The mannitol cycle in *Eimeria*

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SUMMARY

A metabolic pathway known as the mannitol cycle has been identified in Eimerian parasites. The pathway is a shunt off of the glycolytic pathway at fructose-6-phosphate (F6P). Two enzymes convert F6P to mannitol and two other enzymes are responsible for converting mannitol back to F6P when it is utilized. Although the pathway is present in various stages of the parasite the most apparent role of this pathway is in the sexual portion of the life cycle, particularly in the formation of oocysts. Extremely high concentrations of mannitol, approaching 0.3 M, are present in unsporulated oocysts. Mannitol functions as the endogenous energy source for oocysts to sporulate in the environment outside of the host. An inhibitory protein which inactivates the first enzyme of the mannitol cycle has been isolated from an oocyst derived inhibited enzyme complex and is believed to prevent the futile cycling of F6P during the maturation of oocysts. Evidence of the vital role of mannitol in the development and maturation of *Eimeria tenella* oocysts has been facilitated through the use of the drug Nitrophenide™, a known anticoccidial which has now been found to be an inhibitor of one of the enzymes responsible for the biosynthesis of mannitol in the parasite. This compound prevents the formation of oocysts and at lower doses reduces mannitol levels in shed oocysts. In addition, oocysts with reduced mannitol levels fail to complete the sporulation process lending further evidence for this polyol's role in the parasite.

INTRODUCTION

Protozoan parasites of the genus *Eimeria* are the causative agents of coccidiosis, an intestinal disease which occurs in a large number of domesticated and wild animals. By far the most economically important eimerian infections occur in broiler chickens. Coccidiosis outbreaks in poultry houses spread very rapidly and cause severe morbidity and mortality throughout the infected flock. Eradication of coccidian parasites in a poultry operation is currently not achievable and as a consequence all major poultry producers use anticoccidial agents in the feed to avoid the economic risks of a coccidiosis outbreak. Because of the consistent use of these prophylactic agents, the high density housing of broilers and the persistence of infectious organisms, resistance to coccidiostats is an ongoing problem. No major new class of anticoccidial agent has been introduced since the discovery of the potent activity of ionophores in the early 1970s and, as a consequence, ionophores have been the mainstay of coccidiosis control since then. However, ionophore resistance is becoming widespread and a new anticoccidial agent with a novel mechanism of action is needed to overcome this growing resistance problem. Currently, poultry producers are using shuttle approaches in which they sequentially treat with a variety of anticoccidial agents. This has had some success, but is also beginning to produce multidrug-resistant parasite populations.

The ability of eimerian parasites to survive in the poultry house for long periods of time under quite adverse conditions is attributable to the oocyst stage of the parasite's life cycle. The oocyst is responsible for the spread of the infection to new hosts and has an impervious outer wall to protect it in the outside environment. The resilience of this stage of the parasite is demonstrated by the methodology used to prepare oocysts for laboratory studies. Oocysts present in infected tissue and faecal material can be purified by digestion with pepsin at pH 2·0 and surface sterilization with 5·25% sodium hypochlorite (Clorox) to eliminate bacterial/fungal contamination (Jackson, 1964). Following this treatment, the parasites within the oocyst can remain viable for years if protected from desiccation.

The most widely studied eimerian parasite of poultry is *Eimeria tenella*. An *E. tenella* infection is initiated when mature (sporulated) oocysts contaminating the litter are ingested by a chicken (Fig. 1). The oocyst wall is ruptured by the grinding action of the gizzard and 4 sporocysts are released from each fractured oocyst. As these sporocysts migrate down the small intestine, proteolytic enzymes and bile salts stimulate the release of 2 sporozoites from each sporocyst. These sporozoites migrate to the caecal pouches in the chicken and specifically infect epithelial cells lining this region of the gut. The intracellular sporozoites go through a process known as merogony producing approximately 100 new organisms per host cell infection. These parasites, known as merozoites, infect other caecal epithelial cells and undergo another multiplication phase, known as second generation merogony, which produces another 60–70 progeny per host cell. This second round of asexual replication is responsible for most of the intestinal damage seen from an *E. tenella*

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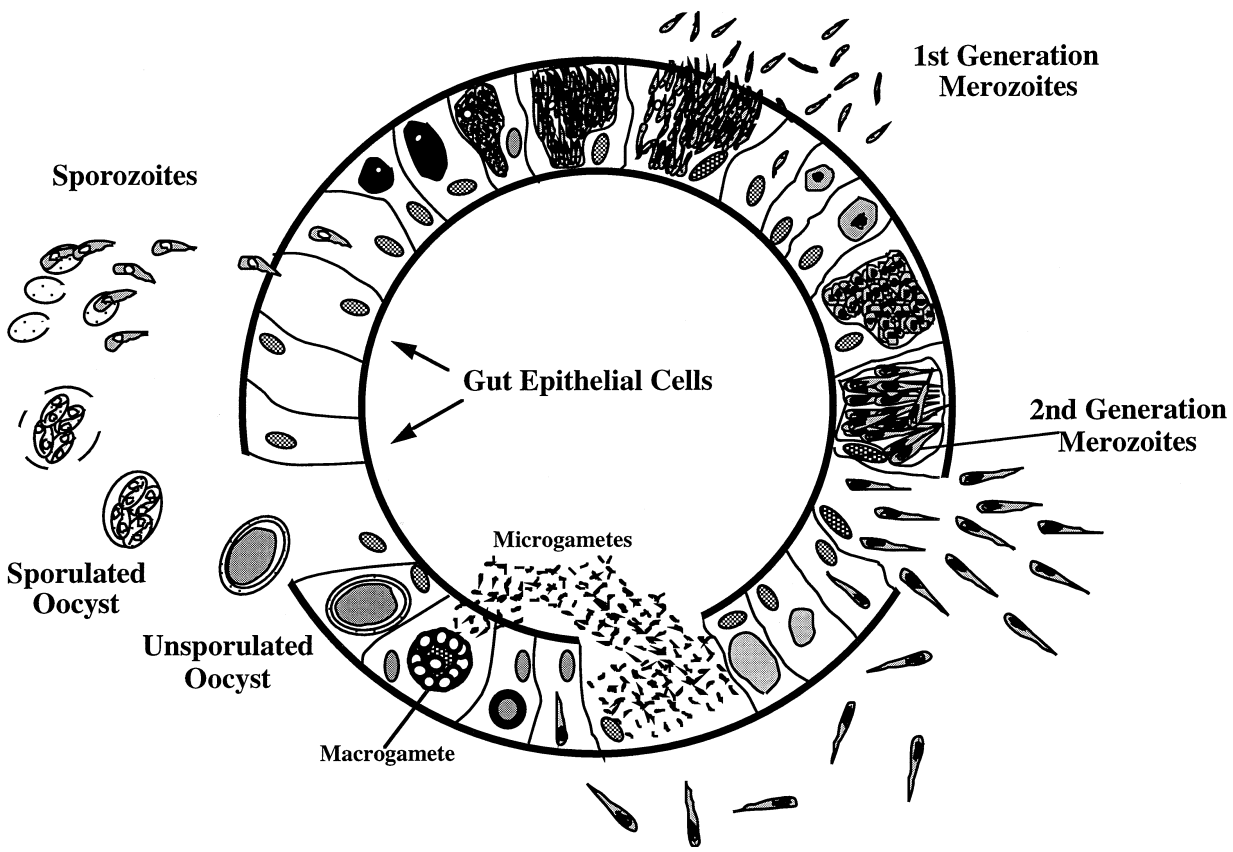


Fig. 1. Life Cycle of *Eimeria tenella* in the chicken. A coccidial infection is initiated by the ingestion of sporulated oocysts from contaminated litter in the poultry house. The outer wall of the oocyst is cracked by the grinding action of the chicken's gizzard and 4 sporocysts are released, each of which contains 2 sporozoites. As the sporocysts migrate down the intestines of the host, proteolytic enzymes and bile salts digest the stiedia body at one end of the sporocyst and sporozoites become activated and emerge through this opening. The sporozoites penetrate epithelial cells lining the gut and go through a process known as merogony which yields approximately 100 new parasites per infected host cell. The parasites, known as merozoites, infect other epithelial cells and go through another round of merogony to yield another 60–70 parasites per infected host cell. The second generation merozoites infect epithelial cells to initiate the sexual cycle of the parasite. The infections with second generation merozoites produce either a large number of microgametes which emerge from their host cell or a macrogamont which remains in the host cell. Microgametocytes will penetrate the host cells containing macrogamonts and initiate fertilization. The resultant zygote will then go through a maturation process to yield an unsporulated oocyst. The unsporulated oocysts are released from the dying host cell and are passed out of the gut through the faeces. Once outside the host, the oocyst will sporulate and initiate another new life cycle when it is ingested by a new host.

infection. The second generation merozoites infect additional epithelial cells and generate either several hundred microgametes (male form) or a single intracellular macrogamete (female form). The microgametes are released from infected host cells and enter host cells containing macrogametes. Fertilized macrogametes mature into unsporulated oocysts which are released from the degenerated host cells and shed in the faeces. These oocysts must undergo sporulation in the external environment before they are infectious to a new host, a process which takes approximately 2 days. There are 6 economically important species of *Eimeria* that infect chickens. All of these are very host specific, infect distinct regions of the gut, and have a similar life cycle to *E. tenella*.

Other than oxygen and water, unsporulated oocysts do not appear to require any exogenous nutrients to sporulate. As a result, they are entirely dependent on endogenous sources of energy for sporulation and to remain viable. Early studies monitoring total carbohydrate levels in oocysts found that the total glucose level in oocysts was maintained throughout sporulation and it was theorized that the carbon/energy source for this process must come from lipids or some other unidentified source in the 25% of the oocyst mass that was uncharacterized (Wilson & Fairbairn, 1961). It is now known that the source of this energy is the alcohol sugar mannitol (Schmatz *et al*. 1988) which went undetected in the earlier characterization studies. Approximately 25% of the

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total dry weight of an unsporulated *E. tenella* oocyst is mannitol with concentrations as high as 0·3 M. The role of mannitol as a major carbon source is apparent from the greater than 90% reduction in total mannitol concentration in an oocyst by the completion of sporulation. Amylopectin, a branched polysaccharide found as granules in almost all stages of the parasite, had previously been thought to be the only major carbohydrate reserve in oocysts (Vetterling & Doran, 1969). However, the consumption of amylopectin during the early stages of sporulation is followed by re-synthesis of new amylopectin granules in the newly formed sporozoites and it was never clear how the net consumed amylopectin could support the sporulation process.

MANNITOL METABOLISM

The metabolic pathway in *E. tenella* that is responsible for the biosynthesis and utilization of mannitol has been identified (Schmatz, Baginsky & Turner, 1989) (Fig. 2) and is similar to a pathway originally identified in fungi, known as the mannitol cycle (Hult & Gatenbeck, 1978). The role of mannitol in fungi is unclear but it has been speculated to be an osmoregulator (Touster & Shaw, 1962; Brown & Simpson, 1972), an intermediate in sugar metabolism (Manners, MacLean & Scott, 1984), a carbon energy reserve (Horikoshi, Iida & Ikeda, 1965) and a scavenger of free radicals (Blumenthal, 1976). Recent studies by Chaturvedi *et al*. (1996) suggest that mannitol is important to the pathogenicity of the fungus *Cryptococcus neoformans* in mice by aiding in the tolerance to heat and osmotic stress. The mannitol cycle in coccidian parasites is an attractive drug target since higher eukaryotic hosts do not biosynthesize or catabolize mannitol. The pathway is present in the 6 major species of *Eimeria* which infect chickens and is also present in *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts (Schmatz, 1989). In *E. tenella*, fructose-6-phosphate (F6P) from the glycolytic pathway is shunted into the mannitol cycle (Fig. 2) by mannitol-1-phosphate dehydrogenase (M1PDH, 1). The resulting mannitol-1-phosphate (M1P) is subsequently converted to mannitol by mannitol-1-phosphatase (M1Pase, 2). These first two enzymes are responsible for the biosynthesis of the mannitol which accumulates during parasite oogenesis in the gut tissue of the host. Once oocysts are shed into the outside environment, the reduced temperature and the presence of oxygen result in the initiation of sporulation and the utilization of the mannitol stores. The consumption of mannitol is initiated by the enzyme mannitol dehydrogenase (MDH, 3) which converts mannitol to fructose and a hexokinase (4) subsequently converts fructose into F6P.

One distinct difference between the mannitol cycle in *E. tenella* and the fungal pathway is the co-enzyme requirements for the dehydrogenases. In the fungal system, M1PDH requires NADH and MDH can utilize either NAD or NADP. In the eimerian pathway, M1PDH can utilize either NADH or NADPH, and MDH can only utilize NAD. Since one of the major functions of the mannitol cycle in fungi, in addition to energy storage, is thought to be the net conversion of NADH to NADPH to support the biosynthesis of fungal metabolites (Ramstedt *et al*. 1986), there appears to be a distinct difference in the role of this pathway in the coccidia. The fact that only NADH is generated by mannitol catabolism in *Eimeria* indicates that the parasite may be using mannitol catabolism exclusively for the generation of ATP. Some bacteria also have the mannitol cycle enzymes M1PDH and MDH but do not appear to have M1Pase (Marmur & Hotchkiss, 1955; Wolff & Kaplan, 1956). Instead they have a membrane associated phosphophenolpyruvate-dependent mannitol phosphotransferase and appear to use mannitol as a carbon source rather than as a storage polyol (Dills *et al*. 1980).

When mannitol is converted back to F6P in *E. tenella* theoretically it can either enter glycolysis or gluconeogenesis, or re-enter the mannitol cycle through M1PDH (Fig. 2). Utilization of F6P to generate ATP is probably occurring throughout sporulation and is achieved by the initial conversion of F6P to fructose-1,6-biphosphate by a unique pyrophosphate-dependent phosphofructokinase (Denton, Thong & Coombs, 1994; Dulski, Schmatz & Gurnett, 1997). Gluconeogenesis is likely to occur later in the sporulation process since amylopectin granules are found in all of the newly formed sporozoites. However, re-entry of the F6P into the mannitol cycle is not an economical route for the parasite's metabolism and would result in a futile cycle. Enzymological studies now indicate that a biochemical mechanism is probably in place to prevent this occurrence (Myers *et al*. 1997).

REGULATION OF MANNITOL METABOLISM

When M1PDH is purified from unsporulated oocysts it is as a protein complex having an unusual, asymmetric quaternary structure composed of 2 proteins, a 67 kDa catalytic protein and a homodimer of 35 kDa subunits which is regulatory and inhibitory (Myers *et al*. 1997). Activation of M1PDH can be achieved by dissociation of the complex with polybasic polypeptides (e.g. polyLys, polyArg, protamine and histones), 1·0 M urea, or shifts in pH to either < 5 or $> 9{\cdot}5$, suggesting that the 67/35 kDa interaction is largely ionic. Protamine, among other activators, stimulates M1PDH activity about 14-fold after a pronounced lag phase (of 5–7 min). This stimulation can also be achieved using rabbit polyclonal antibody raised against the native 35 kDa inhibitory protein (Myers *et al*. 1997). The fact that

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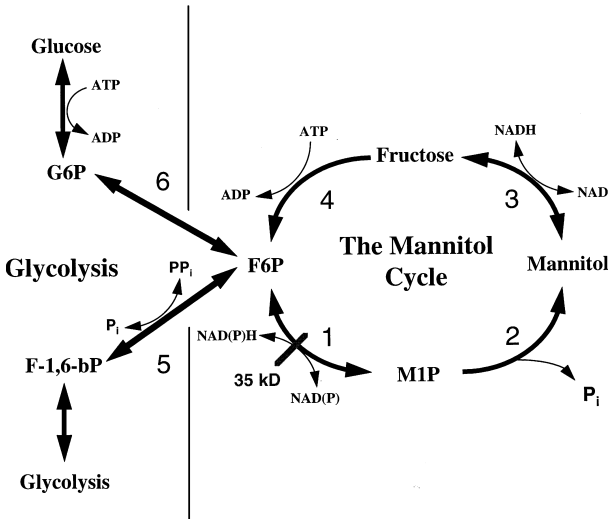


Fig. 2. The mannitol cycle in *Eimeria tenella*. The mannitol cycle is a shunt off of the glycolytic pathway in *Eimeria*. The first committed step of the pathway is mannitol-1-phosphate dehydrogenase (M1PDH, 1) which converts fructose-6-phosphate (F6P) to mannitol-1-phosphate (M1P). This enzyme appears to be regulated by a 35 kDa 14-3-3 protein that exists as a dimer. Mannitol-1-phosphate is converted to mannitol by a unidirectional mannitol-1-phosphatase (M1P, 2). Mannitol appears to accumulate during oogenesis and to be consumed during maturation (sporulation) of the oocysts. During the consumption period, mannitol is converted to fructose by mannitol dehydrogenase (MDH, 3) and then to fructose-6-phosphate (F6P) by a hexokinase (4). Based on the above schematic, there are at least three ways in which F6P can be utilized. It can continue down the glycolytic pathway via a unique pyrophosphate (PPi)-dependent phosphofructokinase (5) to generate ATP, go toward the generation of glucose-6-phosphate (G6P) and ultimately new amylopectin via a phosphoglucoisomerase (6) or re-enter the mannitol cycle via M1PDH (1). It is known that the 14.3.3/M1PDH complex is inactive, and it is believed that this complex forms at the completion of mannitol biosynthesis and prior to the consumption of mannitol at sporulation to prevent futile cycling of F6P through the mannitol cycle. Other abbreviations: F-1,6-bP, fructose-1,6-bisphosphate.

M1PDH is present as an inhibited complex in unsporulated oocysts suggests that the biosynthesis of mannitol is inhibited during this stage of the parasite's life cycle. This seems rational since the mannitol for sporulation is already present in shed oocysts and mannitol is consumed, not synthesized, during sporulation. One hypothesis is that the 67 kDa catalytic protein is uncomplexed and active during the sexual cycle of the parasite in the gut and that the inhibited complex with the 35 kDa dimer forms at the completion of oogenesis to prevent the futile cycling of mannitol during sporulation. There is currently no evidence, however, that the inhibited complex dissociates back to the active form of the enzyme during the parasite's life cycle and at present an endogenous activation mechanism for the complex has not been identified.

Attempts to show that the 67 kDa protein and the 35 kDa homodimer are co-localized in the unsporulated oocyst have been hindered by the inability to conduct effective electron microscopic (EM) studies on oocysts since they are impermeable to the fixatives used for this process. However, immature intracellular oocysts can be examined in intestinal tissue from infected chickens. Immunogold EM studies using antibodies generated to *E. tenella* M1PDH and the 35 kDa protein have been conducted on immature oocysts and it has been found that M1PDH appears most abundant in the amylopectin granules of this stage (Myers *et al.* 1997). Under the same conditions, minimal amounts of the 35 kDa protein can be seen. Serial sections of a few mature oocysts that have been successfully fixed for immunogold EM show the presence of both the 67 and 35 kDa proteins in amylopectin granules. The co-localization of these two proteins can be confirmed by examining intracellular sporozoites in tissue culture where the catalytic sub-unit of M1PDH appears to be abundant in amylopectin granules and the inhibitory 35 kDa subunit is present both in, and around, these granules (R. Myers *et al.* unpublished).

Both the 67 kDa and 35 kDa proteins from *E. tenella* have been purified to homogeneity, cloned and placed into various expression systems. While the functional expression of the 67 kDa protein has been very limited in *Escherichia coli* and COS cells, robust functional expression of the 67 kDa protein has recently been achieved in a baculovirus system (Liberator *et al.* 1997*a*). Efficient expression of the recombinant 35 kDa inhibitory polypeptide has been achieved in *E. coli* (Liberator *et al.* 1997*b*). Purified recombinant 35 kDa protein forms an approximately 72 kDa homodimer (as determined by gel filtration) and potently inhibits native M1PDH in a dose-dependent, saturable manner (IC_{50}: 300 pM) (Myers *et al.* 1997). Based on sequence homology, the 35 kDa inhibitory protein appears to be a member of a family of eukaryotic proteins known as the 14-3-3 proteins (Liberator *et al.* 1997*a*, *b*, Myers *et al.* 1997). These proteins are highly conserved in eukaryotes and, like the *Eimeria* protein, exist as homodimers having a subunit molecular mass of approximately 30 kDa and a pI of around 5·0 (Aitken, 1996). The *Eimeria* 14-3-3 protein has the greatest sequence homology to that of the protozoan parasite *Neospora caninum* (78% identity, 87% similarity) (Liberator *et al.* 1997*a*, *b*). Several reports in the literature have demonstrated that distinct 14-3-3 isoforms are involved in signal transduction, regulation of transcription, and cell cycle control in eukaryotic cells (Aitken, 1996). A 14-3-3 protein is thought to have a role in the Raf-Ras mitogenic pathway in mammalian cells by binding to Raf in a

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bivalent fashion at both the N and C termini. Upon Ras-mediated activation of Raf, the 14-3-3 protein appears to be displaced from the N-terminus while the C-terminal interaction remains intact. The fact that the 14-3-3 is still associated with Raf at the C-terminus may facilitate a rapid reversion to the bivalent inactive state (Bruder, Heidecker & Rapp, 1992; Li *et al*. 1995; Rommel *et al*. 1995). In yeast, 14-3-3 protein knockouts affect mitosis, and a double knockout of the two known isoforms is lethal (Ford *et al*. 1994). There have also been reports of enzyme inhibition by 14-3-3 proteins, including a plant nitrate reductase (Moorhead *et al*. 1996). Unlike most eukaryotes which have several isoforms, it appears that a single 14-3-3 isoform is present in *E. tenella* and it is therefore possible that this protein may serve multiple functions (Liberator *et al*. 1997*a*, *b*).

To explore the interaction of the *E. tenella* 14-3-3 protein and its target protein(s), experiments have been conducted using fluorescent-labeled recombinant *E. tenella* 14-3-3 protein (ETr35) and cryosections of *E. tenella*-infected gut tissue (J. Allocco *et al*. unpublished). The ETr35 protein was found to bind specifically to macrogamonts and developing oocysts, but not host tissue. The same was seen when the fluorescent-tagged Etr35 protein was incubated with sections of *T. gondii*-infected cat gut, where it specifically bound to developing oocysts. These findings show that despite the high level of homology between eukaryotic 14-3-3 proteins, variants of this protein can be highly specific for their targets. Studies focused on identifying 14-3-3 target proteins in the parasite may provide additional insight into cellular regulation. It is also interesting to note that when recombinant *E. tenella* M1PDH was expressed in invertebrate Sf9 cells using a baculovirus system, the expressed protein when isolated was associated with a 14-3-3 protein from the Sf9 host cells (Liberator *et al*. 1997*a*, *b*).

The enzyme responsible for converting mannitol-1-phosphate to mannitol is mannitol-1-phosphatase (M1Pase). M1Pase is irreversible, very substrate-specific and does not appear to require a metal or any co-factors. The enzyme is also unaffected by mannitol levels and has a high specific activity for mannitol-1-phosphate (M1P) (approximately 700 units/mg protein). *E. tenella* M1Pase has been purified to homogeneity and is a monomeric 34·7 kDa protein (Feiglin *et al*. 1997). To date, M1Pase has not been purified from any other source. Functional expression of cloned *E. tenella* M1Pase has been accomplished in *E. coli* and the enzyme kinetics of the cloned protein are indistinguishable from the native protein (Anderson *et al*. 1997). Biochemical studies (Feiglin *et al*. 1997) suggest that M1Pase is a member of histidine phosphatase family of proteins which have a unique reaction mechanism in which a phosphohistidinyl-enzyme intermediate is formed (Van Etten, 1982; Bazan, Fletterick & Pilkis, 1989). Members of this groups of enzymes include phosphoglycerate mutase (PGM), acid phosphatase and the bifunctional enzyme 6-phosphofructo-2-kinase/fructoase-2,6-bisphosphatase (F2,6BP). All of these enzymes have a characteristic group of active site amino acid residues including a signature RHG sequence (Bazan *et al*. 1989). Although it has very limited homology to the other enzymes in this group, the deduced amino acid sequence of *E. tenella* M1Pase contains the signature RHG sequence and other key residues for this class of enzymes (Anderson *et al*. 1997; Liberator *et al*. 1997*b*). The reaction mechanisms for both yeast PGM and F2,6BP have been extensively studied by enzymological studies and site-directed mutagenesis (Lin *et al*. 1992; Ostanin *et al*. 1992, 1993, 1994). More recently, the theoretical catalytic mechanism has been reinforced by solving crystal structures (Fothergill-Gilmore & Watson, 1990; Schneider, Lindqvist & Vihko, 1993; Lee *et al*. 1996). Site directed mutagenesis of these residues in *E. tenella* M1Pase and the establishment of a crystal structure should lend further evidence to support the relationship between these enzymes.

The enzymes responsible for mannitol catabolism in *Eimeria* have not been as extensively studied. Mannitol dehydrogenase (MDH) has a much higher apparent K_m for mannitol (15·4 mM) than M1PDH (0·3 mM for F6P) and M1Pase (0·2 mM for M1P) have for their substrates which may account for the accumulation of mannitol. MDH has a K_m of $>$ 0·3 M for fructose under a variety of conditions, suggesting that the reduction of fructose to mannitol is not likely to occur (Schmatz *et al*. 1989). This, in addition to the fact that M1Pase is irreversible and a mannitol kinase has not been found in *E. tenella*, demonstrates that the mannitol cycle is likely to be unidirectional. It is also interesting to note that M1PDH and M1Pase, the enzymes required for the mannitol biosynthesis, are highly active at 41 °C, the chicken's body temperature, while having minimal activity at 25 °C, the temperature at which oocysts sporulate. In contrast, MDH and the hexokinase are substantially more active at 25 °C than the mannitol-producing enzymes (Schmatz *et al*. 1989). This correlates well with the proposed role of the enzymes, where mannitol is biosynthesized within the host (41 °C) and catabolized outside the host at ambient temperatures (25 °C).

While the first three enzymes of the *E. tenella* mannitol cycle are highly substrate-specific, the fourth enzyme, a hexokinase, is capable of phosphorylating both fructose or glucose with the same efficiency (K_m of 0·5 mM) and combination experiments with both substrates suggests that there is only one type of glucose/fructose hexokinase present in oocyst extracts (Schmatz *et al*. 1989). Therefore the enzyme is likely to also be the hexokinase in the

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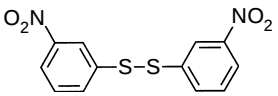


Fig. 3. Structure of 3-nitrophenyl disulphide (nitrophenide, Megasul™). A compound which was sold as a coccidiostat in the 1950s was known to effect the sporulation of *Eimeria* oocysts from treated birds and to have minimal effects on the early part of the parasites life cycle in the chicken. Studies now show that this compound is an active site-directed, irreversible inhibitor of *E. tenella* mannitol-1-phosphate dehydrogenase (M1PDH).

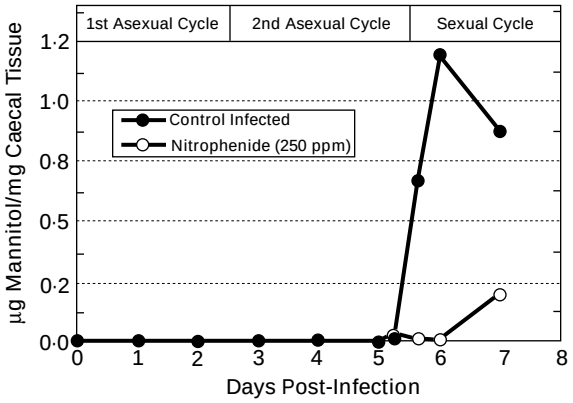


Fig. 4. Mannitol levels in the caeca of nitrophenide-treated and untreated infected chickens. Mannitol levels in the caecal tissue of *Eimeria tenella* infected chickens, in the presence or absence of nitrophenide, was determined by capillary GLC analysis of infected gut extracts. Mannitol biosynthesis was apparent after day 5 of the parasite's life cycle. It has been found to be a highly regulated process that appears to be linked to oogenesis. Nitrophenide inhibits the biosynthesis of mannitol when at 250 ppm in the feed.

glycolytic pathway that converts glucose to glucose-6-phosphate.

Although the mannitol cycle may be functional throughout the life cycle of the parasite and there is evidence of mannitol biosynthesis in free sporozoites (Michalski, Edgar & Prowse, 1992), clearly the most apparent role of this pathway is in the formation and sporulation of oocysts. Since the sexual cycle of *E. tenella* cannot be efficiently reproduced in cell culture, infected gut tissue has been used to explore the regulation of mannitol biosynthesis during oogenesis. By using GLC analysis of extracted, infected tissue and a quantitative DNA probe method for measuring parasite load it has been found that the bulk of mannitol biosynthesis occurs during the sexual cycle, reaching levels in the range of 1–2 μg mannitol per mg of infected caecal tissue (Allocco *et al*. 1997). This increase in mannitol is not occurring as a result of increased parasite load in the gut and the production of mannitol appears to be a highly regulated process, which is probably initiated in macrogamonts at fertilization.

MANNITOL METABOLISM AS A DRUG TARGET

A clearer understanding of the role of the mannitol cycle has been established as a result of the discovery that 3-nitrophenyl disulphide (nitrophenide, Megasul™, Fig. 3), a coccidiostat sold in the 1950s (Waletzky, Hughes & Brandt, 1949; Peterson & Hymas 1950), is an active site-directed, irreversible inhibitor of *E. tenella* M1PDH (IC_{50} 2·5 μM) (Allocco *et al*. 1997). Although this compound is not very potent and may not be entirely selective for M1PDH, it has proven to be useful in looking at the effects of inhibiting M1PDH *in vivo* and the impact of reducing mannitol biosynthesis on oocyst maturation. When *E. tenella*-infected chickens are treated continuously with 500 ppm of nitrophenide in the feed, oocyst shedding is reduced by more than 99 %, and those oocysts which are shed are incapable of complete sporulation. When the dose of nitrophenide is reduced, the level of mannitol in shed oocysts is inversely proportional to the dose of nitrophenide. Also, the levels of mannitol in oocysts collected from nitrophenide-treated birds correlates directly with their ability to sporulate, confirming that mannitol is required for sporulation (Allocco *et al*. 1997). This agrees well with historical reports that sporulation is impaired in oocysts shed by nitrophenide-treated birds (Brackett & Bliznick, 1949; Gardiner, Farr & Wehr, 1952).

Additional information has been provided by evaluating caecal tissue from *E. tenella*-infected birds treated with nitrophenide at 250 ppm in the feed. While mannitol levels are significantly reduced with nitrophenide treatment (Fig. 4), the overall parasite numbers in the gut are unaffected by the compound (Fig. 5). Furthermore, M1PDH enzymatic activity in nitrophenide-treated tissues is much lower than control levels, but equal amounts of the M1PDH (67 kDa) protein are present in both tissues (as determined using rabbit anti-M1PDH antibody). This confirms that the enzyme is present but inhibited. In contrast, M1Pase enzyme activity is equivalent for both groups. This would be expected since this enzyme is not inhibited by nitrophenide and the parasite load is the same (Allocco *et al*. 1997).

These results demonstrate that nitrophenide reduces caecal and oocyst mannitol levels by specifically inhibiting M1PDH rather than as an indirect result of killing the parasites by an unrelated mechanism. It also suggests that an M1PDH inhibitor will not prevent parasite proliferation in the gut and the resultant lesions, but would prevent oocyst shedding. Since the sporulated oocyst is the infectious form of the parasite responsible for transmitting the disease, prevention of oocyst shedding and/or their maturation (sporulation)

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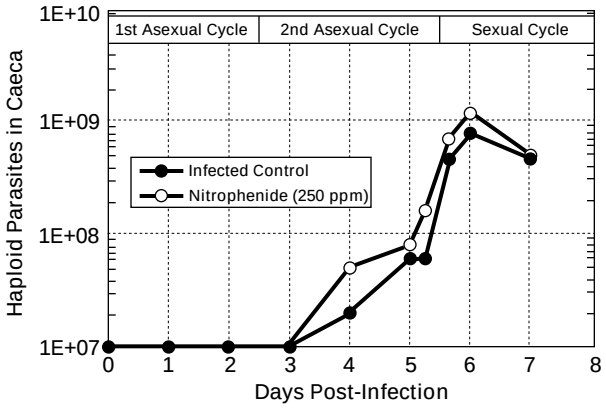


Fig. 5. Parasite load in the caeca of nitrophenide-treated and untreated infected chickens. The number of parasites present in caecal tissue was determined by extracting total genomic DNA from the tissue and quantitating with a parasite-specific DNA probe. As expected, parasite numbers increase with subsequent rounds of asexual multiplication and plateau during the sexual phase when no significant replication occurs. The data in this graph are from tissue samples from the same birds used in the experiment presented in Fig. 4. Note that despite the clear inhibition of mannitol biosynthesis in nitrophenide-treated birds, the parasite load in these birds is indistinguishable from the untreated birds. This demonstrates that the drop in mannitol biosynthesis is not occurring due to a reduction of the overall parasite load but as a result of inhibition of the mannitol cycle.

would be an attractive method of disease control, especially as the major disease problem is not the early focal infections in the poultry house but rather the spread of coccidiosis. Coccidiosis outbreaks in poultry houses routinely occur during the 3rd or 4th week of a 'grow out' when several cycles of *Eimeria* infections have already occurred and the density of oocyst in the facility has substantially increased. A mannitol cycle inhibitor would be expected to prevent the logarithmic expansion of the oocyst population during the first three weeks of the grow out and an acute coccidiosis outbreak should therefore be avoided.

CONCLUSIONS

Further studies are needed to elucidate fully the role of mannitol metabolism in the apicomplexan parasites. Definitive evidence of the vital role of the mannitol cycle in the coccidia could come from studies of organisms in which M1PDH or M1Pase expression had been eliminated. With the recent development of transfection models in apicomplexan parasites experiments in which the gene for one of these enzymes is disrupted or eliminated may now be achievable. The most advanced transfection systems for apicomplexans is in *T. gondii*. If the elimination of mannitol biosynthesis is not detrimental to the tachyzoite stage, tachyzoites lacking a functional gene for M1PDH and/or M1Pase could be developed *in vitro*. These tachyzoites could then be used to infect cats to determine the consequences of the inability to synthesize mannitol on the development and sporulation of *T. gondii* oocysts. At present an experiment of this type cannot be conducted with eimerian parasites due to the lack of a method for continuously propagating these parasites *in vitro*. Currently the only method for conducting genetic studies with eimerian parasites is by *in vivo* passage.

Studies are also needed to explore the role of this pathway in other apicomplexan parasites. Exploitation of this pathway for developing chemotherapeutics for other parasitic infections may be limited if the impact of inhibiting the mannitol cycle is exclusively on the sexual stage of the life cycle, especially in diseases such as toxoplasmosis and malaria in humans where the sexual cycle is present in the intermediate host rather than the definitive host. In contrast, prevention of the sexual cycle would be expected to have significant effects on pathogens like *Isospora* and *Cryptosporidium* where oocyst cycling is responsible for the continued transmission of the infection between hosts.

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