salt solution and the procedure repeated. All the oocysts collected in the upper water layers were pooled together. These were then washed twice in water and sedimented by centrifugation (1000 x g for 10 min) at 4°C and resuspended in potassium dichromate (2.5%, w/v) at a density of $10^6$-$10^7$ oocysts $ml^{-1}$.

### 2.2.2. Washing oocysts

#### 2.2.2.1. *Cryptosporidium*

The required quantity of oocysts in potassium dichromate (2.5%, w/v) was centrifuged at 1000 x g for 10 min at 4°C. The pelleted oocysts were resuspended in RPMI and this procedure was repeated until the pink colour of RPMI remained after resuspension of the pellet (rather than turning yellow due to the residual dichromate).

#### 2.2.2.2. *Eimeria tenella*

Sporulated oocysts of the RET 5 strain of *E. tenella* were grown in chickens and purified as previously described by Horton-Smith and Long (1959) after which they were stored in potassium dichromate (2.5%, w/v) at a density of ~1 x $10^6$ oocysts $ml^{-1}$. These were obtained from Animal Health Discovery, Pfizer Central Research, Kent. Before use the oocysts were pelleted (200 x g for 5 min) and washed three times using phosphate buffered saline (PBS, pH 7.3) to remove the potassium dichromate. Sporocysts were released by vortexing oocysts with glass beads (3 mm, BDH) for approximately 2-3 min. The sporocysts were then excysted in a solution of PBS containing 2% taurodeoxycholic acid (Calbiochem), 0.1% trypsin, 0.01% chymotrypsin and 20 mM magnesium chloride for 60 min at 44°C in a shaking water bath. The released sporozoites were then washed into RPMI.

### 2.2.3. Excystation of *C. parvum* and *C. muris*

Two reagents commonly employed to trigger excystation of *Cryptosporidium* are the bile salt taurodeoxycholic acid (TDC) and sodium hypochlorite (Robertson *et al.*, 1992). Both of these were used and compared in the following studies.

(a) bile salt

Taurodeoxycholic acid was added to oocysts in RPMI to a concentration of 0.8% and mixed well. The mixture was incubated at 37°C for 60 min in an incubator or water bath.

(b) bleach

Washed oocysts were centrifuged at 1000 x g for 10 min at 4°C and resuspended in 10% (v/v) bleach (Tesco, 30-40% sodium hypochlorite (v/v)) in water. The mixture was placed on ice for 10 min before the parasites were washed 3 times in ice cold water, once in RPMI, resuspended in 1 ml RPMI and incubated at 37°C for 60 min in an incubator or water bath.

To calculate the percent excystation for both *Cryptosporidium* and *Eimeria* the following equation was used for all the studies in this chapter:

$$\frac{\text{Original Oocyst Number - Remaining Oocyst Number}}{\text{Original Oocyst Number}} \times 100$$

**2.2.4. pH buffers**

The buffers used to study the effect of pH on the excystation process were as follows: pH 2, 0.2 M potassium chloride; pH 4, 0.1 M sodium acetate; pH 6 and 8 a phosphate buffer; and pH 9.5, 0.1 M sodium bicarbonate (McKenzie and Dawson, 1969). The oocysts were resuspended in 0.33 x RPMI and adjusted to the appropriate pH using the above buffers.

## 2.3. Results

### 2.3.1. Comparison of bile salt and bleach for triggering excystation

The bile salt taurodeoxycholic acid and sodium hypochlorite procedures were tested for efficacy on *C. parvum* and *C. muris* excystations as described in 2.2.3. The percent excystation for each sample was calculated (see 2.2.3.) and the results are shown in Figure 2.3.1. and Table 2.3.1. for *C. parvum* and *C. muris*, respectively. In the case of *C. parvum* it was observed that the oocysts incubated with 0.8% bile salt achieved a greater initial rate of excystation which finally reached approximately 80% excystation in the 80 min incubation period. The oocysts treated with the bleach showed a much slower initial rate of excystation, although this did rapidly increase after 20 min to reach a final value of approximately 60% excystation. These results indicate that the bile salt was the preferred excystation stimulus for *C. parvum* ($0.05<P<0.02$).

The results obtained for *C. muris* are shown in Table 2.3.1. As was the situation for *C. parvum* the bleach treatment (approximately 33% excystation after the 80 min incubation period) was not as effective as the bile salt (approximately 44% excystation) in stimulating excystation ($0.05<P<0.02$). However, *in vivo C. muris* excysts in the stomach where bile is not present and, as the results show, the absence of both bile salt or bleach treatment does not effect the excystation process of this species (approximately 45% excystation). Nevertheless, sporozoites from both of the *Cryptosporidium* species appeared more motile in the presence of bile salt and it was therefore decided to use bile salt in the excystation mixture of both *C. parvum* and *C. muris*.

### 2.3.2. Varying bile concentrations on *C. parvum* excystation

Bile salt was found to be a better excysting agent than bleach for *C. parvum*, hence the optimum concentration of bile salt was determined. Oocysts were incubated with a range of bile salt concentrations, samples were removed at 20 min intervals and the percent excystation was determined by microscopic observation. The results are shown in Figure 2.3.2. The results show that the lower concentrations of bile salt - 0.08% and 0.16% - resulted in a relatively low percent of excystation at approximately 50% and 70% respectively after the 80 min incubation time. As the concentration of bile salt was increased to 0.4% or 1.0% the degree of excystation also increased; however these respective concentrations resulted in the same level of excystation after the incubation period. It was decided therefore to use the lower concentration (0.4%) of bile salt in order to keep any detrimental effects this material may have to a minimum.

### 2.3.3. Varying incubation conditions

Once the most suitable bile salt concentration had been determined, other optimal conditions for excystation were investigated. The effect of shaking the oocyst suspension during a 60 min incubation and the effect of storing the bile salt solution were studied. The percent excystation of oocysts incubated either with a freshly prepared or a frozen bile salt (-20°) solution (0.4%) were compared using the standard procedure. In addition samples were either placed on a shaker (350 rotations $min^{-1}$) during excystation and others were stationary. The results shown in Figure 2.3.3. indicated that the use of fresh bile and keeping the excysting mixture on a shaker during the incubation resulted in the highest excystation rate.

### 2.3.4. Time course of excystation

To determine the end point of the excystation process, samples of the excystation medium were removed every 20 min and percent excystation calculated. The results are shown in Figure 2.3.4. The number of released sporozoites increased until 80 min of the incubation and then rapidly decreased in number due to their low survival time *in vitro*. The number of shells counted, that is the excysted oocysts, did not equal the initial number of unexcysted oocysts, possibly due to the shells being hard to discern under the microscope since they have a transparent appearance in the haemocytometer. Thus it was seen that the minimum time required to excyst the majority of *C. parvum* oocysts was found to be approximately 80 min at 37°C.

### 2.3.5. Standard excystation conditions used

Taking into account the results from these preceding experiments all subsequent studies employed the following standard excystation procedure for both *C. parvum* and *C. muris*: 0.4% bile salt, on a shaker (350 rotations $min^{-1}$) at 37°C for 80 min.

### 2.3.6. Effect of gaseous conditions on excystation

Four samples of oocysts and bile salt were prepared for excystation. Three were transferred to bijoux bottles and gassed by bubbling with the appropriate gas mixture for 2 min (2 $lmin^{-1}$); one with 95% nitrogen/5% carbon dioxide and two with nitrogen. The other sample was not gassed and so was under aerobic conditions with air as the gas phase. All four samples were incubated on a shaker at 37°C for 80 min whereupon samples were removed and percent excystation

determined. The results shown in Table 2.3.2. revealed that the greatest percent excystation of *C. parvum* occurred with the samples gassed with 95% nitrogen/5% carbon dioxide ($0.05<P<0.02$) or nitrogen ($0.01<P<0.002$), that is the anaerobic conditions. However when observing the sporozoites they were equally motile in the anaerobic samples and aerobic samples, and in future experiments aerobic excystation was used since this method was less time consuming.

### 2.3.7. Effect of oocyst age on excystation

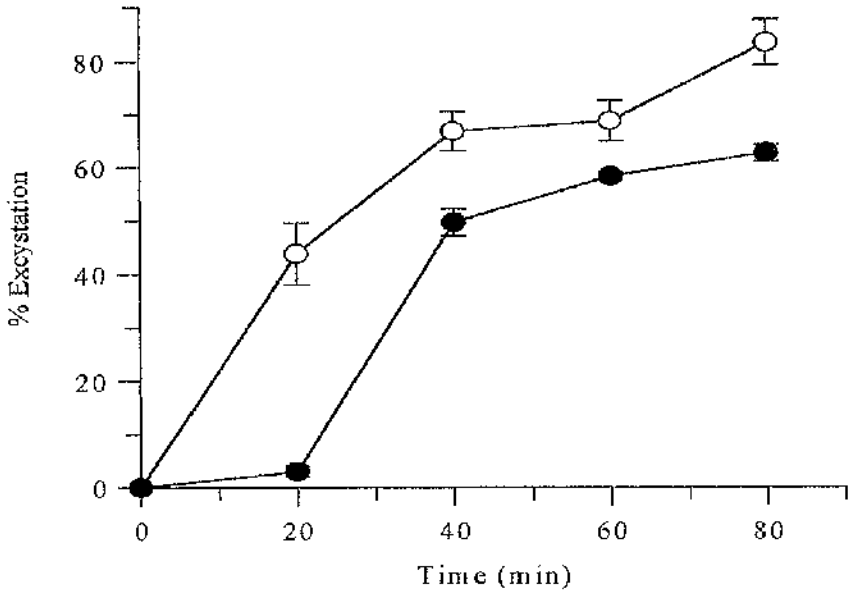
To determine whether the age of the oocysts used effected excystation, sets of oocysts collected at different times were subjected to the standard excystation procedure and the percent excystation determined. The results are shown in Table 2.3.3. It was observed that the older oocysts had a greater percent excystation after the incubation time, but fewer numbers of visible sporozoites were observed. The younger oocysts, although excysting less well, produced many more sporozoites which were much more motile than those released by the older sample.

### 2.3.8. Effect of pH on excystation

Oocysts were washed free of dichromate using non-buffered RPMI and resuspended in RPMI medium at different pH values: pH 2, pH 4, pH 6, pH 8 and pH 9.5 (as described in 2.2.4.). The samples were excysted and the results are shown in Table 2.3.4. Both *C. parvum* and *E. tenella* excysted better at higher pH values in the range of 6.0-9.5 and 8.0-9.5 respectively. Yet both of these parasites had very low or no excystation rates at pH 2.0. Alternatively *C. muris* - known to excyst in the acidic environment of the stomach (approximately pH 2.0) - was

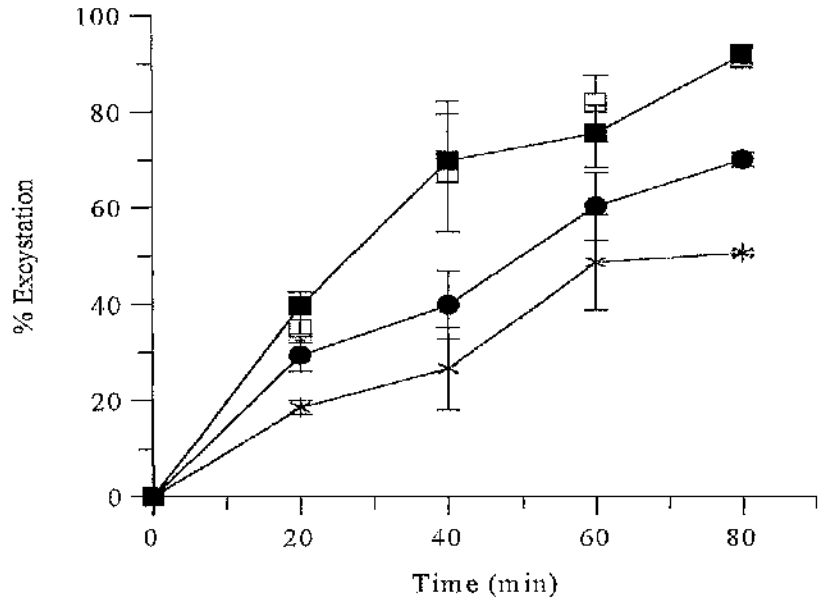
seen to excyst at high rates over the whole range of pH values tested, especially at the extreme ends of the range, that is, pH 2 and pH 9.5.

**Figure 2.3.1: Comparison of bile salt and bleach as stimulus of *C. parvum* excystation**



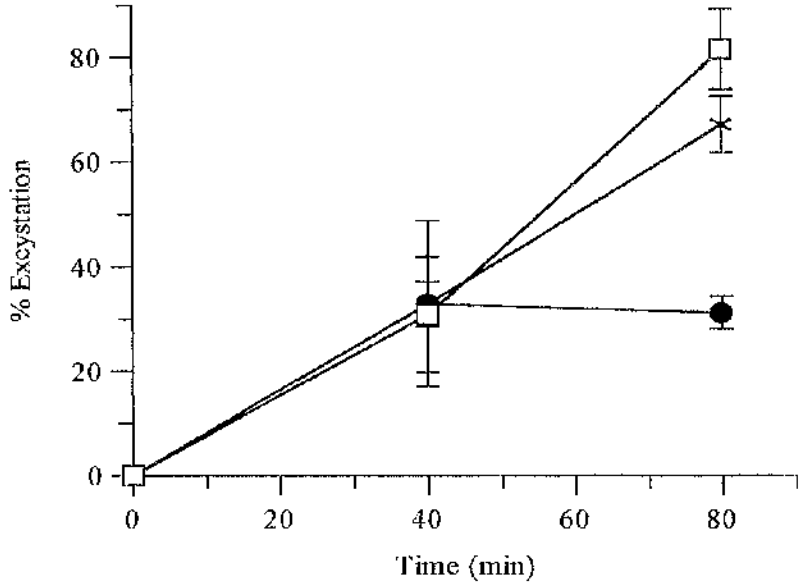

Means ± SE from three experiments
Excystation performed using the bile salt (0.8%, open circles) or bleach treatment (10%, closed circles)

**Figure 2.3.2: Efficacy of different bile salt concentrations as triggers for *C. parvum* excystation**



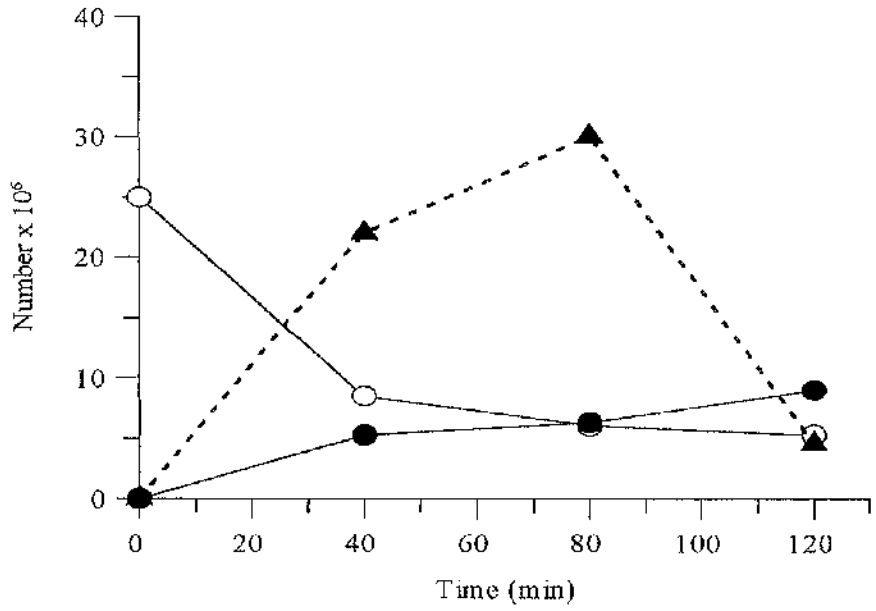

Means ± SE from three experiments
Excystation performed using range of bile salt concentrations 0.08% (star), 0.16% (closed circle), 0.4% (open square) and 1.0 % (closed square)

**Figure 2.3.3: Effect of different conditions on *C. parvum* excystation**




Means ± SE from three experiments
Excystation performed under different conditions: fresh bile (0.4%, star), frozen bile (0.4%, closed circle) and shaken sample with fresh bile (350 rotations $min^{-1}$, open square)

**Figure 2.3.4: Time course of *C. parvum* excystation**




Means from two experiments
Excystation time course monitoring number of oocysts (open circles), shells (closed circles) and sporozoites (closed triangles)

**Table 2.3.1: Comparison of bile salt and bleach on *C. muris* excystation.**

| | % EXCYSTATION |
|---|---|
| Bile | 43.6 ± 5.6 |
| Bleach | 33.3 ± 3.3 |
| Neither | 45.2 ± 5.6 |

Means ± SE from three experiments

**Table 2.3.2: Percent excystment of *C. parvum* oocysts present in various gaseous conditions.**

| | % EXCYSTATION |
|---|---|
| Nitrogen | 86.0 ± 5.9 |
| Nitrogen/Carbon dioxide | 91.3 ± 2.0 |
| Air | 71.0 ± 2.1 |

Means ± SE from three experiments

**Table 2.3.3: Percent excystment of *C. parvum* oocysts of different ages.**

| WEEKS SINCE ISOLATION OF OOCYSTS | % EXCYSTATION |
|---|---|
| 10 | 85 (1) |
| 2 | 66 (3-4) |

Mean of two experiments
Numbers in brackets represent numbers of sporozoites released per oocyst.

**Table 2.3.4: Percent excystation of oocysts in different pH buffers.**

| | % EXCYSTATION | | | | |
|---|---|---|---|---|---|
| pH | 2.0 | 4.0 | 6.0 | 8.0 | 9.5 |
| *C. parvum* | 27.7 ± 8.2 | 36.3 ± 10.4 | 48.3 ± 2.6 | 54.3 ± 6.3 | 61.3 ± 10.5 |
| *C. muris* | 83.0 ± 3.6 | 82.0 ± 4.0 | 84.3 ± 3.9 | 87.7 ± 3.5 | 94.0 ± 1.7 |
| *E. tenella* | 0.0 ± 0.0 | 40.0 ± 11.5 | 40.0 ± 4.0 | 76.3 ± 6.4 | 76.7 ± 5.7 |

Means ± SE from three experiments

## 2.4. Discussion

The purified oocysts from each species of *Cryptosporidium* were used in a number of experiments designed to both optimise the *in vitro* conditions for the excystation process and also to provide more data on the factors influencing this process.

When comparing the bile salt and bleach methods, results obtained from *C. parvum* showed that there was only a slightly higher percent excystation when bile salt was used. However, it was observed that the freshly excysted sporozoites were much more motile than those that had been excysted using the bleach method, suggesting that the bleach may have damaged the sporozoites in some manner. Additionally, *C. muris* also showed a reduction in excystation when the bleach treatment was used, again perhaps indicating that this method may have a detrimental effect either in the process of excystation itself, or on the resulting sporozoites. Having established that the bile salt method was the one to opt for the concentration to be used was determined. Results indicated that both 0.4% and 1.0% both gave approximately the same percent excystation after an eighty minute incubation at 37°C. To minimise any adverse effects that the bile salt may have on the sporozoites once excysted as previously reported (Woodmansee, 1987), the lower concentration of bile salt was used - 0.4% - to excyst *C. parvum*. This chosen concentration lies within the documented range of between 0.15% (Woodmansee, 1987) and 0.75% (Fayer and Ungar, 1986). *C. muris*, known to excyst in the stomach of the host where no bile is present, appears to be unaffected whether bile is present or not, indicating that this species does not require bile to excyst, which merely illustrates this parasite's adaptations to its *in vivo* environment. However bile salt was added to the excystation mixture of *C. muris* since it was observed that the presence of bile salt seemed to increase sporozoite motility in both species, as previously reported for *C. parvum*

(Reduker and Speer, 1985; Woodmansee *et al.*, 1987), suggesting that bile may be advantageous to sporozoite survival. Alternatively the bleach treatment appeared to have an adverse effect on *C. muris* excystation, possibly highlighting the fact that this procedure may damage the oocysts or sporozoites in some way ($P<0.02$).

Investigating variations on the incubation conditions involved shaking the sample, comparing fresh to bile salt previously stored at -20°C and monitoring a time course to determine the endpoint of the excystation process. Shaking the sample and using fresh bile salt both increased excystation markedly. The time course indicated that sporozoite numbers reached a maximum after an eighty minute incubation. Additionally to these results, excystation was found to be better under anaerobic conditions ($P<0.02$) reflecting the fact that *C. parvum* and indeed other coccidia excyst *in vivo* in the gastrointestinal tract where a low oxygen tension environment is present. Yet for *E. tenella* excystation, it has been reported that this process is unaffected and can proceed under both aerobic and anaerobic atmospheres (Wang, 1976). Once the sporozoites have been released from the oocyst the gaseous environment has no effect on *C. parvum* sporozoite survival (see Chapter 3), which is in agreement with results obtained from studies with *Eimeria* showing that the oxygen concentration only affects host cell invasion and not the sporozoites themselves (Wrede *et al.*, 1993).

The importance of oocyst age in determining excystation efficiency is a factor that has not been studied to any great extent, though reports have stated that excystation declines with the age of the oocysts (Fayer and Leek, 1984; Speer and Reduker, 1986). However the results obtained here showed that the newer oocyst sample had a lower percent excystation when compared with the older sample. Yet, in terms of number of sporozoites released, a higher yield was given by the new sample of oocysts. These results confirm those of a previous study which

also investigated oocyst age and excystation. Results allowed the authors to conclude that the number of released sporozoites is a much more accurate criterion with which to measure excystation rather than empty oocysts (shells), since they also found more oocysts excysted in the older batches but released fewer sporozoites (Speer and Reduker, 1985). This may be due to the older oocysts being more permeable and thus excysting easier. However, whether the low yield was due to the sporozoites simply not leaving the oocysts, or leaving and then lysing, has not been determined. It was concluded that oocysts should be used when relatively fresh to maximise the number of sporozoites obtained.

The three species used in the pH studies: *E. tenella, C. parvum* and *C. muris* infect different host species as well as invading different regions of the gastrointestinal tract. *E. tenella* and *C. parvum* excyst in the small intestine (Strout *et al.*, 1994; Current, 1989) whereas *C. muris* excysts in the stomach (Current, 1989), thus the oocysts are exposed to different environments. Many factors may be involved in this site specific excystation, for example in *Eimeria* different species, known to infect different regions of the gastrointestinal tract, possess lectins on their sporozoite surface which have different sugar specificities and may be involved in determining where each species infects (Strout *et al.*, 1994). In terms of pH, the gastrointestinal tract differs quite widely, from approximately pH 2 in the stomach to pH 6-8 in the upper and middle intestine. From the results obtained in this chapter the higher pH values gave optimal excystations for both *E. tenella* and *C. parvum* whereas *C. muris* was capable of excysting over the range of pH values tested. More interestingly *C. muris*, unlike the other two species, was able to excyst extremely well at pH 2 and the resulting sporozoites could survive for up to sixty minutes in these conditions (see Chapter 3) - ample time for invasion to occur.

These results have shown how well these parasites are adapted to excysting in the environment they face *in vivo*. However, exactly how the different species arrive at the correct location for excystation and thus infection remains unknown. For *Eimeria* sporozoites it has been proposed that, once excysted, intraepithelial leukocytes transport sporozoites to their specific site of infection (Lawn and Rose, 1982; Pakandl *et al.*, 1995), though for *Cryptosporidium* no research has been performed in this area. Yet this process of excystation must be investigated further if we are to understand more fully this initial step to the infection of the host.

# CHAPTER 3

# STUDIES ON SPOROZOITE VIABILITY

## 3.1. Introduction

The ways in which coccidian sporozoites are biochemically adapted to their environment in the gut have not been fully elucidated. Most studies to date have focused on events after host cell invasion, little has been reported on events preceding this process and the factors that influence sporozoite viability.

Previous studies have assessed viability using phase contrast microscopy and judging whether the sporozoites are thought to be healthy and motile (Woodmansee *et al.*, 1987; Upton and Tilley, 1989). Using these criteria, serum components like fetuin and albumin also appeared to enhance *in vitro* development and sporozoite motility of *Eimeria* as well as the presence of trypsin and cholic acid in the sporozoite medium (Upton and Tilley, 1992). Trypsin has also been seen to increase *T. gondii* tachyzoite and *C. parvum* sporozoite motility (Robertson *et al.*, 1993; Mondragon *et al.*, 1994). Motility studies performed on *E. nieschulzi* also indicated that high concentrations of mucin present increased the sporozoite motility (Upton and Tilley, 1992).

Length of time of sporozoite survival has also been used as a measure of viability. It was reported that the presence of glucose in the incubation medium increased the length of time that *E. tenella* sporozoites survived at 41°C (Nakai and Ogimoto, 1983). The addition of bile to the excystation fluid has been

reported to enhance *Cryptosporidium* sporozoite survival (Reduker and Speer, 1985), which is in agreement with the observation that the presence of bile maintained sporozoite motility and morphology (Woodmansee *et al.*, 1987). In contrast reduced oxygen tensions - akin to what the sporozoites experience *in vivo* - had no positive short term effects on *E. nieschulzi* and only increased sporozoite survival after an initial 15 hour incubation. For *C. parvum*, viability has been reported to decrease rapidly at pH values below 5.0 or above 8.0 (Hamer *et al.*, 1986), with sporozoite lysis observed at pH values under 6.2 (Woodmansee *et al.*, 1987), though just how long the sporozoites survived was not stated.

However, these methods using microscopic observation have significant limitations, the foremost being that they are, to an extent, subjective. Additionally motility is often intermittent, and difficult to assess with *C. parvum* due to its small size. Alternatively, methods to determine the viability of the oocyst stage of the life cycle have been well documented. The methods employed are either monitoring the oocyst infectivity in mice (Campbell *et al.*, 1982; Fayer and Nerad, 1996), the use of fluorogenic dyes (Smith *et al.*, 1991; Campbell *et al.*, 1992), or both of the afore mentioned methods (Black *et al.*, 1996). A quick, easy and non-subjective method for determining sporozoite viability is required, to aid researchers in monitoring the effects of various physical and chemical factors including the effects of anticoccidial drugs.

The following experiments in this chapter were firstly designed to develop a viability assay for sporozoites, and then to use this technique to provide more information on the metabolism and survival of the sporozoite stages of *Eimeria*

and *Cryptosporidium.*

## 3.2. Materials and Methods

### 3.2.1. Separation of sporozoites from oocysts and shells

To separate *C. parvum* sporozoites from the unexcysted oocysts and oocysts shells, 800 µl samples containing a maximum of $2 \times 10^7$ sporozoites $ml^{-1}$ were filtered using 5 µm filters (Flowpore D, Flow Laboratories). The filters were moistened with 0.5 ml RPMI before the excystation mix was passed through slowly, the filters were then subsequently washed through with 0.5 ml RPMI. The recovery rate of sporozoites in the effluent was approximately 70%.

Similarly *E. tenella* sporozoites (RET 5) were filtered through cotton wool prewet with PBS. Having placed approximately 10 ml cotton wool in a 20 ml syringe the excystation mix was passed through, after which the cotton wool was subsequently rinsed with PBS.

### 3.2.2. Method of determining sporozoite survival

#### 3.2.2.1. Motility studies

The sporozoite suspension was placed in a haemocytometer where the numbers of sporozoites could be counted. 100 sporozoites were counted in total with the number of 'healthy' sporozoites recorded as a percentage, that is those that have the curved shape and are motile as described previously (Russell and Sinden, 1981). A simple method involving microscopic (x40) observation of individual sporozoites was devised as a means of quantifying percent motility of the population. This involved observing each sporozoite individually (50 in total)

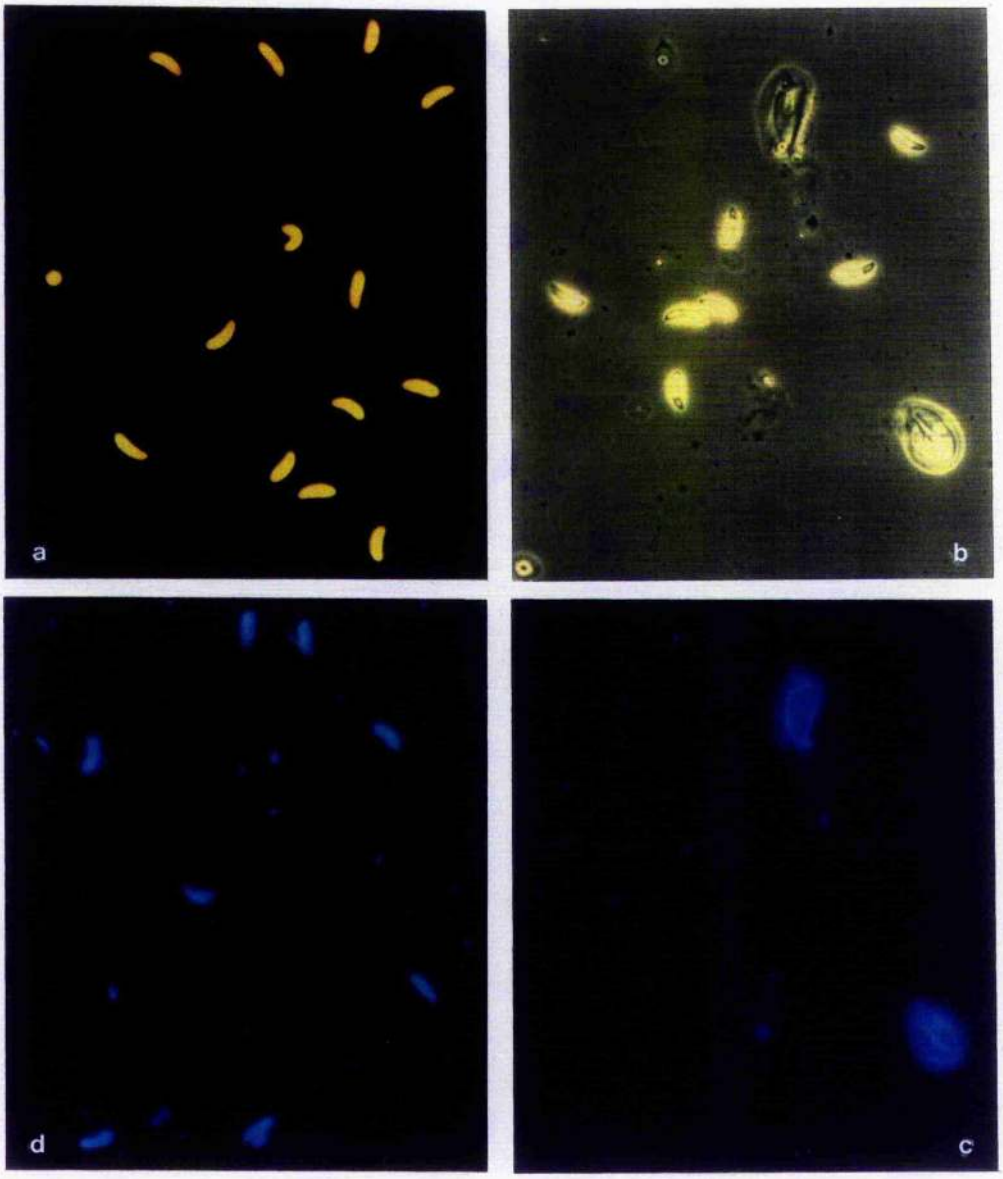
for 20 seconds and judging if there was movement other than Brownian motion. The sporozoites move in two ways: 'flexing' and 'wiggling' (Russell and Sinden, 1981) and if either of these was observed then the sporozoite was classed as motile. These studies were performed at 37°C in an aerobic environment.

### 3.2.2.2. Vital stains

To confirm the results obtained from the motility studies, a more objective method was required to determine sporozoite viability. Thus a number of vital stains were tested on *C. parvum* sporozoites. Trypan blue and DNA intercalating dye 4,6,-diamino-2-phenylindole (DAPI) did not provide a clear result due to the size of the sporozoites. Trypan blue did not appear to stain the sporozoites, whereas those stained with DAPI were very hard to distinguish from the background fluorescence.

Eventually the vital stains acridine orange (Edward Gurr Ltd.) and bis-benzimide (Sigma) were used. The sporozoites were excysted and washed into RPMI or TBS for *Cryptosporidium* and *Eimeria* respectively and, after adding any compound being studied, bis-benzimide (final concentration 0.2 mM, v/v) and acridine orange (final concentration 0.005%, v/v) were mixed into the sporozoite suspension. Slides were then prepared and viewed using a Zeiss fluorescence microscope (Carl Zeiss Inc., New York) with the relevant filters (acridine orange fluorescence: excitation filter, BP450-490 nm; mirror, FT510 nm; barrier filter, LP520 nm; bis-benzimide: excitation filter, G365 nm; mirror, FT510 nm; barrier filter, LP520 nm). With these two stains live and dead sporozoites differ between filters as is illustrated in Figure 3.2.1.

**Figure 3.2.1: Vital stains on *E. tenella* sporozoites.** With the two stains bis-benzimide and acridine orange. Live sporozoites (freshly excysted and washed into RPMI or TBS) fluoresce brightly with the fluorescein filter (see Figure 3.2.1 (a)); compared with bright field microscopy where live sporozoites are clearly visible (Figure 3.2.1. (b)), live sporozoites are barely distinguishable with the bis-benzimide filter (see Figure 3.2.1 (c)). In contrast, dead sporozoites (killed by 10% formaldehyde) have a characteristic bright blue fluorescence when viewed with the bis-benzimide filter (see Figure 3.2.1 (d)).


a
b
d
c

### 3.2.3. Metabolic inhibitors and pH buffers

The metabolic inhibitors used with their final concentrations are as follows: 10 mM potassium cyanide, 10 mM sodium azide, 0.5 mM antimycin A, 1.0 mM Rotenone and 10% formaldehyde as the negative control. *Cryptosporidium* and *Eimeria* sporozoites were suspended in RPMI and PBS, respectively, and incubated at 37°C.

The pH buffers used to adjust the sporozoites suspended in 0.33x RPMI to the required pH, are as follows: pH 2, 0.2 M potassium chloride, pH 4, 0.1 M sodium acetate, pH 6 and 8 a phosphate buffer and pH 9.5, 0.1 M sodium bicarbonate (McKenzie and Dawson, 1969). For the further pH studies on *C. muris* a 0.2 M Glycine-HCl pH buffer was used (McKenzie and Dawson, 1969).

## 3.3. Results

### 3.3.1. Effect of bile salt on sporozoite survival

To determine whether bile salt had a detrimental effect on excysted sporozoites, *C. parvum* oocysts were subjected to the standard excystation procedure and the sporozoites obtained were either washed twice in RPMI to remove the bile salt, or not, and incubated at 37°C on a shaker (350 rotations $min^{-1}$). Samples were taken after a 60 min incubation and motile sporozoites were recorded as described in 3.2.2.1. (see Table 3.3.1.). The results suggest that, using motility as an indicator of viability, bile salt has a positive effect on motility of *C. parvum* sporozoites ($P<0.10$).

### 3.3.2. Effect of gaseous conditions on sporozoite survival

Samples of *C. parvum* oocysts were excysted for 80 min whereupon they were filtered. The sporozoites obtained were gassed (2 $lmin^{-1}$): one sample with 95% nitrogen/5% carbon dioxide and one with nitrogen for two minutes. The other sample was left in aerobic conditions. The samples were placed on a shaker (350 rotations $min^{-1}$) and sporozoite survival was then monitored by removing aliquots at certain time points and counting numbers of sporozoites. The results are shown in Figure 3.3.1. The trend that was observed was that the optimum conditions of those tested here for sporozoite viability, was an anaerobic environment with $CO_2$ present; however this time course needs to be repeated for statistical analysis to be performed.

### 3.3.3. Effect of metabolic inhibitors on sporozoites

Samples of *C. parvum* and *C. muris* oocysts and *E. tenella* sporocysts were excysted and filtered to obtain sporozoites. Metabolic inhibitors were then added to duplicate sporozoite suspensions as described in 3.2.3. The samples were then left on a shaker (350 rotations $min^{-1}$) at 37°C for 60 min. Aliquots were then removed and viability was assessed using the standard procedures (see 3.2.2.1. and 3.2.2.2.) with the results listed in Table 3.3.2. Both *C. parvum* and *E. tenella* sporozoites were unaffected by any of the metabolic inhibitors, whereas viability of *C. muris* appeared to be marginally decreased.

### 3.3.4. Effect of potassium cyanide on *C. muris* sporozoites

To determine the sensitivity of *C. muris* sporozoites to potassium cyanide the sporozoites were incubated for 60 min in a range of concentrations, after which the viability was assessed using the vital stain technique (see Table 3.3.3). The effect of the potassium cyanide was seen to be concentration related, that is, the higher the concentration the greater the detrimental effect on the sporozoites.

### 3.3.5. Salicylhydroxamic acid (SHAM) and potassium cyanide on *E. tenella* sporozoites

SHAM - the alternative oxidase inhibitor of trypanosomes (Wang, 1988; Grady *et al.*, 1993) - was used in conjunction with potassium cyanide with the final concentrations being 2 mM and 10 mM respectively. Having incubated the sporozoites for 60 min with these inhibitors the viability was assessed using the

vital stains technique (see Table 3.3.4). The results indicated that SHAM when used alone and in conjunction with potassium cyanide had no adverse effects on *E. tenella* sporozoites.

### 3.3.6. Effect of varying pH on sporozoite viability

Sporozoites were resuspended 0.33x RPMI and adjusted to the following pH values using the appropriate buffers (see 3.2.3.): pH 2, 4, 6, 8 and 9.5. The sporozoites were then incubated for 60 min at 37°C. The percent viability of the sporozoites was then examined and calculated using the vital stain technique (see 3.2.2.) and listed in Table 3.3.5. Both *C. parvum* and *E. tenella* survived better at the higher pH levels with no viable sporozoites found at pH 2 after 60 min. However 20% of *C. muris* sporozoites were seen to be able to survive at pH 2 for 60 min.

### 3.3.7. Effect of pH 2.0-3.0 on *C. muris*

Having established that some *C. muris* sporozoites could survive at pH 2 for 60 min, their ability to withstand low pH was analysed further by using suspension media of differing pH values. *C. muris* oocysts were excysted and the resulting sporozoites resuspended in 0.33x RPMI before being adjusted to pH values ranging from 2.0 - 3.0 using the appropriate buffer (see 3.2.3.). After a 60 min incubation at 37°C the percent viability was recorded for each sample using the vital stains technique (see Table 3.3.6). From the results obtained it was observed that small fluctuations in pH dramatically affected the percentage of viable sporozoites. For example when compared with pH 2.0, pH 2.4 had

approximately twice as many sporozoites viable after the 60 min incubation. This may be a reflection of the *in vivo* situation, where small fluctuations in pH are caused by a number of factors like diet, immune status etc. (Johnson, 1987), increasing the infection chances of *C. muris*.

### 3.3.8. Time course of *C. muris* at pH 2.0

To analyse further the ability of *C. muris* sporozoites to survive at low pH, differing incubation periods were used. *C. muris* oocysts were excysted and the resulting sporozoites resuspended in 0.33x RPMI before being adjusted to pH 2.0 using the appropriate buffer (see 3.2.3.). At 15 min intervals the number of viable sporozoites was recorded using the vital stains technique (see Table 3.3.6). It was observed that approximately 50% of the sporozoites were still viable after 15 min at pH 2. These numbers decreased quite rapidly during the 60 min incubation with approximately 20% left viable after a 60 min incubation. However these results demonstrate that *C. muris* can survive these extremes in pH long enough to invade the gastric epithelium in the *in vivo* situation.

### 3.3.9. Effect of anticoccidial drugs on sporozoites

*C. parvum, C. muris* and *E. tenella* oocysts were excysted and resuspended in RPMI medium at a density of $10^6$-$10^7$ $ml^{-1}$. To 100 µl volumes the following anticoccidial drugs were added prior to a 60 min incubation at 37°C: monensin (4 µg $ml^{-1}$) and halofuginone (4 µg $ml^{-1}$) with 10% formaldehyde as a negative control. After the 60 min incubation the percent viability of the sporozoites was quantified using the vital stain technique as described in 3.2.2. and recorded in

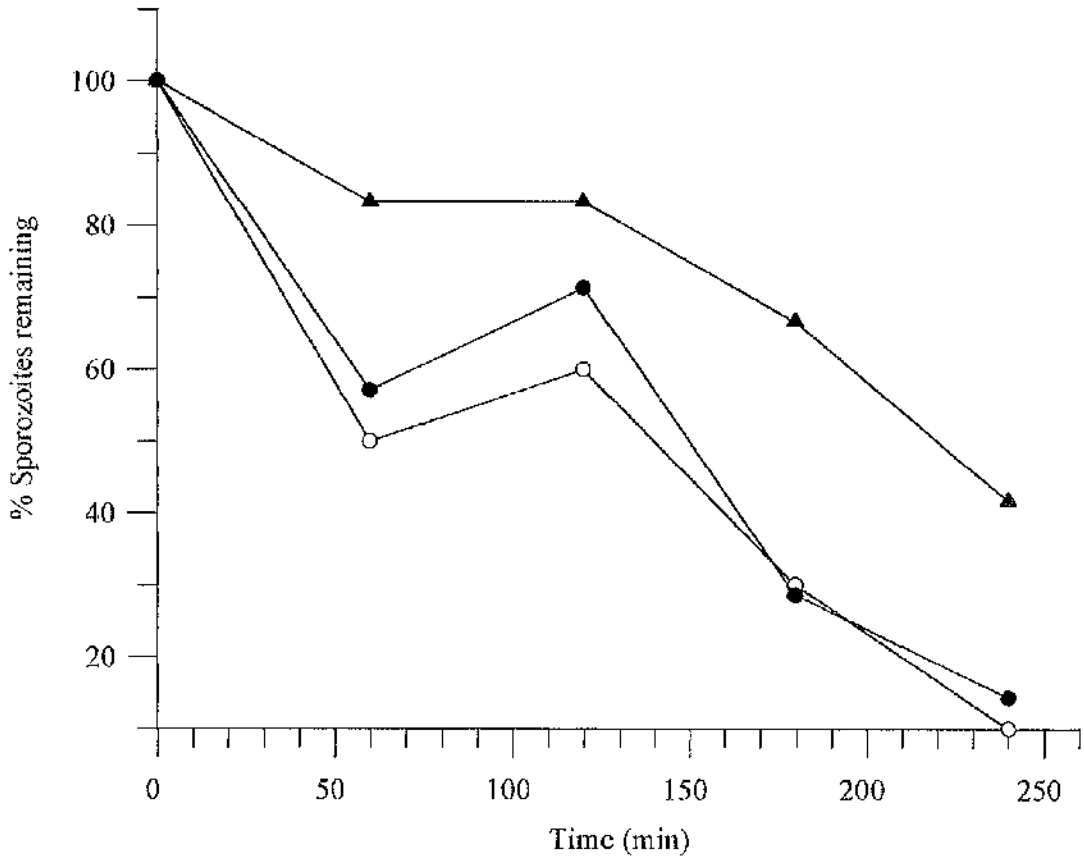
Table 3.3.7. Not one of the compounds was seen to have any detrimental effects on *E. tenella* sporozoites. Alternatively halofuginone and monensin did have a small adverse effect on *C. muris* ($0.02<P>0.01$) and *C. parvum* ($P>0.05$) sporozoites respectively.

**Table 3.3.1: Effect of bile on *C. parvum* sporozoite survival**

| | % MOTILITY |
|---|---|
| WASHED | 69.2 ± 15.8 |
| UNWASHED | 96.3 ± 3.7 |

Means ± SE from three experiments

**Figure 3.3.1:Effect of gaseous conditions on *C. parvum* sporozoite survival**




Means of two experiments
Time course measuring numbers of sporozoites under different gaseous conditions: air (open circle), nitrogen (closed circle) and nitrogen/5% carbon dioxide (closed triangle).

**Table 3.3.2: Effects of metabolic inhibitors on the viability of sporozoites**

| | % APPARENTLY VIABLE | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CONTROL | | POTASSIUM CYANIDE | | SODIUM AZIDE | | ROTENONE | | FORMALDEHYDE | |
| | MOTILITY | STAINS | MOTILITY | STAINS | MOTILITY | STAINS | MOTILITY | STAINS | MOTILITY | STAINS |
| *C. parvum* | 66.5 ± 5.5 | 96.7 ± 0.8 | 57.4 ± 6.6 | 95.7 ± 0.3 | 70.8 ± 3.2 | 96.0 ± 1.5 | ND | 95.0 ± 1.4 | 4.2 ± 0.9 | 0.0 ± 0.0 |
| *C. muris* | 48.7 ± 3.9 | 86.6 ± 2.4 | 32.4 ± 2.8 | 62.8 ± 10.3 | 41.8 ± 8.3 | 72.4 ± 5.0 | ND | 98.3 ± 0.6 | 16.7 ± 3.5 | 0.0 ± 0.0 |
| *E. tenella* | 95.2 ± 1.3 | 96.7 ±1.6 | 94.3 ± 1.2 | 94.2 ± 2.2 | 95.8 ± 1.3 | 97.2 ± 1.0 | ND | 100.0 ± 0.0 | 0.0 ± 0.0 | 0.2 ± 0.2 |

Means ± SE from three experiments
Concentrations of inhibitors: cyanide, 10 mM; azide, 10 mM; rotenone, 1mM; formaldehyde, 10 % (v/v)
'STAINS' refers to the % viable as determined using the vital stain method.
*Leishmania mexicana* promastigotes were used as a positive control; all were killed (judged on motility) by treatment with the inhibitors.
ND: not done.

**Table 3.3.3: Effect of potassium cyanide on *C. muris* sporozoites**

| | % APPARENTLY VIABLE | | | | |
|---|---|---|---|---|---|
| | CONTROL | 1mM KCN | 3mM KCN | 10mM KCN | 10% FORMALDEHYDE |
| *C. muris* | 80.8 ± 1.7 | 79.0 ± 5.7 | 64.2 ± 7.1 | 59.1 ± 10.4 | 0.0 ± 0.0 |

Mean ± SE from three experiments
Viability was assessed using the vital stain method.

**Table 3.3.4: Effect of SHAM and potassium cyanide on *E. tenella* sporozoites**

| | % APPARENTLY VIABLE | | | | |
|---|---|---|---|---|---|
| | CONTROL | 10 mM KCN | 2 mM SHAM | 10 mM KCN + 2 mM SHAM | 10% FORMALDEHYDE |
| *E. tenella* | 98.0 ± 1.2 | 96.7 ± 1.8 | 96.7 ± 1.8 | 99.3 ± 0.7 | 0.0 ± 0.0 |

Means ± SE from three experiments
Viability was assessed using the vital stain method.

**Table 3.3.5: Effect of pH on the viability of sporozoites**

| | % APPARENTLY VIABLE | | | | |
|---|---|---|---|---|---|
| pH | 2.0 | 4.0 | 6.0 | 8.0 | 9.5 |
| *C. parvum* | 0.0 ± 0.0 | 85.3 ± 2.3 | 89.8 ± 1.9 | 99.0 ± 1.0 | 99.3 ± 0.7 |
| *C. muris* | 23.2 ± 4.8 | 88.3 ± 7.7 | 94.0 ± 1.0 | 95.3 ± 2.2 | 90.0 ± 3.2 |
| *E. tenella* | 0.0 ± 0.0 | 94.0 ± 1.2 | 99.0 ± 1.0 | 99.3 ± 0.7 | 99.3 ± 0.4 |

Means ± SE from three experiments
Viability was assessed using the vital stain method.

**Table 3.3.6.: Effects of pH and incubation times upon the viability of *C. muris* sporozoites**

| | % APPARENTLY VIABLE | | | |
|---|---|---|---|---|
| 60 min at pH | 2.0 | 2.4 | 2.8 | 3.0 |
| % Viable | 21.6 ± 6.1 | 44.0 ± 12.2 | 49.7 ± 6.7 | 69.0 ± 9.6 |
| | | | | |
| Min at pH 2.0 | 15 | 30 | 45 | 60 |
| % Viable | 51.0 ± 5.4 | 30.5 ± 5.8 | 28.0 ± 2.3 | 20.8 ± 3.4 |

Means ± SE from three experiments
Viability was assessed using the vital stain method.

**Table 3.3.7.: Effect of anticoccidial drugs on sporozoites**

| % APPARENTLY VIABLE | | | | |
|---|---|---|---|---|
| | CONTROL | HALOFUGINONE | MONENSIN | FORMALDEHYDE |
| *C. parvum* | 97.1 ± 1.0 | 93.0 ± 2.2 | 82.7 ± 7.5 | 0.0 ± 0.0 |
| *C. muris* | 78.4 ± 3.3 | 62.3 ± 6.4 | 73.3 ± 4.3 | 0.0 ± 0.0 |
| *E. tenella* | 97.7 ± 1.6 | 100.0 ± 0.0 | 97.7 ± 2.0 | 0.0 ± 0.0 |

Means ± SE of at least three experiments

Concentrations of drugs: halofuginone, 4 $\mu g ml^{-1}$; monensin, 4 $\mu g ml^{-1}$; formaldehyde, 10% (v/v)

### 3.4. Discussion

The preceding experiments were designed to provide more information on how *Cryptosporidium* and *Eimeria* sporozoites survive in their *in vivo* environments. The pH studies and metabolic inhibitors indicated how the parasites are adapted for living in the gastrointestinal tract. The presence of bile salt increased the motility of *C. parvum* sporozoites and did not appear to have any adverse effects on survival, which is in agreement with previous reports (Reduker and Speer, 1985; Woodmansee *et al.*, 1987). Additionally *C. parvum* sporozoites survived for a longer period of time under anaerobic conditions as in the *in vivo* situation where they are exposed to a low oxygen tension environment. The sporozoites of *Cryptosporidium* do not survive for any reasonable length of time under *in vitro* conditions, and the general consensus, though no research has been performed in this area, is that they are thought to lyse and disappear. The reason for this phenomenon is unknown, although it may be that the sporozoites simply exhaust their internal energy supplies. However this lysis must be taken into account when using *Cryptosporidium* sporozoites for *in vitro* studies, since the results obtained may not be a true reflection of the experimental results if the sporozoites are dying anyway. For this reason incubations with *Cryptosporidium* sporozoites were kept to a maximum of 60 minutes in my studies.

To analyse further the ability of the different sporozoite species to survive in a largely anaerobic environment, a variety of metabolic inhibitors was used. However, since assessment of motility - which has been widely used as a viability test in the past (Upton and Tilley, 1992, 1995) - is an extremely subjective method influenced by various external factors, for example temperature, a new method of determining viability was developed. This

involved the use of the two vital stains, acridine orange and bis-benzimide, used in conjunction with a fluorescence microscope as described in 3.2.2.2. The acridine orange crosses the cell membrane and stains the sporozoite which fluoresces a yellow/orange colour. This stain was used to locate the sporozoites under the fluorescent microscope. The other stain - bis-benzimide - only stains dead sporozoites, since it is unable to cross intact membranes. However once dead, the bis-benzimide crosses the cell membrane and the sporozoites fluoresce a distinctive bright blue. This two-stain method provided a quicker and more reliable method when compared with motility as can be seen from the comparative results in Table 3.3.2. The two-stain method is also a very fast technique which does not require an incubation period. This is an important feature of the assay since - as previously discussed - the sporozoites of *Cryptosporidium* do not survive for a long period of time *in vitro*.

The inhibitors potassium cyanide, sodium azide and rotenone had no effect on either *C. parvum* or *E. tenella* as assessed by both methods to assess viability. These results indicated that sporozoites of these two species did not require a functional respiratory chain. This is in agreement with previous reports which have stated, on evidence from enzyme studies, that they are adapted for anaerobiosis (Denton *et al.*, 1994; Denton *et al.*, 1996). In fact *C. parvum* sporozoites have been reported not to possess a mitochondrion (Current, 1989). In contrast potassium cyanide and sodium azide did have a detrimental effect on *C. muris*, although only a minority of sporozoites were affected. A previous report on the ultrastructure of *C. muris* stated that this species does contain a mitochondrion (Uni *et al.*, 1987); thus if active this would certainly explain the adverse effects of the cyanide and azide. However the fact that only a small number of sporozoites in the *C. muris* population was affected (even at 10 mM

potassium cyanide, see Table 3.3.3) raises the question of whether a functional respiratory chain is actually necessary, and further studies are needed to clarify this. It may be that the potassium cyanide is having some effect other than on the respiratory chain on the sporozoites of this species, possibly on the surface of the parasites. However the results from rotenone are not unusual since another related coccidian *Plasmodium* has also been reported to be unaffected by this inhibitor (Fry, 1991), suggesting that NADH-Q reductase is either absent or has unusual properties.

Salicylhydroxamic acid (SHAM) is an inhibitor of the alternative oxidase involved in reoxidising NADH formed by glycolysis in trypanosomes (Van der Meer *et al.*, 1979). The oxidase is not inhibited by any of the mitochondrial inhibitors, for example cyanide and azide, however SHAM is known to inhibit the trypanosomal oxidase *in vitro* at a concentration of 6 μM even after a short incubation time of 30 min (Opperdoes *et al.*, 1976). However as can be seen from the results in Table 3.3.4, SHAM appeared to have no detrimental effect on coccidian sporozoites and it may be concluded that these parasites may have no alternative oxidase pathway via this system. Recent research has revealed that these coccidian parasites contain plastids. The plastid may contain proteins involved in photosynthesis or an electron-transport chain (Jeffries and Johnson, 1996), possibly enabling these parasites to use a plastid oxidase if their own respiratory chain is inhibited.

The effect of pH on the three species was also studied. As mentioned in Chapter 2, these species not only excyst and infect different hosts but also different regions of the gastrointestinal tract. *E. tenella* and *C. parvum* infect the intestine (Current, 1989; Strout *et al.*, 1994), whereas *C. muris* infects the

stomach (Current, 1989). Thus the species are exposed to different environments including pH. The upper and middle intestine have a pH of 6-8 which, as the results illustrate in Table 3.3.6, are optimal for *C. parvum* and *E. tenella* survival. However the results for *C. parvum* indicate that the sporozoites used in these experiments were not as sensitive to pH changes as previously reported. Hamer *et al.* (1986) and Woodmansee *et al.* (1987) stated that *C. parvum* sporozoite survival was reduced at pH values below 5.0 or above 8.0, and below 6.2 respectively. The results obtained here showed that *C. parvum* can survive at a pH as low as 4.0 and as high as 9.5. The pH of the mammalian stomach is known to be approximately 2.0 (Johnson, 1987), and from Tables 3.3.5. and 3.3.6. the results show that *C. muris* sporozoites are viable over the whole pH range tested including pH 2. Additionally minor increases in pH - possibly akin to normal fluctuations in the stomach - result in significantly larger numbers of viable sporozoites. A time course of *C. muris* at pH 2.0 (see Table 3.3.7.) shows that approximately half of the sporozoites survived for fifteen minutes, which is reported to be sufficient time for *Cryptosporidium* to invade a host cell (Lumb *et al.*, 1988). The mechanisms that enable these sporozoites to withstand the strong acid conditions present in the stomach are unknown. However, other microorganisms are known to have either structural (surface resistance) or metabolic (to reduce the acidity in the immediate environment) mechanisms to aid them (Chan *et al.*, 1992; Batt *et al.*, 1996).

The anticoccidial drugs used appeared to have no effect on *E. tenella* or *C. parvum* sporozoites (see Table 3.3.7.). It should be noted however that the strain of *E. tenella* used in the project is a strain known to be more resistant to ionophorous drugs than other strains, and this may have affected the outcome of

the experiment, at least with monensin. Yet for both *C. parvum* and *C. muris* monensin and halofuginone were seen to have a slight detrimental effect. This relatively small effect may have been due to the short length of time the parasites were exposed to the drugs before viability was assessed. Possibly with a longer incubation, a greater reduction in viability would be observed. Indeed these drugs are thought to inhibit parasite development within the host cell (Yvore and Naciri, 1989; McDonald *et al.*, 1990; Rehg, 1995), and may not affect the sporozoite viability to any great extent.

The results obtained in this chapter have illustrated how these sporozoites are adapted to living in the *in vivo* environment of low oxygen tension and extremes of conditions. However, more studies must be performed, for example, whether or not *C. muris* does possess a mitochondrion in the sporozoite stage, before we can attempt to fully understand how these sporozoites are capable of initiating the infection process.

# CHAPTER 4

# ENZYME AND PROTEINASE STUDIES

## 4.1. Introduction

Studies on the enzymes of *Cryptosporidium* have, in comparison with other coccidia such as *Eimeria* and *Toxoplasma*, been scarce. The enzymes that have been detected in the coccidial organisms are listed in Tables 4.1.1 (a), (b) and (c). My study has concerned three groups of enzymes in particular: proteinases, sialidase and glycolytic enzymes. The aims were to provide more information on the roles these enzymes play in enabling the parasite to survive in the gastrointestinal tract and invade their host cells.

Coccidian proteinases, enzymes that catalyse the splitting of peptide bonds in proteins, have been little researched, and only recently for *Cryptosporidium*. In contrast, many studies have been performed on other parasites to elucidate the role(s) of proteinases in various stages of their life cycles. Putative functions of some parasite proteinases include host cell invasion and survival once inside the cell, as is the case for *Plasmodium* (McKerrow *et al.*, 1993) and amoebae where mRNA levels for cysteine proteinases are higher in pathogenic strains than in non-pathogenic strains (McKerrow, 1993); evasion of the immune system; and changes between life cycle stages involving the activation of various enzymes and proteins for differentiation as is the case for *Trypanosoma cruzi* (McKerrow *et*

**Table 4.1.1(a): Enzymes of energy metabolism in coccidia**

| | *Eimeria* | | | | | | | *Toxo-plasma* | *Sarco-cystis* | *Crypto-sporidium* |
|---|---|---|---|---|---|---|---|---|---|---|
| | Unsporulated oocysts | Sporulated oocysts | Sporozoites | Merozoites | Schizonts | Micro-gametocytes | Macro-gametocytes | | | |
| ***Glycolysis:*** | | | | | | | | | | |
| Hexokinase | ✓ 1,7 | ✓ 1,6 | ✓ 1 | | | | | ✓ 29,30,31 | ✓ 38 | ✗ 1, 42 |
| Glucosephosphate isomerase | ✓ 7,8 | ✓ 6,8,25 | ✓ 1 | | | | | ✓ 27,30,31 | | ✓ 32,33 |
| Pyrophosphate-phosphofructokinase | ✓ 1 | ✓ 1 | ✓ 1 | | | | | ✓ 1,22 | | ✓ 1, 42 |
| Aldolase | ✓ 1,3,7 | ✓ 6 | | | | | | ✓ 30 | ✓ 38 | |
| Triose phosphate isomerase | ✓ 7 | ✗ 6 | | | | | | | | ✓ 42 |
| Glyceraldehyde 3-phosphate dehydrogenase | | ✓ 6 | | | | | | | | ✓ 42 |
| Phosphoglycerate kinase | ✓ 7 | | | | | | | | | ✓ 42 |
| Phosphoglucose mutase | ✓ 7,9 | ✓ 6,25 | ✓ 1 | | | | | ✓ 30,31 | ✓ 34 | ✓ 32,33,42 |
| Enolase | ✓ 7 | ✓ 6 | | | | | | | | ✓ 42 |
| Pyruvate kinase | ✓ 1,7 | ✓ 1,6 | ✓ 1 | | | | | ✓ 1 | ✓ 38 | ✓ 1,42 |
| | | | | | | | | | | |
| Lactate dehydrogenase | ✓ 1,2,7,8 | ✓ 1,6,8,25 | ✓ 1, 19 | | | | | ✓ 30,31 | ✓ 38 | ✓ 1,33,42 |
| | | | | | | | | | | |
| ***TCA cycle:*** | | | | | | | | | | |
| Citrate synthetase | | ✗ 6 | | | | | | | | |
| Aconitase | | | | | | | | ✓ 30 | | |
| Isocitrate dehydrogenase | | ✗ 6 | | | | | | ✓ 30,31 | | ✗ 1,42 |
| α-Ketoglutarate dehydrogenase | | ✗ 6 | | | | | | | | |
| Succinyl CoA synthetase | | ✗ 6 | | | | | | | | |
| Succinate dehydrogenase | ✓ 4,15 ✗ 1 | ✗ 6,1 ✓ 4 | | ✓ 4 | ✓ 4 | ✗ 4 | ✗ 4,15 | ✓ 1 | | ✗ 1 |
| Fumarase | | ✗ 6 | | | | | | ✓ 29,30 | | ✓ 33 |
| Malate dehydrogenase | | ✓ 6 | | | | | | | | ✓ 42 |

**Table 4.1.1(b): Enzymes of carbohydrate metabolism in coccidia**

| | *Eimeria* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Unsporulated oocysts | Sporulated oocysts | Sporozoites | Merozoites | Schizonts | Micro-gametocytes | Macro-gametocytes | *Toxo-plasma* | *Sarco-cystis* | *Crypto-sporidium* |
| ***Pentose phosphate pathway:*** | | | | | | | | | | |
| Glucose 6-phosphate dehydrogenase | ✓ 5 | ✓ 6,8 | ✓ 1,19 | | | | | ✓ 29,30,31 | ✓ 38 | ✓ 42 |
| 6-Phosphogluconate dehydrogenase | ✓ 8 | ✓ 8 | | | | | | ✓ 30 | ✓ 34,38 | |
| | | | | | | | | | | |
| ***Gluconeogenesis:*** | | | | | | | | | | |
| Glucose 6-phosphatase | ✓ 4 | ✓ 4,25 | | ✓ 4 | ✓ 4 | ? 4 | ✓ 4 | | | |
| Fructose 1,6-bisphosphatase | ✓ 7 | | | | | | | | | |
| Pyruvate carboxylase | | | | | | | | ✓ 29 | | |
| | | | | | | | | | | |
| ***Mannitol cycle:*** | | | | | | | | | | |
| Mannitol 1-phosphate dehydrogenase | ✓ 20,21 | ✓ 21 | ✓ 21 | | | | | ✓ 20 | | ✓ 20 |
| Mannitol 1-phosphatase | ✓ 20,21 | ✓ 21 | ✓ 21 | ✓ 21 | | | | | | ✓ 20 |
| Mannitol dehydrogenase | ✓ 20,21 | ✓ 21 | ✓ 21 | ✓ 21 | | | | | | |
| (Hexokinase) | ✓ 20,21 | ✓ 21 | ✓ 21 | | | | | | | |
| | | | | | | | | | | |
| ***Miscellaneous carbohydrate metabolism:*** | | | | | | | | | | |
| Phosphoglucomutase | ✓ 7 | ✓ 6 | | | | | | ✓ 30,31 | ✓ 34 | ✓ 32,33 |
| Amylopectin phosphorylase | ✓ 16 | ✓ 16 | | | | | | | | |
| Amylopectin synthase | ✓ 17 | | | | | | | | | |
| α-Glycerophospate dehydrogenase | ✓ 15 | | | | | | ✓ 15 | | | |
| Phosphoenolpyruvate carboxykinase | | ✓ 6 | | | | | | | | × 42 |
| Malic enzyme | | ✓ 6 | | | | | | | | |
| Glycerol 3-phosphate dehydrogenase | ✓ 1 | | | | | | | ✓ 1,30 | | ✓ 42 |
| Amylase | | | | | | | | ✓ 4 | | |

**Table 4.1.1(c): Enzymes of amino acid metabolism and some hydrolases in coccidia**

| | *Eimeria* | | | | | | | *Toxo-plasma* | *Sarco-cystis* | *Crypto-sporidium* |
|---|---|---|---|---|---|---|---|---|---|---|
| | Unsporulated oocyts | Sporulated oocysts | Sporozoites | Merozoites | Schizonts | Micro-gametocytes | Macro gametocytes | | | |
| | | | | | | | | | | |
| Glutamate dehydrogenase | ✓ 14 | ✓ 14 | | | | | | | | |
| Aspartate aminotranferase | ✓ 14 | ✓ 14 | | | | | | ✓ 27,30 | ✓ 39 | |
| | | | | | | | | | | |
| | | | | | | | | | | |
| ATPase | ✓ 4 | ✓ 4 | | ✓ 4 | ✓ 4, 26 | ✓ 4 | ✓ 4 | | | |
| 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 | ✓ 27,28,41 | ✓ 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 | | |
| | | | | | | | | | | |
| Adenosylmethionine decarboxylase | | ✓ 12 | | | | | | | | |
| Arylsulphatase | | | ✓ 13 | | | | | | | |
| β-Galactosidase | | | ✗ 19 | ✓ 19 | | ✓ 19 | ✓ 19 | ✓ 28 | ✓ 35 | |
| β-Glucosidase | | | ✗ 19 | ✗ 19 | | ✗ 19 | ✗ 19 | | | |
| Carboxylic ester hydrolases | | | ✗ 19 | | | | | | | |
| | | | | | | | | | | |
| 5'-Nucleotidase | | | | | ✓ 26 | ✓ 26 | ✓ 26 | ✓ 23 | | |

**Tables 4.1.1.: Key and references**

✓ : detected
x : apparently absent

1 Denton *et al.* (1994, 1996a and unpublished)
2 Fransden and Cooper (1972)
3 Mitchell and Daron (1982)
4 Micheal and Hodges (1973)
5 Fransden (1976, 1978)
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 (1987)
13 Farooqui and Hanson (1987)
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)
22 Peng and Mansour (1992)
23 Sibley *et al.* (1994a)
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 Barnert *et al.* (1988)
32 Awad-El-Kariem *et al.* (1993, 1995)
33 Ogunkolade *et al.* (1993)
34 Atkinson and Collins, (1981)
35 Farooqui *et al.* (1987)
36 Chaudry *et al.* (1985)
37 Chaudry *et al.* (1986b)
38 Gupta *et al.* (1992)
39 Gupta *et al.* (1993)
40 Fulton and Spooner (1960)
41 Metsis *et al.* (1995)
42 Entrala and Mascaró, (1997)

*al.*, 1995). Out of all of these functions, the one that has drawn the most attention for the coccidia is the first, that is, host cell invasion.

The first stage in infection for both *Cryptosporidium* and *Eimeria* is the excystation of ingested oocysts. This stage has been studied, though more for the latter parasite. Very low proteinase activities were found in sporulated or unsporulated oocysts of *E. tenella* (Wang and Stotish, 1975, 1978), and were completely inhibited by PMSF thus implicating a serine proteinase. Later studies detected high levels of proteinase activity in the order sporozoites > sporulated oocysts > unsporulated oocysts (Farooqui and Hanson, 1983). Indeed, more recently a serine proteinase from *E. tenella* sporulated oocysts was purified and characterised (Michalski *et al.*, 1994).

Additionally three leucine aminopeptidase activities located primarily in the cytoplasm surrounding the sporocysts in *E. tenella* sporulated oocysts were detected (Wang and Stotish, 1978). These activities were greatly reduced in the absence of metal ions and inhibited by chelating agents, thus indicating a metallo-proteinase. These activities were not found in either sporozoites or merozoites.

The role of proteinases in host cell invasion has been analysed in a number of ways, the foremost with the use of specific inhibitors. The proteinase inhibitors $\alpha_1$-antitrypsin, antipain, aprotinin, leupeptin, soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF) all reduced *C. parvum* infection of bovine fallopian tube cells (Forney *et al.*, 1996a). Similarly, *E. vermiformis* sporozoite host cell invasion of Madin Darby Bovine Kidney (MDBK) cells was inhibited by the proteinase inhibitors: antipain, leupeptin, aprotinin, L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone (TPCK) and N-α-p-tosyl-L-lysine

chloromethyl ketone (TLCK) (Fuller and McDougald, 1990). The serine proteinase inhibitor PMSF appeared to be most effective at reducing invasion (Adams and Bushell, 1988; Fuller and McDougald, 1990). It should be noted that PMSF is toxic to both *C. parvum* and *E. tenella* sporozoites (see Chapter 3), and may be adversely effecting the outcome of these studies.

Another approach adopted is to study the invasive stage(s) themselves. Low levels of proteinase activity detected in *E. tenella* invasive sporozoites and merozoites appeared to be sensitive to inhibition by PMSF and to a lesser degree by TPCK and TLCK (Fuller and McDougald, 1990). These activities associated with the sporozoites and merozoites had different pH optima and inhibitor sensitivities, implying stage-specific proteinases. In *Cryptosporidium*, an arginine aminopeptidase activity was seen to be localised in *C. parvum* sporozoite membranes (Okhuysen *et al.*, 1994) and was suggested to be involved in excystation. This arginine peptidase was not detected in intact *Cryptosporidium* oocysts or excysted shells. More recently, a metallo-dependent cysteine proteinase was identified again on *C. parvum* sporozoite surfaces (Nesterenko *et al.*, 1995), and the human serine proteinase inhibitor - α-1-antitrypsin - was seen to bind to *C. parvum* sporozoite membranes (Forney *et al.*, 1996b). For *E. tenella*, a gene with high homology to those encoding aspartic proteinases in other organisms has been reported (Laurent *et al.*, 1993). Immunolocalisation studies suggested that the enzyme was associated with the refractile bodies of the invasive sporozoite.

Coccidial invasion is accompanied by the release of material from the rhoptries, dense granules and micronemes (Strobel *et al.*, 1992; Petersen, 1993; Sterling and Arrowood, 1993) and is described in greater detail in Chapter 6.

The dense granules of *Sarcocystis muris* contain a proteinase which has been suggested to play a role in host cell invasion, possibly by modifying the parasitophorous vacuole in some way (Strobel *et al.*, 1992).

Aside from proteinase studies other enzymes have been analysed in the coccidia. Sialidases (neuraminidases) are enzymes which hydrolyse and thus cleave the O-glycosidic linkage between sialic acids and underlying glycoproteins or glycolipids. Sialic acid residues are thought to act as receptors on cell surfaces for a number of micro-organisms, including the influenza virus (Pedroso de Lima, 1995). Many studies have been performed on trypanosomes which are now known to contain a trans-sialidase activity which transfers sialic acid residues on the host cell to parasite glycoproteins used in host cell invasion with infective forms possessing higher levels of this activity (Schenkman and Eichinger, 1993). Similarly the apicomplexan parasite *P. falciparum* invades erythrocytes and was previously reported to use sialic acid on the erythrocyte surface as a binding site. However, a recent report was unable to detect a parasite sialidase activity as well as the finding that the influenza virus sialidase inhibitor had no effect on parasite invasion or development (Clough *et al.*, 1996). A past study also reported the detection of sialidase on the surface of invasive stages of *E. tenella*, the activity being some 20-fold higher on merozoites than sporozoites (Pellegrin *et al.*, 1993). It has been suggested that the enzyme plays a role in desialylating intestinal mucins and so reducing the viscosity of the environment and aiding migration of the parasite. The enzyme could also be involved in modifying the surface of the host cell prior to and during invasion.

Glycolytic enzymes have raised great interest in *Eimeria* and *Cryptosporidium* since these parasites face a largely anaerobic environment in the gastrointestinal tract. Many enzymes have been detected as shown in Table 4.1.1., and the exact pathways involving these enzymes are described more fully in Chapter 1. Possibly the most interesting result is that *C. parvum, E. tenella* and *T. gondii* contain, at least in the stages investigated, a pyrophosphate-linked phosphofructokinase ($PP_i$-PFK) instead of the conventional ADP-linked enzyme (Peng and Mansour, 1992; Denton *et al.*, 1994, 1996a). This conclusion was confirmed for *Cryptosporidium* in a more recent study (Entrala and Mascaró, 1997). Additionally, the pyruvate kinase (PK) from *C. parvum* shows no evidence of regulatory properties (Denton *et al.*, 1996a).

However, research techniques and subsequent results have varied widely. The following experiments in this chapter have utilised a number of techniques in an attempt to firstly detect these activities, and then in the case of the proteinases, to localise where these enzymes are present in the parasite.

## 4.2. Materials and Methods

### 4.2.1. Survey of enzyme activities

#### 4.2.1.1. Parasites

*C. parvum* and *E. tenella* oocysts and sporozoites were obtained as described previously (see Chapter 2). The oocysts were homogenised by vortexing with glass beads (425-600 µm, Sigma for *C. parvum* and 3 mm, BDH for *E. tenella*) in lysis buffer (50 mM HEPES, pH7, with 20% (v/v) glycerol and 0.25% (v/v) Triton X-100) at a density of $1x10^8$ $ml^{-1}$ and $1.5x10^7$ $ml^{-1}$ for *C. parvum* and *E. tenella* oocysts respectively. Sporozoites were suspended in lysis buffer at a density of $1x10^6$ $ml^{-1}$ for both parasites and vortexed briefly.

Lysates were aliquoted and stored at -20°C until required.

#### 4.2.1.2. Detection of activities

The commercially available APIzym kit (API Systems SA) compares semiquantitative assays for nineteen enzyme activities including three aminopeptidases, six glycosidases, two esterases, one alkaline phosphatase, one lipase, trypsin and chymotrypsin, one acid phosphatase, one α-mannosidase and one α-fucosidase. The assays involve the use of chromogenic enzyme substrates and the activities are detected using the two reagents provided: A and B. Reagent A contained Tris and sodium dodecyl sulphate (SDS); reagent B contained fast blue BB salt in 2-methoxyethanol. The principle of detection is based on the liberation of β-napthol from the substrates which is detected by the indicator.

The enzyme strip was placed in a moist incubation tray and 100 µl of the parasite lysates were added to each reaction well. The strip was then incubated

for 24 h at 37°C, after which the two reagents A and B were used to detect the enzyme activities. One drop of each reagent was added to each reaction well, after which the strip was exposed to a 100 W light source to destroy excess reagent B. Having allowed any colour to develop for 10 min, the positive reactions were graded according to their intensity using the APIzym colour reaction chart.

### 4.2.2. Proteinase detection

#### 4.2.2.1. Preparation of samples

*C. parvum* oocysts were washed in RPMI (Labtech, 1640) to remove the potassium dichromate (1000 x g for 10 min), resuspended in lysis buffer and homogenised using glass beads (see section 4.2.1.1.). The lysate was aliquoted and stored at -20 C.

*Eimeria tenella* oocysts (sporulated and unsporulated) were washed in phosphate buffered saline (PBS: 0.01 M phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) to remove the potassium dichromate (200 x g for 5 min). The sample was then resuspended in lysis buffer (as above) and homogenised using (3 mm) glass beads (BDH). The lysate was aliquoted and stored at -20°C.

*Eimeria tenella* sporozoites were obtained as described previously 2.2.2.2, resuspended in lysis buffer (as above), aliquoted and stored at -20°C until required.

*Toxoplasma gondii* tachyzoites were lysed simply by adding lysis buffer (as above) and the lysate was stored at -20°C.

*Leishmania mexicana* amastigotes were harvested and washed three times in a 0.25 M sucrose and centrifuged to a pellet and stored at -70°C. When required, the pellet was resuspended in 0.25 M sucrose with 0.1% Triton X-100 to an equivalent of 1 x $10^9$ $ml^{-1}$. The lysate was then spun in a microfuge for 5 min and the supernatant removed and stored at -20°C.

Samples were diluted 1:1 with sample buffer (0.5 M Tris/HCl pH 6.8, 5% (v/v) glycerol, 10% (v/v) SDS, 5% (v/v) mercaptoethanol and bromophenol blue, as per Laemmli, 1970), with the addition (for the protein gels only) of proteinase inhibitors: E-64 (1 mM), pepstatin (1 μM) and PMSF (200 mM). Samples were then boiled for 10 min. Some samples, as indicated, were incubated with the appropriate inhibitor for 30 min at 37°C before the sample buffer was added.

**4.2.2.2. Preparation and running of gels**

SDS - polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the protein content of the samples. The separating gels (10% or 12%) and the stacking gels (~5%) were prepared (according to Laemmli, 1970) using stock solutions: 30% acrylamide solution, 1.5 M Tris buffer (pH 8.8), 0.5 M Tris buffer (pH 6.8), 10% ammonium persulphate solution, with the addition of N,N,N',N'-Tetramethylethylenediamine (TEMED) and distilled water. Gels were run for approximately 40 min at 200 volts. They were either stained for proteins using

Coomassie Blue (0.05% (w/v) Coomassie Brilliant Blue Colloidal R-250, 25% (v/v) isopropanol and 10% (v/v), acetic acid) for 60 min before being placed in destain (10% (v/v) acetic acid and 12.5% (v/v) methanol), or subjected to Western blotting (see 4.2.2.4.).

#### 4.2.2.3. Determination of molecular weights

Apparent molecular weight markers of the proteins were determined by comparing their mobility with those of Wide Range Rainbow Markers (Amersham): myosin (220 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa).

#### 4.2.2.4. Western blot transfer

After SDS-PAGE the gel and a hybond-C nitrocellulose membrane were sandwiched between filter papers soaked in Transfer buffer (20 mM Tris, 150 mM glycine, 20% (v/v) methanol) to aid current conductance, and placed in a wet blotter (BioRad). The apparatus was set at 100 volts for 60 min with a maintained temperature of 4°C.

#### 4.2.2.5. Proteinase detection using biotinylated inhibitors and Western blotting

For studies on proteinases, samples were lysed as described in section 4.2.2.1. and stored at -20 C. A stock solution of the biotinylated peptidyl

diazomethane probe (Biosyn) was used initially in a 3 mM solution in dimethylfluoride and incubated with the sample for 30 min at 37°C before adding sample buffer and boiling (see 4.2.1.). The biotinylated inhibitors used were: biotin-Phe-Ala-$CHN_2$ for cysteine proteinase detection; biotin-Arg-Arg-$CH_2Cl$ for the detection of cathepsin B-like enzymes, and subtilisin-like processing enzymes; biotin-Arg-$CH_2Cl$, recommended for detecting trypsin-like activities; and biotin-Phe-$CH_2Cl$, recommended for detecting chymotrypsin-like activities.

Samples were then electrophoresed on a 10% separating gel along with pre-stained Rainbow markers (Amersham). The protein bands were then transferred to nitrocellulose as described in 4.2.2.4. After protein transfer, the blot was blocked overnight at 4°C in a 3% (w/v) bovine serum albumin (BSA) solution in Tris-buffered saline (TBS: 20 mM-Tris, 1 M HCl, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 and 0.01% merthiolate. The nitrocellulose was then incubated with streptavidin/alkaline phosphatase (1:500 stock dilution in TBS) (Biosyn) in a solution containing 0.25% BSA for 120 min at room temperature with gentle shaking. The blot was then washed six times with TBS (pH 9.5) before alkaline phosphatase activity was detected using one BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablet (Sigma) per blot dissolved in 10 ml of distilled water.

#### 4.2.2.6. Localisation of proteinases using a biotinylated inhibitor and fluorescence microscopy

To determine where the proteinases were located in the sporozoite stage itself fluorescence microscopy was used. *E. tenella* sporozoites were obtained as described previously in 2.2.2.2, and air-dried on a glass microscope slide. The

slides were immersed in methanol (100%) for 10 min, after which they were rehydrated in PBS (pH 7.4 containing 10% (v/v) foetal calf serum, Labtech) for 30 min at room temeperature. Having washed the slides twice in PBS (containing 0.05% (v/v) Tween-20), one slide was preincubated with either E64 (1.5 mM), PMSF (1.5 mM), or neither for 10 min. The slides were washed three times and immersed in PBS containing biotin-Arg-Arg-$CH_2Cl$ (100 μM). After an incubation for 30 min at 37°C, the slides were washed twice and a streptavidin-FITC conjugate was placed on the slides for 15 min at room temperature. The slides were washed briefly twice with PBS-Tween-20 and viewed using a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York) with either phase contrast microscopy or the FITC filter (fluorescence: excitation filter, BP450-490 nm; mirror, FT510 nm; barrier filter, LP520 nm).

#### 4.2.2.7. Detection using antisera raised against *L. mexicana* cysteine proteinases

Attempts were made to detect proteinases in the coccidia samples using antibodies raised against Types I and II *L. mexicana* cysteine proteinases. 10% gels were run and blotted as described in 4.2.2.4. The nitrocellulose membranes were then blocked in TBS (pH 7.6) containing 10% (v/v) horse serum and 5% (w/v) dried milk before being incubated with an anti-*lmcpa* primary antibody (Mottram *et al.*, 1992) (1:500 in TBS with 10% horse serum and 5% dried milk). The membranes were washed three times in high volumes of TBS (pH 7.6) before incubating with a secondary anti-rabbit-Horse Radish Peroxidase (HRP) conjugate (1:2000 in TBS, pH 7.6 with 10% (v/v) horse serum and 5% (w/v) dried milk). The membranes were washed and the HRP activity detected using the ECL

(Amersham) detection method and autoradiography film.

### 4.2.3. Sialidase detection

#### 4.2.3.1 Parasites

*E. tenella* merozoites were prepared using a drug-sensitive strain of the parasite - Wis F96. Madin Darby Bovine Kidney (MDBK) cells were inoculated onto glass coverslips at a density of $5 \times 10^4$ per well in a 24-well plate and grown overnight in 0.5 ml RPMI medium (Labtech, 1640) containing 10% heat inactivated foetal calf serum (Labtech), 2 mM L-Glutamine (Sigma), 25 $\mu g\ ml^{-1}$ gentamycin (Sigma) and 2.5 $\mu g\ ml^{-1}$ amphotericin B in a moist incubator with 5% $CO_2$/95% air. The sporozoites of *E. tenella* were excysted and filtered as described in 2.2.2.2. and 3.2.1 and resuspended in RPMI containing 10% heat inactivated foetal calf serum, 2 mM L-glutamine and 0.25 mg $ml^{-1}$ gentamycin at a density of $8 \times 10^5$ sporozoites $ml^{-1}$. Once the MDBK cells had been rinsed three times with serum-free RPMI, 200 µl volumes of the sporozoite suspension were added to each well. The 24-well plate was then incubated at 41°C for 5 h in a 5% $CO_2$/95% air environment, after which the medium was replaced with 0.5 ml fresh medium. The cells were then incubated for 42-46 h in identical conditions after which the first generation merozoites could be collected as they were released. The collected merozoites were then resuspended in lysis buffer (as previously described) with the following proteinase inhibitors: E64 (1 mM), PMSF (2 mM) and pepstatin (10 µM). After a brief vortex, the samples were aliquoted and stored at -20°C until required.

#### 4.2.3.2. Fluorometric assay of sialidase

Sialidase was detected as described previously for *E. tenella* (Pellegrin *et al.*, 1993). Briefly 2'-(4-methylumbelliferyl)-α-N-acetyl-D-neuraminic acid (4-MU-Neu5Ac, Sigma) was used as a substrate which is hydrolysed by the sialidase to release a fluorogenic product which can be measured using a fluorescence spectrophotometer (Potier *et al.*, 1979). The final concentration of the substrate in the assay was 0.25 mM in 0.2 M sodium acetate buffer, pH 5.0 in a total volume of 200 µl. After a 60 min incubation at 37°C, the reaction was stopped by adding 1.5 ml 0.5 M glycine-NaOH, pH 10.5, and the fluorescence was measured on a fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

#### 4.2.3.3 Fluorescent staining of sialidase in polyacrylamide gel electrophoresis

An alternative method for detecting sialidases has been previously described using polyacrylamide gel electrophoresis (PAGE) (Berg *et al.*, 1985). Samples of *E. tenella* merozoite lysate and *Clostridium perfringens* sialidase (Sigma) were loaded onto 10% gels prepared as in 4.2.2.2 after which they were run at 150 volts for approximately 40 min. The gel was then preincubated in substrate buffer (0.2 M acetate buffer, pH 5.0, containing 5 mM calcium chloride) for 30 min. The gel was then placed in solutions of substrate buffer containing 0.5 mM 4-MU-Neu5Ac and incubated at 37°C for 60 min. The presence of fluorescence was observed by irradiation with UV light of 366 nm. After documentation of the fluorescent band the same gel was stained with Coomassie brilliant blue as previously described in 4.2.2.2.

## 4.3. Results

### 4.3.1. Survey of enzyme activities

The APIzym kit was considered to be a convenient method to investigate a large number of enzyme activities rapidly and with relatively small amounts of material. The results of the APIzym kit (see Table 4.3.1.) indicate that a variety of enzymes are present in *Cryptosporidium* and *Eimeria* including alkaline phosphatase, esterases, lipases, arylamidases, acid phosphatase and a phosphohydrolase. Trypsin was detected only in *E. tenella* sporozoites and chymotrypsin detected only in *C. parvum* oocysts. However none of the parasite stages was positive for any of the following: α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

### 4.3.2. Proteinase detection using biotinylated inhibitors

#### 4.3.2.1. Proteinase detection using biotin-Phe-Ala-diazomethane

For the detection of cysteine proteinases, biotin-Phe-Ala-$CHN_2$ was used at a concentration of 100 μM with *E. tenella* unsporulated oocysts, *C. parvum* oocysts and *T. gondii* tachyzoites as described in 4.2.2.5. (typical results are shown in Figures 4.3.1., 4.3.2. and 4.3.3. respectively). Many bands were detected with each lysate. To confirm the bands which were due to a cysteine proteinase, samples were preincubated with the irreversible cysteine proteinase

**Table 4.3.1: Enzyme activities detected by the APIzym kit. + positive; ++: intensely positive; -: apparently absent**

| ENZYME | *C. parvum* oocysts | *C. parvum* sporozoites | *E. tenella* sporulated oocysts | *E. tenella* sporozoites | *E. tenella* unsporulated oocysts | *C. muris* oocysts |
|---|---|---|---|---|---|---|
| Alkaline phosphatase | + | + | + | + | ++ | + |
| Esterase | + | ++ | + | + | + | + |
| Esterase lipase | ++ | ++ | + | + | + | + |
| Lipase | + | + | + | + | + | + |
| Leucine arylamidase | ++ | + | ++ | + | ++ | ++ |
| Valine arylamidase | + | + | + | + | ++ | + |
| Cysteine arylamidase | + | - | ++ | + | ++ | + |
| Trypsin | - | - | - | + | - | - |
| Chymotrypsin | + | - | - | - | - | - |
| Acid phosphatase | ++ | + | + | + | ++ | ++ |
| Napthol-AS-BI-phosphohydrolase | ++ | + | ++ | + | ++ | ++ |
| α-galactosidase | - | - | - | - | - | - |
| β-galactosidase | - | - | - | - | - | - |
| β-glucuronidase | - | - | - | - | - | - |
| α-glucosidase | - | - | - | - | - | - |
| β-glucosidase | - | - | - | - | - | - |
| N-acetyl-β-glucosaminidase | - | - | - | - | - | - |
| α-mannosidase | - | - | - | - | - | - |
| α-fucosidase | - | - | - | - | - | - |

inhibitor E64 (1.5 mM) for 10 min at room temperature prior to adding the biotinylated inhibitor. E64 binds and inhibits all cysteine proteinase activity, thus preventing the biotinylated probe from binding and resulting in the loss or inhibition of the band(s) due specifically to cysteine proteinase activity. The results reveal a cysteine proteinase activity detected in all organisms used - including *L. mexicana* used here as a positive indicator that the method is working. A single band of 46 kDa for *E. tenella*, ~48 kDa for *C. parvum* and ~35 kDa for *T. gondii* was detected and seen to be inhibited by preincubation with E64 (shown by arrows in the corresponding figures).

#### 4.3.2.2. Effect of removing excess inhibitor

To determine whether any of the bands being detected were due to excess biotinylated probe, lysates incubated with biotin- Phe-Ala- $CHN_2$ were centrifuged in a Microcon concentrator (Micon) thus removing the unbound biotinylated probe from the proteins. The samples were then run on gels and blotted as described in 4.2.5. The results (see Figure 4.3.4.) demonstrate that the bands detected were similar in samples containing unbound inhibitor and those from which the unbound inhibitor had been removed.

#### 4.3.2.3. Proteinase detection using biotin-Arg-Arg-diazomethane

To detect cathepsin B- and subtilisin-like processing enzymes the biotin-Arg-Arg-$CH_2Cl$ was used at a concentration of 10 μM, as recommended by the manufacturer. To observe which of the proteins detected were cysteine proteinases the lysate was also preincubated with E64 (1.5 mM) as in 4.3.1.1. The results for the different lysates are shown in Figures 4.3.5, 4.3.6. and 4.3.7. Many bands were detected for each sample, yet the preincubation with E64 indicated that for *E. tenella, C. parvum* and *T. gondii* a 46 kDa, 48 kDa and 35 kDa band cysteine proteinase activity was detected. Additionally to the

preincubation with E64, the serine proteinase inhibitor PMSF was used. The lysates were preincubated with this inhibitor to determine whether any of the activities detected were due to a serine proteinase activity. A number of bands were seen to be partially inhibited, yet the one thought to represent the cysteine proteinase activity was not, thus confirming the results.

#### 4.3.2.4. Localisation of cysteine proteinases in *E. tenella*

To give an indication of where the proteinases are located in the intact parasites, live *E. tenella* sporozoites were incubated with E64 for 10 min at room temperature. The inhibitor cannot cross the cell membrane and should therefore bind only to cysteine proteinases present on the sporozoite surface. Having been washed three times in PBS (pH 7.4, 1000 x g for 5 min) the sporozoites were lysed as described in 4.2.2.1. Sporozoites which had not been preincubated with E64 were also lysed and both of these samples were run on gels and blotted (see 4.2.2.4.). The results are shown in Figure 4.3.8. A couple of bands, present in the lysate, were not present when the live sporozoites preincubated with E64 were lysed (indicated by small arrows). However the band of 46 kDa, detected in the sporozoite lysate - as with the unsporulated oocysts lysate (see Figure 4.3.5, indicated by large arrow) - was inhibited by preincubation with E64. This band was not present when intact sporozoites were incubated with E64, thus indicating that the activity is present on the surface of the sporozoites.

#### 4.3.2.5. Proteinase detection using biotin-Phe-diazomethane and biotin-Arg-diazomethane

The detection of chymotrypsin-like and trypsin-like proteinases was performed using biotin-Phe-$CH_2Cl$ and biotin-Arg-$CH_2Cl$ respectively at final concentrations of 10 μM. To ensure that binding was detecting the presence of these serine proteinases, lysates were also preincubated with PMSF (1 mM final concentration) as for E64 in 4.3.2.1. For the biotin-Phe-$CH_2Cl$ (chymotrypsin-

like) probe the results are shown in Figures 4.3.9, 4.3.10. and 4.3.11. for *E. tenella* sporulated oocysts, *C. parvum* oocysts and *T. gondii* tachyzoites respectively. The biotin-Arg-$CH_2Cl$ (trypsin-like) probe gave typical results as shown in Figures 4.3.12, 4.3.13. and 4.3.14. for *E. tenella* sporulated oocysts, *C. parvum* oocysts and *T. gondii* tachyzoites respectively. With *E. tenella*, a band of ~50 kDa was due to a serine proteinase activity as it was inhibited by PMSF (but not by E64). With *C. parvum* a number of bands were inhibited by PMSF. However, two bands (~51 kDa and ~48 kDa) were consistently inhibited using this method of detection (see Figure 4.3.10.). Similarly with *T. gondii*, the PMSF-preincubated lysate showed an overall reduction in band intensity (see Figure 4.3.14.). However, consistently a band of ~64 kDa was seen to be inhibited by the serine proteinase inhibitor. The results obtained with the inhibitor that binds to trypsin-like enzymes were similar to those using the detection of chymotrysin-like enzymes for both *E. tenella* and *C. parvum*. A band of ~50 kDa for *E. tenella* (see Figure 4.3.12.) and a band of ~48 kDa for *C. parvum* (see Figure 4.3.13.) were detected. However, for *T. gondii* the band found to be inhibited by PMSF was ~58 kDa in size (see Figure 4.3.14.).

#### 4.3.2.6. Localisation of proteinases using biotin-Arg-Arg-peptidyl diazomethane

Typical results of labelling *E. tenella* sporozoites with biotin-Arg-Arg-$CH_2$ Cl and streptavidin-FITC (as described in section 4.2.2.6.) are shown in Figure 4.3.15. Preincubation with proteinase inhibitors did not inhibit the labelling, indicating that this was due to non-specific binding.

### 4.3.3. Detection of proteinases using antisera raised against *L. mexicana* cysteine proteinases

#### 4.3.3.1. Detection of the Type I cysteine proteinases of *L. mexicana*

Western blots of samples of *E. tenella* unsporulated oocysts and sporozoites (see Figure 4.3.16.) and *T. gondii* tachyzoites and *C. parvum* oocysts (see Figure 4.3.17.) did not detect any proteins cross reacting with the antibody raised against the *L. mexicana* Type I cysteine proteinase. The *L. mexicana* sample did show a band of ~30 kDa, as in agreement with previous documented studies and thus confirming that the method was working.

#### 4.3.3.2. Detection of the Type II cysteine proteinases of *L. mexicana*

Western blots of *E. tenella* unsporulated oocysts and sporozoites (see Figure 4.3.18.) did cross react with the antibody raised against the *L. mexicana* Type II cysteine proteinase. A band of ~47 kDa was seen for the sporozoite lysate, but not for the oocyst stage. For *T. gondii* tachyzoites and *C. parvum* oocysts (see Figure 4.3.19.) no cross reaction was observed.

### 4.3.4. Sialidase detection

#### 4.3.4.1. Fluorometric assay

*Clostridium perfringens* sialidase (Sigma) was used to ensure the assay was working and to provide a standard curve for a reference using the method described in 4.2.3.2. (see Figure 4.3.20.). The sialidase inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminc acid (5NeuAc2en) was also used in increasing concentrations to ensure the assay was working (see Figure 4.3.21.).

Having established the assay, *E. tenella* merozoite lysates were assayed for activity. The merozoites were used at a final concentration of $1.25 \times 10^7$ in the

incubation mixture however no activity was seen in these lysate samples (data not shown).

### 4.3.4.2. Fluorescent staining of sialidase in polyacrylamide gel electrophoresis

0.1 U of enzyme activity of the *Clostridium perfringens* sialidase was run on a 10% gel along with *E. tenella* merozoites (3.6 x $10^4$ per well) - the samples were not boiled before the addition of the sample buffer (see 4.2.2.1.) - thus ensuring the preservation of enzyme activity. Once the gels were run and incubated as described in 4.2.3.3, photographs were taken of the gel for both the fluorescent staining and the following coomassie brilliant blue staining (see Figures 4.3.22 (a) and (b)). The results showed a band of fluorescence for the *Clostridium perfringens* sample at approximately 33 kDa, yet the *E. tenella* sample showed no activity.

Arrow indicates band consistently inhibited by preincubation with E64, thus indicating cysteine proteinase activity in *C. parvum* oocysts.

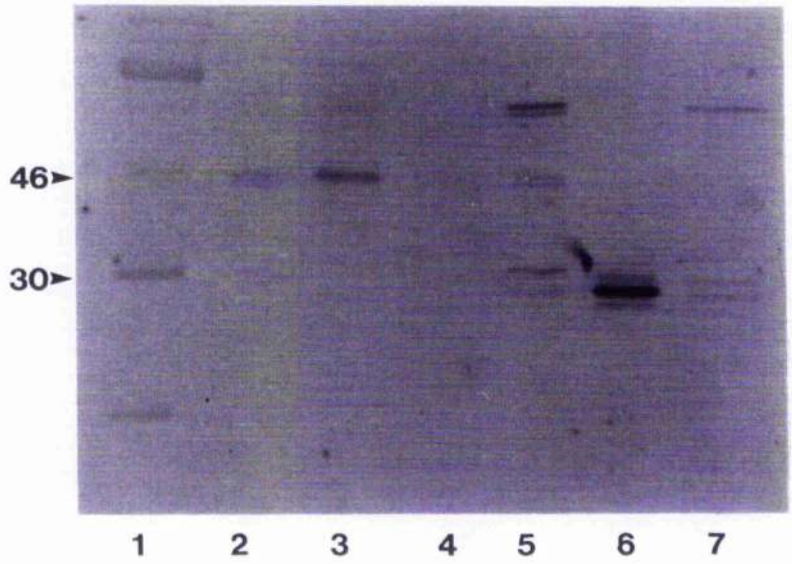



Figure 4.3.1.: Proteinase detection in lysates of *E. tenella* unsporulated oocysts (equivalent to 2 x $10^5$ per well) and *L. mexicana* amastigotes (1 x $10^7$ per well) using biotin-Phe-Ala-$CHN_2$. Lane 1 - Rainbow markers (molecular weights indicated on the left of picture); Lane 2 - *E. tenella* preincubated with E64 (1.5 mM); Lane 3 - *E. tenella* with biotinylated inhibitor (100 μM); Lane 4 - *E. tenella* lysate; Lane 5 - *L. mexicana* preincubated with E64 (1.5 mM); Lane 6 - *L. mexicana* with biotinylated inhibitor; Lane 7 - *L. mexicana* supernatant fraction.

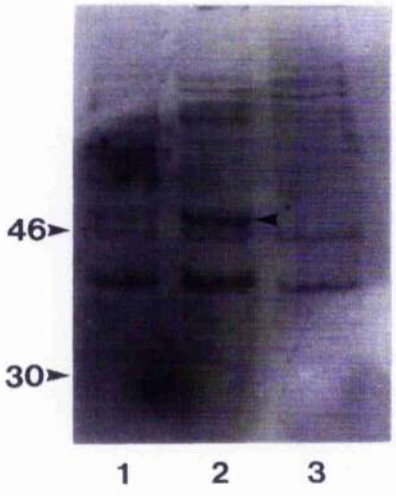



Figure 4.3.2.: Proteinase detection in lysates of *C. parvum* oocysts (3 x $10^4$ per well) using biotin-Phe-Ala-$CHN_2$. Lane 1 - lysate preincubated with E64 (1.5 mM); Lane 2 - lysate with biotinylated inhibitor; Lane 3 - lysate. Molecular weights markers indicated on the left of picture.

Arrow indicates band that is consistently inhibited by preincubation with E64, thus indicating a cysteine proteinase activity in *T. gondii* tachyzoites.

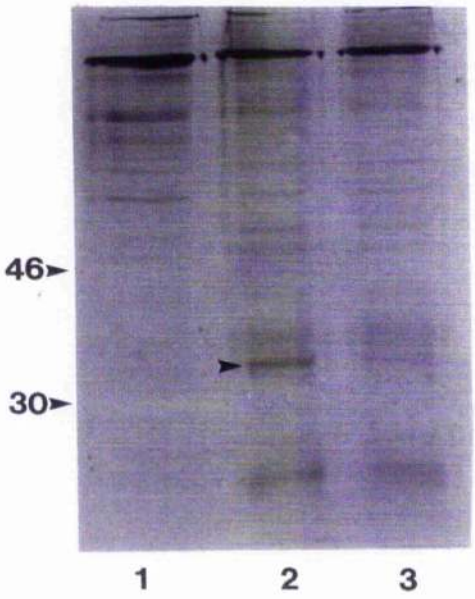



Figure 4.3.3.: Proteinase detection in lysates of *T. gondii* tachyzoites (3 x $10^6$ per well) Bio-Phe-Ala-$CHN_2$. Lane 1 - lysate; Lane 2 - lysate with biotinylated inhibitor; Lane 3 - lysate preincubated with E64 (1.5 mM).

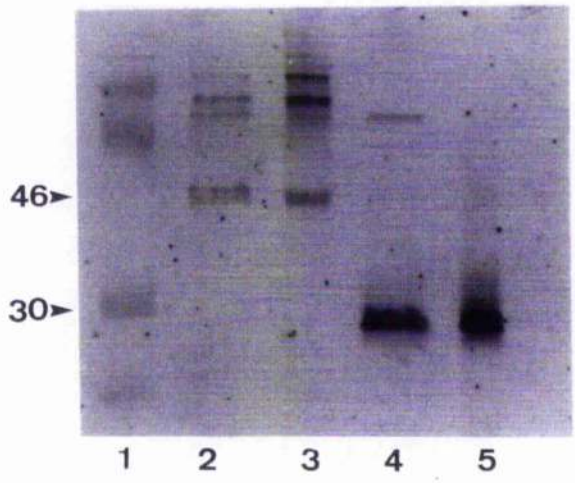



Figure 4.3.4.: Effect of removing excess biotin-Phe-Ala-$CHN_2$ from samples of *E. tenella* unsporulated oocyst lysates (4 x $10^5$ per well) and *L. mexicana* amastigotes lysates ( 2 x $10^7$ per well). Lane 1 - Rainbow markers (molecular weights indicated on the left of picture); Lane 2 - *E. tenella* from which probe has been removed; Lane 3 -*E. tenella* lysate with excess probe present; Lane 4 - *L. mexicana* sample from which excess probe has been removed; Lane 5 - *L. mexicana* lysate with excess probe present.

Arrows indicate bands that are consistently inhibited by preincubation with E64, thus indicating cysteine proteinase activities in *E. tenella* sporulated oocysts and *C. parvum* oocysts.

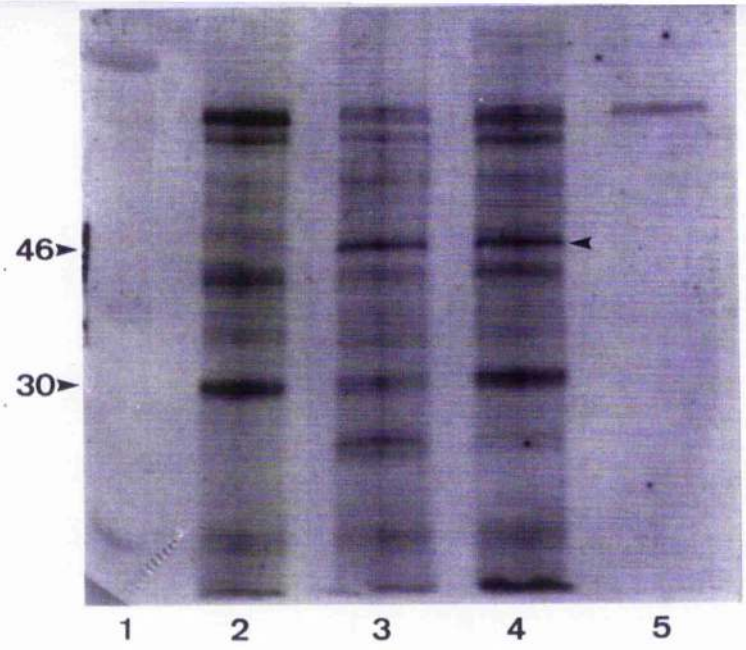



Figure 4.3.5.: Proteinase detection in lysates of *E. tenella* unsporulated oocysts ($2.5 \times 10^6$ per well) using biotin-Arg-Arg-$CH_2Cl$ (10 μM). Lane 1 - Rainbow markers (molecular weights indicated on the left of picture); Lane 2 - lysate preincubated with E64 (1.5 mM); Lane 3 - lysate preincubated with PMSF(1.5 mM); Lane 4 - lysate with biotinylated inhibitor; Lane 5 - lysate.

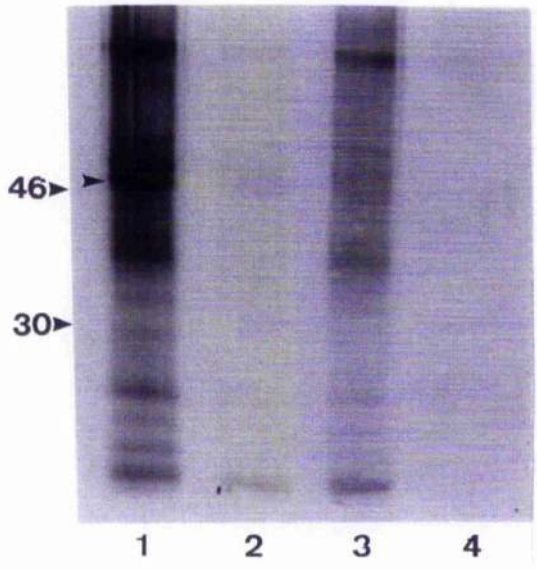



Figure 4.3.6.: Proteinase detection of *C. parvum* oocysts ($3.9 \times 10^5$ per well) using biotin-Arg-Arg-$CH_2Cl$ (10 μM). Lane 1 - lysate with biotinylated inhibitor; Lane 2 - lysate; Lane 3 - lysate preincubated with E64; Lane 4 - Rainbow markers (molecular weights indicated on left side of picture).

Arrowhead indicates band consistently inhibited by preincubation with E64, thus indicating cysteine proteinase activity in *T. gondii* tachyzoites.

Large arrowhead indicates band consistently removed by preincubating *E. tenella* sporozoite lysate or live sporozoites (before lysing) with E64, thus indicating cysteine proteinase activity may be present on *E. tenella* sporozoite surface. Small arrowheads indicate bands inhibited when live sporozoites were preincubated with E64.

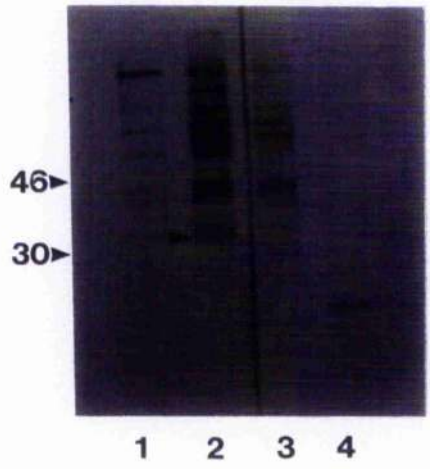



Figure 4.3.7.: Proteinase detection in lysates of *T. gondii* tachyzoites ($1.6 \times 10^5$ per well) using biotin-Arg-Arg-$CH_2Cl$. Lane 1 - lysate; Lane 2 - lysate with biotinylated inhibitor; Lane 3 - lysate preincubated with E64; Lane 4 - Rainbow markers (molecular weights indicated on left side of picture).

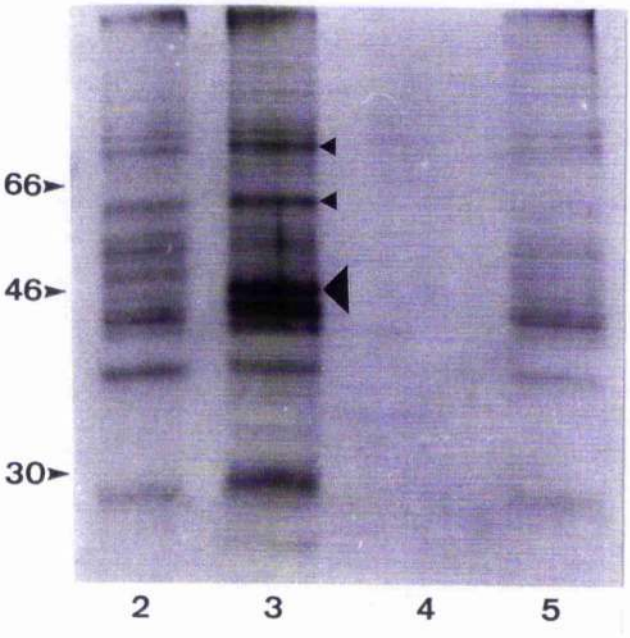



Figure 4.3.8.: Localisation of proteinases in lysates of *E. tenella* sporozoites ($5 \times 10^4$ per well) using biotin-Arg-Arg-$CH_2Cl$. Lane 2 - *E. tenella* lysate preincubated with E64; Lane 3 - *E. tenella* lysate with biotinylated inhibitor; Lane 4 - *E. tenella* lysate; Lane 5 - *E. tenella* sporozoites preincubated with E64 before lysis.

Arrows indicate bands consistently inhibited by preincubation with PMSF, thus indicating serine proteinase activities in *E. tenella* sporulated oocysts and *C. parvum* oocysts.

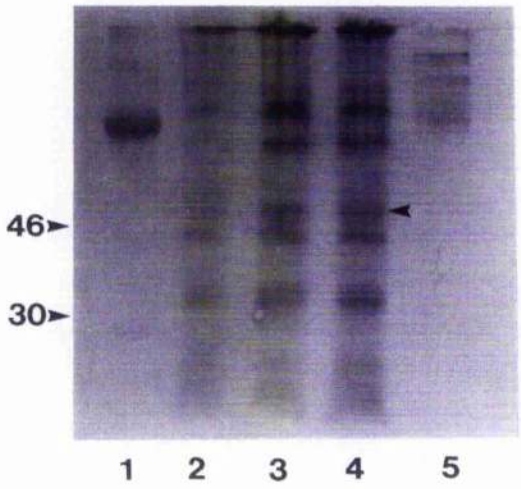



Figure 4.3.9.: Proteinase detection in lysates of *E. tenella* sporulated oocysts (3.9 x $10^5$ per well) using biotin- Phe-$CH_2Cl$ (10 μM). Lane 1 - Rainbow markers (molecular weights indicated on left side of picture); Lane 2 - Lysate preincubated with PMSF (1.5 mM); Lane 3 - lysate preincubated with E64 (1 mM); Lane 4 - lysate with biotinylated inhibitor; Lane 5 - lysate.

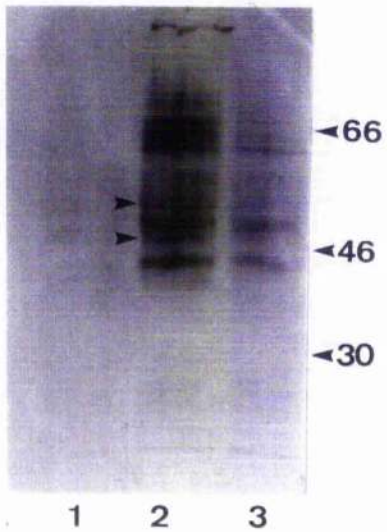



Figure 4.3.10.: Proteinase detection in lysates of *C. parvum* oocysts (3 x $10^6$ per well) using biotin-Phe-$CH_2Cl$ (10 μM). Lane 1 - lysate; Lane 2 - lysate with biotinylated inhibitor; Lane 3 - lysate preincubated with PMSF (1.5 mM).

Arrow indicates bands consistently inhibited by preincubation with PMSF, thus indicating a serine proteinase activity in *T. gondii* tachyzoites.

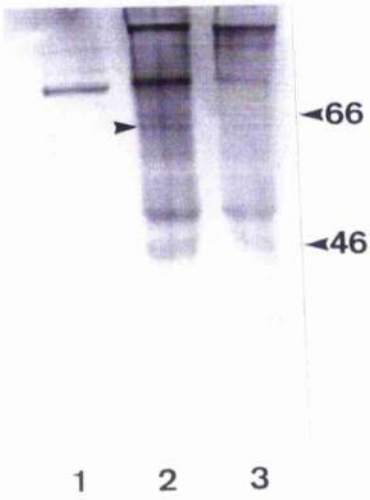



Figure 4.3.11.: Proteinase detection in lysates of *T. gondii* tachyzoites ($1.6 \times 10^4$ per well) using biotin-Phe-$CH_2Cl$ (10 μM). Lane 1 - lysate; Lane 2 - lysate with biotinylated inhibitor; Lane 3 - lysate preincubated with PMSF (1.5 mM).

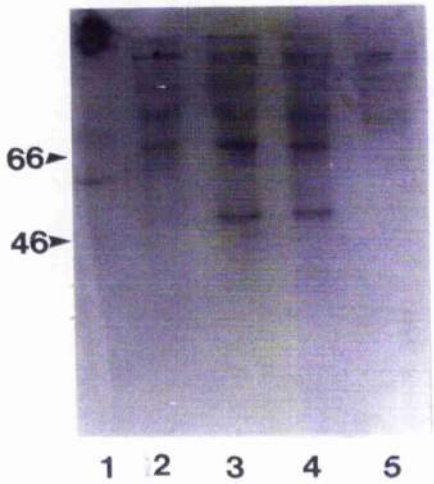



Figure 4.3.12.: Proteinase detection in lysates of *E. tenella* sporulated oocysts ($5.9 \times 10^5$ per well) using biotin-Arg-$CH_2Cl$ (10 μM). Lane 1 - Rainbow markers (molecular weights indicated on left of picture); Lane 2 - lysate preincubated with PMSF (1.5 mM); Lane 3 - lysate preincubated with E64 (1.5 mM); Lane 4 - lysate with biotinylated inhibitor; Lane 5 - lysate.

Arrows indicate bands consistently inhibited by preincubation with PMSF, thus indicating serine proteinase activities in *C. parvum* oocysts and *T. gondii* tachyzoites.

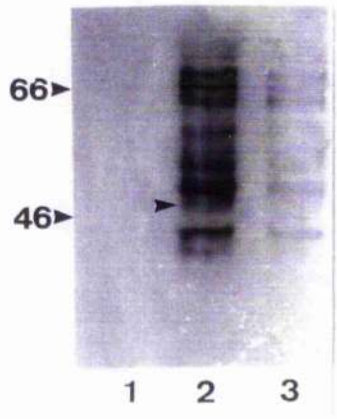



Figure 4.3.13.: Proteinase detection in lysates of *C. parvum* oocysts (1.2 x $10^6$ per well) using biotin-Arg-$CH_2Cl$ (10 μM). Lane 1 - lysate; Lane 2 - lysate with biotinylated inhibitor; Lane 3 - lysate preincubated with PMSF (1.5 mM). Molecular weights indicated on left of picture.

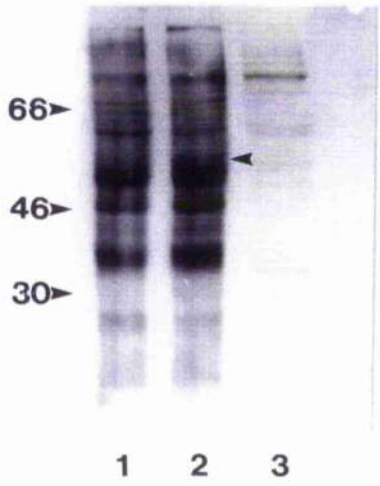



Figure 4.3.14.: Proteinase detection in lysates of *T. gondii* tachyzoites (3.1 x $10^4$ per well) using biotin-Arg-$CH_2Cl$ (10 μM). Lane 1 - lysate preincubated with PMSF (1.5 mM); Lane 2 - lysate with biotinylated inhibitor; Lane 3 lysate . Molecular weights indicated on left side of picture.

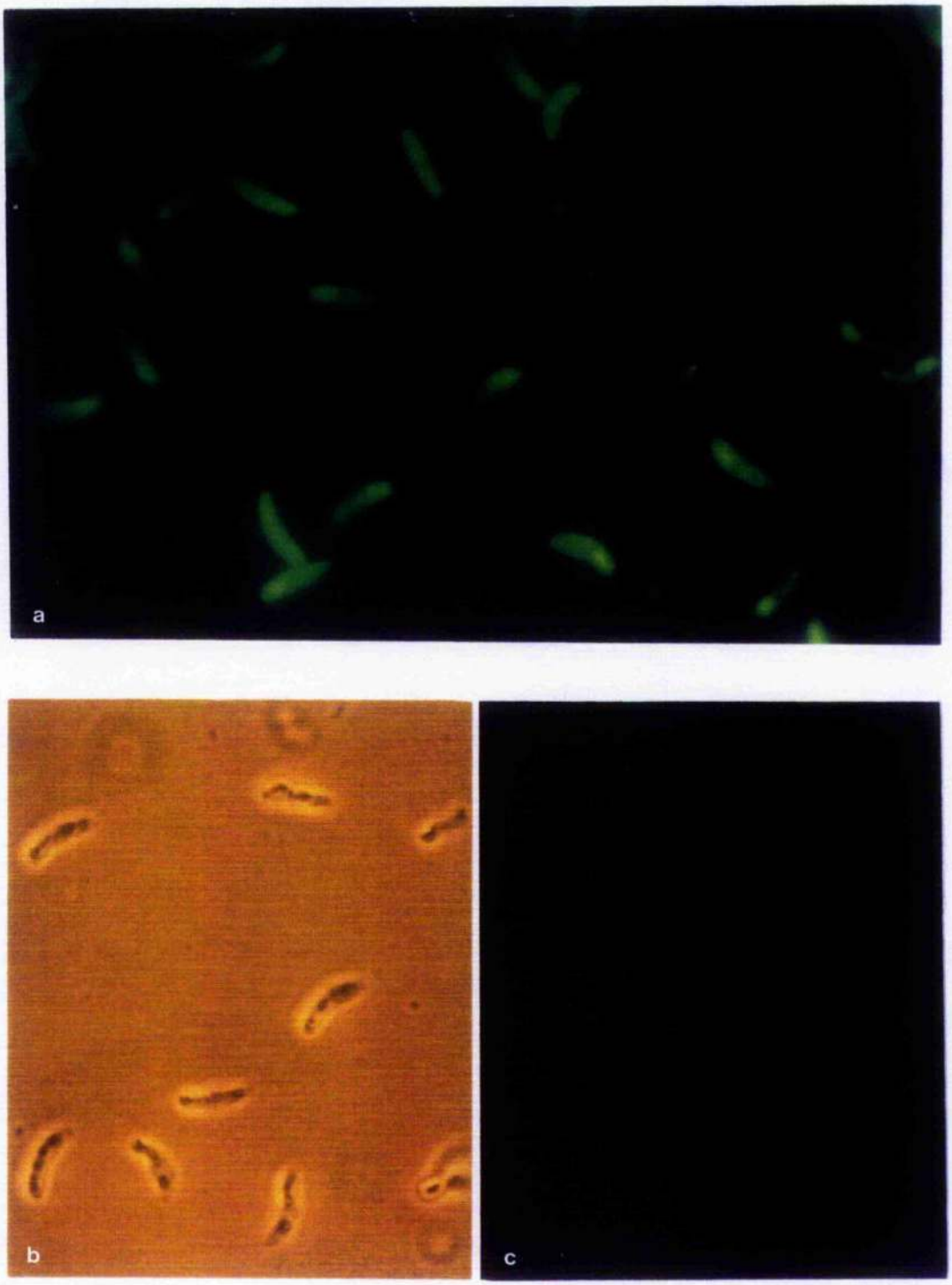



Figure 4.3.15.: Localisation of proteinases using biotin-Arg-Arg-$CH_2Cl$.
(a) *E. tenella* sporozoites incubated with biotin-Arg-Arg-$CH_2Cl$ (100 μM) as seen under the FITC filter of a fluorescence microscope; (b) and (c) (same field) *E. tenella* sporozoites without biotin-Arg-Arg-$CH_2Cl$ as seen under phase contrast microscopy and the FITC filter respectively.

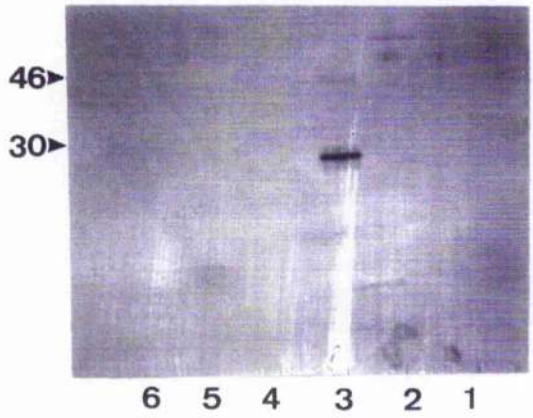



Figure 4.3.16.: Proteinase detection using antisera raised against Type I *L. mexicana* cysteine proteinases in lysates of *E. tenella* unsporulated oocysts and sporozoites. Lane 1 - *E. tenella* unsporulated oocysts (2 x $10^5$ per well); Lane 2 - *E. tenella* sporozoites (3 x $10^5$ per well);Lane 3 - *L. mexicana* amastigotes ( 5 x $10^6$ per well). Lanes 4 - 6 as before but with preimmune serum. Molecular weights indicated on left side of picture.

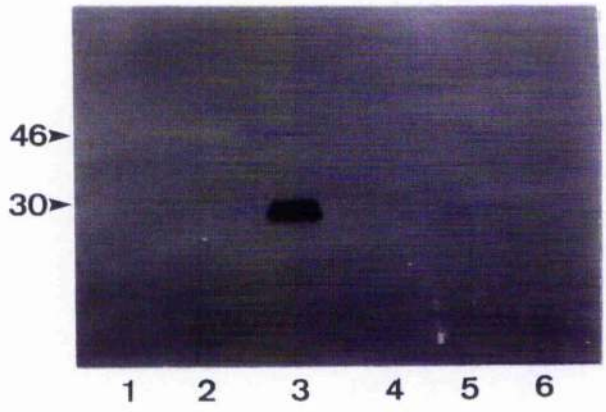



Figure 4.3.17.: Proteinase detection using antisera raised against Type I *L. mexicana* cysteine proteinases in lysates of *T. gondii* tachyzoites and *C. parvum* oocysts. Lane 1 - *T. gondii* tachyzoites (2 x $10^6$ per well); Lane 2 - *C. parvum* oocysts (4 x $10^6$ per well); Lane 3 - *L. mexicana* amastigotes (5 x $10^6$ per well). Lanes 4-6 as before but with preimmune serum. Molecular weights indicated on left side of picture.

Arrow indicates band of activity detected in *E. tenella* sporozoites detected by anti-serum raised against Type II *L. mexicana* cysteine proteinases.

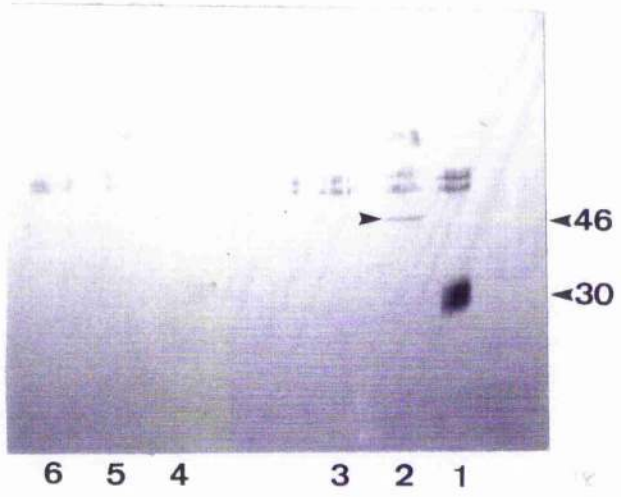



Figure 4.3.18.: Proteinase detection using antisera raised against Type II *L. mexicana* cysteine proteinases in lysates of *E. tenella* oocysts and sporozoites. Lane 1 - *L. mexicana* amastigotes ($5 \times 10^6$ per well); Lane 2 - *E. tenella* sporozoites ($3 \times 10^5$ per well); Lane 3 - *E. tenella* unsporulated oocysts ($2 \times 10^5$ per well). Lanes 4-6 as before but with preimmune serum. Molecular weights indicated on right side of picture.

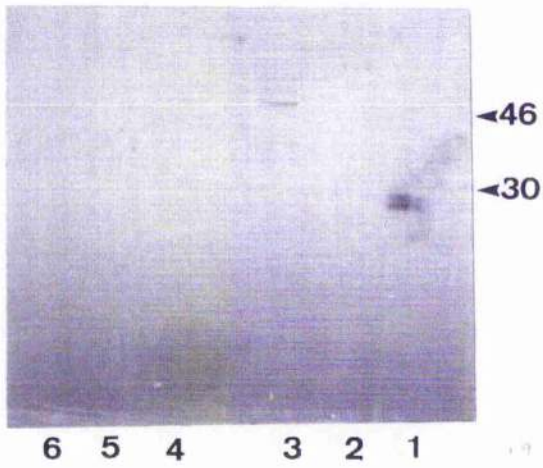
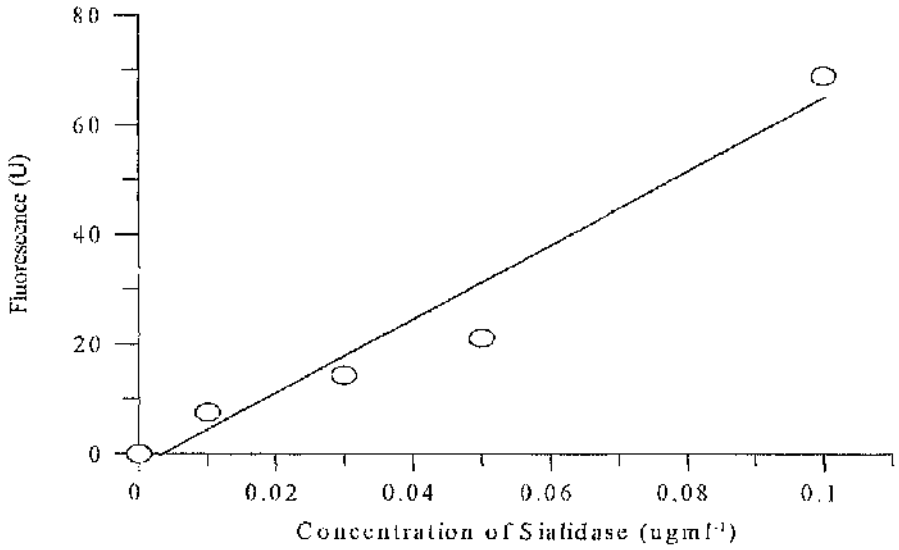



Figure 4.3.19.: Proteinase detection using antisera raised against Type II *L. mexicana* cysteine proteinases in lysates of *T. gondii* tachyzoites and *C. parvum* oocysts. Lane 1 - *L. mexicana* amastigotes ($5 \times 10^6$ per well); Lane 2 - *C. parvum* ($4 \times 10^6$ per well); Lane 3 - *T. gondii* ($2 \times 10^6$ per well); Lane 4 - Rainbow markers (molecular weights indicated on right of picture). Lane 5-8 as before but with preimmune serum.

**Figure 4.3.20: Standard curve using *Clostridium perfringens* sialidase**




Hydrolysis of 4-MU-Neu5Ac by *C. perfringens* sialidase
Results from one experiment

**Figure 4.3.21: Effect of sialidase inhibitor on *Clostridium perfringens* sialidase activity**


5NeuAc2en inhibition of *C. perfringens* sialidase
Results from one experiment

Arrow indicates fluorescent band of sialidase activity in *C. perfringens*.

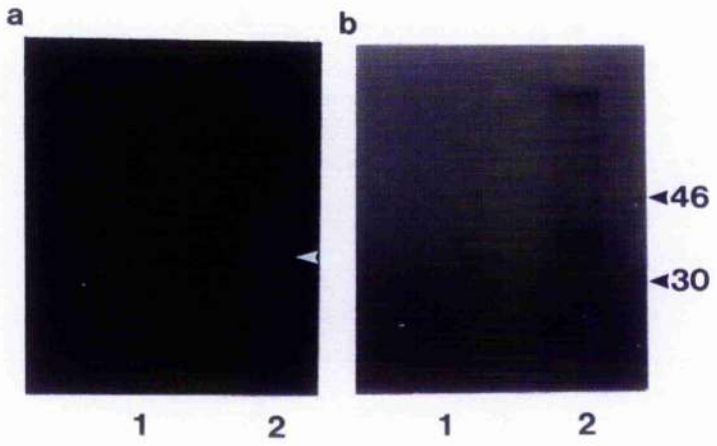



Figure 4.3.22.: Fluorescent staining of sialidases in polyacrylamide gel electrophoresis. Lane 1 - *E. tenella* merozoite lysate ($3.6 \times 10^4$ per well); Lane 2 - *Clostridium perfringens* purified enzyme (0.1 U). (a) fluorescent staining; (b) coomassie brilliant blue staining of same gel.

**4.3 Discussion**

The results from the APIzym kit (see Table 4.3.1.) indicated that a variety of enzymes are present in the *Cryptosporidium* and *Eimeria*, and that life cycle stage variations may be present. However, none of the parasite stages examined contained any of the following: α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase all of which were reported absent in *T. gondii* (with the exceptions of β-galactosidase and β-glucuronidase, Manafi *et al.*, 1993). Trypsin was detected in the sporozoite stage of *E. tenella* (though not present in the oocyst lysate), but this result should be treated with caution since it may be possible that the trypsin used in the *in vitro* excystation procedure may not have been totally removed from the parasite lysate.

Establishing these enzymatic profiles enables us to understand more about the parasites themselves as well as providing a means of characterising these microorganisms. It may also provide a way of determining differences between strains or isolates of the parasites. Indeed Manafi *et al.* (1993) did see differences in the enzymatic profiles in a number of *Toxoplasma* strains, and with isoenzyme differences having been reported in different *Cryptosporidium* isolates (Awad-el-Kariem *et al.*, 1995), this method may also be able to distinguish between strains and isolates, thus making it easier to identify the isolates being studied.

Detection of proteinase activity in coccidia has mainly been performed

using techniques like gelatin gels (Michalski *et al.*, 1994; Forney *et al.*, 1996). However these methods are relatively insensitive, requiring a reasonable amount of parasite material. When the amount of material is limiting, as it is for these parasites, a more sensitive method is needed. Recently developed biotinylated probes (McGinty *et al.*, 1993) were reported to be highly sensitive in detecting proteinase activity. The results shown here employed four biotinylated probes that were thought to bind to and inhibit a number of proteinase activities.

The first two probes, biotin-Phe-Ala-$CHN_2$ and biotin-Arg-Arg-$CH_2Cl$, were specific for cysteine proteinase activities. Many bands were seen using this detection system, so to ensure that the biotinylated probe was binding specifically to cysteine proteinases, we preincubated lysates with E64, an irreversible cysteine proteinase inhibitor. This inhibitor will bind to the cysteine proteinase active site, thus preventing the biotinylated probe from binding, and therefore enabling us to see the bands that are actually due to cysteine proteinases and not just nonspecific binding. Indeed, at least one band was consistently inhibited by the preincubation process, indicating that this method (including the preincubation of the lysates) was a valid one. For all three parasites studied it was observed that both the probes detected bands of the same molecular weight: for *E. tenella* and for *C. parvum* a 46 kDa band was seen to be present (see Figures 4.3.1.and 4.3.5. for *E. tenella*; and Figures 4.3.2. and 4.3.6. for *C. parvum*); and for *T. gondii* a band of approximately 35 kDa was observed (see Figures 4.3.3. and 4.3.7.). These results indicated that there was at least one cysteine proteinase present in these apicomplexan parasites. Whether the activity was present on the invasive stage remained unknown. Using the Bio-Arg-Arg-$CH_2Cl$ probe for cysteine proteinase

detection, localisation of the activity on *E. tenella* sporozoites was attempted (see Figure 4.3.8.). Since E64 does not pass through intact membranes it will bind and block any surface cysteine proteinase activity. The sporozoites preincubated with E64 before lysis showed that the same 46 kDa band was removed along with a couple of others, thus indicating that this cysteine proteinase activity, for *E. tenella*, does appear to be present on the invasive sporozoite surface. This is similar to a recent report on *C. parvum* where a metallo-dependent cysteine proteinase of ~24 kDa was detected and partially purified (Nesterenko *et al.*, 1995). These results, along with the finding that cysteine proteinase inhibitors inhibited mucus penetration and host cell invasion (see Chapter 5 and 6), indicate cysteine proteinases may play a role in these processes.

Serine proteinase activity was detected using a chymotrypsin-like probe biotin-Phe-$CH_2Cl$ and a trypsin-like probe biotin-Arg-$CH_2Cl$. Again to ensure that the bands detected were due to a serine proteinase activity the lysates were preincubated with PMSF, the irreversible serine proteinase inhibitor (as described before for E64). Similar blots were seen using both the probes for *C. parvum* and *E. tenella* with bands of activity seen at ~48 kDa for both of these parasites (see Figures 4.3.9. and 4.3.12. for *E. tenella*; Figures 4.3.10. and 4.3.13. for *C. parvum*) thus indicating that, indeed, a serine proteinase activity has been detected. Recently, a serine proteinase was purified and characterised from *E. tenella* sporulated oocysts. The molecular weight reported was ~ 20 kDa, and this activity was suggested to be involved in host cell invasion (Michalski *et al.*, 1994). Indeed, serine proteinase inhibitors were seen to inhibit mucus penetration

and host cell invasion of sporozoites (see Chapters 5 and 6 respectively, suggesting an involvement of a serine proteinase(s) in these processes.

For *T. gondii* the chymotrypsin-like label detected an activity of ~ 65 kDa (see Figure 4.3.11.), whereas the trypsin-like label detected one at ~58 kDa (see Figure 4.3.14.) indicating that the probes are detecting two distinct activities.

In addition to the Western blotting procedure, the biotinylated probes were used to attempt to localise the proteinase activities on live sporozoites, using fluorescence microscopy. However these experiments were unsuccessful. Preincubation with proteinase inhibitors did not inhibit the fluorescence, suggesting that this was simply due to non-specific binding.

Antisera raised against *L. mexicana* Type I and Type II cysteine proteinases were also used to probe the lysates. Antisera against Type I proteinases did not cross react with any activity in any of the coccidia. The Type II antisera did not cross react with any activity in *Cryptosporidium*, *Toxoplasma* or *E. tenella* unsporulated oocysts, but did show an activity in *E. tenella* sporozoites at approximately 46 kDa - the same molecular weight of the band being detected by the biotinylated probes specific for cysteine proteinases. This indicates stage specificity in proteinases present in *Eimeria*, as is the case for *L. mexicana* (Lockwood *et al.*, 1987). Since it is the invasive sporozoite stage that possesses this activity, it may be that this activity is necessary for this life cycle stage, and may be used in penetration of mucus and invasion of host cells.

The failure to detect sialidase activity using the protocol previously described for *E. tenella* (Pellegrin *et al.*, 1993) may be due to a number of

reasons. For example not enough parasite material present, or the fluorescence spectrophotometer not being sensitive enough. The sialidase inhibitor strongly inhibited mucus penetration (see Chapter 5) (although host cell invasion was not affected), thus suggesting that sialidase is important in the penetration process. However, until it is determined that this inhibitor is specifically inhibiting the parasite enzyme, no definite conclusions cannot be drawn, since this inhibitor may be affecting the parasite in some other way. It would be useful to obtain antibodies, raised against the sialidases of other organisms, to try to detect the enzyme. Immunofluorescence or gold particle labelling could be used on sporozoites and merozoites to localise the enzyme in these invasive stages and to see if the sialidase inhibitor did inhibit this labelling process.

The studies performed in this chapter have attempted to provide more information on the proteinases and sialidase of the coccidia. Cysteine proteinase and serine proteinase activity was detected in all of the coccidia, but localisation of these activities remains to be determined. Sialidase was not detected using methods previously described, and other methods of detection should be attempted.

# CHAPTER FIVE

# MUCUS PENETRATION

## 5.1. Introduction

Mammalian intestinal epithelial cells are covered by a mucus layer of on average 400 μm thick (Cohen and Laux, 1995). The presence of this gel network in the gastrointestinal tract has been suggested to have a variety of functions including a protective barrier against enteric pathogens including bacteria and parasites. Many studies have investigated how organisms penetrate the mucus layer in both the gastrointestinal and respiratory tracts; for example *Escherichia coli* (Smith, 1992) and *Streptococcus pneumoniae* (Rayner *et al.*, 1995) respectively and along with a number of reviews (Cohen and Laux, 1995; Freter, 1988). How interaction with the mucus layer occurs has not been elucidated for many organisms, but for *E. coli* (Smith, 1992) and *Entamoeba histolytica* (Tse and Chadee, 1991) mucus is thought to contain receptors for attachment. Yet for many gut-parasites, although interactions with the host cells have been extensively studied, the first step in host infection, the penetration of the mucus layer, has been largely ignored.

Epithelial goblet cells secrete mucins which form a gel network (mucus). This consists of a mixture of glycoproteins, peptides, lipids, water, electrolytes and various serum and cellular macromolecules (for example IgA) (Tse and

Chadee, 1991). Two distinct physical forms of mucus have been described in the intestine, a water-insoluble gel layer attached to the mucosal surface of the epithelial cells and a water-soluble viscous solution in the lumen (Tse and Chadee, 1991). Mucins or mucus glycoproteins consist of a polypeptide backbone attached to which are oligosaccharide side chains which contain varying amounts of five major sugars: galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine and sialic acid. These mucins are characterised by their large extended structure and high carbohydrate content (>80% w/w) found attached to the protein core via O-glycosidic links (Carlstedt *et al.*, 1985; Tse and Chadee, 1991). Eight different core regions of mucin glycoproteins have been described (Hounsell *et al.*, 1996). The mucus layer is thought to have two main roles: firstly, it plays a non-specific role by providing a physicochemical barrier against enteric pathogens; and secondly, it has specific roles like providing selective adhesion sites for various microorganisms (Neutra and Forstner, 1987; Van Klinken *et al.*, 1995; Batt *et al.*, 1996; Hounsell *et al.*, 1996). These functions are the focus of my attention in this chapter.

The methods used to study the effects that microorganisms have on the mucus have been numerous. One example has been to measure mucin lysis using agar plates and colonies of *Candida albicans* with mucus incorporated into the medium (Colina *et al.*, 1996). Another method involved observing the movement of *Trichomonas vaginalis* through mucus having placed the mucus on slides to monitor the distance travelled by the pathogen over certain time periods (Paget and James, 1994). However *in vitro* studies have not been performed to any great extent on any of the mucus penetrating microorganisms. Indeed most studies

performed have focused on the histology and morphology of mucus goblet cells in the *in vivo* infection.

Certain molecules on cell surfaces are known to be involved in cell-cell recognition. One such molecule is sialic acid - a negatively charged carbohydrate found in mucus almost exclusively at the terminal ends of the mucin oligosaccharides - and it is thought to play a role in cell-surface recognition (Carlstedt *et al.*, 1985; Schenkman and Eichinger, 1993). In rat intestinal mucus sialic acid was estimated to be 13.2% of the total carbohydrate composition (Neutra and Forstner, 1987). Sialic acid has been implicated in host invasion by various organisms including the influenza virus (Pedroso de Lima *et al.*, 1995;), *Trypanosoma cruzi* (Schenkman *et al.*, 1991) and *Plasmodium falciparum* (Cross and Takle, 1993). *T. cruzi* trypomastigotes have a trans-sialidase activity which transfers host sialic acids onto the parasite surface thus creating an epitope involved in host cell invasion, with infectivity correlating with higher activities (Schenkman *et al.*, 1991; Cross and Takle, 1993). Studies have shown that glycosidases and proteinases can act more effectively on glycoproteins, and so degrade the mucus layer, after the removal of terminal sugars such as sialic acids (Engstler and Schauer, 1993). Thus sialidase can play a part in penetration of mucus layers by microorganisms. Indeed, there is some evidence that *Eimeria* may have an effect on mucus since a recent study on three species (*E. tenella, E. maxima* and *E. necatrix*) detected sialidase activity in both the sporozoite and merozoite stages of the parasites - the latter possessing 10-20 times higher activity (Pellegrin *et al.*, 1993). The sialidase was observed to have optimal activity at

40°C (chicken intestine is 40-43°C) and was resistant to the proteinases normally present in the intestine. It was suggested - but with no experimental data - that the sialidase desialylates the mucus thus reducing the viscosity of the environment and facilitating parasite migration (Pellegrin *et al.*, 1993). The sialidase could also be involved in modifying the surface of the host cell prior to and during invasion (Pellegrin *et al.*, 1993).

It was also reported recently that an aspartyl proteinase of *Candida albicans* appears to be involved in mucin proteolysis (Colina *et al.*, 1996). Motility studies performed on *E. nieschulzi* also indicated that high concentrations of mucin present increased the sporozoite motility (Upton and Tilley, 1992). Additionally *C. parvum* infections were seen to alter the mucosal viscosity with high amounts of mucus becoming dislodged - possibly due to enzyme degradation by the parasite (Hill *et al.*, 1991).

Although there were these few reports suggesting that coccidian parasites may be able to affect the mucus lining, there have been no reports on the ability of the parasites to penetrate the mucus and, if so, if it is by way of mechanical movement by the sporozoite and/or due to the release of parasite enzymes, for example sialidase, to break down the mucin network. The following experiments were designed to give a greater insight into the process of mucus penetration.

## 5.2. Materials and Methods

### 5.2.1. Parasites

#### 5.2.1.1. Sporozoites

Sporozoites were excysted as described in 2.3.5. for *C. parvum* and 2.2.2.2. for *E. tenella.* These were then separated from oocysts as described in 3.2.1 and used immediately.

#### 5.2.1.2. *E. tenella* merozoites

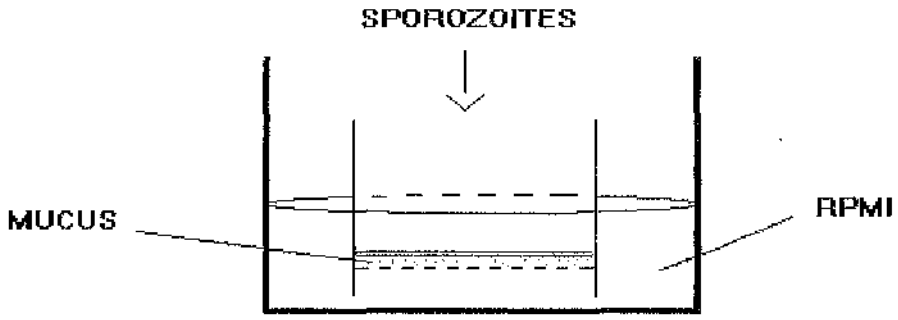
*E. tenella* merozoites were prepared as described previously in section 4.2.3.1.

### 5.2.2. Preparation of mucus layers

To study mucus penetration, desiccated porcine gastric mucus (Sigma) was reconstituted by mixing with RPMI. The mucus was then added to 12 μm culture plate inserts (Millipore) which had already been placed in 400 μl RPMI in wells of a 24 well plate. The mucus was added to a depth of 400 μm, 500 μm or 700 μm for *C. parvum* sporozoites *E. tenella* merozoites and *E. tenella* sporozoites respectively, according to the differences in size between the parasites

(see Figure 5.2.1).

**Figure 5.2.1: Preparation of mucus layers for mucus penetration studies.**




Having allowed the mucus to set for at least 30 min at room temperature, sporozoites were added to the inserts as illustrated in Figure 5.2.1 at concentrations ranging from $10^6$-$10^7$ $ml^{-1}$ in 200 µl volumes. The inserts were incubated at 37°C in 24-well culture plates for 60 or 120 min in an aerobic environment. The number of parasites that had penetrated the mucus layer, thus ending up in the RPMI under the culture plate insert, could then be counted by microscopic observation and expressed as percentage penetration.

### 5.2.3. Enzyme inhibitors and additional compounds

To try and elucidate the mechanisms that may be involved in parasite penetration both sporozoites and merozoites were incubated with a range of inhibitors and/or additional compounds before being added to the mucus.

The enzyme inhibitors used were as follows: cysteine proteinase inhibitors, trans-epoxysuccinyl-leucylamido-[4-guanidino]butane (E64), 1.5 mM; N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK), 25 μM; Z-phenylalanine diazomethane (ZFA), 10 μM; a metallo-proteinase inhibitor: ethylenediaminetetraacetic acid (EDTA), 1 mM; an aspartic proteinase inhibitor (pepstatin, 1 μM); serine proteinase inhibitors: aprotinin, 100 μM; leupeptin, 100 μM (which also inhibits some cysteine proteinases); $\alpha_1$-antitrypsin, 0.5 mg $ml^{-1}$; the polyamine biosynthesis inhibitor DFMO (a specific inhibitor of ornithine decarboxylase, 2 mM); a sialidase inhibitor (2-deoxy-2,3-dehydro-N-acetylneuraminic acid (5NeuAc2en), 0.1 mM; the actin microfilament inhibitor cytochalasin D, 10 μM; and, as a negative control, formaldehyde (10%, v/v).

To further analyse the sialidase activity a lectin was employed which was obtained from the legume plant *Maackia amuriensis* (Vector laboratories). This lectin is known to specifically bind sialic acid residues and was used at a final concentration of 0.1 mg $ml^{-1}$ and preincubated with the sporozoites for 30 min at 37°C.

To further investigate the effect of polyamines on mucus penetration, the mucus was reconstituted with RPMI containing a final concentration of 1 mM putrescine.

## 5.3. Results

### 5.3.1. Effect of various mucus concentrations on sporozoite penetration

A range of mucus concentrations was prepared and placed in prewetted culture plate inserts. *C. parvum* and *E. tenella* sporozoites were then added as illustrated in Figure 5.2.1 and incubated at 37°C for 60 and 120 min for *C. parvum* and *E. tenella* respectively. The percentage of *C. parvum* and *E. tenella* sporozoites that penetrated the mucus is illustrated in Figures 5.3.1, and 5.3.2, respectively. It was observed that for both parasites, as the mucus concentration increased the percentage penetration decreased (after 9% for *E. tenella*) to approximately 2% at a 12% mucus concentration. For *C. parvum* it was decided that the 60 min incubation time should be used since the sporozoites of this species are known to die very quickly in *in vitro* conditions (see Chapter 3), whereas for *E. tenella* the 120 min incubation was chosen. 0.4 mm and 0.7 mm depths of mucus were used for *C. parvum* and *E. tenella* sporozoites, respectively, which simply reflects the size difference between these two parasites. For *C. parvum* 6% mucus was used since this concentration has been used previously for *T. vaginalis* (Paget and James, 1994), which resulted in approximately 4% penetration after a 60 min incubation. Yet for *E. tenella* the concentration of the mucus did not appear to affect sporozoite penetration until after 9%, which was chosen and gave an approximate percentage penetration of 60% after a 120 min incubation.

These conditions were used in the following experiments.

### 5.3.2. Effect of media supplements on the penetration of mucus by *C. parvum*

1% glucose and 10% foetal calf serum (FCS) (heat inactivated) were added to the RPMI into which the *C. parvum* sporozoites penetrate. After a 60 min incubation the results were recorded and are shown in Table 5.3.1. Compared with the control both glucose (though not statistically significant) and foetal calf serum ($P<0.02$) appeared to inhibit penetration.

### 5.3.3. Effect of enzyme inhibitors on mucus penetration

*C. parvum* and *E. tenella* sporozoites were filtered and resuspended in RPMI at a density of $10^6$-$10^7$ $ml^{-1}$. Enzyme inhibitors were added to the sporozoite suspensions as described in 5.2.3. and were preincubated for 10 min at room temperature after which sporozoites were either given three washes in RPMI (10 min and 5 min at 1000 x g for *C. parvum* and *E. tenella* respectively) or not. The sporozoites were added to the mucus (6% and 9% mucus concentrations for *C. parvum* and *E. tenella* respectively) and incubated for 60 min or 120 min for *C. parvum* and *E. tenella,* respectively, whereupon the number of sporozoites that had penetrated was determined. For the sporozoites that were washed free of the inhibitors the results are shown in Figures 5.3.3. and 5.3.5. respectively for *C. parvum* and *E. tenella*. The results obtained with the inhibitors present are shown in Figures 5.3.4. and 5.3.6. for *C. parvum* and *E. tenella* respectively.

For both parasites, the negative control formaldehyde, cytochalasin D, the

sialidase inhibitor (5NeuAc2en), and the serine proteinase (aprotinin, leupeptin, antitrypsin) and cysteine proteinase (E64, TLCK, ZFA) inhibitors all greatly inhibited penetration. However the metallo- (EDTA) and aspartic proteinase (pepstatin) inhibitors had very little - if any effect - on the penetration process. These results were also true when the inhibitors had been washed off from the sporozoites, although these results were only repeated twice and further experimental data would be needed for statistical analysis.

### 5.3.4. Effect of the *Maackia amurensis* lectin II on *E. tenella* penetration

Freshly excysted *E. tenella* sporozoites were preincubated with a *Maackia amurensis* lectin (Vector laboratories, California) which specifically binds sialic acids (Fischer and Brossmer, 1995). The final concentration of lectin in the sporozoite suspension was 0.1 mg $ml^{-1}$ (as described in 5.2.3.). After a 30 min incubation sporozoites were either washed three times in RPMI (1000 x g for 5 min) to remove unbound lectin, or the sporozoites were not washed and therefore the lectin remained in the sporozoite suspension. The sporozoites were then added to the mucus layers and incubated for 120 min, whereupon the penetration of the sporozoites through the mucus was determined.

The results (Figure 5.3.7.) show that the lectin inhibited parasite penetration when it was present throughout the experiment ($P<0.10$). However when the sporozoites were washed, there was no inhibitory effect. This would

suggest that the effect was on the mucus layer when the lectin was present throughout the experiment.

### 5.3.5. Competition between the *M. amurensis* lectin II and sialidase

To determine to what extent the lectin competed with the sialidase activity, the following experiment was performed. *E. tenella* sporozoites were preincubated with the *M. amurensis* lectin as described in 5.2.3. after which sialidase of *Clostridium perfringens* (0.5 mg $ml^{-1}$) was added to the sporozoite suspension which was then added to the mucus layer. Samples of sporozoites were also incubated with the lectin or sialidase alone, at the same concentrations and conditions as in the combined sample. Parasite penetration was allowed to proceed for 120 min at 37°C and the penetration determined and the percent inhibition calculated (see Figure 5.3.8.). It was observed that the addition of sialidase increased mucus penetration by cleaving the sialic acid residues from the mucus and therefore decreasing viscosity. As before in 5.3.4. the presence of the *M. amurensis* lectin inhibited mucus penetration by the sporozoites. When the sialidase and lectin were used together the penetration was increased, suggesting that the lectin did indeed compete or inhibit the action of the sialidase on the mucus layer, possibly by binding to, and therefore protecting, the sialic acid residues in the mucus from the sialidase activity.

### 5.3.6. Reversal of sialidase inhibition

Whether enzyme inhibition could be reversed by adding the enzyme back to the suspension was investigated. *E. tenella* sporozoites were incubated with the sialidase inhibitor 5NeuAc2en (0.1 mM, as described in 5.2.3.) for 10 min at room temperature, and then sialidase (0.5 mg $ml^{-1}$) was added to one sample of sporozoites. The sporozoites were then added to the mucus layer and the results recorded after the 120 min incubation at 37°C (see Figure 5.3.9.). The results show that the sialidase added to the sporozoite suspension when added to the mucus layer did reverse the effect of the sialidase inhibitor action, thus suggesting that 5NeuAc2en was inhibiting the *E. tenella* sialidase enzyme, rather than having another non-specific effect.

### 5.3.7 Effect of polyamines on *E. tenella* mucus penetration

Having observed that the polyamine biosynthesis inhibitor DFMO inhibited mucus penetration, the extent to which polyamines affected this process was analysed with *E. tenella* sporozoites. In these experiments 11% mucus (0.7 mm depth) was used; sporozoite penetration was less than maximal with this concentration and therefore it was possible to observe increases in penetration rates. To determine the effect that polyamines may have on the mucus, it was reconstituted with putrescine as described in 5.2.3. in the samples stated (see Figure 5.3.10.). DFMO and formaldehyde were also used where stated at

concentrations of 2 mM and 10% respectively as described in 5.2.3. The percentage penetration was increased when the mucus was reconstituted with putrescine, indicating that the polyamine has a mucolytic effect. This effect is greatly decreased in the presence of DFMO.

### 5.3.8 *E. tenella* penetration of chicken mucus from different regions of the GIT

Mucus (Pfizer) collected from different regions of the chicken gastrointestinal tract (duodenum, jejunum, ileum/jejunum) was placed on culture plate insert filters as described in 5.2.2 at depths of 0.7 mm. *E. tenella* sporozoites were then added and, after the usual 120 min incubation period, the percentage penetrations calculated (Figure 5.3.11.). The results indicate a difference in the penetration of the different mucus samples, with there being significantly lower penetration through the duodenum sample than those from the jejunum ($P<0.01$) and ileum/jejunum ($P<0.01$) areas.

### 5.3.9. Effect of inhibitors on *E. tenella* merozoite penetration

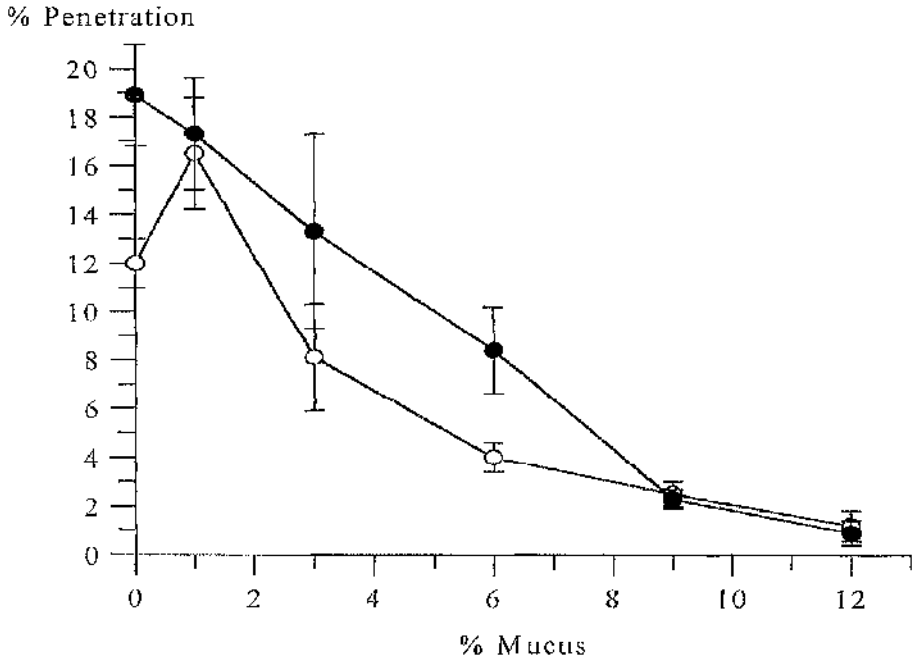
*E. tenella* merozoites were prepared as described in 5.2.1.2. and washed into serum-free RPMI (1000 x g for 10 min) and resuspended at a density of 1 x $10^6$ $ml^{-1}$. Mucus layers were prepared as described in 5.2.2. with a mucus concentration of 9% as for *E. tenella* sporozoites but a depth of 0.5 mm to allow

for the smaller size of the merozoite stage. The merozoites were incubated with a number of the enzyme inhibitors as described in 5.2.3.: E64, 5NeuAc2en, aprotinin, DFMO and formaldehyde for 10 min at room temperature after which the suspensions were added to the mucus layers in 200 µl volumes and incubated at 37°C for 120 min. Experiments using washed merozoite samples were not performed since the merozoites themselves are very fragile. The percentage penetrations were calculated (see Figure 5.3.12.). The control percentage penetration was on average approximately 30-40%. It was observed that the negative control inhibited penetration greatly (~80%) with DFMO, the serine proteinase inhibitor aprotinin and the sialidase inhibitor (5NeuAc2en) also showing large inhibition. E64 however did not have any inhibitory effect on penetration.

### 5.3.10. Effect of increasing sialidase inhibitor concentration on *E. tenella* merozoite mucus penetration

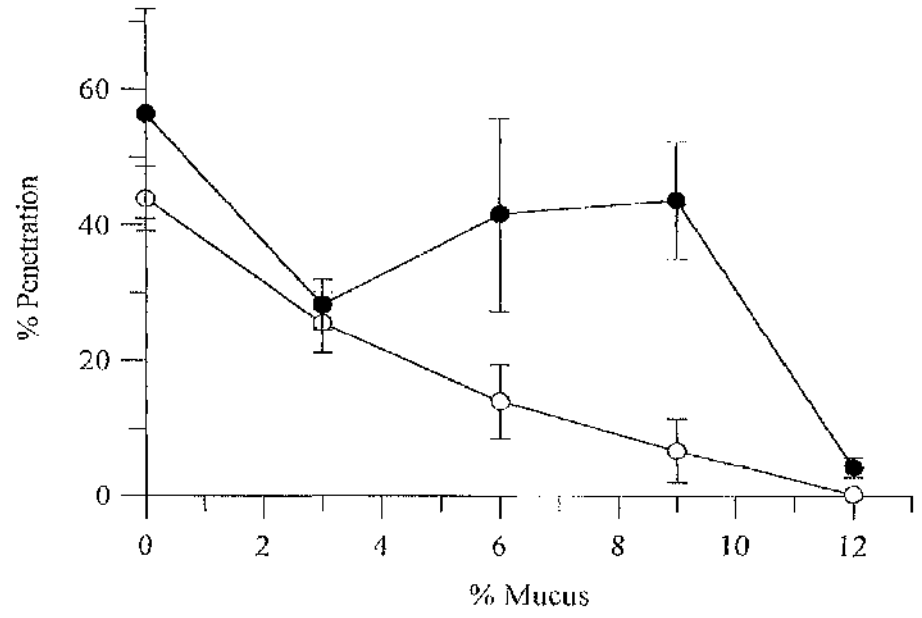
*E. tenella* merozoites were obtained and prepared along with the mucus layers as described in 5.3.10. The merozoites were incubated for 10 min and then added to the mucus layers with 0.1, 0.2 and 0.3 mM 5NeuAc2en, as well as formaldehyde (10%, v/v) as the negative control. The percentage inhibitions were calculated (see Figure 5.3.13.). From the results it was observed that as the sialidase inhibitor concentration increased so did the percent inhibition, as might have been expected.

**Figure 5.3.1: Penetration of *C. parvum* through different mucus concentrations**




Means ± SE from three experiments
Mucus penetration during 60 min (open circles) and 120 min (closed circles)

**Figure 5.3.2.: Penetration of *E. tenella* through different mucus concentrations**



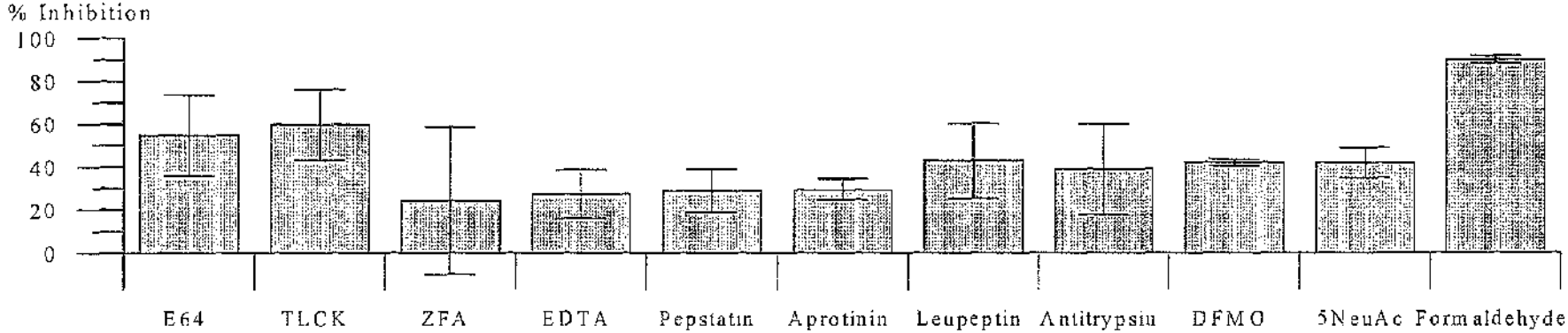

Means ± SE from three experiments
Mucus penetration during 60 min (open circles) and 120 min (closed circles)

**Table 5.3.1: Effect of additional compounds in the medium on *C. parvum* mucus penetration**

| | % PENETRATION | | |
|---|---|---|---|
| | CONTROL | 1% GLUCOSE | 10% FCS |
| 60 min | 4.8 ± 0.9 | 2.9 ± 0.6 | 1.4 ± 0.5 |

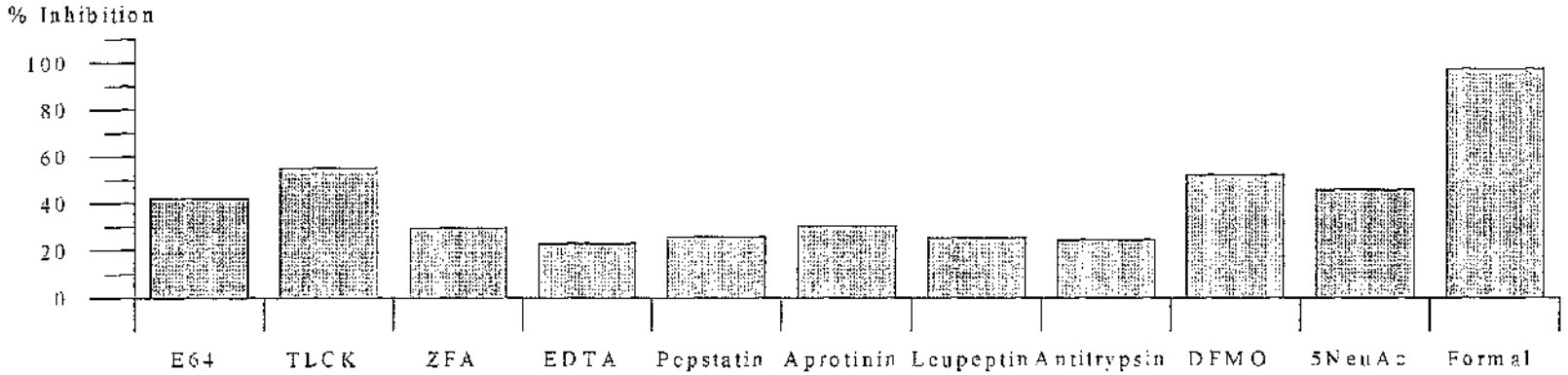
Mean ± three repetitions

**Figure 5.3.3.: Inhibitors on *C. parvum* mucus penetration after washing**




Means ± SE from at least three experiments

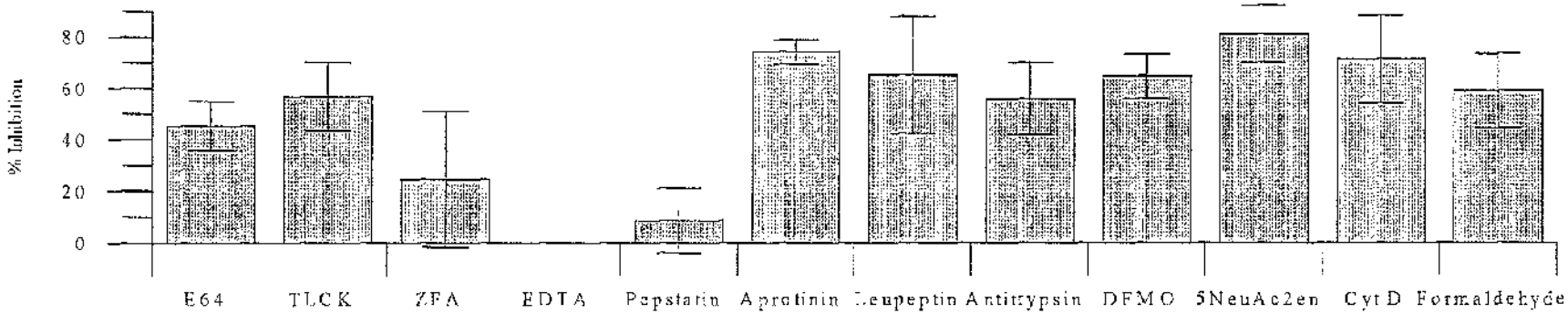
**Figure 5.3.4.: Inhibitors on *C. parvum* mucus penetration without washing**




Means from two experiments

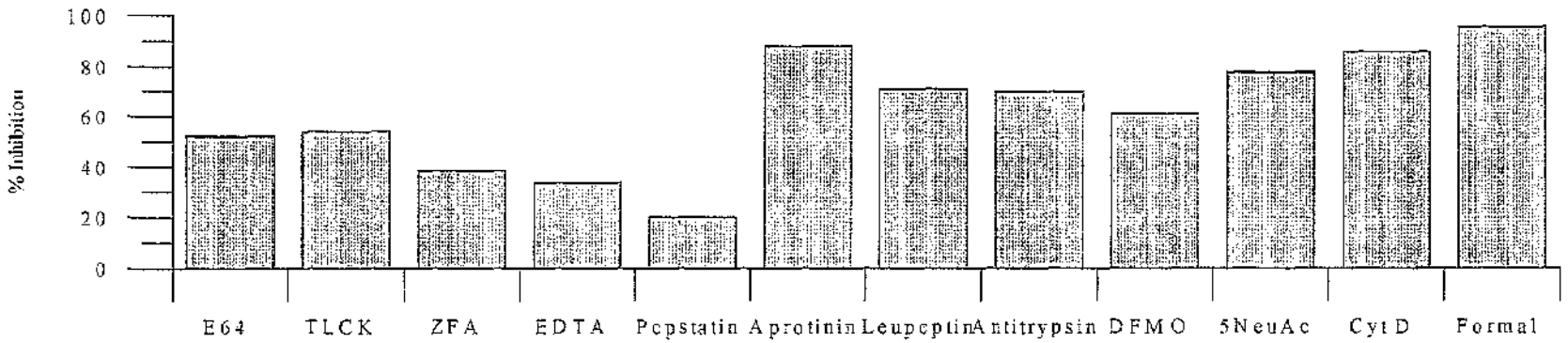
Concentrations of inhibitors: E64,1.5 mM; TLCK, 25 µM; ZFA, 10 µM; EDTA, 1 mM; pepstatin, 1 µM; aprotinin, 100 µM; leupeptin, 100 µM; $\alpha_1$-antitrypsin, 0.5 mg $ml^{-1}$; DFMO, 2 mM; 5NeuAc2en, 0.1 mM; formaldehyde, 10% (v/v)

**Figure 5.3.5.: Inhibitors on *E. tenella* mucus penetration after washing**




Means ± SE from at least three experiments

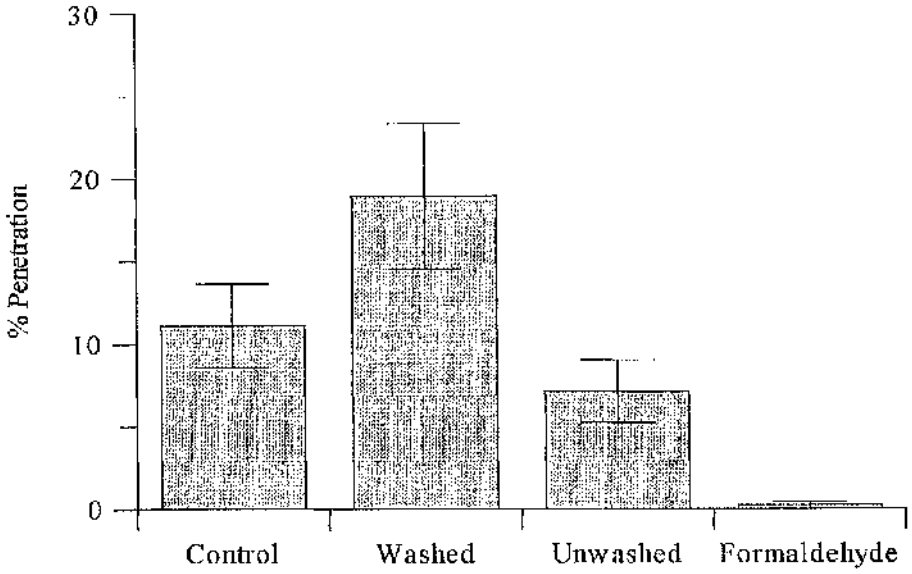
**Figure 5.3.6.: Inhibitors on *E. tenella* mucus penetration without washing**




Means from two experiments

Concentrations of inhibitors: E64,1.5 mM; TLCK, 25 µM; ZFA, 10 µM; EDTA, 1 mM; pepstatin, 1 µM; aprotinin, 100 µM; leupeptin, 100 µM; $\alpha_1$-antitrypsin, 0.5 mg $ml^{-1}$; DFMO, 2 mM; 5NeuAc2en, 0.1 mM; cytochalasin D, 10 µM; formaldehyde, 10% (v/v)

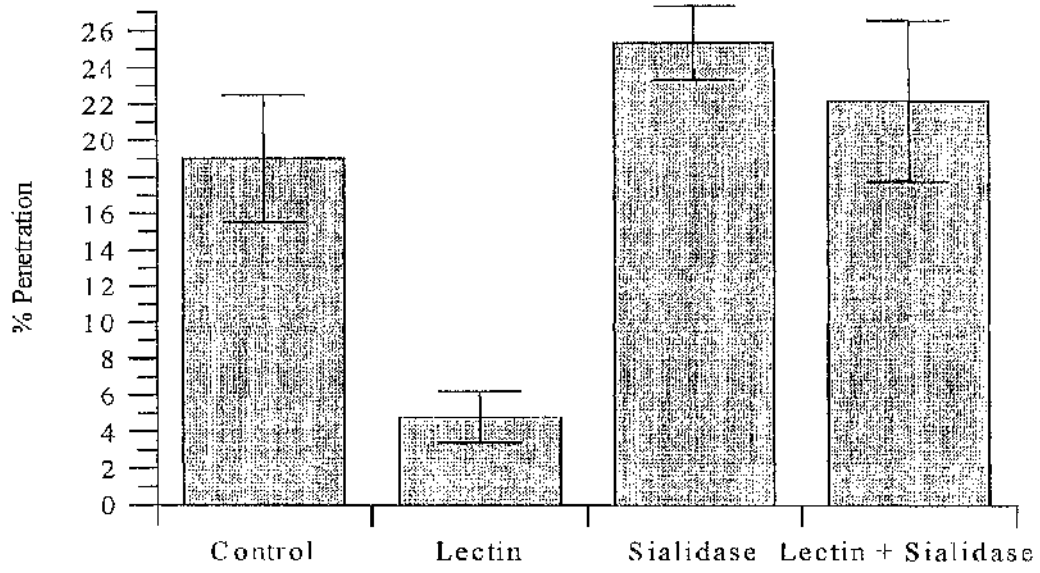
**Figure 5.3.7.: *E. tenella* mucus penetration with or without the *Maackia amurensis* lectin**




Means ± SE from three experiments

Mucus penetration with or without the presence of the *M. amurensis* lectin (0.1 mg $ml^{-1}$)

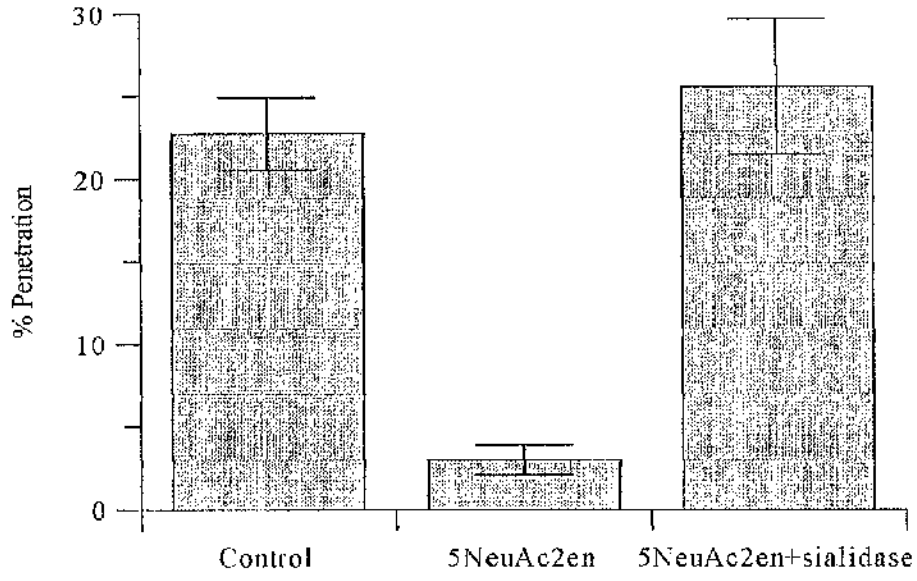
**Figure 5.3.8.: Competition between lectin and sialidase**




Means ± SE from three experiments

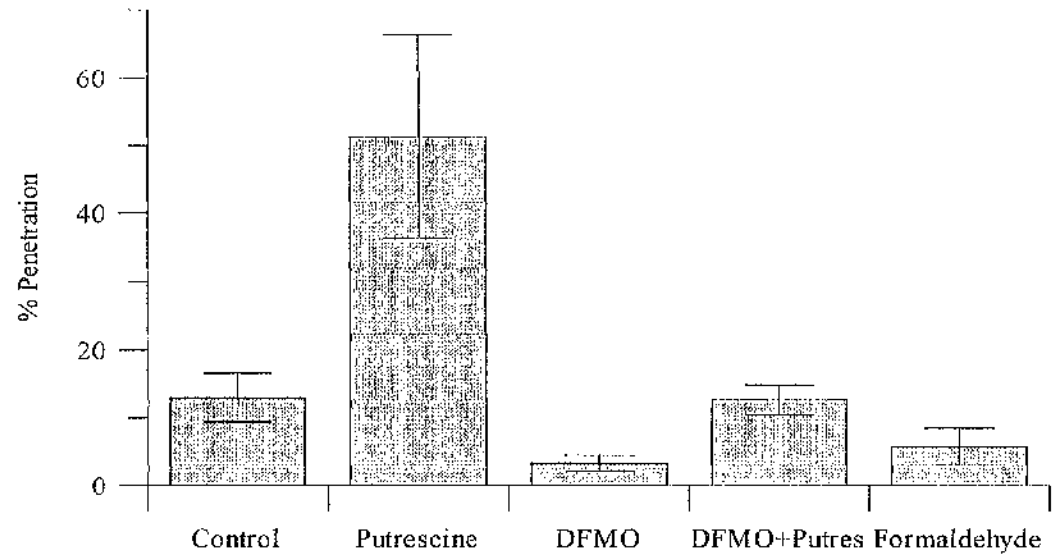
Competition between *M. amurensis* lectin (0.1 mg $ml^{-1}$) and *Clostridium perfringens* sialidase (0.5 mg $ml^{-1}$)

**Figure 5.3.9.: Effect of sialidase on 5NeuAc2en inhibition**



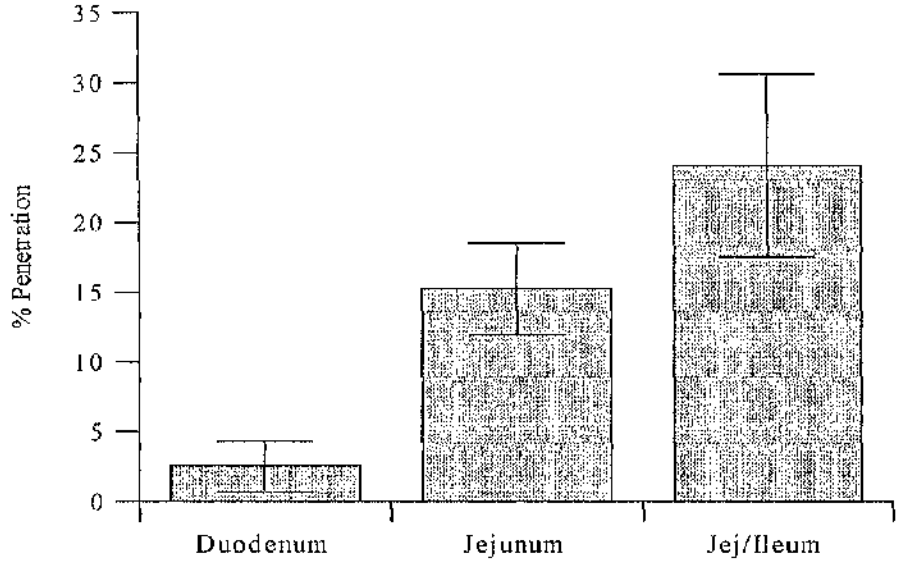

Means ± SE from three experiments
Reversal of sialidase inhibition from the the sialidase inhibitor 5NeuAc2en (0.1 mM) using *C. perfringens* sialidase (0.5 mg $ml^{-1}$)

**Figure 5.3.10.: Effect of polyamines on mucus and DFMO inhibition**



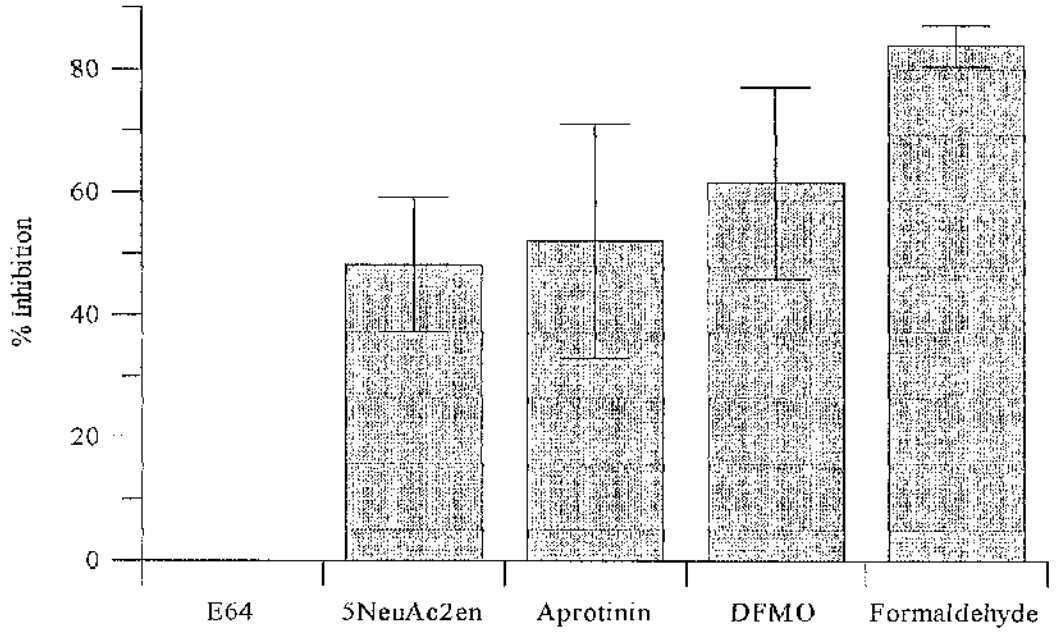

Means ± SE from three experiments
Comparison of mucus reconstituted with putrescine (1 mM) with or without the presence of DFMO (2 mM)

**Figure 5.3.11.: Penetration rates of *E. tenella* through chicken mucus from different regions of the gastrointestinal tract**



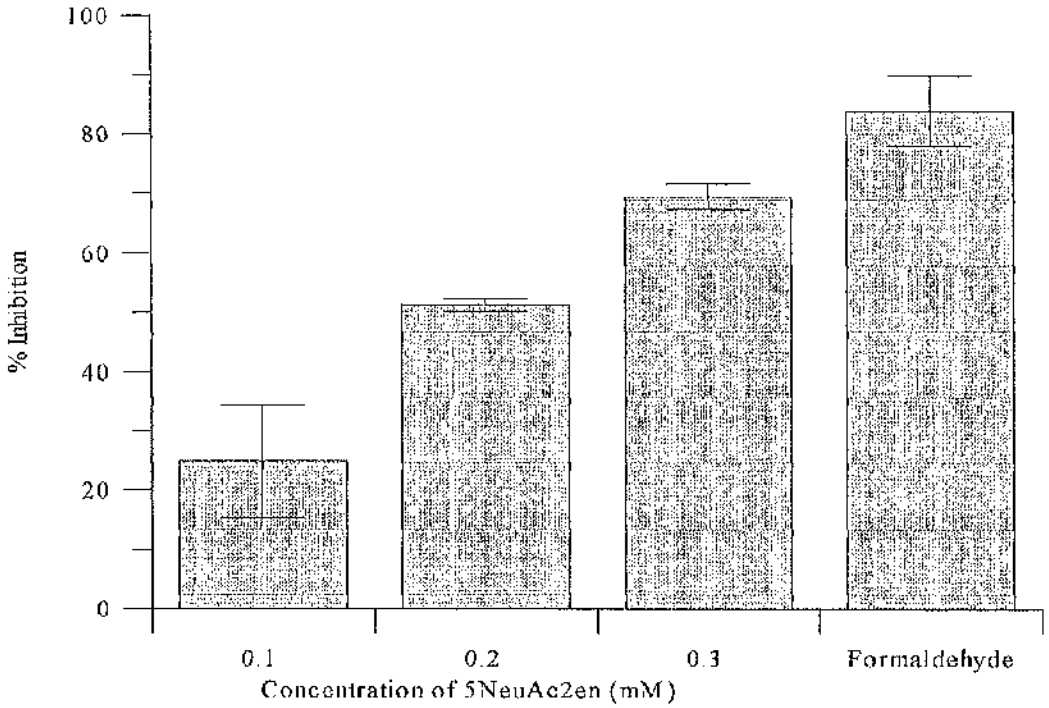

Means ± SE from three experiments

**Figure 5.3.12.: Effect of inhibitors on *E. tenella* merozoites**




Means ± SE from three experiments
Concentrations of inhibitors: E64,1.5 mM; 5NeuAc2en, 0.1 mM; aprotinin, 100 µ M; DFMO, 2 mM; formaldehyde, 10% (v/v)

**Figure 5.3.13.: Effect of increasing sialidase inhibitor concentration on *E. tenella* merozoite mucus penetration**




Means ± SE from three experiments

## 5.4 Discussion

The experiments performed showed that both *C. parvum* and *E. tenella* sporozoites are able to penetrate mucus layers *in vitro*. The *in vivo* situation that the parasites face however probably varies considerably from the conditions in these studies. The mucus layer of the gastrointestinal tract is known to vary in thickness and viscosity due to factors like diet, stress and immune status (Neutra and Forstner, 1987). Additionally other environmental factors are also involved, for example, fluctuations in pH throughout the gastrointestinal tract. Yet this *in vitro* method gives us an indication of mechanisms that may be involved in mucus penetration by these parasites, and enables research in an area which has not previously been studied.

The presence of media supplements was investigated to determine whether two common agents may result in chemotaxis of the sporozoites through the mucus layers. The results shown in Table 5.3.1. indicated that the presence of glucose in the underlying medium did not positively effect parasite penetration. This may imply that, at least for nutrients, chemotaxis does not play an important role in this process of penetrating to the host cells - unlike in the case of bacteria (Freter, 1988) - indicating another means by which the parasites know where to migrate to.

The use of inhibitors provides some clues as to the mechanisms involved in mucus penetration. The formaldehyde-killed parasites were seen to be greatly inhibited in penetrating the mucus layers: ~90% inhibition for *C. parvum* and

~60% inhibition for *E. tenella,* which supports the hypothesis that the parasites actively penetrate the mucus layer. The lower inhibition of *E. tenella* probably reflects the fact that these sporozoites are larger and therefore even some dead sporozoites pass through the mucus due to gravity. The actin microfilament inhibitor cytochalasin D inhibited *E. tenella* sporozoite penetration by ~70% and it can be concluded that motility, plays a role in the penetration process as it has been reported to do in host cell invasion (Russell and Sinden, 1981).

The sialidase inhibitor used inhibited penetration of both parasites, although to a greater extent with *E. tenella.* Sialidase activity has been detected in both sporozoites and merozoites of *E. tenella* (Pellegrin *et al.,* 1993), thus these results are consistent with this finding. Sialidase has not been reported in *Cryptosporidium*, and my studies failed to detect any activity with either parasite (see Chapter 4), however the finding that the sialidase inhibitor does inhibit *C. parvum* penetration may indicate the presence of this activity, though more studies on the enzyme itself are necessary. Pellegrin *et al.*(1993) suggested that the sialidase may be desialylating the mucus gel network and thus enabling the parasites to penetrate more easily, and my results provide the first evidence that the parasite's enzyme activity is directly involved in interacting with mucus.

The ornithine decarboxylase inhibitor DFMO inhibited mucus penetration in both *C. parvum* and *E. tenella,* by ~40% and ~65% respectively. Indeed polyamines have been suggested to have a 'mucolytic' effect, thus enabling organisms to penetrate mucus more easily (Paget and James, 1994) possibly by disrupting the mucin gel-network in some way. DFMO was used in a study of *E. tenella* infections in broiler chickens (Hanson *et al.,* 1981), and found to be effective in preventing the symptoms. If

cured with DFMO, immunity to infection was gained (Hanson *et al.*, 1981). It was concluded that DFMO acted specifically by blocking putrescine production of the parasite (Hanson *et al.*,1981) (see 1.2.5.). This finding along with my evidence that DFMO inhibits mucus penetration suggests that putrescine secretion may be involved in mucus penetration. However for *C. parvum* it has been reported that polyamine metabolism does not occur via ODC but via the enzyme arginine decarboxylase (ADC) and that DFMO has no effect on growth (Yarlett *et al.*, 1996). Yet in a study on AIDS patients that had *Cryptosporidium* infections, 10 of 17 patients had complete resolution when treated with DFMO (Rolston *et al.*, 1989). These results together suggest that DFMO is having an adverse effect on the parasite but not through the inhibition of ODC, possibly by damaging the sporozoite surface.

The serine proteinase inhibitors used were $\alpha_1$-antitrypsin, leupeptin (which also inhibits a number of cysteine proteinases) and aprotinin. With both parasites, inhibition was seen using all of these inhibitors, thus indicating that serine proteinase activity is important in mucus penetration. This is in direct contrast with the results obtained when using the aspartic proteinase inhibitor pepstatin and the metalloproteinase inhibitor EDTA which, for *E. tenella*, showed no inhibition. Yet EDTA is known to be reversible and would be removed during washing. Indeed, when present in the sporozoite suspension, *E. tenella* mucus penetration was inhibited. For *C. parvum*, both pepstatin and EDTA showed an inhibition of ~30% indicating a possible role for aspartic and metallo- proteinase activities in mucus penetration, which is similar to reports on *Candida albicans* which is reported to possess an aspartyl proteinase involved in mucin proteolysis (Colina *et al.*, 1996). These results also suggest that there may be differences in the mechanisms of *E. tenella* and *C. parvum* mucus penetration.

The cysteine proteinase inhibitor results were similar for both the parasites. The peptidyl diazomethane Z-phenylalanine appeared to vary widely in its effect on penetration. However both E64 and TLCK inhibited this process for both *C. parvum* and *E. tenella* at ~50-60%. These results indicate that there is a surface cysteine proteinase activity, since E64 - which cannot cross intact cell membranes - inhibited mucus penetration. These results are consistent with the findings of the cysteine proteinase (Nesterenko *et al.*, 1995) of *C. parvum* which appears to be surface located as well as the aminopeptidase (Okhuysen et al., 1994) which were thought possibly to aid the parasite's penetration of the mucus layer and/or invasion of a host cell.

To further investigate the role that the sialidase activity was playing in this process of mucus penetration, a *Maackia amurensis* lectin specific for sialic acid residues was used (see Figure 5.3.7.). If the lectin remained in the sporozoite suspension when added to the mucus layer (compared to the washed sample) an inhibition of approximately 30% was seen. The results suggest that the lectin bound to the sialic acid residues known to terminate a high proportion of the mucin glycoproteins, and in doing so protected the mucin network from the action of the parasite sialidase and therefore inhibited the penetration by the parasite. However the action of the lectin may not be specific. It may be possible that the lectin has an effect on the parasites themselves by being reversibly bound and removed during the washing, therefore removing the inhibitory effect. More experiments would have to be performed to determine if the lectin was binding to the sporozoites, possibly by labelling the lectin with a fluorescent marker and using microscopic observation to determine firstly if the lectin binds to the sporozoites, and then if washing removes it. To investigate the effect of the lectin

further we chose to see whether the *C. perfringens* sialidase competed with the lectin on the mucus layer (see Figure 5.3.8.). When *E. tenella* sporozoites were added with sialidase present no inhibition was seen, unlike when the lectin was present and a high percentage inhibition was observed in the mucus penetration. However when the lectin and sialidase were both present in the sporozoite suspension the inhibition of the lectin was abolished. This indicates that, although the lectin was bound to a number of sialic acid residues, the sialidase overcame this effect due to the concentration of both the lectin and sialidase present.

Similarly, whether the effect of the sialidase inhibition could be reversed simply by adding the enzyme back into the experiment was investigated (see Figure 5.3.9.). *E. tenella* sporozoites incubated either with the sialidase inhibitor or with the sialidase inhibitor followed by the addition of external sialidase (*C. perfringens*) were observed. The inhibition by the sialidase inhibitor (~60%) was indeed reversed when the sialidase enzyme was present. These results give an indication that the sialidase inhibitor in these studies is inhibiting the parasite sialidase. However to prove that the sialidase inhibitor does specifically inhibit the *E. tenella* enzyme, enzyme studies would need to be performed. These were attempted in Chapter 4 but any sialidase activity from *E. tenella* sporozoites or merozoites was not detected.

The effect of polyamines on the mucus layer was also investigated further with the use of the polyamine putrescine (see Figure 5.3.10.). When the mucus layer was reconstituted with 1 mM putrescine there was approximately a five times increase in mucus penetration by *E. tenella* sporozoites, thus confirming the suggestion that polyamines may act as mucolytic agents. However if the sporozoites were incubated

with DFMO before adding to the mucus layer reconstituted with putrescine, the percentage penetration was greatly decreased to approximately equal the control. The results reveal that if the parasite polyamine production is inhibited using DFMO, the addition of external putrescine abolishes - to a limited extent - this inhibitory effect. Thus confirming the fact that polyamines may be involved in mucus penetration as has been found for *Trichomonas vaginalis* (Paget and James1994).

The differences between the various regions of the gastrointestinal tract are due to a number of factors, including pH and the molecules/chemicals present in these regions. Indeed different species of *Eimeria* are known to preferentially invade specific regions of the intestine (Strout *et al.*, 1994). From our results obtained using the different chicken mucus samples collected from the duodenum, the jejunum and the ileum/jejunum it was observed that the percent penetration by *E. tenella* was seen to vary between the samples (see Figure 5.3.11.). This would therefore indicate that there are differences in the mucus composition throughout the gastrointestinal tract, possibly contributing to the species-specific penetration and subsequent invasion of the different areas. However any conclusions drawn from these results should be treated with caution since the composition of the mucus samples used was not known and these samples had been stored for a number of years prior to use in these experiments. Whether the concentration of the mucus was similar in each sample is not known and obviously the number of times the samples had been freeze-thawed, therefore disrupting the glycoprotein network, is also unknown.

*E. tenella* merozoite mucus penetration was also studied using a number of specific enzyme inhibitors (see Figure 5.3.12.). Unlike with the sporozoite stage, E64 did not inhibit penetration, suggesting that surface cysteine proteinase activity is not involved and indicating possible differences between life cycle stages of *E. tenella*. Both the serine proteinase inhibitor and DFMO inhibited mucus penetration as observed for the sporozoite stage, but the sialidase inhibitor only gave an inhibition of ~45%, compared to an inhibition of ~80% with the sporozoites at the same final concentration. This would suggest that the merozoites do contain higher amounts of sialidase activity as was reported by Pellegrin *et al.* (1993). Even three times the concentration of inhibitor used on the sporozoites did not achieve the same degree of inhibition on merozoites (see Figure 5.3.13.) and resulted in approximately 70% inhibition.

The results obtained in this chapter derived from use of a novel method, showed that *C. parvum* sporozoites and *E. tenella* sporozoites and merozoites are able to penetrate mucus layers; and elucidated some of the activities or processes that are involved. The use of a variety of enzyme activities namely cysteine and serine proteinases, polyamines and sialidase in both parasites, along with motility and site specificity enable the parasites to penetrate the area of the gastrointestinal tract that they infect. However many questions remain unanswered and these studies simply represent a start in understanding the process of mucus penetration and the mechanisms involved.

# CHAPTER SIX

# HOST CELL INVASION

## 6.1. Introduction

An important area of research, when any pathogenic intracellular micro-organism is concerned, is studying attachment to and invasion of host cells. Knowing the mechanisms and processes involved aids researchers to understand how the parasite is adapted to growing in its specific host and, perhaps more importantly, develop methods to prevent the infection. Studies on host cell invasion by the coccidia have varied extensively, both the approaches and methods used. The attachment and invasion process has been monitored with respect to time for each of the coccidia with *C. parvum, E. magna* and *T. gondii*, taking fifteen minutes, five to ten minutes and fifteen to thirty seconds, respectively, (Jensen and Edgar, 1976; Lumb *et al.*, 1988) while the host cell membrane remained intact throughout the whole process (Jensen and Edgar, 1976; Lumb *et al.*, 1988; Moriaski *et al.*, 1995). However one feature that *Cryptosporidium* apart from the other coccidia is that the parasitophorous vacuole remains at the host cell surface rather than deep within the cytoplasm (Lumb *et al.*, 1988; Sterling and Arrowood, 1993).

How coccidia travel to the host cell for attachment and invasion has not been studied to any great extent. Lawn and Rose (1982) demonstrated using electron microscopy that *E. tenella* sporozoites did not directly enter crypt epithelial cells, in fact they were shown to be carried in the cytoplasm of intraepithelial lymphocytes (IELs). Once at the crypts, a process likened to

"reversed phagocytosis" was reported to occur. This involved the sporozoites coming out of the IELs and subsequently being able to then invade the crypt enterocytes (Lawn and Rose, 1982). More recently a study involving *E. magna* reported that sporozoites were observed in IELs at the specific site of infection in the host (Pakandl *et al.*, 1995). However Vervelde *et al.*, (1995) reported that IELs were rarely seen to contain *E. tenella* sporozoites. The reason for this discrepancy may be due to variations in experimental procedures between researchers; for example, different strains and routes of inoculating *Eimeria*.

Motility has often been a focus of attention in coccidian biology and has been implicated in penetration and host cell invasion. Colchicine (a microtubule inhibitor) and cytochalasin D (an actin inhibitor) have been reported to inhibit invasion by both *C. parvum* and *Eimeria* (Russell and Sinden, 1981; Russell, 1983; Wiest *et al.*, 1993; Wiest *et al.*, 1994), *T. gondii* (Aguirre-Cruz *et al.*, 1996; Dobrowolski and Sibley, 1996) and *Neospora caninum* (Hemphill *et al.*, 1996), supporting the hypothesis that motility plays a role in the invasion process. Indeed a point mutation in the single copy actin gene ACT1 of *T. gondii* abolished the inhibitory effect of cytochalasin on host cell invasion by tachyzoites (Dobrowolski and Sibley, 1996) thus suggesting that cytochalasin may inhibit actin by binding to the protein encoded by its gene and that actin has a major role in the host cell invasion process. *C. parvum* has also been reported to contain an actin gene (Kim *et al.*, 1992) and actin and actomyosin have been detected in *Eimeria* sporozoites (Baines and King, 1989; Preston and King, 1992). Myosin has been located at the anterior pole of *Toxoplasma* (Schwartzman and Pfefferkorn, 1983), and microtubules have been detected in the pellicle (Schwartzman and Krug, 1985).

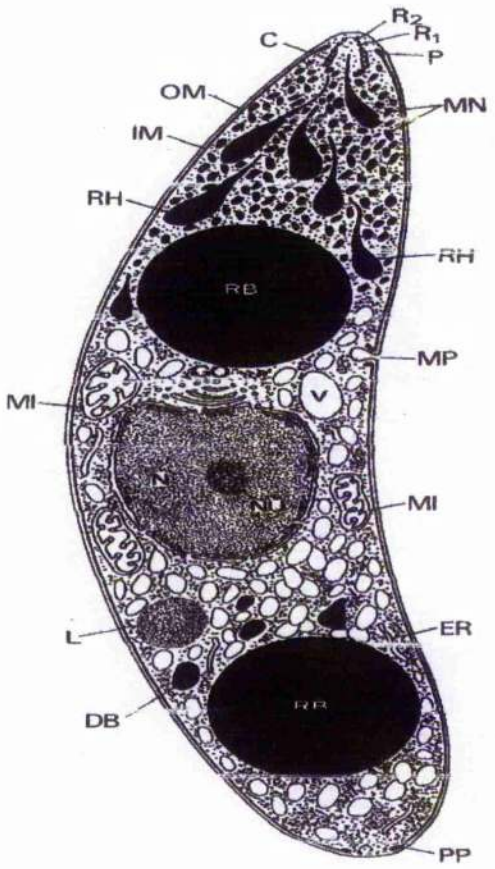
The parasite's attachment to the host cell is the initial step in the invasion process. Structures present on both the parasites and the host cells and the

attachment mechanisms involved have been studied. The findings vary considerably according to the particular cell type used (see section 1.1.7.). For example, it was found that with *C. parvum*, differentiated HT29.74 cells were five times more susceptible to infection than undifferentiated cells. This suggests that the process of differentiation may produce structures that facilitate parasite infection (Flanigan *et al.*, 1991).

The receptor sites used in attachment and therefore invasion have been studied. Using immunofluorescence and immunoprecipitation, *T. gondii* tachyzoites were seen to be covered in a laminin layer (Furtado *et al.*, 1992), and antibodies against both laminin and $\beta_1$-integrins blocked attachment (Joiner, 1991; Furtado *et al.*, 1992). It was suggested that laminin may create a bridge between tachyzoites and host cells thus facilitating host cell attachment. Anti-fibronectin serum was seen to reduce *T. gondii* host cell invasion as well as cycloheximide - the protein synthesis inhibitor (Rosales *et al.*, 1995). This suggested that fibronectin host cell receptors and protein synthesis (possibly by the parasite and host cell) are involved in the attachment and invasion process (Rosales *et al.*, 1995). In contrast when glycosylated receptors on *T. gondii* tachyzoites were blocked, host cell invasion was not affected (Mack *et al.*, 1994).

The Apicomplexa were given their name as a group of parasites because they possess an apical complex (illustrated in Figure 6.1.1.). This is thought to be involved in host cell attachment and invasion. Organelles present at the apical complex include rhoptries, micronemes and the larger dense granules. Unusually *C. parvum* has a single rhoptry whilst other coccidians have at least two (Tetley *et*

**Figure 6.1.1: Diagram of a sporozoite of *Eimeria* to show the principal structures.**




Key to Figure 6.1.1: 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; PL, plastid-like organelle; PP, posterior polar ring; $R_{1,2}$, preconoidal rings; RB, refractile bodies; RH, rhoptries; V, vesicle. The apical complex also characteristically contains dense granules (not shown). Modified from Scholtyseck (1979).

*al.*, 1995). It is now widely agreed that these organelles are indeed involved in host cell attachment and invasion (Jensen and Edgar, 1976; Joiner, 1991; Perkins, 1992; Jacobson and Doyle, 1996). In terms of these organelles, host cell invasion has been described as a series of events for *T. gondii*: attachment to the host cell triggers the micronemes to release their contents; the invagination of the host cell membrane to finally form the parasitophorous vacuole (PV) is initiated by the release of the rhoptry contents; and the dense granule release appears to be delayed until the parasite is fully within the PV (Carruthers and Sibley, 1997).

For *C. parvum* two monoclonal antibodies raised against the oocysts and sporozoites were seen to label the subset of small dense granules in the sporozoites and also to label the parasitophorous vacuole membrane. This labelling suggested that these granules may be exocytosed during host cell invasion (Bonnin *et al.*, 1995). Similarly antibodies raised against a *N. caninum* antigen Nc-p43, usually located on the parasite surface and within the dense granules and rhoptries, were seen to inhibit parasite attachment and subsequent invasion (Hemphill, 1996).

Some analysis of the apical complex organelles has been performed. *T. gondii* tachyzoite rhoptries were seen to be composed of 75% phosphatidylcholine with a cholesterol to phospholipid ratio of 1.5:1 (Joiner, 1991), and *Sarcocystis muris* dense granules were reported to contain a cysteine proteinase (Strobel *et al.*, 1992). However, antibodies raised to rhoptries, micronemes and dense granules of *T. gondii* had no effect on invasion of the tachyzoite stage yet this may be due to the fact that the antibodies could not access these organelles and may therefore not be a true result (Grimwood and Smith, 1996).

Homologues of the heat shock proteins (hsp 70) have been detected in *E. tenella* sporozoites and, using monoclonal antibodies, have been seen to be occasionally located at apical complexes (Laurent *et al.*, 1994; Dunn *et al.*, 1995).

It was suggested that this cytoplasmic hsp may be involved in targetting proteins to the apical complex (Laurent *et al.*, 1994).

Another approach used to analyse invasion is to use specific enzyme inhibitors in an attempt to elucidate the mechanisms involved in the host cell invasion process. Various compounds have been tested and conflicting results have been reported. The addition of exogenous phospholipase $A_2$ ($PLA_2$) increased *T. gondii* host cell invasion, although exactly how this enzyme may influence invasion remains to be defined (Saffer and Schwartzman, 1991). In contrast exposing *E. tenella* sporozoites to $PLA_2$ reduced their capacity to invade (Crane and McGaley, 1991), indicating that different mechanisms may be involved in host cell invasion by these two parasites.

Proteinases are another area of major research in parasites and have been suggested to play roles in survival in the host cell in other parasites (McKerrow *et al.*, 1993; Coombs and Mottram, 1997). Proteinase inhibitors have been investigated previously in terms of their effect on invasion of host cells by various coccidia; however, most investigators failed to test the toxicity of these compounds on the sporozoites themselves (Adams and Bushell, 1988; Fuller and McDougald, 1990: Forney *et al.*, 1996). *E. vermiformis* sporozoite invasion was reported to be inhibited by 5 mM phenylmethylsulfonyl fluoride (PMSF) but not by antipain, leupeptin, chymostatin, tosyl-lysine chloromethyl ketone (TLCK), tosylamido-2-phenylethyl chloromethyl ketone (TPCK) or pepstatin (Adams and Bushell, 1988). These results are similar to findings with *C. parvum* infection on bovine fallopian tube cells, in that serine proteinase inhibitors including PMSF inhibited host cell infection (Forney *et al.*, 1996). In contrast, *E. tenella* sporozoite invasion was inhibited by most of the afore-mentioned inhibitors (chymostatin was not tested) and aprotinin (Fuller and McDougald, 1990).

There have been few reports concerning the importance of environmental conditions for host cell invasion. Under reduced oxygen conditions, *E. nieschulzi* sporozoite motility and invasion was seen to be unaltered although survival time of the parasites in MDBK cells did seem to be increased (Upton and Tilley, 1995). The presence of divalent cations including manganese, calcium and zinc increased attachment of *C. parvum* sporozoites (Hamer *et al.*, 1994). More recently, an increase in calcium concentration in the parasitophorous vacuole has been suggested to be the signal for *Toxoplasma* to exit the host cell (Pingret *et al.*, 1996).

One aspect of host cell invasion that appears to have been largely overlooked is the effect of parasitism on the host cell. It has been reported that when *E. tenella* infects chick kidney cells, a number of morphological changes were observed in the host cells, including a decrease in cell-cell contact and cell-substrate contact (Urquhart, 1981a). It was concluded that the parasite must disrupt the actin microfilament bundles of the host cells, thus possibly resulting in the loss of surface glycoproteins known to be directly linked to these microfilaments and therefore, in turn, resulting in a loss of adhesion by the cells (Urquhart, 1981a). However this phenomenon did not merely occur in parasitised cells. Non-parasitised cells - when present in the same culture - also exhibited these morphological changes. It was further noted that parasitised cells underwent an increase in DNA synthesis, possibly due to the demands on the host cell by the parasite (Urquhart, 1981b). Non-parasitised cells in the same culture also showed an increase in DNA synthesis (though not as large as those that were parasitised), thus indicating that a factor was released into the medium either by the parasite or parasitised cell and that this induced other cells to progress into S

phase (Urquhart, 1981b). Notably, however, parasitised cells also remained in S phase longer than non-parasitised cells suggesting the presence of another factor that is not released into the medium since this phenomenon is only seen with parasitised cells (Urquhart, 1981b). Studies involving *E. necatrix* have revealed that invasion with this species also induces a number of changes in chick crypt cells: an overall increase in surface area (~ 5 fold); an increase in DNA replication; an increase in mechanical resistance of the plasma membrane; an increase in gel-phase lipid in the plasma membrane; infected cells become migratory; and a major protein in the plasma membrane appears (~ 36 kDa) (Fernando and Pasternak, 1983). Additionally to these changes, the host cell membrane also becomes extremely sensitive to proteolytic attack possibly due to the membrane becoming 'leaky' as the infection progresses (Fernando and Pasternak, 1983).

A study in 1977 (De Laat *et al.*) investigated the lipid layers of the cell membrane of C1300 mouse neuroblastoma cells. The microviscosity of the lipid varied throughout the cell cycle reaching a maximum during mitosis, then decreasing to remain at a low level during S phase. Thus, the cell membrane of mitotic cells was reported to be more rigid than that of cells in the interphase. Indeed as cells progress through the cell cycle major changes occur in morphology, geometry and mechanical properties (Needham, 1991). Perhaps not surprisingly then, two studies on *T. gondii* attachment (Grimwood *et al.*, 1996) and invasion (Dvorak and Crane, 1981) have found that host cells are more susceptible to infection during the S phase of their mitotic cycle. This is consistent with other researcher's observations that non-confluent cells (30-60% in S phase) are invaded more readily than confluent cells (majority in $G_1$) (Dvorak and Crane, 1981).

The aims of the experiments in this chapter were initially to set up an *in vitro* invasion assay to monitor parasite invasion of the host cells. Once established, the use of non-toxic enzyme inhibitors (as assessed by the vital stains technique, see Chapter 3) could be used to observe the effects they may have on the invasion process. The MDBK cell cycle was mapped out and its effect on parasite invasion analysed.

## 6.2. Materials and Methods

### 6.2.1. Parasites

1.5 x $10^6$ *C. parvum* oocysts were washed three times in RPMI (Labtech, 1640) to remove the potassium dichromate and then excysted as described in 2.2.3. The resulting sporozoites were separated from unexcysted oocysts and shells by micropore filtration (see 3.2.1.) and used immediately.

1.0 x $10^6$ *E. tenella* sporulated oocysts were washed free of potassium dichromate and excysted as described in 2.2.2.2. The excystation mixture was then filtered through pre-wetted cotton wool and the purified sporozoites were resuspended in RPMI and used immediately.

*E. tenella* merozoites were cultured as described in section 4.2.3.1. These were collected and resuspended in RPMI at a density of 2-3 x $10^6$ merozoites $ml^{-1}$.

### 6.2.2. Host cell culture

Madin Darby Canine Kidney (MDCK) and Madin Darby Bovine Kidney (MDBK) cells were seeded at 5 x $10^4$ per well in 0.5 ml RPMI containing 10% (v/v) heat inactivated foetal calf serum (Labtech), 2 mM L-glutamine (Sigma), 25 $\mu g\ ml^{-1}$ gentamycin (Sigma) and 2.5 $\mu g\ ml^{-1}$ amphotericin B (GIBCO) in a 24-well plate on glass coverslips (Surgipath, 13 mm). These were incubated for 24 h at 37°C in a 5% $CO_2$ moist incubator and then used immediately.

Monkey kidney (COS) cells (Peters *et al.*, 1995) were cultured as described for MDBK cells with the omission of amphotericin B from the medium, since this appeared to have a detrimental effect on growth.

### 6.2.3. Invasion assay

The MDCK, MDBK or COS cells on coverslips in wells were rinsed three times in serum-free RPMI and the freshly excysted sporozoites were added to each well in 0.2 ml volumes. The 24-well plates were then incubated at 37°C in a 5% $CO_2$ moist incubator for 60 min, after which the coverslips were washed twice in serum-free RPMI. Having added 0.25 ml serum-free RPMI to each well, the cells were incubated for a further 60 min to allow attached sporozoites to invade. The RPMI was then removed from the wells which were then washed twice in serum-free RPMI, the cells fixed in 75% ethanol for 60 min at room temperature and the coverslips air dried. The coverslips were then stained in 10% Giemsa's stain (BDH) in Giemsa buffer (3 g/L anhydrous disodium hydrogen orthophosphate and 0.6 g/L potassium dihydrogen orthophosphate, pH 7.2) for 60 min at room temperature. The preparations were rinsed twice with deionised water before being allowed to air dry. The coverslips were then mounted onto glass slides using canada balsam (Sigma). 200 host cells were observed in fields from one side of the coverslip to the other, under oil immersion microscopy, and the number of parasites that had invaded them counted. The results were expressed as the number of parasites per 100 host cells.

### 6.2.4. Enzyme inhibitors

To study the mechanisms that may be involved in host cell invasion, sporozoites and merozoites were incubated with a range of inhibitors before being added to the host cells.

The inhibitors were as follows: the cysteine proteinase inhibitors: trans-

epoxysuccinyl-L-leucincamido(4-guanido)butane (E64, 1.5 mM), N-p-tosyl-L-lysine chloromethyl ketone (TLCK, 25 μM) and Z-phenylalanine (ZFA - a peptidyl diazomethane, 10 μM); the serine proteinase inhibitors aprotinin (100 μM), leupeptin (also known to inhibit a number of cysteine proteinases (100 μM)) and $\alpha_1$-antitrypsin (0.5 mg $ml^{-1}$); the polyamine biosynthesis inhibitor difluoromethyl ornithine (DFMO, 2 mM); a sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (5NeuAc2en, 0.1 mM); a metalloproteinase inhibitor ethylenediaminetetraacetic acid (EDTA, 1 mM); an aspartic proteinase inhibitor pepstatin (1 μM) and the negative control formaldehyde (10%, v/v). With these final concentrations of inhibitors parasites were incubated for 10 min at room temperature.

### 6.2.5. 4,6,-diamino-2-phenylindole (DAPI) staining of MDBK cells

MDBK cells were fixed with 75% ethanol for 60 min. The ethanol was removed and the cells allowed to air-dry before the coverslips were stored at -20°C until processed. For processing the cells were thawed to room temperature and rehydrated with PBS containing 10% (v/v) foetal calf serum for 30 min at room temperature. Having washed the cells twice with PBS, the cells were incubated with the DNA intercalating dye 4,6,-diamino-2-phenylindole (DAPI) (0.01 mg $ml^{-1}$ in PBS) for 10 min at room temperature. They were then rinsed twice in PBS and the coverslips mounted on slides with non-fluorescing glycerol. Cells containing DNA that are stained with DAPI fluoresce brightly with a characteristic blue fluorescence when viewed with a Zeiss fluorescence microscope (Carl Zeiss Inc., New York) with the following filters: excitation filter, G365 nm; mirror, FT510 nm; barrier filter, LP520 nm).

### 6.2.6. Bromodeoxyuridine (BrdU) labelling for cells in S phase

Cells were rinsed three times in serum-free RPMI after which medium containing 40 μM bromodeoxyuridine (BrdU) and an equimolar concentration of 2-deoxycytidine was added to the cells which were incubated at 37°C for at least 60 min. Medium was then removed and the cells fixed with 75% ethanol for 60 min. The ethanol was removed and the wells were allowed to air dry before the coverslips were stored at -20°C until processing. Once defrosted, the fixed cells were denatured with 1.5 M HCl for 20 min at room temperature to allow antibody access. The cells were then washed ten times in PBS (pH 7.2) before adding the anti-BrdU antibody supplied as a working strength solution (Amersham) and incubating for 70 min at room temperature. Having been washed twice in PBS, the cells were incubated with the secondary anti-mouse antibody linked to fluorescein isothiocyanate (FITC) for 45 min at room temperature, after which they were rinsed briefly with PBS (pH 7.2). The cells were then counter-stained with DAPI (as described in 7.2.4.) for 10 min and, after two rinses, were placed on slides with non-fluorescing glycerol. The MDBK cells could then be observed for labelling using the relevant filters: [DAPI (as described above in 6.2.4.); BrdU:excitation filter BP450-490 nm; mirror, FT510 nm; barrier filter, LP520 nm] on the Zeiss fluorescence microscope, and the percentage of cells labelled with BrdU (the cells in S phase) could be calculated.

### 6.2.7. Analysis of MDBK cell cycle

The MDBK cell cycle could be mapped once the periods of mitosis, S

phase, $G_1$ and $G_2$ were established. To do this the cells were synchronised by incubating with 2 mM hydroxyurea (Sigma) in growth medium for 24 h. This arrests cells at the $G_1$/S interphase (Krakoff *et al.*, 1968) by inhibiting the reduction of deoxyribonucleotides and thus DNA synthesis. Once released from this block by washing the cells three times in serum-free RPMI, the cells progress through the cell cycle reasonably synchronously enabling their cell cycle stages to be monitored. The periods of mitosis and S phase were monitored using DAPI staining and BrdU labelling as previously described in 6.2.4. and 6.2.5. respectively. Cells were fixed and stained at half hourly intervals for mitosis, and hourly for BrdU labelling.

## 6.3. Results

### 6.3.1. *C. parvum and E. tenella* sporozoite invasion of MDBK cells

Typical results for both *C. parvum* and *E. tenella* MDBK cell invasion as described in 6.2.3. are illustrated in Figures 6.3.1. and 6.3.2.

### 6.3.2. BrdU labelling of MDBK cells

Typical staining results using DAPI and BrdU labelling on MDBK cells as described in 6.2.6. are illustrated in Figure 6.3.3.

### 6.3.3. Varying the number of sporozoites in the invasion assay

Freshly excysted and filtered *E. tenella* and *C. parvum* sporozoites were added to MDCK and MDBK cells in different numbers to determine the optimum number to use in future experiments. The results are shown in Figures 6.3.4. and 6.3.5. for MDCK cell invasion; and 6.3.6. and 6.3.7. for MDBK cell invasion. Assuming the cells have progressed through one cell cycle in the 24 h attachment incubation prior to the addition of the parasites, a 20:1 sporozoite to cell ratio (2 x $10^6$ sporozoites per well) resulted in the optimum invasion rates for both host cell types (higher ratios resulted in damage to the host cells). MDCK cells appeared to be more susceptible to infection than MDBK cells. However the use of microscopic observation as the method of determining parasite infection required being able to distinguish between parasites that were attached and those that had

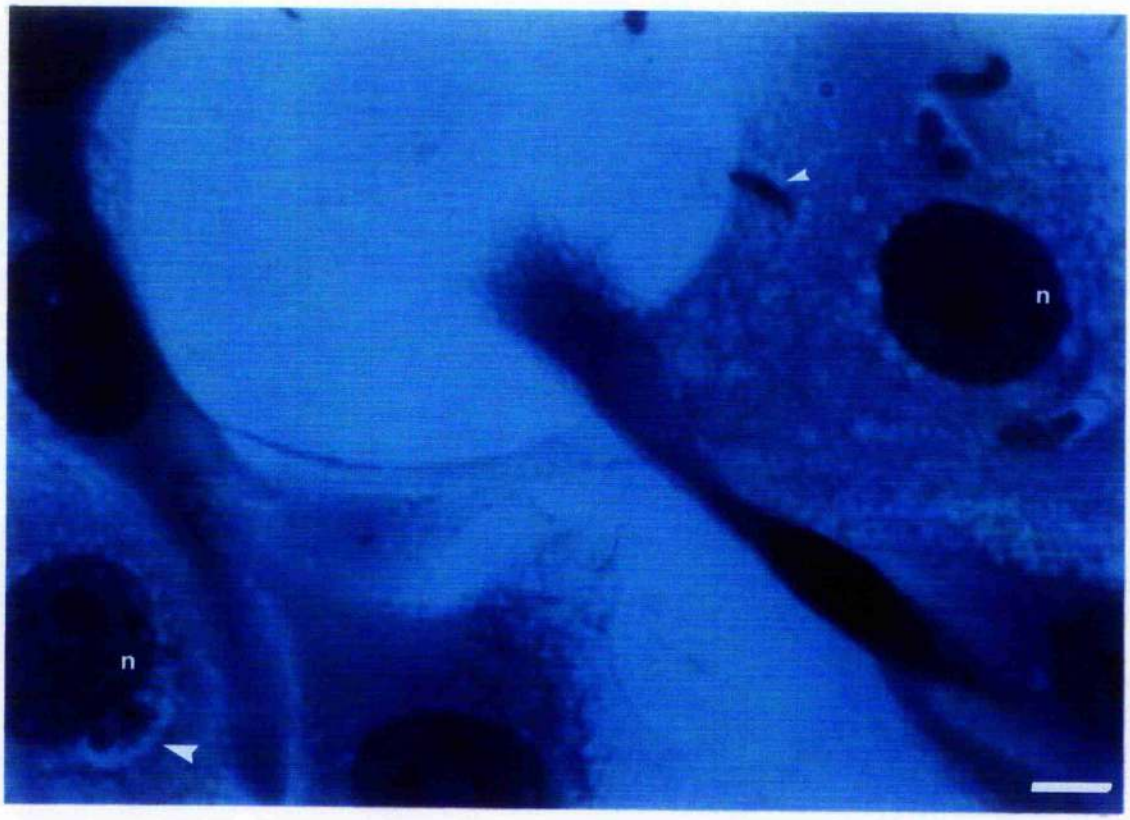
**Figure 6.3.1.: *C. parvum* invasion of MDBK cells**

As seen under x 100 oil immersion microscopy after Giemsa's staining of the cell preparation. The arrow indicates a *C. parvum* sporozoite attaching to a MDBK cell prior to invasion; n-MDBK cell nucleus; scale bar = 10 μm.

**Figure 6.3.2.: *E. tenella* invasion of MDBK cells**

As seen under x100 oil immersion microscopy after Giemsa's staining of the cell preparation. The large arrow illustrates an invaded *E. tenella* sporozoite clearly surrounded by a parasitophorous vacuole; the small arrow points to an attached sporozoite (not invaded); n-MDBK cell nucleus; scale bar = 10 μm


n


n
n

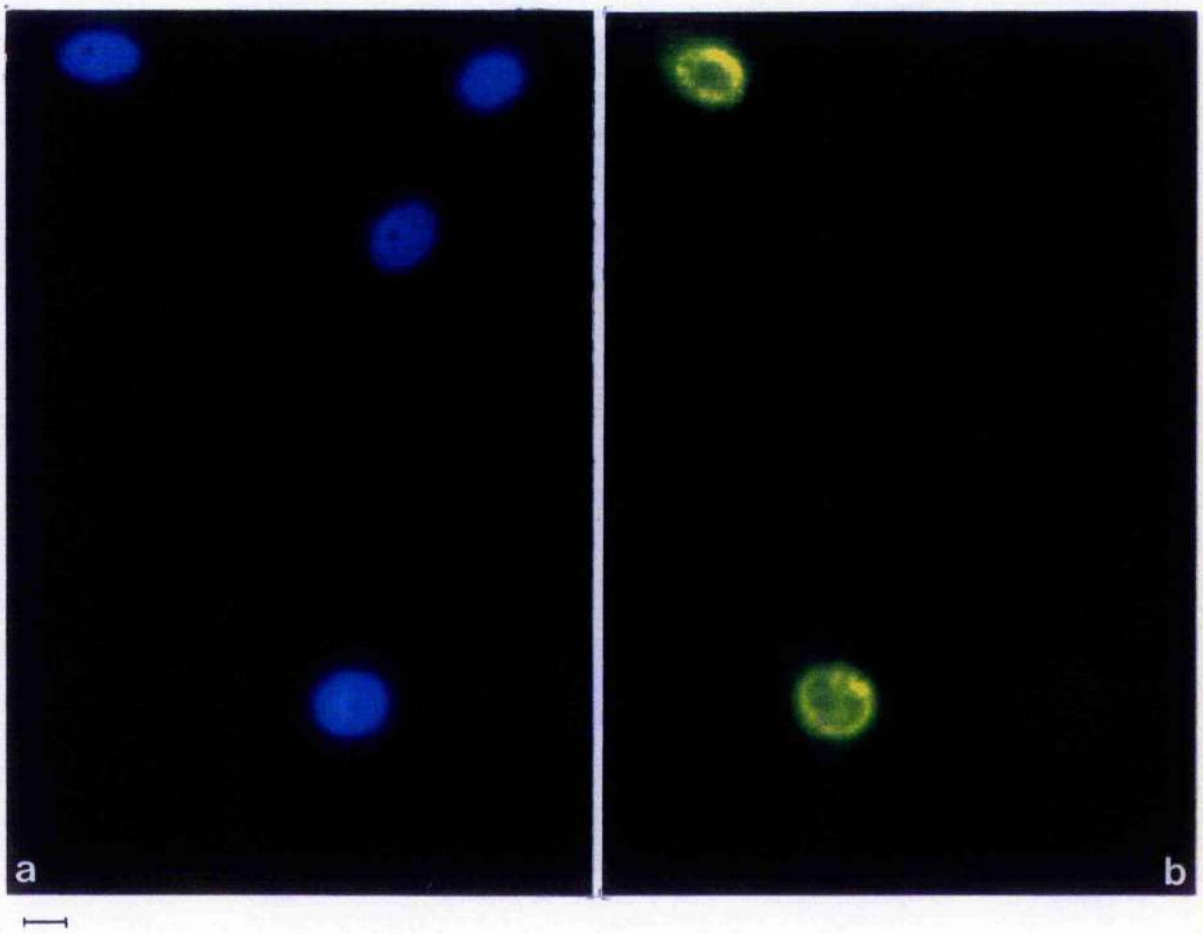



**Figure 6.3.3.: BrdU labelling of MDBK cells**

Figure 6.3.3.(a) x40 DAPI stained MDBK cells under the DAPI filter of a Zeiss fluorescence microscope; Figure 6.3.3.(b) (mirror image of Figure 6.3.3.(a)) BrdU labelling of same field as Figure 6.3.3.(a). Two MDBK cells clearly show BrdU incorporation shown by fluorescence with the FITC filter indicating that these cells are in S phase compared to two cells which are not labelled; scale bar = 10 μm.

invaded. As the MDBK cells appeared to spread out to a greater degree than MDCK cells on the substrate, it was far easier to distinguish invasion from attachment with this cell line, and therefore the combination of MDBK cells with $2 \times 10^6$ sporozoites per well was chosen for future experiments.

### 6.3.4. Effects of inhibitors on invasion

Freshly excysted and filtered sporozoites or freshly collected merozoites were incubated with a range of inhibitors for 10 min at room temperature (see 6.2.4.). None of the inhibitors used had any adverse effects on the sporozoites themselves as determined using the vital stain viability test (see Chapter 3), and these samples were accompanied with appropriate controls, for example, if solvents had been used to solubilise the inhibitors. The sporozoites were then washed three times after which they were added to the appropriate MDBK wells in 0.2 ml volumes for the invasion assay. The results are presented in Figures 6.3.8. and 6.3.9. for *C. parvum* and *E. tenella* sporozoites and 6.3.10. for *E. tenella* merozoites respectively. Invasion by the negative controls (incubated with 10% formaldehyde), was inhibited by approximately 90%. This validates the assay used, confirming that intracellular and extracellular parasites could be distinguished reasonably efficiently. The sialidase inhibitor did not have any effect on *C. parvum* or *E. tenella* sporozoite host cell invasion, but did appear to be involved in *E. tenella* merozoite invasion where it inhibited this process by ~ 30%. The ornithine decarboxylase inhibitor DFMO gave ~40% inhibition for both *C. parvum* sporozoites and *E. tenella* sporozoites and merozoites. All of the proteinase inhibitors except $\alpha_1$-antitrypsin (which had no effect on *C. parvum* host cell invasion) and aprotinin (which did not appear to have any effect on *E. tenella*

merozoite invasion) inhibited the invasion by both parasites to varying degrees.

### 6.3.5. Effect of the *Maackia amurensis* lectin on *E. tenella* host cell invasion

To confirm the results that sialidase was not important for *E. tenella* host cell invasion, the *Maackia amurensis* lectin - which is specific for sialic acid residues - was employed. Freshly excysted *E. tenella* sporozoites were prepared and incubated with the *M. amurensis* lectin (Mal II, Vector laboratories; final concentration 0.1 mg $ml^{-1}$) for 30 min at 37°C, with 10% formaldehyde used as a negative control. After this time, sporozoites were either washed three times in RPMI medium to remove the lectin and formaldehyde, or the sporozoites were unwashed so that the lectin was still present in the sporozoite suspension when they were added to the host MDBK cells for the invasion assay. The results are shown in Figure 6.3.11. and they indicate that the lectin had no effect on the *E. tenella* host cell invasion ($P>0.10$).

### 6.3.6. Analysis of MDBK cell cycle

To determine the duration of the various phases in the cell cycle of the MDBK cells (that is, mitosis, M phase; $G_1$; DNA synthesis, S phase and $G_2$), two methods were employed: DAPI staining (as described in 6.2.5.) to monitor the nucleus and therefore mitosis, and 5-bromodeoxyuridine (BrdU) labelling (as described in 6.2.6.) to monitor the occurrence of DNA synthesis and so the S phase of the cells.

The S phase of the MDBK cells lasted for approximately 5 h after the release from the hydroxyurea block, and mitosis was observed to occur at

approximately 10 h. Having calculated the doubling time of the cells from the rate of growth in the cultures, the cell cycle could be mapped and is illustrated in Figure 6.3.12. The whole cycle was calculated to take approximately 20 h.

### 6.3.7. Influence of MDBK cell cycle on the invasion by *C. parvum* and *E. tenella* sporozoites

MDBK cells were inoculated onto glass coverslips as described in 6.2.2. To determine the extent to which the host cell cycle influences parasite invasion, the invasion of sporozoites into synchronised MDBK cells was compared with invasion of an asynchronous population. The cells were then rinsed three times in serum-free RPMI to remove the hydroxyurea and growth medium was added (see 6.2.2.) to allow the cells to progress into S phase. $2 \times 10^6$ *E. tenella* or *C. parvum* sporozoites were then added at specific time intervals after the hydroxyurea release to both the synchronous and asynchronous cell populations for invasion assays as described before in 6.2.3. The results are illustrated in Figures 6.3.13. and 6.3.14. for both *E. tenella* and *C. parvum* respectively. The results obtained with *E. tenella* were from analysing the whole cell cycle time of the MDBK cells. No difference was seen in the invasion rates except for one time point - 4 h into S phase ($P>0.10$). This increase in MDBK cell invasion is a rapid one which appears to last only one hour (see Figure 6.3.12.). Similar results were obtained with *C. parvum* sporozoites when the MDBK S phase was analysed for invasion. A large increase in invasion at the 4 h time point was observed ($0.01<P>0.002$). These results suggest that MDBK cells are more susceptible to invasion by the parasites 4 hours into S phase (by approximately 3x and 2x for *C. parvum* and *E. tenella* respectively).

The initial invasion rates at time 0 h for *E. tenella* are much higher for

both synchronous and asynchronous cultures. This may be due to the fact that the host cells at this time point are rinsed three times in serum-free RPMI to remove the hydroxyurea block. After this point the host cells are only rinsed twice before the sporozoites are added, possibly leaving a residue of the growth medium on the cells, which may affect sporozoite invasion. Indeed it has been mentioned by other researchers that foetal calf serum inhibits sporozoite invasion, probably due to a number of components thought to be present in the serum, for example proteinase inhibitors (personal communications).

### 6.3.8. Influence of cell cycle of COS cells on their susceptibility to invasion by *E. tenella* sporozoites

To investigate whether the phenomenon found with MDBK cells, which are more susceptible to infection by *E. tenella* sporozoites 4 h into S phase, also occurred with other mammalian cells, COS cells were employed. Synchronous cultures were prepared using hydroxyurea (as described in section 6.2.2.) and, having removed the hydroxyurea by washing the cells three times, *E. tenella* sporozoites were added to the cultures as described for MDBK cells (section 6.3.7.). The results are given in Figure 6.3.15. Although there was a significant increase at 4 h in the synchronous cells ($P<0.01$), there was no significant difference between these and the asynchronous cells, indicating that this feature is one that is not shared with MDBK cells.

### 6.3.9. Effect on MDBK cell cycle by infection with *E. tenella* and *C. parvum*

To determine whether *E. tenella* and *C. parvum* invasion induces MDBK

cells to enter and remain in S phase (as previously reported for *E. tenella*, Urquhart, 1981b), infected cultures were compared with non-infected cultures with respect to the numbers of host cells in S phase. MDBK cultures were grown and infected with *E. tenella and C. parvum* sporozoites as described in sections 6.2.2. and 6.2.3., respectively. Wells either had 2 x $10^6$ sporozoites added or not and incubated at 37°C for 20 h (the time taken for one cell cycle in normal cultures). In addition, the supernatant of infected cultures was used to see if this alone induced the host cells to enter S phase. For this, supernatants from MDBK cell cultures that had been incubating with sporozoites for 120 min at 37°C were collected. These were centrifuged to remove any sporozoites and the supernatant added to non-infected cultures which were also then incubated for 20 h at 37°C. After the incubations, the cells were processed for BrdU labelling as described in section 6.2.6.

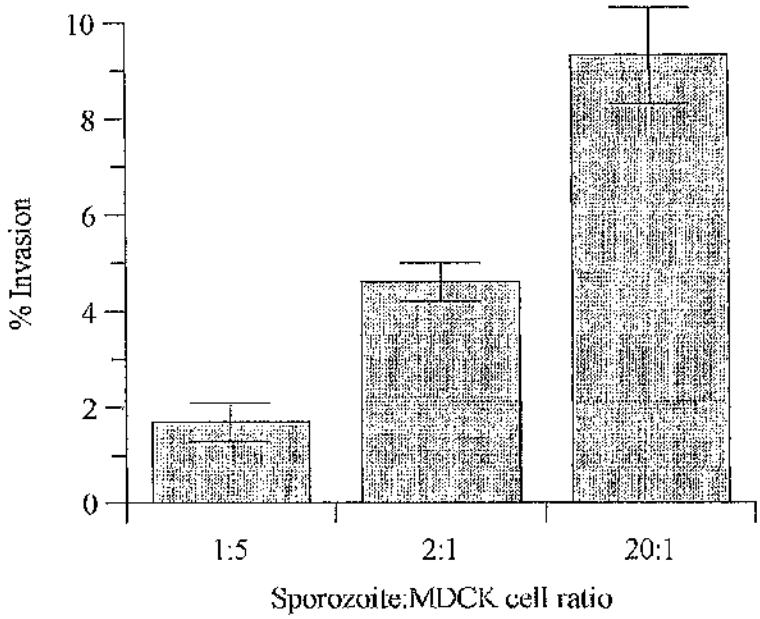
The results for *E. tenella* and *C. parvum* are shown in Figures 6.3.16. and 6.3.17, respectively. The results for *E. tenella* indicate that there may be an increase in the number of cells in S phase in the infected cultures compared with non-infected cultures. However, this result is not statistically significant. The supernatant from infected cultures had no effect when used alone. With *C. parvum*, no difference was seen between infected and non-infected cultures. This may reflect the fact that *C. parvum* invades at a very low rate (~6%, 1 sporozoite per cell) into MDBK cells when compared with *E. tenella* (~40%, 2 sporozoites per cell), thus *C. parvum* may not produce a noticeable difference.

### 6.3.10. Effect of *E. tenella* invasion on MDBK cell proliferation

If the invasion of *E. tenella* was inducing MDBK cells to remain in S

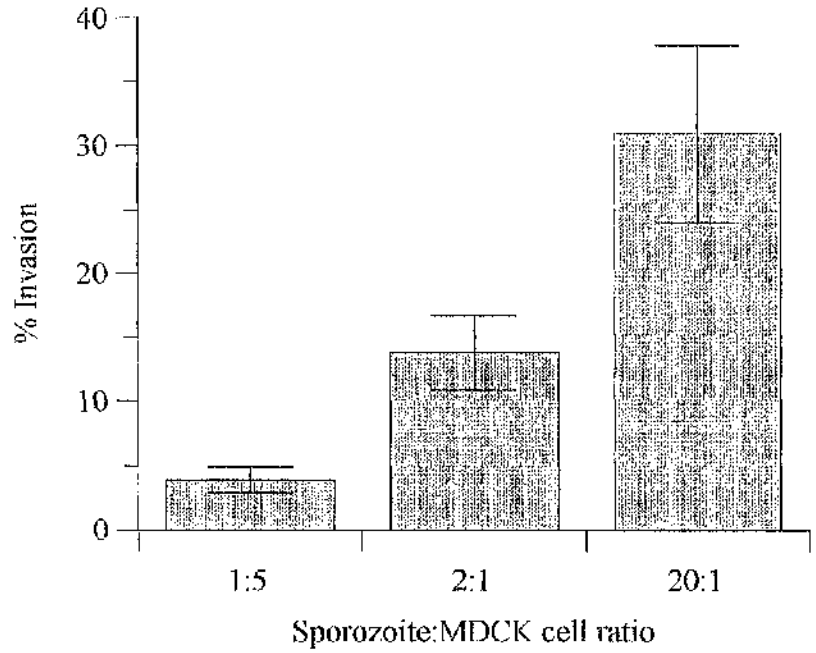
phase, the rates of MDBK proliferation in infected cultures should, theoretically, be lower than those in non-infected cultures. To study this, MDBK cells were either infected with *E. tenella* sporozoites (as described in 6.2.3.) or not, and incubated for 20 h (the time for one cell cycle in normal cultures) at 37°C. After this incubation, host cells were trypsinised from the glass coverslips by incubating for 10 min with HEPES 10 mM (pH 7.5 containing sodium chloride, 140 mM; potassium chloride, 5 mM; calcium chloride, 1 mM; magnesium chloride, 1 mM; D-glucose, 10 mM and trypsin, 0.05%) and the number of cells determined using a haemocytometer. The results shown in Table 6.3.1. indicate that infected MDBK cells proliferated significantly more slowly than non-infected cultures ($P<0.10$).

**Figure 6.3.4.: MDCK invasion using different numbers of *C. parvum* sporozoites**



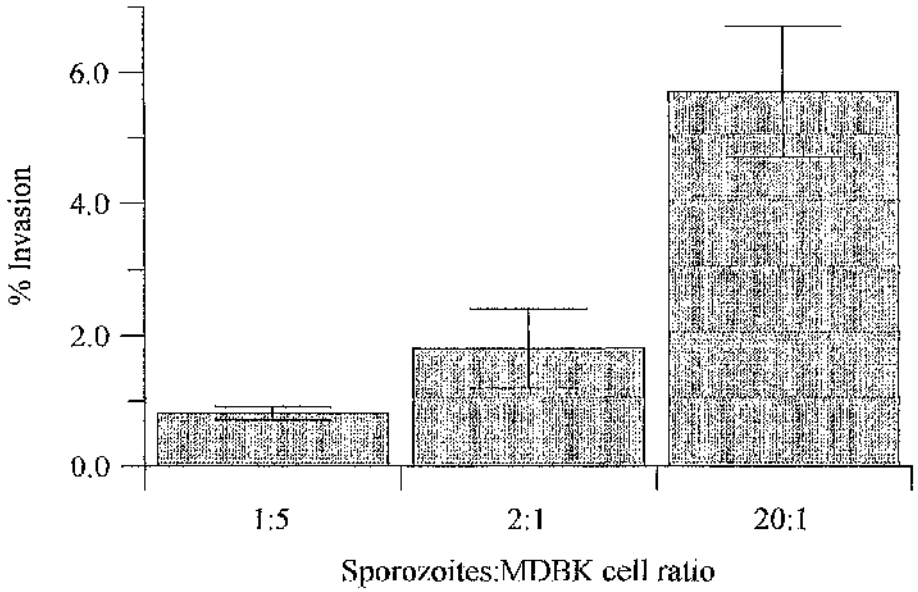

Means ± SE from three experiments

**Figure 6.3.5.: MDCK invasion using different numbers of *E. tenella* sporozoites**



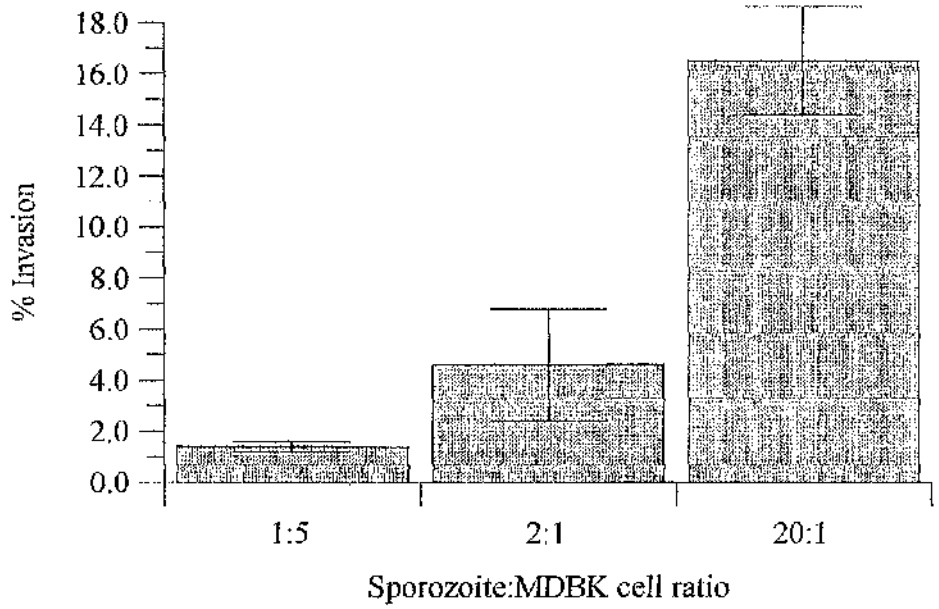

Means ± SE from three experiments

**Figure 6.3.6.: MDBK invasion using different numbers of *C. parvum* sporozoites**




Means ± SE from three experiments

**Figure 6.3.7.: MDBK invasion using different numbers of *E. tenella* sporozoites**




Means ± SE from three experiments

**Figure 6.3.8.: Inhibitors on *C. parvum* MDBK cell invasion**

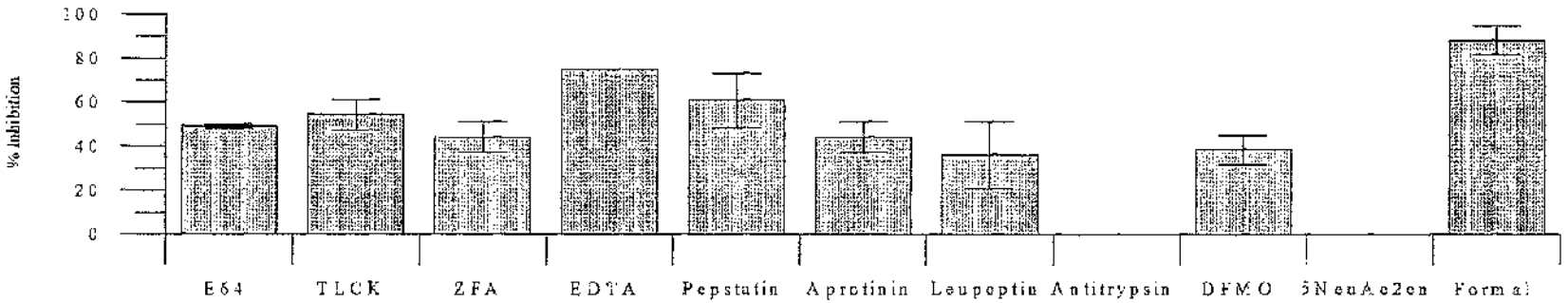
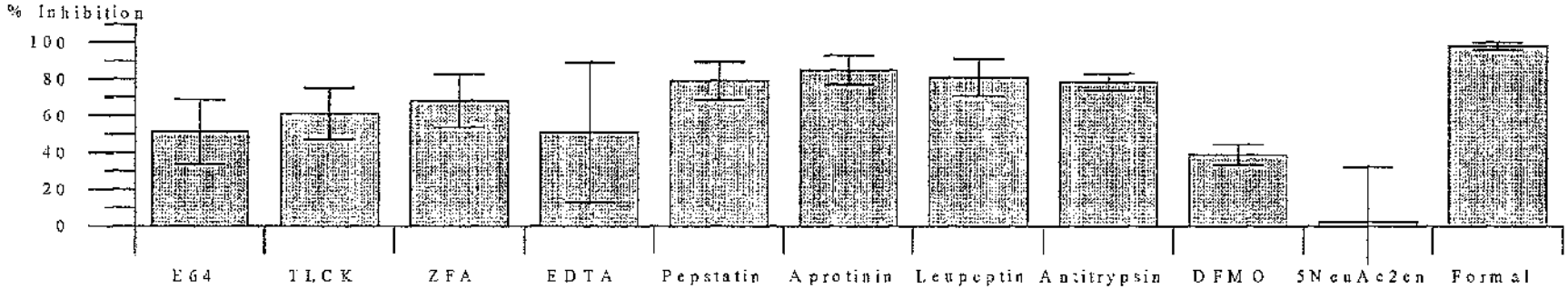


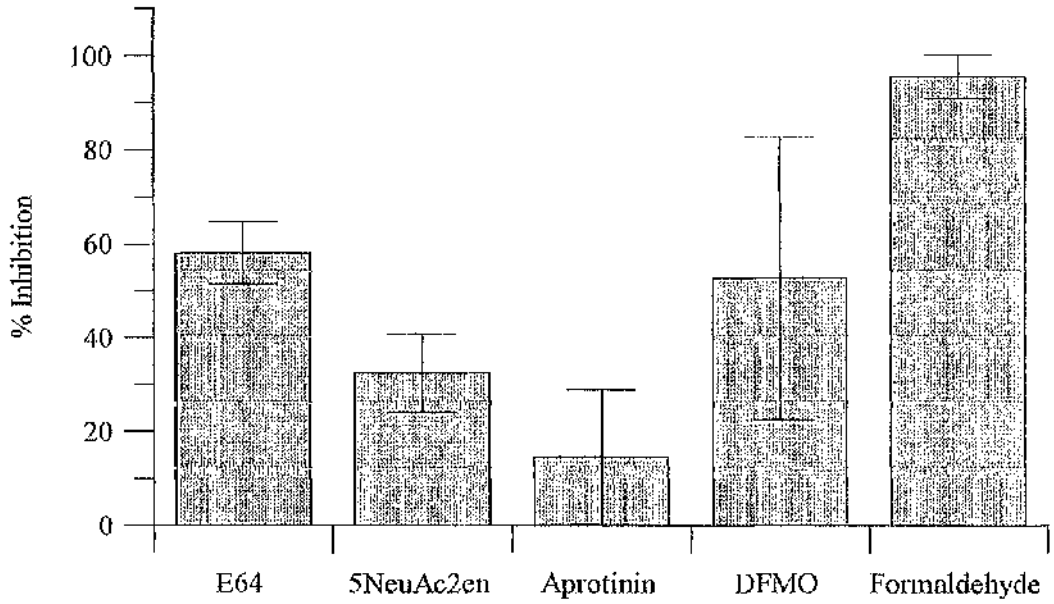

**Figure 6.3.9.: Effect of inhibitors on *E. tenella* sporozoite MDBK cell invasion**




Means ± SE from three experiments

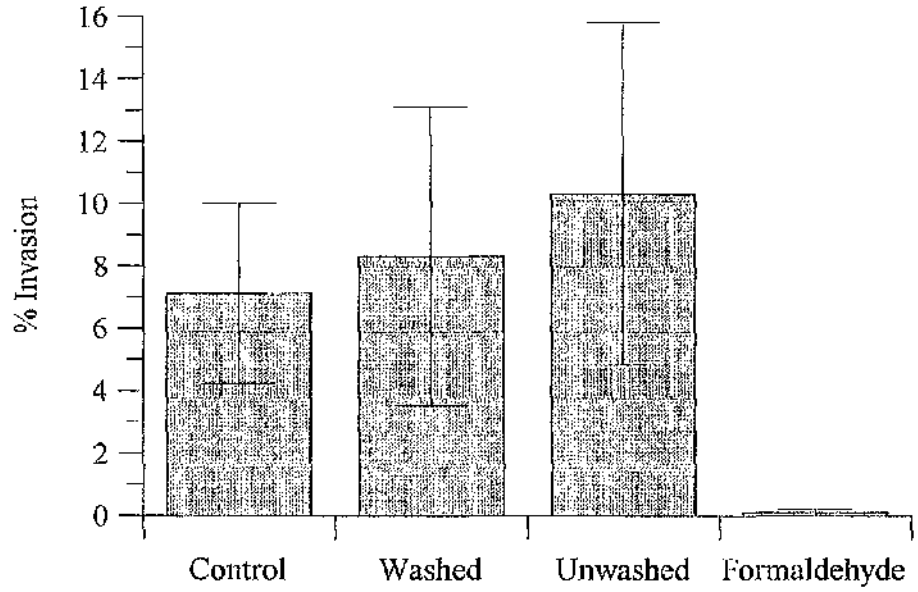
Concentrations of inhibitors: E64, 1.5 mM; TLCK, 25 μM; ZFA, 10 μM; EDTA, 1 mM; Pepstatin, 1 μM; Aprotinin, 100 μM; Leupeptin, 100 μM; Antitrypsin, 0.5 mg $ml^{-1}$; DFMO, 2 mM; 5NeuAc2en, 0.1 mM and formaldehyde, 10%.

**Figure 6.3.10.: Effect of inhibitors on *E. tenella* merozoite COS cell invasion**



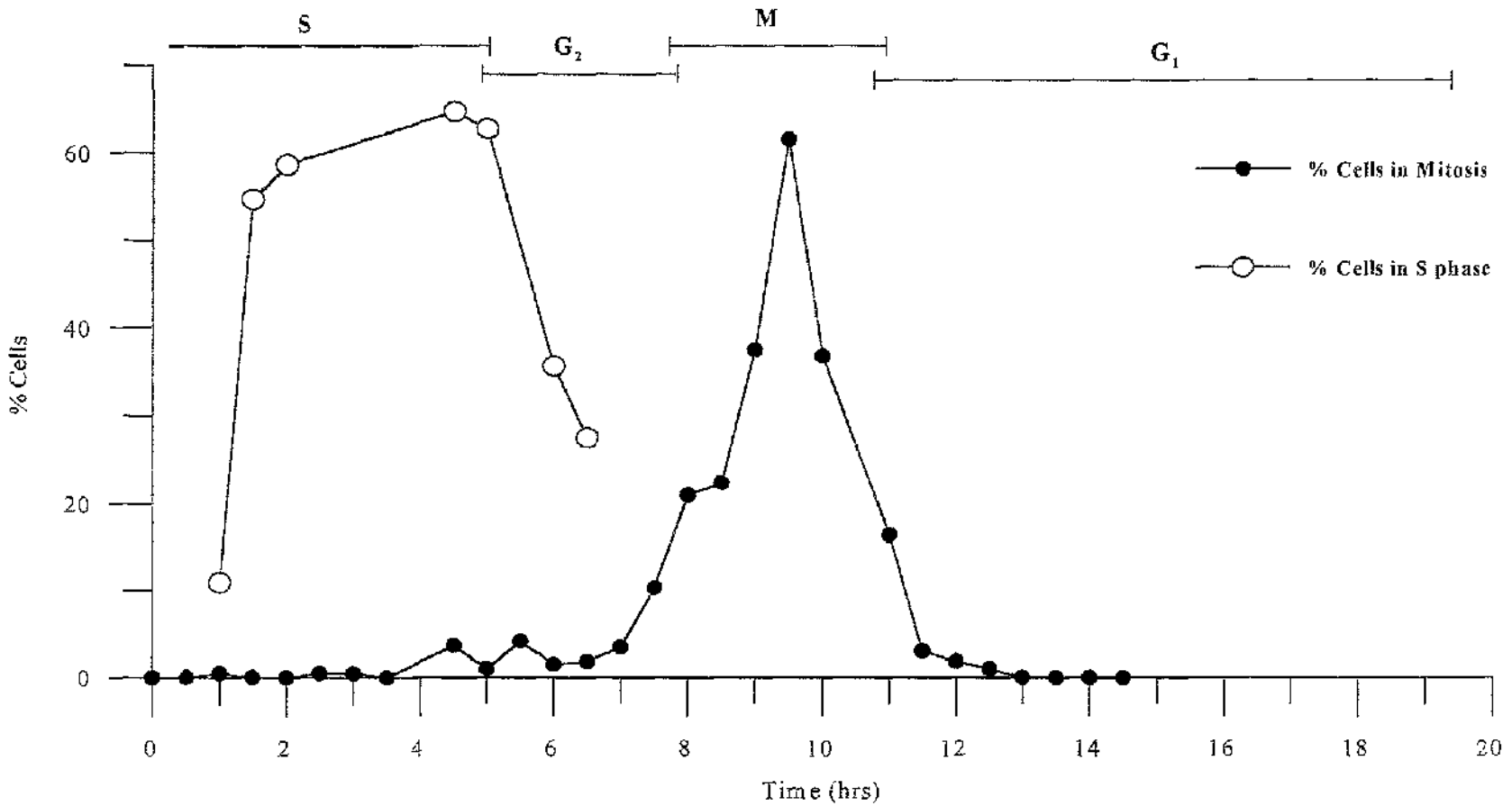

Means ± SE from three experiments
Concentrations of inhibitors: E64, 1.5 mM; Aprotinin, 100 µM; DFMO, 2 mM; 5NeuAc2en, 0.1 mM and formaldehyde, 10%.

**Figure 6.3.11.: *E. tenella* MDBK cell invasion with and without the presence of the *M. amurensis* lectin**



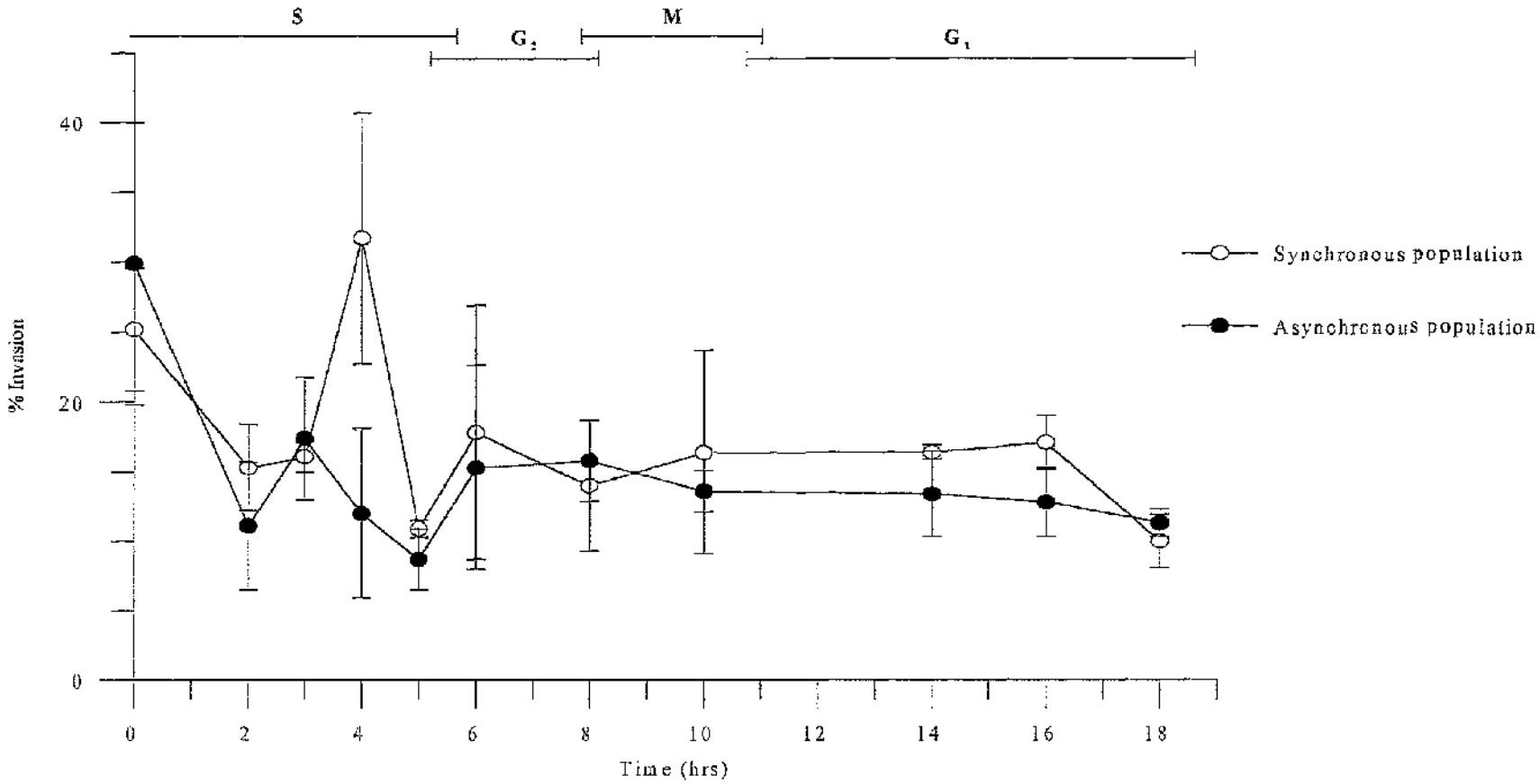

Means ± SE from three experiments

**Figure 6.3.12.:MDBK cell cycle**



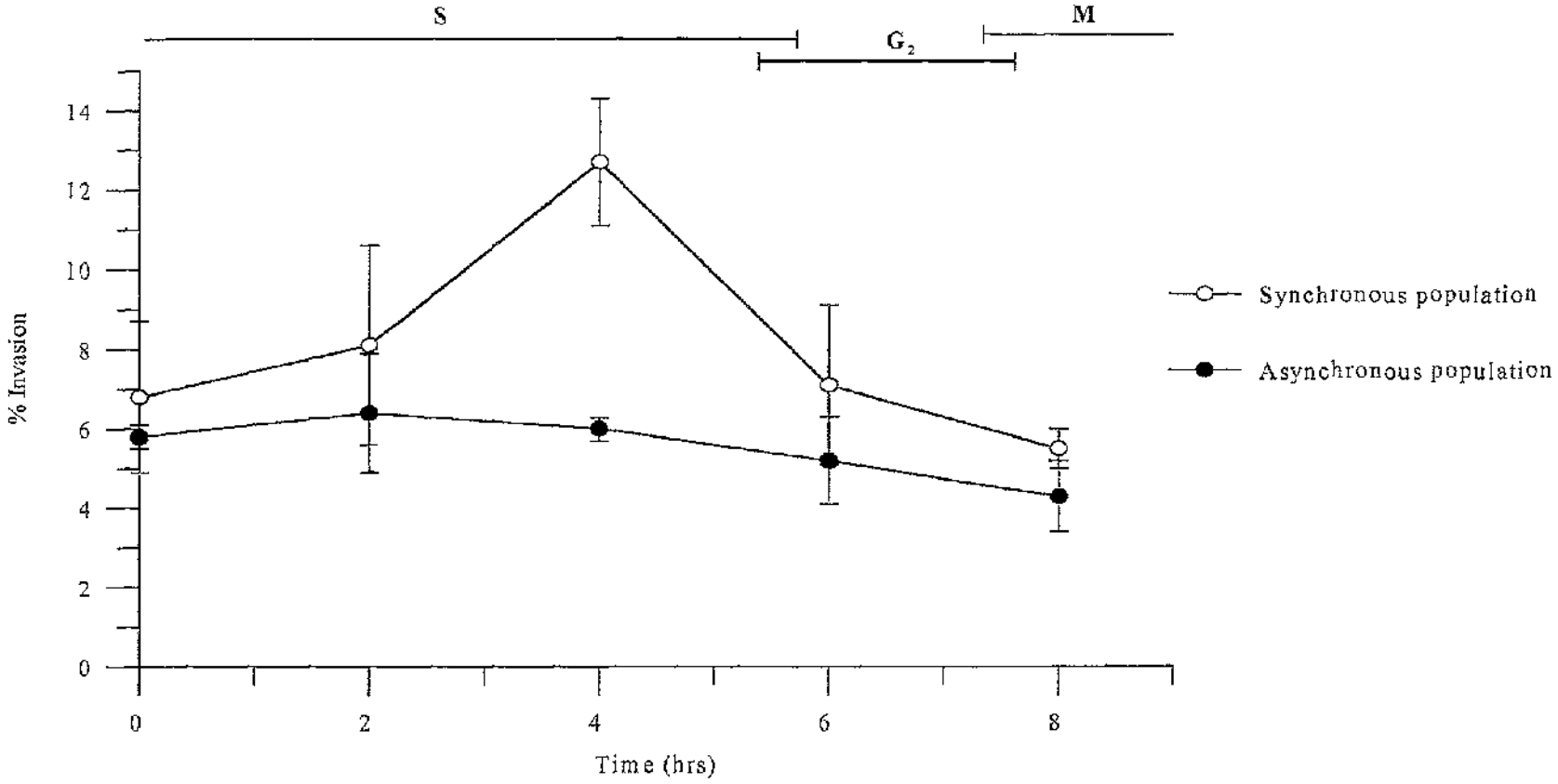

Results from at least one experiment

**Figure 6.3.13.: Influence of MDBK cell cycle on *E. tenella* invasion**



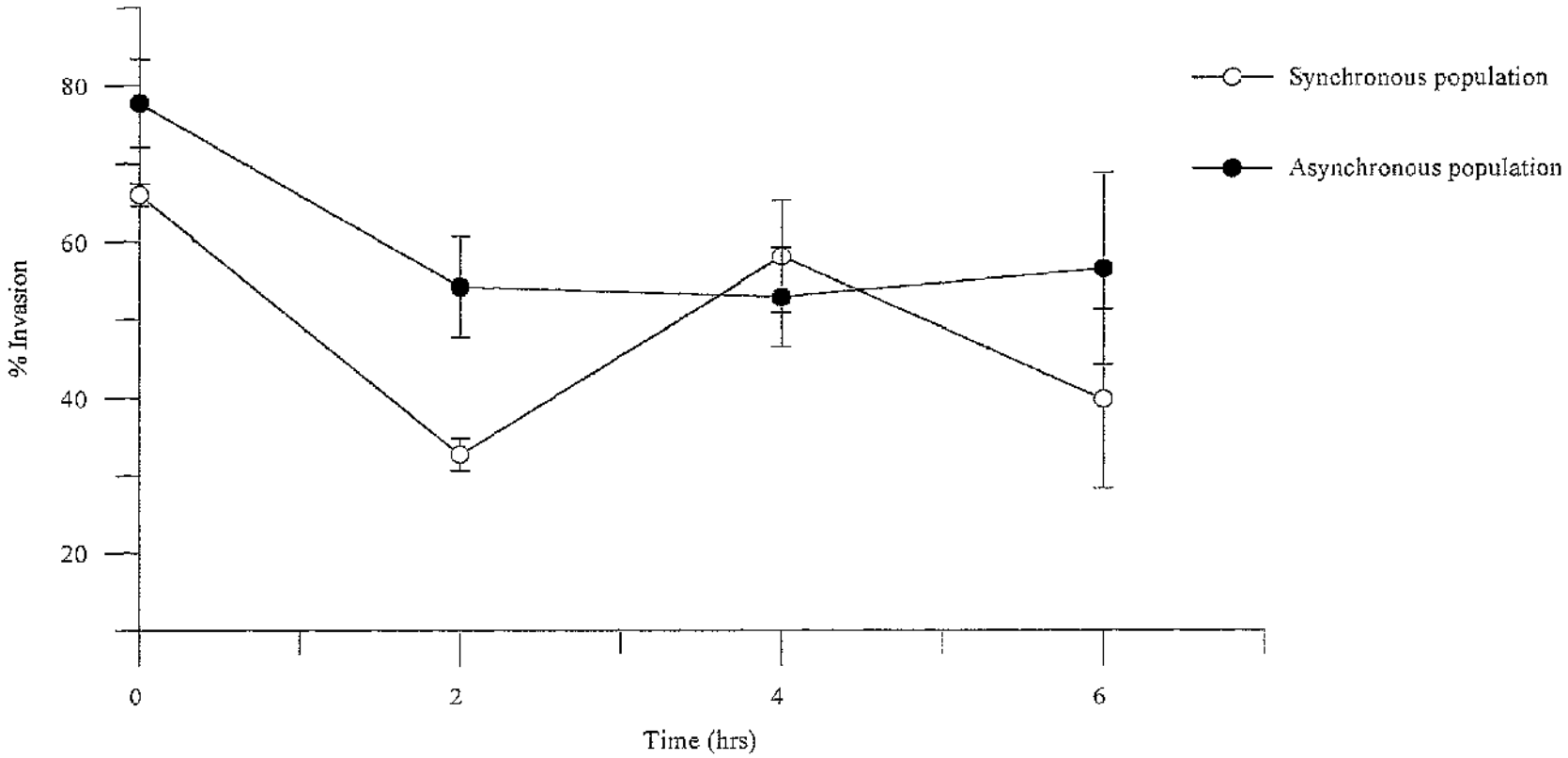

Means ± SE from three experiments

**Figure 6.3.14.: Influence of MDBK cell cycle on *C. parvum* invasion**



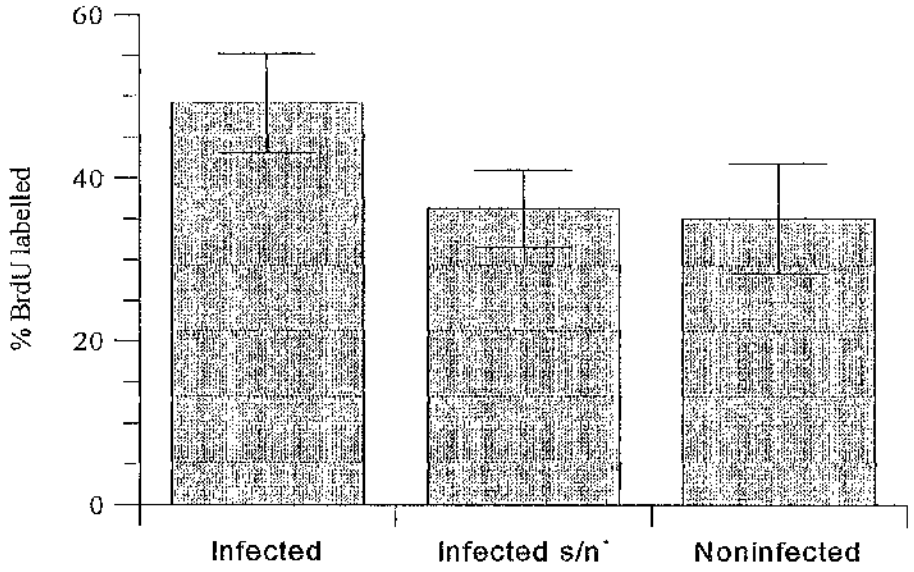

Means ± SE from three experiments

### 6.3.15.: Influence of COS cell cycle on *E. tenella* invasion



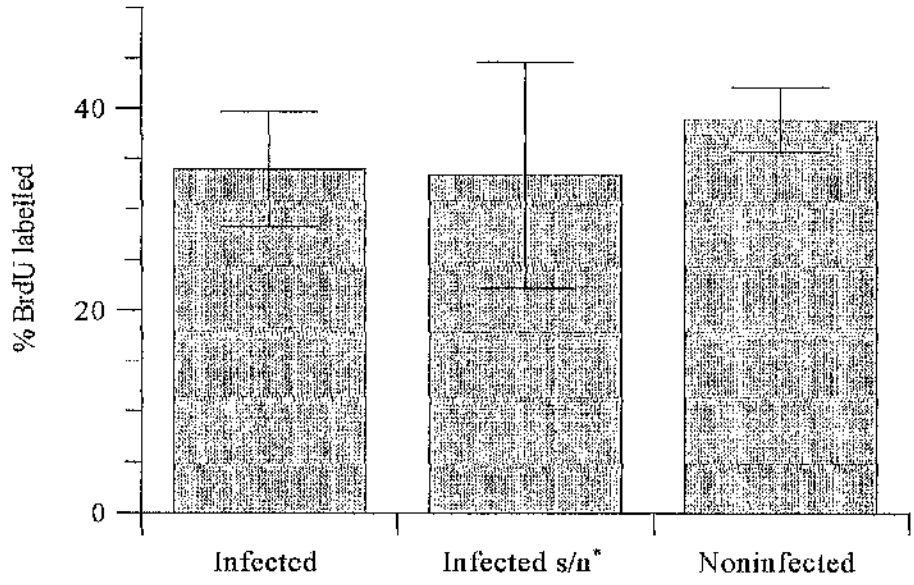

Means ± SE from three experiments

**Figure 6.3.16.: Effect on MDBK cell cycle by infection with *E. tenella***




Means ± SE from three experiments
Infection rate is approximately 20% of MDBK cells infected with 2 sporozoites
*s/n - supernatant from infected cultures added to noninfected cultures

**Figure 6.3.17.: Effect on MDBK cell cycle by infection with *C. parvum***




Means ± SE from three experiments
Infection rate is approximately 6% of MDBK cells infected with 1 sporozoite
*s/n - supernatant from infected cultures added to noninfected cultures

**Table 6.3.1.: Effect of *E. tenella* invasion on MDBK cell proliferation**

| | NUMBER OF CELLS PER WELL AFTER 20 h ($x 10^4$) |
|---|---|
| INFECTED CULTURE | $6.5 \pm 4.3$ |
| NON-INFECTED CULTURE | $16.0 \pm 6.9$ |

Means ± SE from three experiments
Initial density of MDBK cells: $5 \times 10^4$; number of sporozoites added: $2 \times 10^6$

## 6.4 Discussion

The invasion assay and a method of measuring host cell invasion using Giemsa's staining and microscopic observation proved a successful and rapid technique for both *Cryptosporidium* and *Eimeria* compared, for example, with other studies that have used longer incubations for the *Cryptosporidium* invasion process (Rosales *et al.*, 1995; Forney *et al.*, 1996). The method was validated since invaded parasites could be distinguished from those that were merely attached to the cells. MDBK cells were used routinely for sporozoite invasion assays, but *E. tenella* merozoites did not invade these cells to any measurable extent (on average 1%), so COS cells were used for this life cycle stage. The results obtained therefore should be treated with caution when comparing *E. tenella* sporozoites and merozoites, since the host cell line may have affected the results due to differences in, for example, receptors (number and type) and differentiation of the host cell (known to affect *C. parvum* invasion, Flanigan *et al.*, 1991).

To investigate the enzymes that might be involved in host cell invasion a number of enzyme inhibitors were used, as was done for the mucus penetration studies (see Chapter 5). The negative controls, that is the sporozoites killed with 10% formaldehyde, were seen to be inhibited by 90-100% for *C. parvum* and *E. tenella* sporozoites and merozoites. These results validate the assay and lead to the conclusion that dead parasites did not invade the cells and suggests that host cell invasion therefore required an active process by the parasites themselves. This is consistent with data reported for *T. gondii* where it was also concluded that invasion was an active process (Morisaki *et al.*, 1995).

An interesting result was that, in direct contrast to the findings that

sialidase activity appears to be important in mucus penetration (see Chapter 5), the sialidase inhibitor had no effect on host cell invasion of sporozoites. This result was confirmed when, in a similar experiment to that involving the mucus in 5.3.4., the *Maackia amurensis* lectin, which specifically binds sialic acids, was used. The presence of this lectin did not inhibit host cell invasion by the parasites (see Figure 6.3.11.), thus indicating that sialic acids and sialidase do not play a role in this invasive process by sporozoites. This is in direct contrast with another apicomplexan parasite *Plasmodium* which is known to possess a sialidase activity and to use sialidase receptors on the host erythrocyte cell surface to invade (Clough *et al.*, 1996). Alternatively, *E. tenella* merozoite invasion of COS cells was seen to be inhibited by approximately 30% by the sialidase inhibitor. This stage of the *E. tenella* life cycle has been reported to have twenty times more sialidase activity than sporozoites (Pellegrin *et al.*, 1993), and it could be that there are stage-specific differences in the mechanisms used to invade the host cells. It should be noted however that the difference in host cell lines used for the sporozoite and merozoite invasion assays may well affect these results. COS cells may have more sialic acid residues on their surface thus requiring sialidase activity to cleave these and possibly reveal attachment sites for the invading microorganism.

The polyamine biosynthesis inhibitor DFMO inhibited host cell invasion by approximately 40% for both *E. tenella* and *C. parvum* sporozoites, and approximately 55% for *E. tenella* merozoites. It has been reported that polyamines are involved in differentiation in *Trypanosoma brucei* (Pegg and McCann, 1988) and the release of the polyamine putrescine may also provide more protective roles since it is known to act as an anti-inflammatory agent and has antioxidant functions (Yarlett and Bacchi, 1991). Yet the role that

polyamines play in *C. parvum* and *E. tenella* host cell invasions remains unclear. The report that *C. parvum* does not possess a functional ornithine decarboxylase suggests that the DFMO (Yarlett *et al.*, 1996) must be having some other effect at least on this parasite. However more research is required before anything can be concluded, for example electron microscopy may reveal alterations to the sporozoite surface induced by DFMO.

The serine proteinase inhibitors that were used inhibited host cell invasion by both *C. parvum* and *E. tenella* sporozoites, though to a greater extent in the latter parasite. No effect was seen when aprotinin was used on *E. tenella* merozoites, again possibly highlighting stage-specific differences. $\alpha_1$-antitrypsin did not inhibit *C. parvum* at all, possibly indicating that this inhibitor due to its high specificity, inhibits fewer serine proteinases. These results confirm previous findings by Fuller and McDougald (1990) and Forney *et al.* (1996) on *E. tenella* and *C. parvum* sporozoites respectively, and hopefully resolve the conflicting results that other researchers have reported. It should be noted, however, that the widely used serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) was excluded from the studies. This was due to this inhibitor proving to be toxic to the sporozoites after a 60 min incubation whereupon it lowered viability by approximately 60%, as tested using the vital stains method.

Although they had no effect on mucus penetration (see 5.3.3.), the inhibitors of aspartic proteinases (pepstatin) and metallo-proteinases (EDTA) did have a large effect on sporozoite host cell invasion, again highlighting the different mechanisms that appear to be involved in these two procedures. Yet EDTA is known to be a reversible inhibitor and would be removed from the sporozoites during the washing. This inhibitor must therefore have an effect on the sporozoites during the ten minute incubation, possibly by affecting calcium

ion concentrations. The result from the aspartic proteinase confirms the report that *Eimeria* has a gene with high homology to those encoding aspartic proteinases in other organisms (Laurent *et al.*, 1993). This proteinase is thought to be associated with the refractile bodies of the sporozoite (Laurent *et al.*, 1993), and antibodies raised against the recombinant protein inhibited invasion by sporozoites.

The cysteine proteinase inhibitors also inhibited host cell invasion by sporozoites of *E. tenella* and *C. parvum* and merozoites of *E. tenella*, thus indicating a role for these activities in this process. Other parasites are thought to utilise proteinases to invade the host cell (McKerrow, 1993), and indeed a metallo-dependent cysteine proteinase has been detected on the surface of the invasive *C. parvum* sporozoite. Indeed, the use of biotinylated probes specific for cysteine and serine proteinases indicated that both of these activities were detected in *C. parvum, E. tenella and T. gondii* extracts, with the apparent localisation of a cysteine proteinase activity on the surface of *E. tenella* sporozoites (see Chapter 4). The roles of these proteinase activities may be to modify the host cell membrane or parasitophorous vacuole in some way, though more research needs to be performed (Strobel *et al.*, 1992; McKerrow *et al.*, 1993).

Most previous studies have focused on the means by which the parasite may effect infection. Another area of interest however, is whether the host cells play a role in determining the extent to which they are invaded. Clearly the cell cycle of the host cell itself could be important in this process.

The cell cycle of the MDBK cells was mapped out having already established the cell cycle time as approximately twenty hours from doubling times

in culture. Once this was completed a comparison of invasion of asynchronous and synchronous cultures, was performed for both *C. parvum* and *E. tenella* (see Figures 6.3.9. and 6.3.10. respectively).

For both parasites it was seen that, with synchronous cultures there was a peak of invasion four hours into S phase. Further analysis revealed that this peak is very narrow and only appears to last for one hour. This suggests that there are differences in the host cell at this four hour point in S phase compared with the rest of the cell cycle. Interestingly *T. gondii* tachyzoites were also seen to attach (Grimwood *et al.*, 1996) and invade (Dvorak and Crane, 1981) host cells to a greater extent in host cell S phase. The possibility that the host cell membrane is easier to penetrate in S phase may be a plausible explanation for this phenomenon. Certainly the microviscosity of host cell membranes was reported to be at a low level in S phase (De Laat *et al.*, 1977). There is also the possibility that surface receptors on the host cells are developmentally regulated and that one(s) important for parasite invasion are upregulated at S phase, allowing more parasites to attach and consequently invade. Yet when this experiment was performed with *E. tenella* invasion in synchronous COS (monkey kidney) cells there was no increase in invasion four hours into S phase, indicating that this phenomenon is not shared amongst mammalian cells, thus raising questions on the situation *in vivo*. However not all of the COS cell cycle was analysed. It may be that different mammalian cells are more susceptible to invasion at different times of their life cycle according to the parasite in question.

Invasion of MDBK cells by *E. tenella* induced the host cells to enter and remain in S phase as has been previously reported for *E. tenella* (Urquhart, 1981b). On average approximately 20% of MDBK cells were invaded with two sporozoites. Compared with the mean value of cells in S phase in the noninfected

culture there was approximately a 15% increase, possibly correlating with the number of cells invaded and thus induced into S phase. When the supernatant from infected cultures was added to non-infected cultures no increase in cells in S phase was observed. This disagrees with previous reports that stated a possible factor(s) may be released into the medium, either by the host cell or by the parasite, to induce non-infected cells to also enter S phase (Urquhart, 1981b). However, the incubation periods used in my studies were much shorter than those previously reported which may explain the discrepancy. The reason for this induction is not clear, it may be that, since the parasite finds it easier to enter the cells during S phase, it is in the parasites best interest to induce the host cells into this optimal invasion stage. Indeed during microscopic observation of the infected cultures, it was frequently noted that in an infected cell more than one parasite was present (as mentioned above). This may be due to the first parasite entering the cell (since invasion occurs throughout the host cell cycle) and inducing the host cell to enter S phase thus facilitating other sporozoite invasion. However, no difference was seen with *C. parvum*. This could be due to the low invasion rates in this cell line (approximately 6%), which would cause very few cells to enter S phase and be distinguishable from the non-infected culture.

The effect of *E. tenella* invasion also dramatically reduced proliferation of MDBK cells (see Table 6.3.1.). This could perhaps be attributed to cell death in the infected cultures. However, only 20% of the MDBK cells were infected, and even if these cells were killed, it still does not account for the extent of the reduction in proliferation of these cultures. This result is consistent with the findings that invasion induces the host cells to enter and remain in S phase, thus disrupting the cell cycle and inhibiting proliferation.

The results obtained from these studies provide more information on the invasion process by *C. parvum* sporozoites and *E. tenella* sporozoites and merozoites. Proteinases, polyamines and sialidase (for merozoites) all appear to play a role in this process, along with the influence of the host cell cycle.

# CHAPTER SEVEN

## GENERAL DISCUSSION

The general aims of the project were to study how the coccidian parasites of the *Cryptosporidium* species and *Eimeria tenella* are adapted to surviving and infecting the host, focusing on three aspects, in particular: excystation, penetration to and invasion of the host epithelial cells.

Excystation for *Cryptosporidium* was optimised before ensuing experiments were performed. The studies indicated that a number of factors may affect the excystation process, including the age of the oocysts used. As the age of the oocysts increased, the number of viable sporozoites released decreased. Consequently a decision was made to use oocysts up to a maximum age of 8 weeks after the initial date of preparation to minimise, and possibly eliminate, any influence that the age of the sporozoites will have on the experimental findings. Environmental factors were also seen to affect the excystation process. For *C. parvum,* anaerobic reducing conditions were seen to be optimal, along with a pH similar to that found in the *in vivo* situation for *C. parvum, C. muris* and *E. tenella.* This is in agreement with previous reports on *C. parvum* (Fayer and Leek, 1984; Sundermann *et al.,* 1987).

Studies performed on the sporozoite stage, at least for *Cryptosporidium parvum*, had to be limited to sixty minute incubations due to the very short survival time for the sporozoites of this species. Some of the inhibitors/compounds may not have been fully effective in this short time period and this must be taken into account when the results are examined. Similarly other researchers should beware of this problem of the short *in vitro* life of *C. parvum* sporozoites lest their studies are affected by this phenomenon. Additionally, motility is not an easily well defined criterion of viability for *Cryptosporidium* or *Eimeria* sporozoites, which has led to the necessity to develop a fast and non-subjective viability assay. This assay involved the use of the vital stains acridine orange and bis-benzimide which, when used in conjunction with a fluorescence microscope, enabled the researcher to distinguish easily between viable and non-viable sporozoites. The assay was then used to assess the effect of the various inhibitors/compounds, and to ensure that the variety of enzyme inhibitors used in the mucus penetration and host cell invasion assays were not toxic to the sporozoites themselves.

The metabolic inhibitors potassium cyanide, sodium azide and rotenone did not affect the viability of either *C. parvum* or *E. tenella* sporozoites, indicating that neither of these parasites requires an active respiratory chain in this life cycle stage. Indeed there is now good evidence that *C. parvum* sporozoites do not have a recognisable mitochondrion (see Appendix I) and the parasite is thought to be anaerobically adapted (Denton *et al.*, 1994, 1996a). It may be useful, however, to measure oxygen uptake by these parasites after reports on the findings of plastids

- containing extrachromosomal DNA - which .may themselves contain an electron transport system (Jeffries and Johnson, 1996). However the equipment required to measure the fluctuations in oxygen concentrations would have to be highly sensitive, and a large amount of parasite material would be required - an overriding factor when working with these parasites. It may also be interesting to investigate other stages of the parasites to see if the plastid is functional in these. An alternative approach would be possibly to inhibit the plastid electron transport chain and examine to what extent the parasite is affected. It has already been reported that certain herbicides - thought to inhibit the plastid respiratory components - have anticoccidial activity (Hackstein *et al.*, 1995).

Salicylhydroxamic acid (SHAM), an inhibitor of the alternative oxidase system known to occur in Trypanosomes (Opperdoes *et al.*, 1976), was used on *E. tenella* sporozoites. However no inhibition was seen.

*C. muris* viability was marginally reduced by potassium cyanide and sodium azide. Indeed a mitochondrion has been reported to be present in *C. muris* sporozoites (Uni *et al.*, 1987), though whether it is functional in this stage has not been ascertained. However the finding that these respiratory inhibitors only reduced the viability of the sporozoites to a certain degree, raises questions on how dependent these sporozoites are on a respiratory chain. It may be possible that the inhibitors are having an alternative effect on *C. muris* sporozoites, possibly damaging the surface in some way. Or perhaps the length of the sixty minute incubation is too short to reflect the true effect of the inhibitors.

Studies on the effect of pH indicated that the sporozoites of each species are adapted for surviving in the areas of the gastrointestinal tract that each infects *in vivo*. This was particularly evident for the gastric parasite *C. muris*, which was able to survive pH 2.0 - similar to that of the stomach. The conclusion drawn was that this species must have some structural or metabolic adaptation(s) enabling it to survive these extreme conditions. Further studies, possibly at the electron microscope level, may reveal ultrastructural differences, such as an extra membrane surrounding the sporozoite. The pH of the incubation fluid was tested before and after the incubation with the sporozoites, yet the method used is not sensitive enough to monitor small fluctuations, which as the studies reveal, increase survival greatly. The reason for this lack of sensitivity was simply due to the fact that the pH meters available could not be used on such small volumes of fluid.

Enzyme and proteinase studies were performed to a limited extent. Use of an enzyme kit (APIzym) indicated the presence of stage-specific enzymes (though non-quantifiably), possibly resulting from the switching on and off of certain genes throughout the life cycle. Biotinylated probes (as used before for *Fasciola hepatica*, McGinty *et al.*, 1993) and antibodies raised against *L. mexicana* cysteine proteinases were used to probe for proteinases in the coccidial lysates. Both cysteine proteinase and serine proteinase activities were detected, which is consistent with the results obtained from the mucus penetration and host cell invasion experiments in which proteinases were seen to play a role in both these processes. However these data do not allow us to pinpoint which enzymes are

involved in penetration and invasion, and a great deal more work is required, such as isolation and purification of the proteinases and enzyme assays. Observing the localisation of the proteinases was attempted using the biotinylated probes on live sporozoites with an alkaline phosphatase-fluoroscein isothiocyanate (FITC) conjugate. Yet there was no apparent difference when the sporozoites were preincubated with proteinase inhibitors, suggesting that the binding of the probe was non-specific to the live sporozoites. Alternatively it could possibly be due to the proteinase inhibitors not causing a large enough decrease in fluorescence for the naked eye to distinguish, and it may be useful to measure the fluorescence with an extremely sensitive device. Another way would be the use of specific antibodies raised against a variety of proteinases. These could be used to label live or fixed parasites and examine them under the microscope, and by employing immuno-gold particle or fluorescence try to localise the activities in the invasive stages.

Sialidase is an activity that has been previously reported in *E. tenella* merozoites and sporozoites (Pellegrin *et al.*, 1993). The results obtained indicated that sialidase was used by *C. parvum* sporozoites and *E. tenella* sporozoites and merozoites in mucus penetration (Chapter 5); and by *E. tenella* merozoites in host cell invasion (Chapter 6). Yet the attempt to detect sialidase using fluorogenic substrates was not successful. This may have been due to the limited amount of material available for the assay systems. Another method would be to try and detect this activity with the use of specific antibodies raised against the sialidase

of other organisms. But it is necessary to prove that the sialidase inhibitor used in these studies does inhibit the parasite enzyme and is not having some other effect.

The sialidase inhibitor inhibited *E. tenella* sporozoite mucus significantly, whereas the merozoite stage was not inhibited as greatly. This is consistent with the evidence suggesting that merozoites have 20-fold more sialidase than the sporozoite stage (Pellegrin *et al.*, 1993). The presence of this larger amount of sialidase indicated that it may be important for merozoite survival, and is possibly involved in the interactions with the environment. Merozoites have to invade, leave and reinvade epithelial cells, possibly coming into contact - if not having to traverse - the mucus layer, thus requiring more sialidase. Alternately the sialidase may be required internally - as is the case for *Trypansoma cruzi* (Schenkman and Eichinger, 1993). Indeed COS cell invasion by merozoites was seen to be inhibited by the sialidase inhibitor unlike sporozoite invasion of MDBK cells, indicating further roles for sialidase in the merozoite stage. However the difference in the host cell type used may influence the results obtained and, until work can be performed with the same host cell type, these results must be treated with caution.

The host cell cycle was mapped and, in terms of invasion, was more susceptible four hours into S phase for both *C. parvum* and *E. tenella*. This is consistent with results obtained using MDBK cells and *T. gondii* attachment which was seen to be optimal at five hours into S phase (Dvorak and Crane, 1981). Yet this feature is not shared amongst mammalian cells as was observed when using COS cells.

In conclusion these experiments have studied the mechanisms employed by *Cryptosporidium* and *Eimeria tenella* to survive, penetrate to and invade the host. Many problems were faced whilst working with these parasites, the primary one being the limited amount of parasite material available. The development of an *in vitro* cultivation system appears to be the solution to this lack of material, and research is continuing on this area in many laboratories. Once established, this will provide all the life cycle stages, allowing ultrastructural, biochemical and molecular comparisons between stages of each parasite, and between the different apicomplexans.

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# APPENDIX I: A 3-D RECONSTRUCTION OF *CRYPTOSPORIDIUM PARVUM*

## INTRODUCTION

Coccidian protozoan parasites - a subclass of the Apicomplexa - include organisms responsible for several important diseases of man and animals. Prevalence of these infections - due to drug resistance and other factors - is increasing, highlighting the economic and social implications. The coccidia include the gastrointestinal parasite *Cryptosporidium parvum,* which infects susceptible domestic stock and man. Water-borne cysts are ingested from which invasive sporozoites emerge and invade gut epithelial cells. The parasites proliferate causing primarily a diarrhoeal disease. In the immunocompetent humans, illness resolves within 3–20 days (Current and Garcia, 1991). However, cryptosporidiosis is a major and potentially life-threatening complication for AIDS patients and other immunocompromised individuals.

The process of cell invasion by the sporozoite is a dynamic event of much interest in cell biology. The sequential attachment and entry into the host cell involves recognition molecules as well as specifically timed secretion of the contents of discrete compartments from within the sporozoite. The structures thought to mediate attachment and invasion are collectively housed in the anterior region of the sporozoite and termed the apical complex (Jensen and Edgar, 1976; Joiner, 1991; Perkins, 1992; Jacobson and Doyle, 1996). To date no detailed description of the 3 dimensional structure of the infective stages of *C. parvum*

have been reported. This may be due to the difficulties in preserving integrity using conventional chemical fixation approaches, and the technical tedium of analysing multiple serial thin sections for 3D reconstructions. By using cryotechniques and EFTEM to image thick sections, unique images of this parasite have been generated, permitting new insights into the organisation of the cellular and macromolecular components involved.

## MATERIALS AND METHODS

*Parasites*

*C. parvum* oocysts were washed three times in RPMI (Gibco, 1000 x g for 10 min at 4°C) and pelleted. These were either processed whole, or excysted at 37°C using 0.4% taurodeoxycholic acid (Calbiochem), on a shaker (350 rotations $min^{-1}$) for 80 min after which the liberated sporozoites were pelleted by centrifugation (1000 x g for 10 min at 4°C). Concentrated parasites were then resuspended in 50 µl of medium and transfered immediately to carriers for cryofixation

*Cryopreparation*

Live parasites were impact frozen on a copper mirror using a Leica MM80 cryofixation system and then held at below -190°C under liquid nitrogen prior to transfer to a dewar-based cryo substitution system, where specimens were slowly dehydrated in 1% osmium tetroxide in acetone for 6 days at -85°C. After controlled overnight warm-up to 0°C (5°C/hr) and removal of cryosubstitution medium by exchange with fresh acetone, the retrieved specimens were infiltrated with increasing concentrations of Spurrs resin in acetone and finally polymerised at 60°C for 24 hours.

*Energy Filtering Electron Microscopy (EFTEM) and 3D Reconstruction*

0.5 μm or 0.15 μm thick sections were collected in ribbons of 12 onto carbon reinforced, Formvar-coated slot grids and stained in methanolic uranyl acetate for 15 min followed by lead citrate for 10 min to fully contrast the entire specimen thickness. Specimen contrast was also selected by EFTEM .
Image series were photographically recorded at 80kV in a Zeiss 902 EFTEM at 100 eV energy loss to optimise contrast for 0.5 μm sections and at 30 eV for 0.15 μm sections.

Colour coded acetate traces performed on prints were used to derive digitised reconstruction profile data which was input via bit pad using "3D-HVEM" PC software (developed at Univ of Boulder, Colorado, USA). Reconstructions were photographed from the monitor screen with an SLR camera using Fuji 400 daylight reversal film.

## RESULTS

The distribution of organelles in the sporozoite is depicted in a longitudinal section (see Fig.1), showing the characteristic conical shaped apical complex region and the posteriorly located nucleus of the invasive sporozoite. Between the nucleus and apical complex are found the various elements of the secretory machinery such as the small spherical micronemes and larger dense granules. Centrally located in the apical region is the single rhoptry. One or 2 large crystalline bodies - 2 in this sporozoite - are found to either side of, or posterior to, the nucleus. The spacing of the sub-unit structures in these organelles was 35 nm. In the non-dividing sporozoites, the nucleus exhibits peripheral condensed chromatin and a central nucleolus, and has distinct widely spaced nuclear membranes - the outer of which is ribosome-studded. On the anterior aspect of the nucleus the outer nuclear membrane extends into the cytoplasm, excluding ribosomes from this region. The plastid body is found anterior to the nucleus close to this region. No conventional Golgi apparatus or mitochondrion have been identified in cryoprocessed sporozoites (or in oocyst precursor stages).

The whole apical complex of *C. parvum* sporozoites stereo imaged in a 0.5 μm thick section revealed only a single rhoptry (Fig.2). Also seen in this view - though not common to all sporozoites examined - was a microporc structure which appeared to connect with the suface membrane just below the conoid. Serial 0.15 μm sections [see Fig.3 (a), (b) & (c)] through the same region as in

Fig.2, confirmed the existence of a single rhoptry, and showed details of the conoid, polar rings and the distribution of micronemes. Spherical dense granules are absent from this part of the sporozoite. The flask-shaped rhoptry consists of a bulb attached to the thin membrane at the tip of the conoid by a long tubular neck. A conspicuous differentiated region in the centre of the bulb was observed [Fig.3 (a), arrow] of paracrystalline appearance detailed in Fig. 3 (d).

Structures associated with the nuclear region include the tongue-like extension of the outer nuclear membrane (see Fig.1) found on the anterior aspect of the nucleus which may have a specialised as yet unknown function. Favourable sections show this structure to contain nuclear pores or similar membrane fenestrations [Fig. 4(a)]. Separate from the nucleus, but always closely apposed to it, lies a ribosome-studded 300 nm diameter spherical organelle interpreted as being a double membrane-bound plastid body, similar to that recently described in other Coccidian parasites [see Fig. 4(b)] (Köhler *et al.*, 1997). The plastid body appears to lie usually to the posterior of the nucleus - but may be anteriorly located and is present in encysted parasites [Fig 4(c)].

3D analysis of serial sectioned parasites [see Fig. 5 (a), (b)] shows the relative shape, size and distribution of organelles, and provides a means of estimating relative volume percentages. A sporozoite's internal organisation can be visualised by stereo viewing as in Fig. 5(a). A reconstruction of an 8 section series part is shown in Fig. 3 (a) - (c). Similarly, the organisation of nascent sporozoites within the thick-walled oocyst is seen stereoscopically in Fig. 5 (b).

To simplify visualisation, the upper profiles of the reconstruction, the considerable volume occupied by the oocyst residual body and the outline of individual sporozoites have been omitted. The alignment of sporozoites within the oocyst is seen with their anterior end pointing towards the region containing the suture (not shown) through which the parasites emerge (Reduker *et al.*, 1985).

The disposition of the 4 nascent sporozoites within the thick-walled sporulated oocyst is shown in Fig. 6(a). There are 4 profiles of individual sporozoites and the remainder of the central and peripheral area are occupied by the residual body from which the sporozoites arise. The cyst wall suture through which the sporozoites will eventually emerge is not visible in this section. Dense granules, micronemes, rhoptry and a nucleus are all clearly preserved and appear typical of organelles described for excysted sporozoites. The nature of the thick walled oocyst is visible in Fig. 6(b) and displays a regularly spaced triple layer structure in addition to an underlying diffuse granular layer.

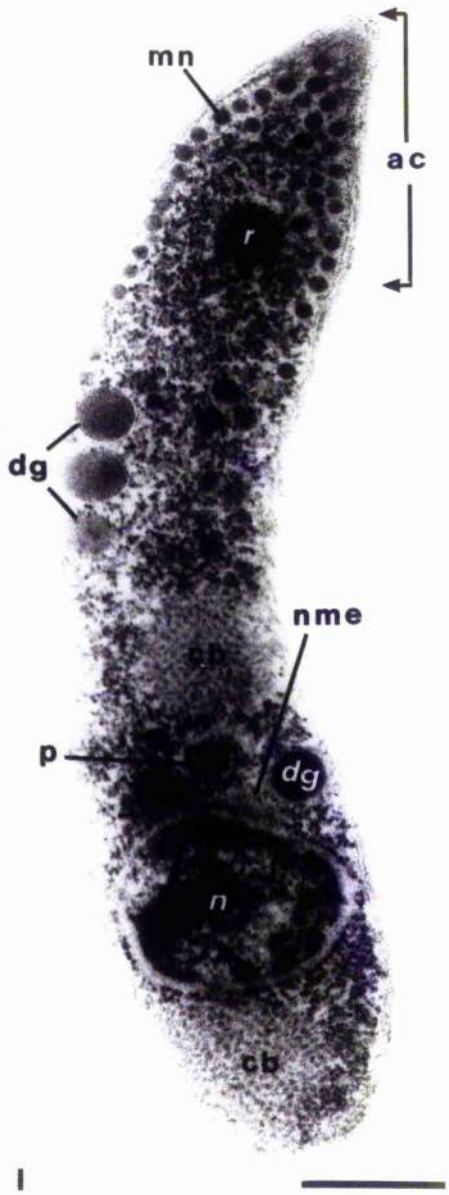



**Figure 1**: Longitudinal section (0.15 μm) through a sporozoite showing the distribution of internal organelles. The prominent apical complex (ac) containing the micronemes (mn) and rhoptry (r) are found at the tapering anterior of the cell with the nucleus (n) and adjacent crystalloid bodies (cb) at the posterior rounded end. Dense granules (dg) are found mostly in the centre portion of the cell. Visible also are the plastid (p) and extended nuclear membrane region (nme). Scale bar = 0.5 μm

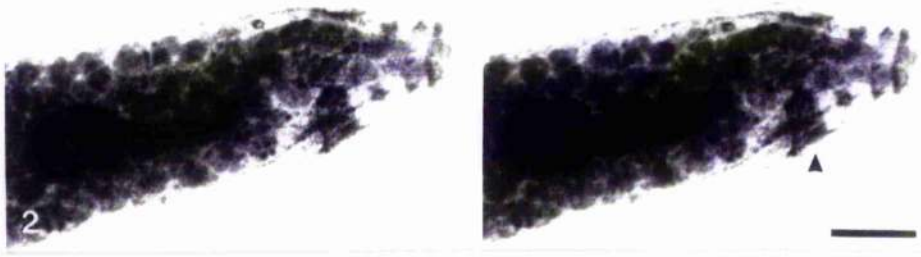


**Figure 2:** Stereo pair of 0.5 μm thick sections of the entire apical complex region of a sporozoite. The fused image reveals the single rhoptry, its tubular neck coursing through numerous micronemes to terminate at the apical complex tip. The neck of the rhoptry arches over a micropore (arrowhead) an invaginated specialisation of the cell membrane, thought to be a feeding structure.
Scale bar = 0.25 μm

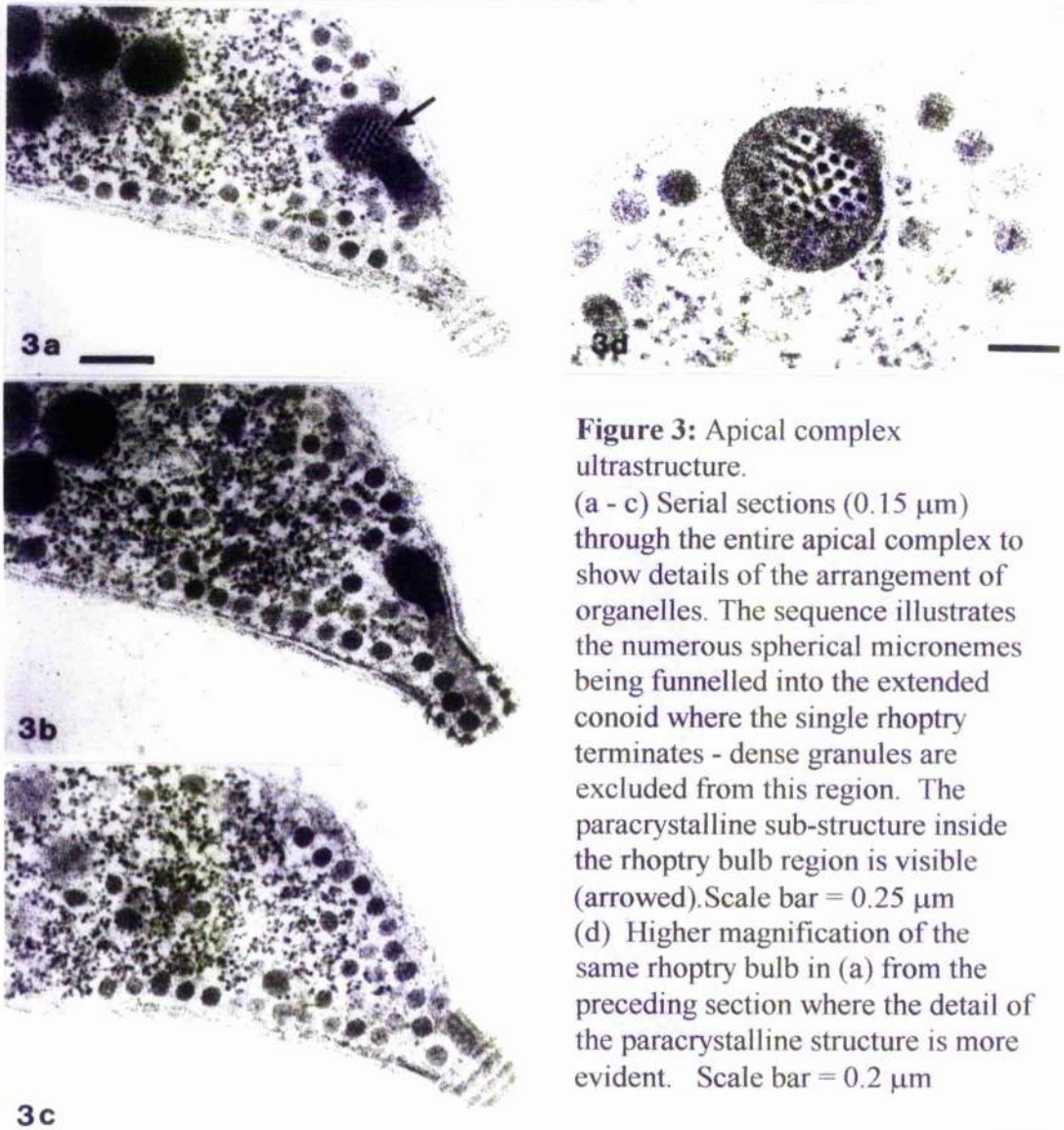
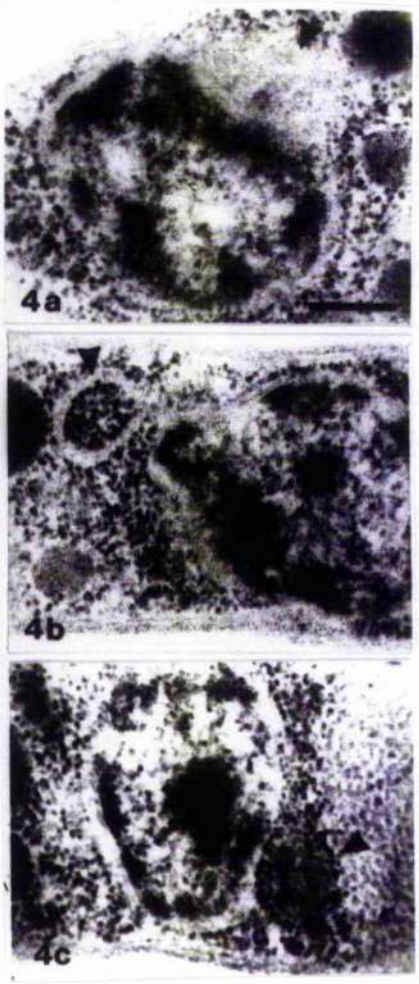


**Figure 3:** Apical complex ultrastructure.
(a - c) Serial sections (0.15 μm) through the entire apical complex to show details of the arrangement of organelles. The sequence illustrates the numerous spherical micronemes being funnelled into the extended conoid where the single rhoptry terminates - dense granules are excluded from this region. The paracrystalline sub-structure inside the rhoptry bulb region is visible (arrowed).Scale bar = 0.25 μm
(d) Higher magnification of the same rhoptry bulb in (a) from the preceding section where the detail of the paracrystalline structure is more evident. Scale bar = 0.2 μm

**Figure 4:** Nucleus-associated ultrastructure (0.15 μm sections)




(a) Specialisation of the outer nuclear membrane on the anterior aspect of the sporozoite nucleus. The membrane tongue-like extension (arrowhead) exhibits circular profiles resembling nuclear pores in glancing section. Scale bar = 0.25 μm

(b) Spheroidal membrane-bound plastid body (arrowhead) anterior to the sporozoite nucleus.

(c) Ribosome-studded nuclear associated organelle (arrowhead) which has been observed both anterior or posterior to the nucleus. This organelle of unknown function is seen in an encysted sporozoite closely apposed to the posterior aspect of the nucleus.

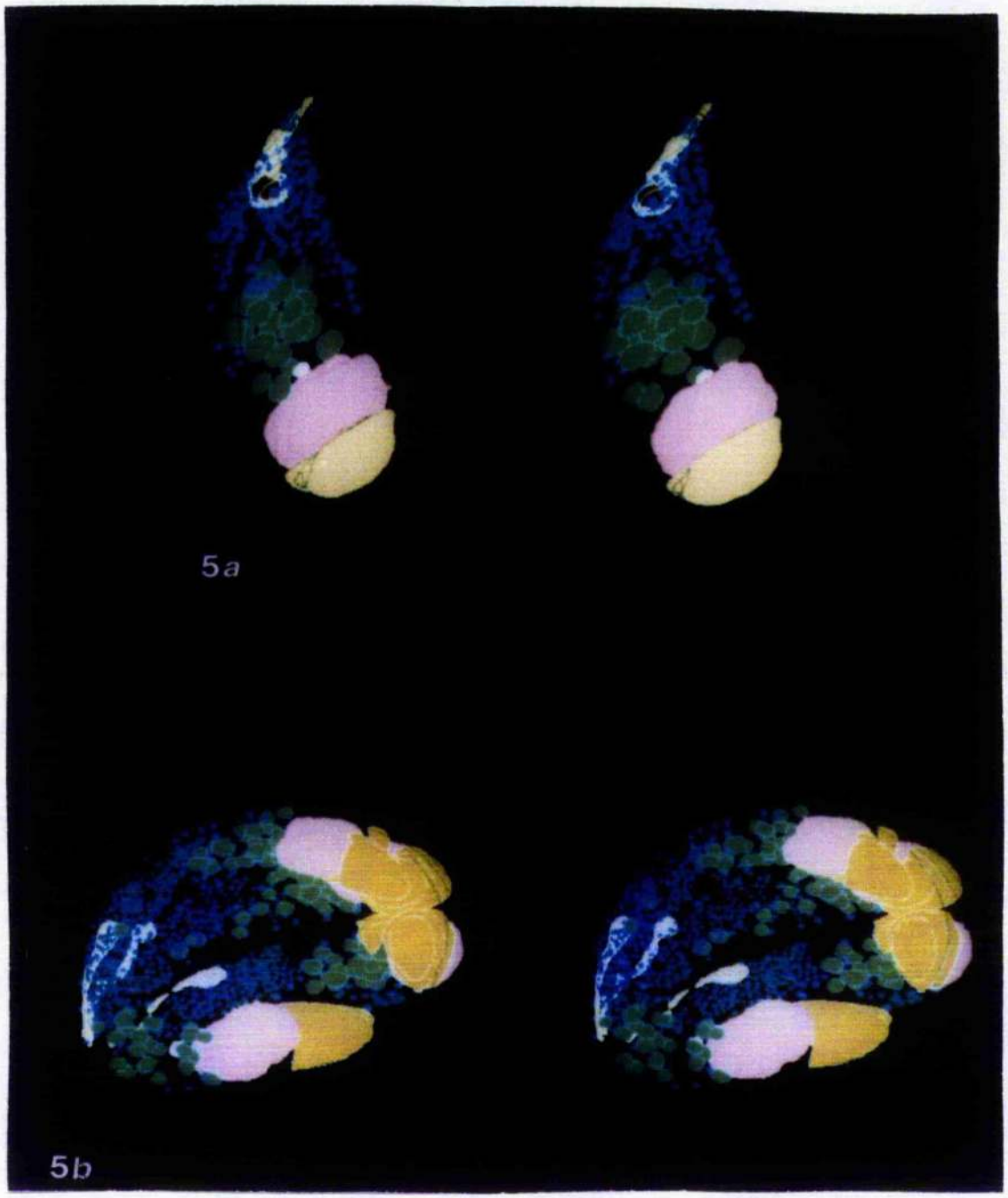



**Figure 5:** Stereo images of (a) reconstructed sporozoite and (b) partial reconstruction of an oocyst. Spherical dense granules, micronemes and the plastid structure are represented in the section plane occupied by their equatorial profile. Larger organelles are represented in all section planes in which they occur. Images are rotated 180° between views and elements in the display which would be hidden from view by the colour filling procedure are normally overwritten. Visualisation of the internal arrangement of the oocyst has been assisted removing the first 4 section planes.

Colour coding : cell boundary/oocyst wall - green; micronemes - blue; rhoptry - yellow; rhoptry sub-structure - red; dense granules - dark green; nucleus - pink; crystalloid body - orange; plastid - white.

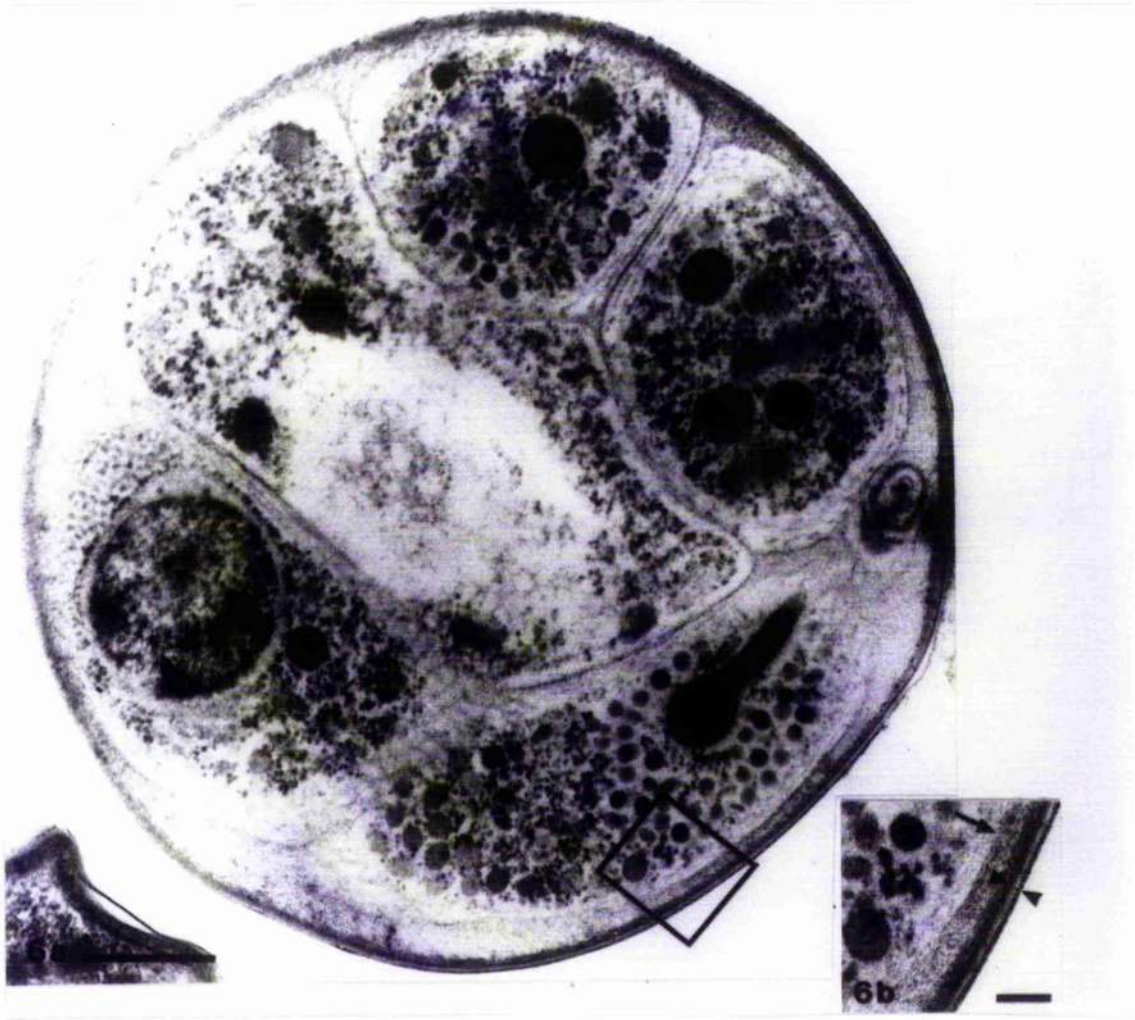



**Figure 6:**

(a) Equatorial section showing 4 sporozoite profiles and the residual body fully formed in the thick-walled oocyst. Integrity of the oocyst content is not compromised by the presence of a the thick wall structure when cryofixation is employed.
Scale bar = 0.5 μm

(b) Enlargement of a region of the oocyst wall showing three layer structure (arrowheads) underlying diffuse material (arrow). The intervening material between cyst wall and sporozoite fills all space unoccupied by cellular structure. Scale bar = 100 nm

## DISCUSSION

The rationale behind applying any form of cellular fixation must include the requirement to stabilise cellular constituents in as near to the natural state as possible. However, artefacts associated with chemical fixation, are now well documented (Lee *et al.*, 1982). Slow penetration of fixatives and continued activity of metabolic processes after their administration (Kellenberger *et al.*, 1992), are important considerations - especially in relation to resistant stages such as oocysts. Retention of the integrity of cellular architecture in labile and short lived stages such as sporozoites, may be compromised if chemically-fixed membranes remain osmotically active as is reported for other cells (Bowers & Masters, 1988). In order to avoid such problems the cryofixation route to specimen preparation was employed.

The *Cryptosporidium parvum* sporozoite is a motile, short lived organism whose function is to invade gut epithelial cells after emerging from the oocyst. A flexing movement normally visible by DIC interference microscopy presumably assists parasite invasion which is acheived by anterior end attachment to the host cell membrane, followed by a secretion process involving the organelles of the apical complex invasion (Jensen and Edgar, 1976; Joiner, 1991; Perkins, 1992; Jacobson and Doyle, 1996). The micropore (or micropyle) seen below the conoid in the stereo pair images is considered to be a feeding structure (Current, 1989) but was infrequently encountered in this study.

*Rhoptry*

Two to twenty elongated flask-shaped rhoptry structures are said to be found in motile apicomplexan stages (Scholtyseck, 1979) and 2 rhoptries are reported as being present in *C. parvum* merozoites (Current, 1989). Only one of these secretory structures has been demonstrated here by stereo thick section and 3D analysis in the *C parvum* sporozoite. The periodic structure within the single rhoptry bulb region gives an indication of a pentameric sub-unit arrangement and suggests an ordered lipid or lipoprotein nature judging by high contrast, stemming from an apparent affinity for osmium teroxide. In the rhoptries of other sporozoites or merozoites, processed by conventional aldehyde and osmium fixation at higher temperature (ie above 4°C), such organisational order is not observed. This is presumably due to the absence of solvent effects which may disorganise lipid arrays at higher temperatures.

*Micronemes*

A large number of micronemes (167 counted in one reconstructed sporozoite) - secretory granules - thought to be involved in penetration of the target cell membrane - occupied the anterior one third of the sporozoites. The few published micrographs of sporozoites of *Cryptosporidium spp* (Uni *et al.*, 1987; Lumb *et al.*, 1988) show few micronemes, suggesting premature discharge of these structures. The name "microneme" means "thread-like structure" and derives from past observations on other invasive stage apicomplexans where these organelles appear elongated (Scholtyseck, 1979). In this study, the micronemes are spherical, constant sized and regularly spaced within the cytoplasm, and line

up to the tip of the apical complex alongside the rhoptry neck. Some doubt must be expressed as to the reported shape of microneme structures in conventionally fixed preparations where an elongate appearance may be artefactual: it has been observed that micronemes isolated from *Eimeria* parasites when exposed to glutaraldehyde assume an elongate shape (Tomley, unpublished).

*Dense granules*

A population of spherical, 300 nm diameter, darkly stained granules were found in the region extending between nucleus and apical complex, varying somewhat in electron density. However, this may be a feature of variable stain penetration through the thick section or only partial inclusion of the entire granule. Sub-populations of dense granules although not morphologically distinguishable have been defined using immunoelectron microscopy by Bonnin et al (1995). The proteins localised in this specific dense granule sub-set were also found on the host cell parasitophorous vacuolar membrane confirming the view that dense granules are exocytosed during establishment of the infection.

*Crystalloid Bodies*

Prominent, nonmembrane-bound crystalloid bodies occurring either behind or to either side of the nucleus are a feature of the sporozoite, but are without defined function. Although possibly serving as storage or energy reserve sites in the cell. Nothing is known of their composition, yet the regularly spaced lattice appearance suggests a protein or lipoprotein crystalloid nature.

*Plastid body*

A single plastid - characterised by the double membrane spaced halo surrounding a ribosome-filled spheroid - is identified here in *C.parvum*. The size and position of this structure fits the description of other plastid bodies which have been extensively studied in *Plasmodium* (Williamson *et al.*, 1994; Wilson, 1997) and *Toxoplasma gondii* (Köhler *et al.*, 1997). The juxtanuclear site facilitating partitioning during division of the organelle thought to accompany parasite nuclear division. The discovery and intense interest in these plant-like organelles (as indicated from DNA sequencing), stems from the observations made on the parasite sensitivity to dinitroaniline herbicides (Hackstein *et al.*, 1995) whose effect is thought to be inhibiting components of the respiratory chain encoded by the plastid. Thus a therapeutic approach based on differential sensitivity to herbicide derivatives may be expected to show promise.
The plastid may contain an electron transport chain essential to sporozoite metabolism and may tie in with the lack of an identifiable mitochondrion in this stage .

This report represents the first study on the 3 dimensional organisation of the infective sporozoite stage of *C. parvum*. A single rhoptry was found to be present in this stage of the parasite; and evidence of a plastid. Future research to compare other life cycle stages of this parasite; and different species of *Cryptosporidium* would be of great interest.