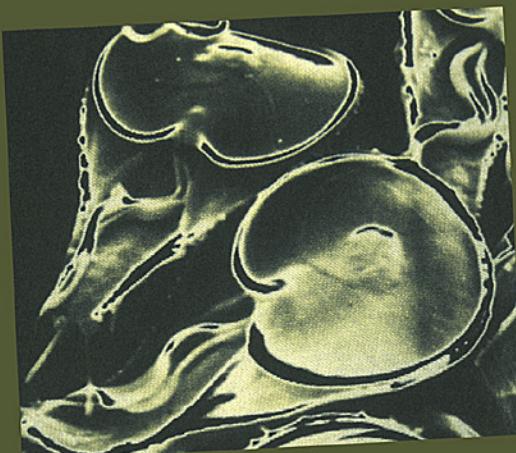


Biochemical Protozoology

Edited by Graham Coombs and Michael North



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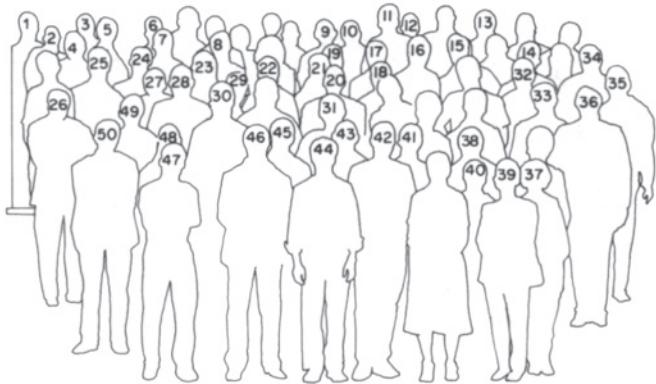


Figure 1. Some of the participants at the Symposium on Biochemical Protozoology as a Basis for Drug Design. The contributors to this book in the photograph are: 1, B.Ullman; 2, A.M.Gero; 3, J.J.Marr; 4, J.J.Cazzulo; 5, R.L.Krauth-Siegel; 6, R.H.Schirmer; 7, J.A. Reynoldson; 8, T.A.Paget; 9, M.Fry; 10, P.J.Schofield; 11,J.J.Blum; 12, K.Tanabe; 13, B.H.TerKuile; 14, M.K.Riscoe; 15, F.R. Opperdoes; 16, G.H.Coombs; 17, C.D.Ginger; 18, P.F.L.Boreham; 19, W.E.Gutteridge; 20, J. Schrével; 21, P.J.Rosenthal; 22, J.H.McKerrow; 23, J.A.Sakanari; 24, J.C.Mottram; 25, A.G. Williams; 26, M.Müller; 27, N.Yarlett; 28, R.Zidovetski; 29, S.Krishna; 30, C.D.Robertson; 31, E.L. Jarroll; 32, R.W.Mason; 33, H.Scholze; 34, B.C.Elford; 35, H.Ginsburg; 36, M.J.North; 37, B.C. Lockwood; 38, I.W.Sherman; 39, M.Robert-Gero; 40, F.Lawrence; 41, T.Bakker-Grunwald; 42, R. Etges; 43, D.G.Lindmark; 44, S.Turco; 45, P.A.Bates; 46, J.D.Lonsdale-Eccles; 47, M.J.McConville; 48, P.A.Haughan; 49, R.L.Blakeley; 50, K.Smith.

Preface

Knowledge and understanding of the biochemistry of parasitic protozoa has advanced considerably during the 15 years since the publication of the most recent introductory text devoted to the subject (Gutteridge and Coombs, 1977). There has not been an attempt to cover the subject comprehensively in a single text since the now classic *Biochemistry of Parasites* compiled by von Brand (1973). A large number of reviews dealing with individual or groups of organisms or particular aspects of their biochemistry have appeared during the 1980s, but the time seemed right to gather together in a single volume a set of reviews that cover the entire range of the subject and give details of current knowledge and an insight into the present status of research and the ideas that are being formulated. This book is an attempt to do this. It is not totally comprehensive and some areas of current interest are under-represented. We have tried to reduce these deficiencies by giving some appropriate references in Chapter 1.

The book is intended for scientists who have little or no familiarity with the biochemistry of parasitic protozoa but should also prove valuable as an introductory source for committed biochemical protozoologists wishing to delve into other topics. All the reviews give a good number of references to the primary literature and so should provide good starting points for information gathering. The chapters were written by experts and then edited in order to ensure that there is not excessive overlap, there is helpful cross-referencing of reviews and there is reasonable consistency between chapters. It was decided, however, not to restrict individuality by imposing a set format for each review. Thus each chapter is an individual read.

The reviews provide little or no information on the general biology of the organisms or the diseases they cause. It is not necessary, however, for the reader to have a foundation in parasitology to understand and profit from the reviews, although it is likely that some such knowledge will aid understanding. No attempt has been made to obtain absolute consistency in nomenclature of organisms or indeed enzymes, mainly because there is no consensus view on some issues. We have attempted to ensure that there is no ambiguity and that confusion should not arise but the reader should consult the primary literature or recent books if interested in obtaining information on the current status of nomenclature. Similarly, we have avoided entering into the debate on taxonomy and phylogenetic relationships. Recent findings suggest that the Protozoa is not a meaningful grouping (see Sogin *et*

al., 1989). Whilst not contesting this, our view is that many still associate the term Protozoa with the important parasites with which this book is mainly concerned. Our aim is to provide a body of information on the biochemistry of organisms that have a variety of features in common as well as having distinct differences. A compendium of such knowledge may aid in formulating views on the relationships between the organisms but, more importantly, should help promote greater understanding of how the organisms are adapted at the molecular level to their environments and so lead to new therapies against the diseases. There is frequent reference in the book to antiprotozoal drugs and finding new drug targets, which is a major driving force in current research. The book does not, however, deal with chemotherapy *per se*. Readers should consult other sources (for example, Campbell and Rew, 1986) for comprehensive coverage of this.

As an adjunct to the preparation of this book, we organised a 3-day symposium entitled *Biochemical Protozoology as a Basis for Drug Design*. This was held at the University of Stirling, Scotland, and many of the authors attended (see Figure 1). This meeting was made possible through the generous support of The Wellcome Trust and the following companies: Smith Kline & French Laboratories Ltd; Merrell Dow Research Institute; F.Hoffmann-La Roche Ltd; and The Wellcome Research Laboratories. It is hoped that such a meeting concentrating on the biochemistry of parasitic protists will become a regular occurrence.

G.H.Coombs
M.J.North

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1. The current status of biochemical protozoology

G.H.Coombs and M.J.North

Protozoa differ biochemically from other eukaryotic cells, including one another, in many ways and the information gained from studies so far has revealed fascinating insights into how different species are adapted to their particular niche. It has also provided further proof of the enormous diversity of cells at the biochemical level. A number of features that appear to be unique to some protozoa have already been discovered and there is little doubt that others will be found in the future. It could be considered that the contribution studies such as these make to our understanding of life at the biochemical level is reason enough for their existence, but it has to be accepted that such purely academic justifications are often not considered sufficiently compelling in the eyes of those controlling the finances. Thus it is not surprising to find that current biochemical research is largely directed towards those protozoa that are causative agents of major human and, to a lesser extent, veterinary diseases and that the main thrust is for the discovery of biochemical peculiarities that offer opportunities for chemotherapeutic exploitation. The contents of this book reflect this state of affairs.

One approach being pursued by many is the characterization and analysis of parasite-specific molecules using crystallography and computer graphics. The expectation is that this, in combination with other techniques, will provide sufficient information to allow the design of specific inhibitors (see Hart *et al.*, 1989). This goal has not yet been achieved, however, and all of the currently used antiprotozoal drugs were discovered and developed using mainly empirical methods. Moreover, the modes of action of the vast majority of antiprotozoal drugs are still unknown, even though most have been in use for decades. Elucidation of exactly how a drug works is not easy; nevertheless, this does seem a worthwhile pursuit because it is one way of discovering a parasite feature that definitely can be exploited by a drug. Unfortunately, relatively little work is in progress in this area.

A second factor that directs current biochemical research effort is the availability, or otherwise, of parasite material for study. Some parasitic protozoa and some stages in their life cycle offer far greater opportunities than others. Thus the multiplicative bloodstream form of *Trypanosoma brucei* can be obtained pure and in large quantities

and has been studied extensively with most exciting results. These investigations have been pursued even though this particular cattle parasite is not a major problem, whereas *T. vivax* and *T. congolense* are, and despite there being relatively few cases of sleeping sickness. In contrast, malaria is responsible for more human deaths than any other parasite but the difficulties in obtaining parasite material that is pure and in sufficient quantity have severely limited biochemical investigations. Other problems with malaria parasites are their host specificity, which means that animal models are not generally available for *Plasmodium falciparum*, and our inability to grow the parasites axenically *in vitro*. The development of a method for culturing the blood stages of *P. falciparum* intraerythrocytically *in vitro* (Trager and Jenson, 1976) was a notable advance, it is perhaps surprising that this did not precipitate the avalanche of biochemical investigations that had been predicted. Despite the inherent difficulties, there is now a considerable body of knowledge on the biochemistry of the asexual intraerythrocytic forms of *P. falciparum* (see Chapter 2 and others). In contrast, virtually nothing is known about the biochemistry of the other stages in the life cycle, including those that occur in the liver and several stages in the mosquito. This situation, where only one development stage (and frequently not the one responsible for disease) has been studied in detail, is common for protozoa with complex life cycles.

Most parasitic protozoa have been studied less than malaria parasites and trypanosomes. In some cases, for instance trichomonads, material for study can be obtained easily, but for others, e.g. *Cryptosporidium* species, it is the main factor limiting advance. A problem with some protozoa is that investigations have been carried out using populations of cells containing more than one parasite form. For instance, leishmanias grow *in vitro* at 25°C as promastigotes, but metacyclic promastigotes are also present and can be abundant in stationary phase populations (see Mallinson and Coombs, 1989). Indeed, the lack of suitable methodology can be a limitation with all parasitic protozoa.

Traditionally, biochemical studies on parasitic protozoa have involved the application of standard biochemical techniques, developed in connection with other systems, to the parasitological problems being addressed. This is still the usual situation but the position has changed a little in recent years with the realization that protozoa provide one of the better models for some studies. For instance, glycosomes present a marvellous system with which to study the mechanisms of targetting proteins into microbodies. Not only are they particularly abundant in some trypanosomatids but they contain enzymes with isoenzymes in the cytosol and with cytosolic counterparts in other eukaryotes. Other aspects particularly suitable for investigation include the mechanisms of adaptation to anaerobiosis (and the role of hydrogenosomes) and the features determining the substrate specificity of enzymes (as revealed by studying trypanothione-metabolizing enzymes and the purine salvage enzymes that handle allopurinol).

The majority of topics that are currently under investigation or have aroused particular interest during recent years are covered by one or more reviews in this book. There are, however, a few exceptions. There is no profound reason for their omission. It was decided that the book would deal solely with biochemistry as

opposed to molecular biology *per se*. Clearly there is a much overlap between the two and the differences will undoubtedly become more blurred in the future. Indeed we are convinced that a joint approach is essential if success is to be achieved more rapidly. Analyses of gene sequences will provide extensive data on the structure of protozoan proteins. In addition, some of the most exciting advances that have come out of molecular biology, such as *trans* splicing and RNA editing, are inevitably going to merge into biochemistry. Perhaps, indeed, molecular biology will return to being considered as an aspect of biochemistry! *Trans* splicing and RNA editing, together with some other 'molecular biology' topics, are mentioned (and lead-in references given) in the latter half of Chapter 55. There is little or no coverage of other enzymes involved with nucleic acids (such as polymerases, topoisomerases, gyrase and endonucleases), and so some lead-in references are given in the Bibliography at the end of this chapter. One aspect that is under-represented is pyrimidine metabolism, a recent review (Hassan and Coombs, 1988) details the current knowledge and relevant literature.

There are a number of important parasitic protozoa on which the book contains relatively little information, although in many cases this is simply a reflection of the lack of studies. There has been an enormous upsurge in interest in protozoa associated with human immunodeficiency virus (HIV) infections, but this is yet to include biochemical investigations of *Cryptosporidium*; *Toxoplasma* is still under-researched and we include the first comprehensive review on the biochemistry of *Pneumocystis* (Chapter 3). Research on *Eimeria* has declined greatly in recent years as a consequence of pharmaceutical companies apparently deciding that current chemotherapy cannot be bettered economically. One recent finding of particular note concerns the presence of the mannitol cycle in *Eimeria* (Schmatz, 1989). It will be interesting to see whether this cycle also occurs in other protozoa.

The reference list below contains recent papers that should provide a lead-in to the primary literature on aspects not dealt with in the main chapters of this book. Also listed are a variety of recent reviews on aspects related to the biochemistry and mode of action of antiprotozoal drugs. The literature cited is a selected sample and certainly is not intended to be comprehensive. Readers are also advised to consult the last few years' issues of the journals that specialize in the biochemistry of parasitic protozoa. Top of the list is *Molecular and Biochemical Parasitology*, whereas *Parasitology Today* has published many excellent short summaries of topical issues.

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2. The biochemistry of malaria: an overview

I.W.Sherman

Biochemical studies with *Plasmodium* began with the work of J.D.Fulton and S.R. Christophers in 1938, and, in the half-century since, there have been more than a dozen reviews summarizing the progress made (Fulton, 1951; McKee, 1951; Fulton and Spooner, 1955; Moulder, 1948, 1962; Honigberg, 1967; Peters, 1969; Trager, 1970; Fletcher and Maegraith, 1972; Oelshlegel and Brewer, 1975; Homewood, 1978; Homewood and Neame, 1980; Sherman, 1979, 1984; Scheibel, 1988; Scheibel and Sherman, 1988; Trigg, 1988; Fairlamb, 1989). Although the present review is based on these earlier works, it is not intended to be a recapitulation of what has already been written. Rather, this overview is an attempt to summarize and critically evaluate the published work on the biochemistry of the blood stages of malaria that appeared in print between 1984 and July 1990, with special emphasis on the human malarial parasite *P. falciparum*.

CARBOHYDRATE METABOLISM

Glycolysis and glycolytic enzymes

The intraerythrocytic stages of malaria lack carbohydrate reserves and, consequently, the blood glucose serves as their primary energy source. (This is not entirely unexpected since these parasites live in an environment in which glucose is readily available). Despite living in oxygen rich surroundings, *P. falciparum* does not oxidize glucose completely to carbon dioxide and water; instead, it is a micro-aerophilic homolactate fermenter. (This inefficient utilization of carbohydrate is of no handicap to the parasite since it lives in a food-rich environment and can rely on the host to reprocess the partially oxidized waste product, lactate). Human red cells (10^9) consumed 4.6 ± 1.5 fmol glucose/24h, whereas a similar number of cells from an asynchronous culture utilized 122 ± 34 μmol in the same time period (Jensen *et al.*, 1983); the schizont stages produced most of the lactate (Pfaller *et al.*, 1982). During *in vitro* growth, the parasites prospered when the oxygen concentration was 3 per cent, but levels as low as 0.5 per cent could be tolerated. *In vitro*, oxygen levels

such as that found in ambient air (21 per cent) were deleterious to parasite development (Scheibel *et al.*, 1979), as were concentrations of lactate >12mM, and a pH lower than 7.2 (Jensen *et al.*, 1983).

Although all of the enzymes of the Embden-Meyerhoff glycolytic pathway have been identified in extracts of *P. falciparum* (Roth *et al.*, 1988), only lactic dehydrogenase (LDH) (Vander Jagt *et al.*, 1981; Simmons *et al.*, 1985), phosphofructokinase (Buckwitz *et al.*, 1988, 1990a) and hexokinase (Roth, 1987) have been characterized in any detail. *P. falciparum* also showed activity for the non-glycolytic enzymes glutamic dehydrogenase (GDH), isocitric dehydrogenase (IDH), malic dehydrogenase and glutamic-oxaloacetic transaminase. The NADP-specific glutamic dehydrogenase is not found in uninfected cells; therefore, it is frequently used as a convenient marker for the cytosol of *Plasmodium* (Vander Jagt *et al.*, 1982). Monoclonal antibodies prepared against *Proteus* sp. GDH cross-reacted with the enzyme from *P. falciparum*, but not with the GDH from other species of malaria (Ling *et al.*, 1986).

The phosphofructokinase and lactic dehydrogenase of *Plasmodium* appeared to be less sensitive to high concentrations of substrate and low pH than those of the red cell, suggesting a different means for regulating glycolysis in host cell and parasite. Confirmation of these findings has been made using the non-invasive and non-destructive technique of high resolution ^{31}P NMR; *P. berghei* infected red cells showed a deline in 2, 3-diphosphoglycerate and ATP. Furthermore, while normal rodent erythrocytes utilized glucose at a low rate, this was increased about 25-fold upon parasitization (Deslauriers *et al.*, 1982). Quinine, quinidine, quinacrine and DDS (dapsone), but not chloroquine, were inhibitors of glucose utilization (Deslauriers *et al.*, 1983). Unfortunately, NMR has not been used to study the metabolism of *P. falciparum* or any species other than *P. berghei*.

Pentose phosphate pathway

The hexose monophosphate shunt pathway permits the red cell to produce ribose, as well as NADPH, and while this shunt pathway may play only a minor role in a non-reproducing cell such as the erythrocyte, it could be of considerable significance in merozoite formation, which for nucleic acid synthesis requires not only purines and pyrimidines, but ribose as well. It was once believed that malaria parasites lacked the first enzyme of the shunt, glucose-6-phosphate dehydrogenase (G6PD). As a consequence, despite the easily demonstrated presence of the second enzyme of the pathway, 6-phosphogluconate dehydrogenase, the shunt pathway of the parasite was thought to be inoperative. Recent work, however, has demonstrated a parasite-encoded G6PD in *P. falciparum* (Yoshida and Roth, 1987; Ling and Wilson, 1988; Kurdi-Haidar and Luzzatto, 1990), *P. knowlesi* (Ling and Wilson, 1988) and *P. berghei* (Buckwitz *et al.*, 1990b). The activity of the parasite G6PD is about 5 per cent of that of the normal human erythrocyte. The *P. falciparum* G6PD had a higher affinity for glucose-6-phosphate and NADP than did the host enzyme. The contribution of the shunt to the ribose pool of the parasite appears to be minor (Roth *et al.*, 1986b). Most of the pentoses appear to arise either from the transaldolase and transketolase

catalysed condensation of fructose 6-phosphate and glyceraldehyde 3-phosphate, or phosphorolysis of inosine followed by isomerization to ribose 5-phosphate.

Erythrocyte deficiency in G6PD—an X-linked abnormality—often coincides with a high incidence of falciparum malaria. It has been hypothesized that such a deficiency offers a selective advantage against lethal malaria infections and, as a consequence, the gene is more prevalent in malarious regions of the world. The *in vitro* growth of *P. falciparum* has been shown to be impaired in G6PD deficient cells and, since retardation of parasite growth was enhanced by exposure to oxidants (Golenser *et al.*, 1988; Roth and Schulman 1988), this has provided increased support for the ‘malaria hypothesis’. But, how does red cell G6PD deficiency protect? The biochemical mechanism for the impaired growth of *P. falciparum* in G6PD-deficient red cells has been suggested to be due to low levels of reduced glutathione and inhibition of PRPP (phosphoribosylpyrophosphate) synthetase activity rather than increased oxidant sensitivity of the parasite itself or a reduction in the availability of ribose (Roth *et al.*, 1986a). Indeed, inhibitors, of glutathione reductase, such as nitrosoureas, inhibited the *in vitro* growth of *P. falciparum* (Zhang *et al.*, 1988).

The malarial parasite is exquisitely sensitive to active forms of oxygen, such as superoxide, hydrogen peroxide and peroxide radicals. However, both *P. berghei* and *P. falciparum* have been shown not to synthesize their own superoxide dismutase (SOD), an enzyme important in protecting against activated oxygen. Instead, the plasmodia appear to take up this enzyme from their host cells and concentrate it in lysosomes (Fairfield *et al.*, 1986, 1988; Arias and Walter, 1988). A minor cyanide insensitive SOD, probably localized in the mitochondrion, has been reported from *P. falciparum* (Ranz and Meshnick, 1989).

Carbon dioxide fixation

All of the malarial parasites studied, including *P. falciparum* (Blum and Ginsburg, 1984), are capable of fixing carbon dioxide. In *P. berghei*, *P. knowlesi* and *P. lophurae* (see Sherman, 1979, 1984), the end-products of fixation were identified as alanine, malate, citrate, aspartate and glutamate, with α -ketoglutarate and oxaloacetate as intermediates; unfortunately, no products were identified in the studies with *P. falciparum*. It remains unclear how much of a contribution this pathway makes to the overall economy of the parasite.

Pyridine nucleotides

Nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) play key roles in glycolysis and in the hexose monophosphate shunt pathway. NADPH may provide reducing equivalents for the reduction of oxidized glutathione. The recent report of a 10-fold increase in the NAD content of *P. falciparum* infected cells (Zerez *et al.*, 1990) confirms earlier values for rodent and bird malarias (Nagarajan, 1964; Sherman, 1966). The increase in levels of these pyridine nucleotides appears to be due to synthesis from both nicotinamide and nicotinic acid, since levels of nicotinic acid phosphoribosyltransferase (NAPRT), nicotinamide phosphoribosyltransferase

(NPRT) and nicotinamide deamidase, enzymes involved in the biosynthesis of NAD, were found to be elevated in *P. falciparum* infected cells. The antagonists, 6-amino nicotinic acid, 6-amino nicotinamide and 3-acetylpyridine, when added to *in vitro* cultures of *P. falciparum*, significantly reduced the amount of NAD, but did not affect parasite growth. Although no increase in the level of NADP was found with *P. falciparum* (Zerez *et al.*, 1990), both NADP and NADH were elevated in *P. lophurae* and *P. berghei* infected cells (Nagarajan, 1964; Sherman, 1966).

NADH can be oxidized via LDH, whereas NADPH can be oxidized via GDH or IDH (Vander Jagt *et al.*, 1989). Most malarias demonstrate LDH and GDH activity and, in the bird malarias a citric acid cycle is present. *P. falciparum* (unlike the bird malarias), however, does not possess a citric acid cycle; therefore, the source of isocitrate for IDH remains unknown. NADPH is possibly formed from NADH via an ATP-requiring transhydrogenase, or isocitrate could conceivably result from carbon dioxide fixation; this, however, is yet to be demonstrated.

OXYGEN UTILIZATION AND ELECTRON TRANSPORT

The favouring effect of oxygen on the *in vitro* growth of *P. lophurae*, *P. knowlesi* and *P. falciparum* (see Scheibel *et al.*, 1979; Sherman, 1984) suggests a parasite requirement for oxygen, albeit at a low level. Apart from the presence of cytochrome oxidase, however, cytochromes have not been detected in plasmodia (Scheibel, 1988) and only recently has the gene for cytochrome *b* been described for the bird malaria *P. gallinaceum* (Aldritt *et al.*, 1989). What, then is the role of oxygen?

In the *de novo* formation of pyrimidines (see below), a key step is the conversion of dihydroorotate to orotate. This step, catalysed by the enzyme dihydroorotate dehydrogenase, is mitochondrial in mammalian cells and is closely linked to the cytochrome chain to which electrons are passed directly via the ubiquinones, and for which oxygen is the final acceptor. Gutteridge *et al.* (1979) proposed that the oxygen utilized by *Plasmodium* sp. may be coupled to pyrimidine biosynthesis. In support of this is the finding of dihydroorotate dehydrogenase in extracts of plasmodia, and inhibition of such activity by CN and antimycin. A depression of enzyme activity also occurred when parasites were cultured in the presence of tetracycline and with this antibiotic there was also a decrease in rhodamine 123 fluorescence (Prapunwattana *et al.*, 1988; Kiatfuenfoo *et al.*, 1989). (Rhodamine 123 is a positively charged dye that is selectively accumulated by the mitochondria of living cells because of a highly negative mitochondrial membrane potential (see Divo *et al.*, 1985 b.) Hudson *et al.* (1985) found that the hydroxynaphthoquinones, BW58C and BW568C, were 200-fold more active against the respiratory-linked dihydroorotate dehydrogenase of *P. yoelii* than liver mitochondria. Gutteridge (1990) reported that BW566C80, a drug targeted at the mitochondrial respiratory chain, cured *P. falciparum* infections in *Aotus* with a single oral dose of 10 mg kg⁻¹. Thus, the evidence strongly suggests that the plasmodial dihydroorotate dehydrogenase is mitochondrial.

Morphologic descriptions of the mitochondria of several species of *Plasmodium* have been published (Aikawa, 1988) and, by reconstruction from serial sections, it was demonstrated that *P. falciparum* contains only a single mitochondrion (Slomianny and Prensier, 1986). Only rarely, however, has the organelle itself been isolated (Kilejian, 1975; and see Chapter 13). Despite this, mitochondrial DNA (mtDNA) has been obtained from *P. lophurae*, *P. knowlesi* and *P. falciparum* (Gardner *et al.*, 1988; Aldritt *et al.*, 1989; Joseph *et al.*, 1989). Vaidya *et al.* (1989) reported the complete DNA sequence of a 5984-base-pair repeating unit in *P. yoelii* that had significant sequence homology with mitochondrial-encoded proteins such as cytochrome *c* oxidase subunit I, and cytochrome *b*. Two regions showed extensive similarity to the conserved central loop of the peptidyl transferase domain of the large rRNA of mitochondria and chloroplasts, suggesting that mtDNA in *Plasmodium* has less homology with the cytoplasmic rRNA of other eukaryotes. In addition Joseph *et al.* (1989) isolated an extrachromosomal DNA from *P. gallinaceum* with sequence similarities to the large mitochondrial rRNA and cytochrome *b*.

After qinghaosu treatment mitochondrial swelling was seen in monkeys infected with *P. inui* (Jiang *et al.*, 1985) and in rodents infected with *P. berghei* (Ellis *et al.*, 1985); such findings are different from the effects of chloroquine and mefloquine on *P. falciparum* (Jacobs *et al.*, 1987, 1988), *P. gallinaceum* (Aikawa and Beaudoin, 1969) and *P. berghei* (Warhurst and Hockley, 1967), where the primary site of action was the food vacuole.

POLYAMINES

Polyamines (i.e. spermine and spermidine) are ubiquitous in biological materials and increased levels are often associated with rapidly growing cells. They are involved in protein synthesis and contribute to ribosome and possibly membrane stability, as well as a number of other metabolic reactions. The main route of biosynthesis is from methionine and ornithine. Methionine is phosphorylated and combined with adenosine to form (*S*)-adenosylmethionine. Spermidine and spermine are derived from the decarboxylated (*S*)-adenosylmethionine (via the enzyme, (*S*)-adenosylmethionine decarboxylase) and from putrescine, which arises from the decarboxylation of ornithine by ornithine decarboxylase, the rate-limiting step in polyamine biosynthesis. (*S*)-Adenosylmethionine decarboxylase and ornithine decarboxylase activities have been identified in *P. falciparum* (Heidrich *et al.*, 1983; Königk and Putfarken, 1985) and Assaraf *et al.* (1988) purified the latter enzyme. The *in vivo* and *in vitro* growth of *Plasmodium* is inhibited by (difluoromethylornithine (DFMO)), a specific suicide substrate of ornithine decarboxylase (Assaraf *et al.*, 1987; Bitonti *et al.*, 1987). DFMO, in combination with bis (benzyl) polyamine analogues, inhibited the *in vitro* growth of chloroquine resistant *P. falciparum* and cured *P. berghei* infected mice (Bitonti *et al.*, 1989). The precise mode of action of these analogues is unknown. For further information see Chapters 42, 43, 46, and 47.

PERMEABILITY

Unlike the metabolically sluggish red cell, the rapidly growing plasmodium requires increased amounts of materials from the extracellular milieu. Therefore, a boost in the flux rate across the erythrocyte membrane may be essential for the survival of the intracellular parasite. Changes in the permeability of the malaria-infected red cell for a variety of substances have been described during the past two decades (reviewed by Sherman, 1988; Ginsburg, 1990; Cabantchik, 1990) and reviewed elsewhere in this volume (see Chapters 35 and 36). What remains controversial, and as yet unresolved, is a clear biochemical description of the mechanism that underlies the observed changes in the permeability of the malaria-infected red cell.

DE NOVO BIOSYNTHESIS OF PYRIMIDINES AND THE ROLE OF FOLATES

The maturation of the mammalian erythrocyte is accompanied by the loss of its capacity to synthesize pyrimidines *de novo*. By contrast, the various species of *Plasmodium*, during asexual intraerythrocyte development, contain a functional *de novo* pathway (reviewed by Scheibel and Sherman, 1988).

Evidence for a functional *de novo* pyrimidine biosynthetic pathway comes from three lines of investigation:

1. incorporation of radioactive bicarbonate into pyrimidine bases of RNA and DNA (Walsh and Sherman, 1968);
2. the presence of folate enzymes of the thymidylate cycle (Platzer, 1972); and
3. the detection of all the enzymes necessary for the *de novo* synthesis of UMP (Hill *et al.*, 1981; Gero *et al.*, 1984).

The conversion of carbamylaspartate to UMP is catalysed by the five enzymes of the *de novo* pathway: aspartate transcarbamylase, dihydroorotase, dihydroorotate dehydrogenase, orotate phosphoribosyltransferase, and orotidine-5'-phosphate decarboxylase. The latter three enzymes were identified in extracts of *P. falciparum* (Gero *et al.*, 1981; Reyes *et al.*, 1982); the plasmodial enzymes, unlike those of mammals, did not comprise a multi-functional complex and the decarboxylase was far more sensitive to inhibition by mercurials than the host cell enzyme (Rathod and Reyes, 1983). Dihydroorotase, purified from *P. berghei*, was unlike the mammalian enzyme in that it was a relatively small monomeric protein (Krungkrai *et al.*, 1990). Pyrazofurin, an inhibitor of orotidine-5'-phosphate decarboxylase, retarded the *in vitro* growth of *P. falciparum* (Scott *et al.*, 1986) and BW58C, a ubiquinone antagonist, blocked parasite pyrimidine synthesis (Hammond *et al.*, 1985; and see Chapter 13).

Thymidylate synthetase is a distinct enzyme when obtained from bacteria, yeast and mammals, and consists of a dimer with a native molecular weight of 70kDa. However, in protozoa, including *Plasmodium*, thymidylate synthetase exists as a bifunctional protein in combination with dihydrofolate reductase (DHFR), and has a

native molecular weight of 140 kDa, with a subunit molecular weight of 70 kDa for the synthetase (Garrett *et al.*, 1984). The DNA sequence of the bifunctional protein from *P. falciparum* has been determined (Bzik *et al.*, 1987). The significance of a bifunctional protein in malaria parasites probably resides in the fact that it allows for coordinate control and metabolic channelling of dihydrofolate, thereby, permitting continued dTMP synthesis (Ivanetich and Santi, 1990).

Pyrimethamine, by binding to DHFR, acts as an antimalarial; pyrimethamine-resistant strains of *P. falciparum* and *P. chabaudi* do exist and these show a decreased binding of the inhibitor to the enzyme. In *P. chabaudi*, resistance was related to gene duplication, allowing at least a two-fold expression of the enzyme (Cowman and Lew, 1989), whereas in *P. falciparum*, resistance was related to point mutations. (However, see also Tanaka *et al.*, 1990.) In all pyrimethamine-resistant isolates examined, the Thr/Ser-108 of the sensitive strain was converted to Asn. Other resistant strains also have an Asn-51 to Ile mutation and/or a Cys-59 to Arg mutation. Resistance to cycloguanil appeared to result from mutations of Ser-108 to Thr, in addition to an alanine-valine change at position 16. These mutations are found in the region in which the enzyme binds to the inhibitor, suggesting that conformational changes contribute to the resistant phenotype (Cowman *et al.*, 1988; Snewin *et al.*, 1989; Foote *et al.*, 1990).

PURINE SALVAGE

A functional *de novo* purine biosynthetic pathway has not been demonstrated for any malaria parasite; consequently, the plasmodia require a supply of preformed purines (Sherman, 1984; Scheibel and Sherman, 1988). Infected erythrocytes show a high uptake of adenosine, inosine and hypoxanthine. Tracy and Sherman (1972) suggested that the infected red cell membrane has a common 6-oxypurine site which would transport hypoxanthine and inosine (derived from deamination of adenosine). Indeed, a recent report claims that adenosine deaminase activity is localized on the external surface of the red cell (Franco *et al.*, 1990). The parasite-preferred purine for both *P. lophurae* and *P. falciparum* appears to be hypoxanthine and is probably derived intraerythrocytically by: ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow inosine \rightarrow hypoxanthine (Yamada and Sherman, 1980, 1981; Webster *et al.*, 1984; Roth *et al.*, 1989). Parenthetically, it is of interest to note that the *in vitro* growth of some strains of *P. falciparum* could be enhanced by the addition of hypoxanthine ($50\text{ }\mu\text{g ml}^{-1}$) to the medium (Zolg *et al.*, 1982), and this may also permit one to postpone a change of medium for up to 72 h (Reber-Liske, 1983).

P. falciparum isolated from the human erythrocyte was found to possess the following purine salvage pathway enzymes: adenosine deaminase, purine nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase and adenosine kinase (Reyes *et al.*, 1982).

Schimandle and Sherman (1983) purified the adenosine deaminase from *P. lophurae* and, while most of its physical properties were similar to those of the red cell, the parasite enzyme was unique in its lack of sensitivity to the inhibitor EHNA

(erythro-9(2-hydroxy-3-nonyladcnine)). Daddona *et al.* (1984) found a two-fold increase in the adenosine deaminase content of red cells infected with *P. falciparum* (8 per cent parasitemia); the parasite adenosine deaminase did not cross-react with an antibody prepared against the human erythrocyte enzyme and, like the *P. lophurae* deaminase, was insensitive to EHNA.

The purified adenine phosphoribosyltransferase (APRT) of *P. falciparum* had a molecular weight smaller than that of the host cell enzyme, and was not significantly inhibited by sulphydryl reagents; 6-mercaptopurine and 2, 6-diaminopurine were competitive inhibitors of the parasite enzyme, but not of the red cell enzyme. The activity of APRT was 1/1000 that of HGPRT (Queen *et al.*, 1989).

Schimandle *et al.* (1986), purified and characterized purine nucleoside phosphorylase (PNP) from *P. lophurae*. This enzyme had several unique properties: it was a pentamer with a molecular weight of 125kDa and it showed Michaelis-Menten kinetics. Daddona *et al.* (1986) found PNP expression for *P. falciparum* in enzyme deficient red cells. The parasite enzyme had a molecular weight of 147 kDa and was inhibited in a similar fashion by 8-amino-5-deoxychloroguanosine and 8-amino-9-benzylguanine.

The purified HGRT of *P. lophurae* was found to be distinctly different from that of the red cell in several kinetic parameters using allopurinol and 6-thioinosine as substrates (Schimandle *et al.*, 1987) and the parasite enzyme was competitively inhibited by formycin B. The complete nucleotide sequence for the HGPRT from *P. falciparum* has been reported, and shows *c.* 48 per cent overall homology to the mouse enzyme (King and Melton, 1987; Vasanthakumar *et al.*, 1989; Shahabuddin and Scaife, 1990). The gene product has been successfully expressed in an *Escherichia coli* strain deficient in purine salvage. The putative 20 residue catalytic domain showed considerable homology with phosphoribosyl transferases from other sources.

Activity for IMP dehydrogenase and adenylosuccinate synthetase has also been identified in extracts of *P. lophurae* (Sherman, unpublished). Consequently, the various species of *Plasmodium* appear to contain the complete enzymatic machinery for purine salvage. The unique properties of such enzymes, should they occur, could provide potential targets for chemotherapeutic agents. Indeed, 50 μ M bredinin, an inhibitor of IMP dehydrogenase, was found to arrest the *in vitro* growth of *P. falciparum* (Webster and Whaun, 1982). Furthermore, mycophenolic acid inhibited both the *in vitro* growth and the IMP dehydrogenase activity of *Eimeria tenella* (Hupe *et al.*, 1986), which would suggest that this inhibitor might also be active against *Plasmodium*.

NUCLEIC ACIDS

DNA synthesis, base composition and chromosomes

A merozoite contains *c.* 1×10^{-13} g of DNA and, after schizogonic development, the amount is increased 10–20 fold. DNA synthesis in *P. falciparum* begins at the early trophozoite stage (*c.* 29.5 h) and increases logarithmically until late

schizogony (44–48 h). Synthesis is inhibited by both hydroxyurea and aphidocolin (Inselburg and Banyal, 1984). The nuclear DNA base composition of *Plasmodium* can be separated into those species with high and low percentage G+C. Simian malarias and *P. vivax* belong to the first category (c.30 per cent); rodent malarias, *P. falciparum* and *P. lophurae*, have a lower (c.18 per cent) G+C content (McCUTCHAN *et al.*, 1984; Janse and Mons, 1987; Weber, 1988). The plasmodial genome consists of 2.5×10^7 base pairs, organized into 14 discrete chromosomes. (By comparison, the human genome consists of 3×10^9 base pairs, organized into 46 chromosomes.) There is considerable geographic variation in chromosome size in *P. falciparum*, most likely due to chromosome breakage and ‘healing’ by direct addition of telomeric repeats (Ravetch, 1989; Foote and Kemp, 1989). Riou *et al.* (1986) purified and characterized the DNA topoisomerases of *P. berghei*, and found the currently used antimalarials to be poor inhibitors of these enzymes.

RNA synthesis, base composition and ribosomes

The RNA content of the intraerythrocytic stage of malaria is about five times greater than the DNA content. Most of this RNA is localized in cytoplasmic ribosomes. Indeed, because of its abundance, it has been suggested by Waters and McCutchan (1990) that species-specific sequences of ribosomal RNA could be used as a molecular probe for the detection of malaria parasites in blood, thereby eliminating some of the problems associated with microscopic examination of large numbers of blood specimens. When *in vitro* cultures of *P. falciparum* were pulse labelled with tritiated hypoxanthine, RNA synthesis was shown to increase from the onset of the trophozoite state (c.18h), reach a maximum at 24–30 h, and then decrease as the parasites became mature schizonts (Waki *et al.*, 1985; DeRojas and Wasserman, 1985).

In *P. falciparum*, and in other malarias, the ribosomes sediment at 80S and the ribosome can be dissociated into two subunits of 60S and 40S. The larger subunit with a molecular mass of 1.49×10^6 , and the smaller subunit, with a mass of 0.78×10^6 , lack substantial poly-A sequences. An early report claimed that at least a part of the ribosomal subunit was provided by the host (Tokuyasu *et al.*, 1969); however, this has now been shown to be incorrect (Sherman *et al.*, 1975; Trigg *et al.*, 1975; Sherman and Jones, 1977; Miller and Ilan, 1978).

The base composition of plasmodial ribosomal RNA (rRNA) is typically protozoan (35–37 per cent G+C) and is distinctly different from that found in other eukaryotes. In *P. falciparum*, the rRNAs were found to be 25S, 17S, 5.8S and 5S, again differing from those of other eukaryotes in which the rRNAs are typically 28S, 18S, 5.8S and 5S. In most eukaryotes, the larger rRNAs are transcribed from one multigene family as a single precursor and then processed to yield the mature rRNAs; the 5S RNA is transcribed from a separate tandemly arranged multigene family. Malaria parasites differ from other eukaryotes in their rRNA gene organization: the rRNA genes are not amplified (which may correlate with the absence of a clearly defined nucleolus in some species of malaria) and the large rRNA transcription units are not clustered but instead are dispersed in the genome (McCUTCHAN, 1986). In the rodent malaria *P. berghei*, there are only four rRNA genes, and these are neither identical in length,

nor in sequence. The two classes of rRNA transcription units of *P. berghei* appear to be differentially regulated (Gunderson *et al.*, 1987). In the avian malaria *P. lophurae*, there are six to eight rRNA genes which, by restriction analysis, can be separated into four distinct classes with unique flanking sequences; in this malaria, the 25S rRNA is interrupted twice by non-coding sequences, whereas the 17S rRNA gene is interrupted once by a non-coding sequence. In *P. falciparum*, there are eight different rRNA genes with only three 5S rRNA genes, the lowest number described for any eukaryote (Shippen-Lentz and Vezza, 1988). This parasite possesses at least five classes of rRNA transcription units (Shippen-Lentz *et al.*, 1990), and recent studies have shown that only one of the two rRNA genes is expressed during erythrocytic development (McCutchan *et al.*, 1988). The stability and turnover of plasmoidal ribosomes is unknown, as is the manner of transcription regulation of the rRNA genes.

PROTEIN SYNTHESIS

The available evidence indicates that the molecular mechanism involved in plasmoidal protein synthesis are typically eukaryotic. Protein synthesis is sensitive to cycloheximide and puromycin, but not to chloramphenicol (Sherman and Jones, 1976, 1977; Gershon and Howells, 1986). Mitochondrial protein synthesis represents less than 0.25 per cent of the total protein synthesis by *P. falciparum*. Several antibiotics in addition to chloramphenicol (e.g. clindamycin, pirlimycin, tetracyclines, thiamphenicol and erythromycin), and which affect 70S (prokaryotic or mitochondrial) ribosomes, were found to be inhibitory to the *in vitro* growth of *P. falciparum* (Divo *et al.*, 1985a; Ginsburg *et al.*, 1986).

The first successful attempt at cell free protein synthesis with product identification was that of Eggit *et al.* (1979), who use mRNA from *P. knowlesi* and a rabbit reticulocyte cell-free system. Wallach and Kilejian (1982) developed a homologous system for *P. lophurae* using plasmoidal ribosomes, a pH 5 fraction from the parasite cell sap and *P. lophurae* transfer RNA (tRNA). The presence of plasmoidal or duck reticulocyte tRNA appeared to be critical for efficient translation. Wallach *et al.* (1984) also described methods for the efficient extraction of mRNA from *P. falciparum*. However, no histidine-rich proteins appeared in the translation products using mRNA's from either *P. lophurae* or *P. falciparum* and, surprisingly, the proteins synthesized by both plasmoidal mRNAs appeared to be identical.

The histidine-rich protein, a major protein of the dense granules of *P. lophurae*, with a molecular size of 58 kDa, was successfully translated *in vitro* in a wheat germ cell free system using poly A containing *lophurae* mRNA (Feder and Blobel, 1983). The translation product was synthesized as a large unglycosylated precursor of 63 kDa, but in the presence of dog pancreas microsomal membranes a larger form (66 kDa) was found. The larger form, which was core glycosylated, was subsequently proteolytically processed to the mature lower molecular weight upon transport from the rough endoplasmic reticulum to the dense granule. Discrepant and less clear-cut

findings on the cell-free translation of *lophurae* mRNA and the synthesis of the histidine-rich protein have been reported by Wallach and Boeke (1983).

FEEDING, FOOD VACUOLES AND PIGMENT FORMATION

The intraerythrocytic stages of *Plasmodium* can effect nutrient uptake by pinocytosis, diffusion and/or transport as well as by bulk ingestion of host cell cytoplasm via a specialized organelle, the cytostome. Under the electron-microscope, this organelle appears as a depression in the plasma membrane surrounded by two dense rings. In the avian malarias (*P. lophurae*, *P. fallax*, *P. cathemerium* and *P. gallinaceum*), as well as in some simian malarias (*P. knowlesi* and *P. cynomolgi*), the bolus of engulfed erythrocyte cytoplasm is pinched off from the wall of the enlarged cytostomal cavity. As the bulge forms, the cytostomal opening is sealed by a membrane together with the cytostomal wall and, in this way, the food vacuole is formed. In *P. chabaudi*, a rodent malaria, a cytostomal tube extends backward from the cytostome proper and, at its distal end, food vacuoles bud off. The double limiting membranes become tightly adherent and, as haemoglobin digestion begins, one of the membranes disappears (Slomianny *et al.*, 1985; Aikawa and Seed, 1980).

Ring-stage parasites contain tiny food vacuoles bounded by a single membrane and have small granules of malarial pigment (haemozoin). As the parasite grows, the food vacuoles appear to coalesce, the pigment granules become larger in size and there is a tendency for the granules to be stacked one upon the other. With further maturation of the parasite, the contents of the food vacuole become less electron dense and the amount of pigment is increased. Based on ultrastructural studies, the food vacuole appears to be the primary target of the antimalarials, chloroquine, pyronardine and mefloquine.

By histochemical techniques, acid phosphatase, aminopeptidase and endoarylamidase were demonstrated in the food vacuoles of the rodent malaria *P. chabaudi* (Slomianny *et al.*, 1983); however, the isolated digestive vacuoles of *P. falciparum* did not contain the hydrolases (i.e. acid phosphatase, β -glucuronidase and β -galactosidase) commonly found in lysosomes, but did show haemoglobinase and ATPase activities (Choi and Mego, 1988; Goldberg *et al.*, 1990). At pH 4, haemoglobinase activity was pepstatin inhibitable, whereas at a pH equivalent to the *in vivo* state of the food vacuole (pH 5) haemoglobin proteolysis was inhibited by about 30 per cent by E-64, an inhibitor of cysteine proteases (Rosenthal *et al.*, 1988; Goldberg *et al.*, 1990; and see Chapter 24). Serine and metalloproteases were also in evidence and it was claimed that these function after aspartic protease action. In many respects, these results with *P. falciparum* confirm the report of cathepsin D (an aspartic protease) in *P. lophurae* (Sherman and Tanigoshi, 1983). Other reviews on the proteinases of *Plasmodium* are given elsewhere in this volume (Chapters 24 and 25). It would be of considerable interest to localize, at the ultrastructure level, the various proteinases in the *P. falciparum* infected cell using immunohisto-chemical methods and/or electron-dense labelled inhibitors.

The end product of haemoglobin degradation is a blackish brown pigment, called haemozoin. The chemical composition of haemozoin has been the subject of much controversy. Initially, it was believed to be melanin and, later, it was claimed to be haematin or haematin coupled to a polypeptide (see Scheibel and Sherman, 1988). Morselt *et al.* (1973) utilized microspectrophotometry to analyse pigment granules in intact cells and found a gradual transition of the absorption maximum from that of haemoglobin at 416 nm to that of a compound with an absorption maximum at 442 nm. They concluded that the exact nature of this pigment was not clear, but it was neither pure haematin nor bilirubin. Mössbauer effect spectroscopy showed that the iron in the pigment was trivalent and high spin with spectral characteristics unlike any known iron-porphyrin compound (Yayon *et al.*, 1984b).

Yamada and Sherman (1979) extracted malaria pigment from *P. lophurae* and found it to consist of insoluble monomers and dimers of haematin, ferriprotoporphyrin (FP) coupled to a plasmodial protein, and insoluble methaemoglobin. Similar results were obtained with *P. chabaudi* and *P. berghei* haemozoin when studied by photoacoustic spectroscopy (Balasubramanian *et al.*, 1984). Based on the properties of haemozoin extracted from *P. falciparum*, Fitch and Kanjananngulpan (1987) claimed it was an aggregate of insoluble haematin, whereas Ashong *et al.* (1989) contended that haemozoin was ferriprotoporphyrin coupled to a plasmodial protein. It is difficult to state with certainty that the characteristics of such extracted pigments are identical to the haemozoin that is formed within the food vacuoles, since it is known that haematin may bind avidly to certain proteins and, during parasite lysis, some cytoplasmic constituents may be released and then be bound to the vacuolar contents. In addition, the reagents used to extract and purify the pigment may have altered it. The use of haemozoin from isolated food vacuoles, hopefully, may help to resolve these contradictions.

One cannot discuss malaria pigment without mentioning that it, or ferriprotoporphyrin IX, have been suggested to be involved in chloroquine resistance and that the toxicity of chloroquine has been attributed to a FP-chloroquine complex (Schueler and Cantrell, 1963; Phifer *et al.*, 1966; Orjih *et al.*, 1985; Fitch, 1989). There are, however, several limitations to this model (see, for example, Sherman *et al.*, 1965; Yayon *et al.*, 1984a, 1985; Zhang and Hempelmann, 1987; Schlesinger *et al.*, 1988).

MEMBRANE LIPIDS

During the intracellular development of *Plasmodium*, many membrane-containing organelles such as the nucleus, endoplasmic reticulum, mitochondrion, cytostome, food vacuoles, rhoptries, micronemes, pellicular complex, and the ever-enlarging parasitophorous vacuolar membrane are formed. Associated with such membrane biogenesis is a marked increase in the lipid content of the infected red cell (reviewed by Holz, 1977; Sherman, 1979, 1984). The plasmodium, like its host cell, is incapable of synthesizing fatty acids or cholesterol *de novo*, but it can form phosphatidylcholine (PC) and phosphatidylethanolamine (PE) via *de novo*

pathways, or by methylation of PE or decarboxylation of phosphatidylserine (PS), respectively (Vial *et al.*, 1990). Plasmodial membranes, in comparison to those of the host cell, appear to be low in cholesterol, sphingomyelin (SM) and PS, but are enriched in PC, PE and phosphatidylinositol (PI).

The blood plasma serves as the primary source of the fatty acids required for plasmodial membrane formation. Activation of fatty acids to acyl-CoA thioesters occurs via acyl-CoA synthetase, an enzyme activity increased 20-fold in *P. knowlesi* infected cells (Beaumelle *et al.*, 1988). Subsequent steps involve the formation of phosphatidic acid and diacylglycerol (DAG). Several pathways for the biosynthesis of PC have been described: the *de novo* pathway involves condensation of DAG with CDP-choline (CDP-choline arises by choline transport from the plasma into the red cell, followed by activation via a parasite cytosolic enzyme, choline kinase). Further activation of CDP-choline occurs via a choline-phosphate cytidyltransferase that is both cytosolic and membrane bound. Synthesis is completed by the reaction of CDP-choline with DAG, catalysed by the membrane-bound enzyme, CDP-choline: 1, 2-DAG choline phosphotransferase (Vial *et al.*, 1984). PC can also arise by methylation of PE, which itself may be formed from PS decarboxylation via PS decarboxylase or by a *de novo* pathway analogous to that for PC, but instead involving ethanolamine kinase and ethanolamine transferase (Ancelin *et al.*, 1986). Since the membrane-bound enzymes, choline phosphotransferase and ethanolamine transferase, are parasite specific, these can be powerful indicators of the purity of plasmodial membranes (Vial *et al.*, 1984; Ancelin *et al.*, 1986). The pathways for PS and PI formation have not been studied in detail, but it is presumed that reactions of the CDP-DAG pathways are involved.

Since plasmodial phospholipid (PL) synthesis is critical to membrane biogenesis, it would be expected that interference with parasite-specific pathways could lead to impaired parasite growth. Analogue s of choline and ethanolamine, as well as inhibitors of acylation, have been shown to be inhibitory to *P. falciparum* *in vitro* (Vial *et al.*, 1984a; Ancelin *et al.*, 1985; Ancelin and Vial, 1986). However, because of their potential *in vivo* host toxicity, such an approach leaves much to be desired.

We (Maguire and Sherman, 1990; Maguire *et al.*, unpublished) have shown unique and dramatic alterations in the distribution of PL in the erythrocyte membrane of red cells infected with *P. falciparum*. Using high parasitaemias and purified host cell membranes, after treatment of intact cells with phospholipase A₂ of sphingomyelinase, we were able to demonstrate not only an increased exposure of PE, but also a decreased accessibility and/or reactivity of SM and PC in the outer leaflet of the bilayer. In addition, using an anti-PS monoclonal antibody, as well as a sensitive prothrombin to thrombin assay system, PS exposure on the surface of the infected cell could be shown. The changes in SM and PS were correlated with parasitaemia as well as degree of maturation of the intracellular parasite. Although no differences were found in the content of PE, and there were only small changes in the PC and PS contents, the SM and cholesterol contents of red cell membranes from both trophozoite- and schizont-infected cells were sharply reduced. Infected cells also had a reduced cholesterol exchange rate when compared to uninfected erythrocytes. These changes in the erythrocyte membrane of the malaria-infected cell could affect transport,

antigenicity, endothelial adhesiveness, reactivity with phagocytes, and susceptibility to polyethylene and virus-induced fusion (see also Chapter 31).

ERYTHROCYTE INVASION

Invasion of a red cell by the malaria parasite follows a definite sequence: recognition and attachment by the merozoite stage to specific receptors on the erythrocyte surface; junction formation between the merozoite apical region and the red cell membrane; discharge of the contents of the apical organelles (rhoptries and micronemes); invagination of the erythrocyte membrane to enclose the merozoite within a vacuole; and resealing of the red cell membrane after parasite entry is complete, thereby re-establishing continuity of the erythrocyte membrane (see reviews by Pasvol, 1984; Breuer, 1985; Mitchell and Bannister, 1988; Hadley and Miller, 1988; Perkins, 1989).

Recognition

The putative receptors on human red cells for three species of *Plasmodium* have been described. For *P. knowlesi* and *P. vivax*, the major receptor appears to be the Duffy glycoprotein (Barnwell *et al.*, 1989; Wertheimer and Barnwell, 1989) and, for *P. falciparum*, the sialic acid rich glycophorins appear to serve as the principal receptors. However, recently, sialic acid independent invasion has been shown for a variety of strains of *P. falciparum* and, under the appropriate circumstances, merozoites of *P. falciparum* can invade mouse erythrocytes (Klotz *et al.*, 1987) with glycophorins different from those of human red cells. *P. knowlesi*, which ordinarily does not invade Duffy-negative red cells, will do so after chymotrypsin treatment. Thus, there appear to be primary as well as secondary receptors involved in merozoite invasion.

Putative merozoite surface proteins involved in binding to the red cell receptor are reported to be: for *P. falciparum*, a glycophorin binding protein ((GBP, also called gp130 and Pf200, also called gp195 and an erythrocyte binding protein Pf175 (Sim, 1990)); for *P. vivax*, a 135 kDa protein; and for *P. knowlesi*, two proteins of 135 and 155 kDa (see Perkins, 1989). The proteins involved in sialic acid independent invasion, as well as those of the mouse erythrocyte, have not been identified, but Pf200 appears not to be involved, since this protein does not bind to mouse red cells. Prior to 1988, it was claimed that the GBP was the primary *P. falciparum* merozoite ligand. However, recent evidence suggests that gp130 does not serve to recognize the sialic acid containing receptor. This protein is secreted by schizonts (Bianco *et al.*, 1987); it adheres non-specifically to a blank column of amino ethyl-Bio Gel, i.e. lacking coupled glycophorin (van Schravendijk *et al.*, 1987), and it binds only weakly to human red cells; furthermore, its binding to red cells is not affected by neuraminidase. Thus, GBP is neither an n-acetylneurameric acid dependent binding protein (Perkins and Holt, 1988), nor does it recognize the surface exposed regions of glycophorin (Perkins, 1989).

Junction formation

The biochemical events involved in junction formation are unknown but electron microscopic observations shows adhesion via short (*c.* 4 nm) filaments at the apical prominence-red cell surface (Bannister and Mitchell, 1988; Aikawa, 1988). During this phase, intramembranous particles (IMPs) in the red cell membrane become closely aggregated in the area of merozoite attachment, forming a dense junction.

Apical organelles

Three types of membrane-bound organelles are found at the apical end of the merozoite: rhoptries, micronemes and microspheres. Rhoptries are paired, teardrop-shaped organelles that are believed to be attached to smaller, elongated membrane-bound organelles called micronemes, and these form an interconnected complex. Rhoptries and micronemes contain a granular material; they give a positive reaction for protein and stain with basic dyes. When merozoites are fixed in the presence of tannic acid or ruthenium red the rhoptry tips show membranous whorls and, with glutaraldehyde fixed samples, blebs containing membranous lamellae appear at the apex (Bannister and Mitchell, 1988). The precise chemical nature of these lamellar structures is not known, but they have been suggested to be lipidic. Indeed, Mikkelsen *et al.*, (1988) claimed that invasion and formation of the parasitophorous vacuolar membrane involved the insertion of merozoite phospholipids derived from the apical organelles.

A large number of antigens have been assigned to the rhoptry on the basis of antibody reactivity (Aikawa and Atkinson, 1990). For example, *P. falciparum* has two families of rhoptry proteins: a complex of 140/110/105 proteins (Coppel *et al.*, 1987; Sam-Yellowe and Perkins, 1990), and antigens of 82, 65 and 40kDa (Crewther *et al.*, 1990; Ridley *et al.*, 1990). Antibodies that react with these antigens often inhibit invasion *in vitro* and, therefore, these antigens are vaccine candidates. The precise biochemical role of rhoptry of microneme proteins, and what triggers their release, is unknown. Another antigen of *P. falciparum*, Pf155, is transferred from the merozoite to the red cell surface and is clearly in evidence when cells contain ring-stage parasites. For this reason, it has been called RESA (ring infected erythrocyte surface antigen). RESA has been localized in micronemes of the merozoite as well as in the membrane of ring-stage infected erythrocytes (Brown *et al.*, 1985; Kemp *et al.*, 1990).

The gene for a 41 kDa polypeptide, localized to the rhoptry, has been cloned, sequenced and expressed in *E.coli* (Knapp *et al.*, 1990). The translation of this gene was initiated as a UAG stop codon (Ghersa *et al.*, 1990). The 41 kDa protein has been identified as aldolase and has been shown to be protective against a malaria infection in *Saimiri* monkeys (Perrin *et al.*, 1985; Certa *et al.*, 1988). Antisera against this protein specifically inhibited plasmodial aldolase activity and did not cross-react with human or rabbit muscle aldolases (Srivastava *et al.*, 1990).

By electron microscopy, Torri *et al.* (1989) have shown that the contents of the microspheres are discharged into the vacuolar space and this leads to the formation of channels from the parasitophorous vacuole. The significance of the channels is

not clear. However, it appears the microspheres are not involved in the initial invasion process.

Invagination

Invagination of the red cell surface is associated with an IMP-depleted parasitophorous vacuolar membrane; the lipid bilayer freed of cytoskeletal restraints flows into the depression leaving the IMP around its rim as the junction. Red cell ghosts can be depleted of as much as two-thirds of their haemoglobin and still sustain invasion. However, when the ATP level of red cell ghosts drops below a critical level, or cytoskeletal proteins are cross-linked, merozoite invasion is inhibited (summarized by Rangachari *et al.*, 1989). Since merozoites treated with cytochalasin B attach but do not invade (Miller *et al.*, 1979), it has been suggested that entry requires merozoite motility and that invasion is not a process driven solely by red cell membrane movement (reviewed by Mitchell and Bannister, 1988). Proteinases have also been shown to be involved in merozoite invasion of the erythrocyte and this is described in Chapter 25.

The parasitophorous vacuolar membrane (PVM)

The PVM has not been isolated from any species of *Plasmodium*. What little we know of this membrane is that: it enlarges with the intracellular growth of the parasite; it differs in its IMP composition and in its reactivity with cationized ferritin (CF); and it is lacking in the red cell membrane proteins, spectrin, glycophorin, band 3 and ankyrin (Sherman *et al.*, 1988; Dluzewski *et al.*, 1989). The vacuolar space, but not the PVM itself, contains several antigens, such as Pf 101 (=ABRA) and Pf 126 (Coppel *et al.*, 1988; Delplace *et al.*, 1988; Kara *et al.*, 1988, 1990; Weber *et al.*, 1988), whereas, Pf 19 is both within and around vesicles of the PVM. Maurer's clefts are membranous vesicles found in the erythrocyte cytoplasm of the *P. falciparum* infected cell and form as evaginations of the PVM. A 46kDa antigen, localized to the membrane of Maurer's clefts, has recently been described (Hui and Siddiqui, 1988; Etzion and Perkins, 1989). Since these vesicles have been suggested to be involved in the trafficking of proteins from the plasmodium to the erythrocyte surface (but this is by no means proven!), antigen markers such as these could be invaluable in the isolation and characterization of Maurer's clefts and the other membrane-bound vesicles.

MEMBRANE PROTEINS

Biochemical research in the 1940s was devoted primarily to enzymology and the deciphering of metabolic pathways; the 1950s and 1960s began an era that concerned itself with informational molecules; and, in the 1970s, there was a shift in emphasis toward membranes. Since the early 1980s, research activity in malaria 'membranology' has been explosive and an overview such as this cannot begin to

deal effectively with such a wealth of information. However, the findings on the structure and function of the membrane proteins of both the red cell and the parasite have been reviewed during the past decade. Those interested in this area should read: Howard (1982, 1987, 1988, 1989); Howard and Barnwell (1984); Sherman (1985); Hommel and Semoff (1988); Trigg (1988); Howard and Gilladoga (1989); and Jungery and Griffiths (1989).

GENES AND ANTIGENS

During the past decade, molecular biologists have studied the genes of several species of *Plasmodium*, but most notably those of *P. falciparum*, in order to obtain quantities of materials that might serve as the basis of a vaccine, to produce species-specific diagnostic tools, to examine key enzymes in order to design better chemotherapeutic agents and/or to provide for a clearer understanding of drug resistance. It is beyond the scope of this overview to treat the wealth of information that has been acquired on the structural characteristics of the genes encoding malaria antigens. However, for those interested in this subject, there are several review articles that summarize this rapidly developing field of study: Kemp *et al.* (1986, 1987, 1990); Scaife *et al.* (1986); and Anders and Smythe (1989). Parenthetically, it should be noted that, although a bewildering array of antigens, most being referred to by their acronym (e.g. ABRA, GARP, TRAP, SERA, MESA, RESA, FIRA, SHARP, Pf, EMP, KAHRP, S, CS, PMMSA and HSP), exists, only a few have had their function defined.

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3. *Pneumocystis carinii*: potential targets for chemotherapeutic attack

W.E.Gutteridge

INTRODUCTION

As in any other parasite, potential targets for chemotherapeutic attack in *Pneumocystis carinii* can be identified from two types of experimental investigation:

1. studies on the biochemical mode of action of compounds active clinically or even only experimentally; and
2. analysis of the comparative metabolism of the organism and its mammalian host.

In this review (literature surveyed to July 1990), I first provide key background information on the biology of the organism. I then deal in turn with the above approaches. Finally, in the light of this information, I present a list of potential targets and comment on the controversial taxonomic position of *P. carinii*. More detailed information on the biology of *P. carinii* can be found in Hughes (1987b).

BIOLOGY

Introduction

The protozoan that was later to be called *P. carinii* was first described by Chagas (1909) in the lungs of guinea-pigs and humans and by Carini (1910) in the lungs of rodents. Both investigators described the structures they observed as being stages in the life cycles of trypanosomes. It was not until later that Delanoe and Delanoe (1912) produced evidence that these forms in fact represented a species in its own right and called it *Pneumocystis carinii*. Later still, it emerged that latent infections of this parasite occur frequently in the majority of apparently healthy mammals (reviewed in Matsumoto and Yoshida, 1986).

Aetiology

Vaneck and Jirovec (1952) were the first to establish the aetiological relationship of *P. carinii* to pneumonitis, in their case interstitial plasma cell pneumonitis of premature and marasmic infants. *P. carinii* pneumonitis (PCP) has an almost 100 per cent mortality rate if untreated. It is now known to be likely to occur in man in a number of high-risk groups (Matsumoto and Yoshida, 1986):

1. babies born prematurely and children suffering from malnutrition;
2. children with primary immunodeficiency disorders;
3. patients of all ages receiving immunosuppressive agents for the treatment of various cancers; and
4. patients with acquired immune deficiency syndrome (AIDS).

The vast majority of those suffering from PCP today are AIDS patients. More than half of these will succumb to this disease, unless they are given chemoprophylaxis (Matsumoto and Yoshida, 1986; Hughes, 1987a). Because PCP has only come to prominence with the arrival of AIDS, its biological data base and that on the organism that causes it are still fragmentary.

Morphology

What is believed to be the main vegetative form of *P. carinii*, the so-called trophozoite, is 2–5 µm in diameter and highly pleomorphic. Trophozoites often occur in clumps. Light microscopy shows little detail of structure; e.g. in Giemsastained preparations, only a dark purple nucleus and a light blue cytoplasm can be seen. Transmission electron microscopy reveals a eukaryotic type of cellular organisation (Ruffolo *et al.* 1989; Dei-Cas *et al.*, 1989). There is a single nucleus with nucleolus and pores in the nuclear membrane. Mitochondria with well developed cristae can be seen, together with rough endoplasmic reticulum and structures which might be lipid and glycogen granules. The presence of cytoplasmic microtubules and microfilaments and of lysosomes has not been clearly established. The outer pellicle is thin (20–30 nm) and consists of a plasmalemma and an electron dense outer layer. Many tubular expansions arise from it, often described as filopodia.

The cyst stage is usually spherical, 4–6 µm in diameter and has a thick pellicle (70–140nm) consisting of plasmalemma, an electron lucent middle layer and an electron dense outer layer. Within the mature cysts, there are eight intracystic bodies, initially spherical in shape, each with a nucleus and mitochondria (Ruffolo *et al.*, 1989; Dei-Cas *et al.*, 1989).

Freeze fracture studies indicate that the density of intramembranous particles is lower in the cysts than in the trophozoites, a feature which has been interpreted as being indicative of a resting stage (Yoshikawa and Yoshida, 1989).

Location pathology

Organisms are found usually only in the lungs, though disseminated infections have been described, especially in AIDS patients on aerosolized pentamidine

prophylaxis. These tend to involve the lymph nodes, bone marrow, spleen and liver, in that order, but other organs are sometimes involved (Telzak *et al.*, 1990).

In low level infections, trophozoites grow in close adherence to, rather than intracellularly in, the type 1 pneumocytes of the alveolar epithelial lining. It is not clear whether this feature serves as a mechanism of anchorage or nutrition or both. As the infection develops, more alveoli become involved and eventually many of them become filled with organisms and cell debris. By now, cysts are also present, there being about 1 for every 10 trophozoites. The presence of parasites in large numbers leads to alveolar lining hypertrophy and increased prominence of alveolar macro-phages (Hughes, 1987b). As a consequence of all these changes, severe lung dysfunction occurs, resulting, if untreated, in death.

Life cycle/transmission/serology

This is not known in detail, though many hypotheses have been put forward. The simplest suggestion involves the trophozoite obligatorily becoming a cyst in which eight intracystic bodies are produced by a process akin to protozoal schizogony and from which ultimately eight new trophozoites emerge through a pore, leaving behind a collapsed and near empty structure (Vanek and Jirovec, 1952). A more complex version allows binary fission or budding of trophozoites and thus direct replication of this stage, as well as cyst formation (Campbell, 1972). Most elaborate of all are schemes involving sexual reproduction (Matsumoto and Yoshida, 1986). There is now some evidence for binary fission of trophozoites (Richardson *et al.*, 1989) and there is no evidence for genetic recombination or exchange, so that it begins to look as if the more complex version is correct.

Transmission from animal to animal (including man to man) is believed to occur solely by the airborne route and to most likely involve the cyst stage (Hughes, 1987b). Congenital transmission is not likely to be of great significance, though it does possibly occur in some instances. The trophozoite is most likely to be involved here. There may be barriers to the transmission of *P. carinii* between different species of mammal.

Serological studies of children have revealed that nearly 100 per cent of normal children have acquired antibodies to *P. carinii* during the first 2 years of life (Meuwissen *et al.*, 1977; Pifer *et al.*, 1978). Natural and presumably asymptomatic infection seems, therefore, to be universal. Immunosuppression, for whatever reason, provokes residual latent infection or allows reinfection. Which is the subject of current investigation.

Diagnosis

This is made principally on the basis of chest radiograph, coupled with histopathological demonstration of the presence of cysts in specimens obtained by bronchial lavage or induced sputum (Hughes, 1987b; Hopkin and Wakefield, 1989). The latter usually involves the use of Gomori's methamine silver nitrate stain, though more recently immunofluorescent antibodies have been described, and an

immunofluorescence antibody test (IFAT) kit has been developed (Northumbria) which appears to have greater sensitivity and specificity than the silver stain method. Serum antibody and antigen detection tests are not thought to be reliable (Walzer *et al.*, 1987). DNA probes, to be used either on their own or with polymerase chain reaction (PCR) amplification, are in development (Wakefield *et al.*, 1988).

Culture

P. carinii cannot be cultured serially in the laboratory, even in the presence of mammalian cell lines. The systems now available mostly use an inoculum obtained from rat lung. The size of the inoculum is not well defined because of the difficulty in estimating trophozoite numbers, but appears to be large. Commonly used media are M199 or Eagles MEM, together with fetal calf serum. Many cell lines have been used. Popular ones are WI-38, MRC-5, Vero, Chang liver, AH-1, and CEL. Organisms seem to prefer highish carbon dioxide and lowish oxygen tensions (Smith and Bartlett, 1984). Such systems support some initial multiplication of the parasite, but give little or no growth on subsequent transfer (Trager and Daggett, 1989). They thus resemble malaria parasite cultures of pre-1976. Nevertheless, they are of value, even now, especially in providing systems for assessing the effects of potential chemotherapeutic agents. Effects have mostly been monitored by direct counts either of parasites, which is difficult to do accurately because of parasite clumping, or of their nuclei (Smith and Bartlett, 1984). A potentially valuable way of avoiding this problem has been described recently: the use of tritiated *p*-aminobenzoate incorporation into folates, since such incorporation is not affected by the presence of contaminating host cellular or enzymatic material (Kovacs *et al.*, 1989).

In vivo models

The standard here is the immunocompromised rat model, first developed by Weller (1955). This uses dexamethasone to immunosuppress the rats and hence provoke latent infections. The limitations of this model are the size of the rats (200g initially), which is a problem in experimental chemotherapy since a large quantity of drug is required, adverse reactions to dexamethasone, the need to co-administer tetracycline to prevent overwhelming bacterial infections in rats, which might potentiate/antagonize experimental compounds, and the lack of non-invasive means for monitoring development of the infection. Nevertheless, the model is widely used in experimental chemotherapy and, increasingly, as in our own laboratory, provides a source of material for biochemical investigation. It has proved particularly useful in predicting experimentally the likely efficacy of compounds in the clinic (Hughes, 1987a).

As indicated above, most mammals are infected naturally with *P. carinii* so that it is perhaps surprising that no other models are used routinely in the laboratory. In most cases, this is because infection does not develop sufficiently and/or it is too variable, animal to animal, to be useful. This is very much the case with mice, where even nudes have proved disappointing (Walzer, 1984). There are reports, however, to suggest that *scid/scid* mice are very susceptible to *P. carinii* (Schultz *et al.*, 1989).

Isolation techniques

Many methods have been published for the isolation of *P. carinii* from infected (usually rat) lung (Walzer, 1984). Mostly these methods involve homogenization, coarse filtration to remove large debris, enzymatic digestion to reduce the size of small debris, and filtration and/or density gradient centrifugation (especially Percoll) to separate out the organisms. There are two main problems with these methods. The first relates to the fact that the product is a mixture of trophozoites and cysts. The second relates to contamination with host, especially cellular, material. Because host cells are more than an order of magnitude larger than trophozoites and we do not know their metabolic rates relative to *P. carinii*, levels of contamination as low as 1 per cent (cell/cell) are unacceptable, although they are often used. A recent breakthrough involves the use of unit gravity rather than density gradient sedimentation (Taylor and Easmon, 1990). In our experience (Stubberfield and Gutteridge, unpublished), this routinely yields levels of contamination substantially below the critical 0.1 per cent value.

CHEMOTHERAPY

Introduction

Large numbers of compounds, especially drugs developed originally for other therapeutic indications, have been tested experimentally, usually *in vivo* in the rat model, and/or clinically for activity against *P. carinii*. Most of these compounds showed no impact on the course of the disease (see Hughes, 1987b for list). A number have, however, been identified as active (see Table 3.1). Some of these are used in the clinic, including cotrimoxazole (trimethoprim+sulphamethoxazole) and pentamidine, which are the mainstays of current efforts to control PCP in man.

Table 3.1. Compounds with activity against *P. carinii*.

Chemical series	Examples	References
Diamidines	Pentamidine	Hughes (1987a, 1987b)
	Diminazine	Jones <i>et al.</i> (1990) Tidwell <i>et al.</i> (1990) Walzer <i>et al.</i> (1988)
Quinaldines	Quinapyramine	Walzer <i>et al.</i> (1988)
2,4-Diaminopyrimidines	Trimethoprim Pyrimethamine	Hughes (1987a, 1987b)
Folate analogues	Trimetrexate Piritrexim	Queener <i>et al.</i> (1987)
Sulphonamides	Sulphamethoxazole Sulphadoxine	Hughes (1987a, 1987b)
Sulphones	Dapsone	Hughes and Smith (1984)
Sulphonylureas	Carbutamide	Hughes and Smith-McCain (1986)
Sulphone derivatives	4,4-Sulphonyl-bisformanilide	Hughes <i>et al.</i> (1986)
Ornithine analogues	α -Difluoromethylornithine	Clarkson <i>et al.</i> (1988)
Purine analogues	9-Deazainosine	Smith <i>et al.</i> (1987)
Hydroxynaphthoquinones	566C80	Hughes <i>et al.</i> (1990)
8-Aminoquinolines	Primaquine	Queener <i>et al.</i> (1988)
Lincomycin analogues	Clindamycin	Queener <i>et al.</i> (1988)

Analysis of the biochemical mode of action of such compounds provides, at least in theory, a reliable means of identifying potential targets for chemotherapeutic attack; by definition, inhibition of the functioning of such targets blocks the replication of the organism and screening in cell-free assays of them represents a potentially rapid means of identifying novel chemical leads with which to attack them. The problem of applying this approach to *P. carinii* is that little is known about antimicrobial modes of action of the compounds listed in Table 3.1 in general, let alone their specific effects on *P. carinii* itself. What is known is summarized in the following sections.

Diamidines

One of the best examples of a chemical series where little is known about its mode of action is the aromatic diamidines, even though representatives of the series have been in use as trypanocides for over 40 years. Nothing is known about their effects on *P. carinii*; their antitrypanosomal action is still far from resolved. They appear to exert a much more marked effect on the biosynthesis of DNA, RNA, protein and phospholipid than they do on catabolism (Gutteridge, 1969), but whether such effects are primary is still not clear. All aromatic amidines are positively charged at physiological pH and, as such, bind readily to DNA, though not apparently by an intercalative mechanism (Newton, 1974). Such binding would explain their effects on nucleic acid synthesis, but not why the synthesis of kinetoplast (mitochondrial) DNA is particularly sensitive to disruption (Newton, 1974). The antitumour effects of pentamidine also appear to relate to effects on biosynthesis, though the inhibitory concentrations used were very high ($>2\text{mg ml}^{-1}$) and the authors interpreted their data as indicating specific effects on nucleotide metabolism (Bornstein and Yarbro, 1970). There have been suggestions that these compounds interfere with polyamine synthesis (see Chapter 43).

Quinaldines

Quinapyramine is used as a cattle trypanocide. In trypanosomatid flagellates, it blocks protein synthesis, seemingly by displacing magnesium ions and polyamines from the cytoplasmic ribosomes (see Newton, 1974). Its action against *P. carinii* has not been investigated.

2, 4-Diaminopyrimidines and folate analogues

There are hard data to indicate that the antibacterial and antimalarial action of trimethoprim and pyrimethamine and the antitumour action of trimetrexate and piritrexim are all mediated through inhibition of the enzyme dihydrofolate reductase (see Franklin and Snow, 1989). Data have been obtained with partially purified (Allegra *et al.*, 1987) and genetically engineered (Edman *et al.*, 1989c) *P. carinii* dihydrofolate reductase to suggest that such effects are likely to occur in this organism also. Note, however, that the K_i of the *P. carinii* enzyme for trimethoprim

is *c.* 10 μM , a high figure compared with those for its bacterial and malarial counterparts (*c.* 10 nm). This suggests that the trimethoprim in cotrimoxazole does not contribute much to the anti-PCP activity of the mixture. There is experimental evidence that this is the case (Hughes, 1987a).

Sulphonamides, sulphones, sulphonylureas and sulphone derivatives

These compounds are considered together since it seems reasonable to suppose that they are all inhibitors of dihydropteroate synthase, though the sulphonylureas and sulphone derivatives might have to be metabolized first to the active species. Certainly there are good data to support this conclusion as far as the antibacterial and antimalarial effects of the sulphonamides and the sulphones are concerned (Franklin and Snow, 1989). There are also recent data to suggest that the *P. carinii* enzyme is sensitive to inhibition by dapsone and sulphamethoxazole, though the K_i values (9 and 59 μM respectively) are higher than might have been anticipated (Merali *et al.*, 1990). For more information on the activities of these compounds and 2, 4-diaminopyrimidines see Chapter 51.

Ornithine analogues

Biochemical studies on the effects of α -difluoromethylornithine (DFMO) on trypanosomes have shown that it works by blocking polyamide biosynthesis at the rate-limiting ornithine decarboxylase step (Bacchi *et al.*, 1980: and see Chapters 43 and 46). Unlike mammalian ornithine decarboxylase, which has a rapid turnover, the trypanosome enzyme lacks the amino acid sequence referred to as PEST and turns over relatively slowly. As a result, DFMO, an irreversible inhibitor, permanently blocks the trypanosome ornithine decarboxylase reaction (Phillips *et al.*, 1987). There is evidence that DFMO works in the rat model of PCP and in man (see Table 3.1 for references), but, surprisingly, Persanti *et al.* (1988) were unable to detect ornithine decarboxylase activity in cell extracts.

Purine analogues

Against trypanosomes and leishmania, compounds such as 9-deazainosine appear to work, after phosphorylation to nucleotides, by blocking purine interconversion reactions and/or nucleic acid synthesis (Marr *et al.*, 1984; and see Chapter 48). There is no information on how they work against *P. carinii*.

Hydroxynaphthoquinones and 8-aminoquinolines

Hydroxynaphthoquinones act as antiprotozoal agents by blocking respiratory chainlinked electron transport at complex III (Fry *et al.*, 1984), probably by functioning as analogues of ubiquinone (Gutteridge and Coombs, 1977; and see Chapter 13). In malaria, since the respiratory chain is not significantly involved in energy metabolism, the major consequence of inhibition is blockage of pyrimidine

bio-synthesis *de novo* (Hammond *et al.*, 1985)—the dihydroorotate dehydrogenase step of that pathway is intimately linked to the respiratory chain (Gutteridge *et al.*, 1979). There is as yet little evidence that hydroxynaphthoquinones work against *P. carinii* in a similar fashion. However, it has been noted that the IC₅₀ for 566C80 against an oxygen consumption of unit gravity-purified preparations of *P. carinii* (43 nM), suggesting a primary effect on oxygen-utilizing, cyanide-sensitive reactions (Stubberfield and Gutteridge, unpublished).

Antimalarial 8-aminoquinolines such as primaquine are believed to be metabolized to products including 5, 6-quinoline diquinone, which have structural similarities to hydroxynaphthaquinones. It has been suggested that these metabolites are also ubiquinone analogues which act by disrupting the respiratory chain of the parasite (Gutteridge and Coombs, 1977). Such a mechanism could carry over to *P. carinii*.

Lincomycin analogues

As an antibacterial, clindamycin works by blocking protein synthesis at the ribosome level. The lincomycin-binding site is on the L15 ribosomal protein of the 50S ribosomal subunit. It is proposed that the analogues work by stimulating the dissociation of peptidyl-tRNA from ribosomes, possibly during an attempted translocation step, thereby interrupting the competition of the peptide chain (Franklin and Snow, 1989; and see Chapter 52). There is no information on their action against *P. carinii*.

BIOCHEMISTRY

Introduction

Little information is available about the biochemical structure and metabolism of *P. carinii* and what is known has all too often been derived from isolated preparations of mixtures of trophozoites and cysts of dubious purity. My task in preparing this section felt like trying to put together two jigsaw puzzles from a mixture of the two with most of the pieces missing and an indeterminant number of those actually present belonging to a third puzzle!

Nucleic acids

Quantitative fluorescence microscopy using 4', 6-diamidino-2-phenylindole (DAPI) has indicated that *P. carinii* contains between one and two units of DNA per nucleus, with peaks of distribution at one and two units. Similar studies with mature cysts containing eight intracystic bodies showed the presence of eight times more DNA than in trophozoites (Yamada *et al.*, 1986). A method has been developed to isolate DNA from *P. carinii*. Analysis of this indicated a DNA content per nucleus of 0.22–0.34 pg, well within the range seen in other parasitic protozoa, but an order of magnitude higher than that seen in fungi (Gradus *et al.*, 1988). Isolated *P. carinii*

DNA has been characterized by melting point analysis and shown to have a G+C content of 33 per cent (Worley *et al.*, 1989). There is no information on the buoyant density of the DNA and, therefore, on whether or not unusual bases may be present and no information on the likely presence of a mitochondrial DNA fraction.

Initial attempts using pulsed-field gel electrophoresis to determine the karyotype of *P. carinii* suggested 16–20 chromosome bands ranging in size from 320–1500 kb, indicating a total genome complexity of 800–16000 kb (Yoganathan *et al.*, 1989). Ribosomal RNA and histone genes were located on two distinct chromosomes. More detailed investigation suggested the presence of 13 chromosomes ranging in size from 300–700 kb, giving a minimum genome size of 7000 kb. Genetic heterogeneity among different *P. carinii* isolates was documented by demonstration of chromosomal size variability. By hybridization studies, the genes for topoisomerase I, dihydrofolate reductase, rRNA, actin and thymidylate synthase were mapped to single chromosomes of about 650, 590, 550, 460 and 350 kb (Lundgren *et al.*, 1990).

A restriction map of the cloned rRNA gene of *P. carinii* has been published (Edman *et al.*, 1989a). This gene is ultimately transcribed into three rRNA species of 18S, 5.8S and 26S. A novel feature of the gene is the presence of a 390 bp intron 31 nucleotides from the predicted 3' end of the small subunit rRNA coding sequence (Sogin and Edman, 1989).

The sequence of the 16S (18S) rRNA of *P. carinii* has been published and interpreted as indicating a close phylogenetic relationship to mainstream fungi (Edman *et al.*, 1988). Similar conclusions have been drawn by Stringer *et al.* (1989) and Cushion *et al.* (1989). In contrast, sequencing studies by Watanabe *et al.* (1989) on 5S (5.8S) rRNA sequences suggest a closer phylogeny to the 'Rhizopoda/Myxomycota/Zygomycota' group of fungi, rather than to common fungi such as Ascomycota and Basidiomycota or to protozoa.

Carbohydrates and proteins

Studies with colloidal gold-labelled concanavalin A have demonstrated binding sites for this lectin on the electron dense outer layer of the pellicle of the trophozoite (Yoshikawa *et al.*, 1988). Experiments with a panel of fluorescein isothiocyanate conjugated lectins used by Cushion *et al.* (1988) indicated that both trophozoites and cysts have mannose, *N*-acetylglucosamine and *N*-acetylgalactosamine as predominant surface carbohydrates. No evidence for sialic acid or β -galactose was found. Use of biotin conjugated lectins, followed by reaction with avidinperoxidase, was used to demonstrate the presence of glycoproteins on the surface of whole organisms (Persanti and Shanley, 1988). A number were detected which behaved electrophoretically quite distinctly from those of rat cells. Detailed analyses of these have indicated that the major surface glycoproteins of *P. carinii* are in the range 105–120 kDa and that they are mannose rich. Variation in their electrophoretic mobilities may reflect degrees of glycosylation. The majority of sera tested from humans with diagnosed PCP reacted strongly with these glycoproteins (Tanabe *et al.*, 1989; Radding *et al.*, 1989).

It would seem from the above that one component of the electron dense outer layer of the *P. carinii* pellicle is mannose rich glycoprotein. Another, both in trophozoites and cysts, is probably chitin ($\beta(1, 4)$ -linked *N*-acetylglucosamine residues) (Walker *et al.*, 1990). There is some evidence that the electron-lucent middle layer of the cyst contains $\beta(1, 3)$ -glucan since this can be digested by (partially-purified) $\beta(1, 3)$ -glucan laminaripentohydrolase (Matsumoto *et al.*, 1989). Both these polymers are typical components of the surface structures of fungi.

Lipids

The major ester linked fatty acids in the total lipids extractable from *P. carinii* isolated from rats were 16:0 (palmitate), 18:0 (stearate), 18:1 (oleate), 18:2 (linoleate) and 20:4 (archidonate). Others detected included 14:0 (myristate), 16:1 (palmitoleate) and 24:1. The major sphingolipid fatty acids were 16:0, 18:0, 22:0 (behenate), 24:0 (lingocerate) and 24:1. Others included 14:0, 18:1, 20:0 (arachidate), 23:0, 24:2 and 26:0 (Kaneshiro *et al.*, 1989). The total lipid fatty acid composition of the lipids extracted from control lung material were similar. The expected phospholipids are apparently present: phosphatidylglycerol, cardiolipin, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylinositol (Persanti, 1987). Freeze fracture localization of filipin-sterol complexes indicates the presence of sterol in the plasmalemma but does not indicate which sterol is present (Yoshikawa *et al.*, 1987; Yoshikawa and Yoshida, 1988). Since then, however, cholesterol has been identified as the major sterol present, contrasting with fungi where it is ergosterol (Kaneshiro *et al.*, 1989).

Metabolism

Although there is some ultrastructural evidence for glycogen, there is no biochemical information for the presence of it, or indeed any other energy reserve. Glucose can be catabolized to CO₂, and such metabolism is blocked by mannose, fructose and 2-deoxyglucose, suggesting that other sugars can be taken up and/or metabolized (Persanti and Cox, 1981). However, it is neither clear whether or not other substrates such as amino acids or fatty acids can also be utilized, nor what is the preferred substrate for catabolism. In this connection, the presence of a glutamate dehydrogenase, often associated with amino acid catabolism, might be significant (Mazer *et al.*, 1987). Lactate dehydrogenase activity has been detected (Mazer *et al.*, 1987; Taylor and Easmon, 1990), but it is unlikely that the organism is a homolactate fermenter, since succinate dehydrogenase (Mazer *et al.*, 1987) and the malic enzyme (Persanti, 1989) are present, possibly indicating a functional TCA cycle, and cyanide sensitive oxygen consumption occurs (Persanti, 1984). This last finding, together with the occurrence of cristate mitochondria, suggests the presence of a functional cytochrome containing respiratory chain. Glucose-6-phosphate dehydrogenase (G6PD) has been detected (Mazer *et al.*, 1987; Persanti, 1989); this might indicate a functional pentose phosphate shunt.

Organisms have some protection against oxygen mediated damage in that they make their own superoxide dismutase and catalase, though they probably do not

contain a glutathione peroxidase (Persanti and Cox, 1981; Persanti, 1989). However, the levels of catalase are very low, suggesting that such defences are minimal. In agreement with this suggestion is Persanti's (1984) finding that *P. carinii* is susceptible to the lethal action of hydrogen peroxide and superoxide, though not to a hydroxyl radical generating system.

On the anabolic side, uracil and uridine can apparently be salvaged and incorporated into nucleic acids (Persanti and Cox, 1981; Cushion and Ebbets, 1990). However, Persanti (1989) could not detect utilization of either hypoxanthine or thymidine, whereas Cushion and Ebbets (1990) could. Such differences may reflect on the different incubation/culture conditions used. Labelled amino acids are readily utilized for *P. carinii* specific protein synthesis and such synthesis is sensitive to cycloheximide but not tetracycline (Persanti and Cox, 1981; Cushion and Ebbets, 1990). *N*-Acetylglucosamine is also utilized (Cushion and Ebbets, 1990) and labelled choline and possibly acetate are incorporated into phospholipids (Persanti, 1989). It is considered unlikely that the organism can synthesize fatty acids or sterols *de novo*, though some chain elongation of existing fatty acids may occur (Kaneshiro *et al.*, 1989). Lack of sterol biosynthesis is consistent with the observed lack of sensitivity to ketoconazole (Hughes, 1989), which blocks sterol biosynthesis in other microbes (Franklin and Snow, 1989; and see Chapter 29).

Dihydrofolate is synthesized *de novo*: tritiated *p*-aminobenzoate incorporation into folates has been demonstrated (Kovacs *et al.*, 1989), as has been the presence of the enzyme dihydropteroate synthase (Merali *et al.*, 1990), and the organism is sensitive to sulphonamides and sulphones (see above). The dihydrofolate reductase-deficient gene has been cloned and expressed in a dihydrofolate reductase *Escherichia coli* (Edman *et al.*, 1989c). The genomic sequence predicts a protein of 206 amino acids with an M_r of 23868. The gene is not physically linked to thymidylate synthase, unlike in protozoa generally; indeed it is located on a different chromosome. Its sequence has most homology with fungal (yeast) dihydrofolate reductase. Detailed kinetic parameters for the enzyme have been published (Kovacs *et al.*, 1990). The thymidylate synthase gene has also been isolated and expressed in *E. coli* (Edman *et al.*, 1989b). The gene encodes 297 amino acids with a predicted M_r of 34269. The deduced amino acid sequence is similar to the isofunctional enzymes from most other organisms; it is closest to yeast (65 per cent homology).

Clearly, the metabolic map of *P. carinii* is very far from complete. However, what is already clear is that the organism shows every sign of being metabolically competent: the close physical relationship that it has with type I pneumocytes is likely to be significant only in the contexts of anchorage and/or nutrition.

CONCLUSIONS

Targets

On the basis of what is known about the biochemistry of *P. carinii* and the biochemical mode of action of compounds active against it, an initial list of 'possible'

targets can be constructed along the lines of the one shown in Table 3.2. Not all the entries in the table represent serious targets:

1. neither lactate dehydrogenase nor succinate dehydrogenase are likely to be of great significance in an organism which apparently has an active respiratory chain;
2. actin, histones, glucose-6-phosphate dehydrogenase and phospholipid synthesis have not yet been established as viable targets for clinically useful chemotherapy in any organism; and
3. thymidylate synthase inhibition could be bypassed by the salvage of thymidine, likely to be present at high levels in lungs full of the products of cell lysis.

Those that remain on the list after such analysis represent 'potential targets' (Table 3.2). Note that the list is far from complete. It will not be possible to extend the list until further biochemical data on the organism are available. This is urgently required if real progress is to be made in developing novel therapies specifically for PCP.

Table 3.2. Chemotherapeutic targets in *P. carinii*.

Target	Possible	Potential	Proven
<i>Macromolecules</i>			
DNA (especially mtDNA)	T	T	T
Surface glycoproteins	T	T	
Cholesterol	T	T	
Ribosome	T	T	T
Histones	T		
Actin	T		
<i>Enzymes/enzyme systems</i>			
β 1,3-Glucan	T	T	
Chitin	T	T	
Glycolysis	T		
Lactate dehydrogenase	T		
Succinate dehydrogenase	T		
Electron transport	T	T	T
G6PD	T		
Purine metabolism	T	T	
Topoisomerase I	T	T	
Dihydrofolate reductase	T	T	
Dihydropteroate synthase	T	T	T
Thymidylate synthase	T		
Ornithine decarboxylase	T	T	T
Phospholipid biosynthesis	T		
<i>Other approaches</i>			
Oxygen radical generation	T	T	

T, target.

It is of course possible to select from the list a set of 'proven' targets by pulling out those where the therapeutic experience with *P. carinii* already suggests that drug interaction is a cidal or static event (Table 3.2):

1. DNA (if indeed it is the diamidine target);
2. ribosome;

3. electron transport;
4. dihydrofolate reductase; and
5. dihydropteroate synthase.
6. ornithine decarboxylase

These may not, however, represent the best targets for novel chemotherapies. The best clinical means for disrupting them may already have been identified. If resistance with the existing therapies were ever to become a problem, which is likely now that primary and secondary prophylaxis figures predominantly in the management of AIDS patients, cross-resistance would be a problem for the novel compounds too. The 'potential' targets listed in Table 3.2 probably represent the best compromise between the 'possibles' and the 'provens'.

Taxonomy

Consideration of the above information about the biochemistry and chemotherapeutic sensitivity of *P. carinii* highlights the enigma of its taxonomic position. Most of the gene and protein sequence data suggest closer affinities to fungi than to protozoa; the pattern of drug sensitivity suggests the reverse (Table 3.3). The bottom line surely is that *P. carinii* is a taxonomic unit in its own right, which has probably evolved independently from a common ancestor of the fungi and the protozoa. Students of *P. carinii* research would do well to bear this in mind and be prepared for either eventuality. This applies even to those interested in chemotherapy. While it is true at present that all the existing clinical therapies have their origins as antiprotozoals, this may not always be true (see Schmatz, 1990).

Table 3.3. Taxonomy of *P. carinii*.

Fungal characteristics	Protozoal characteristics
18S rRNA sequence	DNA content
5.8S rRNA sequence	Cholesterol
β -(1,3)-Glucan	Size of rRNA species
Chitin	Sensitivity to: diamidines, quinaldines, 2,4-diaminopyrimidines, sulphonamides/sulphones, DFMO, 566C80, 9-deazoinosine, primaquine/clindamycin
Separate DHFR/TS genes	Lack of sensitivity to: amphotericin B, ketoconazole
DHFR sequence	
TS sequence	

DHFR, dihydrofolate reductase; TS, thymidylate synthase; DFMO, α -difluoromethylornithine.

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4. *Giardia* cysts: their biochemistry and metabolism

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INTRODUCTION

Ribosomal gene sequence comparisons suggest that one of the most primitive eukaryotic cells studied to date belongs to the genus *Giardia* (Sogin *et al.*, 1989). *Giardia* has ribosomal genes which much more closely approximate the sizes of those in prokaryotes than those in eukaryotes (Sogin *et al.*, 1989), and they lack mitochondria, microbodies and a detectable Golgi apparatus (Friend, 1966). These apparently ancient flagellates have successfully parasitized a wide variety of vertebrates including humans (Meyer and Jarroll, 1980). In humans, giardiasis is considered one of the most important water-borne infectious diseases (Craun, 1986).

Giardia exhibits a simple and direct life cycle which includes a vegetative trophozoite and a resistant cyst. Information on the biochemistry and physiology of trophozoites (Jarroll *et al.*, 1989a; Jarroll and Lindmark, 1990) is more abundant than it is for cysts since trophozoites have been in axenic culture for nearly 20 years and large quantities are easily obtained for study. Although less abundant than that for trophozoites, information concerning cyst biochemistry and physiology is increasing and is important because it is as cysts that *Giardia* survive in the external environment and infect hosts.

Large quantities of viable *Giardia* cysts, free from microbial contamination, are much more difficult to acquire than large quantities of axenic trophozoites. Even though recent attempts at *in vitro* encystment of axenically cultured trophozoites have been somewhat successful (Gillin *et al.*, 1987; Sterling *et al.*, 1988; Schupp *et al.*, 1988), the number of viable cysts produced *in vitro* appears to be much lower than the number of intact cysts which appear in the cultures (Schupp *et al.*, 1988). Furthermore, the number of intact cysts which appear in encysting cultures is much lower than the number of trophozoites which produce cyst wall antigen containing vesicles (Berrada *et al.*, 1990). However, such encysting cultures do make the study of the encystment process possible (Reiner *et al.*, 1989; Berrada *et al.*, 1990; Schupp *et al.*, 1990).

Because of the paucity of viable *Giardia* cysts available from *in vitro* sources, studies that do exist concerning the disinfection of water containing cysts (Jarroll,

1988) and the studies on cyst physiology and biochemistry (Paget *et al.*, 1989; Jarroll *et al.*, 1989b) have had to rely on cysts from mammalian faeces. Fortunately, studies involving water disinfection do not require cysts completely free of microbial contamination; unfortunately, those for physiological studies do. In 1984, Sauch reported that *Giardia* cysts could be separated from most of the microbial contaminants remaining after sucrose gradient purification of cysts by subsequent velocity sedimentation of the sucrose purified cysts on a percoll gradient. This procedure has made initial studies on *Giardia* cyst biochemistry and metabolism possible since it does appear to reduce microbial contaminants to levels below biochemical detection (Jarroll *et al.*, 1989b).

CYST MORPHOLOGY

A *Giardia* cyst measures approximately 6–10 µm in length and is composed of a pair of trophozoites (incomplete cytokinesis) encased within a cyst wall. The cyst wall is composed of an outer filamentous and an inner membranous portion (Feely *et al.*, 1984; Erlandsen *et al.*, 1989). The inner cyst wall consists of two membranes: the inner membrane borders the peritrophic space (the space between the cyst wall and the trophozoite), and the outer membrane appears to serve as an attachment site for the outer cyst wall. The outer cyst wall is composed of filaments which measure 7–20 nm in diameter and which are arranged in a tightly packed meshwork (Erlandsen *et al.*, 1989, 1990). The outer cyst wall appears, by high-resolution scanning electron microscopy, to be morphologically unchanged by sodium dodecylsulphate (SDS) treatment (Jarroll *et al.*, 1989b) and is approximately 0.3–0.5 µm thick. The inner cyst wall is apparently completely removed by the SDS treatment as is virtually all trophozoite material (Jarroll *et al.*, 1989b).

CYST WALL BIOCHEMISTRY

Although no studies have directly assessed the biochemical composition of the inner cyst wall, the ultrastructure suggests that it is membranous (Erlandsen *et al.*, 1989). Disappearance of the inner cyst wall following extraction of cysts with either SDS or chloroform-methanol (Jarroll *et al.*, 1989b; Jarroll, unpublished observation) supports this observation. Jarroll *et al.* (1989a) also reported that there were no obvious differences detected between the lipids extracted from trophozoites and those extracted from cysts.

Long before ultrastructural studies which demonstrated the inner and outer components were done, curiosity about *Giardia* cyst wall chemical composition existed. It is clear that early workers, using light microscopes, could have only observed the outer cyst wall. Thus, when they referred to the cyst wall they were really describing only the outer cyst wall. In this review, the term outer cyst wall is used to refer to the meshwork of fibrils remaining after SDS treatment of a cyst.

Most studies on the composition of the *Giardia* outer cyst wall have been histochemical. Among the first of these was one done by Kofoed *et al.* (1932) who assessed, histochemically, the composition of protozoan cyst walls, i.e. those of *Entamoeba histolytica*, *Endolimax nana*, *Councilmania lafleuri*, *C. dissimilis*, and *Giardia lamblia*. These investigators disputed even earlier speculations that these outer cyst walls were either chitin, pseudo-chitin or cellulose. The lack of chitin reported by Kofoed *et al.* (1932) was based on negative results with Zander's iodine-zinc chloride, Kuhnelt's sulphuric acid-iodine, and the picronigrosine tests. Cellulose was eliminated because of a negative amyloid test. Lipids were unlikely since the outer cyst walls were not soluble in ether, chloroform, alcohol or xylol, and were microscopically unaffected by heating for 2 h at 140°C in 2 per cent KCN. These authors believed that the outer cyst wall was proteinaceous since it gave a positive xanthoproteic reaction. Furthermore, they observed that the outer cyst wall was insoluble in dilute acetic, lactic, hydrochloric, sulphuric acids, and that it was only soluble after boiling in strong hydrochloric, nitric or sulphuric acids. Outer cyst walls were also insoluble in either potassium, sodium, or ammonium hydroxides. Kofoed *et al.* (1932) observed that pepsin (but not trypsin or bactotrypsin) digestion of outer cyst walls made a distinctly visible pore. Based on all their observations, this group proposed that the outer cyst walls of these protozoans were probably keratinlike proteins. In 1952, Filice stated that the cyst wall (i.e. outer cyst wall) of *Giardia* from the laboratory rat: (a) did not contain chitin since it was Feulgen negative after hydrolysis with hydrochloric acid (no details were given); (b) was not composed primarily of lipids since it did not stain with Sudan IV; and (c) did not contain a simple protein or saccharide since the outer cyst wall was apparently unaffected by pepsin, trypsin, papain, animal diastase or plant amylase. Evidence from Dutta (1965) suggested that, because the *G. lamblia* outer cyst wall was acridine orange positive after sulphation and periodic acid-Schiff (PAS) positive, that it was composed of polysaccharides in combination with proteins. Ward *et al.* (1985) proposed that the *Giardia* outer cyst wall is composed largely of chitin ($\alpha\beta(1,4)$ -homopolymer of *N*-acetylglucosamine (GlcNAc). Their claim was based mainly on the binding of fluorescein isothiocyanate (FITC) conjugated wheat germ agglutinin (FITC-WGA, putative binding affinity for GlcNAc and sialic acid (SA) and FITC-succinylated WGA (sWGA, putative binding affinity for GlcNAc) to *G. lamblia* and *G. muris* outer cyst walls. This binding was abrogated following treatment of the cyst with chitinase. The possibility that chitin was a component of the outer cyst wall was enhanced by a report from Gillin *et al.* (1987) that they had detected the activity of chitin synthetase in cultures of encysting *Giardia* trophozoites.

The fact that chitinase treatment abrogated WGA binding is difficult to interpret because: (1) no provision was made to obviate the possible activity of other glycosidases and proteinases often associated with purified chitinase (Roberts and Cabib, 1982); and (2) WGA will bind a greater number of substances than GlcNAc and SA (Monsigny *et al.*, 1980). The presence of chitin synthetase activity (Gillin *et al.*, 1987) is also difficult to interpret since it was assessed by measuring the incorporation of [³H]UDP-GlcNAc by encysting trophozoite lysates into a trichloroacetic acid (TCA) precipitate. Unfortunately, the chemical composition of

the TCA precipitate, into which the UDP-GlcNAc was incorporated, was not confirmed.

The first biochemical study of the *Giardia* outer cyst wall failed to confirm the presence of chitin as a major *Giardia* outer cyst wall component (Jarroll *et al.*, 1989b). Gas chromatography (GC), mass spectrometry (MS), lectin binding (*Phaseolus limensis*, LBA; putative binding affinity for *N*-acetylgalactosamine [GalNAc]) and enzyme (chitinase) analyses were used to investigate the carbohydrate composition of *in vivo* derived cysts, outer cyst walls and trophozoites (grown in medium without added bile). The only detectable amino sugar associated with the *Giardia* outer cyst wall was galactosamine (GalN) (Jarroll *et al.*, 1989b). GalN was second only to glucose in abundance in cysts. Unlike glucose, GalN was detected in cysts (*c.* 41 nmol per 10^6 cysts) and outer cyst walls (*c.* 63 nmol per 10^6 cysts), but not in trophozoites. This observation suggested that GalN is a *Giardia* cyst wall specific sugar. Localization of this sugar by staining cysts with FITC-LBA lectin suggested that GalN is confined to the outer cyst wall, at least once the mature cyst is formed. Based on the putative binding affinity of LBA, GalN probably exists as GalNAc. Glucosamine (GlcN), SA and muramic acid (MA) were below the limits of detection in *Giardia* trophozoites, cysts or outer cyst walls, but GlcN and MA were detected in hydrolysates of *Staphylococcus aureus* used as a control for these amino sugars. In addition to the lack of detectable levels of GlcN, *Giardia* outer cyst walls failed to stain with Calcofluor M2R (which stains $\beta(1, 4)$ —and $\beta(1, 3)$ -linked polysaccharides). Furthermore, *G. lamblia* trophozoites, *G. muris* cysts and *G. muris* cysts which had been induced to excyst were assayed for chitinase activity with and without exogenous *N*-acetylglucosaminidase (NAGase). Chitinase activity (measured by assaying for liberated GlcNAc from chitin) was below the limits of detection (0.3 nmol GlcNAc) in all these cases. When 10^8 *G. muris* cysts were substituted for purified chitin (crab shell chitin was used as a control) in assays using purified chitinase, the liberation of GlcNAc from the outer cyst wall was also below the limits of detection. Taken together, these data indicate that chitin is not a major outer cyst wall component.

Glucose detected by GC and verified by MS represented the most abundant sugar in HCl hydrolysates or methanolysates of cysts, outer cyst walls and trophozoites (between 63 and 75 nmol per 10^6 cells). Whether glucose was associated with the cyst wall or with glycogen was not determined by Jarroll *et al.* (1989b). In a later study, Manning *et al.* (1990) demonstrated that glucose is present in cysts primarily as glycogen. Their observations were based on the fact that PAS staining of both cysts and outer cyst walls was abrogated by treatment of these structures by amyloglycosidase (γ -amylase, degrades both $\alpha(1, 4)$ —and $\alpha(1, 6)$ -glycosidic bonds). This histochemical observation was supported by a demonstrable reduction of glucose from about 63 nmol per 10^6 outer cyst walls to about 2 nmol per 10^6 outer cyst walls treated with proteinase-free γ -amylase. These results indicate that glycogen is not removed from cysts or outer cyst walls by SDS treatment. The histochemical and biochemical data presented by Manning *et al.* (1990) support the histochemical observation made by Dutta (1965) that *G. intestinalis* (syn. *G. duodenalis* and *G. lamblia*) cysts contain amylase degradable glucose (probably as

glycogen). Furthermore, this study also confirmed the presence of GalN as the major *Giardia* outer cyst wall sugar (*c.* 37 per cent of the outer cyst wall dry weight after glycogen is removed). GC/MS analysis of cyst and outer cyst wall hydrolysates prior to amylase treatment showed approximately equivalent amounts of glucose and GalN (each represents *c.* 47 per cent of the total sugars detected). Following amylase treatment of outer cyst walls, GalN represents *c.* 86 per cent of the total sugars detected while glucose decreased to *c.* 3 per cent.

Jarroll *et al.* (1989b) also detected galactose (*c.* 10 nmol per 10^6 cysts) and ribose (*c.* 5 nmol per 10^6 cysts) in *Giardia*. Ribose, but not galactose, was detected in *G. lamblia* trophozoites after methanolysis.

Manning *et al.* (1990) expanded the list of sugars associated with *G. muris* cysts to include (numbers represent micrograms of sugar per milligram dry weight and an individual sugar's percentage of weight of total sugar): tetrose (either erythrose or threose), 12, 2.8; ribose, 11, 2.6; arabinose, 10, 2.3; mannose, 5, 1.2; galactose, 4, 0.9; and xylose, 3, 0.7. Whether or not these sugars are associated with the outer cyst wall remains to be determined.

Although Kofoid *et al.* (1932) first suggested that the *Giardia* outer cyst wall is proteinaceous, it was not until recently that more convincing biochemical, rather than histochemical, evidence for this premise was found. Reiner *et al.* (1989) immunized rabbits with human source *G. lamblia* cysts separated from faeces and used this antiserum to probe Western blots of an analogous strain of encysting trophozoites for the expression of antigens unique to the encystment process. Their results suggest that cyst wall specific material (trypsin sensitive on Western blots) is, at least in part, protein. Furthermore, they detected the cyst wall material in encysting trophozoites within vesicles and they noted its appearance on Western blots as soon as 19 h after encystment was induced. Berrada *et al.* (1990) made rabbit anti-*G. duodenalis* (MR4 strain) cyst wall from cysts produced *in vitro*. They demonstrated by indirect immunofluorescence that anti-cyst-wall recognized only cyst walls and vesicles containing cyst wall material in homologous encysting MR4 trophozoites. The cyst wall vesicles were detected as early as 2 h after trophozoites were placed in encystment medium; and the number of trophozoites expressing cyst wall vesicles, as well as the number of vesicles per trophozoite, increased with time. The number of cells expressing the cyst wall vesicles or a cyst wall reached a maximum of about 50 per cent of the cells within 24 h. Furthermore, these authors demonstrated that the vesicles containing cyst wall material react specifically with anti-cyst-wall and with antisera eluted from Western blots probed by anti-cyst-wall. Berrada *et al.* (1990) also demonstrated that cyst wall proteins appeared on Western blots as soon as 12 h post-induction. Both groups of investigators (Reiner *et al.*, 1989; Berrada *et al.*, 1990) demonstrated that the first bands to appear were a group of low molecular weight proteins ranging in M_r from 29000 to 45000. The fact that cyst wall antigens can be detected on Western blots during the encystment process suggests that components of the outer cyst wall are SDS soluble at least during a portion of their synthesis. Neither group has conclusively demonstrated whether or not these proteins are glycosylated or, if glycosylated, when glycosylation occurs.

It seems likely, considering the present body of data, that the *Giardia* outer cyst

wall is, as was suggested by Dutta (1965), some combination of carbohydrate in association with proteins. Just what that combination is remains to be determined.

CYST PHYSIOLOGY AND METABOLISM

One of the first suggestions that *Giardia* cysts are metabolically active came indirectly from the work of Bingham *et al.* (1979). These authors showed that the viability of cysts purified from faeces decreased with storage time, regardless of the storage temperature. However, the viability decreased more rapidly as the storage temperature was increased. That *Giardia* cysts have the enzymes necessary to carry out glycolytic processes was not shown until several years later. Lindmark and Miller (1988) demonstrated that *G. lamblia* cysts and *G. muris* cysts and trophozoites exhibit the specific activities of glycolytic enzymes equivalent to those of *G. lamblia* trophozoites grow *in vitro*. Paget *et al.* (1989, 1990) showed that *G. muris* and *G. lamblia* cysts exhibit endogenous respiration. The cysts of both organisms exhibit respiratory rates of c. 10–20 per cent that of their respective trophozoites, but cyst respiration is not stimulated by exogenous glucose. Furthermore, cyst and trophozoite respiratory activity decrease when O₂ concentrations reach a critical threshold. In the case of *G. muris* cysts, respiration increases with increasing O₂ concentration over the range 0–25 µMO₂; respiration decreases at O₂ concentrations above 25 µM. For *G. lamblia*, respiration decreased above 30 µMO₂, and it was irreversible. Lowering the temperature from 37°C to 17°C lowers the respiratory activity by c. 80 per cent; at 7°C the respiratory activity would be negligible. These observations could explain, at least in part, why lower water temperatures favour *Giardia* cyst viability. Interestingly, Paget *et al.* (1989, 1990) reported that metronidazole, the drug most commonly used to treat giardiasis, had no effect on cyst respiration or excystation. However, menadione, a redox cycling naphthoquinone, potently inhibited respiration and excystation. Both menadione and metronidazole inhibited flagellar motility and respiration in *G. lamblia* and *G. muris* trophozoites.

Giardia trophozoites appear to perform little, if any, *de novo* synthesis of various necessary biochemicals. They rely instead on salvage of lipids, purines and pyrimidines (Jarroll and Lindmark, 1990). This great dependence on salvage for critical cellular requirements coupled with the fact that a major outer cyst wall sugar, GalNAc, is below the limits of GC detection in non-encysting trophozoites begs the question of whether GalNAc is salvaged or synthesized during the encystment process. Macechko and Jarroll (1990) have presented evidence to suggest that encysting *Giardia* trophozoites synthesize GalNAc mainly from glucose rather than salvaging GalNAc directly from the medium. Their assertion was based on two lines of evidence: First, when trophozoites were encysted in the presence of ¹⁴C precursors of GalNAc (e.g. glucose, GalN, GlcN, galactose, mannose and UDP-GlcNAc) only glucose was incorporated significantly by encysting trophozoites and subsequently converted to GalN (c. 68 per cent of the glucose incorporated as sugar was detected as GalN). Secondly, activity of a key enzyme in the synthesis of UDP-GalNAc from glucose has been detected in crude homogenates of encysting trophozoites. This enzyme,

UDP-GlcNAc 4'-epimerase (E.C.5.1.3.7; UG4E) catalyses the reversible epimerization of UDP-GlcNAc to UDP-GalNAc (Glaser, 1959).

Macechko and Jarroll (1990) assayed for UG4E activity in homogenates of encysting *G. duodenalis* (MR4 strain) using an assay system which measures the conversion of [¹⁴C]UDP-GalNAc to [¹⁴C]UDP-GlcNAc (Kingsley *et al.*, 1986). While in some systems this enzyme appears to be able to catalyse the conversion of UDP-galactose to UDP-glucose, this does not appear to be the case in *Giardia*. Activity of UG4E was below the limits of detection in nonencysting trophozoites, but was detected in cultures of encysting trophozoites after approximately 8 h in encystment medium. From this time onwards, the activity increased dramatically until approximately 48 h. The activity dropped only slightly by 72 h. UG4E activity was enhanced two-fold in crude homogenates by the addition of Triton X-100, and its activity increased linearly with time and with increasing trophozoite protein. Preliminary evidence indicates that UG4E is reversible when UDP-GlcNAc is used as substrate. These preliminary studies suggest that the synthesis of UG4E is induced during the encystment process. Of course, the isotope incorporation studies and the activity of UG4E suggests that GalNAc is synthesized from glucose via GlcN. Since GlcN was below our limits of GC detection in non-encysting trophozoites (grown in medium without added bile) and in cysts generated *in vivo* (Jarroll *et al.*, 1989b), it suggests that GlcN is present in *Giardia* as a transient intermediate during GalNAc synthesis.

SUMMARY

A *Giardia* cyst represents a pair of incompletely divided trophozoites encased within a cyst wall. This cyst wall is composed of an inner membranous portion and an outer fibrous portion. The latter appears to be protein associated in some fashion with a large quantity of GalNAc; chitin is not a demonstrable component. The GalNAc component appears to be synthesized from glucose with a glucosamine intermediate during the encystment process. The synthesis of at least one of the enzymes in that pathway, UDP-GlcNAc 4'-epimerase, appears to be induced during the encystment process.

Current evidence suggests that *Giardia* cysts are metabolically active and not true cryptobiotic forms; it is likely that the cysts catabolize endogenous glycogen reserves. Low temperature appears to slow their metabolic activity, and thus, favours longer cyst survival in the environment. The chemotherapeutic agent most commonly used against giardiasis, metronidazole, does not measurably inhibit cyst respiration or excystation, but respiration is sensitive to oxygen concentrations above *c.* 25–30 µM.

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5. The biochemical activities and importance of the ciliate protozoa in the rumen ecosystem

A.G. Williams

RUMEN ECOSYSTEM

The principal sites of microbial fermentative activity in the intestinal tract of herbivorous mammals are located either in an enlarged forestomach or in the large intestine (Bauchop, 1977; McBee, 1977). Evolutionary anatomical and physiological adaptations have occurred at these sites to aid the retention of digesta and encourage microbial colonization. The conditions that prevail within these eco-systems allow diverse protozoa-containing microbial populations to develop; these intestinal protozoal populations are host specific (Dehority, 1986; Hungate, 1988).

Herbivores classified in the suborder Ruminantia have an extensive pre-gastric fermentation. The ruminant forestomach is structurally complex comprising the reticulum, rumen, omasum and abomasum; the latter portion corresponds to the stomach of monogastric animals (Church, 1976). Functionally, food enters the reticulum from the oesophagus and passes to the rumen; the ruminoreticulum, which is commonly referred to as the rumen, is the major site of microbial activity. Digesta leaving the rumen passes through the omasum, where further microbial digestion can occur (Smith, 1984), before entering the acid-secreting abomasum. Large particles are retained at the reticulo-omasal orifice and, under certain conditions, some liquids can bypass the rumen by flowing directly from the oesophageal groove.

An important feature of the ruminal environment is its relative constancy. Conditions in the rumen are normally within the range 39–40°C, pH 6–7 and with an E_h value of approximately -350mV (Hungate, 1966). Ambient oxygen concentrations in ruminal contents are low (<5 µM; Scott *et al.*, 1983) and the major fermentation gases are carbon dioxide and methane. The ecosystem is well supplied with nutrients and, in consequence, a diverse microbial population, comprising bacteria, protozoa and fungi, becomes established (Hobson, 1988).

The host ruminant is dependent upon the rumen population to pre-digest its feed constituents into a usable form. Although many intermediates may be formed, the principal fermentation end products are short chain volatile fatty acids (VFA). These acid metabolites and the microbial cells, after digestion in the lower gut, represent an important nutrient source for the ruminant; VFA, for example, contribute approximately two-thirds of the dietary energy of the host animal (Church, 1976). The metabolic activities and significance of the ciliate protozoa in the rumen ecosystem are discussed in this chapter.

THE RUMEN PROTOZOA

The rumen protozoa are well adapted to conditions in the ruminal environment and a protozoal population develops in all wild and domesticated ruminants. The majority

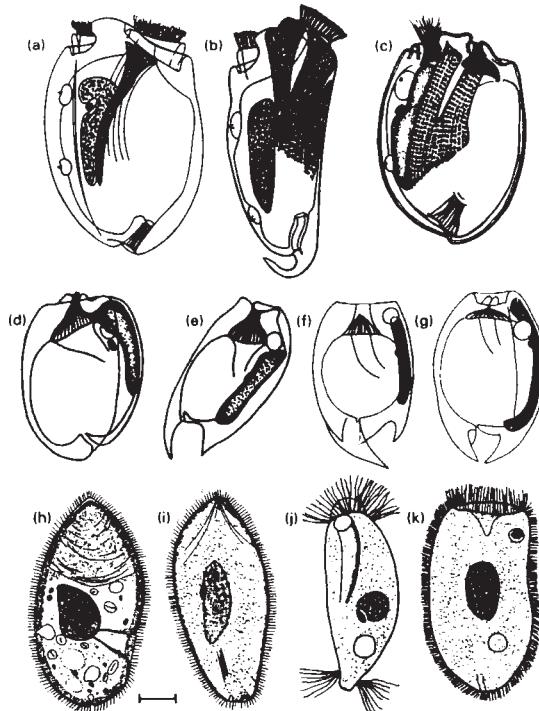


Figure 5.1. Examples of some ruminal entodiniomorphid and holotrich ciliate protozoa, (a-c) Genera of the large entodiniomorphid ciliates that are representative of a B-type protozoal population (Williams and Coleman, 1991): (a) *Eudiplodinium maggi*, scale bar=25 µm; (b) *Epidinium caudatum*, scale bar=15 µm; (c) *Metadinium* (syn. *Ostracodinium*) *minorum*, scale bar=20 µm.

Entodinium spp. and holotrich ciliates are common to A- and B-type populations, (d-f) Four species of *Entodinium*; scale bar=10 µm for all four species: (d) *Entodinium ovibos*, (e) *Ent. ciculum*, (f) *Ent. tsunodai*, (g) *Ent. longinucleatum*.

The holotrich ciliates are from the families Isotrichidae (h,i), Blepharocorythidae (j) and Buetschliidae (k); (h) *Isotricha intestinalis*, scale bar=20 µm; (i) *Dasytricha ruminantium*, scale bar=10 µm; (j) *Charonina ventriculi*, scale bar=5 µm; (k) *Buetelia parva*, scale bar=10 µm.

Drawings (c), (d) and (e) reproduced with permission of Burk Dehority and the *Journal of Protozoology*.

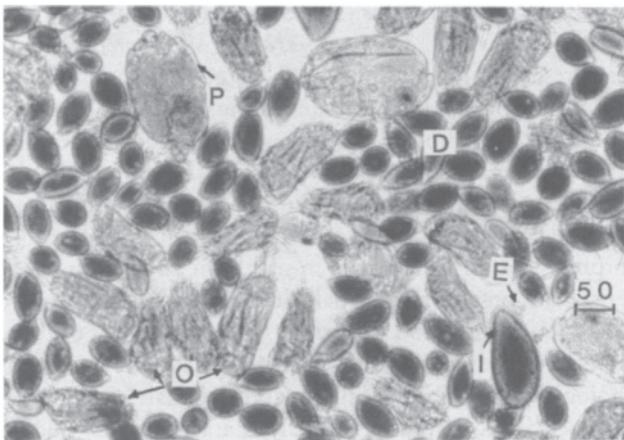


Figure 5.2. Mixed protozoa (A-type population) isolated from the ruminal contents of a sheep fed a ration of hay and molassed sugar beet pulp. The preparation was prepared by differential filtration of the separated protozoal population (Williams and Coleman, 1991) and represents the fraction retained by a 40 µm porosity filter, as seen using phase contrast microscopy. The entodiniomorphid genera indicated are *Polyplastron* (P), *Ophryoscolex* (O) and *Entodinium* (E). The isotrichid holotrich genera *Isotricha* and *Dasytricha* are also indicated. Scale bar=50 µm.



Figure 5.3. *Entodinium* spp. as observed under phase contrast microscopy. Scale bar=10 µm. The preparation was isolated by differential filtration from the ruminal contents of a hay-fed sheep that had been chemically defaunated and in which a mixed population of *Entodinium* spp. and *Polyplastron multivesiculatum* had been re-established.

of the protozoa are ciliates (approximately 10^4 – 10^6 g⁻¹ rumen contents), although some flagellates (e.g. *Trichomonas* spp., *Monocercomonas* sp. and *Chilomastix* sp.) are present in low numbers (10^3 – 10^4 g⁻¹). There is little known about the biochemistry and role of the flagellates in the rumen (Williams and Coleman, 1988, 1991); and the discussion in this review is confined to the ciliates.

Two groups of ciliate protozoa usually occur in the rumen (Figures 5.1 to 5.3). The majority of these ciliates belong to the family Ophryoscolecidae (order Entodiniomorphida), are unique to ruminants and camelids and are known as entodiniomorphid ciliates. They range in size from 25×15 μm to $>200 \times 120$ μm and characteristically possess a rigid pellicle and specialized bands of cilia which function in locomotion and food ingestion. Over 250 species of rumen entodiniomorphid ciliates have been described, although speciation has often been based on minor morphological differences (Williams and Coleman, 1991). The second group of ciliates are described as holotrichs and are characterized by body flexibility and complete surface ciliation. They occur within one of the four families Isotrichidae, Blepharocorythidae, Buetschliidae and Paraisotrichidae. Although the subclass Holotrichia has been superseded, the terminology holotrich is still used widely. The holotrich group is thus diverse, containing 15 different genera. Detailed studies, however, have only been undertaken with species from the isotrichid genera *Dasytricha* and *Isotricha* (Williams, 1986). The other holotrich genera occur infrequently and with low population densities. Detailed taxonomic and morphological descriptions, with photomicrographs, are given for both groups in recent reviews and monographs (Ogimoto and Imai, 1981; Williams and Coleman, 1988, 1991).

The numbers and types of ciliates present in the rumen are determined by various interacting factors. Some species are widely distributed in various hosts, whereas others are more host specific (Clarke, 1977; Williams and Coleman, 1991). The more important factors that affect the generic profile of the protozoal population are the type of host and its geographical location, the nature of the ration and the feeding habit of the host (grazer/browser), the level and frequency of feeding and interspecies protozoal antagonisms. The number of protozoa in the rumen changes during the diurnal cycle and in animals fed once daily the numbers are highest during the feeding period and begin to decline thereafter. The relative proportions of the two ciliate groups present also change as the post-prandial variations in the population size of the holotrichs and the entodiniomorphs differ (Coleman, 1980; Williams and Coleman, 1988, 1991).

EFFECTS OF RUMEN PROTOZOA ON THE HOST RUMINANT

The protozoa directly affect the productivity and well-being of the host ruminant, both through their metabolic activities and as a consequence of the post-ruminal degradation and utilization of the protozoal cellular components. In addition to this direct involvement, the presence of protozoa in the rumen produces changes in the environmental conditions and microbial population with resultant indirect effects on the rate, extent and site of the digestive processes (Williams and Coleman, 1991).

Table 5.1. Some effects of ciliate protozoa on ruminal characteristics and ruminant performance.^a

Change in parameter when protozoa present	
Increased	Decreased
pH	Size of bacterial population
VFA concentration	Lactic acid concentration
Ammonia concentration	Flow of nitrogen to lower tract
Organic matter breakdown	Microbial protein synthesis
Fibre breakdown	Plasma glucose concentration
Starch digestion	Plasma polyunsaturated fatty acid levels
Proteolytic activity	Plasma and hepatic copper levels
Methanogenesis	Live-weight gain
Plasma saturated fatty acid levels	Wool growth
Susceptibility to bloat	Food conversion efficiency
Physical appearance of host improved	Susceptibility to copper toxicity
	Susceptibility to lactic acid acidosis

^a These effects of protozoa were assessed by comparison of faunated and defaunated (ciliate free) animals. Not all studies reached the same conclusion and the effects given above are those most frequently reported. More comprehensive surveys of the findings in the literature are given by Williams and Coleman (1988, 1991).

Despite the metabolic diversity of the ciliates and their large biomass in the rumen, the protozoa, unlike the bacteria, are not essential for the survival and development of the host ruminant. Indeed, under certain dietary situations the elimination of the ciliate population (defaunation) can result in increased animal growth and productivity (Nolan *et al.*, 1989).

Over 60 years ago Becker *et al.* (1929) concluded that ciliates were commensals which were of little obvious benefit to the host. Although there is now much more known about the effects of the protozoa (Veira, 1986; Jouany *et al.*, 1988; Williams and Coleman, 1988, 1991), a common consensus on the value of the protozoa has still not been achieved. It is apparent from Table 5.1 that some effects of the protozoa are beneficial to the host; however, if the consequences of protozoal action on protein supply to the host are considered, the ciliates may be regarded as parasites, since the host benefits from their elimination. Although animal productivity can be influenced by defaunation, it has not yet been established if an improvement in animal performance can be achieved by manipulating the size or generic composition of the protozoal population.

Although most diets support a large ruminal protozoal population, the ciliates sequester in the rumen so that the proportion of protozoal biomass in the rumen digesta is considerably greater than that in the ruminal outflow material reaching the lower tract (Leng, 1989). The protozoal contribution to the nutrition of the host is dependent on the biomass reaching the lower tract and thus the full potential nutritional value of the ciliates is not gained by the host. Despite protozoal sequestration in the rumen, approximately 25 per cent of the microbial protein available to the host is protozoal in origin (Coleman, 1979b).

Comparative studies with faunated and protozoa-free animals have shown that the ciliates not only contribute to the metabolic processes within the rumen but also suggest that the protozoa are able to modulate the physico-chemical characteristics

of the ecosystem. The protozoa have also been implicated in the aetiology of certain digestive disorders and thus affect the health and general well-being of the ruminant (Williams and Coleman, 1991).

The presence of protozoa in the rumen has also been shown to influence the volume of the rumen and digesta retention, the composition and activities of the microbial population present, the concentrations and proportions of microbial fermentation end products and the environmental pH (Table 5.1). Changes in any of these parameters can influence ruminal function and, in consequence, ruminal digestion of dietary protein, organic matter and fibre is higher in faunated animals. Conversely, net microbial synthesis and the flow of protein to the lower tract is reduced when protozoa are present in the rumen (Veira, 1986; Jouany *et al.*, 1988; Williams and Coleman, 1988, 1991; Nolan *et al.*, 1989). Defaunation offers the potential to improve the efficiency of live-weight gain, milk production and wool production as these biological processes may be constrained by protein limitation (Bird, 1989). Some of the recognized effects of the rumen protozoal population on the host are also summarized in Table 5.1. Many of the differences between the ruminal fermentation in faunated and ciliate-free animals can, however, be attributed directly to the metabolic activities of the protozoa. As these effects may have important consequences for animal productivity a detailed knowledge of the bio-chemical capabilities of the rumen protozoa is an essential prerequisite to understanding the biological significance of the ciliates in the rumen ecosystem.

BIOCHEMICAL ACTIVITIES

The protozoa are dependent on the host for their food supply, but are able to transform an array of plant and bacterial constituents to cell components and metabolites that are utilized by the host animal. Biochemical studies on the ciliates have been hampered by the difficulties encountered in establishing laboratory cultures and because of the presence of ingested and attached viable bacteria in the protozoal preparations. It has not been possible to cultivate the ciliates in the absence of living bacteria for extended periods. Many entodiniomorphid protozoa have, however, been grown *in vitro* successfully in cultures with viable bacteria (Coleman, 1987), whereas the holotrichs have only been maintained in laboratory culture for relatively short periods (Williams, 1986; Williams and Coleman, 1991). Media used for cultivation of the ciliates are complex and resemble ruminal conditions in that the redox potential is low and particulate matter (e.g. plant material, starch grains and bacteria) is more readily available than rapidly metabolizable soluble compounds. Under these conditions added food is engulfed by the protozoa and bacterial overgrowth does not occur. Many of the metabolic studies have been undertaken using cells that have been isolated directly from the rumen contents of conventional or specifically faunated animals by either sedimentation, centrifugation or differential filtration techniques (Williams and Coleman, 1991).

Because of these technical constraints, the protozoa have been studied less extensively than the ruminal bacteria and only limited information is available on

the metabolic capabilities of many of the ciliates. In many instances mixed populations of ciliate species have been studied or only one species within a genus has been investigated. There have been no biochemical studies on the holotrich and entodiniomorphid ciliates that occur less frequently or at a low population density. The ciliates are biochemically versatile but the two groups occupy different metabolic niches in the ruminal ecosystem. The holotrichs utilize soluble carbohydrates whereas the entodiniomorphid ciliates ingest and metabolize particulate material. Bacteria are the most important source of nitrogenous compounds for the ciliates and are actively engulfed by both groups. The biochemistry of the protozoa has been reviewed comprehensively (Clarke, 1977; Coleman, 1979b, 1980, 1986a; Williams, 1986, 1989a; Williams and Coleman, 1988, 1991) and therefore, in the following sections only the principal biochemical attributes of the protozoa that directly affect ruminant performance are considered.

Carbohydrate degradation

The concentration of soluble sugars in the rumen is relatively low and is only elevated for relatively short periods immediately after feeding. The principal carbohydrates available for microbial fermentation are thus the plant structural and storage polysaccharides. The protozoa are attracted by chemotaxis to plant material entering the rumen (Orpin, 1984, 1985). The large plant fragments are colonized rapidly (Bauchop, 1989); however, attachment is not essential for the ingestion and intracellular digestion of plant fragments to occur. By feeding directly on the plant tissues and cellular contents the protozoa participate directly in the degradative processes. The ability of the protozoa to degrade specific plant polymers is considered in the following sections.

Cellulose

The presence of cellulolytic activity in the rumen ciliates was first suggested in growth experiments in which it was observed that some of the larger entodiniomorphid ciliates were able to engulf and digest cellulose (Hungate, 1942, 1943; Coleman *et al.*, 1976). Although *Entodinium* spp. and the holotrichs possess little or no cellulase activity (Bonhomme, 1975; Williams, 1986), active cellulosedegrading enzymes have been detected in other entodiniomorphid genera (Coleman, 1985). Comparative measurements have indicated that the specific activities of cellulolytic enzymes are higher in the rumen protozoal population than in the bacterial population (Williams and Strachan, 1984), and Coleman (1986b) estimated that over 60 per cent of the ruminal cellulase activity was associated with the protozoa. However, despite the potential importance of the protozoal enzymes in ruminal cellulolysis, little is known about their characteristics or mode of action (Williams, 1989a), and doubts are still expressed as to the origin of the enzymes. Thines-Sempoux *et al.* (1980) suggested from electron microscopic studies that the cellulase was produced by bacteria in intracellular vesicles. However, bacteria-free, subcellular membrane-bound structures containing cellulolytic activity have been

isolated from *Polyplastron multivesiculatum* (Williams and Ellis, 1985), and further evidence confirming the protozoal origin of the cellulases in this ciliate was obtained by Bonhomme *et al* (1986) who isolated cellulose-degrading enzymes from protozoa that had been cultivated in the absence of cellulolytic bacteria. Bonhomme (1988) has also demonstrated the presence of cellulase, xylanase, β -glucosidase and β -xylosidase activities in cell-free extracts of *Epidinium ecaudatum* grown *in vitro* in the absence of cellulolytic and hemicellulolytic bacteria. However, it is inevitable that the origin of the enzymes will remain equivocal until the cellulolytic ciliates can be grown in axenic culture.

Hemicellulose

The activities of hemicellulose-degrading enzymes in the rumen microbial population and ruminal hemicellulolysis are reduced after defaunation (Jouany, 1989; Williams and Withers, 1991). Hemicellulolytic activity has been detected widely in the ruminal ciliates (Williams and Coleman, 1985; Williams, 1989a, b). Although enzyme activities are higher in the cellulolytic species, extracts of non-cellulolytic holotrichs and entodinia are also able to degrade isolated hemicellulosic polysaccharides. The hemicellulase complex of *Epi. ecaudatum* has been examined in most detail. The partially purified enzyme had maximal activity at 40°C and close to pH 6 (Bailey *et al.*, 1962; Hayer *et al.*, 1976; Rao *et al.*, 1977; Williams, 1989a, b).

Hemicelluloses are heteropolysaccharides and depolymerization requires the intervention of both polysaccharidase and glycoside hydrolase enzymes to release the component monosaccharides. The rumen ciliates form a wide range of glycoside hydrolases (Williams *et al.*, 1984) that have an important function in protozoal hemicellulolysis (Bailey *et al.*, 1962; Bailey and Gaillard, 1965, 1969).

The glycosidase profiles of the holotrich and entodiniomorphid ciliates are similar. Studies using carbohydrate and chromogenic glycosides have indicated activities in both groups against glycosides of pentoses, hexoses, hexuronic acids and hexosamines (Williams *et al.*, 1984). Invertase and other holotrich glycosidases are secreted extracellularly, possibly to enhance carbohydrate release when nutrients are depleted (Williams, 1979a). However, the acid hydrolases of both ciliate groups are predominantly intracellular enzymes that are located in one or more populations of membrane-bound subcellular vesicles (Williams *et al.*, 1986). The acid hydrolase and polysaccharidase containing subcellular structures have been isolated from *Dasytricha ruminantium* and *Polyplastron multivesiculatum* by isopycnic density centrifugation and had mean densities in sucrose gradients of 1.13 and 1.19 g ml⁻¹, respectively (Williams and Ellis, 1985; Yarlett *et al.*, 1985).

There is relatively little information available on the characteristics of the protozoal glycosidases (see Williams, 1989a,b). They are typical acid hydrolases having acidic pH optima and transglycosylase activity. The glycosidases involved in storage polymer breakdown have been characterized more fully than those having a role in fibrolysis (Williams, 1986, 1989a).

Pectin

Many rumen ciliates form pectin esterase and one or both of the depolymerizing enzymes polygalacturonase and endopectate lyase (Abou Akkada and Howard, 1961; Coleman *et al.*, 1980). Highest activities in the entodiniomorphid ciliates were detected in the more actively cellulolytic species, although *P. multivesiculatum* differed in that it contained no endopectate lysase. The intracellular pectolytic enzymes are located in the same subcellular fractions as the acid hydrolases and other polysaccharolytic enzymes (Williams *et al.*, 1986). The enzymes, unlike the other fibrolytic enzymes, however, have alkaline pH optima (Coleman *et al.*, 1980; Williams, 1989a). Although both the holotrich and entodiniomorphid ciliates are able to degrade hemicellulose and pectin, there is no evidence that they can metabolize the released pentose sugars or galacturonic acid. These degradative activities may render the cellulose fibrils in the cell wall structures more accessible to attack or aid the release of fermentable intracellular carbohydrates.

Starch

The two principal reserve carbohydrates stored in plant cells are starch and inulin. With the exception of the smallest *Entodinium* spp. and possibly *D. ruminantium*, starch grains are ingested by the rumen ciliates, although the rate of uptake, digestion rate and metabolic products are species dependent (Coleman, 1980, 1986a). All of the protozoal species that have been examined contain amylase and a-glucosidase (Williams, 1989a); the activity of these enzymes in the entodiniomorphs is regulated by maltose and glucose, respectively, preventing an over-accumulation of intracellular sugars. Starch breakdown is also effected by the phosphorylase mechanism in some of the entodiniomorphs and there is evidence to indicate that the phosphorolytic pathway is more important in *Epi. ecaudatum* and *P. multivesiculatum* (Coleman and Laurie, 1976, 1977).

The protozoal amylolytic activity is important in stabilizing the ruminal fermentation. In the ovine rumen, 35–57 per cent of the total amylase activity is located in the protozoal population (Coleman, 1986c). The rapid ingestion and controlled metabolism of starch to VFA by the protozoa suppresses the potentially harmful bacterial amylolytic fermentation to lactic acid. The protozoa not only reduce the rate of lactic acid formation but they also have an important role in the ruminal metabolism of lactic acid and in preventing the onset of the nutritional disorder lactic acid acidosis (Newbold *et al.*, 1986, 1987; Williams and Coleman, 1991).

Inulin

Although fructose and fructosans are apparently not utilized for growth by the entodiniomorphid ciliates, fructose-containing saccharides are rapidly metabolized by the isotrichid holotrichs *D. ruminantium* and *Isotricha* spp. (Williams, 1986). *Dasytricha* is the more active of the isotrichid genera in degrading plant fructosans (Thomas, 1960). Extracts of both holotrichs and the entodiniomorphs *Epi.*

e caudatum, *P. multivesiculatum* and *Entodinium* spp. contain invertase activity and, with the exception of the epidinal preparation, will degrade both sucrose and inulin (Williams, 1989a). The properties of the holotrich enzyme are well characterized (see Williams, 1986, 1989a) and the invertase of *Isotricha prostoma* has recently been purified (Dauvrin and Thines-Sempoux, 1989). The purified enzyme is a heterogeneous tetramer having an M_r of 350000 and maximum activity at pH 5.5–6 and 50°C. The enzyme also has transglycosylase properties. The characteristics of the invertase in extracts of *D. ruminantium* and the entodiniomorphid ciliates are similar (Thomas, 1960; Abou Akkada *et al.*, 1963; Williams, 1979a). Although it was initially believed that a single enzyme was responsible for the degradation of all fructose-containing saccharides, the purified enzyme from *I. prostoma* was found to have only restricted activity against raffinose and negligible activity on inulin. There were, however, a minimum of five distinct forms of the monomeric unit and this heterogeneity may result in differing substrate specificities.

Invertase is the most active of the holotrich extracellular enzymes and the ciliates are responsible for much of the free invertase activity in the rumen ecosystem (Thomas, 1960; Williams, 1979a).

Carbohydrate fermentation

The isotrichid ciliates *D. ruminantium*, *I. prostoma* and *I. intestinalis* utilize the monosaccharides fructose, glucose, galactose and a variety of soluble oligomers composed of one or more of these monosaccharides. The range and rate of carbohydrate utilization is genus dependent (Williams, 1986; Williams and Coleman, 1991). The rate of sugar utilization is affected by the nature and concentration of the carbohydrate, environmental pH, temperature and the proportion of carbon dioxide and oxygen in the gas phase. *D. ruminantium* is not selective in the uptake of available carbohydrates and does not regulate sugar entry by preferential or sequential utilization. A concomitant uptake thus occurs irrespective of molecular size or ultimate metabolic effects (Williams, 1979b). This absence of control may allow the protozoa to compete more successfully for the substrates in the rumen. The rapid assimilation of soluble carbohydrates by the holotrichs could facilitate ruminal bacterial cellulolysis (Ryle and Ørskov, 1987), as the formation of fibrolytic enzymes by bacteria is reduced in the presence of soluble carbohydrates (Williams, 1989b).

Although the entodiniomorphid protozoa are primarily amylolytic, all the species tested would also take up glucose and maltose (Coleman, 1980, 1986a; Williams and Coleman, 1991). The active uptake of maltose by *Ent. caudatum* is four-fold faster than that of glucose. The calculated rates of glucose uptake for *Entodinium* spp., large entodiniomorphid ciliates and the isotrichid ciliate *D. ruminantium* are 0.05–2.4, 15–40 and 120–150 pmol/cell/h, respectively. The uptake of soluble sugars by the entodiniomorphid ciliates occurs by an active process at low concentrations and by a passive process at higher levels. There is little information available on the range of sugars metabolized by the entodiniomorphs. The evidence that these protozoa can use sugars other than glucose and glucose polymers is poor.

Ent. caudatum will take up and metabolize ribose, sucrose and galactose, whilst some of the larger entodiniomorphid genera might use xylan and polygalacturonic acid to a limited extent.

The principal products of carbohydrate utilization by *D. ruminantium* and *Isotricha* spp. are lactic acid, butyric acid, acetic acid, carbon dioxide, hydrogen and a reserve polysaccharide amylopectin (Williams, 1986; Williams and Coleman, 1991). The production of formic acid and propionic acid as minor products is equivocal as these may arise from bacterial contamination. Alanine has been identified recently by ^{13}C NMR spectroscopy as a product of glucose metabolism by *D. ruminantium* (Ellis, Lloyd and Williams, unpublished data). The rates of fermentation are dependent on the ciliate and the nature of the substrate.

An important feature of the holotrichs is their propensity to synthesize and store an intracellular reserve polysaccharide during the limited periods in which soluble sugars are available in the rumen. Approximately 75–80 per cent of the sugar taken up is converted to amylopectin. Simultaneous breakdown and synthesis of the glucan occurs in both genera; maximum deposition rates determined for *D. ruminantium* and *I. prostoma* were 130 and 1300 pmol amylopectin/cell/h (Prins and Van Hoven, 1977; Van Hoven and Prins, 1977), although deposition rates are dependent on the nature and concentration of the sugar available. The products formed from amylopectin breakdown are lactate, acetate, butyrate, hydrogen and carbon dioxide. The maximum endogenous rates of amylopectin breakdown are 12 and 85 pmol/cell/h for *Dasytricha* and *Isotricha* spp., respectively.

Glucose is taken up by the entodiniomorphid ciliates principally into the cell pool, polysaccharide granules and intracellular bacteria. The metabolites ultimately formed from glucose and its polymers by the entodiniomorphs are hydrogen, carbon dioxide, acetic and butyric acid with smaller amounts of lactic, formic and propionic acids. Glucose or maltose uptake, however, does not stimulate the rates of gas and acid production, as both sugars regulate enzymes involved in starch breakdown and thus the intracellular sugar levels remain constant.

Protozoal assimilation of soluble sugars into amylopectin decreases the extent of saccharolytic bacterial activity in the rumen in the immediate post-feed period. The subsequent protozoal fermentation of their reserve polysaccharide stabilizes the rate of acid formation and extends the post-prandial period of VF A synthesis. Coleman (1979a) calculated that approximately 30 per cent of dietary sugars were converted into amylopectin by the protozoal population.

Pathways of product formation

The principal metabolic routes of metabolite formation in the holotrichs have been identified (Williams, 1986; Williams and Coleman, 1988, 1991). The pathways in *D. ruminantium* and *Isotricha* spp. are the same and the enzymes involved are hydrogenosomal and cytosolic in location (Yarlett *et al.*, 1981, 1983, 1985). Pyruvate is generated by glycolysis and, in the absence of formate dehydrogenase, hydrogen is formed in a series of hydrogenosomal reactions (see Chapter 6). Additional adenosine triphosphate (ATP) is generated through substrate level phosphorylation reactions

during the formation of acetate and butyrate (Yarlett *et al.*, 1982, 1985). Rate limiting reactions in glycolysis and butyrate formation have been identified and factors regulating the activity of lactate dehydrogenase in *I. prostoma* have been described (Counotte *et al.*, 1980; Yarlett *et al.*, 1985; Mertens *et al.*, 1989).

There is relatively little known about intermediary metabolism of the entodiniomorphs. The evidence for the existence of glycolysis in members of the genus *Entodinium* is based on the inhibitory action of iodoacetate, the detection of aldolase and other enzymes from the pathway in crude homogenates, and the fate of radio-active label in tracer experiments (Coleman, 1964; 1981; Hoshino *et al.*, 1982). Some properties of pyruvate kinase from *Entodinium* spp. have recently been described (Wakita and Hoshino, 1989). Sedimentable hydrogenase and pyruvate synthase (pyruvate: ferredoxin oxidoreductase) activities have been detected in some entodiniomorphid ciliates, and, as with the isotrichid ciliates, the enzymes of hydrogen formation are located in hydrogenosomes (Yarlett *et al.*, 1984; Paul *et al.*, 1990). For further details on hydrogenosomes, see Chapter 6.

The ruminal ciliates that possess hydrogenosomes are not strictly anaerobic organisms and exhibit some characteristics of aerotolerance (Lloyd *et al.*, 1982; Yarlett *et al.*, 1982). The rumen ciliates have an important role in ruminal oxygen utilization (Ellis *et al.*, 1989) and VFA formation. These two metabolic features are, however, inextricably inter-related as the fermentation profiles of the ciliates are determined by physiological oxygen concentrations (Hillman *et al.*, 1985), which in turn are partially modulated by the hydrogenosome-containing rumen ciliates (Lloyd *et al.*, 1989).

Nitrogen metabolism

Protozoal digestion of dietary and microbial proteins has important effects on the nitrogen economy of the ruminant host. When protozoa are present in the rumen the efficiency of protein utilization by the ruminant is reduced. In general, the release of ammonia, the proteolytic activity of the ruminal population and ruminal protein degradation are all higher in faunated animals (Nolan, 1989). Although the rumen ciliates are able to synthesize amino acids *de novo* the most important sources of amino acids for protozoal protein synthesis are from the digestion of bacteria; plant proteins and free amino acids also represent a valuable nitrogen source for some species.

Bacterial ingestion

Rumen ciliates engulf bacteria, sometimes selectively, with maximum uptake rates approaching 10^5 bacterial cells/protozoan/h (Coleman, 1986a). The rate of bacterial uptake is species dependent and is influenced by the density of the bacterial population, the physical form of the bacteria, the nutritional status of the protozoa and environmental factors such as pH and salt concentration (Coleman, 1975, 1989).

The bacteria are engulfed into cytoplasmic vesicles and most are rapidly killed and digested with some release of soluble digestion products into the medium. The

predatory activities of the protozoa reduce the size of the total ruminal bacterial population and can influence the relative proportions of the bacterial types present. For example, the number of cellulolytic bacteria probably increases on faunation whereas the number of amylolytic bacteria falls (Coleman, 1989; Jouany, 1989; Williams and Coleman, 1991).

Ingested bacteria are digested in endoplasmic vesicles; the rate of digestion is dependent on both the type of protozoa and bacterial species involved. Similarly, cellular components differ in their rate and extent of digestion. The cell contents, peptidoglycan and proteins are rapidly degraded, whereas the lipopolysaccharide component of the cell wall of Gram-negative bacteria is more recalcitrant. Amino acids derived from the bacterial protein are incorporated unchanged into protozoal protein with only limited degradation. Bacterial purines and pyrimidines are also incorporated directly into protozoal nucleic acids. Bacterial predation and release of digestion products by the ciliates results in appreciable turnover of bacterial carbon and nitrogen in the rumen, and is important in ruminal nitrogen cycling as released amino acids are utilized for bacterial growth with subsequent release and loss of ammonia.

Some ingested bacteria are, however, resistant to degradation and viable bacteria can also be isolated from the protozoa. The presence of free bacteria within the cytoplasm of entodiniomorphid and holotrich ciliates has been revealed by electron microscopy (Williams and Butler, unpublished data), although it remains to be established whether these endobionts are parasitic or symbiotic. Intracellular bacteria do have an important role in the metabolism of soluble substances ingested by the protozoa. Many ciliates also have bacteria attached to outer surfaces of their pellicles. Some of the adherent bacteria have been shown to be methanogenic by epifluorescent microscopy (Stumm *et al.*, 1982; Coleman, 1989; Williams and Coleman, 1991).

Proteolysis

Although there is only limited information available on the uptake of protein by the ruminal ciliates (see Williams, 1989a), studies with mixed protozoal preparations (Prins *et al.*, 1983; Forsberg *et al.*, 1984) and single species (Coleman, 1983; Lockwood *et al.*, 1988) have provided information on their proteolytic enzymes. The protozoa contain several different proteinases. The enzymes are active over a wide pH range but the optimum pH in most species examined was between 3 and 4.5; the rate of breakdown was found to be substrate dependent (Williams, 1989a). Inhibitor effects indicated that the protozoal proteinases were predominantly of the aspartic and cysteine types (see Chapter 15) with M_r values ranging from 25000 to 200000 (Coleman, 1983; Lockwood *et al.*, 1988). The molecular weight distribution and inhibitor susceptibilities of the proteinases varied in protozoa from different genera. These variations and the variations in the proteinase profiles of the apparently closely related isotrichid genera, and within the genus *Entodinium*, would indicate that a wide range of proteinase activities are present within the protozoal population.

Amino acid metabolism

All of the rumen ciliates studied are able to take up free amino acids by both active and passive mechanisms. The rate of amino acid uptake varies between species and individual amino acids. The rates of amino acid uptake determined *in vitro* would not meet the demand for protein synthesis if other sources of amino acids were not available. As most protozoa do not synthesize quantitatively significant amounts of amino acids from starch or glucose, the acids required for protein synthesis must be obtained from the ingestion and digestion of microbial cells and feed materials (plant matter, protein supplements, etc.) consumed by the host ruminant.

After uptake or release, the subsequent metabolism of amino acids is species dependent, but, in general, few interconversions occur and the amino acids are not extensively degraded but are incorporated directly into protozoal protein. Some catabolism of amino acids does, however occur. Unfortunately most investigations have been undertaken using mixed protozoal preparations (Coleman, 1986a; Williams, 1986, 1989a; Williams and Coleman, 1991) and hence the degradative activities of the individual ciliates are unknown. Many amino acids are *N*-acetylated or formylated prior to excretion, whilst a small proportion are oxidatively deaminated with the loss of the C1 moiety as carbon dioxide to form the corresponding branched-chain fatty acid. Decreased ruminal ammonia levels consistently accompany defaunation and the deaminase activity of the microbial population is increased in the presence of protozoa. Extracts of mixed rumen ciliates deaminate several amino acids in the presence of nicotinamide adenine dinucleotide (NAD) and deamination is enhanced in the presence of 2-ketoglutarate, suggesting that glutamate dehydrogenase is involved following the transamination reactions (Hino and Russell, 1985). A wide range of amino acids are transaminated by mixed protozoal enzyme preparations and a pyridoxal phosphate requiring branched-chain amino acid aminotransferase has been partially purified from an *Entodinium* spp. preparation (Wakita and Hoshino, 1975). Pyridoxal phosphate is also a co-factor during the dethioalkylation of sulphur containing amino acids (see Chapter 9). The decarboxylation of diaminopimelic acid produces lysine which can be further metabolized to pipecolate. Ornithine, citrulline and proline are intermediates in the sequential conversion of arginine to δ -aminovalerate. The ciliates also produce 2-aminobutyrate from threonine and methionine; the decarboxylation of the 2-ketobutyrate intermediate results in some propionate formation (Williams, 1989a). The energetic significance of amino acid catabolism to the rumen ciliate is not known.

Lipid metabolism

The role of protozoa in ruminal lipid metabolism is poorly resolved as there have been relatively few investigations into the metabolism of lipids by the ruminal holotrich and entodiniomorphid ciliates (Coleman, 1986a; Williams, 1986). The ciliates are able to synthesize long-chain fatty acids and phospholipids from short-chain precursors (Demeyer *et al.*, 1987) and ciliate phospholipids are a potentially

important source of choline for the host as free choline is readily degraded by the rumen bacteria. The protozoa, however, have only a limited ability to assimilate and transform dietary lipid, although the ciliates contribute significantly to total ruminal lipolytic activity (estimates range from 20 to 75 per cent). Their involvement in the biohydrogenation of unsaturated lipids has not been conclusively established, although it is indicated from biochemical and defaunation studies. The capacity of rumen contents to hydrogenate unsaturated lipids is only slightly reduced by defaunation, nevertheless the proportion of unsaturated fatty acids is lower in the plasma of faunated animals. The majority of studies undertaken have confirmed that the entodiniomorphid ciliates are able to hydrogenate linoleic acid and other free unsaturated long-chain fatty acids (Coleman, 1986a; Williams, 1989a; Williams and Coleman, 1991). The involvement of the holotrichs in the process is, however, equivocal; although hydrogenation of oleic acid and linoleic acid has been observed, other studies failed to detect holotrich hydrogenation and the involvement of associated bacteria in the process was implicated (Williams, 1986).

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6. Energy metabolism of anaerobic parasitic protists

M.Müller

INTRODUCTION

A number of parasitic and endosymbiotic protists and helminths have a fermentative metabolism, i.e. produce organic acids and alcohols from hexoses used as energy source, instead of completely oxidizing them to CO₂ and H₂O. Several thrive in the absence of O₂ and can be termed 'anaerobic'. The degree of independence from O₂ varies from species to species and some could be termed more properly 'microaerophilic'. For convenience, however, only the term 'anaerobic' will be used here with full realization of its ambiguity.

This chapter is a brief and selective overview of some aspects of the energy metabolism of anaerobic protists. This topic has been reviewed repeatedly either from a comparative point of view (e.g. Müller, 1988; Bryant and Behm, 1989; Fairlamb, 1989; Coombs, 1990) or as related to specific organisms (e.g. Reeves, 1984; McLaughlin and Aley, 1985; Williams, 1986; Orpin, 1988; Bauchop, 1989; Jarroll *et al.*, 1989; Müller, 1989). Other chapters in this volume also deal with some of the problems raised here (see Chapters 5 and 7–9). These papers contain references to data supporting a number of general statements made below. Only those few organisms on which sufficient biochemical information is available for a detailed discussion are considered (Table 6.1).

The view on anaerobiosis in parasitic organisms has been radically transformed in the past decade and a half. It is now recognized that fundamental organizational and enzymatic differences in the nature of the energy metabolism exist between

1. anaerobic protists and aerobic protists; and
2. anaerobic protists and anaerobic helminths.

Major groups of anaerobic protists do not contain mitochondria and their energy metabolism is either entirely cytosolic or is compartmentalized between the cytosol and hydrogenosomes (Müller, 1988). Several key enzymes of this metabolism are markedly different from enzymes in aerobic protists, just as hydrogenosomes are different from mitochondria. This means that anaerobic protists express a number of

Table 6.1. Systematic position and some characteristics of the anaerobic protists discussed.

Species ^a	Habitat	Hydrogenosome	Major end products	
			Anaerobic	Aerobic
Phylum Rhizopoda				
<i>Entamoeba histolytica</i>	Human large intestine	No	Ethanol, CO ₂	Ethanol, acetate, CO ₂
Phylum Zoomastigina				
<i>Giardia lamblia</i>	Human small intestine	No	Ethanol, acetate, CO ₂	Ethanol, acetate, CO ₂
<i>Trichomonas vaginalis</i>	Human genitourinary tract	Yes	Glycerol, lactate, acetate, H ₂ , CO ₂	Glycerol, lactate, acetate, CO ₂
<i>Tritrichomonas foetus</i>	Bovine genitourinary tract	Yes	Glycerol, succinate, acetate, H ₂ , CO ₂	Glycerol, succinate, acetate, CO ₂
Phylum Ciliophora				
<i>Dasytricha ruminantium</i>	Ovine rumen	Yes	Lactate, acetate, butyrate, H ₂ , CO ₂	Not viable
<i>Isotricha</i> species	Ovine and bovine rumen	Yes	Lactate, acetate, butyrate, H ₂ , CO ₂	Not viable
Phylum Chytridiomycota				
<i>Neocallimastix patriciarum</i>	Ovine rumen	Yes	Lactate, acetate, formate, ethanol, H ₂ , CO ₂	Not viable

^a Designation of phyla is for orientation only without any commitment to the numerous macrosystems of protists proposed currently. Phylogenetic distances between the flagellates listed are certainly greater than implied by their assignment to the same 'phylum'.

genes which are not expressed or are not present in the aerobes. In contrast, anaerobic helminths rely on the usual cytosol/mitochondrion compartmentation of eukaryotes (Köhler, 1985) and many of them, at least in certain stages of their life cycle, do express genes of proteins characteristic of aerobic metabolism (Fairbairn, 1970).

The main energy substrates for anaerobic protists are carbohydrates. Certain amino acids can also serve as energy substrates (see Chapters 8 and 9), but their role has been insufficiently explored so far, and it is not known to what extent they can support energy metabolism in the absence of carbohydrates. Recent data indicate that fatty acids are not utilized as energy substrates.

This chapter is restricted to a discussion of carbohydrate catabolism. Glucose or glycogen are catabolized via glycolysis leading to the formation of phosphoenolpyruvate and pyruvate. Although in some species pyruvate is reduced to lactate as a major pathway of end product formation, all organisms are able to convert pyruvate and phosphoenolpyruvate to additional end products, such as acetate and ethanol. The reactions responsible for their production can be regarded as extensions of the glycolytic pathway. It needs to be emphasized that these organisms do not contain a complete tricarboxylic acid cycle; thus they cannot oxidize acetyl-CoA to CO₂, nor do they link oxidative steps to electron transportlinked, membrane-dependent phosphorylation of ADP to ATP. In essence,

their energy metabolism is short and lacks several key components of carbohydrate metabolism which enable aerobic eukaryotes to obtain further energy from nutrients.

The organisms in question tolerate the presence of O₂, although some only in low concentrations. This is not unexpected since their natural habitats almost always contain some O₂. It needs emphasizing, however, that their metabolism remains fermentative whether O₂ is absent or present. If present, O₂ serves as terminal electron acceptor; thus all these organisms respire. The presence of O₂ affects significantly the overall energy metabolism, the quantities and also the nature of organic end products. This was recognized from early studies in which the effects of more or less strict anaerobiosis and of full aerobiosis were compared. Recent developments in instrumentation permit the control of O₂ partial pressure at low levels and have provided new insights into the regulation of metabolism under conditions likely to occur in the natural habitats of anaerobic protists (Lloyd *et al.*, 1987; Paget and Lloyd, 1990; Paget *et al.*, in press; Chapter 7). The mechanisms of these controls remain to be elucidated but a major factor is probably the ratio of reduced and oxidized pyridine nucleotides (Paget and Lloyd, 1990). Though these developments are of great importance, for the sake of simplicity I deal here only with genuinely anaerobic processes.

GIARDIA LAMBLIA AND TRICHOMONAS VAGINALIS

The carbohydrate catabolism of two reasonably well-studied species can be used as paradigms of two main types of anaerobic energy metabolism of protists. Their presentation is, by necessity, simplified and some details might need revision in the future, e.g. the findings reported in Chapter 8 suggest that the pathway leading to acetate in *Giardia lamblia* is incompletely understood. The two species are *G. lamblia*, which does not contain hydrogenosomes (Figure 6.1) and *Trichomonas vaginalis*, which does (Figures 6.2 and 6.3). As ‘typical’ anaerobic protists, both show the hallmarks of such organisms; i.e. their metabolism is fermentative and they contain a number of enzymes which differ from those present in aerobic protists.

The common metabolic characteristic of the two species is the presence of an extended glycolytic pathway from hexose to acetate (Lindmark, 1980; Steinbüchel and Müller, 1986a). The pyruvate produced in glycolysis is decarboxylated to acetylCoA which yields acetate as a metabolic end product while the energy of the thioester bond is conserved in a substrate level phosphorylation reaction. In both organisms, enzymes have been detected which can provide a bypass of unknown significance from phosphoenolpyruvate to pyruvate through oxalacetate and malate. Pyruvate is also reduced to other products, ethanol in *G. lamblia* and lactate in *T. vaginalis*. Energy conservation in both organisms occurs only by substrate level phosphorylation and the number of possible steps of phosphorylation is identical for any given end product, whether the metabolism is compartmentalized or not.

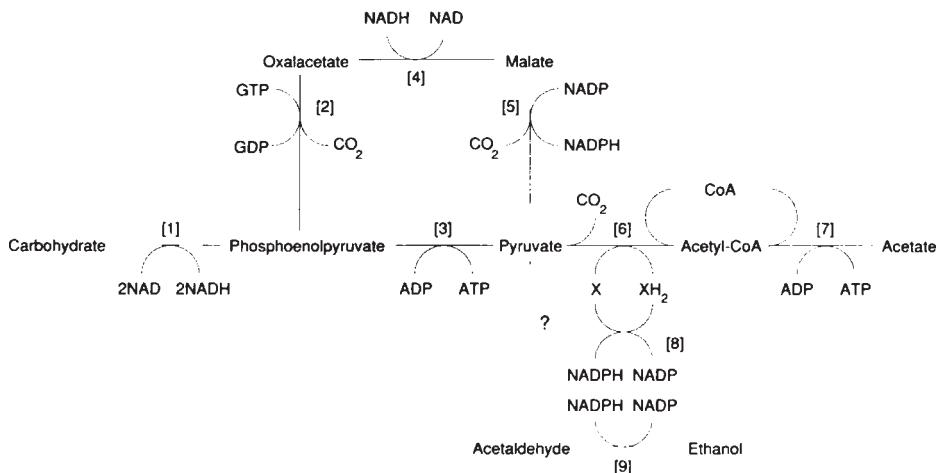


Figure 6.1. Map of anaerobic carbohydrate catabolism on *G. lamblia*. [1] Glycolytic enzymes; [2] phosphoenolpyruvate carboxykinase (GDP); [3] pyruvate kinase (ADP); [4] malate dehydrogenase (NAD); [5] malate dehydrogenase (decarboxylating) (NADP); [6] pyruvate: ferredoxin oxidoreductase; [7] acetate thiokinase; [8] NADPH: acceptor oxidoreductase; [9] alcohol dehydrogenase (NADP); X, electron transport protein, probably ferredoxin. (Reproduced with permission from Lindmark, 1980.)

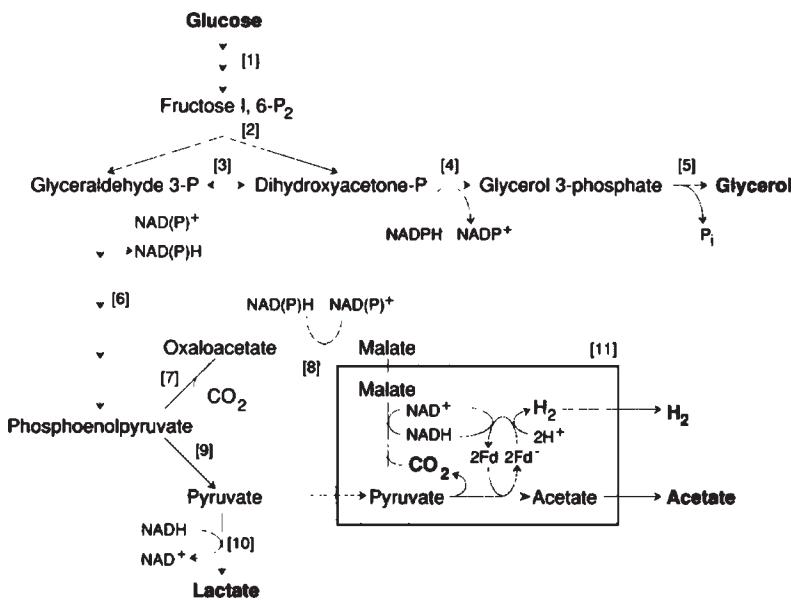


Figure 6.2. Map of anaerobic carbohydrate catabolism of *Trichomonas vaginalis*. [1] Enzymes forming fructose-1, 6-biphosphate; [2] aldolase; [3] triosephosphateisomerase; [4] glycerol 3-phosphate dehydrogenase (NADP); [5] glycerol 3-phosphatase; [6] enzymes of the C3 part of glycolysis; [7] phosphoenolpyruvate carboxykinase (?); [8] malate dehydrogenase [NAD(P)]; [9] pyruvate kinase; [10] lactate dehydrogenase; [11] hydrogenosomal enzymes (see Figure 6.3). (Reproduced with permission from Steinbüchel and Müller, 1986a.)

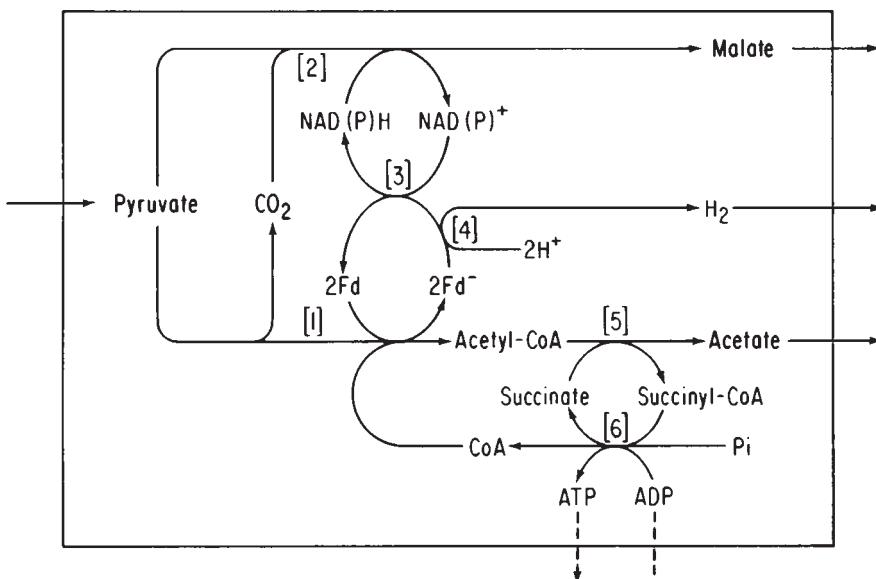
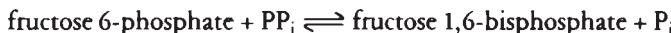


Figure 6.3. Map of hydrogenosomal metabolism of *Trichomonas vaginalis*. [1] Pyruvate:ferredoxin oxidoreductase; [2] malate dehydrogenase (decatboxylating) (NADP); [3] NAD (P): ferredoxin oxidoreductase; [4] H₂: ferredoxin oxidoreductase; [5] acetate:succinate CoA-transferase; [6] succinate thiokinase. Dashed arrows indicate a postulated adenyl nucleotide transfer. (Reproduced with permission from Steinbüchel and Müller, 1986b.)

There are, however, differences in the overall scheme between the two organisms. First, the reducing equivalents from pyruvate oxidation are utilized in *G. lamblia* in the formation of reduced end products and are not released as H₂, while the latter process occurs in *T. vaginalis*. Second, the energy of the thioester bond is conserved by a one-step reaction in *G. lamblia* and by a two-step reaction in *T. vaginalis*. In *T. vaginalis*, glycerol is also produced in glycolysis.

The two species also differ from aerobic eukaryotes in the nature of several key enzymes and their subcellular location. In the glycolytic sequence, both contain, instead of an ATP-linked enzyme, an inorganic pyrophosphate (PP_i) linked phosphofructokinase (Mertens *et al.*, 1989; Mertens, 1990):



which is unaffected by the major regulatory compound of glycolysis, fructose-2, 6-bisphosphate and other effector compounds. The replacement of ATP by PP_i as phosphoryl donor in this enzymatic step renders the reaction freely reversible. Since ATP-phosphofructokinase is the major regulatory enzyme of glycolysis in most organisms, its absence from *G. lamblia* and *T. vaginalis* indicates a different mechanism of glycolytic regulation. The use of PP_i as phosphoryl donor is likely to

benefit an organism which obtains its ATP primarily from glycolysis. PP_i is a 'waste product' of biosynthetic reactions and the energy of the pyrophosphate bond is usually lost by hydrolysis. PP_i linked phosphofructokinase salvages the energy of the pyrophosphate bond and thus decreases the input of ATP needed for glycolysis, in other words it increases the ATP yield. Biosynthetic PP_i production and glycolysis were calculated to proceed at similar rates in *T. vaginalis* (Searle and Müller, 1991) indicating that PP_i production might be a factor regulating glycolysis. Partial amino acid sequence data of *T. vaginalis* PP_i phosphofructokinase show similarities to ATP-phosphofructokinases of both prokaryotes and eukaryotes (E.Mertens and R.G. Kemp, personal communication) suggesting that these enzymes belong to the same protein family and probably originate from a common ancestor.

A most important characteristic of the two species is the nature of the enzyme that catalyses the oxidative decarboxylation of pyruvate, a key step in carbohydrate catabolism. In *G. lamblia* and *T. vaginalis* this enzyme is pyruvate:ferredoxin oxidoreductase, a reversible iron-sulphur enzyme of modest size (240 kDa in *T. vaginalis*) (Lindmark, 1980; Williams *et al.*, 1987), which uses ferredoxin, another iron-sulphur protein, as electron acceptor. Lipoate is not involved in this reaction. In mitochondrion-containing protists—with the known exception of euglenoid flagellates (Inui *et al.*, 1987)—the enzyme catalysing the reaction is the irreversible pyruvate dehydrogenase multienzyme complex with lipoate as cofactor and NAD⁺ as electron acceptor (Wahl and Orme-Johnson, 1987). The mechanisms of the two enzymes are also fundamentally different (Reed, 1974; Wahl and Orme-Johnson, 1987). The reaction mechanism of pyruvate:ferredoxin oxidoreductase has been clarified in the related species, *Tritrichomonas foetus*, revealing a unique process with the involvement of two free radical species (Docampo *et al.*, 1987). The purified *T. vaginalis* enzyme (Williams *et al.*, 1987) is similar in all properties studied so far to prokaryotic pyruvate:ferredoxin (or flavodoxin) oxidoreductases (Kerscher and Oesterhelt, 1982). The *G. lamblia* enzyme has not been purified yet and its identity has been established by studies on cell extracts (Lindmark, 1980).

Partial sequence on the *T. vaginalis* enzyme reveal (unpublished results) a significant homology to the only related enzyme sequenced, pyruvate:flavodoxin oxidoreductase (*nif J*) of *Klebsiella pneumoniae* (Arnold *et al.*, 1988), indicating that these enzymes belong to the same protein family. Neither *nif J* nor the N-terminal sequence of *T. vaginalis* enzyme shows homology with the pyruvate decarboxylase enzyme (E_i) of the pyruvate dehydrogenase complex.

The thioester energy of acetyl-CoA formed by oxidative decarboxylation of pyruvate is conserved as high energy phosphate bond by substrate level phosphorylation of ADP to ATP. The enzymatic steps of this process are insufficiently known. In *G. lamblia* the reaction occurs in a single step, catalysed by acetate thiokinase (Lindmark, 1980).

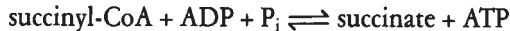


This enzyme seems to be restricted to a few organisms and, besides *G. lamblia* has been noted only in *Entamoeba histolytica* (Reeves *et al.*, 1977) and the bacterium,

Selenomonas ruminantium (Michel and Macy, 1990). In *T. vaginalis* hydrogenosomes, the CoA moiety is first transferred from acetate to succinate:



thus producing the substrate for succinate thiokinase (T.Jenkins, P.D.J.Weitzman and M.Müller, unpublished data), an enzyme well known from the tricarboxylic acid cycle:



In *G. lamblia*, the 'reducing equivalents' generated by pyruvate oxidation are removed via reduced organic compounds (Lindmark, 1980). *T. vaginalis* contains hydrogenase (H_2 ; ferredoxin oxidoreductase) which permits the anaerobic removal of 'reducing equivalents' in the form of molecular hydrogen (Steinbüchel and Müller, 1986b). The electron transfer protein that serves as an acceptor for pyruvate:ferredoxin oxidoreductase is not known in *G. lamblia* but is probably a ferredoxin. A [2Fe-2S] ferredoxin has been purified and characterized from *T. vaginalis* (Gorrell *et al.*, 1984). This protein serves as electron acceptor for pyruvate: ferredoxin oxidoreductase and donor for hydrogenase, a function demonstrated *in vitro* as well (Steinbüchel and Müller, 1986). *T. vaginalis* ferredoxin shows great similarity in its amino acid sequence and in the environment of its iron-sulphur cluster to ferredoxins participating in mixed function oxidase systems of aerobic bacteria and vertebrate mitochondria and in its amino acid sequence also to cyanobacterial ferredoxins (Johnson *et al.*, 1990). This ferredoxin differs markedly in its size and other properties from those 2[4Fe-4S] ferredoxins which usually serve as electron donors for hydrogenase in anaerobic bacteria (Gorrell *et al.*, 1984).

Last but not least, the subcellular location of the pathways must be discussed. No compartmentation exists in *G. lamblia* and all the enzymes mentioned are cytosolic. In *T. vaginalis*, however, a significant part of the hexose to acetate pathway is localized in an organelle (Lindmark *et al.*, 1975), limited by two unit membranes, the hydrogenosome (Figure 6.3). This organelle is the site of the oxidative decarboxylation by pyruvate: ferredoxin oxidoreductase, the conversion of acetyl-CoA to acetate accompanied by substrate level phosphorylation, and the formation of H_2 (Steinbüchel and Müller, 1986b). The complete protein composition of the *T. vaginalis* hydrogenosome has not yet been elucidated and the biological nature remains enigmatic. It probably does not contain DNA (Turner and Müller, 1983).

The contribution of these organelles to the energetics of *T. vaginalis* remains an open question. *T. vaginalis* lacking hydrogenosomal carbon flow, as shown by the absence of acetate and H_2 generation, grows extremely slowly (Čerkasovová *et al.*, 1986), indicating that hydrogenosomal functions are beneficial. At the same time, a consideration of *T. vaginalis* hexose catabolism does not reveal an obvious difference of energy yield between the fermentation of the wild type organism and the hydrogenosome-deficient one. Wild type cells eliminate equivalent molar amounts of

glycerol and acetate. Each mole of acetate produced by hydrogenosomes provides 1 mol of ATP. The formation of each mol of glycerol requires, however, the hydrolysis of 1 mol of glycerol 3-phosphate by cytosolic phosphatase; thus leading to the loss of 1 mol of phosphate ester (Steinbüchel and Müller, 1986a). This ATP yield corresponds to that of the yield of homolactic fermentation observed in hydrogenosome-deficient lines (Čerkasovová *et al.*, 1986).

OTHER SPECIES

The additional organisms listed in Table 6.1 can easily be assigned to one or other of the paradigmatic patterns discussed above. *Entamoeba histolytica* is a typical hydrogenosome-free organism which does not produce hydrogen. Other trichomonad species, the rumen ciliates and the rumen fungus, *Neocallimastix patriciarum*, produce H₂, corresponding to the presence of hydrogenosomes. Certain characteristics of some of these organisms, particularly differences between various species, deserve brief mention. A topic of great interest, but not discussed here, is the presence of hydrogenosomes in a number of free living ciliates from anaerobic sediments (Zwart *et al.*, 1988; Fenchel and Finlay, 1991).

Entamoeba histolytica is the only hydrogenosome-free anaerobic protist other than *Giardia* that has been subjected to great scrutiny. Its metabolism resembles that of *G. lamblia*. This species provided the first detailed insights into one of the major peculiarities of anaerobic protists, notably the privileged role of PP_i (Reeves, 1984). In addition to PP_i-phosphofructokinase, *E. histolytica* contains other PP_i-linked enzymes of extended glycolysis, including an AMP, PP_i-pyruvate dikinase instead of an ATP-linked pyruvate kinase. The significance of the presence of these PP_i-linked enzymes remains unknown. Pyruvate:ferredoxin oxidoreductase and its electron acceptor ferredoxin of *E. histolytica* have been characterized (Reeves *et al.*, 1977, 1980). It is noteworthy that the latter is a 2[4Fe-4S]ferredoxin which is strikingly similar to ferredoxins of *Clostridium* species (Huber *et al.*, 1989). Acetyl-CoA is converted to acetate by the action of acetate thiokinase (Reeves *et al.*, 1977).

The presence of hydrogenosomes has been demonstrated in several trichomonad species (Lindmark and Müller, 1973, 1974; Lindmark *et al.*, 1975; Müller, 1980), rumen ciliates (*Snytis* *et al.*, 1982; *Yalik* *et al.*, 1981, 1983, 1984; Paul *et al.*, 1990) and a rumen fungus (Yarlett *et al.*, 1986). The metabolism of all these organisms is similar to that of *T. vaginalis*. Some of the notable differences are the presence of an ATP-linked phosphofructokinase, instead of a PP_i-linked one, in *N. patriciarum* (E.Mertens, personal communication), a reversed partial tricarboxylic acid cycle in the cytosol of *T. foetus*, leading to the production of succinate (Müller, 1976), and the extension of the hydrogenosomal metabolism of holotrich rumen ciliates leading to the formation of butyrate (Yarlett *et al.*, 1985). The mechanism of hydrogenosomal substrate level phosphorylation in rumen ciliates is not fully elucidated. The detection in rumen ciliates (Yarlett *et al.*, 1981) of enzymes of acetyl phosphate metabolism, previously noted only in prokaryotes, is a finding that needs further study.

GENERAL COMMENTS

The enzymatic and organellar patterns outlined above seem to be in basic register with the environmental conditions that the organisms discussed usually encounter. The adaptive value of any of these properties is however difficult to evaluate. In certain cases there is some evidence suggesting an advantage for the organisms, but in most cases we are still groping in the dark. Recent studies, mentioned below, confirm that the diverse organisms which exhibit an 'anaerobic' metabolic organization belong to several independent branches of the phylogenetic tree. They share only the nature of their habitat without having a common origin. This points strongly to their 'convergent' evolution having been determined largely by ecological factors and also to a significant adaptive value of the 'anaerobic' metabolic organization for protists living in environments of low oxygen content.

It is not known how widespread in nature the basic metabolic pattern discussed here is, and where else hydrogenosomes are still to be found. Even our limited survey reveals, however, that both the absence of mitochondria and the presence of one or another constellation of 'anaerobic' enzymes can be observed in organisms that belong to highly different evolutionary lineages, some more 'ancestral' and some more 'evolved', as assumed for some time and confirmed by recent phylogenetic studies based on rDNA sequence comparisons (Baroin *et al.*, 1988; Sogin, 1989). Such studies also confirmed that the rumen ciliates with hydrogenosomes are close relatives of free-living ciliates (P.Delgado and A.Baroin, personal communication) which contain mitochondria and that rumen organisms of the genus *Neocallimastix* indeed belong to Chytridiomycota (A.G.Brownlee, personal communication; Doré and Stahl, 1991). Members of this group, with the exception of species inhabiting the rumen, all contain mitochondria. The close affinity of *Neocallimastix* species and other chytridiomycetes to other fungal groups is well established (A.G.Brownlee, personal communication; Doré and Stahl, 1991). Whether or not further taxa with similar 'anaerobic' properties will be discovered, current knowledge forces us to reach the conclusion that organisms with 'anaerobic' enzymes and organelles cannot be grouped into a single evolutionary lineage and that some, but probably not all, of them were derived from aerobic, mitochondrion-containing ancestors. These considerations run counter to the interpretation of 'anaerobic' characteristics as primitive or ancestral ones. Even if we disregard the phylogenetic diversity of 'anaerobic' protists, the data can hardly be interpreted in terms of an orthogonal evolution of energy metabolism. The evolutionary history of 'anaerobic' metabolism is a topic which presents an exciting challenge to future investigators.

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7. The effects of environmental factors on the metabolism of *Giardia* and *Trichomonas*

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The human parasites *Giardia lamblia* and *Trichomonas vaginalis* parasitize sites where levels of nutrients, competing organisms and gaseous composition fluctuate with respect to time. In this chapter the influence of environmental variables on parameters such as metabolic flux and drug sensitivity are discussed. Particular attention is focussed on the effects of O₂ and CO₂ on metabolism, as techniques developed in this laboratory have allowed the monitoring of these gases in tandem with other parameters such as intracellular redox potential. The two stage life cycle of *G. lamblia* makes an understanding of the relationship between the parasite and its different environments more complex than for *T. vaginalis*. These studies have provided new insights into possible antiparasite strategies.

METHODS FOR STUDIES AT CONTROLLED GAS CONCENTRATIONS

To maintain organisms at controlled gas concentrations, the experimental system must be open for gases. This technology was originally designed to monitor O₂, using an O₂ electrode (Lloyd *et al.*, 1982). In the closed O₂ electrode, as traditionally employed for respiratory studies, O₂ levels continuously decrease during the experiment and steady-state measurements are not possible. In the open system, however, the level of dissolved O₂ is monitored under a gas phase kept at a fixed O₂ tension. The resulting steady-state level of dissolved O₂ represents a balance between supply and demand. By increasing or decreasing the level of O₂ in the gas phase it is possible to poise the system at any required O₂ concentration. This system was limited in that O₂ was the only gas that could be monitored. Mass spectrometry (MS) provides a method for the separation, identification and quantification of ions using a single detector. Interfacing the vacuum of a quadrupole mass spectrometer through a gas permeable membrane, such as Teflon, provides a method for the

simultaneous and continuous monitoring of many gases in solution (Lloyd *et al.*, 1989). With a silicone membrane, low molecular weight volatile compounds such as ethanol can also be monitored. Replacing the O₂ electrode with the mass spectrometer probe in an open system, produces a versatile method of monitoring the effects of steady-state concentrations of many gases, not just O₂, on biological systems. By modifying the design of the open system, it has been possible to combine MS with spectrophotometry, as well as fluorescence, and chemiluminescence monitoring, and thus allow the simultaneous monitoring of several environmental and intracellular parameters (Lloyd *et al.*, 1989).

GIARDIA LAMBLIA

G. lamblia (syn. *G. intestinalis*, *Lamblia intestinalis*) is a motile, binucleate protozoan; it exhibits two morphologically distinct forms: the 'dormant' cyst and the 'vegetative' trophozoite. Its life cycle is direct, requiring no intermediate host. Trophozoites colonize the upper small intestine and, when triggered, round up and elaborate a cyst wall. The resulting cyst is shed with the faeces. Transmission occurs when viable cysts are ingested. In the intestine the cysts excyst to produce trophozoites; infection results in a severe diarrhoeal disease.

During its life cycle, *Giardia* encounters a wide range of environmental conditions. Trophozoites in the intestine are maintained at a constant temperature, poised around pH6.8 (Evans *et al.*, 1988), at high concentrations of dissolved CO₂ (Atkinson, 1980), and at levels of O₂ that fluctuate between 0 and 60 µM (Atkinson, 1980). *Giardia* trophozoites also encounter the host-derived immune response and must compete with intestinal epithelial cells and gut microflora for available nutrients. Cysts have to survive a wide range of ambient conditions after ejection from the host.

What influences do environmental factors have on the life cycle, morphology and biochemistry of the organism?

Trophozoites

G. lamblia trophozoites are motile, yet they attach to the intestinal epithelium by means of a ventral striated disc (Erlandsen *et al.*, 1978). Attachment is an important event in infection. The organisms must adhere strongly enough to prevent expulsion from the intestine, but they must also be able to detach and reattach so as not to be sloughed off during the constant renewal of cells.

The factors affecting trophozoite attachment to glass *in vitro* have been reviewed by Gillin (1984). Several major requirements were identified. These include serum and thiols, in particular cysteine. The role of cysteine in attachment is unclear; however, the fact that surface proteins are rich in cysteine (Aggarwal *et al.*, 1989) may be significant. The pH and divalent cations such as Ca²⁺ and Mg²⁺ affect attachment, suggesting that this process involves ionic interactions (Munoz *et al.*, 1988). The relative importance of these factors in attachment to mucosal surfaces

has not been determined; local variations in ionic strength, pH and redox potential must affect the process. *In situ*, the trophozoite has to compete for nutrients with the natural microbial flora and the gut mucosal cells. Relationships between organisms in the gut have not been elucidated. Vitamin deficiency and lactose intolerance have been correlated with *Giardia* infections in humans; this may, in part, be related to consequent changes in the gut microbial flora (Tolboom, 1989). *G. lamblia* has complex nutritional requirements. Glucose is the only natural sugar utilized by trophozoites (Jarroll *et al.*, 1989); amino acids may also act as a source of energy (Paget *et al.*, 1991; and see Chapter 8). The organism scavenges purines, pyrimidines and lipids from the environment (Jarroll *et al.*, 1989). Amino acids, other than alanine and valine, are not synthesized *de novo* by *G. lamblia* (Paget *et al.*, 1991; and see Chapter 8). *Giardia* is thus highly dependent on the nutrients available in the gut. Use of ready-formed precursors for biosynthesis allows the organism to minimize its biosynthetic capacity and hence its energy requirements.

It has been shown that O₂ does not stimulate the growth of *G. lamblia* (Gillin, 1984). These results, however, were obtained from cells growing in a medium containing cysteine. Paget and Lloyd (1989) have shown that cysteine in the presence of O₂ is detrimental to the growth of some 'anaerobic' parasites; therefore, the interpretation of the results is not straightforward. Definitive studies to determine the effects of O₂ on growth are further complicated by the fact that cysteine is essential for the growth of this organism. Trophozoites of *Giardia* can survive exposure to air for several hours without loss of viability, this would seem to suggest that *Giardia* can certainly be classed as aerotolerant.

Observations on non-proliferating trophozoites showed that they possess respiratory activity. Although the pathways involved in O₂ uptake have not been fully characterized, flavin and FeS proteins play some role (Jarroll *et al.*, 1989). Trophozoites have a high affinity for O₂, but the O₂ dependence of respiration is unusual; respiration rate increases with increasing O₂ concentration up to a threshold, above which O₂ consumption decreases. This threshold is around 80 μM. The inhibition of O₂ uptake at O₂ concentrations greater than 80 μM was found to be irreversible; no reduction in parasite motility was observed during the course of the experiment (Paget *et al.*, 1989). The presence of thresholds for respiration suggests that O₂ has a deleterious effect on respiratory activity. In other organisms, O₂ inhibition thresholds have been correlated with the production of active oxygen species (Lloyd *et al.*, 1989).

The carbohydrate metabolism of trophozoites is typically fermentative; glucose is converted via the Embden-Meyerhoff-Parnas pathway to pyruvate (Jarroll *et al.*, 1989). This intermediate is further metabolized to acetate, ethanol and CO₂ (see Chapter 6). O₂ affects the nature of the end products excreted by *G. lamblia*. Under anaerobic conditions, ethanol predominates; under aerobic conditions acetate and CO₂ are the major products (Jarroll *et al.*, 1989). Recently it has been shown that *G. lamblia* also produces alanine as an end product of carbohydrate metabolism (Edwards *et al.*, 1989), but only under anaerobic conditions (Paget *et al.*, 1990a). A detailed study of the effects of O₂ on the metabolism of *Giardia* revealed that even low concentrations of O₂ cause profound alterations in the carbon balance of this

Table 7.1. Rates of end product formation by *G. lamblia* trophozoites anaerobically and at various oxygen tensions.

End product	Rate of production ^a (nmol min ⁻¹ (10 ⁷ trophozoites) ⁻¹		
	Anaerobic	0.2 kPa O ₂	0.4 kPa O ₂
Ethanol	5.0 ± 0.2	6.2 ± 0.4	8.8 ± 0.4
Alanine	4.7 ± 0.2	3.2 ± 0.2	1.8 ± 0.2
Acetate	0.6 ± 0.1	1.2 ± 0.3	2.5 ± 0.4
CO ₂	4.5 ± 0.6	5.2 ± 0.4	7.0 ± 0.8
			5.2 ± 0.4
			0.78 ± 0.1
			5.7 ± 0.5
			8.2 ± 0.7

^a Rates of end product formation were determined by high performance liquid chromatography (HPLC) analysis of samples taken from reaction vessels maintained under various gas phases. CO₂ production was monitored continuously by mass spectrometry (MS).

organism (Table 7.1). It was suggested that changes in apportionment of carbon flow are linked to the redox state of the NAD(P)H pool (Paget *et al.*, 1991). The concentration of O₂ in the gut is thought to vary between 0 and 60 μM (Atkinson, 1980); however, the gut is a complex and dynamic ecosystem with varying local O₂ concentrations. The response of *Giardia* to oxygen suggests that its metabolic fluxes are sensitively modulated by localized O₂ levels.

Cysts

The cyst form of *Giardia* is responsible for transmission of giardiasis. Trophozoites must encyst or they will not survive outside the host. Despite the importance of encystation in the infection cycle, little is known about the mechanism(s) involved. Environmental conditions may play a part. Trophozoites colonize the small intestine by attaching to the intestinal epithelium; once they pass down the intestine they will be exposed to alkaline pH, high concentrations of bile and lipolytic products (Evans *et al.*, 1988).

It has been shown that bile at physiological concentrations induces encystment at pH7.8 (Gillin *et al.*, 1988). The cysts produced were observed to be similar in morphology to those isolated from human faeces. Rates of encystment were, however, very variable (from 1 to 95 per cent). Primary bile salts produce high levels of encystment (Gillin *et al.*, 1989). Rates of encystment increase with increasing pH up to 7.8, at values above this encystment decreases. The small intestine has a bacterial population of around 10⁵ ml⁻¹, whereas the large intestine has approximately 10¹⁰ ml⁻¹. Thus, as the trophozoites move down the intestinal tract into the large intestine they will be exposed to elevated concentrations of bacterial products. The role of bacterial products was, therefore, examined; lactic acid stimulates the terminal stages of encystment, consistently high numbers of cysts being produced (Gillin *et al.*, 1988).

Excystation is the primary event in infection. This process occurs when viable cysts are ingested. What factors stimulate this process? Several workers have studied

excystation in various species of *Giardia* *in situ*. The single most important external stimulus is thought to be the decrease in pH that cysts experience as they pass through the stomach (Rice and Schaefer, 1981). Cysts of *G. muris* exposed to acid have excystation rates of approximately 90 per cent; the value for cysts of *G. lamblia*, isolated from gerbils, is usually 40–60 per cent (Feeley, 1986). The effects of bile salts and reducing agents have also been investigated. Alone, none of these induces significant levels of excystation, but in combination with an acid pretreatment step, high levels are observed (Feeley, 1986). Sodium bicarbonate has been shown to play some role in this process (Rice and Schaefer, 1981). Successful excystation and encystation *in vitro* have been obtained by mimicking the succession of conditions occurring along the gastrointestinal tract (see Chapter 4).

There has been a sharp increase in the number of water-borne outbreaks of giardiasis in the past 20 years. Survival of cysts in water has been studied in some detail. Bingham and Meyer (1979) showed that survival of cysts of both *G. muris* and *G. lamblia* is temperature dependent, lower temperatures favouring cyst viability. At temperatures below 8°C, cysts were viable in distilled water for periods up to 5 months, whereas at 37°C, cysts lost viability after 4 days. More recently, De Regnier *et al.* (1989) measured cyst viability in lake, river, tap and distilled waters: their results agreed with those obtained by Bingham and Meyer (1979).

The temperature dependence of cyst viability indicated that cysts are metabolically active. It has now been shown that cysts of both *G. muris* and *G. lamblia* have respiratory activity (Paget *et al.*, 1989; 1990b). It appears, however, that temperature has a negligible effect on respiration below 7°C, and low temperatures favour cyst viability. These two factors are linked: at low temperatures the rate of endogenous substrate utilization is decreased, stored energy reserves are conserved and the viable lifetime of the cyst is prolonged. This may explain the high incidence of giardiasis in populations where untreated water is supplied from cold streams (Craun, 1984).

It is, therefore, evident that environmental factors play major roles in the life cycle of *Giardia*. However, the extent of environmental variability *in situ* is not fully known. More information will help the optimization of *in vitro* growth conditions and may allow the elaboration of a defined growth medium for trophozoites. A complete understanding of the mechanisms involved in encystation and excystation would facilitate laboratory culture of *G. lamblia* through the complete life cycle. The availability of a better experimental model for study may lead to the identification of novel targets for drug action. An economically feasible method of killing cysts in water supplies is also a high-priority goal.

TRICHOMONAS VAGINALIS

Three species of trichomonads infect man; they inhabit the vagina, the mouth, and the intestinal tract. Oxygen is present in all these locations at steady-state concentrations, dependent on supply (from the atmosphere, for instance with food and drink, or from the blood supply) and consumption rate (determined by the

demands of tissue respiration and the local microbial flora). Steep O₂ gradients exist near centres of rapid O₂ consumption. At any point, time-dependent fluctuations in ambient O₂ occur as a consequence of changing rates of supply and demand. The infecting organisms impose their own metabolic burden and, thereby, change the characteristics of the niche. For example, the O₂ consuming capacity of *T. vaginalis* is higher than that of most mammalian cells and even rivals that of brain tissue. As well as exhibiting high O₂ uptake rates, trichomonads have high O₂ affinities, which rival those in organisms with mitochondrial cytochrome oxidase (Lloyd *et al.*, 1982). The dissolved O₂ at the mucosal surface of the vagina has been measured to be about 13 µM (Wagner and Levin, 1978).

In the laboratory, trichomonads are routinely grown to high cell concentrations in the presence of reducing agents in sealed culture vessels (Diamond, 1957). These are the usual conditions that have been employed for the production of organisms for biochemical studies. Parasites grown under such conditions may, however, differ from those that occur naturally. It has been shown that omission of reducing agents from the growth medium results in metabolic and morphological changes (Paget and Lloyd, 1989). Tests designed for evaluation of *in vitro* minimal lethal concentrations of antitrichomonal agents frequently also employ anaerobic conditions, and these can influence the susceptibility of the parasite to the drug (Milne *et al.*, 1978).

Metronidazole is the most commonly used antitrichomonal drug (Breccia *et al.*, 1982). The biological reduction by the parasites of this and other 5-nitroimidazoles, to their corresponding nitro-radical anions, is accomplished rapidly and effectively when O₂ is kept below levels at which it can be readily detected. In the presence of 'excess' O₂, however, oxidation of the drug radical occurs with the production of superoxide radical (Wardman and Clarke, 1976). Most clinical isolates are killed by drug at 1 µg ml⁻¹ when tested under anaerobic conditions (Čerkasovova *et al.*, 1984). Organisms obtained from patients who had failed to respond to treatment are also usually killed by low drug concentrations in the anaerobic *in vitro* test. In aerobic tests, however, many of these isolates are not susceptible to killing by metronidazole (Milne *et al.*, 1978; Meingassner *et al.*, 1978). The importance of O₂ as a determinant of the metronidazole sensitivity of *T. vaginalis* emphasizes that this organism is not a strict anaerobe, that its normal life-style is geared to cope with (and perhaps even utilize) O₂ and that drug-susceptibility tests must take account of this.

Marked differences in the effects of aerobiosis on metronidazole metabolism in drug-susceptible and drug-resistant *T. vaginalis* strains (Beaulieu *et al.*, 1981) led to the demonstration that an O₂-consuming NADH oxidase was present and lowered activity was observed in one resistant isolate (Clarkson and Coombs, 1982); however, eight other resistant strains did not show this correlation (Müller and Gorrell, 1985). The discovery that metronidazole radical anions produced within intact organisms of the cattle parasite *Tritrichomonas foetus* reach concentrations above the limits of detection by electron-spin resonance (Moreno *et al.*, 1983) enabled direct comparison of intracellular accumulation of these toxic bioreduction products in sensitive and resistant strains of *T. vaginalis* (Lloyd and Pedersen, 1985). In this study, the responses of steady-state pools of nitroimidazole radical ions to controlled changes in O₂ levels indicated that radical

production in a metronidazole-resistant clinical isolate (strain CDC 85) was more sensitive to O₂ than radical production in susceptible strains. Thus, it is more difficult to reach and maintain the level of toxic radicals required for cell killing. To determine whether this phenomenon was due to increased intracellular O₂ concentrations, Yarlett *et al.* (1986a) investigated the O₂-dependence of the respiration rates in intact organisms and by subcellular fractions. The metronidazole-resistant organisms all showed increased K_m values for O₂, as did the corresponding hydrogenosomal-enriched suspensions. These results indicate that the hydrogenosomal oxidase in the metronidazole-resistant strains is less effective at scavenging traces of O₂ and, therefore, intracellular levels of O₂ are elevated. Evidence also suggests that ferredoxin (which mediates hydrogenosomal electron transport between pyruvate: ferredoxin oxidoreductase and hydrogenase, see Chapter 6) has different redox properties or is present at a lower concentration in the resistant isolates (Yarlett *et al.*, 1986b). It would seem, therefore, that nitroimidazole resistance in *T. vaginalis* is due in part to elevated intracellular levels of O₂, thereby reducing the efficacy of drug activation, and also to modification or decreased levels of pyruvate:ferredoxin oxidoreductase (Yarlett *et al.*, 1987).

T. vaginalis can be cultured in the presence of a vast excess of O₂, although growth rates decrease by comparison with those achieved anaerobically (Mack and Müller, 1978). In non-proliferating cell suspensions, fermentation product balances are modified to some extent by the presence of O₂ and also by enhanced levels of CO₂ (Steinbüchel and Müller, 1986). *In vivo* ¹³C NMR measurements under controlled conditions, and simultaneous MS monitoring to measure dissolved O₂, confirmed that traces of O₂ (e.g. 1 μM) lead to profound alterations in the ratios of glycerol, acetate and lactate produced (Lloyd *et al.*, 1989).

The effects of O₂ and CO₂ on growth and metabolic characteristics of *T. vaginalis* have been studied using continuous MS control of both gases (Paget and Lloyd, 1990). Organisms grew most rapidly with traces of O₂ (<0.25 μM) and with the CO₂ level controlled at 5 mM (Table 7.2). When CO₂ was not supplemented, or when O₂ was either decreased (by removing residues from N₂) or increased (to 1–3 μM), division times were longer. Under strictly anaerobic conditions and at 5 mM CO₂, equimolar glycerol and lactate accounted for more than 95 per cent of the measured products. Lower CO₂ favoured acetate production. Under microaerobic conditions (O₂<0.25 μM) and with no CO₂ supplementation, acetate became the major product; again 5 mM CO₂ gave increased glycerol and lactate production. Assays of key enzymes in cell-free extracts confirmed that the altered fluxes of carbon containing metabolites appeared to correlate with the different levels of enzyme activities in organisms produced under different gaseous environments. Fine-tuning of metabolic responses by allosteric controls superimposed on altered enzyme levels could account for this observed modulation of fluxes.

Thus, *T. vaginalis* appears to be neither a strict nor an aerotolerant anaerobe, but a microaerophile. It has a life-style that is exquisitely tuned to the fluctuations of its habitat. It proliferates rapidly in the presence of O₂, even at levels of 15–20 μM (Paget and Lloyd, 1990). Presumably, therefore, it can detoxify superoxide radical anions, as can some related trichomonads (Lindmark and Müller, 1974), and deal

Table 7.2. Effect of CO₂ and O₂ on the growth of *Trichomonas vaginalis*.

Conditions	Doubling time ^a (h)	Cell yield (organisms/ml)
N ₂ with traces of O ₂ (less than 0.25 μM)	3.5	1 × 10 ⁶
O ₂ free N ₂ ^b	4.4	2.5 × 10 ⁶
N ₂ with traces of O ₂ (less than 0.25 μM) + 5 mM CO ₂	2.3	1 × 10 ⁷
O ₂ free N ₂ ^b + 5 mM CO ₂	2.9	4 × 10 ⁶

^a Doubling times were determined graphically from growth curves and cell counts were performed using a Coulter counter. CO₂ levels were controlled from the MS via a BBC master computer.

^b Commercially available O₂ free N₂ from which O₂ was removed by passage through an Oxy-trap column (see Lloyd *et al.*, 1989).

with the H₂O₂ that may be produced. It can also reproduce when O₂ is effectively absent and when the only CO₂ available is that produced by its own metabolic activities. Optimum growth, however, requires high CO₂ and traces of O₂. The corresponding phenotype (low activity of pyruvate:ferredoxin oxidoreductase, the enzyme responsible for the reduction of 5-nitroimidazoles) is somewhat more resistant to killing by metronidazole than that produced under strictly anaerobic conditions with low CO₂ concentrations. This highlights the importance of environmental factors to the parasites' metabolism and, consequently, their sensitivity to drugs.

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8. Energy metabolism in *Giardia intestinalis*

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In 1980, Lindmark published observations on the energy metabolism of *Giardia intestinalis* (syn: *Giardia lamblia*) (Lindmark, 1980). This was quickly followed by reports on the nature of respiratory metabolism (Weinbach *et al.*, 1980) and additional observations on carbohydrate and lipid metabolism (Jarroll *et al.*, 1981). Virtually nothing else was reported until our findings in 1989 (Edwards *et al.*, 1989) which suggested that giardial energy metabolism needs to be reappraised.

THE INITIAL STUDIES

Despite its pathogenic nature and the ubiquity of giardiasis, giardial metabolism had not been studied in any detail before 1980, presumably because of the lack of suitable culture conditions. However, with the development of efficient and reliable axenic culture methods, sufficient *G. intestinalis* trophozoites could be grown for metabolic and enzymic studies. Lindmark (1980) took advantage of these techniques and adopted the conventional approach of harvesting cells grown *in vitro*, resuspending them in a minimal medium, and assessing the metabolism of potential fuels during short-term incubations.

Although *G. intestinalis* is an anaerobic protozoan, it is aerotolerant and was found to exhibit a surprisingly high rate of endogenous respiration which was stimulated by about 30 per cent by glucose. Other carbohydrates, tricarboxylic acid cycle intermediates and metabolically important organic acids had no effect on this respiration. From these observations, it was concluded that *G. intestinalis* has no functional tricarboxylic acid cycle, and this is in accord with the lack of intracellular structures which could be recognized as mitochondria. The inability to oxidize carbohydrates (namely, glucose) completely to CO₂ suggested that ATP generation must be provided only by substrate level phosphorylation in the absence of any indication of oxidative phosphorylation (Lindmark, 1980).

The normal respiratory inhibitors (cyanide, dinitrophenol, arsenate, etc.) had no effect on giardial respiration, whereas high concentrations of atebrin, a flavoprotein

inhibitor, did exert an inhibitory effect. This pattern of inhibition (or lack of it) suggested that cytochrome-mediated oxidative phosphorylation is absent, but that flavoproteins are involved in terminal electron transport (Lindmark, 1980).

Since *G. intestinalis* could not completely oxidize glucose to CO₂ and H₂O, the end products of metabolism would be expected to be more reduced than CO₂. When trophozoites were incubated in a simple salt solution (phosphate buffered salt solution containing 33 mM glucose) they produced ethanol, acetate and CO₂. The same non-gaseous end products, i.e. ethanol and acetate, were produced from the trophozoites' endogenous reserves in the absence of glucose, and the relative amounts of each product was affected by oxygen availability. Significantly, no hydrogen was produced.

The enzyme profile was consistent with the proposition that ATP synthesis is via anaerobic glycolysis. A number of enzymes of glycolysis were present (e.g. hexokinase and pyruvate kinase) together with several enzymes conceivably involved in acetate production and in the disposal of reducing equivalents (e.g. pyruvate:ferredoxin oxidoreductase, NADPH oxidoreductase, superoxide dismutase). Absent (or below the limits of detection) were most of the TCA cycle enzymes. Ethanol production was accounted for by a reported NADP-dependent alcohol dehydrogenase.

It was concluded that the energy metabolism of *G. intestinalis* is fermentative, with incomplete oxidation of glucose to ethanol and acetate and that these end products are produced from pyruvate by enzyme systems similar to those that had been reported for trichomonads and *Entamoeba* spp., with the electrons generated in the pyruvate oxidation being transferred to a terminal system involving a flavin, iron-sulphur protein system (Lindmark, 1980).

The involvement of this type of system was established by Weinbach *et al.* (1980), who confirmed the limited number of respiratory substrates which *G. intestinalis* utilizes. Although acid-extractable flavins were found to be present, *G. intestinalis* lacked mitochondria, mitochondrial enzymes and haem proteins but did contain iron-sulphur protein(s) which were reduced by NADPH as an electron donor. In contrast to *E. histolytica*, the giardial respiratory enzymes were found to be largely particulate, and different in that all the carriers that mediate electron transfer from reduced substrates to molecular oxygen are particulate.

Jarroll *et al.* (1981) also soon confirmed, by both enzymatic methods and silicic acid column chromatography, that intact *G. intestinalis* trophozoites produce ethanol and acetate from exogenous glucose. From the incorporation of ¹⁴C into CO₂ from [1-¹⁴C]glucose and [6-¹⁴C]glucose they concluded that the pentose phosphate pathway was present. On the basis of radiorespirometric data from those specifically labelled glucoses and from [3,4-¹⁴C] glucose, Jarroll *et al.* (1981) proposed that *G. intestinalis* trophozoites metabolize glucose at approximately equivalent rates via glycolysis and via the hexose monophosphate pathway. However, it is unwise to draw quantitative conclusions on relative carbon fluxes simply from CO₂ incorporation data; more precise and more specific ¹⁴C incorporation patterns are required to estimate reliably relative fluxes through the two pathways. But there seems little doubt that the hexose monophosphate pathway is present, and

interestingly, of the [^{14}C]glucose that was incorporated into intact trophozoites, almost 90% was found in the nucleic acid fraction. This suggests that there is a considerable flux through the non-oxidative component of the hexose monophosphate pathway providing ribosyl units for replication. We have recently demonstrated activity for the two dehydrogenases of the oxidative segment of the pathway, *viz.* glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, thus verifying at the enzyme level the elevated C1/C6 ratio for $^{14}\text{CO}_2$ observed by Jarroll *et al.* (1981). Surprisingly, glucose was not incorporated into the glycerol skeleton of phospholipids or into sterols, and in general it appears that *G. intestinalis* is incapable of synthesizing the majority of its lipids *de novo* (Jarroll *et al.*, 1981).

In summary, then, since 1981 the accepted view of energy metabolism of *G. intestinalis* has been that the parasite utilizes only glucose, and produces acetate and ethanol with substrate level phosphorylation providing ATP (Jarroll *et al.*, 1989). Iron-sulphur proteins are probably involved in terminal electron transport, but the fine details regarding the terminal electron acceptors, and the intermediates and steps involved in the oxidative production of ethanol and acetate have not been rigorously defined. For further details on glucose catabolism in *Giardia*, see Chapter 6.

THE DISCOVERY OF ALANINE AS A MAJOR END PRODUCT

We have been using nuclear magnetic resonance (NMR) spectroscopy to define metabolism in protozoan parasites originally using *Crithidia luciliae* as a prototype. NMR is now a well established technique for the monitoring of selected biological pathways in biological systems. It has the advantage over other approaches that apart from being non-destructive, a range of metabolites in a complex system can be observed simultaneously and without any *a priori* assumptions as to their nature. In particular, ^1H NMR spectroscopy can be applied to the detection and quantitation of metabolites in the culture medium and to defining the major carbon sources utilized. When we applied this approach to *G. intestinalis* (Edwards *et al.*, 1989) we found to our surprise that alanine was a significant end product of metabolism (Figure 8.1). During the log phase of growth, alanine was the major end product released into the medium, while the formation of ethanol initially lagged behind that of alanine. Ethanol became the major metabolite only after about 4 days of growth when cell numbers were stationary or declining. In contrast, acetate was produced at a more constant rate throughout growth.

The formation of alanine had not been previously reported for *G. intestinalis*, although it had been found in other protozoa (Mackenzie *et al.*, 1983; Darling *et al.*, 1987), and it provides a very good example of the potential of ^1H NMR in monitoring fuel metabolism in protozoan parasites. The only previously reported substantial study of fuel metabolism in *G. intestinalis* used trophozoites harvested in late log phase and resuspended in a simple phosphate-buffered saline with glucose (or other potential substrates). As indicated above ethanol and acetate were

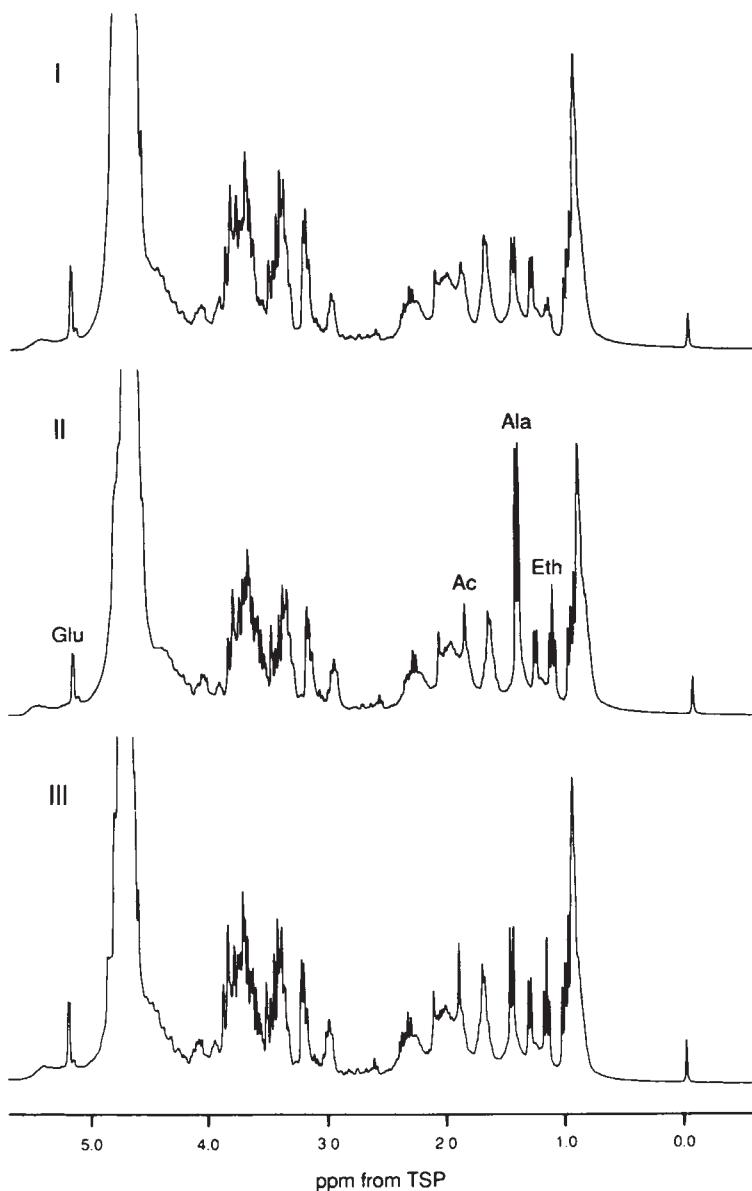


Figure 8.1. 300 MHz ^1H NMR spectra of *G. intestinalis* medium showing metabolite production and utilization during normal growth, and the inhibitory effects of L-cycloserine. Major metabolites produced were alanine (Ala), ethanol (Eth) and acetate (Ac). Glucose (Glu) was the major observable component utilized. I, Fresh medium; II, medium after 4 days' growth of parasites; III, medium to which 0.3 mM L-cycloserine was added prior to 4 days growth of parasites.

Table 8.1. Amino acid composition of *G. intestinalis* culture medium.^a

Amino acid	Concentration in medium (mM)	
	Day 0	Day 4
Alanine	6.1	21.3
Arginine	2.6	0
Aspartate	2.1	2.9
Cysteine	3.6	4.8
Glutamate	5.7	7.5
Glycine	2.4	3.8
Histidine	0.9	1.4
Isoleucine	3.0	4.2
Leucine	9.9	11.3
Lysine	6.3	7.3
Methionine	1.6	2.4
Phenylalanine	3.2	4.1
Proline	0	4.1
Ser/Asn/Gln ^b	4.8	1.1
Threonine	2.7	2.7
Tyrosine	1.1	1.6
Valine	4.9	6.8
NH ₄ ^c /tryptophan ^c	4.6	11.0
NH ₃ ^d	6.4	13.5

^a The amino acid composition of unused culture medium and medium after 4 days growth of parasites was measured using an autoanalyser.

^b Serine, asparagine and glutamine co-eluted and the peak was quantitated as serine.

^c Tryptophan and ammonia co-eluted and the peak was quantitated as ammonium.

^d Ammonia estimated using an ammonia electrode.

identified as end products under these conditions, but alanine was apparently not assayed. This illustrates the intrinsic bias in the conventional approach of using single additions to defined minimal medium. As no nitrogenous substrate was used in the study it is unlikely that significant alanine formation could have occurred. Another striking feature also revealed by the NMR approach was that, during the log phase, *G. intestinalis* released more carbon as alanine than as ethanol and acetate combined. The ratio of ethanol to acetate, depending on the growth stage, was generally similar to that for harvested trophozoites (Landmark, 1980), but overall our results present a more complete picture of the parasite's total fuel metabolism during *in vitro* culture.

The other surprising aspect that arose was that the glucose utilized could not account for the total carbon in the ethanol, alanine and acetate produced. Furthermore, it is reasonable to assume that some CO₂ (which was not measured) was also produced. This imbalance in the carbon economy suggests that another (nitrogen containing) metabolic fuel is utilized in addition to glucose. A possible alternative nitrogenous fuel that was present in the culture medium in substantial amounts was free amino acids. Amino acid analysis of the culture medium

demonstrated that arginine was rapidly utilized, and there were much smaller changes in several other amino acids (Table 8.1). Overall the changes in concentration of these other acids were insufficient to account for the nitrogen in alanine (and in ammonia which was also produced) or the carbon in ethanol, alanine and acetate. Clearly alanine could not be derived from the free amino acids alone, and this in turn suggests that the source of the amino nitrogen and carbon may be polypeptides and/or proteins. The utilization of peptides or protein from the medium is consistent with the observed changes in ^1H NMR spectra. In difference spectra, glucose was the only component that appeared to be utilized, but the conditions for acquiring spectra were such that protons from proteins and peptides were not observable. The recent report of proteolytic enzymes in *G. intestinalis* (Hare *et al.*, 1989; and see Chapter 21) lends support to the proposal that proteins may be utilized.

The importance of alanine in fuel metabolism was also strikingly demonstrated by the use of inhibitors. When *G. intestinalis* was grown in the presence of aminotransferase inhibitors carboxymethoxylamine (amino-oxyacetate) and L-cycloserine, both parasite growth and alanine production were inhibited without concurrent major effects on ethanol or acetate formation (Figure 8.1). L-Cycloserine, which is the more specific inhibitor of the two, was also the more effective inhibitor, with an IC_{50} of approximately $70 \mu\text{M}$. In contrast, the antibiotic D-cycloserine was much less effective, with an IC_{50} an order of magnitude higher than for L-cycloserine, and gave simultaneous inhibition of ethanol and alanine production and of cell growth. Although both carboxymethoxylamine and L-cycloserine may inhibit enzymes other than aminotransferases, and there is no direct evidence as yet for their uptake into the trophozoites, the inference from these observations is that alanine is formed from pyruvate via an aminotransferase. We now have evidence for the existence of a number of aminotransferases, including an alanine aminotransferase with high activity ($1.1 \mu\text{mol min}^{-1} \text{ mg protein}^{-1}$).

Similar activities were observed for both 2-oxoglutarate and oxaloacetate as the keto acid, but no reaction could be detected with 2-oxoisovalerate or 2-oxoisocaprate. The activity with 2-oxoglutarate was higher than for any glycolytic enzyme measured by Lindmark (1980), even allowing for the higher temperature of the aminotransferase assays (37°C as opposed to 30°C). High activities for aspartate and aromatic amino acid (tyrosine, phenylalanine and tryptophan) aminotransferases were also observed. L-Cycloserine, but not D-cycloserine, was a potent irreversible inhibitor of the alanine aminotransferase activity.

THE ARGININE DIHYDROLASE PATHWAY

Amino acid analysis of the culture medium indicated that the arginine was completely utilized within the first 2 days of growth, and that there was a concurrent production of free ammonia together with the appearance of an unexpected peak in the elution profile. This was subsequently shown to be ornithine. This suggested the presence of a functional arginine dihydrolase pathway (see Chapter 9 for diagram)

which we have subsequently confirmed both enzymically (Schofield *et al.*, 1990a) and radiometrically by the liberation of $^{14}\text{CO}_2$ from [^{14}C]guanidino arginine. All three enzymes of the pathway (arginine deiminase, ornithine transcarbamoylase (catabolic) and carbamate kinase) are present in high activity (203, 175 and 2350 nmols $\text{min}^{-1}\text{mg protein}^{-1}$, respectively). Although the pathway has previously been reported in some bacteria (Manca de Nadra *et al.*, 1988) and in *Trichomonas vaginalis* (Linstead and Cranshaw, 1983), there was no previous indication that it was present also in *G. intestinalis*, and indeed, our preliminary studies on the properties of the giardial enzymes suggest that they show substantial differences to the trichomonad enzymes. The activity of the enzymes in *G. intestinalis* is very much greater than that in *T. vaginalis*, and suggests that the flux through the pathway can contribute substantially to giardial energy production by means of the substrate level phosphorylation at the terminal step (carbamate kinase).

It now seems very likely that arginine availability is a limiting factor in giardial growth *in vitro*. The normal concentration of arginine in the medium for *in vitro* growth is 2–3 mM. This is utilized in the first 2 days of growth, even before significant utilization of glucose, with the production of stoichiometric amounts of ammonia and ornithine. Increasing the initial concentration of arginine (by up to an additional 10 mmol $^{-1}$) results in increased growth and ammonia production, while removal of arginine from the medium (by pretreatment with arginase) substantially reduces giardial growth. Additional arginine has no major effect on glucose utilization during the initial growth stage, and it appears that *G. intestinalis* preferentially uses arginine rather than glucose even when presented with high concentrations of the carbohydrate.

Conceivably there are several biological advantages in using arginine rather than glucose. The major one is that, via the dihydrolase pathway, there are no reducing equivalents in the form of NADH or NADPH to be disposed of, and the concurrent substrate level phosphorylation can provide substantial ATP production under conditions of anaerobiosis. One of the major end products, ammonia, can be readily excreted, whereas the other, ornithine, may be required for putrescine synthesis (see Chapter 42). The excretion of the excess nitrogen as ammonia has the additional benefit of not requiring the involvement of aminotransferases or related systems required for the disposal of a-amino nitrogen. The absence of the arginine dihydrolase pathway from the human host makes it a potential target for chemotherapeutic attack on the parasite.

DOES *G. INTESTINALIS* REALLY REQUIRE GLUCOSE?

The observation that arginine is used in preference to glucose, and that the imbalance in glucose utilization and end product formation suggests the utilization of nitrogenous precursors, raises the question as to whether or not glucose is essential for growth of *G. intestinalis*. Most previous experimental studies have involved parasites grown in media containing high concentrations of glucose (normally of the order of 50 mM) and most reported metabolic studies have used

Table 8.2. Comparative carbon fluxes in *G. intestinalis* grown for 4 days in various concentrations of glucose.^a

Nominal glucose concentration (mM)	Actual glucose concentration (mM)	Input	Output				
		Glucose uptake (mM)	Glucose C (mM)	Ethanol C (mM)	Alanine C (mM)	Acetate C (mM)	Total C (mM)
50	48.5	15.5	93	30	81	8	119
25	36.5	15.5	93	26	69	6	101
10	13.3	8.3	49	25	84	8	117
5	8.0	7.0	42	10	57	6	73
1	4.4	3.1	18	2	30	6	38
0	3.2	2.1	12	1	24	8	33

^a The data are representative values of cultures grown for 4 days at varied initial glucose concentrations. The 'nominal glucose concentration' is the concentration of glucose added to the medium. The 'actual glucose concentration' is the glucose concentration as determined and is higher than the theoretical concentration due to medium carry-over and the human serum component of the medium.

similarly high glucose concentrations (typically of the order of 30 mM). These concentrations, however, bear little relationship to the concentrations encountered by *G. intestinalis* in the small intestine *in vivo*.

We found that reducing the glucose concentration of the medium from 50 mM had little effect on growth and product formation until a level of approximately 10mM was reached (Schofield *et al.*, 1991). Below this concentration, the same products (alanine, ethanol and acetate) were still formed, but cell growth and the rate of alanine and ethanol (but not acetate) formation decreased significantly (Table 8.2). What was most surprising was that *G. intestinalis* continued to grow, albeit at only half the normal growth rate, in medium to which no glucose had been added, but which contained 2–3 mM glucose from carryover and from the human serum component (Schofield *et al.*, 1990b). The fact that *G. intestinalis* trophozoites can grow in medium almost devoid of glucose (and in fact the small amount of glucose is completely depleted in the initial phase) undermines the assumption that glucose is the only carbon source that the trophozoites can use. We would not suggest that *Giardia* can grow in the total absence of glucose as a precursor for bio-synthesis. We would, however, propose that glucose is just one of a range of metabolic fuels that contributes significantly to ATP production. The findings suggest that reducing the ambient glucose concentration *in vitro* and supplying sources of nitrogen may provide a better functional model of *G. intestinalis* metabolism *in vivo*.

THE PROBLEMS TO BE RESOLVED

Firstly, there is the problem of acetate production. Conventional wisdom is that both ethanol and acetate are produced via pyruvate as a common intermediate, and that there is a metabolic bifurcation at the level of pyruvate (see Chapter 6). This may not be the case. Whereas ethanol production is sensitive to glucose availability, acetate production is not. Acetate production is constant over a whole range of conditions,

always remaining in the 2–4 mM range for day 4 regardless of glucose concentrations. At nominal zero glucose, acetate production continues unabated, whereas ethanol production is virtually abolished. This constancy of acetate production *vis à vis* large changes in ethanol production suggests that acetate production may be wholly or partially independent of glucose metabolism and that only a small proportion of the acetate carbon may be derived from glucose. Growing the trophozoites in medium containing [1-¹³C]glucose and monitoring the incorporation of ¹³C into the end products by ¹³C NMR spectroscopy indicated approximately 25 per cent ¹³C enrichment of C₁ of both ethanol and alanine. This is consistent with at least half the carbon in the ethanol and alanine being derived from glucose, or an even higher proportion if the pentose phosphate pathway is active. However, no ¹³C enrichment was observed in acetate implying that glucose is not converted into acetate, or at least not by any conventional route.

Secondly, it should be noted that the metabolic route for ethanol production remains ill defined. The original proposition, and one that has never been thoroughly tested subsequently, was that pyruvate is converted to acetaldehyde which is then reduced by an NADP-dependent alcohol dehydrogenase. However, no enzymes were found then that could account for the acetaldehyde synthesis (Lindmark, 1980), and that is still the case. In addition to this gap in the metabolic scheme, it may well be that the following reductive step involves not an NADP-alcohol dehydrogenase, but rather an NADP-dependent aldehyde reductase. We have observed that valproate, a classic inhibitor of mammalian aldehyde reductases, specifically inhibits ethanol production by *G. intestinalis*. The occurrence of an aldehyde reductase in *Giardia* would not be an unusual phenomenon. Aldehyde reductases have been reported for some other parasitic protozoa (Kobayashi, 1982; Arauzo and Cazzulo, 1989) and, as such, they may have potential as new loci for chemotherapeutic attack.

Thirdly, there is the imbalance between glucose utilization and product formation. As the glucose concentration is decreased, the imbalance steadily worsens such that at lower glucose concentrations it can account for less than half the carbon in the products. Under these conditions, which may better reflect the normal *in vivo* milieu, the contribution of other carbon sources becomes increasingly important. What are those other sources? We propose that protein and polypeptides are the most likely.

This imbalance impinges on the fourth major aspect, which relates to alanine production. At all glucose concentrations alanine is always a major product. What is also surprising is that, although the absolute amount of alanine produced decreases as glucose availability decreases, its relative contribution to total end product carbon remains virtually constant. Alanine production always accounts for about two-thirds of the total carbon produced, and this constancy suggests the presence of metabolic regulatory mechanisms which act to maintain a controlled flux through alanine. The nature of these mechanisms is quite unknown at this stage.

The final novel aspect to giardial metabolism relates to giardial phosphate-containing metabolic intermediates. In addition to ¹H and ¹³C NMR spectroscopy, we have also subjected giardial extracts to ³¹P NMR spectroscopy, with surprising

results. The profile of phosphate containing metabolites is very unusual in that phosphoenolpyruvate and pyrophosphate are by far the predominant species. From these observations it has been very tempting to speculate that the high concentration of pyrophosphate is not simply a metabolic artefact, but that it has a central role as an alternative to ATP in kinases, as in *Entamoeba histolytica* (Reeves, 1984) and *Trichomonas vaginalis* (see Chapter 6 for further details). It thus comes as no surprise to us that Mertens (1990) has just demonstrated that the giardial phosphofructokinase is pyrophosphate dependent rather than ATP dependent. It might be expected that there are other pyrophosphate dependent kinases yet to be discovered in *Giardia*.

CONCLUSIONS

Within the area of biochemical parasitology, as in many other areas of contemporary biological sciences, it is the application of newly developed techniques which allows fundamental problems to be addressed from new viewpoints. A decade ago, the development of suitable axenic culture methods allowed the growth of *G. intestinalis* in quantities sufficient to study its energy metabolism. The pioneering studies, particularly of Lindmark (1980) and of Jarroll *et al.*, (1981) provided a substantial base. A decade later, the application of NMR spectroscopy has provided a new and powerful approach of monitoring metabolic pathways in complex systems. This, together with associated analytical techniques, has produced unexpected discoveries which must perforce lead to a re-evaluation of energy metabolism in *G. intestinalis*. It has become very evident that the concept that *G. intestinalis* utilizes only glucose as a metabolic fuel and converts this to ethanol, acetate and CO₂ is no longer tenable. The energy metabolism of *G. intestinalis* is far more complex, involving amino acids, and possibly protein. Central to this is the involvement of alanine, arginine and other nitrogenous metabolites. Parasites may be 'metabolically lazy' as Fairlamb (1989) points out, but that does not necessarily mean that they are metabolically simple. The energy metabolism of *G. intestinalis* is likely to produce many more metabolic surprises.

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9. Amino acids catabolism in anaerobic protists

B.C.Lockwood and G.H.Coombs

Amino acids are required for protein synthesis but many are also involved in other aspects of cell metabolism. It has become increasingly apparent during recent years that several amino acids are catabolized in anaerobic protists including those species (trichomonads, giardias and entamoebae) that are causative agents of disease in man and domestic animals. The aim of this overview is to provide details of the pathways operating in these parasitic organisms and insights into their functional significance.

The anaerobes in which amino acid catabolism has been studied in any detail are the parasitic species *Trichomonas vaginalis*, *Tritrichomonas foetus*, *Giardia lamblia* and *Entamoeba histolytica* and the rumen ciliate protozoa (see Chapter 5). Unfortunately the lack of methods for axenic culture of rumen protozoa has prevented detailed biochemical characterization of individual species. Although these organisms are phylogenetically quite diverse, they possess a number of features in common (Coombs, 1990). Recent results have indicated that the way in which they utilize amino acids may be one such feature. Other protozoa have been reported to withstand periods of anoxia, for example *Trypanosoma brucei* and *Tetrahymena pyriformis*, but these are basically aerobic organisms and will not be considered further in this chapter.

AMINO ACID UTILIZATION

The anaerobes all utilize exogenous amino acids. In addition, they all contain and excrete proteinases at relatively high activities (see Chapters 21–23) and in the absence of free amino acids can obtain their requirements from proteins and peptides present in the growth media. The use of amino acids by *T. vaginalis* has been studied. This parasite rapidly consumes arginine from its environment and catabolizes it through an energy-yielding pathway. It has been suggested that arginine may be an important energy substrate under some circumstances (Linstead and Cranshaw, 1983; Yarlett, 1988), as detailed below. Other amino acids that are

also rapidly consumed and catabolized by *T. vaginalis*, include methionine, leucine and threonine (Lockwood and Coombs, 1989).

All of the species of anaerobic protozoa have been shown to be fermentative and to produce a mixture of acids (lactate, acetate, succinate, butyrate and propionate), alcohols (e.g. ethanol and glycerol) and gases (e.g. carbon dioxide and hydrogen) as the major end products. In all cases energy production is based upon glycolysis with the mixture of end products providing a means of maintaining the redox balance at the same time as generating energy in addition to that resulting from glycolysis (Müller, 1988; and see Chapter 6).

Recent findings, however, have suggested that the overall picture is not this simple and that other metabolic substrates can and must be used by these parasites (see Chapter 8). It has been shown that the environmental conditions, for example the gaseous atmosphere in which the parasites are grown, have profound effects on the parasites' metabolism (Paget and Lloyd, 1990; and see Chapter 7) and that the substrate concentrations available *in vivo* should also be considered when interpreting data obtained *in vitro*. For example, although *G. lamblia* can utilize glucose it does not use glucose exclusively or even preferentially when presented with a variety of metabolic substrates. It has been demonstrated that during anaerobic growth alanine is the major end product released by the parasite into the growth media (Edwards *et al.*, 1989; Paget *et al.*, 1990). To account for this alanine formation, substantial amounts of a nitrogenous compound must be either present in the parasite or obtained from the growth medium. Amino acid analysis of the culture medium before and after growth suggests that arginine is the most likely candidate. The utilization of a combination of glucose and protein and polypeptides would be consistent with the metabolic fuels available to the parasite *in vivo* (see Chapter 8).

There is very little information available on amino acid utilization in *E. histolytica* with a lone report on the effect of amino acids on the aerobic respiration of the parasite. Of 22 amino acids tested, only serine was shown to increase oxygen consumption by the parasite and it was suggested that L-serine may be an important energy source in the aerobic metabolism of this parasite (Takeuchi *et al.*, 1979).

Nitrogen metabolism in the rumen protozoa is important to the overall nitrogen economy of the host. Unfortunately, relatively little is known about the metabolism of amino acids by these protozoa. The information that has been gained by using mixed protozoal preparations containing both holotrich and entodiniomorphic species has been reviewed by Williams (1986; and see Chapter 5) and Coleman (1980), respectively.

AMINOTRANSFERASES AND GLUTAMATE DEHYDROGENASE

The citric acid cycle is largely inoperative in anaerobic protozoa, and so aspartate aminotransferase might be expected to play an important role in the maintenance of α -ketoglutarate and oxaloacetate pools. The aspartate aminotransferase from *T. vaginalis* is of particular interest owing to its unusual substrate specificity (Lowe

and Rowe, 1985, 1986a, 1987). Whereas the mammalian enzyme will only catalyse the transamination of aspartate or glutamate with oxaloacetate or 2-oxoglutarate, the parasite enzyme will also catalyse, at high rates, the transamination of the aromatic amino acids, phenylalanine, tryptophan and tyrosine. Growth of *T. vaginalis* was unaffected by gostatin, an irreversible inhibitor of aspartate aminotransferase, although the enzyme activity within the cell had been completely inhibited and the intracellular aspartate concentration was elevated. This suggests that the enzyme is not important to the parasite under these conditions. In media lacking aspartate and glutamate, the amino acid substrates of the aspartate aminotransferase reaction, gostatin produced a larger increase in the aspartate pool. During incubation of cells with or without gostatin, aspartate and glutamate were produced in the medium, presumably by proteolysis of medium proteins, hence it would appear that any involvement of the aspartate aminotransferase reaction in amino acid synthesis had been bypassed (Rowe and Lowe, 1986).

Lowe and Rowe (1986b) published the results of a survey of aminotransferase activities in *T. vaginalis*. They found α -ketoglutarate to be an effective amino acceptor from 11 amino acids. High activities of aspartate, aromatic amino acid, branched chain amino acid and ω -amino acid aminotransferases were demonstrated. In contrast, no aminotransferase activity with β -alanine, glutamine, glycine or serine was detected, although glutamine and glycine aminotransferases commonly occur in other eukaryotes. The high levels of activity with branched chain and aromatic amino acids and aspartate confirm the earlier findings of Jaroszewicz and Maylszko (1965).

Glutamate dehydrogenase, which generally functions with aminotransferases in transferring amino groups between keto and amino acids, was isolated from *T. vaginalis* and characterized by Turner and Lushbaugh (1988). The animation reaction of glutamate dehydrogenase is probably an insignificant contributor to the cells' supply of NADP, but this is probably satisfied by NADPH-oxidase which is present at high activity in the cytosol. Nevertheless, glutamate dehydogenase may represent an important link in the catabolism of amino acids. Trophozoites of *G. lamblia* have both glutamate dehydrogenase and alanine aminotransferase activity. As glutamate is not seen as an end product, it is suggested that these enzymes cooperate to convert pyruvate to alanine, with the concomitant oxidation of NAD(P)H (Paget *et al.*, 1990).

ARGENTINE AND ORNITHINE METABOLISM

Arginine is rapidly depleted from the medium during the cultivation of *T. vaginalis* in a defined or semi-defined medium. It is broken down by the three enzymes of the dihydrolase pathway: arginine deiminase, catabolic ornithine transcarba moylase and carbamate kinase to ornithine, ammonia and carbon dioxide (see Figure 9.1). Arginase, urease and citrulline hydrolase appear to be absent (Linstead and Cranshaw, 1983). Ornithine, a product of the pathway, was further converted to putrescine by an active ornithine decarboxylase (see Chapter 42). The occurrence of

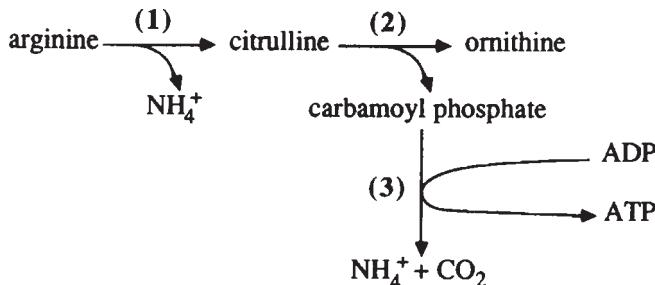


Figure 9.1. The arginine dihydrolase pathway of arginine catabolism. Enzymes: (1) arginine deiminase; (2) catabolic ornithine transcarbamoylase; (3) catbamate kinase.

this dihydrolase pathway is unusual in a eukaryote, although relatively common in prokaryotes including both aerobic and anaerobic bacteria. It is anticipated that the major role of the pathway would be to produce ornithine for biosynthesis of polyamines. The presence of carbamate kinase, however, means that *T. vaginalis* can obtain 1 mol of ATP for every mole of arginine catabolized. This enzyme has a much higher specific activity than the other enzymes of the pathway which is in keeping with conserving the ATP-generating potential of an unstable substrate. The importance of the pathway as a source of ATP in *T. vaginalis* is difficult to estimate. Certainly under the conditions of microaerobic laboratory culture, glucose is utilized at a more rapid rate than that reported for arginine, and glycolysis is usually considered the major source of ATP in *T. vaginalis*. However, *in vivo*, *T. vaginalis* is present on the vaginal mucosa and the nature of its major source of energy is not known. Arginine is present at relatively high levels in the extracellular fluid of mammals and may make a significant contribution to the energy of *T. vaginalis* in its natural habitat. This view is strengthened by the observation that there is a strong correlation between the occurrence of polyamines in the vaginal fluid of women and vaginitis due to infection with *T. vaginalis* (Chen *et al.*, 1982).

It has been recently reported that the dihydrolase pathway is also functional in *Giardia* and serves in energy generation (see Chapter 8).

SULPHUR CONTAINING AMINO ACID METABOLISM AND METHIONINE RECYCLING

The sulphur containing amino acid L-methionine is a precursor for the biosynthesis of S-adenosylmethionine (see Figure 9.2 and Chapters 40 and 42). This compound is involved in many important biological processes including the synthesis of polyamines and transmethylation reactions. In *T. vaginalis*, L-methionine is rapidly catabolized to volatile thiols, including methanethiol (Thong *et al.*, 1987a). Methanethiol is a pungent volatile gas. It is known to be toxic to mammals and pathogenic fungi and to inhibit mammalian mitochondrial respiration and catalase

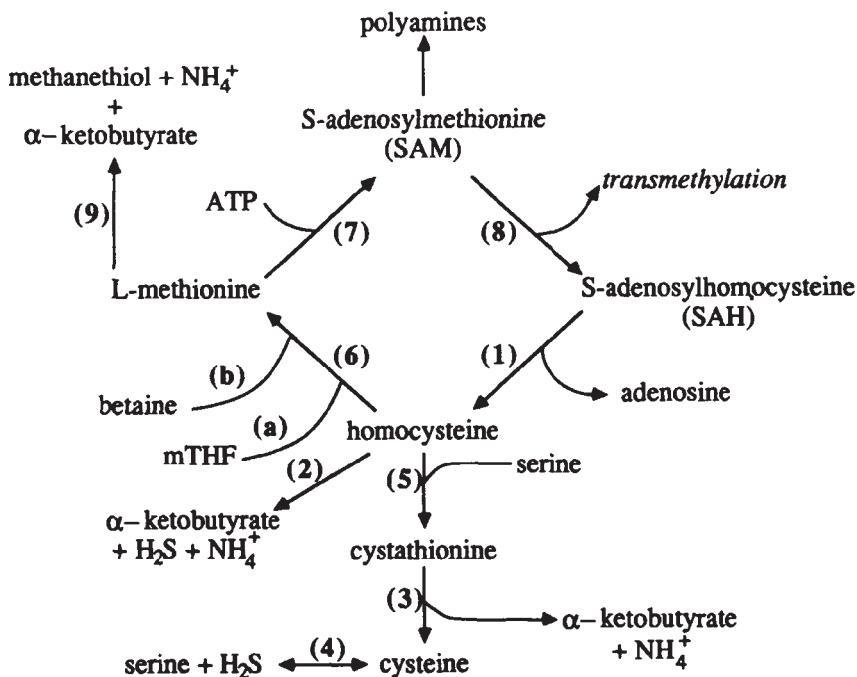


Figure 9.2. Metabolism of sulphur containing amino acids. Enzymes: (1) *S*-adenosylhomocysteine hydrolase; (2) homocysteine desulphurase; (3) γ -cystathionease; (4) serine sulphhydrase; (5) cystathione β -synthetase; (6a) methyltetrahydrofolate: homocysteine methyltransferase; (6b) betaine: homocysteine methyltransferase; (7) *S*-adenosylmethionine synthetase; (8) *S*-adenosylmethionine-dependent methyltransferases; (9) methionine γ -lyase.

activity, although it is reported to be essential for cell division in certain cell lines. It is also known that higher plants produce volatile thiols to repel potential predators and parasites. The significance of the production of volatile thiols by *T. vaginalis* is unknown (for references see Thong *et al.*, 1987a). It is possible that this may play a part in the interactions of the parasite with its host and the other micro-organisms present in the human vagina.

In mammals methanethiol is a by-product of polyamine biosynthesis or produced directly from methionine by transamination to α -ketomethylthiobutyrate followed by dethiomethylation (Cooper, 1983). A similar mechanism has been proposed for aerobic bacteria and fungi. In some anaerobic bacteria, however, methionine is deaminated and dethiomethylated simultaneously. The enzyme that catalyses this reaction is methionine γ -lyase, an equivalent enzyme is present at high activity in *T. vaginalis* (Lockwood and Coombs, 1989). The same enzyme is also responsible for the homocysteine desulphurase activity that catalyses the breakdown of homocysteine to hydrogen sulphide, α -ketobutyrate and ammonia in *T. vaginalis* (Thong and Coombs, 1987a; Lockwood and Coombs, 1989).

The enzyme has been purified to homogeneity and shown to be pyridoxal

5'-phosphate dependent and have properties typical of this group of enzymes (Lockwood and Coombs, unpublished). It has a native molecular mass of approximately 160 kDa and consists of four subunits of molecular mass 43–45 kDa. The enzyme catalyses α , β - and α , γ -elimination reactions of a number of derivatives of methionine and cysteine, respectively. It also catalyses γ -replacement reactions of the thiomethyl group of methionine, homocysteine and ethionine to yield the corresponding *S*-substituted homocysteine. Only L-amino acids are substrates, D-enantiomers are not, nor do they competitively inhibit the enzyme. Amino acids without sulphur containing substituents are not substrates indicating the specificity for the C-S rather than the C-C bond, although both norleucine and La-aminobutyrate inhibit the enzyme and may bind to the active site. Purified methionine γ -lyase has no activity with cystathione and is clearly distinct from the mammalian enzyme and, therefore, may have potential as a target for antitrichomonad chemotherapy. Further information on the sequence and structure of the active site along with the data already obtained on substrate and inhibitor binding should allow the design of compounds (prodrugs) that will be specifically hydrolysed and so activated by this enzyme. Such prodrugs should have a high chemotherapeutic index as they would not be activated by mammals.

A second approach to exploiting the presence of methionine γ -lyase in *T. vaginalis* is to obtain specific inhibitors. This seems less promising, however, because all the enzyme activity in living cells can be irreversibly inhibited by adding propargylglycine at 10^{-5} M to the growth medium and yet this affects cell growth *in vitro* very little. It may be, however, that the enzyme plays some key role in enabling the parasite to survive *in vivo*.

The presence of this enzyme in *T. vaginalis* is particularly interesting since it apparently does not occur in other species of trichomonad or in a range of other protozoan parasites, including *G. lamblia*. It is present in *E. histolytica* and a number of species of rumen ciliate, albeit at much lower levels than in *T. vaginalis* (Lockwood and Coombs, unpublished).

Living trichomonads have been shown to incorporate exogenous labelled L-methionine into intracellular S-adenosylmethionine (SAM) (Thong *et al.*, 1986, 1987b). The labelled methyl carbon was also detected in nucleic acids and lipids, presumably owing to the subsequent role of SAM as one carbon donor in transmethylation reactions (see Chapter 40). These results suggest that trichomonads contain SAM synthetase and SAM-dependent methyltransferases although the former enzyme has not been detected in *T. vaginalis* using methods effective for detecting the enzyme in mouse liver. The presence of methionine γ -lyase and the rapid incorporation of methionine into SAM and subsequently into a range of macromolecules accounts for the high rate of consumption of methionine by *T. vaginalis*. It may also explain the large difference in consumption between *T. vaginalis* and *Tritrichomonas foetus*. The latter neither contains methionine rlyase nor does it incorporate methionine into SAM at a high rate. Another possible explanation is that *T. foetus* may be able to recycle methionine to a greater extent than *Trichomonas vaginalis*. As yet the extent and mechanism of methionine synthesis from homocysteine in trichomonads is unknown.

Methionine can also be salvaged from methylthioadenosine (MTA), a co-product of polyamine biosynthesis. In mammalian cells and some micro-organisms MTA is degraded in one step to adenine and methylthioribose 1-phosphate (MTR-1-P) MTA phosphorylase. However, in other microbes including *E. histolytica* and *G. lamblia* MTA is catabolized in two steps, first to adenine and MTR via MTA nucleosidase followed by conversion of MTR to MTR-1-P via MTR kinase. In both cases MTR-1-P is subsequently recycled into methionine via a diketo intermediate and 2-ketomethylthiobutyrate (Riscoe *et al.*, 1989; and see Chapter 41). This metabolic difference is a potentially exploitable target for antiparasitic chemotherapy, although it is not yet known which pathway operates in trichomonads.

Another thiol producing enzyme that is common to all anaerobic protozoa is serine sulphhydrase, which catalyses the reversible interconversion of serine and cysteine (Thong and Coombs, 1985). Results obtained by Thong *et al.* (1987a) indicate that methionine γ -lyase and serine sulphhydrase are related in function, although their exact physiological role in trichomonads remains uncertain.

LEUCINE AND THREONINE CATABOLISM

The addition of leucine and threonine to cultures of *T. vaginalis* growing *in vitro*, results in the production and release of α -hydroxyisocaproate and α -hydroxybutyrate, respectively (Lockwood and Coombs, 1989). They are synthesized via the corresponding α -keto acid. Interestingly, although methionine is also converted to α -ketobutyrate, addition of methionine to cultures does not result in increased α -hydroxybutyrate excretion. The enzymes involved in the pathways have been detected in *T. vaginalis*. Threonine dehydratase has also been detected in *Tritrichomonas augusta*, *T. foetus*, *E. histolytica*, *E. invadens* and *G. lamblia* (Lockwood and Coombs, unpublished). It is a cytosolic enzyme present at similar levels in all three species of trichomonad, and in both metronidazole-resistant and metronidazole-sensitive lines of *Trichomonas vaginalis*. It is a pyridoxal 5'-phosphate dependent enzyme and the activity is significantly enhanced by addition of the co-factor to the assay. The enzyme is not affected by isoleucine, which indicates that it is involved in the catabolic breakdown of threonine to α -ketobutyrate rather than the biosynthetic pathway to isoleucine. The activity is allosterically activated by AMP and ADP and inhibited by ATP. The AMP-binding ability has been utilized in purifying the enzyme using AMP-Sepharose affinity chromatography. The molecular mass of the purified enzyme was 170 kDa determined by gel filtration with a subunit apparent molecular mass of 45 kDa determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The functional significance of the catabolism of these amino acids is not clear at present, but it is likely that hydroxyacid secretion is simply a reflection of the high concentrations of keto acids that are produced and the broad substrate specificity of lactate dehydrogenase. Isoenzymes of lactate dehydrogenase in *T. vaginalis* have been shown to be active towards both α -ketobutyrate and α -ketoisocaproate (Lockwood and Coombs, 1989). Hydroxy acid secretion has not been detected in

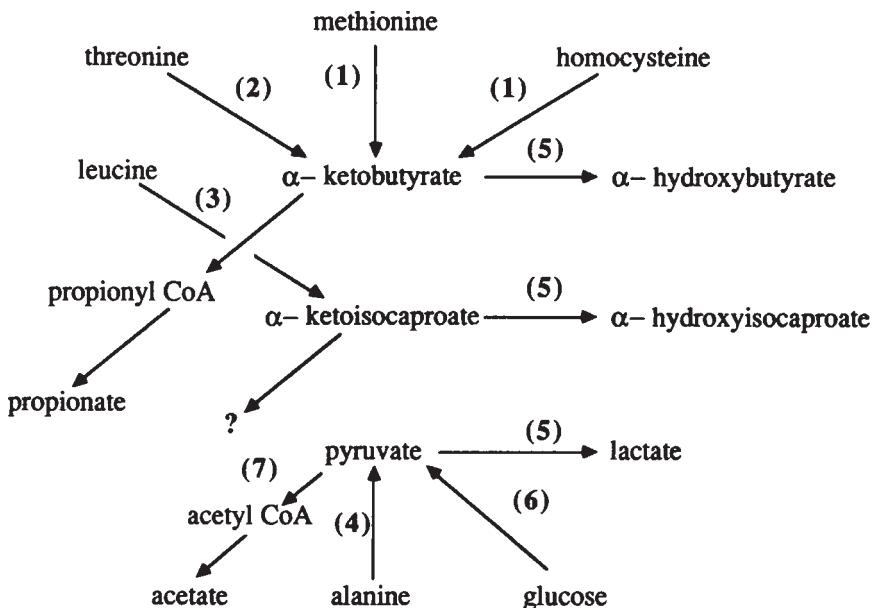


Figure 9.3. Putative pathways of amino acid catabolism in *Trichomonas vaginalis*. Enzymes: (1) methionine γ -lyase/homocysteine desulphurase; (2) threonine dehydratase; (3) leucine aminotransferase; (4) alanine aminotransferase; (5) lactatedehydrogenase; (6) the glycolytic sequence; (7) pyruvate: ferredoxin oxidoreductase.

either *Tritrichomonas augusta* or *T. foetus* and this may simply reflect the absence of lactate dehydrogenase from these two species. Threonine and leucine catabolism may be as important to these species as it appears to be for *Trichomonas vaginalis*.

The finding that methionine catabolism does not lead to detectable hydroxybutyrate release suggests that there must be other routes for α -ketobutyrate metabolism, and these presumably are of some advantage to the parasite. An attractive possibility is that the main route of metabolism of the keto acids is via energygenerating pathways similar to the catabolism of pyruvate (see Figure 9.3). Some support for this proposal comes from the finding that pyruvate: ferredoxin oxidoreductase, the enzyme that catalyses the conversion of pyruvate to acetyl-CoA, will also catalyse the conversion of α -ketobutyrate to propionyl-CoA (Williams *et al.*, 1987). Furthermore, preliminary results suggest that *T. vaginalis* grown in the presence of elevated levels of methionine excrete elevated levels of propionate (unpublished observations). Work is currently in progress to confirm these findings and to provide further insights into the significance of amino acid metabolism to the anaerobic protists in general.

ACKNOWLEDGEMENTS

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10. Intermediary metabolism of *Leishmania*

J.J. Blum

INTRODUCTION

Most parasites have a number of unusual features in their metabolic pathways (Bryant and Behm, 1989), and *Leishmania* sp. are no exception. Examples are the compartmentation of glucose utilization (see Chapter 11), the role of $p\text{CO}_2$ and $p\text{O}_2$ in metabolic regulation, the formation of D-lactate, and the metabolism of alanine and its role in osmoregulation. In this brief review we focus on the current understanding of some of these unusual features of *Leishmania* metabolism. Of necessity, most of these studies have been performed on promastigotes, since it was relatively difficult to obtain sufficient quantities of either the infective metacyclic forms or of amastigotes for metabolic studies. The recent development of methods to obtain sufficient quantities of the metacyclic stages of *L. major* (Sacks *et al.*, 1985), and to grow axenic cultures of the amastigote form of *L. panamensis* (Eperon and McMahon-Pratt, 1989) now make it feasible to undertake metabolic studies on metacyclines and amastigotes such as those that have been performed on promastigotes, and thus to increase our understanding of the metabolic changes that occur as the parasites change their form and habitat.

GLUCOSE CATABOLISM

Initial metabolic studies (reviewed by Marr, 1980) showed that glucose was not completely oxidized. However, the identity of the full complement of organic acids released was not certain, nor was it known how the amounts of each product changed as a function of $p\text{O}_2$, $p\text{CO}_2$, osmotic pressure, or other environmental factors. Recent studies have shown that the products of glucose catabolism by *L. braziliensis panamensis* promastigotes are succinate, glycerol, D-lactate, pyruvate, alanine and, in the presence of oxygen, CO_2 (Darling *et al.*, 1987). Cells incubated with glycerol as sole carbon released acetate, succinate, D-lactate, and CO_2 ; those incubated with alanine released acetate and CO_2 ; those incubated with acetate released only CO_2 (Darling *et al.*, 1989a).

REGULATION OF METABOLISM BY $p\text{CO}_2$ AND $p\text{O}_2$

Early studies on *Trypanosoma rhodesiense* (Ryley, 1962), *T. lewisi* (Ryley, 1951), *L. mexicana* (Hart and Coombs, 1981), and *L. donovani* (Chatterjee and Datta, 1973) indicated that CO₂ played an important role in the anaerobic metabolism of trypanosomatids. The observation that the rate of glucose utilization of *L. b. panamensis* was markedly reduced by hypoxia in the absence of CO₂ (Darling *et al.*, 1987) led us to examine the effect of CO₂ on glucose utilization and product formation. Under anaerobic conditions in the absence of CO₂, glucose consumption by *L. major* almost ceased (a total 'reverse' Pasteur effect). The inclusion of 5 per cent CO₂ in the gas phase, however, restored glucose consumption to the same rate as under aerobic conditions (Darling *et al.*, 1989b). To obtain further insight into the role of $p\text{O}_2$ in regulating glucose metabolism, a study was performed in which $p\text{O}_2$ was varied at constant $p\text{CO}_2$ (5 per cent) (Keegan and Blum, 1990). As the $p\text{O}_2$ decreased from 95 to 6 per cent, there was an appreciable increase in glucose

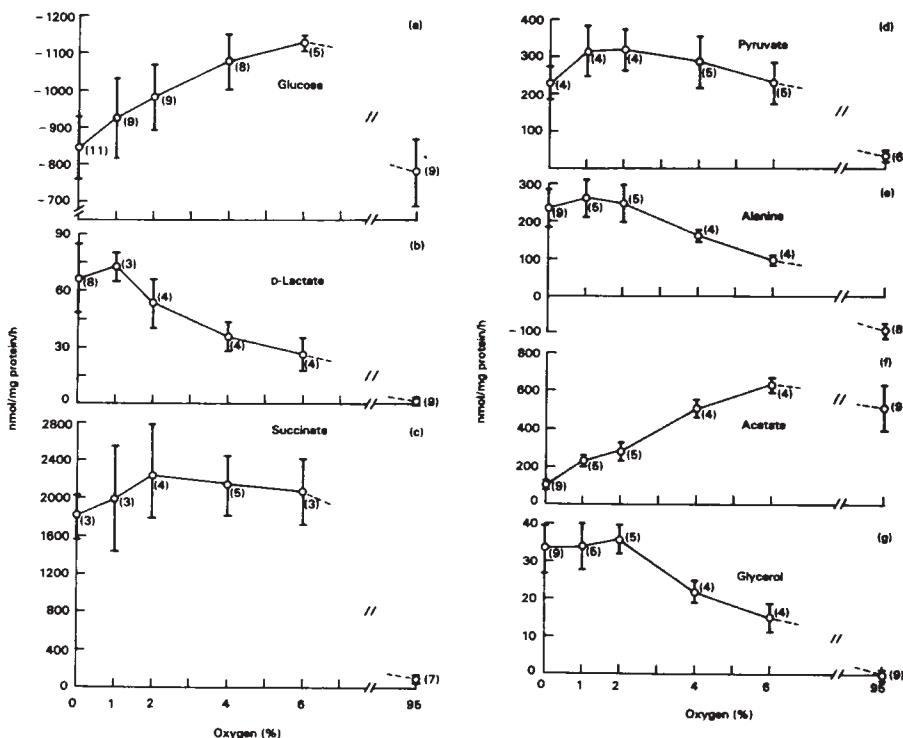


Figure 10.1. Effect of $p\text{O}_2$ on products formed from glucose by *L. major* piomasugotes. Cells from late log phase cultures were washed and resuspended in a buffer containing 8 mM glucose and incubated for 1 h at 26°C under a gas phase containing 5 per cent CO₂ and the oxygen level indicated on the abscissae. The lines at each point indicate the mean \pm SD for the number of measurements shown in parentheses. (Taken from Keegan and Blum, 1990.)

consumption (Figure 10.1(a)) and a large increase in the production of succinate (Figure 10.1(c)) and pyruvate (Figure 10.1(d)). Since there was no difference in glucose consumption or succinate production by promastigotes incubated under air or under 95 per cent O₂/5 per cent CO₂, we have postulated the presence of a low affinity oxygen sensor in *L. major*.

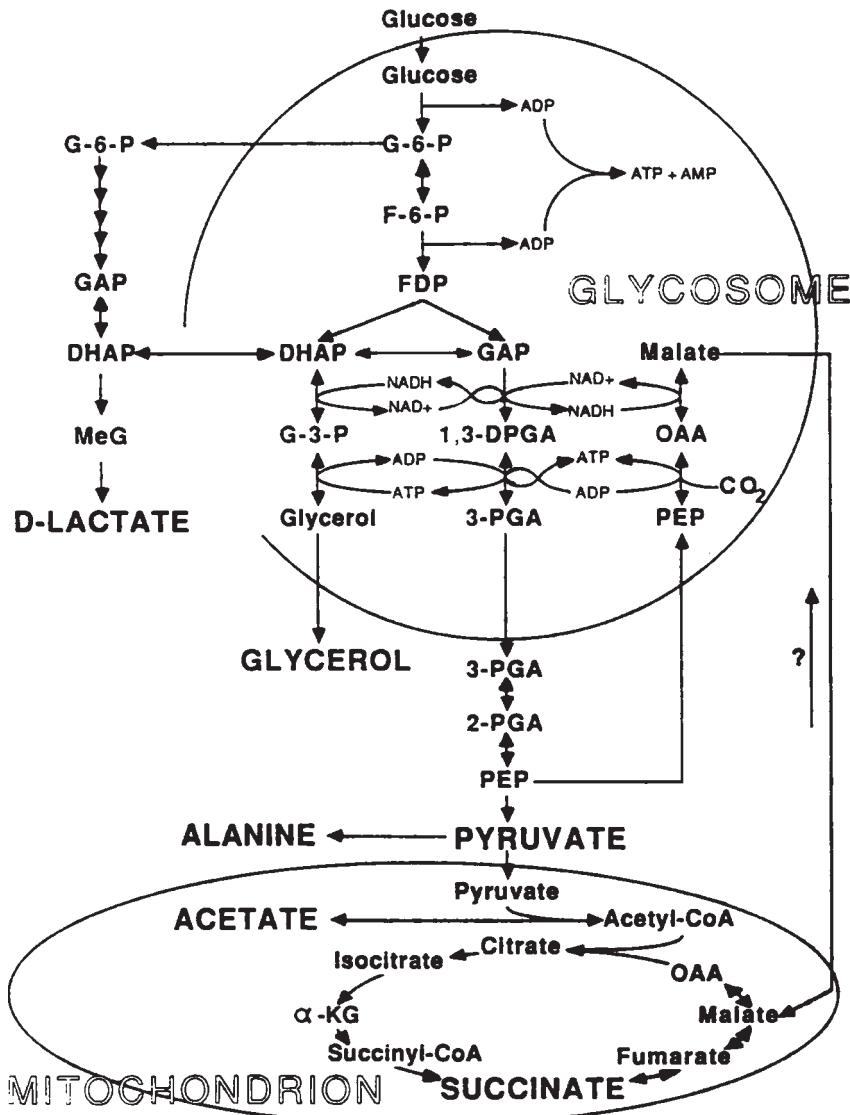


Figure 10.2. Pathways of glucose catabolism in *L. major* promastigotes. This scheme is based on current knowledge, but some of the pathways are yet to be confirmed. For clarity, the pathway for the formation of D-lactate via the methylglyoxal (MeG) pathway is shown as being localized in the cytosol.

The observation that CO₂ is essential for anaerobic glycolysis and that succinate is the major product formed both in *Leishmania* (Figure 10.1) and in several other trypanosomatids requires that CO₂ be fixed onto a C₃ acid. In *Crithidia fasciculata* (de los Santos *et al.*, 1985), *T. brucei* (Broman *et al.*, 1983), and *T. cruzi* (Cannata and Cazzulo, 1984), the evidence suggests that CO₂ fixation onto phosphoenolpyruvate (PEP) via phosphoenolpyruvate carboxykinase (PEPCK) is the primary route. Marr (1980) has summarized evidence suggesting that in *Leishmania* promastigotes fixation of CO₂ onto pyruvate via pyruvate carboxylase may be the primary route. This conclusion was based in part on the failure of several groups to find PEPCK in *Leishmania*. Mottram and Coombs (1985a, b), however, found PEPCK to be present in *L. mexicana* (largely in the glycosomes, as in *T. brucei* (Opperdoes and Cottem, 1982), but only low levels of pyruvate carboxylase. Since [3-¹³C] alanine, which forms [3-¹³C]pyruvate as the first step in its metabolism, did not form enough [¹³C]succinate to be detected in NMR studies of *L. b. panamensis* promastigotes (Darling *et al.*, 1989a), we presently favour PEPCK as the major route for CO₂ fixation in *Leishmania*, but a role for pyruvate carboxylase cannot be eliminated at present. A diagram of our current understanding of the pathways of glucose catabolism in *L. major* promastigotes is shown in Figure 10.2.

CO₂ fixation results in the formation of oxaloacetate (OAA), which can be reduced to malate via malate dehydrogenase. Upon entering the mitochondrion, the malate can be converted to fumarate via fumarase and then to succinate via fumarate reductase. This latter enzyme has not been examined in *Leishmania*, but is present in *T. cruzi* (Boveris *et al.*, 1986) and *T. brucei* (Turrens, 1989), where it reoxidizes mitochondrial NADH. In many cells (Kroger, 1978), including some trypanosomatids (Klein *et al.*, 1975), fumarate reduction is linked to a substrate level phosphorylation. It is not known whether this occurs in *Leishmania*, although the ability of *L. major* to maintain its ATP (although at a reduced level) under 95 per cent N₂/5 per cent CO₂ in the presence of glucose suggests that this may occur (Darling and Blum, 1989). Further information on the fumarate reductase of trypanosomes is presented in Chapter 12.

D-LACTATE

Early studies on lactate formation and on the presence of lactate dehydrogenase (LDH) in trypanosomatids produced conflicting results. Several studies reported the presence of LDH and the production of lactate in some species, while other studies failed to show the presence of either LDH or of lactate (for references, see: Bacchi *et al.*, 1970; Darling *et al.*, 1987). The discovery that D-lactate rather than L-lactate was a product of glucose catabolism in *L. b. panamensis* (Darling *et al.*, 1987) led us to examine lactate production in four species of *Leishmania* and two species of *Trypanosomes*. All four species of *Leishmania* examined produced only D-lactate (Darling *et al.*, 1988). *T. brucei gambiense* procyclic forms produced only L-lactate, while *T. lewisi* produced both isomers. These observations raise many interesting questions concerning the adaptive significance of the two pathways. It would be

interesting not only to extend these studies to other trypanosomatids, but also to study the control of the relative rates of formation of the D versus L isomers in *T. lewisi*. Teixeira de Mattos *et al.* (1984) showed that glucose-limited chemostat cultures of the bacteria *Klebsiella aerogenes* responded to the addition of a saturating concentration of glucose by a marked increase in D-lactate output. Under these low growth conditions, the increase in glucose uptake was thus accompanied by only a slight increase in ATP production. These and similar studies (Streekstra *et al.*, 1987) suggest that one function of the methylglyoxal bypass is to allow a high rate of glucose consumption without an excessive rate of ATP synthesis. Whether D-lactate production serves only as a bypass of the energy producing glycolytic pathway of *Leishmania* under anaerobic conditions remains to be determined.

D-lactate is formed via the methylglyoxal pathway in *Leishmania* (Darling and Blum, 1988) and in many mammalian cells (see, e.g. Talesa *et al.*, 1989, and references therein). Since both methylglyoxal and phenylglyoxal are toxic to *Leishmania* (Darling and Blum, 1988; Ghoshal *et al.*, 1989), it is reasonable to inquire whether agents which show a preferential inhibition of the *Leishmania* methylglyoxal pathway relative to the same pathway in cells of the mammalian host might be of therapeutic value. Recent systematic studies on inhibition of glyoxylase I (Barnard and Honek, 1989) and glyoxylase II (Klopman and Dimayuga, 1988) provide a framework for selecting compounds to test the relative susceptibilities of these enzymes in *Leishmania* versus human macrophages.

Very little D-lactate is formed when glycolysis occurs at high pO_2 (Figure 10.1(b); see also Darling *et al.*, 1988). With increasing hypoxia, D-lactate production increases, reaching its maximal level at about 2 per cent O_2 . Glycerol formation, which also begins from dihydroxyacetone phosphate (DHAP) increases in parallel with D-lactate (compare Figure 10.1(g) with 10.1(b)). The rise in pyruvate (and alanine) production roughly parallels the rise in that of D-lactate and of glycerol, suggesting that with increasing hypoxia there is a decrease in pyruvate disposal via the tricarboxylic acid cycle, an increase in intracellular pyruvate levels, and a diversion of carbon flow, initially from PEP or pyruvate to succinate (Figure 10.1(c)) via OAA and malate and then, with increasing hypoxia, from DHAP to D-lactate and glycerol. Recent studies show that whereas the concentration of fructose 2, 6-biphosphate [$F(2, 6)P_2$] rises from about 0.5 to about 10 pmol/mg protein within 5 min of the addition of glucose to washed promastigotes under aerobic conditions (95 per cent O_2 /5 per cent CO_2), it increases to about 3 pmol/mg protein under hypoxic (6 per cent O_2 /5 per cent CO_2) or anaerobic (95 per cent N_2 /5 per cent CO_2) conditions (Keegan and Blum, 1991). $F(2, 6)P_2$ appears to be the major activator of pyruvate kinase in trypanosomatids, including *L. major* (Van Schaftingen *et al.*, 1985). The large rise in $F(2, 6)P_2$ levels under aerobic conditions would allow rapid glycolysis to the level of pyruvate and, since adequate oxygen is present, its oxidation to CO_2 . If the moderate rise in $F(2, 6)P_2$ levels under hypoxic conditions allowed glycolysis to proceed more rapidly than the pyruvate formed could be oxidized in the mitochondrion, this might account for the rise in pyruvate and alanine release, the overflow of glycolytic intermediates such as DHAP to D-lactate and glycerol, and the rise in succinate production from PEP and/or pyruvate.

ALANINE METABOLISM AND ROLE IN RESPONSE TO OSMOTIC STRESS

Leishmania promastigotes oxidize exogenous alanine at a rate comparable to that of glucose (Keegan *et al.*, 1987; Darling *et al.*, 1989a). *Leishmania* also contain a very large intracellular pool of alanine (Simon *et al.*, 1983; Darling *et al.*, 1987; Mallinson and Coombs, 1989). When *L. major* are incubated with glucose under 95 per cent O₂/5 per cent CO₂, some of the alanine pool is consumed (Figure 10.1(e)). When *L. b. panamensis* were incubated with glucose under an air atmosphere, a very small net production of alanine occurred (Darling *et al.*, 1989a). Since consumption of internal alanine by *L. major* changes to production of alanine when the pO₂ is reduced to 6 per cent (Figure 10.1(e)), this suggests that there was a mild degree of hypoxia in the experiments on *L. b. panamensis*, and that the transition from consumption to production is controlled by the presumptive low affinity oxygen sensor. Production of alanine when glucose is the sole exogenous substrate requires a source of amino groups, presumably arising from proteolysis (Simon and Mukkada, 1983) although transamination from other free amino acids present (Simon *et al.*, 1983; Mallinson and Coombs, 1989) could also occur.

Further insight into the role of alanine in *Leishmania* came from a serendipitous observation made during our studies on glucose metabolism. It was observed (Darling and Blum, 1990) that within 20 min of the addition of glucose or 2-deoxyglucose to promastigotes suspended in buffer, the cells shortened and rounded. This shape change could be prevented by raising the osmolarity of the buffer with mannitol, and could be mimicked by reducing the osmolarity of the buffer in the absence of glucose. Since alanine was known to be released in response to hypo-osmotic stress in many eukaryotic species (Chamberlin and Strange, 1989), it was of interest to ascertain whether the shortening and rounding of the cells in response to acute hypo-osmotic stress was accompanied by a release of alanine and/or other ninhydrin-positive substances (NPS). We found that with increasing hypo-osmotic stress, increasing amounts of alanine and other NPS were released. The release is very rapid; the entire intracellular pool of alanine, which comprises about 40 per cent of the NPS, is released within 1 min after sufficient H₂O is added to reduce the osmolarity of the buffer from 305 to 102 mOsm kg⁻¹ (Darling *et al.*, 1990b). It was also found that within 1 min after reduction of the osmolarity from 305 to 153 mOsm kg⁻¹ the average length decreased from 11.5 to 7 µm while the diameter increased from about 3 to 4.4 µm (Figure 10.3). If the cells are assumed to be prolate spheroids, then the effect of the reduction in osmolarity is a rapid swelling (from about 52 to 75 µm³), followed by a return to the original volume (regulatory volume decrease) within about 10 min (Darling *et al.*, 1990). The rapid release of alanine and other NPS serves to prevent excessive swelling and possible damage to the cell. The nature of the intracellular signal(s) responsible for initiating the rapid shape changes and release of osmolytes is at present unknown. Furthermore, nothing is known about the cytoskeletal elements involved in the shape change.

The central role of alanine both in the metabolism of *L. major* and in its ability to respond to osmotic stress is further indicated by experiments in which

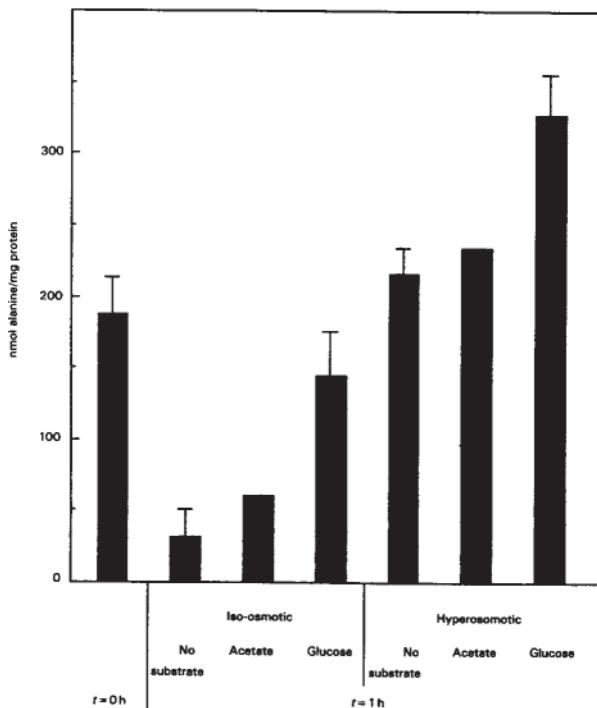


Figure 10.3. Changes in alanine content of promastigotes during incubation under iso-osmotic and hyperosmotic conditions. *L. major* promastigotes were washed twice and resuspended in iso-osmotic buffer (305 mOsm kg^{-1}) or in that buffer supplemented with mannitol to yield 614 mOsm kg^{-1} . Cells were incubated under an air atmosphere in a shaker bath at 26°C with no exogenous substrate or with 2.0 mM acetate or 5.5 mM glucose. At $t=0$ and after 1 h of incubation perchloric acid was added and total alanine content was measured in the neutralized supernatants. Results are mean \pm SD for five experiments except for the two experiments in which acetate was present. (Burrows and Blum, unpublished.)

promastigotes in iso-osmotic buffer were compared with the same cells incubated under hyperosmotic conditions (Figure 10.3). Under iso-osmotic conditions, the internal pool of alanine was largely consumed. The presence of glucose markedly reduced the amount of alanine consumed, but the presence of acetate had little effect. When the cells were placed into a hyperosmotic buffer, the alanine pool did not decrease either in the absence of an exogenous carbon source or in the presence of acetate. In the presence of glucose, however, the alanine pool size increased about 1.8-fold.

We have seen that *L. major* promastigotes respond to acute hypo-osmotic stress by releasing alanine and other osmolytes, while in response to an acute hyperosmotic stress the alanine pool is either maintained or, in the presence of glucose, increased. This latter observation suggested that alanine oxidation was inhibited by hyperosmotic conditions. To test this, cells were incubated with [$1-^{14}\text{C}$]alanine and $^{14}\text{CO}_2$ production was measured as a function of osmolarity. It was

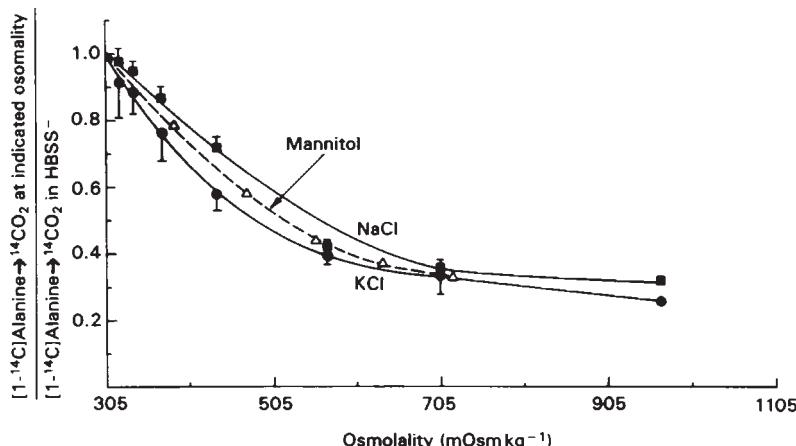


Figure 10.4. $^{14}\text{CO}_2$ production from $1\text{-}[^{14}\text{C}]$ alanine as a function of osmolarity. *L. major* promastigotes from late log phase cultures were washed and resuspended in Hanks' balanced salt solution (305 mOsm kg^{-1}) containing 3 mM $1\text{-}[^{14}\text{C}]$ -L-alanine. The suspension was incubated in a shaker bath at 26°C and $^{14}\text{CO}_2$ release was determined after 1h. The osmolarity was increased by adding NaCl (■), KCl (●), or mannitol (△). For the experiments using NaCl or KCl, values are the mean \pm SD ($n=3$). For the experiment using mannitol, the values are the mean of triplicate assays in a single experiment.

found that, as the osmolarity increased, $^{14}\text{CO}_2$ production decreased markedly (Figure 10.4). Essentially the same inhibition occurred whether the increase in osmolarity was caused by the addition of NaCl, KCl, or mannitol, indicating that the inhibition was primarily caused by the increase in osmolarity. Similar results have been observed for $[1\text{-}^{14}\text{C}]$ glutamate, $[1(3)\text{-}^{14}\text{C}]$ glycerol, $[1\text{-}^{14}\text{C}]$ acetate, and $[6\text{-}^{14}\text{C}]$ glucose. Since the only pathway common to all these substrates is the oxidative portion of the Krebs cycle, it appears that an acute hyperosmotic stress causes an inhibition of mitochondrial oxidation which prevents the utilization of cytoplasmic alanine. The increase in osmolarity also initiates proteolysis and/or other mechanisms which lead to an increase in alanine pool size, thus reducing the rate of water loss from the cell. Thus the regulation of alanine metabolism and of its permeability appear to play key roles in determining the ability of *L. major* promastigotes to cope with osmotic stress.

PHYSIOLOGICAL IMPLICATIONS

L. major promastigotes develop in the midgut and upper portions of the sandfly intestinal tract (Lainson and Shaw, 1987). Although it is not known whether the $p\text{O}_2$ in these locations drops below that of air when the sandfly is above ground, it is likely that some degree of hypoxia occurs when the sandflies enter rodent burrows or other poorly ventilated places. If so, it might be advantageous for promastigotes to have a low affinity oxygen sensor that, in response to hypoxia, would accelerate glycolysis and thus maintain ATP levels. In addition to possible changes in $p\text{O}_2$ with

changes in microhabitat, there will surely be changes in the osmolarity of the contents of the upper intestinal tract due to differences in the plant sap (or blood meal) that the sandfly ingests. One may suppose that the ability to respond rapidly to changes in osmolarity allows promastigotes to remain viable and to develop into infectious metacyclic forms. The discovery that promastigotes are sensitive to changes in pO_2 and osmolarity raises many new and interesting questions about the control of intermediary metabolism and cell shape in these cells.

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11. Glycosomes

F.R.Opperdoes

INTRODUCTION

Members of the Kinetoplastida possess a number of features unique in nature. These include the kinetoplast and the editing of mitochondrial DNA, a mini-exon sequence that is spliced to all messenger RNAs, trypanothione (see Chapter 44) and the glycosome. The glycosome is a peculiar microbody-like organelle, resembling the peroxisomes of other eukaryotic cells, which was described for the first time by Opperdoes and Borst (1977). The glycosome has been the subject of extensive study during recent years, not only the organelle in the African trypanosome *Trypanosoma brucei*, but also those in a number of other representatives of the order Kinetoplastida. Because an extensive review has appeared recently (Opperdoes, 1987), this chapter provides only a short overview of the latest findings related to the glycosome. It includes also some data on the organelle from organisms other than *T. brucei*, such as *Leishmania*, *T. cruzi*, *Phytomonas* and *Trypanoplasma* sp.

THE GLYCOSOME

In morphology, glycosomes strongly resemble the microbodies or peroxisomes of other eukaryotic cells. However, the organelle that was first described in *T. brucei* lacks such typical peroxisomal enzymes as catalase and hydrogen peroxide producing oxidases. Instead it was shown to contain nine enzymes involved in the conversion of glucose and glycerol to phosphoglycerate, and this was the reason why the name 'glycosome' rather than peroxisome was chosen for this organelle (see Figure 11.1). Subsequently, organelles with similar functional properties have been reported in other trypanosome species and related genera, such as *T. cruzi*, *Crithidia* spp. and *Leishmania* spp. Together they constitute the major representatives of the trypanosomatid family. With a recent finding of glycosomes in the bodonid organism *Trypanoplasma borelli*, a parasite of fish (Opperdoes *et al.*, 1988), it is now generally accepted that glycosomes occur not only in members of the Trypanosomatidae, but are also a general feature of all members of the Kinetoplastida, which comprise both the Trypanosomatidae and the Bodonidae.

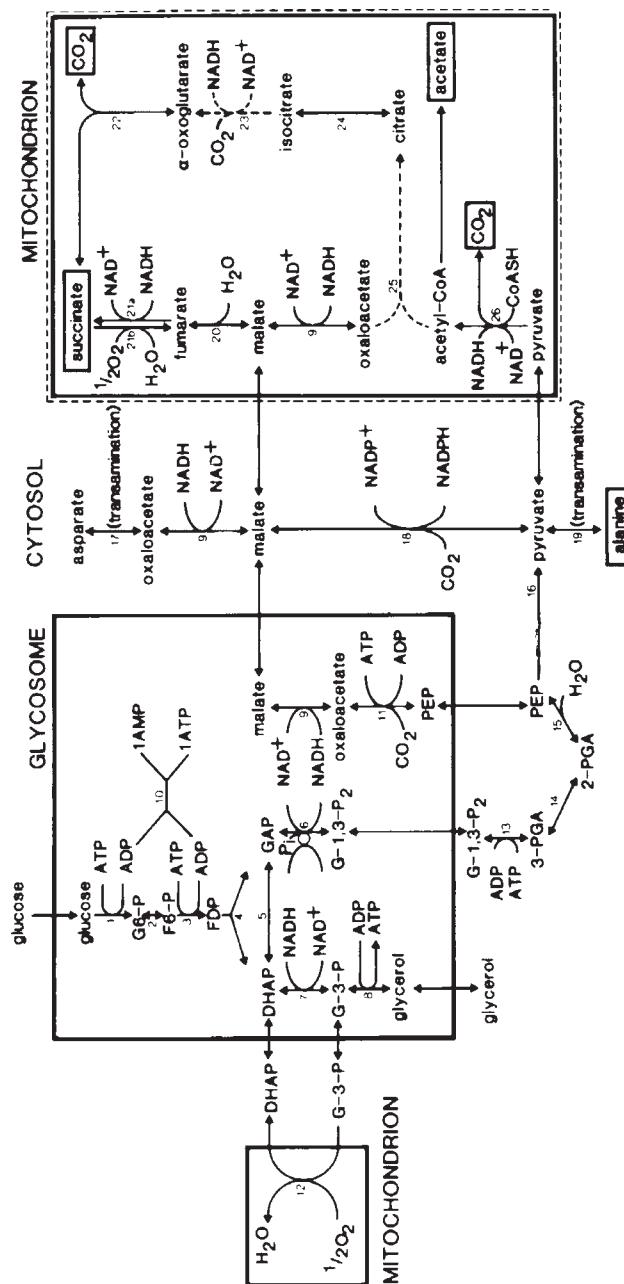


Figure 11.1. Pathways of glucose metabolism in procyclic trypanastigotes of *T. brucei*. The enzyme locations in the glyosome and cytosol have been established, but the location of the mitochondrial enzymes (except for glycerophosphate oxidase and malate dehydrogenase) have not yet been clearly demonstrated. End products of aerobic or anaerobic metabolism are enclosed in boxes. The dashed lines indicate enzymes whose presence remains uncertain. 1, Hexokinase; 2, phosphoglucone isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, glycerol-3-phosphate dehydrogenase; 8, glycerokinase; 9, malate dehydrogenase; 10, adenylate kinase; 11, phosphoenolpyruvate carboxykinase; 12, glycerol-3-phosphate oxidase; 13, phosphoglycerate kinase; 14, phosphoglycerate mutase; 15, enolase; 16, pyruvate kinase; 17, aspartate aminotransferase; 18, malic enzyme; 19, alanine aminotransferase; 20, fumarate hydratase; 21a, fumarate reductase; 21b, succinate dehydrogenase; 22, α -oxoglutarate decarboxylase; 23, isocitrate dehydrogenase; 24, aconitase; 25, citrate synthetase; 26, pyruvate dehydrogenase. (Modified from Fairlamb and Opperman (1986)).

Catalase has been found to be associated with the glycosomes of some representatives of the order Kinetoplastida, such as *Crithidia* sp. (Opperdoes *et al.*, 1976), *Phytomonas* sp. (Opperdoes, unpublished) and *Trypanoplasma* sp. (Opperdoes *et al.*, 1988). In addition, enzymes involved in the β -oxidation of fatty acids and the biosynthesis of plasmalogens were later found in glycosomes (Opperdoes, 1984; Hart and Opperdoes, 1984). This strongly suggests a relationship with the peroxisomes of other eukaryotes that all contain catalase and are involved in fatty acid metabolism. None of the human pathogens (*Trypanosoma* and *Leishmania* sp.) contain detectable amounts of catalase in their glycosomes.

The African trypanosome, *T. brucei*, is entirely dependent on glycolysis for its energy production. Its glycolytic flux is amongst the highest measured. In the vertebrate stage of *T. brucei* the glycosome is highly specialized in glycolysis. Ninety per cent or more of its protein content is involved in the pathway. Nevertheless, only about 9 per cent of the total protein of *T. brucei* is devoted to glycolysis, whereas in skeletal muscle, or in fermenting yeast, this may be as high at 65 per cent. In *T. brucei* the glycosomes represent more than 4 per cent of the total cell volume, while in *Phytomonas* this may be as high as 7 per cent (DeSouza, personal communication). Apparently this unique compartmentalization of the glycolytic pathway within an organelle allows the trypanosome to carry out glycolysis much more efficiently than in any other cell type studied to date. In the insect-stage trypanosome and in other representatives of the order Kinetoplastida, glycolysis plays a less important role, because these organisms are capable of oxidizing amino acids in addition to glucose, owing to the presence of a more or less well developed mitochondrial respiratory chain and Krebs' cycle (see Chapters 10 and 12).

From a comparison of the enzymes and pathways associated with glycosomes from various organisms, the following picture emerges. Only the glycosomes of the vertebrate stage of the African trypanosomes, and possibly some *Phytomonas* species (unpublished results), are highly specialized in glycolysis, while those of the insect stage of the same organism and those of the other genera are probably much less specialized and have additional functions.

Other functions are carbon dioxide fixation (Opperdoes and Cottem, 1982; Cannata *et al.*, 1982), pyrimidine biosynthesis (Hammond *et al.*, 1981), purine salvage (Hassan *et al.*, 1985), β -oxidation of fatty acids (Hart and Opperdoes, 1984) and the biosynthesis of ether lipids (Opperdoes, 1984; Hart and Opperdoes, 1984).

In *T. brucei* bloodstream forms it was estimated that some 240 glycosomes are present per cell (Opperdoes *et al.*, 1984). Although such data are not yet available for the procyclic insect stage, purification factors obtained for glycosomes from this stage are similar to those of the vertebrate stages (Hart *et al.*, 1984), which suggest that in this stage of the life cycle the situation is probably not too different from that in the bloodstream form. In *Leishmania major* promastigotes 50 to 100 glycosomes were estimated to be present per cell (Hart and Opperdoes, 1984), while serial sections of *L. mexicana* amastigotes revealed only 10 glycosomes (Coombs *et al.*, 1986). This seems a small number compared with the promastigote stage or the various stages of *T. brucei*, although one must take into account the relatively small

size of the intracellular amastigote and it was calculated that glycosomes accounted for just under 1 per cent of the total cell volume. In contrast, a *Phytomonas* sp. isolated from the plant *Euphorbia characias* seems to be exceptionally rich in glycosomes (Attias *et al.*, 1988), and their volume may represent as much as 7 per cent of the total cell (W.DeSouza, personal communication).

Subcellular fractionation experiments carried out on both the bloodstream and insect stages of *T. brucei*, the promastigote forms of the various representatives of the genus *Leishmania*, epimastigotes of *T. cruzi*, promastigotes of *Phytomonas* sp. and *Crithidia luciliae* (Opperdoes, unpublished) have shown that the glycosomes of the different species resemble each other in their function.

Some marked differences, however, have been detected, for instance between the enzyme activities of glycosomes from bloodstream and insect stage *T. brucei* (see Opperdoes, 1987). Nevertheless, all glycosomes contained the early enzymes of the glycolytic pathway from hexokinase to phosphoglycerate kinase and two enzymes of the glycerol pathway (glycerol kinase and glycerol-3-phosphate dehydrogenase), an enzyme involved in CO₂ fixation (phosphoenolpyruvate carboxykinase) and possibly malate dehydrogenase. Some evidence is available to suggest that in certain *Leishmania* species the enzymes enolase and phosphoglycerate mutase are also associated with glycosomes (Froment *et al.*, 1989). Several enzymes of the β -oxidation of fatty acids were partly associated with glycosomes and partly with the mitochondrial fraction. In addition, it has been demonstrated that dihydroxyacetone-phosphate acyltransferase (Opperdoes, 1984), orotate decarboxylase and orotidine phosphoribosyltransferase (Hammond *et al.*, 1981) are associated with these organelles.

Little is known about the biochemical composition of the glycosomes of the amastigote stages of *Leishmania* and *T. cruzi*. This is mainly due to the great difficulty of breaking the amastigotes prior to cell fractionation. It is likely, however, that the enzyme content of amastigote glycosomes will only differ quantitatively from those of promastigotes and epimastigotes, since it is thought that the metabolism in these life cycle stages is similar. Some quantitative differences between promastigotes and amastigote stages of *Leishmania* that have been observed (Hart *et al.*, 1981) suggest that the intracellular stages are more dependent on the oxidation of fatty acids than on that of carbohydrates. The rate of fatty acid oxidation in amastigotes of *L. mexicana* was found to be 10-fold higher than in promastigotes stages of the same strain. It would be of interest to know whether the part of the β -oxidation associated with the glycosomes rather than the mitochondrion is entirely responsible for this increased activity, as is also the case for the clofibrate-induced peroxisomes of mammals.

THE ENZYMES

Misset and co-workers (Misset and Opperdoes, 1984; Misset *et al.*, 1986) have isolated nine glycosomal enzymes and two of their cytosolic isoenzymes (Misset *et al.*, 1987; Misset and Opperdoes, 1987. Note nomenclature; the prefixes g and c

refer to glycosomal and cytosolic, respectively) from bloodstream-form *T. brucei* and have compared them with their homologues from other organisms. For two of these, triosephosphate isomerase (TIM) and glyceraldehyde-phosphate dehydrogenase (GAPDH), crystals of sufficiently high quality have been grown to allow the resolution of their three-dimensional structure to 1.9 and 2.5 Å, respectively. The cytosolic enzyme pyruvate kinase (PK) has also been purified (Barnard and Pedersen, 1988; Callens *et al.*, unpublished). The genes encoding the enzymes glucosephosphate isomerase (PGI) (Marchand *et al.*, 1989), aldolase (ALDO) (Marchand *et al.*, 1988), TIM (Swinkels *et al.*, 1986), gGAPDH (Michels *et al.*, 1986), cGAPDH, glycosomal phosphoglycerate kinase (gPGK), cPGK (Osinga *et al.*, 1985) and PK from *T. brucei*, the gGAPDH from *T. cruzi* (Kendall *et al.*, 1990) and *L. mexicana amazonensis* (unpublished), the gPGK and cPGK of *C. fasciculata* (Swinkels *et al.*, 1988), as well as a gene encoding a 60 kDa protein that was originally thought to be a microtubule-associated protein (Kueng *et al.*, 1989) but turned out to be the glycosomal phosphoenolpyruvate carboxykinase (PEPCK) (Parsons and Smith, 1989), have all been cloned and sequenced.

All glycosomal proteins from *T. brucei*, except PGI, are characterized by a very high isoelectric point, as a result of additional positively charged amino acids, most of which are accommodated in unique amino acid extensions or insertions that are responsible for the slightly greater molecular mass of these glycosomal proteins. The cytosolic isoenzymes all have a more neutral pH, and lack the specific insertions or extensions. Based on these observations and the fact that the positive charges often clustered together on the surface of the proteins, Wierenga *et al.*, (1987) postulated that these 'hot spots' would be involved in the import of the glycosomal proteins into the glycosome. However, when the amino acid sequences of gGAPDH from *T. cruzi* and *L. m. amazonensis* and the gPGK from *C. fasciculata* became available, it turned out that, although the insertions and extensions were all conserved between species, the high overall positive charge was not. It is not yet clear what the function of the high positive charge of the glycosomal enzymes in *T. brucei* would be. The most likely explanation is that it plays an essential role in the neutralization of the negative charges of the glycolytic metabolites that serve as substrate and product of the glycosomal enzymes and that are present at particularly high concentrations similar to that of the enzymes' active sites (Misset *et al.*, 1986).

It is not yet clear how the enzymes are imported into the glycosome. The amino acid insertions and/or extensions may play an essential role, since they are absent from all other homologous glycolytic enzymes studied so far. The enzymes PGI and GAPDH contain a C-terminal tripeptide that resembles the SKL peptide (Gould *et al.*, 1988) that has been shown to be involved in the targeting of some, but not all, peroxisomal proteins (Table 11.1). Apart from two amino acid substitutions the cytosolic and glycosomal PGKs from *C. fasciculata* only differ by the presence of a 40 amino acid long C-terminal extension in the glycosomal enzyme. This makes this extension a likely candidate for the topogenic signal. However, a C-terminal SKL-like peptide is absent. The *T. brucei* enzyme has a similar extension of which the C-terminal tripeptide contains S and L, but a positively charged amino acid (H, K, R), thought to be essential for targeting of peroxisomal proteins, is absent.

Table 11.1. Comparison of some C-terminal amino acid sequences of microbody enzymes (modified from Miyazawa *et al.* (1989))

Acyl-CoA oxidase (rat)	K	H	L	K	P	L	Q	S	K	L
Bifunctional enzyme (rat)	S	L	A	G	P	H	G	S	K	L
Luciferase (firefly)	K	A	K	K	G	G	K	S	K	L
Uricase (soybean)	A	S	L	S	R	L	W	S	K	L
Malate synthase (cucumber)	I	H	H	P	R	E	L	S	K	L
D Amino acid oxidase (pig)	N	L	L	T	M	P	P	S	H	L
Glucosephosphate isomerase (<i>T.b</i>)	I	N	M	F	N	E	L	S	H	L
Glyceraldehyde-phosphate dehydrogenase (<i>T.b</i>)	A	A	R	D	R	—	A	A	K	L
Glyceraldehyde-phosphate dehydrogenase (<i>T.c</i>)	A	S	K	D	R	—	S	A	R	L
Glyceraldehyde-phosphate dehydrogenase (<i>L.m</i>)	A	A	K	D	A	A	S	S	K	M
Phosphoglycerate kinase (<i>T.b</i>)	G	T	L	S	N	R	W	S	S	L
Phosphoglycerate kinase (<i>C.f</i>)	A	T	V	S	M	V	L	A	S	P

T.b, *Trypanosoma brucei*; *T.c*, *Trypanosoma cruzi*; *L.m*, *Leishmania mexicana amazonensis*; *C.f*, *Crithidia fasciculata*

Transformation of trypanosomes, using hybrid genes that code for proteins consisting of a cytosolic enzyme to which a putative glycosomal targetting signal is attached, eventually should provide us with more information on the nature of the glycosomal targetting signal.

EVOLUTIONARY ORIGIN

The two (i.e. g and c) GAPDH isoenzymes from *T. brucei* are remarkably different with only 50–60 per cent sequence identity between them (Michels *et al.*, 1991). This indicates that the two isoenzymes are not more related to each other than any GAPDH from a prokaryote and eukaryote would be. This suggests that the two trypanosome isoenzymes must have had a separate origin. It is of interest to note that the cytosolic isoenzyme is closely related to the *Escherichia coli* enzyme (about 80 per cent sequence identity) (Michels *et al.*, 1991). This may not, however, be interpreted as an indication for a prokaryotic origin of this trypanosome cytosolic isoenzyme, since amongst all the known bacterial GAPDH sequences, the *E. coli* enzyme itself is anomalous in that it is the only prokaryotic sequence with a typical eukaryotic character (Branlant and Branlant, 1985). This suggests that *E. coli*, or its ancestor, may have gained the gene for GAPDH from a eukaryotic host by an event of horizontal gene transfer. Similarly, the trypanosomatid ancestor could have obtained an additional gene by horizontal transfer of DNA, either from an endosymbiont, or by other means. Phylogenetic analyses of all available GAPDH sequences using both a distance matrix and a parsimony method, predict that the Trypanosomatidae diverged very early from the main stream of eukaryotic evolution, well before the separation of the Plants and Fungi Kingdoms (Figure 11.2). Similar analyses based on the sequence information available for cytochrome c, PGK and TIM (Opperdoes, unpublished), and the small ribosomal RNAs (Sogin *et al.*, 1986) all suggest a branching that better coincides with that inferred from the

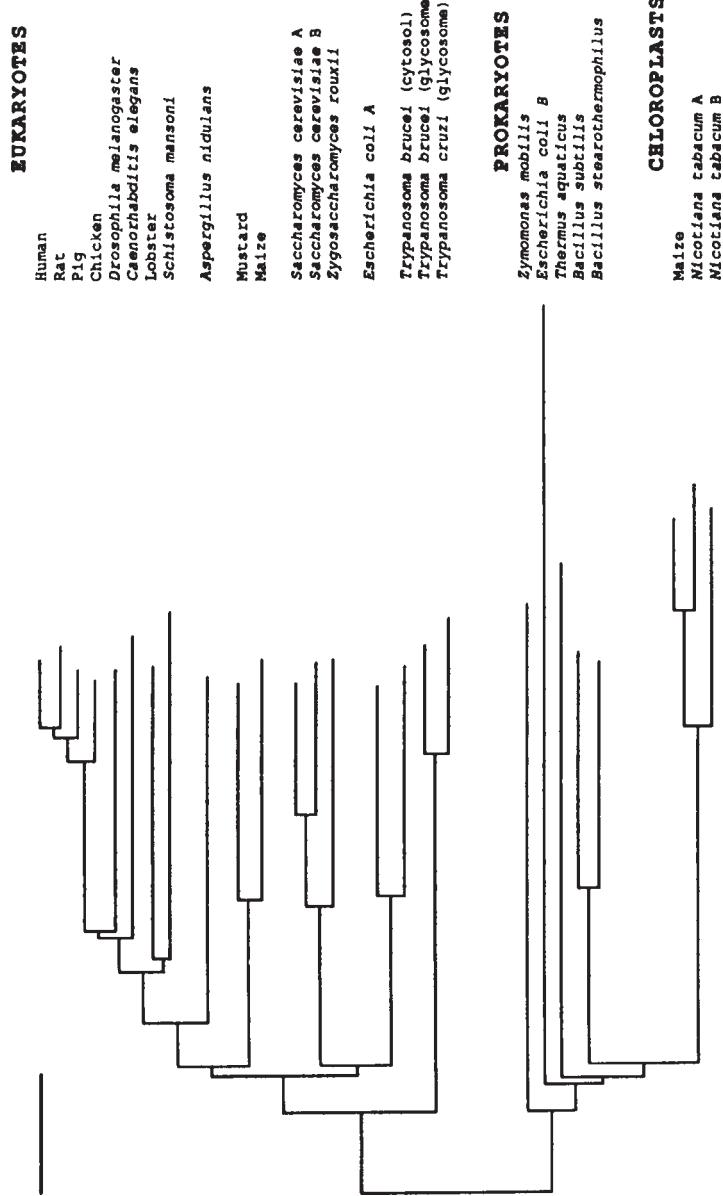


Figure 11.2. Phylogenetic tree showing the evolutionary relationships between various glyceraldehyde-phosphate dehydrogenase from both prokaryotic and eukaryotic origin. The horizontal bar represents five accepted point mutations per 100 amino acids.

gGAPDH, than from the cGAPDH sequence. Together these data suggest that the gene which codes for the gGAPDH is the gene that was originally present in the trypanosome's ancestor when it diverged from the other eukaryotes. The fact that gGAPDH, gPGK and TIM (Borst, 1986) are all of a typical eukaryotic nature makes it unlikely that the glycosome, in its entirety, arose from a prokaryotic endosymbiont. Such analyses may not, however, be interpreted as absolute proof for the eukaryotic origin of the glycosome. With only three enzymes analysed to date, it cannot be excluded that the glycosome entered as a prokaryote, but that the original enzymes were gradually replaced by their homologous counterparts already present in the eukaryotic cell. Clearly many more sequences must be analysed before a definite answer can be given as to the evolutionary origin of this intriguing organelle.

REGULATION OF GLYCOLYTIC FLUX

Pyruvate kinase from *T. brucei* resembles the PKs from other eukaryotes in that it is activated by fructose 1, 6-bisphosphate ($\text{Fru}(1, 6)\text{P}_2$) and glucose 1, 6-bisphosphate ($\text{Glc}(1, 6)\text{P}_2$), while its activity is modulated by adenine nucleotides and inorganic phosphate. However, the trypanosome enzyme differs from most other PKs in that it is insensitive to regulation by most of the metabolites that have been reported to modulate PK activity from other sources (Callens *et al.*, unpublished).

T. brucei PK, together with the PKs from the other glycosome-containing members of the Kinetoplastida (i.e. the insect trypanosomatid *C. luciliae*, the human trypanosomatid *L. donovani*, and the parasitic Bodonid organism *T. borelli* (Van Schaftingen, 1985; Opperdoes *et al.*, 1988)) all differ from their homologous counterparts in other eukaryotes in that they constitute the only class of PKs that are activated by fructose 2, 6-bisphosphate ($\text{Fru}(2, 6)\text{P}_2$) (Van Schaftingen *et al.*, 1985, 1987; Opperdoes *et al.*, 1988). The fact that in these organisms $\text{Fru}(2, 6)\text{P}_2$ modulates the activity of PK rather than that of phosphofructokinase (PFK), as is the case in most other eukaryotic cells, is most likely related to the fact that PFK in the Kinetoplastida is not a cytosolic enzyme, but is sequestered inside the glycosome. This sequestration most likely renders it inaccessible to $\text{Fru}(2, 6)\text{P}_2$ which is synthesized from fructose 6-phosphate (Fru 6-P) by the cytosolic PFK2 (Van Schaftingen *et al.*, 1987). This compartmentation has probably led to the eventual loss of regulation of glycosomal PFK by $\text{Fru}(2, 6)\text{P}_2$, in favour of an adaptation of the original $\text{Fru}(1, 6)\text{P}_1$ -binding site of cytosolic PK to accommodate $\text{Fru}(2, 6)\text{P}_2$.

$\text{Fru}(2, 6)\text{P}_2$ -regulated PK is active in both the vertebrate and insect stages of *T. brucei*; no evidence has been found for the presence of a second PK isoenzyme and only two identical tandemly repeated genes have been detected (Michels *et al.*, unpublished). This indicates that one and the same enzyme must be involved in the regulation of two such different pathways of carbohydrate metabolism as is the case in the two life cycle stages of *T. brucei* (see Opperdoes, 1987).

How does PK adapt to two such completely different situations? Interestingly, Van Schaftingen *et al.* (1987) have shown that in the vertebrate stage, both with

aerobic and anaerobic glycolysis, the cellular concentrations of Fru(2, 6)P₂ and phosphoenolpyruvate (PEP) were inversely related. Since the conversion of PEP to pyruvate is the only known fate of this glycolytic metabolite in the vertebrate stage of *T. brucei* it is not unexpected that a decrease in the concentration of Fru(2, 6)P₂ will be compensated for by an increase in PEP. This indicates the presence of regulation of PK activity. The advantage, however, for the vertebrate-stage trypanosome of being controlled at this level by Fru(2, 6)P₂ is not clear at present, especially since several authors have reported that in this life cycle stage the ratelimiting step of glycolysis is located at the very beginning of the pathway (see Chapter 33).

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12. Mitochondrial metabolism of African trypanosomes

J.F.Turrens

INTRODUCTION

The survival of any form of life depends upon a continuous supply of energy, usually provided by oxidation of nutrients. In most eukaryotic organisms, oxygen is the final acceptor for the reducing equivalents produced during these oxidations, becoming completely reduced to water in a reaction catalysed by the mitochondrial cytochrome oxidase. One exception to this rule is found in the bloodstream forms of African trypanosomes, in which electron transfer to oxygen is carried out by a KCN insensitive *sn*-glycerol-3-phosphate oxidase (also known as alternative oxidase), without cytochromes. Energy production in these cells depends only on glycolysis, and the role of the alternative oxidase is to oxidize glycerol 3-phosphate produced in the glycosome during the reoxidation of NADH (Grant and Sargent, 1960; and see Chapter 11). This system is located in the mitochondrial membrane (Fairlamb and Opperdoes, 1986), and uses ubiquinone Q₉ as a redox intermediate between the glycerol-phosphate dehydrogenase and the terminal oxidase (Tielens and Hill, 1985; Turrens *et al.*, 1986; Clarkson *et al.*, 1989a).

When bloodstream African trypanosomes are cultured at 26 °C, they transform into procyclic trypomastigotes (also found in the midgut of tsetse flies). These forms develop a KCN sensitive respiratory chain in which oxygen is reduced to water by a haem-containing cytochrome *c* oxidase. Glycerol 3-phosphate is also among the substrates for the cytochrome-containing respiratory chain (giving electrons to ubiquinone), but in this case its oxidation is inhibitable by antimycin and KCN. Thus, although both oxidases have some components in common (glycerol 3-phosphate dehydrogenase and ubiquinone Q₉ (Clarkson *et al.*, 1989b)) the segments between ubiquinone and the terminal oxidases are entirely different.

Even though the mitochondrial respiratory chain of procyclic trypomastigotes of African trypanosomes is similar to the mammalian counterpart, there are several important differences between the two organelles. This review describes some of the differences regarding the production of metabolic intermediates and their oxidation by the respiratory chain.

KREBS' CYCLE

Glycolysis in mammalian cells leads to the formation of pyruvate which is decarboxylated, oxidized and activated to acetyl-CoA by the enzyme pyruvate dehydrogenase inside the mitochondrion. In the first reaction of the Krebs' cycle, acetyl-CoA reacts with oxaloacetate producing citrate, followed by a series of reactions equivalent to a complete oxidation of the acetate group, producing NADH, CO₂ and regenerating oxaloacetate. NADH is in turn oxidized by the first complex in the respiratory chain (NADH-ubiquinone reductase), transferring electrons to ubiquinone, ultimately producing water at the cytochrome oxidase level (Figure 12.1). A few other oxidizable substrates (i.e. succinate, fatty acyl-CoA, glycerol phosphate, etc.) may transfer electrons directly to ubiquinone, skipping the first respiratory complex (Figure 12.1). The combined stoichiometry indicates that 4 mol NADH and 1 mol succinate are produced per mole of pyruvate. Therefore, the mammalian respiratory chain oxidizes much more NADH than succinate and the inhibition of succinate oxidation (i.e. by malonate) has little or no effect on the rate of oxygen consumption, changing only the steady-state concentration of NADH (Turrens, 1989).

Carbohydrate metabolism in African trypanosomatids varies throughout their life cycle. Bloodstream forms of *Trypanosoma brucei* (long slender trypomastigote forms) produce pyruvate as the final product of glycolysis (Fairlamb and Opperdoes, 1986), while *T. brucei* procyclic trypomastigotes (culture forms with an active respiratory chain) produce oxaloacetate instead of pyruvate. Oxaloacetate is transformed into malate regenerating the oxidized NAD⁺ required for glycolysis (Fairlamb and Opperdoes, 1986) (Figure 12.2). Although pyruvate is not the final product of glycolysis, it still may be formed by the malic enzyme, in the cytoplasm. The next step, catalysed by pyruvate dehydrogenase (producing acetyl-CoA), has also been identified, at least in *T. rhodesiense* (Ryley, 1962).

The Krebs' cycle in African trypanosomes presents significant differences when compared with mammalian cells. Although several Krebs' cycle enzymes have been identified in *T. brucei* procyclic trypomastigotes (Klein *et al.*, 1975) (i.e. malate dehydrogenase, succinate dehydrogenase and fumarase, all using 4-carbon dicarboxylic acids), the first enzyme of the cycle (citrate synthase) appears to be missing (Fairlamb and Opperdoes, 1986). Therefore, although acetyl-CoA is formed, it cannot be utilized for producing citrate in the Krebs' cycle. In summary, oxidation of glucose to CO₂ does not take place in these cells as in mammalian systems.

In 1975 Klein *et al.* (1975) identified the enzyme NADH-fumarate reductase in trypanosomatids, which can reverse the Krebs' cycle by reducing fumarate to succinate. Therefore, even though the cycle cannot operate through the same reactions identified in mammalian cells, the reduction of fumarate by fumarate reductase provides an alternative route for converting malate into succinate (Klein *et al.*, 1975) (Figure 12.2). Upon entering the mitochondrion, malate may either be oxidized to oxaloacetate or dehydrated to fumarate. In the absence of citrate synthase, oxaloacetate may be only eliminated by transamination, decreasing the rate of

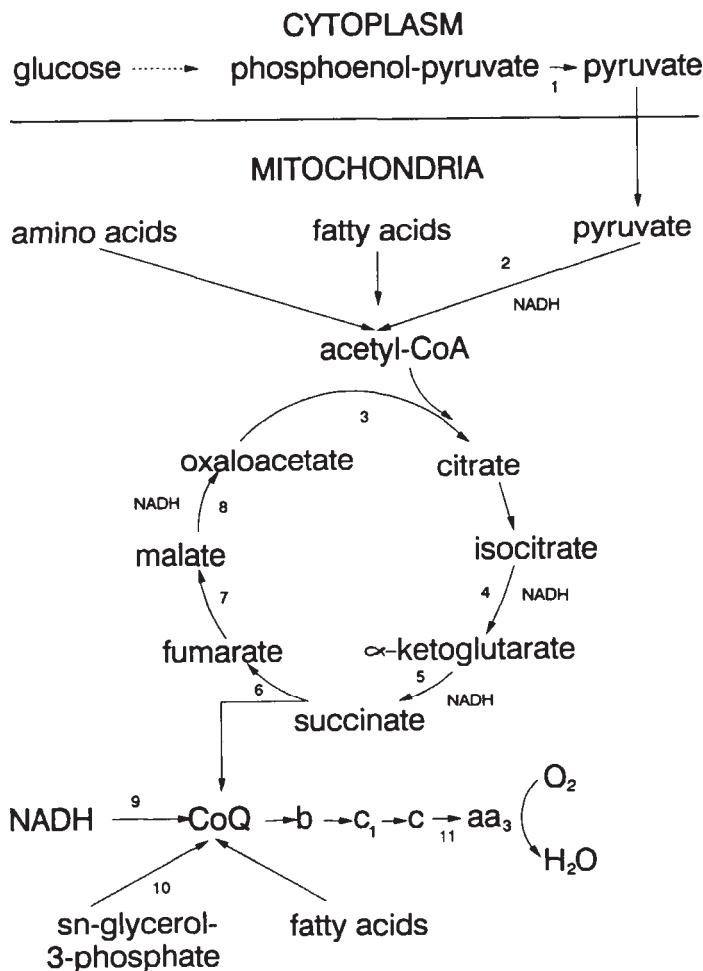


Figure 12.1 Brief scheme representing glycolysis, tricarboxylic acid cycle and respiratory chain in mammalian cells. The dotted arrow from glucose to phosphoenol-pyruvate summarizes the glycolytic reactions which are not included in the scheme as they are not relevant to the areas discussed in this paper. Enzymes: 1, pyruvate kinase; 2, pyruvate dehydrogenase; 3, citrate synthetase; 4, isocitrate dehydrogenase; 5, α -ketoglutarate dehydrogenase; 6, succinate dehydrogenase; 7, fumarase; 8, malate dehydrogenase; 9, NADH-dehydrogenase; 10, glycerol-3-phosphate dehydrogenase; 11, cytochrome oxidase.

oxaloacetate utilization. Since the equilibrium between malate and oxaloacetate is shifted towards malate ($\Delta G^\circ = +8.1 \text{ kcal mol}^{-1}$), malate will be hydrated to fumarate. As NADH is not efficiently oxidized by the respiratory chain (see below), we postulated that fumarate can oxidize NADH through the NADH-fumarate reductase, producing succinate to be oxidized in the respiratory chain (Figure 12.2).

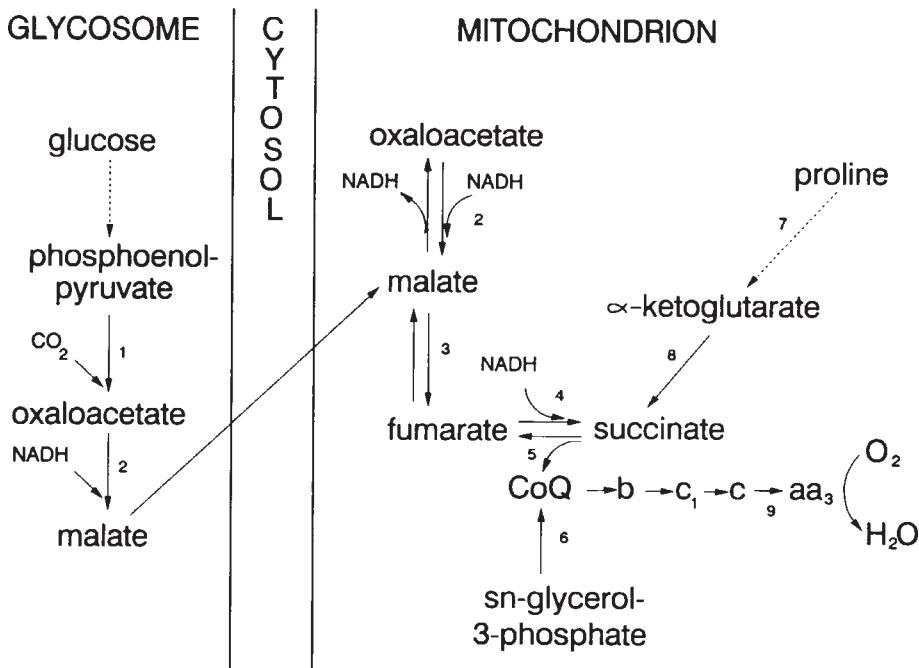


Figure 12.2 Glycolysis, proline oxidation and mitochondrial metabolism in *T. brucei* ptocyclic trypanosomes. In trypanosomatids glycolysis takes place largely inside a specific organelle (glycosome, see Chapter 11 for details). Malate may be transformed into pyruvate by the malic enzyme in the cytoplasm (not shown). Enzymes: 1, phosphoenolpyruvate carboxykinase; 2, malate dehydrogenase; 3, fumarase; 4, NADH-fumarate reductase; 5, succinate dehydrogenase; 6, glycerol-3-phosphate dehydrogenase; 7, proline oxidase; 8, α -ketoglutarate dehydrogenase; 9, cytochrome oxidase.

In summary, succinate could be considered the main mitochondrial product of malate metabolism, ultimately the last product from glucose catabolism. NADH is not produced at high rates as the enzymes citrate synthase and NAD-dependent isocitrate dehydrogenase appear not to be active in African trypanosomes (Fairlamb and Opperdoes, 1986). Thus, NADH cannot compete efficiently with succinate as an electron donor for the respiratory chain, and this explains why malonate (an inhibitor of succinate oxidation), is a potent inhibitor of glucose-dependent oxygen consumption by intact cells in *T. brucei* procyclic trypanomastigotes (Turrens, 1989).

RESPIRATORY CHAIN

The cytochrome-containing mitochondrial electron transport chain of trypanosomes is also quite different from that found in other eukaryotes. The respiratory chain of trypanosomatids is insensitive to rotenone, an inhibitor of complex I in mammalian mitochondria (Table 12.1). In addition, myxothiazol, an inhibitor of the cytochrome

Table 12.1. Comparison between the respiratory chain in mitochondria from *T.brucei* procyclic trypomastigotes and mammalian cells.

Component	Mammalian cells	<i>T. brucei</i>
NADH-ubiquinone reductase	Very active	Undetectable
Typical inhibitors	Rotenone, antimycin, KCN	Antimycin, KCN
K _i for myxothiazol	0.02 μM ^a	0.25 μM
α Band for cytochrome c ₂ +	550 nm	555 nm

^a Determined in rat liver mitochondria.

*bc*₁ region (complex III) that covalently binds cytochrome *b* (Di Rago *et al.*, 1989), is about 12-fold less potent in trypanosomatids than in mammalian mitochondria (Table 12.1), suggesting that there may be major differences in the polypeptide sequence of this cytochrome in trypanosomes. There are also differences in the structure of cytochrome *c*, with a maximal absorption band for the reduced form at 555nm instead of 550nm (Table 12.1). The spectral differences with the mammalian cytochrome *c* reflect major differences in protein structure, to the extent that reduced mammalian cytochrome *c* cannot be used as an electron donor by the KCN sensitive cytochrome oxidase of trypanosomes (Boveris *et al.*, 1986).

As previously indicated, respiration in *T. brucei* procyclic trypomastigotes (Turrens, 1989) (as well as in *T. cruzi* epimastigotes (Sylvester and Krassner, 1976)) may be inhibited by malonate. This inhibition can be reversed by adding excess succinate, indicating that malonate inhibits respiration by competing with succinate and that succinate is its primary substrate. From these results one may conclude that either NADH is not efficiently oxidized or it is not supplied at a rate fast enough to compensate for the demand of electrons in the respiratory chain in the absence of succinate oxidation.

Succinate is not only produced from a reversal of the Krebs' cycle, but is also the end product of proline catabolism, which is the preferred oxidizable substrate for culture forms of African trypanosomes (Evans and Brown, 1972) and tsetse flies (Konji *et al.*, 1988). Proline catabolism includes the opening of the ring and its oxidation to *a*-ketoglutarate, subsequently producing succinate to be oxidized in the respiratory chain.

Isolation of intact mitochondria from *T. brucei* procyclic trypomastigotes is very difficult, as the forces required to open the cell membrane also disrupt the very large mitochondrion present in trypanosomatids. For this reason we have developed a procedure based on permeating intact cells with digitonin without disrupting the mitochondrial integrity (Turrens, 1989). In these preparations, we investigated whether other NADH dependent mitochondrial substrates (i.e. glutamate, β-hydroxybutyrate, malate, pyruvate, etc.) could stimulate oxygen consumption. None of these substrates had any effect on oxygen consumption; only succinate and glycerol 3-phosphate (both feeding electrons at the ubiquinone level of the chain) were able to stimulate respiration (Turrens, 1989). Further studies looking at oxygen consumption by subcellular fragments of *T. brucei* procyclic trypomastigotes indicated that the isolated respiratory chain does not efficiently oxidize NADH,

Table 12.2. Different enzymatic activities in membrane fragments^a of *T. brucei* procyclic trypomastigotes.

Enzyme	Specific activity (nmol min ⁻¹ min/mg protein)
NADH-oxidase + antimycin (1 μM)	14 ± 2 12 ± 1
NADH-cytochrome <i>c</i> reductase + antimycin (1 μM) + fumarate (4 mM)	74 ± 7 71 17
Succinate-cytochrome <i>c</i> reductase + antimycin (1 μM)	38 ± 4 0
Fumarate reductase Membrane-bound Soluble	52 ± 3 6.5 ± 0.6

^a Prior to homogenization, the cells were resuspended in buffer containing mannitol (0.23 M), sucrose (70 mM), EDTA (0.1 mM) and Tris-HCl (5 mM, pH 7.5) at a final concentration of around 20 mg ml⁻¹. The suspension was frozen and thawed, and homogenized by passage three times through a 27 gauge needle. The membranes were collected in a table top Eppendorf Microfuge at 12000 g. The soluble fraction was separated from small membrane fragments by ultracentrifugation at 105000g.

indicating that the segment between complexes I and III is not very active in *T. brucei* procyclic trypomastigotes (Table 12.2).

Spectrophotometric studies indicated that NADH oxidation occurred at a slow rate, and it was antimycin (Table 12.2) and KCN insensitive. H₂O₂ rather than water was the final product of NADH oxidation (Turrens, 1987). H₂O₂ formation could be blocked by fumarate, suggesting that this substrate could be the acceptor for the electrons coming from NADH oxidation. The concentration of fumarate which inhibited H₂O₂ formation by 50 per cent was very similar to the *K_m* of the enzyme NADH-fumarate reductase for this substrate. In addition, the *K_m* for NADH in both activities was very similar, suggesting that the H₂O₂ was being produced by the membrane bound NADH-fumarate reductase (Turrens, 1987).

We speculated that the lack of KCN sensitive NADH-supported respiration in mitochondrial membranes could have been the consequence of cytochrome *c* being washed from the membrane during centrifugation, thus inhibiting electron transport between cytochrome *c₁* cytochrome oxidase (complexes III to IV). We then determined the activity of both succinate—and NADH-cytochrome *c* reductase, to measure the integrity of the electron transport chain between the electron donor and cytochrome *c*. Although both substrates were capable of reducing cytochrome *c*, NADH dependent cytochrome *c* reduction was insensitive to antimycin, while succinate reduced cytochrome *c* in a reaction inhibitible by antimycin (Table 12.2). This indicated that the electrons from NADH were unable to reach the respiratory complex III, but rather reduced cytochrome *c* directly. The NADH dependent reaction was also inhibitible by fumarate, suggesting that it was a side reaction of NADH-fumarate reductase similar to the partial reduction of oxygen to H₂O₂. The same results were obtained in experiments using *T. congolense* procyclic trypomastigotes mitochondrial membranes (Bienen and Turrens, unpublished results).

Our results strongly suggest that the respiratory chain in African trypanosomes depends mostly on succinate, and that the segment between NADH-dehydrogenase and ubiquinone is not active. This explains why malonate inhibits respiration *in vivo*, as these cells should depend mostly on succinate for respiration. We have postulated that the combined action of NADH-fumarate reductase and succinate dehydrogenase provides an alternative route for the segment NADH-dehydrogenase ubiquinone found in the mammalian respiratory chain (Figure 12.2).

The transcription product of a gene that may code for the NADH-dehydrogenase from maxicircles of *T. brucei* kinetoplasts has been isolated from different forms of the parasite (Jasmer *et al.*, 1985; Feagin *et al.*, 1986). This suggests that there should be a NADH dehydrogenase activity in the mitochondrion of these parasites. Our evidence does not support the traditional concept of an active mitochondrial complex I in trypanosomatids. We wonder if this gene fragment may actually encode the NADH-dehydrogenase segment of fumarate reductase or another NADH-binding enzyme.

SUCCINATE AS A KEY INTERMEDIATE METABOLITE

In addition to being a major respiratory substrate, succinate is also a final metabolic product exported to the extracellular medium. *T. rhodesiense* procyclic trypomastigotes under aerobic conditions convert 4 per cent of the utilized glucose into succinate. Under anaerobic conditions (in the presence of CO₂) more than 60 per cent of glucose is transformed into succinate, without any lactate formation (Ryley, 1962; but see Chapter 10). These results indicate that succinate is being used as a final sink for electrons, in order to obtain sufficient NAD⁺ to continue with anaerobic glycolysis. These cells do not contain lactic dehydrogenase (Fairlamb and Opperdoes, 1986) to catalyse NADH reoxidation and depend on malate dehydrogenase for regenerating NAD⁺ under anaerobic conditions. We have recently identified a soluble NADH-fumarate reductase (Table 12.2), which may act in tandem with malate dehydrogenase replacing the mammalian lactic dehydrogenase, producing succinate to be secreted to the extracellular environment.

With the results discussed in this review we have constructed the following hypothesis. It appears as if the mitochondrion of African trypanosomes had evolved in a different direction than its mammalian counterpart, becoming adapted to a different environment. The mammalian cell depends on either glucose or fatty acids oxidation for energy production, both producing acetyl-CoA, requiring a fully operational Krebs' cycle for its final oxidation to CO₂. Those African trypanosomes that use tsetse flies as a vector depend on proline for their subsistence, and are better adapted for oxidizing succinate than NADH. By developing the enzyme NADH-fumarate reductase, these cells may oxidize NADH producing succinate, one of the central metabolites in African trypanosomes. Succinate may be used either as the main respiratory substrate or as an electron sink to be excreted outside the cell under anaerobic conditions.

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13. Mitochondria of *Plasmodium*

M.Fry

INTRODUCTION

The directly utilizable energy source for the intraerythrocytic stages of the malarial parasite is glucose; glucose is readily metabolized by the infected red cell, often at rates of about 100 times that of the uninfected red cell. Parasite-infected red cells also show enhanced O₂ uptake with glucose by comparison with uninfected red cells, and the favourable effects of low O₂ levels on the *in vitro* growth of infected cells suggest that the parasite is microaerophilic. There is suggestive evidence for a citric acid cycle in avian *Plasmodium*, but only malate dehydrogenase has been confirmed in the mammalian parasite. It has been proposed that the citric acid 'cycle' of the mammalian parasite branches at the level of oxaloacetate. Comprehensive reviews on the metabolism of *Plasmodium* have been given by Sherman (1979, 1983a, b; and see Chapter 2).

Whereas electron microscopy of avian *Plasmodium* has consistently shown the presence of cristate mitochondria, the mitochondria of mammalian *Plasmodium* have been variously described as cristate or acristate, or as being absent altogether. The mitochondria of *P. falciparum* have been shown to contain at least some cristae in all stages of their intraerythrocytic life cycle (Langreth *et al.*, 1978).

The terminal oxidase of the classical mitochondrial respiratory chain, cytochrome oxidase, has been assayed in platelet-free preparations of *P. knowlesi*, *P. berghei*, *P. cynomolgi* and *P. falciparum* (Scheibel and Miller, 1969a, b); electron microscope cytochemistry localized this activity to the acristate mitochondria of *P. berghei* and *P. gallinaceum* (Theakston *et al.*, 1969).

Over the past decade more evidence has accumulated to suggest an active role of the intraerythrocytic *Plasmodium* mitochondrion. Mitochondrial inhibitors have been shown to arrest *in vitro* parasite growth (Ginsburg *et al.*, 1986; Fry *et al.*, 1990); inhibitors of mitochondrial protein synthesis are potent antimalarial agents (Geary and Jensen, 1983), and the malarial parasite mitochondrion has been shown to maintain a transmembrane electrical potential (Divo *et al.*, 1985; Izumo *et al.*, 1988). However, with only circumstantial evidence available, the idea of an abbreviated parasite respiratory chain serving as an electron sink for pyrimidine biosynthesis, *via* dihydroorotate dehydrogenase, has persisted (Gutteridge *et al.*,

1979), although recent studies by Kanaani and Ginsburg (1989) suggest the parasite mitochondrion may be important in the supply of ATP to both the parasite and host erythrocyte. Our own studies failed to provide convincing evidence for ATP provided by parasite oxidative phosphorylation (Fry *et al.*, 1990).

The isolation and characterization of *Plasmodium* mitochondria from any species was not achieved until recently (Fry and Beesley, 1991). Obvious reasons for this include the quantities of parasite material required, the expense of producing them, and the lack of any major commitment to take the biochemical approach towards describing the mitochondrion. The commitment required has come from the pharmaceutical industry and, in particular, the desire to elucidate the site and mode of action of a new class of antimalarial hydroxynaphthoquinone (the Wellcome hydroxynaphthoquinone, 566C80; see Chapters 3 and 55). The antimalarial activity of similar compounds has been known since 1942 (hydrolapachol) and structurally similar quinones with antimalarial activity stemmed largely from the work of Fieser and Leffler, particularly the hydroxynaphthoquinones. As early as 1945, Wendell described certain hydroxynaphthoquinones as potent respiratory inhibitors, and in 1948 Whitman demonstrated the inhibition of respiration in *P. lophurae*-infected erythrocytes. In 1974, Porter and Folkers described a new fundamental approach to malarial chemotherapy based on the biochemical rationale of inhibition of the electron transfer mechanisms in the metabolism of plasmodia by antimetabolites of coenzyme Q (see Porter and Folkers, 1974, and references therein). This biochemical rationale has at long last proved to be well-founded and, along with the development of the antimalarial hydroxynaphthoquinone 566C80, the isolation and characterization of the *Plasmodium* mitochondrion has begun (Fry and Beesley, 1991).

THE APPEARANCE OF *PLASMODIUM* MITOCHONDRIA

As mentioned already, the mitochondrion in a mammalian malarial parasite appears to be of a variable morphology, whereas the mitochondrion of their avian counterparts is generally considered to be cristate and 'typical' in appearance. Studies by Slomianny and Prensier (1986) suggest that the mitochondrion in *P. falciparum* is single. We experienced no problems in visualizing the mitochondria of isolated *P. yoelii* (rodent) or *P. falciparum* (human) trophozoites. The use of intact saponin-released parasites probably overcomes problems of incomplete fixation that can be inherent in processing the parasite while still contained within the erythrocyte. The mitochondria of *P. yoelii* trophozoites were readily identifiable with a typical cristate morphology. Mitochondria of *P. falciparum* trophozoites are best identified by their pronounced double membrane; the extent of their internal structure is variable, and only occasionally can well-defined cristae or membrane whorls be readily distinguished (Fry and Beesley, 1991).

ISOLATION OF MITOCHONDRIA

Investigations that have sought to assay subcellular enzyme activities of the intraerythrocytic malarial parasite have used whole cell homogenates or crude particulate fractions. Notable among these are those studies involving the mitochondrial enzyme, dihydroorotate dehydrogenase, and *de novo* pyrimidine biosynthesis of the malarial parasite (Gutteridge *et al.*, 1979; Gero *et al.*, 1981, 1984).

Relatively pure mitochondrial fractions are, however, required for characterization purposes, and to this end we have employed the isolation procedure outlined in Figure 13.1. There are two aspects of this procedure which should be emphasized. In the first place we used homogenization to break the saponin-released parasites. The parasites were found to be surprisingly tough and, although fairly vigorous and prolonged homogenization was employed, only a proportion of

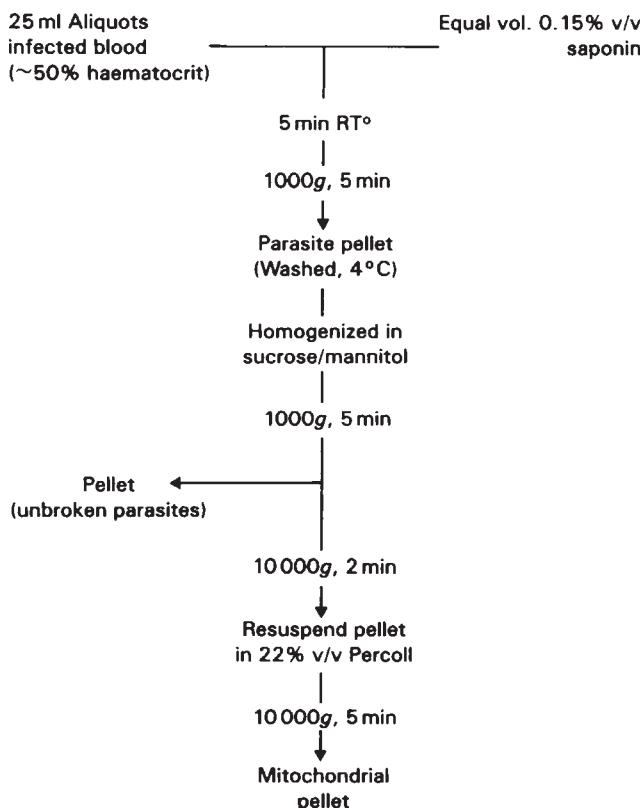


Figure 13.1. Scheme for isolation of *Plasmodium* mitochondria. Isolation medium contained 0.07 M sucrose, 0.21 M mannitol, 1 mM EGTA, 5 mM MgCl₂, 5 mM KH₂PO₄, and 4 mM, pH 7.4. R.T.[°], room temperature. Homogenization was in a pre-cooled tight-fitting Teflon pestle and glass homogenizer operated at 2000 rpm. Fifty complete strokes of the pestle were employed for disruption of the parasites.

the parasite starting material was broken. Examination of the lowspeed pellet showed many intact parasites and suggested that it was principally the trophozoite/schizont stages which are disrupted. Thus, this method of cell breakage can be stage-specific; modifications or other methods may need to be used if isolation of subcellular components from, for example, the ring stages are required. A major purification step in the scheme shown in Figure 13.1 is the centrifugation of the 10000g particulate fraction through 22 per cent Percoll. This step results in a pellet of highly purified mitochondria, essentially free of the parasites haemozoin pigment which is found towards the top of the Percoll. The amount of haemozoin present within the Percoll appears to reflect the severity of the cell breakage procedure, i.e. longer homogenization, sonication or freeze-thawing results in a more even distribution of haemozoin throughout the Percoll, including the mitochondrial pellet. It would appear that controlled breakage of the parasite by homogenization can liberate the haemozoin-containing vacuoles intact. Electron micrographs of the top fraction taken from the 22 per cent Percoll purification step (scheme of Figure 13.1) have shown the haemozoin pigment to be membrane bound (unpublished results of the author). Cathepsin L like proteinase activity was shown to be concentrated in this fraction with a specific activity of 6.7nmol min⁻¹/mg protein, using the substrate Z-Phe-Arg-AMC. This method of cell fractionation may, therefore, prove of interest to those investigators interested in, for example, lysosomal proteinases.

Using the scheme shown in Figure 13.1, the yield of isolated mitochondria is small, comprising about 4 per cent total homogenate protein. *P. yoelii* mitochondria are more convenient to work with since these preparations can be stored frozen with little subsequent loss of activity on thawing. *P. falciparum* mitochondria decrease appreciably in activity upon freeze-thawing. Judged by electron microscopy, the mitochondrial fraction from either parasite is highly purified (Fry and Beesley, 1991).

RESPIRATORY ACTIVITY OF MITOCHONDRIA

We have assessed the respiratory activity of mitochondrial preparations from *P. yoelii* and *P. falciparum* using a number of the more common mitochondrial substrates. The preferred assay is spectrophotometric, following the reduction of cytochrome *c*; this assay is convenient, rapid, reproducible and sparing on mitochondrial material. About 0.1 mg of mitochondrial protein per assay is desirable, but for substrates such as NADH or α -glycerophosphate, much less protein is required. Although polarographic assays can be employed, our earliest use of this method indicated problems in maintaining the linearity of oxygen uptake. A gradual decrease in activity, particularly with NADH as substrate, was observed; addition of superoxide dismutase and catalase to the assay were beneficial. Quite possibly there is a deleterious effect of high O₂ tension on these mitochondria, which might result in the production of oxygen radicals. This method also requires more mitochondrial material per assay and is inherently less sensitive than the spectrophotometric assay.

Table 13.1 Effect of various substrates on respiration of isolated *Plasmodium yoelii* and *P. falciparum* mitochondria. Reactions were followed by reduction of cytochrome *c* monitored at 550 nm in a 1 ml reaction volume containing 0.1 mg mitochondrial protein. Activities are expressed as the means of triplicate readings on each of a number of different mitochondrial preparations (figures in parentheses)±the standard deviation. N.D., not detectable, i.e. activity less or equal to that observed in the absence of added substrate. All activities are corrected for endogenous rate without added substrate.

Substrate [mM]	Specific activity (nmoles substrate oxidized/min/mg protein)	
	<i>P. yoelii</i>	<i>P. falciparum</i>
None	7 ± 3 (3)	5 ± 3 (4)
NADH [0.3]	470 ± 45 (3)	385 ± 37 (5)
α-Glycerophosphate [5]	230 ± 31 (3)	176 ± 24 (4)
Succinate [5]	150 ± 24 (3)	112 ± 13 (4)
Proline [1]	42 ± 8 (2)	* 31 ± 7 (4)
Dihydroorotate [0.5]	18 ± 3 (2)	* 11 ± 3 (3)
Glutamate [5]	N.D.	* 27 ± 12 (3)
α-Ketoglutarate [5]	N.D.	N.D.
Malate [5]	N.D.	N.D.
Pyruvate [5]	N.D.	N.D.
β-Hydroxybutyrate [5]	N.D.	N.D.
Fumarate [5]	N.D.	N.D.
Lactate [1]	N.D.	N.D.

*Rates with these substrates were linear for only the first minute of assay.

Table 13.1 shows the respiratory rates obtained with a number of substrates for *P. yoelii* and *P. falciparum* mitochondrial preparations. Three substrates, namely NADH, α-glycerophosphate and succinate, were readily utilized by these mitochondria. It is important to note that these results indicate the potential of such mitochondria to oxidize a particular substrate, and need not necessarily reflect the situation *in situ*. Specific activities given in Table 13.1 are about 15 per cent (succinate), 33 per cent (α-glycerophosphate) and 45 per cent (NADH) of the corresponding rates given by rat liver mitochondria. Lower respiratory rates were found for proline, dihydroorotate and glutamate (*P. falciparum* only); assay with these latter substrates was difficult, not just because of the lower activities, but also because the reduction of cytochrome *c* remained linear for less than the first minute of measurement (possibly due to product inhibition). It was notable that for both parasite preparations there was no detectable respiratory activity with most NAD⁺-linked substrates, such as malate or pyruvate, although glutamate was oxidized by *P. falciparum* mitochondria. Given that the mammalian malarial parasite apparently lacks a complete citric acid cycle (Sherman, 1979, 1983a), this finding is perhaps not altogether unexpected. Furthermore, although NADH is readily utilized by these mitochondria, its oxidation is apparently mediated by an electron transport pathway quite different from that which mediates the oxidation of succinate or α-glycerophosphate, and by a dehydrogenase insensitive to inhibitors of the normal mammalian NADH-dehydrogenase (see Table 13.2).

The testing of various standard mitochondrial inhibitors (Table 13.2) indicates that oxidation of α-glycerophosphate and succinate proceeds through a 'classical'

Table 13.2 Respiratory inhibitors of *Plasmodium yoelii* and *P. falciparum* mitochondria. Assays as in Table 13.1. Inhibitors were added 1 min following addition of substrate. Inhibitor concentrations in brackets are micromolar, and superoxide dismutase (SOD) and catalase (CAT) were added at 200 units each. HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; SHAM, salicylhydroxamic acid; TTFA, the enoyl trifluoroacetone; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

Respiratory substrate	Inhibitor	Specific activity (nmoles substrate oxidized/min/mg protein)	
		<i>P. yoelii</i>	<i>P. falciparum</i>
α -Glycerophosphate [5 mM]	None	230 ± 31 (3)	176 ± 24 (4)
	Antimycin A [2]	112 ± 11 (3)	92 ± 8 (4)
	Myxothiazol [30]	124 ± 13 (3)	94 ± 7 (3)
	UHDBT [4]	115 ± 12 (2)	89 ± 6 (3)
	HQNO [4]	128 ± 13 (2)	85 ± 6 (3)
	Funiculosin [3]	112 ± 14 (2)	92 ± 8 (2)
	SHAM [500]	102 ± 16 (2)	81 ± 10 (2)
	Rotenone [80]	165 ± 22 (2)	124 ± 18 (2)
Succinate [5 mM]	Agaric acid [20]	131 ± 18 (2)	98 ± 13 (2)
	Indomethacin [60]	109 ± 12 (2)	88 ± 9 (2)
	SOD + CAT	227 ± 27 (2)	169 ± 21 (2)
	None	150 ± 24 (3)	112 ± 13 (4)
	Antimycin A [2]	73 ± 9 (3)	66 ± 5 (4)
	TTFA [20]	69 ± 10 (2)	68 ± 9 (3)
NADH [0.3 mM]	Malonate [5 mM]	72 ± 8 (2)	77 ± 10 (2)
	Rotenone [80]	85 ± 11 (2)	78 ± 8 (2)
	SOD + CAT	146 ± 18 (2)	103 ± 9 (2)
	None	470 ± 45 (3)	385 ± 37 (5)
	Antimycin A [20]	476 ± 41 (3)	389 ± 35 (4)
	Rotenone [80]	459 ± 38 (2)	368 ± 27 (3)
	Piericidin [100]	463 ± 32 (2)	365 ± 24 (2)
	Dicumarol [50]	464 ± 38 (2)	361 ± 25 (2)
	Flavone [200]	268 ± 21 (2)	204 ± 12 (2)
	Mersalyl [10]	238 ± 15 (2)	188 ± 10 (2)
	SOD ± CAT	380 ± 26 (2)	302 ± 18 (2)

electron transport chain. Of particular interest are the cytochrome bc_1 (complex III) inhibitors: antimycin A, myxothiazol, UHDBT, HQNO and funiculosin. At higher concentrations any one of these inhibitors afforded 95–99 per cent inhibition in the cytochrome *c* reductase assay with α -glycerophosphate or succinate as substrate. Such complete inhibition would suggest a linear electron transport chain, and argue against a branch to an ‘alternative’ respiratory chain or ‘alternative oxidase’. However, in complete contrast, the oxidation of NADH was insensitive to most inhibitors; inhibitors of complex III were without effect, as were inhibitors of the mammalian mitochondrial complex I (rotenone and piericidin). Only the relatively non-specific inhibitors, flavone and mersalyl, were found to inhibit the NADH-cytochrome *c* reductase. Flavone has been reported to inhibit the rotenone-insensitive plant mitochondrial NADH-dehydrogenase (Cook and Cammack, 1984), and mersalyl to inhibit the mitochondrial outer membrane NADH-cytochrome *b*₅ reductase (Bernardi and Azzone, 1981). Since the inner mitochondrial membrane is

Table 13.3 NADH-reductase activity of *Plasmodium yoelii* and *P. falciparum* mitochondria. Assays as in Table 13.1. All additions were made prior to starting the reaction with NADH. Superoxide dismutase (SOD) and catalase (CAT) were added at 200 units of each. Activities were calculated from the initial rate of absorbance change over the first minute of assay.

Assay conditions	Specific activity (nmoles substrate oxidized/min/mg protein)	
	<i>P. yoelii</i>	<i>P. falciparum</i>
(A) NADH oxidation measured in the absence of cytochrome <i>c</i> by decrease in absorbance at 340 nm		
NADH [0.3 mM]	450 ± 37 (3)	371 ± 23 (4)
NADH (in N ₂ gassed buffer)	428 ± 29 (3)	342 ± 18 (3)
NADH + azide [1 mM]	443 ± 28 (3)	357 ± 21 (3)
NADH + SOD + CAT	468 ± 25 (3)	384 ± 15 (3)
NADH + fumarate [5 mM]	562 ± 35 (3)	457 ± 27 (3)
NADH + fumarate + malonate [5 mM]	461 ± 25 (3)	391 ± 18 (3)
NADH + fumarate + rotenone [80 µM]	532 ± 28 (2)	431 ± 21 (2)
(B) NADH oxidation measured in the presence of 100 µM cytochrome <i>c</i> and 1 mM azide by increase in absorbance at 550 nm		
NADH [0.3 mM]	470 ± 45 (3)	385 ± 37 (5)
NADH + fumarate [5 mM]	142 ± 13 (3)	112 ± 12 (3)
NADH + SOD + CAT	368 ± 12 (3)	312 ± 19 (3)
NADH + fumarate + SOD + CAT	106 ± 11 (3)	85 ± 9 (3)
NADH + fumarate + SOD + CAT + antimycin A [2 µM]	41 ± 8 (3)	37 ± 7 (3)
NADH + fumarate + SOD + CAT + rotenone [80 µM]	59 ± 6 (2)	48 ± 9 (2)

generally assumed to be impermeable to NADH, the ready utilization of NADH by the parasite mitochondrion suggests a number of possibilities. Either the mitochondria of such preparations are severely damaged, or the parasite inner mitochondrial membrane is freely permeable to NADH, or the oxidation of NADH is mediated by an NADH-dehydrogenase either on the outer mitochondrial membrane or on the outer aspect of the inner mitochondrial membrane. At present it is not possible to say which of these is the most likely.

Turrens (1989; and see Chapter 12) has proposed that an NADH-fumarate reductase activity in the mitochondrion of *Trypanosoma brucei* may be the main mechanism by which these organisms reoxidize mitochondrial NADH. That such a mechanism might be operative in the malarial parasite prompted us to investigate further the oxidation of NADH by *P. yoelii* and *P. falciparum* mitochondria. The effects of various agents on NADH oxidation are summarized in Table 13.3. In the absence of added cytochrome *c*, the oxidation of NADH is significantly enhanced by the addition of fumarate, and then inhibited by addition of malonate. In the presence of cytochrome *c*, the NADH-cytochrome *c* reductase activity is significantly depressed by the addition of fumarate, and then inhibited by antimycin

Table 13.4 Respiratory control in mitochondrial preparations from *Plasmodium yoelii* and *P. falciparum*. Assays were made on freshly isolated mitochondrial preparations using 0.5 mg protein in a total volume of 1 ml. Oxygen uptake values given are corrected for uptake observed in the absence of added substrate. CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

Assay	Oxygen uptake (nmoles O ₂ /min/mg protein)	
	<i>P. yoelii</i>	<i>P. falciparum</i>
No substrate	7 ± 3 (3)	5 ± 3 (4)
α-Glycerophosphate [5 mM] (α-GP)	58 ± 9 (2)	42 ± 4 (3)
α-GP + ADP [1 mM]	70 ± 13 (2)	45 ± 5 (3)
α-GP + ADP + oligomycin [1 μM]	59 ± 7 (2)	44 ± 3 (3)
α-GP + CCCP [0.5 μM]	78 ± 5 (2)	49 ± 6 (3)
Succinate [5 mM]	32 ± 6 (2)	27 ± 4 (3)
Succ. + ADP [1 mM]	42 ± 7 (2)	34 ± 6 (3)
Succ. + ADP + oligomycin [1 μM]	34 ± 5 (2)	31 ± 3 (3)
Succ. + CCCP [0.5 μM]	49 ± 5 (2)	38 ± 3 (3)

A. At least two interpretations of these results can be made. Firstly, that the *Plasmodium* mitochondrion contains an NADH-fumarate reductase pathway, generating succinate which is then reoxidized by the antimycin A sensitive classical respiratory chain. Secondly, that in such mitochondria the NADH-cytochrome *c* reductase activity is mediated by an NADH-cytochrome *b*₅ system, as has been described for mammalian mitochondria (Bernardi and Azzone, 1981). The topology of either or both systems with respect to NADH needs to be investigated. Mammalian cells may contain several different 'shuttle systems' for oxidizing cytosolic NADH and transfer of reducing equivalents to the mitochondrial electron transport chain (Dawson, 1979). The α-glycerophosphate shuttle is one example, and the ability of the *Plasmodium* mitochondrion to readily oxidize α-glycerophosphate suggests that the parasite has the potential to operate such a shuttle. The question of why this parasite, with its high level of lactate production, would need additional routes for oxidizing cytosolic NADH needs to be addressed.

MITOCHONDRIAL CYTOCHROMES

The isolation of purified and haemozoin-free mitochondria from *P. yoelii* and *P. falciparum* has for the first time allowed an unambiguous measurement of the cytochrome content of these organelles. The low-temperature spectra of *P. yoelii* and *P. falciparum* mitochondria shown in Figure 13.2 indicate the presence of cytochrome *aa*₃ (601 and 445 nm), *b* cytochromes (560, 562 and 565 nm), cytochrome *c* (547 nm) and cytochrome *c*₁ (554 nm). The presence of these cytochromes testify to the essentially 'classical' nature of the parasite mitochondrial respiratory chain. The cytochrome contents of *P. yoelii* mitochondria is higher than that of *P. falciparum*.

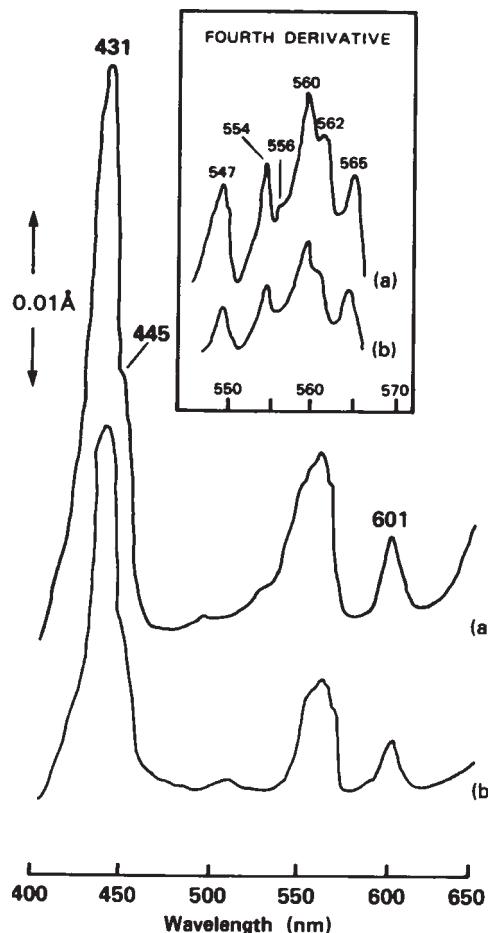


Figure 13.2. Dithionite-reduced minus oxidized low temperature difference spectra of *P. yoelii* (a) and *P. falciparum* (b) mitochondrial fractions. Both fractions contained 1.6 mg protein/ml, spectra being accumulated from 54 repeat scans (Shimadzu UV-3000) using a 2 nm bandwidth.

ENERGY GENERATION

The ability of the *Plasmodium* mitochondrion to generate and maintain a membrane potential is suggested through the use of the laser dye Rhodamine 123 (Divo *et al.*, 1985; Izumo *et al.*, 1988). We have attempted, by assay of oxygen uptake, to measure the respiratory control and oxidative phosphorylation potential of *P. yoelii* and *P. falciparum* isolated mitochondria (Table 13.4). The results are far from definitive and, although ADP can stimulate respiration with succinate or α -glycerophosphate in an oligomycin-sensitive manner, the changes are small, and the respiratory control ratios of such preparations are very low, typically about 1.3.

PERSPECTIVES

Figure 13.3 is an attempt to fit together what we have learnt regarding potential substrate utilization by the *Plasmodium* mitochondrion with the most likely, as well as the more speculative, metabolic interconnections with the cytosol.

Glycolytic conversion of phosphoenolpyruvate (PEP) to pyruvate and lactate probably represents the major carbohydrate catabolic route in mammalian

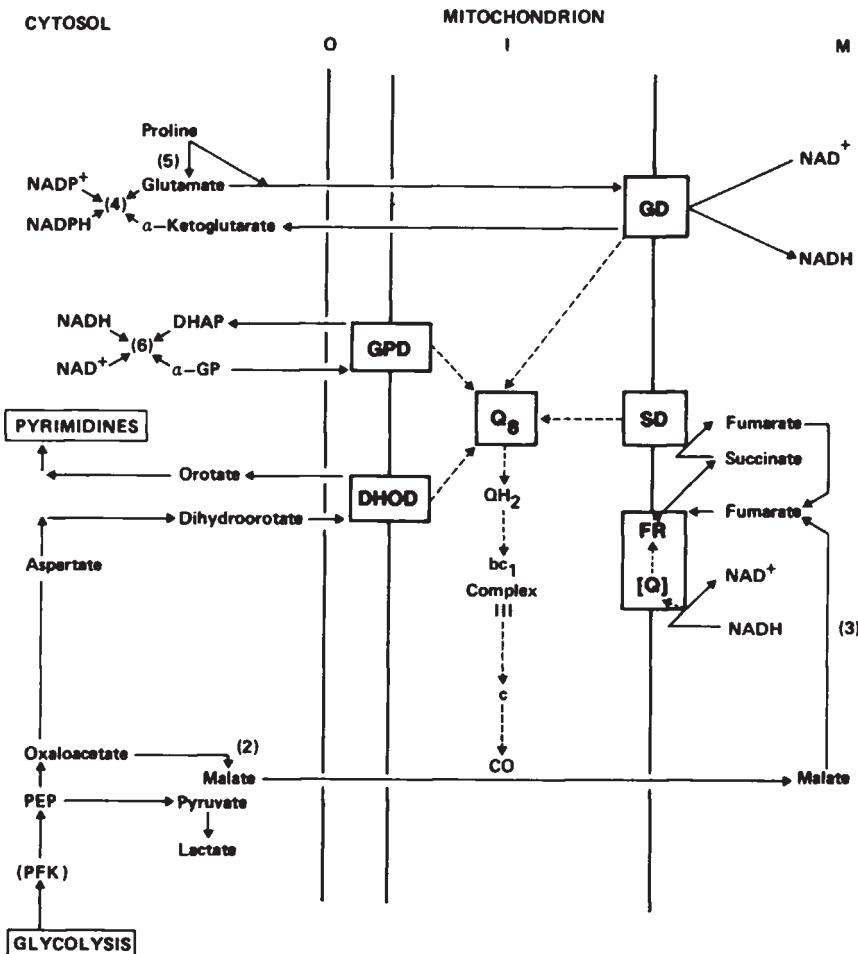


Figure 13.3. Proposed scheme for mitochondrial metabolism in mammalian plasmodia. Interconnections between cytosolic glycolysis, pyrimidine biosynthesis and NAD(P)H reactions with the mitochondrial respiratory chain are suggested. O, I, outer and inner mitochondrial membrane; M, mitochondrial matrix; GPD, α-glycerophosphate dehydrogenase; DHOD, dihydroorotate dehydrogenase; GD, glutamate dehydrogenase; SD, succinate dehydrogenase; FR, fumarate reductase; CO, cytochrome oxidase. 2, malate dehydrogenase; 3, fumarase; 4, cytosolic glutamate dehydrogenase; 5, proline oxidase; 6, cytosolic α-glycerophosphate dehydrogenase.

plasmodia. A key glycolytic enzyme, phosphofructokinase (PFK) has recently been partially purified from *P. berghei* and shown to exhibit appropriate allosteric behaviour with regard to fructose 6-phosphate and ATP and with a shift of the pH dependence to the acidic range, such that the parasite can maintain a high glycolytic flux under conditions in which erythrocyte glycolysis is strongly inhibited (Buckwitz *et al.*, 1990 and references therein). Nevertheless, identification of a PEP carboxylase in *P. berghei* (Siu, 1967) suggests glycolysis may branch at the level of PEP to give rise to oxaloacetate. The only citric acid cycle enzyme identified with any certainty in mammalian plasmodia is malate dehydrogenase (Sherman, 1966; Tsukamoto, 1974; Momen, 1979), and thus oxaloacetate may be converted to malate (2). Indications are that this enzyme may be extramitochondrial and it has therefore been placed outside of the mitochondrion in Figure 13.3. Certainly our own studies on isolated mitochondria have failed to detect respiratory activity with malate. Oxaloacetate may also give rise to aspartate, a precursor of pyrimidine biosynthesis. Dihydroorotate-cytochrome *c* reductase activity is measurable in the isolated plasmodium mitochondrion, and thus we assume the mitochondrial localization of a key pyrimidine biosynthetic enzyme, dihydroorotate dehydrogenase (DHOD). Malate produced in the cytosol, would readily enter the mitochondrion where conversion to fumarate could occur (although there is a lack of evidence regarding the presence of fumarase (3)). However, interconversion of fumarate and succinate is shown to occur in a cyclical manner through the activities of succinate dehydrogenase (SD) and an NADH-fumarate reductase (FR). The role of fumarate as a terminal acceptor of phosphorylative electron transport is usually discussed in the context of an anaerobic energy-generating pathway, although for the malaria parasite this would seem unlikely. Rather, the rationale behind the *Plasmodium* fumarate reductase would be to provide a means for the reoxidation of mitochondrial NADH, particularly given that a site 1 of the respiratory chain appears to be missing from such mitochondria. In some bacteria the fumarate reductase is mediated by menaquinone or desmethylmenaquinone, and in certain eukaryotes by rhodoquinone (Kröger, 1978). Although the major ubiquinone species of *Plasmodium* appears to be Q₈ (Porter and Folkers, 1974), unlike the Q₁₀ that is typical of mammalian mitochondria, no search has yet been made for a quinone species that would satisfy the redox requirements of the NADH-fumarate reductase pathway. It is perhaps also worth mentioning that in some bacteria the fumarate reductase pathway is capable of oxidizing dihydroorotate, thus the possibility that this pathway may play a role in pyrimidine biosynthesis in the malaria parasite cannot be discounted.

A major source of intramitochondrial NADH may be the mitochondrial glutamate dehydrogenase (GD), although this activity was not measurable in *P. yoelii* mitochondria, possibly due to problems of substrate accessibility or latency. The mitochondrial glutamate dehydrogenase is assumed to be distinct from its cytosolic counterpart. Glutamate dehydrogenase from a number of *Plasmodium* species has been shown to be a cytosolic protein, specific for NADP⁺, with the predominant reaction catalysed being the oxidation of glutamate to α -ketoglutarate (4) (Ling *et al.*, 1986). This reaction pathway could be a major provider of cytosolic

NADPH, especially with glutamate forming a major contribution to the free amino acid pool of the infected erythrocyte and parasite (Sherman and Mudd, 1966). Both *P. yoelii* and *P. falciparum* mitochondria appear to be capable of oxidizing proline. The conversion of proline to glutamate (5) would require the enzyme proline oxidase; this enzyme has not been described in *Plasmodium* but is apparently present in the culture promastigotes of *Leishmania donovani* (Krassner and Flory, 1972; and see Chapter 10). It is suggested that in the insect stage of the parasite life cycle, oxidation of proline, which is present in large quantities in the insects haemolymph (Hoyt and Haden, 1971), could provide a major utilizable energy source (proline is a major substrate in insect flight metabolism (Raghupathi and Campbell, 1969)). Respiration of proline by mitochondria from the intraerythrocytic trophozoites of both *P. yoelii* and *P. falciparum* may, therefore, represent an epigenetic adaptation for the insect phase of the life cycle.

α -Glycerophosphate appears to be a major utilizable substrate by mitochondria of both *P. yoelii* and *P. falciparum*. In soluble fractions of *P. yoelii* and *P. falciparum* trophozoites, the dihydroxyacetone phosphate (DHAP) dependent oxidation of NADH (6) can be measured (unpublished results of the author). It is therefore suggested that a major role of such activity would be as a redox shuttle to control cytoplasmic NADH levels and deliver reducing equivalents to the mitochondrion (Dawson, 1979).

In summary then, oxidation of α -glycerophosphate, succinate, dihydroorotate and glutamate (proline) is apparently mediated by an essentially classical electron transport pathway, although deviation from the mammalian picture is expected regarding the ubiquinone component (Porter and Folkers, 1974) and is apparent in the inhibitor sensitivity of the complex III region (Fry and Beesley, 1991). However, the *Plasmodium* respiratory chain is atypical in apparently lacking a site 1, rotenone-sensitive NADH-ubiquinone reductase, but does seem to possess an NADH-fumarate reductase activity mediated by an as yet unidentified quinone component. The evidence available to date does not suggest a branched respiratory chain or alternative oxidase in such mitochondria.

Whether the mitochondrial electron transport chain we have described is an efficient generator of ATP is still uncertain. Such mitochondrial preparations exhibit only low respiratory control. The studies of Kanaani and Ginsburg (1989) can be interpreted to suggest a significant effect of mitochondrial inhibitors on total parasite ATP. Conversely, our own studies have failed to show such effects (Fry *et al.*, 1990). However, this same study did indicate mitochondrial inhibitors, particularly oligomycin, could elevate parasite ADP levels. One interpretation of this would be that, although the parasite might be able to maintain ATP levels (by import from the erythrocyte?), the rise in ADP levels seen in the presence of mitochondrial inhibitors is indicative of the loss of oxidative phosphorylation capability.

Information on the isolated mitochondrion, together with a growing body of more indirect data, emphasizes the active participation of this organelle in the intraerythrocytic life cycle of *Plasmodium*. Indeed, the importance of this organelle to the malaria parasite may prove to be central to the successful chemotherapy of malaria.

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14. Proteinases of mammals: an overview

R.W.Mason

A review on mammalian proteinases may seem out of place in a book on biochemical protozoology, but, if drugs targetted to parasitic proteinases are to be considered useful in the control of parasitic protozoa in man, then it is essential that the host proteinases are not also inactivated by such drugs. In addition, the known similarities between mammalian and parasitic proteinases suggest that it is likely that techniques used to control the activity of mammalian proteinases will also be applicable to parasitic proteinases. This review therefore concentrates on the control of specific proteolytic activity and attempts to direct the reader to consider methods for controlling the activity of individual proteinases, rather than generally inhibiting proteolysis.

There are four major classes of proteinases for which the catalytic mechanism has been defined, and these have been termed aspartic, cysteine, metallo and serine proteinases (Barrett and McDonald, 1980). Other proteinases have been discovered for which the catalytic mechanism is unclear, and these may represent another type of enzyme. In this review I intend to concentrate on the cysteine proteinases but will first briefly discuss the other classes of enzymes.

SERINE PROTEINASES

As their name suggests, the serine proteinases contain an essential serine at the catalytic site. It is the hydroxyl group of this serine that initially attacks the carbonyl group of a peptide bond to form a tetrahedral intermediate prior to hydrolysis. This intermediate is stabilized by hydrogen bonding of the oxygen anion. Deacylation of the intermediate is catalysed by the addition of a proton from the imidazole group of a histidine. These amino acids appear to be essential for hydrolysis and are conserved in all serine proteinases. Many serine proteinases are found in the circulation, and are the class of enzyme involved in the blood coagulation cascade. The specificity of these enzymes is primarily determined by the binding site for the amino acid towards the amino terminus of the substrate protein adjacent to the peptide bond cleaved (termed P_1). Orientation and accessibility of the cleavage site of the substrate peptide bond are important in determining the enhanced specificity

of such proteinases. *In vivo*, uncontrolled proteolysis is prevented by the presence of protein inhibitors (Travis and Salvesen, 1983; see also Barren and Salvesen (1986) for an excellent review on most of the known inhibitors (except for α_2 -macroglobulin) of all classes of proteinases). These are pseudo-substrates which are usually tight-binding reversible inhibitors, although true reversibility has not been demonstrated for all of the inhibitors. The concentration of these inhibitors in biological fluids is usually far greater than the K_i for inhibition of the target enzymes, thus in free solution most of the proteolytic activity of serine proteinases will always be controlled (Travis and Salvesen, 1983).

ASPARTIC PROTEINASES

The major intracellular mammalian aspartic proteinases are cathepsin D, which is a lysosomal enzyme, and renin, which is found in discrete granules in the kidney. Other mammalian aspartic proteinases are found in the intestinal tract (e.g. pepsin and chymosin). There are no known protein inhibitors of the aspartic proteinases, but all are inhibited by pepstatin, a pentapeptide-like inhibitor secreted by *Streptomyces* (Aoyagi *et al.*, 1971; Rich, 1986). The precise mechanism of action of aspartic proteinases is not clear, but may be by a simple acid-base type of mechanism at acidic pH, involving the carboxyl side-chains of the two essential aspartic acids (see Dunn (1989) in a useful practical guide to the study of proteinases edited by Beynon and Bond (1989)). These enzymes do not exhibit any activity above pH 6.0, and hence uncontrolled proteolysis will not occur in extracellular body fluids.

METALLOPROTEINASES

The metalloproteinases are a diverse group of enzymes that coordinate a zinc ion with the aid of glutamic acid and histidine residues. It has been proposed that the zinc ion acts as an electrophile to enhance the reactivity of the carbonyl group of the peptide bond to be hydrolysed to facilitate nucleophilic attack by a water molecule (Powers and Harper, 1986a). This class of enzymes includes the matrix metalloproteinases that are inhibited by a protein called TIMP (tissue inhibitor of metalloproteinases (Cawston *et al.*, 1981)) and plasma membrane-bound metalloproteinases such as endopeptidase 24.11 (enkephalinase) and meprin, for which no natural protein inhibitors are known (Matsas *et al.*, 1984; Butler and Bond, 1988). The activity of the membrane-bound enzymes may be controlled by their restricted location and in the case of enkephalinase, by its restricted specificity. Membrane-bound proteinases are particularly attractive for models for degradation for extracellular proteins in processes such as tumour invasion because they do not suffer from the problem of diffusion away from the site of action, dilution or inhibition/inactivation. Metalloproteinases are the best characterized membranebound proteinases.

UNCLASSIFIED PROTEINASES

Perhaps the major proteinase for which a catalytic mechanism is unknown is mammalian signal peptidase (Evans *et al.*, 1986). This enzyme is usually purified in the presence of a cocktail of the major proteinase inhibitors and is, therefore, clearly not a standard class of proteinase. The mechanism of action of a mitochondrial matrix processing proteinase is also unknown, as this appears to require Mn²⁺ and free thiols, but it is not clear whether these are involved in the catalytic mechanism of this proteinase (Schneider *et al.*, 1990). Furthermore, the sequence of the protein is not homologous to any of the known classes of proteinases. The classification of a major cytoplasmic proteinase, multicatalytic proteinase, is similarly unclear, and has been reported as being a cysteine proteinase but may be an atypical serine proteinase based on inhibition by dichloroisocoumarin (Orlowski and Michaud, 1989; Mason, 1990). The precise mechanism of this proteinase awaits clearer characterization of the amino acids involved in catalysis.

CYSTEINE PROTEINASES

The major mammalian cysteine proteinases are the cytoplasmic calpains and the lysosomal cysteine proteinases, cathepsins B, H and L (Barrett *et al.*, 1988). The calpains are calcium dependent proteinases that consist of two chains of M_r 80000 and 30000. One portion of the heavy chain of this molecule carries a sequence that shows a small degree of identity with papain, but the degree of identity is insufficient to determine whether or not they are evolutionarily related (Ohno *et al.*, 1984).

All of the cysteine proteinases have an essential cysteine, histidine and asparagine. The mechanism of action is similar to that of serine proteinases except that the sulphonium ion of the cysteine provides the nucleophilic attack on the carbonyl group in the peptide bond. Apart from their cellular location, a feature that distinguishes the calpains from cathepsins L and B is their lack of inhibition by Z-Phe-Ala-CHN₂ (Z-N-benzyloxycarbonyl; CHN₂, diazomethane) and Z-Phe-Phe-CHN₂, although they are inhibited by Z-Leu-Leu-Tyr-CHN₂, an inhibitor related to the known substrate specificity of calpain (Crawford *et al.*, 1988). In common with the lysosomal cysteine proteinases, the calpains are also inhibited by the class-specific inhibitor, E-64, and the more general inhibitor, leupeptin. My particular emphasis has been on the lysosomal cysteine proteinases, cathepsins B, H and L.

Cysteine proteinase inhibitors

The major natural inhibitors of mammalian cysteine proteinases are α_2 -macroglobulin, the cystatins and the kininogens. α_2 -Macroglobulin is found in serum and inhibits proteinases by a trap mechanism that is dependent upon proteolytic cleavage, but does not distinguish between mechanistic classes of proteinases. The cystatins are low M_r proteins that are found either in the cytoplasm of cells or are secreted into extracellular body fluids (Barrett, 1987). They are

tightbinding reversible inhibitors that are generally better inhibitors of cathepsin L than cathepsin B, and do not inhibit calpain. The kininogens, as well as being precursors of bradykinin contain three copies of cystatin-like sequences, two of which are inhibitory for cysteine proteinases. One unique feature of the kininogens is that they also inhibit calpain.

Characterization of mammalian lysosomal cysteine proteinases

Cathepsins B, H and L have all been purified from human tissues and found to have quite distinct catalytic properties (Schwartz and Barrett, 1980; Mason *et al.*, 1985; Rich *et al.*, 1986). Thus cathepsin B is both an endopeptidase and a peptidyldipeptidase, cathepsin H is both an endopeptidase and an aminopeptidase whilst cathepsin L is only an endopeptidase. The bimodal activities of cathepsins B and H have been questioned, and it was proposed that they are in fact solely exopeptidases (Takahashi *et al.*, 1986, 1988). This was proved to be erroneous by the demonstration that both enzymes cleaved and were bound by α_2 -macroglobulin, a process that only occurs when internal peptide bonds of this proteinase inhibitor are cleaved (Mason, 1989). However, the relative contribution of the endopeptidase and exopeptidase activities of cathepsins B and H *in vivo* are still not known. Recently, another powerful cysteine proteinase has been discovered in bovine spleen, which has been termed cathepsin S (Kirschke *et al.*, 1989). This enzyme has also been found in human spleen (Mason *et al.*, 1989a) and appears to be the first cysteine proteinase to show any preferred tissue location. Cathepsin S, like cathepsin L, is a powerful proteinase, but has not yet been characterized as extensively as the other mammalian cysteine proteinases.

Proteolytic activities of lysosomal cysteine proteinases

Cathepsin L is generally a much more powerful proteinase than either cathepsin B or H. It can hydrolyse the extracellular matrix proteins, collagen and elastin, even more efficiently than the enzymes better known for this activity, collagenase and neutrophil elastase (Kirschke *et al.*, 1982; Mason *et al.*, 1986a). Cathepsin L can also degrade type IV collagen, a major structural component of basement membranes (Baricos *et al.*, 1988). Thus cathepsin L, and to a lesser extent cathepsin B, have the proteolytic ability to degrade extracellular matrix and, if secreted by tumour cells, could even assist cells to break through basement membranes. Cathepsin L has an additional mechanism by which it could contribute to extracellular matrix degradation in that it inactivates the physiological inhibitor of neutrophil elastase, α_1 -proteinase inhibitor (Johnson *et al.*, 1986). The fragments of α_1 -proteinase produced by such a cleavage are chemotactic for neutrophils (Banda *et al.*, 1986), which would in turn increase the proteolytic load in diseases such as pulmonary emphysema. Furthermore, elastase can inactivate cystatin C and this can result in a proteolytic cascade (Abrahamson *et al.*, 1991). Thus studies to date have shown that the lysosomal cysteine proteinases have the potential to cause extracellular tissue degradation, but this is yet to be proven. Experimental tools are now being developed to approach this problem.

Biosynthesis and location of lysosomal cysteine proteinases

In order to investigate the biosynthesis and location of the lysosomal cysteine proteinases we and others have raised antibodies to the individual enzymes. The first evidence that cathepsin L is synthesized as a precursor came when a cDNA from a mouse macrophage cDNA library had been sequenced and identified from the deduced amino acid sequence as the mouse equivalent of cathepsin L (Portnoy *et al.*, 1986; Mason *et al.*, 1986b). Alignment of the two sequences showed that the protein coded by the mouse cDNA had an additional 113 amino acids at its N-terminus, presumably encoding the pre-pro region of the enzyme (Figure 14.1). Subsequent sequence data showed that this cDNA provides the coding sequence for a protein that had been isolated some 8 years earlier by Gottesman (1978) as the major secreted protein from transformed 3T3 cells, identifying it as procathepsin L. The biosynthesis and processing of this protein had also been shown by pulse-chase experiments (Gal *et al.*, 1985). Thus it became clear that cathepsin L could be secreted by transformed cells, albeit as a pro-enzyme. These cells also synthesize cathepsin B but, interestingly, do not secrete the precursor of this enzyme (Mason *et al.*, 1989b). It has recently been proposed that this secretion of cathepsin L may be due to its having a low affinity for the mannose 6-phosphate receptor that normally targets the enzymes to mammalian lysosomes (Dong and Sahagian, 1990). This has provided the strongest evidence to date that individual lysosomal enzymes might be packaged differently from one another.

The cathepsin B in these transformed cells is synthesized as a precursor of M_r 39000 and then processed to a stable form of M_r 35000 (Mason *et al.*, 1989b). This is unusual; cathepsin B is normally isolated from tissues as a two-chain enzyme of M_r 25000+5000, although it does occasionally appear in some species as a single-chain enzyme of M_r 30000 (Mason, 1986). We have shown that cathepsin B from normal mouse liver is predominantly the two-chain enzyme with some single-chain enzyme of M_r 33000 (Wilcox and Mason, unpublished results). We do not know the reason for the difference in M_r for the single-chain enzyme, but it could be due to a different cleavage of the pro-enzyme, or to different glycosylation. The lack of a two-chain form of the enzyme in the transformed cells suggests that the second

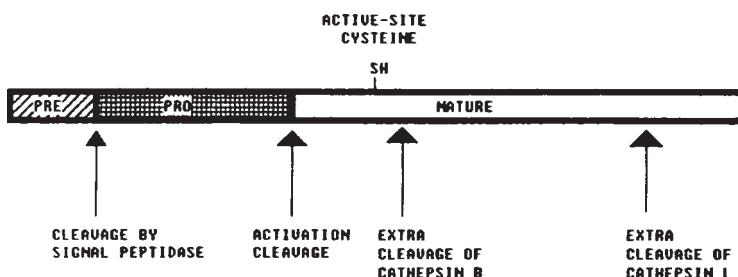


Figure 14.1. Schematic representation of the gene product of a lysosomal cysteine proteinase. Sites of cleavage to give the pro-enzymes (signal peptidase), the single chain mature enzymes (activation) and sites of further cleavage to give the two chain forms of the active enzyme are indicated.

stage in the processing of cathepsin B does not occur. Possible causes of this include: the processing enzyme is not expressed in these cells; the single-chain form of cathepsin B in these cells is resistant to the processing enzyme; or the processing enzyme is not in the same compartment as cathepsin B. We have found a similar processing defect in human macrophages and monocytic cell lines (Reilly *et al.*, 1989; Wilcox and Mason, unpublished results). These results all suggest that the distribution of different lysosomal cysteine proteinases is not the same in all cell types and may not be the same in lysosomes of a given cell.

Development of specific probes for cysteine proteinases

Peptidyl inhibitors have been used extensively to determine the peptide bond specificity of proteinases (Powers and Harper, 1986b; Rich, 1986). The specificity of the inhibitor is determined by the amino acids that bind in the specificity pockets of the active sites of the enzyme(s). In an extensive study with purified mammalian cysteine proteinases, a number of diazomethane inhibitors were identified that can distinguish between the lysosomal proteinases, cathepsins B and L, and the cytoplasmic enzyme, calpain II (Crawford *et al.*, 1988). The information gained by this study was utilized to synthesize a radiolabelled inhibitor, Z-[¹²⁵I] Tyr-Ala-CHN₂. We have used this radiolabelled compound to demonstrate that these inhibitors are truly specific for cysteine proteinases by showing that it only reacts with active forms of cathepsins B and L in Kirsten virus-transformed cells (Mason *et al.*, 1989b). This study also established this type of inhibitor as a useful probe for active cysteine proteinases in living systems. Thus it is possible to study its interaction with enzymes without the need to purify them first. This is a major advantage because when lysosomal proteinases are isolated by conventional techniques, they are often unstable and obtained in poor yields. Furthermore, homogenization results in the destruction of membrane barriers that keep the lysosomal proteinases separated from the endogenous inhibitors, the cystatins; thus masking the activity of many of these enzymes.

I have now established that the radiolabelled inhibitor enters cells by diffusion across lipid bilayers and not by fluid phase pinocytosis as had previously been suggested (Shaw and Dean, 1980). This was done by observing the ability of the inhibitor to react with the proteinases in intact lysosomes. The enzymes in purified lysosomes reacted with the inhibitor, even though the lysosomes were aged for several hours. By contrast, when the lysosomes were lysed, the enzymes would only react with the inhibitor if a reducing agent was added. Using lysed lysosomes, the labelling of the enzymes with Z-[¹²⁵I]Tyr-Ala-CHN₂ could be blocked by pretreating the extracts with other inhibitors of cysteine proteinases, including E-64, leupeptin and Z-Phe-Phe-CHN₂. However, in similar experiments with intact lysosomes, only Z-Phe-Phe-CHN₂, blocked labelling, but not the other two. These results clearly demonstrate that the lysosomal membrane is not a barrier to the diazomethanes used in this study, but is a barrier to E-64 and leupeptin. This was an unexpected discovery, because E-64 and leupeptin have been used in numerous laboratories to demonstrate the role of lysosomal cysteine proteinases in living cells (e.g. Seglen,

1983). We have also demonstrated that pinocytosis is not a significant route for the entry of E-64 or leupeptin into lysosomes of fibroblasts. Given that the concentration of enzymes in lysosomes could be as high as 1 mM (Dean and Barrett, 1976), insufficient inhibitor would be delivered by pinocytotic fusions to inactivate all the proteinases in the cell.

Another important finding of our studies was that the enzymes did not have to be activated in order to bind to the inhibitor in live cells, indicating that the enzymes were already in an active form in the cell. These findings demonstrate that oxidation/reduction of the active site of the proteinases is not a major mechanism for regulation of these enzymes *in vivo*. In contrast, activation with thiols is essential to measure the catalytic activity of the mammalian cysteine proteinases *in vitro* (Barrett and Kirschke, 1981). In fact cathepsins B and L are less efficiently labelled *in vitro* than *in vivo*, probably due to the instability of the enzymes in crude homogenates and their inhibition by exposure to endogenous proteinase inhibitors (Mason *et al.*, 1989a; Wilcox and Mason, unpublished results). This probe has also enabled me to show which of the different molecular forms of lysosomal cysteine proteinases are active *in vivo* (i.e. both of the mature forms (single-and two-chain enzymes), but not the pro forms; Figure 14.1).

Functions of lysosomal enzymes

Lysosomal proteinases are involved in the degradation of proteins taken up by cells by phagocytosis, pinocytosis or receptor-mediated endocytosis. They are also found in extracellular body fluids, which suggests that they may also have a role outside cells. The role of lysosomal proteinases in the degradation of intracellular and extracellular proteins has largely been determined by the use of inhibitors that either block the acidification of lysosomes or block the activity of the proteinases directly (Seglen, 1983). Because the lysosomal proteinases are the most potent of the cellular proteinases, it is likely that the interpretation given to these studies will be correct; however, the specificity of action of the individual inhibitors may not be as clear as suggested in the original publications. We know that the cysteine proteinases cathepsins B and L are in fact active at neutral pH (Mort *et al.*, 1984; Mason *et al.*, 1985) and that cathepsin B is more stable at neutral pH than originally thought. Furthermore, cathepsin S from bovine spleen is completely stable at pH 7.5, even in the presence of thiol activator (Kirschke *et al.*, 1989; Mason, unpublished results). Thus, although abolition of the low pH within the lysosome could have an inhibitory effect on the total proteolytic capacity of lysosomes in cells, it is by no means certain that lysosomotropic agents will be 100 per cent effective. Perturbation of the pH in cells will also affect the delivery of lysosomal enzymes to lysosomes by influencing the binding of these enzymes to the mannose 6-phosphate receptor. Binding of enzymes to this receptor apparently depends upon relatively subtle changes in pH (Tong *et al.*, 1988; Tong and Kornfeld, 1989), so it is also uncertain how effective the lysosomotropic agents will be in blocking delivery of newly synthesized proteins by this mechanism. We have now found that some of the inhibitors of cysteine proteinases used in earlier studies to inhibit cathepsins B

and L do not enter cells freely, and hence it is unclear that their inhibitory effect on cellular proteolysis was actually due to inhibition of the proteinases. Overall, the proteinase inhibition studies that have already been performed indicate that proteinases do play a role in cellular protein turnover, but are insufficiently subtle to provide information on which particular enzymes are involved in degradation of intracellular or extracellular proteins or indeed which of the different processed forms of the enzymes are primarily involved in degradation. It has been proposed that cathepsin B may be involved in the degradation of phagocytosed proteins and that cathepsin L may be involved in the degradation of cellular proteins based on the apparent cellular distribution of the two enzymes in rat liver (Li *et al.*, 1985). Thus higher levels of cathepsin B were found in phagocytic cells than in non-phagocytic cells—the latter cells containing more cathepsin L. Unfortunately this does not agree with experiments with other species that have found a large concentration of cathepsin L in mouse macrophages (Portnoy *et al.*, 1986) and human macrophages (Reilly *et al.*, 1989). Whether or not any particular lysosomal proteinase has a more important role in the degradation of different populations of proteins still remains to be established.

Secreted forms of cysteine proteinases

Extracellular forms of cysteine proteinases have been known for some time, particularly for cathepsin B (Sloane *et al.*, 1982; Baici and Knopfel, 1986; Buttle *et al.*, 1988; Chang *et al.*, 1989). The source of these proteinases and their physiological or pathological action is, however, still obscure. Cathepsin B is poorly inhibited by one of the major circulating cysteine proteinases inhibitors, kininogen (Salvesen *et al.*, 1986) and is therefore the easiest enzyme to measure in body fluids. In addition, because the synthetic substrates used for cathepsin B provide a very sensitive method for assaying the enzyme, fractions need to be diluted in order to measure activity (Barrett and Kirschke, 1981). Large dilutions of fractions also has the effect of diluting the weak reversible inhibitors, causing the release of enzyme from enzyme-inhibitor complexes. One feature of partially purified extracellular forms of cathepsin B is an apparent stability at neutral pH. However, at least one report has suggested that the enzyme becomes more unstable during purification (Buttle *et al.*, 1988). This may mean that the enzyme is stabilized by association with one or more protein in the body fluids, acting as a sort of molecular chaperone. At present the nature of this increased stability is unclear. A second feature of extracellular forms of lysosomal cysteine proteinases is that they are often thought to be of a higher molecular weight than their lysosomal counterparts. The nature of this increased size is unknown, but it seems to be partly due to differences in glycosylation (Buttle *et al.*, 1988).

Inactive precursor forms of the enzymes secreted by cells have been characterized more extensively. We now know that mouse macrophages and fibroblasts, human macrophages and rat hepatocytes secrete the precursor form of cathepsin L (Portnoy *et al.*, 1986; Ishidoh *et al.*, 1987; Mason *et al.*, 1989b; Reilly *et al.*, 1989). Human macrophages also secrete cystatin C, the most potent inhibitor of

cathepsin L (Chapman *et al.*, 1990), and hence it is not clear how cathepsin L activity can be expressed outside these cells. We originally found that the purified precursor of mouse cathepsin L was active against peptide and protein substrates (Mason *et al.*, 1987), but lack of reaction of the unpurified enzyme with an active-site-directed inhibitor has recently led us to the conclusion that the precursor is in fact inactive and that the activity of the purified protein was probably due to a conformational change brought about by the purification process (Mason *et al.*, 1989b). Treatment of the pro-enzyme at pH 3.0 also seemed to result in the exposure of the active site, allowing it to react with an inhibitor and causing autolysis. Thus we have demonstrated two non-physiological mechanisms for activation of the proenzyme, but as yet have no indication as to any potential physiological activation process.

CONCLUSIONS

We can conclude that expression and secretion of lysosomal proteinases is not sufficient to prove that they are necessarily involved in extracellular protein degradation. The enzymes must be active, and this can be demonstrated by the use of radiolabelled peptidyldiazomethane inhibitors. We have demonstrated that such compounds are indeed specific for cysteine proteinases in living systems and that, given a suitable amino acid composition, they can penetrate cell membranes. The challenge now is to design inhibitors that are specific for individual proteinases that may be useful for the therapeutic control of proteinases. Obviously inhibitors designed to control the activity of proteinases from parasitic organisms should be designed such that they do not affect the activity of host proteinases. Specificity of inhibitors for parasite enzymes could theoretically arise out of differences in substrate preference between the parasite and host enzymes. Alternatively, the different amounts of parasite enzyme may mean that the amount of inhibitor that is sufficient to inhibit all of the parasite enzyme will have no significant effect on host proteolysis.

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15. Proteinases of parasitic protozoa: an overview

M.J.North

THE BACKGROUND

The number of chapters on proteinases included in this book reflects the recent upsurge of interest in the proteolytic enzymes of parasitic protozoa. From a mere trickle 10 years ago the flow of publications has now swollen such that over 100 papers on this topic have appeared in the literature in the last 2 years. Much of the earlier work on protozoan proteinases concerned free-living non-pathogenic species such as *Tetrahymena* and *Euglena* (see North, 1982). Indeed, until the late 1970s, the interest in parasite enzymes was largely confined to *Entamoeba* and *Plasmodium*, in which roles for proteinases in host tissue invasion and haemoglobin digestion, respectively, had long been anticipated. There are three main reasons for the current level of interest. First is the realization that proteolytic enzymes can have a wide variety of roles in living cells and that interference with proteolysis could affect parasite functions concerned with the host-parasite relationship and pathogenesis. Second is the development of methods, mostly adapted from procedures used to analyse mammalian proteinases, that have allowed the detection, separation and characterization of the multiple forms of proteinase that occur in parasitic protozoa. Earlier papers often referred to parasite proteinase activity in terms of a single enzyme. It is now apparent that multiple proteinases are the rule, and one of the challenges of proteinase research is to unravel the complexity by identifying individual enzymes and establishing their respective properties and function. Third, and of direct relevance to the development of drugs, is the availability of a range of peptide derivatives which have been designed to inhibit specific proteinases. These now offer the parasitologist the opportunity to use reagents which may selectively inhibit the protozoan proteinases. Much of the recent work has been stimulated by the idea that the proteinases will prove to be appropriate targets for antiparasite drugs.

The discovery and identification of most protozoan proteinases has resulted from studies which set out directly to analyse the enzymes. There have, however, been some surprises which have further stimulated interest. An example is the discovery that the major surface antigen of *Leishmania* species is a proteinase (see Chapter

20). More recently, the sequence of a major antigen of *Plasmodium falciparum*, SERP or SERA, has revealed that this protein contains a region with considerable homology with cysteine proteinases. Although SERP lacks the active-site cysteine, the sequence of a related protein, SERP H, has now been shown to include this residue (Knapp *et al.*, 1991) and the protein would appear to be an unusual and unexpected cysteine proteinase.

The chapters in this book cover all the major aspects of current research on the proteinases of parasitic protozoa. In addition, there has been a small amount of work carried out on *Eimeria* (for recent papers see: Adams and Bushell, 1988; Fuller and McDougald, 1990). Interest in *Theileria* proteinases should now be stimulated by the cloning of a cysteine proteinase gene from *Theileria parva* (Nene *et al.*, 1990). There have been a few reports on the proteinases of *Naegleria* and *Acanthamoeba*, but little characterization of the enzymes has been undertaken. There are some parasitic protozoa for which the proteinases are yet to be described. For example it has not been possible to detect proteinase activity in *Cryptosporidium* or *Toxoplasma* using standard methods (North *et al.*, 1990a), although it seems unlikely that they are totally devoid of proteinase. Nothing has yet been reported on *Pneumocystis* proteinases.

THE ENZYMES

There are two major types of proteolytic enzyme (peptidase), endopeptidases and exopeptidases (Barrett and McDonald, 1986). Historically the term 'proteinase' has been used for proteolytic enzymes which show specificity towards proteins, but Barrett and McDonald (1986) suggested that it ought only apply to endopeptidases and not be used for exopeptidases that degrade proteins. The term 'protease' has often been used interchangeably with 'proteinase', but Barrett and McDonald (1986) suggested it should be used as an alternative to 'peptidase', i.e. for any proteolytic enzyme. The majority of proteolytic enzymes discussed in this book are known to hydrolyse proteins, although their mode of action is not always known, and for simplicity we have standardized by using only the term proteinase. It must be remembered, however, that some of the enzymes may not fall within the stricter definition of the term if they are found to have only exopeptidase activity.

Four major groups of proteinase have been defined: aspartic, cysteine, metallo- and serine (see Chapter 14). Although the parasitic protozoa represent a very diverse group of organisms, the highest activity proteinases are often of the same type, the cysteine (thiol) proteinases (North *et al.*, 1990a). These have been found in most of the important parasites during at least one stage in their life cycle (see Chapters 16–19 and 21–25). Cysteine proteinases belonging to the papain superfamily are present in all groups of eukaryotes, excepting fungi (see North, 1982). Other types of protozoan proteinase have been described, although few have been characterized in detail. A notable exception is the surface metalloproteinase of *Leishmania* species (see Chapter 20). Metalloproteinases have also been detected in trichomonads (see Chapter 21) and *Trypanosoma cruzi* (see Chapter 17) while serine proteinases are

probably present in African trypanosomes (see Chapter 18) and *Plasmodium* (see Chapters 24 and 25). Aspartic proteinases have only been reported in malarial parasites (see Chapters 24 and 25). For a number of parasite enzymes it is still not possible to define the type unequivocally. There are examples of enzymes which have apparent characteristics of both serine and cysteine proteinases (see Chapters 17 and 25), although this might be owing to the fact some of the inhibitors used to determine the proteinase type may not be as specific as originally claimed.

THE GENES

Data from biochemical analyses are now being complemented by structural information provided by proteinase gene sequences. The first complete sequence for a parasitic protozoan cysteine proteinase gene, that from *Trypanosoma brucei*, appeared in 1989 (Mottram *et al.*, 1989; and see Chapters 18 and 19). Since then, sequences have become available for all or part of cysteine proteinases from *T. cruzi* (Chapter 17), *Leishmania mexicana* (Chapter 19), *Entamoeba histolytica* (Chapters 22 and 23), *Plasmodium falciparum* (Chapter 24), *Theileria parva* (Nene *et al.*, 1990) and *T. annulata* (Hall and Mottram, unpublished). The sequences show that all of the protozoan enzymes are more closely related to mammalian cathepsin L than they are to cathepsin B. The proteinases of the trypanosomatids (trypanosomes and *Leishmania*) have the closest relationship to cathepsin L. In contrast, helminth cysteine proteinases are from the cathepsin B-branch of the papain superfamily (Klinkert *et al.*, 1989; Cox *et al.*, 1990). The protozoan sequences have already revealed some unusual features including the long C-terminal extension of trypanosome proteinases (see Chapters 17–19). Analysis of the *Entamoeba* proteinase genes is shedding light on the differences between pathogenic and non-pathogenic strains (see Chapters 22 and 23).

Genes for the surface metalloproteinase of *Leishmania major* and *L. chagasi* have been cloned and sequenced (see Chapter 20). These contain putative zinc-binding sites but otherwise show little resemblance to mammalian metalloproteinases. Use of the polymerase chain reaction (PCR) has revealed a putative serine proteinase gene in *T. cruzi* (see Chapter 16), but a complete sequence has not yet been published and its relationship to other serine proteinases is not yet known.

THE APPROACHES TO PROTEINASE ANALYSIS AND DRUG DESIGN

Current studies have two main aims: firstly to provide an understanding of the function of proteinases in the parasite life cycle; and secondly to find inhibitors and substrates which have potential as antiparasite drugs or prodrugs, respectively. There is further reason why information on proteinases is important to anyone working with parasite proteins. Many of these enzymes can cause unwanted

proteolysis during protein isolation. This can be countered by the use of appropriate inhibitors (see North, 1989).

Proteinase analysis has been enhanced by techniques which have allowed multiple proteinase forms to be detected and analysed. Substrate sodium dodecylsulphate—polyacrylamide gel electrophoresis (SDS-PAGE) has now become a standard technique (see Chapter 19, for example) and has proved particularly useful for establishing the complexity of proteinases in many parasitic protozoa. A wide range of chromogenic and fluorogenic peptide derivatives is now available and their use as substrates allows enzymes with differing specificities to be analysed separately. The combination of gelatin-SDS-PAGE with fluorogenic peptides provides a direct means of determining the specificity of individual proteinases without the need to purify each enzyme (see North *et al.*, 1990b; and Chapters 19 and 21). Inhibitors are used to determine the proteinase type (North, 1982), but peptide derivatives may also be used to gain information on the specificity of the enzymes. Peptidyl diazomethanes and peptidyl fluoromethanes (fluoromethyl ketones) are now essential tools for probing the subsite requirements of proteinases. Those working with protozoa, however, have had to rely in the main on inhibitors designed for mammalian proteinases (see Chapter 14). It is hoped that the expanding interest in the protozoan enzymes will encourage chemists to synthesize inhibitors tailored for the latter proteinases.

One of the approaches being adopted for the development of proteinase inhibitors as drugs is described in Chapter 16. In outline, it involves cloning the gene for a selected proteinase, using the sequence information to model the proteinase, designing inhibitors which fit the proteinase and testing these on recombinant proteinase. If inhibitors are to be useful, it will be important to focus on enzymes whose function is essential to the parasite and which differ sufficiently from host enzymes. Many of the proteinases studied to date are high-activity enzymes which have been detected with non-specific substrates or ones designed for mammalian proteinases such as cathepsins B and L. There is, therefore, an inbuilt bias towards characterizing enzymes which are more closely related to host proteinases. In such cases it will be important to exploit the unusual structural features revealed by sequencing, such as the C-terminal extension of the trypanosome cysteine proteinases. This same approach could be extended to the design of prodrugs. The use of proteinase substrates as prodrugs has received little attention to date (but see Chapters 19 and 25), but may prove particularly useful with high activity parasite enzymes.

To increase the chances of finding suitable target enzymes, it will be important to widen the range of proteinases studied. Those with a narrow specificity will be of special interest but prove more difficult to detect initially. Enzymes involved in protein processing may be particularly appropriate drug targets, especially if the details of the processing site are known. Potent inhibitors of the HIV proteinase, for example, have been obtained by screening many hundreds of compounds based on the structure of the viral polyprotein processing sites. Some of these enzyme inhibitors have proved to be effective against the virus. In malaria parasites, a

number of examples of protein processing have been reported and progress is being made in characterizing the enzymes involved (see Chapters 24 and 25).

It may be that useful inhibitors will be found simply by screening a range of available proteinase inhibitors for activity against the parasite *in vitro*. Such screens may be performed without any prior knowledge of the endogenous proteinases. This approach avoids the necessity for biochemical analysis and has been adopted for a number of parasites. It could lead to the discovery of target proteinases not identified by other procedures but may also prove relatively unsuccessful because of the rationale behind the design of the compounds. Only time will tell.

Ultimately, the success of using proteinase inhibitors and substrates as antiparasitic agents will depend on factors such as the importance and location of the enzymes during parasite infection. Although there are many clues as to the role of individual proteinases in parasitic protozoa, much of the evidence is still circumstantial, depending on correlations between virulence and proteinase and/or antiproteinase antibody levels or on the observed effects of proteinase inhibitors *in vitro*. As yet there has been only one instance in which an *in vivo* effect of a proteinase inhibitor been demonstrated (see Chapter 21). This is undoubtedly a reflection of the fact that such tests have not yet been made. However, the growing list of positive effects of inhibitors tested at nanomolar to micromolar concentrations *in vitro* gives increasing support to the view that proteinases are essential to protozoan parasites, albeit for different reasons, and that some of the enzymes will prove to be useful targets for drugs.

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16. New approaches to molecular analysis of proteinases of parasitic protozoa

J.H.McKerrow and J.A.Sakanari

Over the past decade, studies from a number of laboratories have highlighted the importance of proteolytic enzymes in the metabolism of protozoa, as well as in the pathogenesis of diseases caused by parasitic protozoa (McKerrow, 1989; North *et al.*, 1990). Several of these enzymes are attractive targets for drug design. There are two approaches that can be taken as a first step in drug development. Traditionally, the enzyme of interest is identified and purified from the organism, and detailed biochemical analyses are carried out to determine inhibition profile and substrate specificity.

Recently, a second, computer-based approach has been developed. Large numbers of potential inhibitors are screened by 'docking' compounds within the active site as visualized by three-dimensional computer graphics (Knight, 1990). This approach is being applied in designing inhibitors for a number of different enzymes, and some compounds that have been predicted by such studies are already in clinical trials.

Obviously, this latter approach requires detailed structural data on the enzyme. Ideally, these data would come from X-ray crystallographic analysis, but this requires large quantities (milligrams) of ultrapure enzyme. Not all parasitic protozoa can be readily obtained in large quantities from *in vitro* culture systems; hence, adequate amounts of ultrapure enzymes cannot be obtained via classical protein purification protocols. Furthermore, even when adequate amounts of ultrapure enzyme can be obtained, crystal growing remains as much an art as a science. To circumvent some of these problems, we have developed a strategy to obtain structural information for computer graphics which requires only small amounts of parasite material. This strategy takes advantage of three technological advances that have occurred in the last several years:

1. development of degenerate oligonucleotides, based on conserved structural

- motifs in proteolytic enzymes, that allow amplification of gene fragments using the polymerase chain reaction (PCR) (Sakanari *et al.*, 1989; Eakin *et al.*, 1990);
2. algorithms that allow rational three-dimensional structures of proteins to be modelled according to primary sequence (Cohen *et al.*, 1986a, b); and
 3. new expression vectors that allow expression of active recombinant proteolytic enzymes (Graf *et al.*, 1987).

'GENERIC PRIMERS' FOR ISOLATING PROTEINASE GENES

One of the most powerful new tools in molecular biology is the technique called the polymerase chain reaction (PCR). The ability to amplify target sequences several-million-fold by PCR revolutionized forensic medicine, evolutionary biology, gene cloning, site-directed mutagenesis, and disease diagnosis. To amplify and clone proteinase gene fragments from helminths and parasitic protozoa, primers for PCR were designed according to the consensus sequence of amino acids flanking the active sites of serine and cysteine proteinases (Sakanari *et al.*, 1989; Eakin *et al.*, 1990). Inosines were used to minimize degeneracy and maximize base-pairing promiscuity. In the case of the serine primers, the oligonucleotides were 2048- and 8192-fold degenerate; and for cysteine primers, 1024- and 384-fold degenerate. We reasoned that, under low stringency conditions (whereby primers are annealed at 25°C during the PCR), this technique might overcome the drawbacks of the degeneracy of the primers, since both PCR primers must hybridize for amplification to occur.

To confirm the authenticity of the PCR products, amplified gene fragments were used as probes for Southern blot analysis and then sequenced. The PCR gene fragments were then used as homologous gene probes to screen cDNA or genomic libraries. Proteinase gene fragments isolated by this method have been obtained from *Trypanosoma cruzi* (Eakin *et al.*, 1990), *T. brucei* (Eakin *et al.*, 1990), *Entamoeba histolytica* (Eakin *et al.*, 1990), *Plasmodium falciparum* (see Chapter 23) and other parasitic protozoa (North *et al.*, 1990).

MOLECULAR MODELLING OF PROTEINASES BASED UPON PRIMARY SEQUENCE

The use of generic PCR primers for isolating proteinase genes makes it possible to expand rapidly the data base of sequences for serine and cysteine proteinases. In both cases the structural motifs used to develop the primers occur close enough to the ends of the coding region that 70 per cent or more of the protein sequence can be obtained from the amplified gene fragment alone. Subsequent use of this gene fragment to obtain genomic or cDNA clones can provide the remaining sequence if necessary.

While awaiting crystal growing for X-ray crystallography, or in those cases where it proves impossible to grow usable crystals, molecular modelling based on the primary sequence can be used to visualize the active site. The modelling algorithms that we apply to parasitic proteases were developed by Dr Fred Cohen and his colleagues in the Department of Pharmaceutical Chemistry, University of California, San Francisco, USA (Cohen *et al.*, 1986a, b). Briefly, the position of a polypeptide inhibitor in the binding site is determined by analogy to other known structures (e.g. papain and actinin for cysteine proteinases; trypsin and elastase for serine proteinases). Energy calculations are used to refine the model structure. Global measures of structure quality include solvent accessibility profile, packing homogeneity, and atomic solvation analysis. Once the model has been refined, the predicted binding specificity of the active site is studied by means of computer graphics.

The ability of the molecular model to predict active-site specificity can be tested in two ways. First, a series of synthetic peptides can be used to confirm binding predictions in the P₁ to P₄ subsites. Second, site-directed mutagenesis of residues, predicted to be important in determining binding specificity, can be carried out and analysed.

EXPRESSION VECTORS FOR RECOMBINANT PROTEINASES

The third element of our strategy is to use recombinant enzyme for biochemical studies. This is absolutely necessary in cases where sufficient amounts of natural enzyme cannot be obtained, and, again, exploits the ability to access rapidly genes coding for these enzymes with the generic primer PCR technique. There are several obstacles that must be overcome in order to express active proteolytic enzymes. A major one is the potential toxicity of the proteinases to the vectors being used to express the recombinant product. This problem has been overcome in two ways:

1. the use of endogenous signal peptides to export trypsin from bacteria in the vector pTrap, developed by Charles Craik at the University of California, San Francisco, USA (Graf *et al.*, 1987); and
2. intracellular expression leading to formation of inclusion bodies.

The latter approach was used in the successful expression of the *Trypanosoma brucei* cysteine protease by Eric Pamer, using Studier's vector systems (Pamer *et al.*, manuscript submitted; Studier and Moffatt, 1986). Active enzyme can be rescued from inclusion bodies by a denaturation-renaturation step using urea (Schein, 1989, 1990).

Craik and co-workers have utilized an *Escherichia coli* expression system to express active recombinant vertebrate trypsins (Graf *et al.*, 1987). The principal features of this system are driving expression by the alkaline phosphatase promoter (although more recent modifications have taken advantage of stronger and more tightly regulated promoters), and placing the subcloned trypsin insert in frame with

the endogenous *E. coli* alkaline phosphatase signal peptide. This results in transport of expressed trypsin to the periplasmic space, from which it can be isolated after lysozyme treatment of the bacteria.

In the case of trypsin, active enzyme was produced using the coding region for the fully processed enzyme; however, for other proteinases, expression of the preproform appears necessary to obtain active enzyme. Trypsin has a very short activation peptide. Other serine and cysteine proteinases have activation peptides up to 100 or more amino acids long. David Agard's group found that the absence of the pre-pro region of the α -lytic proteinase was absolutely required for expression of active enzyme (Silem and Agard, 1989). In fact, even when the pre-pro was expressed *in trans* to the rest of the enzyme, active enzyme was obtained. This suggests that the pre-pro region may play a role in providing a template for correct folding of the molecule. In the case of trypsin, such a template is apparently not required. It is worth noting that successful expression of the *T. brucei* active cysteine proteinase required that the entire prepro region be expressed in order to obtain active enzyme (Pamer *et al.*, 1991). Furthermore, in contrast to the case of trypsin, this enzyme was expressed intracellularly, where it formed an insoluble product. The formation of this insoluble product may, in fact, have protected the vector from the effects of the proteinase. Renaturation after urea denaturation provided active enzyme.

Many research groups have spent considerable time attempting to express a variety of proteolytic enzymes in bacteria, yeast, or mammalian cell systems. Their results suggest that, unfortunately, expression may have to be tailored to the individual enzyme. Promoters and vector systems that work for one enzyme may not necessarily work for another. Apart from choosing a system that was previously shown to express a related proteinase (if one exists), successfully, the only other approach is trial and error. Fortunately, despite these difficulties, when active recombinant proteinases have been expressed, their biochemical characteristics appear to be similar, if not identical, to those of the natural product. The advantage is a much more efficient source of enzyme for large scale drug screening, crystallization, and biochemical analyses.

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17. Proteinases of *Trypanosoma cruzi*

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INTRODUCTION

The proteinases of *Trypanosoma cruzi* participate in the nutrition of the parasite at the expense of the host, but also appear to be involved in other aspects of the host-parasite relationship. There is evidence that they might be involved in penetration of the trypomastigote into the host cell, since this process is partially sensitive to several well-known proteinase inhibitors (Piras *et al.*, 1985), as well as in evasion from the immune response of the host, either through a 'fabulation' mechanism (Krettli *et al.*, 1980) or by intracellular digestion of human IgG endocytosed after binding to specific antigens (Teixeira and Santana, 1989). In addition, Avila *et al.*, (1979a) have shown that a protein, catalase, is able to satisfy the amino acid requirements of *T. cruzi* epimastigotes, suggesting that it is taken up by the parasite and degraded by proteinases. Knowledge of the proteinases of the parasite is important for research workers involved in the isolation and purification of antigens and enzymes, so that the enzymes can be inhibited and degradation of the material during preparative procedures prevented.

A number of proteolytic activities have been described in *T. cruzi* epimastigotes, starting with the work of Itow and Camargo (1977) and Avila *et al.* (1979b); it is difficult to tell whether the activities described in these papers belong to different or the same enzymes, since these early studies were performed with crude extracts (for a review, see Cazzulo, 1984). Itow and Camargo (1977) demonstrated that the four enzymes they characterized were different from each other, by several criteria. Cazzulo and Franke de Cazzulo (1982) showed that proteolysis of endogenous substrates present in cell-free extracts of *T. cruzi* epimastigotes followed a pattern of pH response, temperature dependence and effect of inhibitors which suggested that the major activity responsible was one of the enzymes first described by Itow and Camargo (1977), that able to hydrolyse azocasein at pH 5.0. This proteolysis was inhibited by *N*-ethylmaleimide and iodoacetate, which suggested that one or more cysteine proteinases were involved (Cazzulo, 1984).

Recently, the proteinase make-up of the parasite has been studied using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in substrate-containing minigels. Fibrinogen-containing gels show a double band of proteinase activity at an apparent molecular weight of about 60 kDa in

epimastigotes of all parasite stocks and clones tested (Campetella *et al.*, 1990), with traces of activity at higher apparent molecular weights (unpublished results). Gelatin-containing minigels clearly show that, although the 50–60 kDa proteinase is the major activity, there are at least three more bands of higher molecular weight, all belonging to the cysteine proteinase class as shown by response to inhibitors, in addition to a probable metalloproteinase band (Greig and Ashall, 1990). Although it is difficult to assess the molecular weight of the enzyme bands, since the samples are usually not reduced and they are never boiled, it is quite clear that, at variance with the case of *T. brucei* where the major cysteine proteinase has a molecular weight of about 28 kDa (Lonsdale-Eccles and Mpimbaza, 1986), no proteinase bands are seen with apparent molecular weight values lower than 50–60 kDa.

CYSTEINE PROTEINASES

Three proteinases, all of which belong to the cysteine proteinase group, have been purified to homogeneity from epimastigotes. One of them has a high molecular weight (about 200 kDa), and efficiently hydrolyses benzoylarginine-*p*-nitroanilide (Bz-Arg-Nan) (Bongertz and Hungerer, 1978). This proteinase might be the same enzyme as the one recently reported and studied in further detail by Ashall (1990); the other two have molecular weights of about 60 kDa (Rangel *et al.*, 1981a,b; Bontempi *et al.*, 1984) and might be the same enzyme, except for some differences in substrate and inhibitor specificity. It is noteworthy that these enzymes were obtained homogeneously after purification of 60-fold or less from whole soluble extracts of epimastigotes; therefore, they probably make up a substantial proportion of the soluble protein of the epimastigote cell.

The 200 kDa proteinase

Bongertz and Hungerer (1978) purified this enzyme to homogeneity from epimastigotes of the D₁ strain of *T. cruzi*. The molecular weight was shown to be about 200 kDa by gel filtration on Sephadex G-150 and gradient gel electrophoresis. The amino acid composition and the carbohydrate composition (since the enzyme is a glycoprotein containing about 2% carbohydrate) were determined. The proteinase was able to hydrolyse Bz-Arg-Nan 47-fold better than trypsin, on a molar basis, but was less efficient than the serine proteinase for the hydrolysis of haemoglobin. The enzyme also showed esterase and transamidase activities, but lower than those of papain. When tested on small synthetic peptides, the 200 kDa enzyme was able to hydrolyse peptide bonds in which the carboxyl group of Arg, Trp or α -N-substituted Lys were involved. The proteinase activity was strongly inhibited by sulphhydryl reagents like *p*-chloromercuribenzoate (*p*-CMB), a characteristic of cysteine proteinases and also by an inhibitor of both serine and cysteine proteinases, *N*- α -*p*-tosyl-L-lysine chloromethylketone (TLCK). Bongertz and Hungerer (1978) also showed that the enzyme was present with similar activity in tryptomastigotes of the

same parasite strain, and suggested that it might be involved in penetration into the mammalian cell.

Recently, Ashall (1990) has purified to apparent homogeneity an enzyme that is able to digest Bz-Arg-Nan and other synthetic substrates containing Arg (and, to a lesser extent, Lys) at the P₁ position, with an optimal pH of 8.0. The molecular weight was judged by electrophoresis to be higher than 200 kDa under non-reducing conditions, which kept the enzyme active. Under reducing conditions, several bands occurred in the 50–70 kDa region, and all enzyme activity was lost. The enzyme showed highest activity with Bz-Arg-Nan, with lower rates on longer synthetic peptides, and no activity at all with proteins such as myosin, bovine serum albumin, ovalbumin and gelatin; haemoglobin, which would have afforded a direct comparison with Bongertz and Hungerer's enzyme, seems not to have been tested. Ashall (1990) concluded that the enzyme, which was also present in amastigotes and trypomastigotes (although at lower levels) of *T. cruzi*, and apparently in other trypanosomatids as well, is the major peptidase able to hydrolyse Bz-Arg-Nan in these organisms, and might be the same enzyme as that described by Bongertz and Hungerer (1978), even though there are a few discrepancies in the properties of the two preparations. Although the enzyme was little affected by the cysteine proteinase-specific inhibitor L-trans-epoxysuccinyl leucylamido (4-guanidino) butane (E-64), Ashall (1990) assumed it to be a cysteine proteinase, because of its sensitivity to leupeptin, TLCK and Z-Leu-Lys-CHN₂ (Z-N, benzyloxycarbonyl; CHN₂, diazomethane). Further studies are required to confirm the nature of the enzyme.

The 60 kDa proteinase

A cysteine proteinase with an approximate molecular weight of 60 kDa was purified by Rangel *et al.* (1981a,b) from epimastigotes of the Y strain of *T. cruzi*. The enzyme was able to hydrolyse casein and haemoglobin, with optimal pH values of about 6.0 and 3.0, respectively; serum albumin was not a good substrate. The optimal temperature was 37–45°C, the activity being considerably lower at 56°C. Proteolysis was enhanced by EDTA and β-mercaptoethanol, and inhibited by thiol binding reagents, but not by pepstatin, diisopropylfluorophosphate, TLCK or TPCK. The authors concluded that the enzyme was a cysteine proteinase. When the molecular weight was estimated by SDS-PAGE, the value obtained in the absence of dithiothreitol was 60 kDa, but in its presence a second component, of molecular weight 6 kDa, appeared; this was considered to be a product of proteolysis. The molecular weight, as judged by gel filtration on Sephadex G-200, was also 60 kDa, indicating that the enzyme was monomeric. The enzyme was reported as being present at the cell surface in the three main forms of the parasite; the authors suggested that it might be involved in the pathogenesis of Chagas' disease (Rangel *et al.*, 1981b).

Bontempi *et al.* (1984) purified from epimastigotes of the Tul 2 stock another 60 kDa cysteine proteinase, which might be the same as the one previously purified by Rangel *et al.* (1981a, b). There are some important differences, however: the enzyme purified by Bontempi *et al.* was strongly inhibited by TLCK and also by

TPCK, although to a lesser extent; its optimal temperature was 62–64°C and bovine serum albumin was the best protein substrate, with an optimum pH of 3–4. Furthermore, recent studies (see below) have indicated that the major product of self-proteolysis has a molecular weight of about 25 kDa (Hellman *et al.*, 1991), and a 6 kDa product was never observed.

The 60 kDa cysteine proteinase, for which the trivial name ‘cruzipain’ has been proposed (Cazzulo *et al.*, 1990b), has been studied in detail recently; it is a high-mannose type glycoprotein (Cazzulo *et al.*, 1989, 1990b), as shown by binding to Con A-Sepharose and elution with the specific sugars (Cazzulo *et al.*, 1989); by increase in electrophoretic mobility in SDS-PAGE upon hydrolysis with *endo-N*-acetylglucosaminidase H (Cazzulo *et al.*, 1990a), and by determination of the composition of the oligosaccharide chains isolated from the purified enzyme (Cazzulo *et al.*, 1990a). The enzyme is inhibited by E-64, with a second-order rate constant of 20800 M⁻¹s⁻¹; this constant is high enough to allow the use of the reagent for active-site titration (Cazzulo *et al.*, 1990b). Other effective inhibitors, in addition to the thiol binding reagents, TLCK and TPCK (Bontempi *et al.*, 1984), are leupeptin, chymostatin and antipain (Cazzulo *et al.*, 1989). Pepstatin A was completely ineffective, as was phenylmethylsulphonylfluoride (PMSF); not only did EDTA not inhibit the enzyme activity, but it actually caused activation, probably through the chelation of heavy metal ions able to react with the active site SH group.

The above evidence clearly indicated that the enzyme must be a cysteine proteinase; this was confirmed by direct sequencing of the *N*-terminus and an internal tryptic peptide, which presented high homology with cathepsin L and papain (Cazzulo *et al.*, 1989). Recently, sequencing of a DNA fragment obtained by polymerase chain reaction (PCR) amplification (Eakin *et al.*, 1990) has confirmed homology with those proteins, as well as with the cysteine proteinase recently cloned and sequenced in *T. brucei* (Mottram *et al.*, 1989).

Recent experiments have indicated that the enzyme is able to degrade itself (Hellman *et al.*, 1991). This digestion was shown to be the result of self-proteolysis, since it presented the same pattern of response to inhibitors as the enzyme activity on azocasein and other substrates. The part of the enzyme corresponding to the ‘mature enzyme domain’ (Mottram *et al.*, 1989) was completely degraded to small peptides, whereas a 25 kDa proteolysis-resistant, glycosylated moiety was left; considering the sequence evidence available, this fragment must correspond to a C-terminal extension domain, similar to that recently described for the *T. brucei* enzyme (Mottram *et al.*, 1989).

Figure 17.1 summarizes the sequence information available at present, including sequences of the C-terminal extension inferred from DNA sequencing (Åslund *et al.*, 1991), compared with the sequence of the mature enzyme and C-terminal extension domains of the cloned *T. brucei* cysteine proteinase (Mottram *et al.*, 1989). The overall homology is high in the mature enzyme domain (70 per cent identity) and lower, but significant, in the C-terminal extension (36 per cent). The sequenced gene codes for a C-terminal extension with a molecular weight of 14 kDa. The predicted sequence shown in Figure 17.1 includes four potential *N*-glycosylation sites, three of which are, at variance with the *T. brucei* enzyme, in the

*	*	
T.c. (p) APAAUVDWAXGAUTAUVKDQGQGGGCWAFAFSRIG		
T.c. APAAUVDWAA <u>B</u> GAUTAUVKDQGQCGSCWAFAFSRIG <u>N</u> SGQWF <u>L</u> GHPLTMLSEQMLUSCDKTD		60
T.b. APAAUVDW <u>R</u> E <u>K</u> GAUTPUKVQGQCGSCWA <u>F</u> STIG <u>H</u> LEG <u>Q</u> W <u>Q</u> AGNPLUSLSEQMLUSCOTIO		
*		
T.c. (p) IUQ	ISPP	
T.c. SGCGGLMNNHAFEW <u>I</u> U <u>Q</u> ENNGGU <u>T</u> EDS <u>P</u> YAS <u>G</u> EG <u>I</u> SPP <u>C</u> TTSGHT <u>Y</u> AT <u>I</u> T <u>G</u> HUEL <u>P</u> Q		120
T.b. SGCGNGGLMDNA <u>F</u> N <u>H</u> W <u>I</u> <u>U</u> NSNGGU <u>T</u> ERS <u>P</u> Y <u>U</u> SG <u>H</u> GE <u>Q</u> P <u>Q</u> C <u>Q</u> M <u>G</u> HE <u>I</u> G <u>A</u> <u>A</u> <u>I</u> T <u>D</u> H <u>U</u> <u>D</u> <u>L</u> <u>P</u> Q		
*		
T.c. (p)		UPY
T.c. DE <u>Q</u> <u>I</u> AA <u>W</u> LA <u>U</u> NG <u>P</u> U <u>Q</u> U <u>A</u> AH ASS <u>U</u> MTTY <u>T</u> GG <u>U</u> MT <u>S</u> C <u>U</u> SE <u>Q</u> LD <u>H</u> G <u>L</u> LLUG <u>Y</u> ND <u>S</u> AA <u>U</u> PY <u>W</u> <u>I</u>		180
T.b. DE <u>Q</u> <u>I</u> AA <u>Y</u> LA <u>E</u> NG <u>P</u> <u>L</u> <u>A</u> <u>I</u> AU <u>D</u> RES <u>F</u> <u>M</u> Y <u>U</u> NG <u>G</u> <u>L</u> <u>L</u> T <u>S</u> C <u>T</u> <u>S</u> K <u>Q</u> <u>L</u> <u>D</u> <u>H</u> <u>G</u> <u>U</u> <u>L</u> UG <u>Y</u> ND <u>S</u> NP <u>P</u> <u>Y</u> <u>U</u> <u>I</u>		
*		
T.c. (p) MSWTAQWG <u>E</u> D <u>G</u> Y <u>I</u> R	EEASSAUU <u>G</u> GG <u>P</u> G <u>P</u> T <u>P</u> E <u>P</u> XXXXXXSAPXP	
T.c. KNSW <u>I</u> AQWG <u>E</u> D <u>G</u> Y <u>I</u> R <u>I</u> A <u>K</u> G <u>S</u> N <u>Q</u> C <u>L</u> KE <u>E</u> ASSAUU <u>G</u> GG <u>P</u> G <u>P</u> T <u>P</u> E <u>P</u> TTTTTS <u>S</u> P <u>G</u> P <u>S</u> SYFU		240
T.b. KNSW <u>S</u> N <u>M</u> W <u>G</u> E <u>D</u> G <u>Y</u> <u>I</u> R <u>E</u> K <u>G</u> T <u>N</u> Q <u>C</u> L <u>M</u> N <u>Q</u> A <u>S</u> S <u>A</u> U <u>U</u> G GPTPPPPPPPPSATFT <u>Q</u> DFCEG		
*		
T.c. QMSCT <u>D</u> A <u>R</u> <u>C</u> <u>I</u> UG <u>C</u> EN <u>U</u> LT <u>P</u> T <u>G</u> Q <u>C</u> <u>L</u> <u>L</u> T <u>T</u> SG <u>U</u> S <u>A</u> U <u>T</u> C <u>G</u> A <u>E</u> T <u>L</u> <u>T</u> <u>E</u> <u>E</u> <u>Y</u> <u>F</u> FT <u>T</u> <u>S</u> H <u>C</u> GPSUR		300
T.b. KG CTK GCS <u>H</u> AT <u>F</u> PT <u>G</u> EC <u>U</u> QT <u>T</u> GUG <u>S</u> U <u>I</u> AT <u>C</u> G <u>A</u> SN <u>L</u> T <u>Q</u> <u>I</u> <u>I</u> Y <u>P</u> LS <u>R</u> S <u>C</u> GPSUP		
*		
T.c. SS <u>U</u> PL <u>N</u> Q <u>C</u> N <u>A</u> <u>L</u> <u>L</u> R <u>G</u> S <u>E</u> FF <u>C</u> G <u>S</u> <u>S</u> <u>S</u> G <u>A</u> <u>L</u> <u>A</u> <u>D</u> U <u>D</u> R <u>Q</u> R <u>A</u> Y <u>Q</u> P <u>Y</u> H <u>S</u> R <u>H</u> A <u>R</u> <u>L</u>		360
T.b. II <u>U</u> PL <u>D</u> K <u>C</u> <u>I</u> <u>P</u> <u>I</u> <u>L</u> IG <u>S</u> U <u>E</u> Y <u>H</u> C <u>S</u> <u>I</u> <u>N</u> P <u>P</u> T <u>K</u> <u>A</u> <u>R</u> <u>L</u> <u>U</u> <u>P</u> <u>H</u> <u>Q</u>		

Figure 17.1. Comparison of the sequences of cruzipain (T.c.) and a cysteine proteinase from *T. brucei* (T.b.) (Mottram *et al.*, 1989). In the case of T.c., both a composite sequence inferred from DNA sequencing (Eakin *et al.*, 1990) up to amino acid 173, and Aslund *et al.* (1991) up to the C-terminus) and partial sequences obtained by peptide sequencing (T.c. (p), Cazzulo *et al.*, 1989, 1990a; Hellman *et al.*, 1991) are shown. Identities are indicated by vertical lines; conservative substitutions are underlined. Asterisks indicate potential sites for *N*-glycosylation.

mature enzyme domain. The first one, close to the active-site Cys, is very likely to be glycosylated in the native enzyme, since this Asn is the amino acid where the direct sequencing of the whole enzyme by Edman degradation stopped (Cazzulo *et al.*, 1989). Although there is no evidence yet for the presence in cruzipain of pre- and pro-enzyme domains, cleaved to give the mature enzyme, it is very likely that they are present, as in the case of the *T. brucei* cysteine proteinase (Mottram *et al.*, 1989).

Cruzipain seems to be the major cysteine proteinase of the parasite, present not only in epimastigotes, but also in cell-culture trypomastigotes and amastigotes (Campetella *et al.*, 1990). The levels of the proteinase, as shown both by enzyme activity (either assayed with azocasein *in vitro*, or detected in fibrinogen-containing SDS-PAGE gels) and as immunoreactivity in Western blots, are considerably lower

in amastigotes and trypomastigotes than in epimastigotes, which suggests that the expression of the enzyme is developmentally regulated (Campetella *et al.*, 1990). The subcellular localization of cruzipain was studied through two different approaches. Biochemical evidence clearly suggests a lysosomal localization (Bontempi *et al.*, 1989); the cysteine proteinase showed the same response to digitonin extraction from whole cells, the same pattern of fractionation by differential centrifugation and by isopycnic ultracentrifugation in sucrose gradients, and similar latency to α -mannosidase, the only good lysosomal marker available in *T. cruzi* (Bontempi *et al.*, 1989). Recent immunoelectron microscopy experiments (Souto-Padrón *et al.*, 1990) using the same monospecific polyclonal antiserum previously shown to be able to detect the cysteine proteinase specifically in Western blots (Campetella *et al.*, 1990), suggests that, as well as being present in the lysosomes the enzyme might be located at the surface of epimastigotes and amastigotes, and also of the transition trypomastigote-amastigote forms. In the case of the trypomastigotes, evidence for the presence of the enzyme at the flagellar pocket, which is the main site of pinocytosis in the parasite (De Souza, 1984), was obtained (Souto-Padrón *et al.*, 1990). Since we have recently found (unpublished results) that there is immunological cross-reactivity between the cysteine proteinase and some cloned antigens of the parasite, particularly the clone 13 protein (Ibañez *et al.*, 1987), and this is a surface antigen (Souto-Padrón *et al.*, 1989), further experiments will be necessary to confirm or discard a true localization of cruzipain at the cell surface.

Recently, Murta *et al.* (1990) have reported that an antigenic glycoprotein, known as GP 57/51 and purified from *T. cruzi* by Scharfstein *et al.* (1986) has 30 amino acids at the N-terminus almost identical to those of the N-terminus of mature cruzipain (Cazzulo *et al.*, 1989). Moreover, the antigen, purified in the presence of iodoacetamide, has some remaining proteinase activity detectable in IgG-containing polyacrylamide gels, and this activity shows the same mobility as a cysteine proteinase purified by these authors from the same extracts (Murta *et al.*, 1990). These and other similarities make it likely, therefore, that GP 57/51 is cruzipain or a closely related proteinase. Cruzipain is antigenic *in vivo*, being recognized, like GP 57/51, by sera from chronic patients, even when these sera are previously adsorbed with the fusion proteins corresponding to clones 13 and 36 (J. Martinez, O.Campetella, A.C.C.Frasch and J.J.Cazzulo, unpublished results).

Cruzipain is able to degrade both small chromogenic peptides and native proteins. Hydrolysis of small peptides requires the presence of Arg or Lys at the P₁ position; in addition, the NH₂ group of this amino acid must be blocked (therefore, the enzyme is not an aminopeptidase) and at least a second amino acid, preferably Arg, Phe, Val or Leu, must be present at the P₂ position. BAPA, which is an excellent substrate of trypsin, and is the best substrate for the 200 kDa proteinase/peptidase (Bongertz and Hungerer, 1978; Ashall, 1990), is hydrolysed only slightly by cruzipain if at all. Protein substrates such as casein (azocasein), haemoglobin, bovine serum albumin (Bontempi *et al.*, 1984), fibrinogen (Campetella *et al.*, 1990) and human IgG (Bontempi and Cazzulo, 1990) are efficiently hydrolysed. At least in the cases of fibrinogen and IgG, the evidence indicates that degradation must be extensive. This evidence, together with the selfproteolysis experiments, which

yielded very small peptides, some of which were sequenced and shown to be tetra—or penta-peptides (Hellman *et al.*, 1991), as well as experiments which show extensive degradation of oxidized A and B chains of bovine insulin (Hellman, Raimondi and Cazzulo, unpublished results), suggests that cruzipain probably has a wide specificity, similar to that of papain (Glazer and Smith, 1971) and cathepsin L (Barren and Kirschke, 1981).

Cruzipain-catalysed hydrolysis of human IgG was shown to consist of extensive degradation of the Fc moiety, whereas the Fab moiety was little attacked (Bontempi and Cazzulo, 1990); the enzyme is probably responsible for the intracellular digestion of human IgG bound to specific antigens at the parasite surface and taken up by endocytosis, recently described by Teixeira and Santana (1989), and might be involved in the defence mechanisms of the parasite against the immune system of its mammalian host.

PROTEINASES BELONGING TO OTHER CLASSES

In addition to cysteine proteinases, recent evidence indicates the presence of a serine proteinase (Sakanari *et al.*, 1989) and a metalloproteinase (Greig and Ashall, 1990). The former authors used PCR to amplify genomic DNA, using as primers consensus sequences flanking the active-site residues (His, Asp and Ser) from eukaryotic serine proteinases (see Chapter 16). In the case of *T. cruzi*, a 438 base pair fragment was obtained and sequenced, and was found to contain regions that encode the conserved amino acid sequences at the active site; therefore, although no serine proteinase activity has been identified so far in *T. cruzi*, a gene coding for one is likely to be present (Sakanari *et al.*, 1989).

There is a recent report of a 60 kDa metalloproteinase, thus classified because of its sensitivity to 1, 10-phenanthroline; it was shown to be a membrane-associated protein, which was partitioned into the detergent phase in phase-separation experiments with Triton X-114. This proteinase might have some resemblance to the *Leishmania* gp63, although it is not a major surface protein; it was classified as an alkaline peptidase, since it had optimal activity at pH 10, with little activity below pH 7.0, in gelatin-containing SDS-PAGE minigels (Greig and Ashall, 1990).

There is no evidence so far for the possible presence of aspartic proteinases in *T. cruzi*, and high molecular weight, multicatalytic, proteinases have not been reported yet in the parasite, although their presence seems likely, considering their universal distribution (Dahlmann *et al.*, 1989).

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18. Proteinases of African trypanosomes

J.D.Lonsdale-Eccles

INTRODUCTION

The study of proteinases in African trypanosomes, is still in its infancy. The only well-defined class of proteinase identified so far is the cysteine proteinase group (North *et al.*, 1983; Lonsdale-Eccles and Mpimbaza, 1986). There has also been a report of acid (or aspartic) proteinases in African trypanosomes (Venkatesan *et al.*, 1977), but this has not been substantiated. It is likely that the latter activity was a lysosomal cysteine proteinase rather than a cathepsin D-like aspartic enzyme. Nevertheless, the lack of demonstrable aspartic proteinase activity should not be considered as proof of the absence of such enzymes. It should be remembered that the aspartic and cysteine proteinases of the lysosomes of mammalian cells act in concert.

Metalloproteinases have been reported in a variety of American trypanosomes but have not yet been observed in African trypanosomes. Aminopeptidase activity has been detected in extracts of *Trypanosoma brucei* (Lonsdale-Eccles and Grab, 1987a; Knowles *et al.*, 1987) and aminopeptidases and carboxypeptidases are frequently metalloenzymes. It is not yet known, however, whether these trypanosomal aminopeptidases, or the other peptidase activities present in African trypanosomes (Gibson *et al.*, 1980; Letch and Gibson, 1981; Young and Godfrey, 1983), are metalloenzymes.

It is possible that some proteinases play an important role in the pathogenesis of trypanosomiasis. Knowles *et al.* (1987) reported that an aminopeptidase is released by the parasites during infection and *T. brucei* also releases proteinases (Lonsdale-Eccles and Grab, 1987b; Nwagwu *et al.*, 1988; Boutignon *et al.*, 1990).

The aminopeptidases and aspartic proteinases, are not discussed further in this review. Instead I focus on the serine and cysteine proteinases of African trypanosomes.

LYSOSOMAL CYSTEINE PROTEINASES

The major proteolytic activities of *T. brucei* and *T. congolense* have all the usual characteristics of the classical cysteine proteinases, such as papain and cathepsin L.

Zymogram analysis shows that the enzymes of *T. brucei* and *T. congolense* have M_r values of approximately 28000 and 31000, respectively (Rautenberg *et al.*, 1982; Lonsdale-Eccles and Mpimbaza, 1986; Pamer *et al.*, 1989). (The equivalent zymogram pictures to *T. vivax* are much weaker and more complex.) These enzymes are optimally active at acidic pH and their activities are enhanced by thiol reducing agents such as dithiothreitol and inhibited by mercurial compounds and microbial cysteine proteinase inhibitors such as *trans*-epoxysuccinylleucylamidoguanidinobutane (E-64) and leupeptin. The limited amount of data on the substrate specificity of these enzymes suggests that they have both cathepsin L-like and cathepsin B-like properties. However, subtle differences in the specificities of the enzymes are observed and are to be expected. They readily hydrolyse Z-Phe-Arg-NHMec and Pro-Phe-Arg-NHMec (Z, *N*-benzyloxycarbonyl; NHMec, 7-amido-4-methylcoumarin) and are able to hydrolyse Z-Arg-Arg-NHMec slightly (North *et al.*, 1983; Lonsdale-Eccles and Mpimbaza, 1986; Lonsdale-Eccles and Grab, 1987a; Robertson *et al.*, 1990).

In the case of *T. brucei*, the amino acid sequence deduced from cDNA shows extensive similarity with the sequences of *Dictyostelium* cysteine proteinase 1 and human cathepsin L (Mottram *et al.*, 1989). In addition, the cDNA encoding the *T. brucei* enzyme predicts a molecule with a long (108 amino acids) C-terminal extension of unknown function. This extension includes a stretch of nine contiguous proline residues near the junction of the C-terminal domain with the enzyme. Some plant cysteine proteinases have C-terminal extensions, but only a cold-induced tomato proteinase has such a long C-terminal extension. A similar extension has now been found in *T. cruzi* (see Chapter 17). The cDNA sequence also provides evidence for pre—and pro-regions in an extension at the N-terminus of the enzyme. These sequences are typical of similar sequences in other cysteine proteinases from mammals, plants and protozoa, and are presumably involved in the processing of the enzyme during and after synthesis (Neurath, 1989; and see Chapter 14). The pre-region probably corresponds to the signal peptide and may be implicated in targetting the enzyme into the lumen of the endoplasmic reticulum for further post-translational modifications, while the pro-region is probably involved in zymogen processing. Although there are conserved domains within the various cysteine proteinases, the cDNA of *T. brucei* only weakly hybridized to *T. cruzi* genomic DNA, thus indicating that there may be considerable divergence between the proteinases of the two species (Mottram *et al.*, 1989). A comparison of the sequences of trypanosome cysteine proteinases is given in Chapter 17. Although other explanations can be invoked, the limited binding of IgG directed against *T. congolense* cysteine proteinase with the equivalent enzyme from *T. brucei* (Mbawa and Lonsdale-Eccles, unpublished observations) may also be a consequence of such divergence.

Subcellular fractionation studies (Lonsdale-Eccles and Grab, 1987a) indicate that the Z-Phe-Arg-NHMec hydrolytic activity of *T. brucei* is located within organelles that resemble lysosomes. Similar studies have been performed with *T. congolense*, but Percoll® density gradient centrifugation shows that the major band of Z-Phe-Arg-NHMec hydrolytic activity in *T. congolense* is both more dense and more diffuse than that obtained from *T. brucei*. When examined by electron

microscopy the lysosomes appear to form part of a large complex interconnected network of tubules and vacuoles (Webster, 1989; Webster and Fish, 1989; Mbawa *et al.*, unpublished observations). Antibody to the cysteine proteinase of *T. congolense* has been used to localize the enzyme by immunoelectron microscopy in conjunction with studies of the endocytosis of colloidal gold. Although immunoreactivity was observed in the flagellar pocket, with a trace on the surface of the parasite, the majority was observed to be localized within lysosome-like organelles of the endocytic network. (Mbawa *et al.*, unpublished observations). This localization suggests that the enzyme may be involved in the degradation of endocytosed materials. Specific studies to explore this possibility have not yet been performed. However, it is clearly an area of potential importance for immunological and chemotherapeutic studies and it should be readily amenable to study using specific inhibitors. Perhaps pertinent to this observation is the enhancement of the cysteine proteinase activity of lysates of purified African trypanosomes by serum. This increase in proteolytic activity is apparent when trypanosome lysates are subjected to zymogram analysis in the presence of trace amounts of plasma or serum (Lonsdale-Eccles and Grab, 1987a). The molecule responsible for the altered zymogram patterns has been purified from serum and appears to be a glycoprotein (Lonsdale-Eccles and Mpimbaza, unpublished observations). It will be of considerable interest to ascertain whether this molecule modulates the cysteine proteinase activity *in vivo*, as well as *in vitro*.

CYTOSOLIC ALKALINE PEPTIDASE

Serine proteinases appear to be present in African trypanosomes, but the evidence is not as strong as the evidence for the presence of cysteine proteinases. Diisopropylfluorophosphate (DFP), a specific inhibitor of serine esterases (Aldridge and Reiner, 1972), binds to several distinct *T. brucei* proteins that do not appear to be proteinases (Lonsdale-Eccles *et al.*, 1989). However, the soluble fraction of lysates of *T. brucei* clearly contains serine esterase-like hydrolytic activity against small peptide substrates of trypsin such as benzoylarginine *p*-nitroanilide and Z-Gly-Gly-Arg-NHMec (Lonsdale-Eccles and Grab, 1987a). The kinetics of inhibition of the enzyme by DFP and the pH activity profile of the enzyme are similar to those observed with other serine proteinases (Kornblatt *et al.*, unpublished observations). The enzyme may belong in the group of processing enzymes which have a general specificity for basic paired residues (Bond and Butler, 1987) because the best substrate we have found for this cytosolic enzyme is Z-Arg-Arg-NHMec.

This DFP-inhibitable enzyme exhibits several interesting and unusual properties. The enzyme shows pronounced substrate inhibition with a variety of peptide substrates (Kornblatt *et al.*, unpublished observations) and, in addition to being inhibited by serine proteinase inhibitors, the enzyme is inactivated by mercurial compounds and Z-Leu-Lys-CHN₂ (CHN₂, diazomethane) (Lonsdale-Eccles and Grab, 1987a; Ashall, 1990; Kornblatt *et al.*, unpublished observations). Z-Leu-Lys-CHN₂ is

reported to be a specific cysteine proteinase inhibitor (Green and Shaw, 1981; Kirschke *et al.*, 1988). However, the enzyme is not inhibited by E-64, which is also a specific cysteine proteinase inhibitor (Hanada *et al.*, 1983). At present, the question as to whether this enzyme is a serine or cysteine proteinase has not yet been resolved, although we favour its classification as a serine proteinase.

Interestingly, a similar activity is apparently widespread amongst trypanosomatids (Ashall, 1990), although little or none of this enzyme is found in *T. vivax* (Mbawa *et al.*, 1991). Either the pathway served by this enzyme is absent in this evolutionarily relatively primitive African trypanosome (Gardiner, 1989), or a different enzyme assumes its role. We estimate that the apparent M_r of the *T. brucei* enzyme is 80000, but in the case of *T. cruzi* the enzyme is reported to have an approximate M_r of 200000 (Ashall, 1990; and see Chapter 17) which may indicate that they are distinctly different enzymes.

ENZYMES ACTIVE TOWARDS Z-Arg-Arg-NHMec

When analysing crude subcellular fractions, we observed that activity which hydrolysed Z-Arg-Arg-NHMec was distributed in both the soluble and small granule fractions (Lonsdale-Eccles and Grab, 1987a). It is not clear whether the two activities reflect the existence of two distinct enzymes or a single one occurring in two sites. Recent studies, however, provide evidence that there are two enzymes in *T. brucei* that hydrolyse Z-Arg-Arg-NHMec (Robertson *et al.*, 1990). These enzymes may be variants of the soluble enzyme that hydrolyses Z-Gly-Gly-Arg-NHMec and is inhibited by DFP (see above). However, the subcellular fractionation studies indicate that some of the Z-Arg-Arg-NHMec hydrolytic activity is associated with an organellar fraction (Lonsdale-Eccles and Grab, 1987a). It is possible that this enzyme may be similar to that which hydrolyses Z-Phe-Arg-NHMec, in which case the enzyme may be considered to be similar to histolysin from *Entamoeba histolytica* and a variety of other enzymes with different M_r values from *Trichomonas vaginalis* and *Tritrichomonas foetus* (North *et al.*, 1990). All these enzymes appear to be classical cysteine proteinases, but with distinctly different sensitivities to diazomethanes and E-64. Although the Z-Arg-Arg-NHMec hydrolytic activities from *Entamoeba histolytica*, *Trichomonas vaginalis* and *Tritrichomonas foetus* are found both intra—and extra-cellularly, there is no published evidence yet for a secretory form of the enzyme in African trypanosomes. Clearly we are far from having a complete understanding of the nature of the trypanosomal enzymes which hydrolyse Z-Arg-Arg-NHMec.

LIFE CYCLE VARIATIONS

Each of the proteinases is likely to play an important, but as yet undefined, role in the parasite, but do their roles vary with the different life cycle stages of parasites? In the three species of African trypanosome studied, *T. brucei*, *T. congolense* and *T.*

vivax, Z-Phe-Arg-NHMec hydrolytic activity is much higher in the life cycle stages that possess a variable surface glycoprotein (VSG) (i.e. metacyclic and bloodstream forms of the parasites (Lonsdale-Eccles and Mpimbaza, 1986; Pamer *et al.*, 1989; Mbawa *et al.*, 1991)). Although the precise results depend upon the substrates used, in general, bloodstream forms of *T. brucei* and *T. congolense* exhibit the highest levels of hydrolytic activity against azocasein, azocoll, SDS-fibrinogen, SDS-gelatin and Z-Phe-Arg-NHMec. Life cycle forms that lack a VSG also lack the Z-Phe-Arg-NHMec hydrolytic activity. Thus, this hydrolytic activity may be involved in the intracellular processing of endocytosed proteins and/or of the variable surface glycoprotein. Interestingly, while monomorphic bloodstream forms show no change in the ability to hydrolyse Z-Phe-Arg-NHMec or various proteins during the progression of parasitaemia, pleomorphic forms exhibit marked increases (3–12 fold) as the parasites differentiate from long slender forms into short stumpy forms (Pamer *et al.*, 1989; Mbawa *et al.*, 1991). It has been suggested that this induction may be a consequence of polyamine depletion (Pamer *et al.*, 1989).

In general, the converse seems to apply to the Z-Arg-Arg-NHMec activity (Mbawa *et al.*, 1991). This activity is higher in the epimastigote forms of *T. congolense* and the procyclic forms of *T. brucei*, neither of which has a VSG. In *T. vivax*, where activity against Z-Arg-Arg-NHMec is fairly low, it was found in both bloodstream and insect forms of the parasite. The specific activity profiles against Z-Gly-Gly-Arg-NHMec tended to parallel those of Z-Arg-Arg-NHMec. Little or no activity towards this substrate was observed in *T. vivax* and the highest specific activity against Z-Gly-Gly-Arg-NHMec was observed in epimastigotes of *T. congolense* and procyclics of *T. brucei* (Mbawa *et al.*, 1991).

In *T. vivax* the maximum proteolytic and Z-Phe-Arg-NHMec hydrolytic activities are found in different life cycle stages of this parasite; bloodstream forms have the highest Z-Phe-Arg-NHMec specific activity, while epimastigote forms have maximum proteolytic activity towards either fibrinogen incorporated in SDS-PAGE gels, or azocoll or azocasein (Mbawa *et al.*, 1991). The American trypanosome, *T. cruzi*, resembles *T. vivax* in that it also has maximum proteolytic activity in the epimastigote forms with the amastigote and tryomastigote forms having almost 10-fold lower activity (Campetella *et al.*, 1990; and see Chapter 17).

CONCLUSIONS AND PROSPECTS

The stage-specific expression of these various enzyme activities suggests that they play important roles in each of the respective life cycle stages. The unusual inhibitory characteristics exhibited by some of the enzymes may render them suitable for appropriate drug targetting and may provide means of chemotherapeutic attack of the different life cycle stages of the parasites. The lysis of bloodstream forms of *T. brucei*, but not the procyclic forms, by peptidylfluoromethyl ketones (Ashall *et al.*, 1990) provides hope that an appropriately designed inhibitor of trypanosomal proteinases may eventually form the basis of a new class of trypanocidal drugs.

Additional evidence of the importance of controlling trypanosomal proteinases has come from recent observations showing that trypanotolerant N'Dama cattle and buffalo (*Syncretus caffer*) raise antibodies to the M_r 33000 cysteine proteinase of *T. congolense* during their first infection by the parasite (Authie *et al.*, unpublished). In contrast, trypanosusceptible cattle generally fail to do so, although after repeated infections and treatments some of these animals may do so. Although trypanotolerant cattle may still harbour the parasites, they are much less susceptible to the pathogenic effects of the disease.

Finally, it should be stressed that we have not yet begun to address such problems as whether African trypanosomes contain complex proteolytic systems similar to those found in mammalian cells (e.g. the multicatalytic protease complex or the ubiquitin/ATP dependent proteases). Neither do we know how any of the described enzymes are regulated nor even how they are distributed to their various subcellular compartments. It would be of more than passing interest to know if African trypanosomes possess surface proteinases similar to those reported in American trypanosomes and leishmanias. Clearly much remains to be determined in the field of proteinases and their control in African trypanosomes and in their possible involvement in the pathogenesis of the disease.

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19. Cysteine proteinases of leishmanias

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INTRODUCTION

Proteinases of parasites are attracting considerable interest at present, with particular attention being given to the cysteine proteinases (North *et al.*, 1990; and see Chapter 15). The enzymes of *Leishmania mexicana* subspecies are especially notable as they exhibit marked stage specificity and there are multiple high-activity forms in the amastigote. They appear to play key roles in enabling this mammalian stage of the parasite to survive and multiply in macrophages. This review provides an overview of the leishmanial enzymes and the biochemical and molecular methods being used to gain greater insight into their features and peculiarities, their roles in the cell and their potential as drug targets.

CYSTEINE PROTEINASES OF *L. MEXICANA* AMASTIGOTES

The initial finding that amastigotes of *L. m. mexicana* contain unusually high cysteine proteinase activity (Coombs, 1982) led to the discovery that these amastigotes contain multiple soluble enzymes with apparent sizes in the range 16–36 kDa (North and Coombs, 1981; Pupkis and Coombs, 1984; Lockwood *et al.*, 1987; see Figure 19.1). These enzymes accounted for over 90 per cent of the total proteinase activity and were shown to be located within unusual lysosome-like organelles (Pupkis *et al.*, 1986) which have an acidic pH (Antoine *et al.*, 1989). These structures have been termed ‘megasomes’ because many are relatively large. Amastigotes contain as many as 34 of these organelles, which can comprise approximately 15 per cent of the total cell volume (Coombs *et al.*, 1986). Similar structures also occur in amastigotes of *L. m. pifanoi* and *L. m. amazonensis*, which are also characterized by the presence of multiple high-activity cysteine proteinases (Pupkis *et al.*, 1986; McMahon-Pratt *et al.*, 1987; Alfieri *et al.*, 1989), but not in promastigotes or in other *Leishmania* species (Pupkis *et al.*, 1986; Tetley *et al.*, 1989).

The multiple cysteine proteinases of *L. m. mexicana* have been purified, to be apparently free from all other proteins, by sequential affinity chromatography, gel

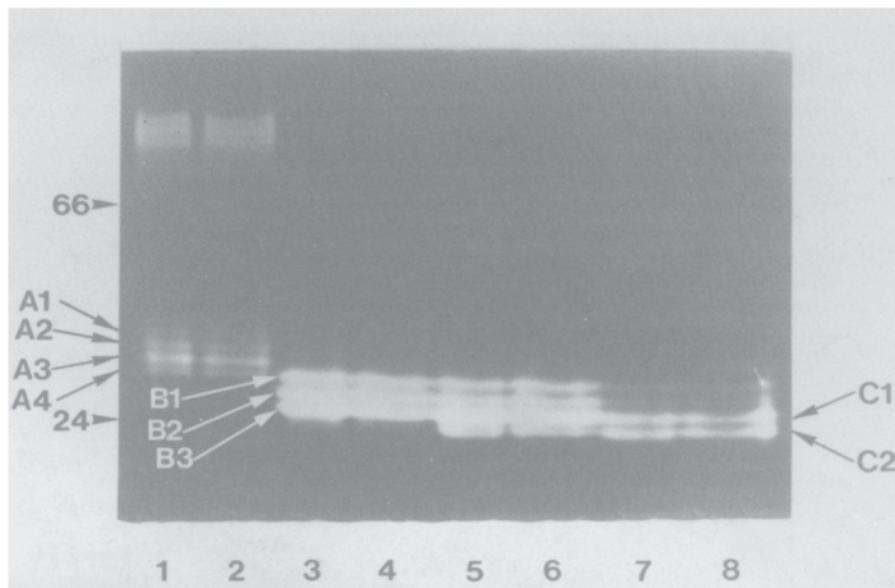


Figure 19.1. Gelatin SDS-PAGE of the three groups of cysteine proteinases in amastigotes of *L. m. mexicana*. Lanes 1 and 2: the enzymes eluted from Concanavalin A-Sepharose CL 4B. The cysteine proteinases are labelled A1–A4. Lanes 3–8: the enzymes eluted from an anion exchange column (mono Q). The two groups of cysteine proteinases are labelled B1–B3 and C1 and C2, respectively. The positions of two molecular-weight markers are shown. (Reproduced from Robertson and Coombs (1990).)

filtration and anion exchange chromatography (Robertson and Coombs, 1990). The use of Concanavalian A-Sepharose was an important step in this purification, removing a group of at least four mannose-containing, glycosylated cysteine proteinases from the other cysteine proteinases (see Figure 19.1). The five proteinases that do not bind to the lectin cannot be totally separated except by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Nevertheless, two groups of enzymes have been distinguished on the basis of their relative affinities for different peptide substrates (see Figure 19.2) and their net negative charge as judged by their elution from an anion exchange column. Thus, the group of three apparently larger proteinases (as judged by gelatin SDS-PAGE) have a lower net negative charge than the two enzymes in the other group and also have relatively greater activity towards peptide substrates with tyrosine in the P_1 position (nomenclature of Schechter and Berger (1967)).

These data on the substrate preferences of the three groups of amastigote cysteine proteinases were obtained by separating the enzymes using SDS-PAGE and subsequently incubating the gels in buffer containing appropriate fluorogenic peptide substrates (Robertson and Coombs, 1990; and see Chapter 15). Hydrolysis of a substrate by a particular enzyme was then detected as a fluorescent band when the gel

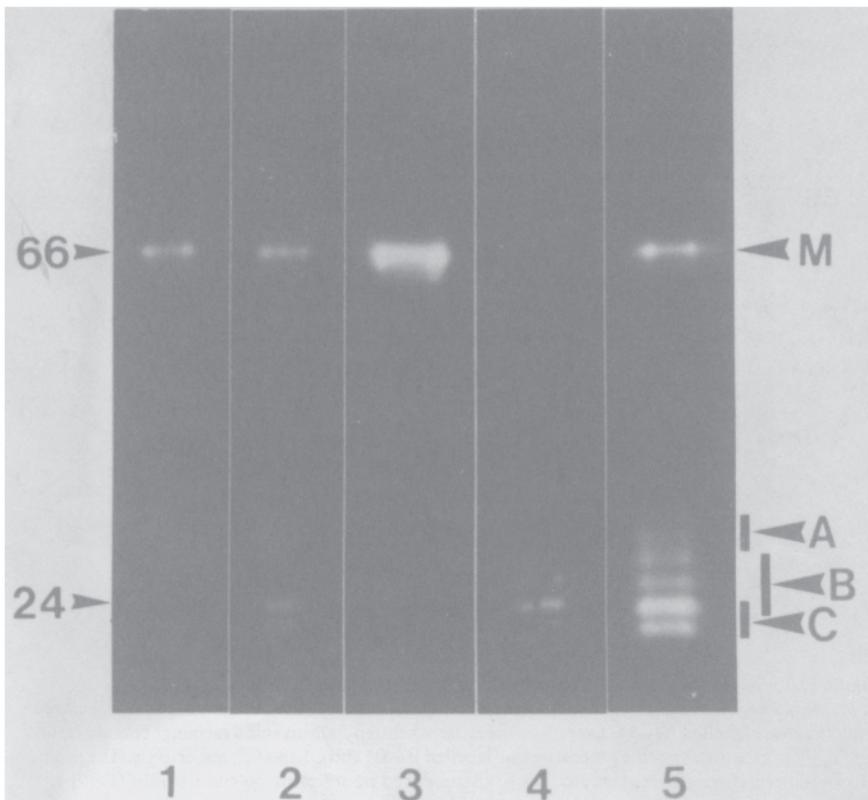


Figure 19.2. Activity of *L. m. mexicana* amastigote proteinases towards peptidyl amidomethylcoumarin substrates after SDS-PAGE. The peptide moieties of the substrates are: lane 1, Phe-Ser-Arg; lane 2, Phe-Val-Arg; lane 3, Gly-Gly-Arg; lane 4, Leu-Val-Tyr; lane 5, Pro-Phe-Arg. The positions of the three groups of cysteine proteinases (A, B and C) are indicated along with a higher molecular weight activity (M) and two molecular-weight markers. (Reproduced from Robertson and Coombs (1990).)

was illuminated with ultraviolet light (Figure 19.2). Thus this method allowed the substrate preferences of the individual proteinases to be studied without the need to purify the enzymes first. The results of this study show that, in general, the *L. mexicana* cysteine proteinases preferentially cleave peptides with a basic P₁ residue (although tyrosine in that position is accepted well by the one group of enzymes) and bulky, hydrophobic amino acids in the P₂ and P₃ positions. In this respect, the leishmanial enzymes resemble more closely mammalian cathepsin L than cathepsin B. One group of amastigote enzymes, however, show good activity towards one substrate of cathepsin B (*N*-benzoylphenylalanylvalylarginylamidomethylcoumarin), but not similar cathepsin B substrates that contain only single or paired arginine residues. Thus there are at least some differences between the substrate specificities of leishmanial and mammalian enzymes: the full extent of the differences must await

further analysis of the parasite enzymes. Clearly, however, the leishmanial enzymes are in many ways typical of cysteine proteinases in general and this similarity is confirmed both by the staining of megasomes of *L. m. amazonensis* by a monoclonal antibody raised against rat cathepsin B (Prina *et al.*, 1990) and the information already obtained from the analysis of cysteine proteinase genes. These latter studies, however, have also highlighted some significant differences between the structures of trypanosomatid and mammalian cysteine proteinases (see below).

The same multiplicity of cysteine proteinase bands was detected after PAGE irrespective of whether the conditions were reducing or non-reducing and whether SDS was present or not (Robertson and Coombs, 1990), showing that the multiple banding is not an artefact of the procedure involving only mildly denaturing SDS treatment. It is not yet known, however, whether all the enzymes are products of different genes or if some are simply subject to differential post-translational modification. We have shown (unpublished) that after boiling in SDS the enzymes that are not bound by Concanavalin A run together during SDS-PAGE, suggesting that their primary structures are very similar. It will, however, be necessary to obtain information on the primary structure of the proteinases and their genes in order to elucidate the extent and ways in which the different enzymes are related to each other.

STAGE SPECIFICITY OF *L. M. MEXICANA* PROTEINASES

The majority of the studies done so far have involved analysis of the soluble cysteine proteinases in amastigotes of *L. m. mexicana*. Early reports showed that promastigotes contained much lower proteinase activity and it has been subsequently discovered that multiplicative promastigotes do not apparently contain the same enzymes as occur in the amastigotes of *L. m. mexicana* (Lockwood *et al.*, 1987). Intriguingly, however, low activities of similar enzymes do occur in populations of stationary phase cells (Lockwood *et al.*, 1987; and see Figure 19.3). Such cell populations are thought to contain metacyclic promastigotes, the stage responsible for infection of mammals, and these forms have been shown to be intermediate between multiplicative promastigotes and amastigotes in a variety of ways (Mallinson and Coombs, 1989). The presence of the cysteine proteinases in these forms is suggestive that the parasite is pre-adapted for survival upon being taken into a mammal and also that the enzymes play an immediate and vital role in this survival. We have recently shown that acid treatment of stationary phase cell extracts subsequent to separation by SDS-PAGE results in the activation of cysteine proteinases of somewhat slower mobility on gels compared to the amastigote enzymes. This suggests that metacyclic promastigotes contain inactive precursor forms of cysteine proteinases. This can be seen as another pre-adaptation of the metacyclic stage, for the proteins could serve as a source of active enzymes that would be immediately available to the parasite after its uptake into a macrophage. The changes that occur during this acid activation have not been determined, but one possibility worth investigating is that involving the removal of the pro-region of the primary translation product.

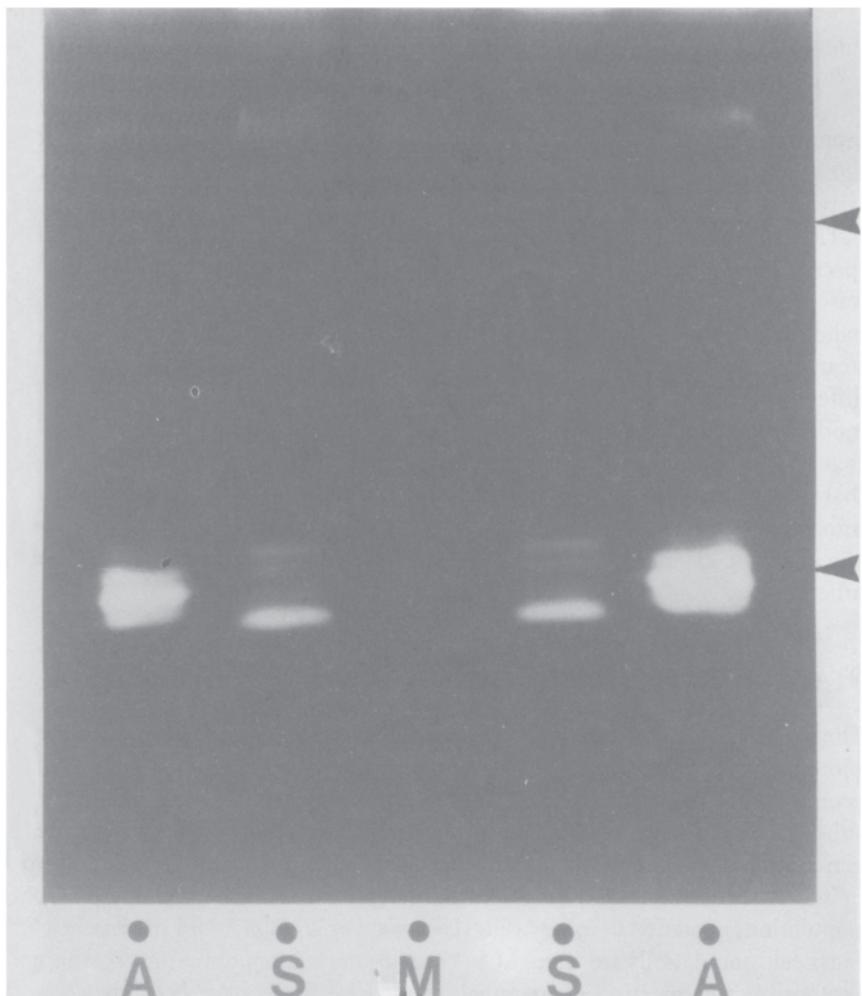


Figure 19.3. Comparison of enzymes in amastigotes and populations of promastigotes of *L. m. mexicana* using gelatin SDS-PAGE. The amastigote samples were in lanes A with stationary phase promastigote samples containing metacyclcs in lanes S and a multiplicative promastigote sample in lane M. The gel was developed to show the differences between the major enzymes of the three samples, hence some of the less active enzymes are not apparent. Arrows indicate the positions of two molecular-weight markers (66 and 24 kDa).

It is notable that the stationary phase promastigote samples contain a cysteine proteinase activity with an electrophoretic mobility greater than that of any of the amastigote enzymes (North and Coombs, 1981; and Figure 19.3), suggesting that the metacyclic promastigote may also have a stage-specific activity. The relationship between this enzyme and those of the amastigote is unknown, but it will be interesting to find out whether it is a precursor to the amastigote enzymes (for instance, a product of the same gene but post-translationally modified in a different

way) or if it serves some particular function in the metacyclic stage. This latter suggestion gains some credence from the finding that the enzyme has somewhat different substrate preferences from the amastigote enzymes, and it may also be relevant that we have recently discovered that one cysteine proteinase gene of *L. m. mexicana* is transcribed most abundantly in stationary phase cells (see below).

Although multiplicative promastigotes are relatively deficient in soluble cysteine proteinase activity in comparison with amastigotes, they do possess other proteinase activities. Some are particulate, whilst some show stage specificity (Lockwood *et al.*, 1987) and differ in their substrate preferences (Robertson and Coombs, 1990). These activities appear similar to those present in other *Leishmania* species and are discussed below.

GENES OF CYSTEINE PROTEINASES

The use of molecular techniques to analyse the cysteine proteinase genes of leishmanias should provide information on the number of genes, the structures of the proteins that they encode and the mechanisms involved in their stage regulation. Such data should give a greater insight into the special adaptations of the leishmanial enzymes and the ways in which they differ from their mammalian counterparts.

The high level of conservation in the active-site regions of eukaryotic cysteine proteinases has allowed the design of degenerate oligonucleotides for the isolation of cysteine proteinase genes or gene fragments from parasites (Mottram *et al.*, 1989; Sakanari *et al.*, 1989; Eakin *et al.*, 1990; and see Chapters 15–17, 22 and 23). Analysis of a cDNA isolated from a *Trypanosoma brucei* library using the oligonucleotides as probes showed that it encoded a protein possessing the key characteristics of a cysteine proteinase but with several unusual features (Mottram *et al.*, 1989). The deduced protein could be divided into four domains, based on similarities with other cysteine proteinases (see Figure 19.4). The pre-, pro—and central domains showed considerable homology with the cathepsin L class of cysteine proteinases, but the trypanosome gene predicted a protein with a long, 108 amino acid C-terminal extension that distinguished it from all mammalian cysteine proteinases described to date. Subsequently it has been reported that *T. cruzi* contains a gene that predicts an enzyme with a similar C-terminal extension (see Chapter 17) and it is possible that such cysteine proteinases are a feature of trypanosomatids. The functional significance of the extension is unknown, but it is likely that it is reflected in the activity and substrate specificity of the proteinase, especially as it has been deduced that at least part remains in the mature enzyme (Mottram *et al.*, 1989). It could also be involved in associating the encoded enzyme to a particular subcellular location, such as a membrane, or in targeting the enzymes to lysosomes. Nothing is known about the mechanisms of lysosomal targeting in trypanosomatids, although there is some evidence that mannose 6-phosphate is not involved (Cazzulo *et al.*, 1990).

A cysteine proteinase gene of *L. m. mexicana* has been isolated by screening an amastigote cDNA library with the *T. brucei* cysteine proteinase cDNA under relaxed

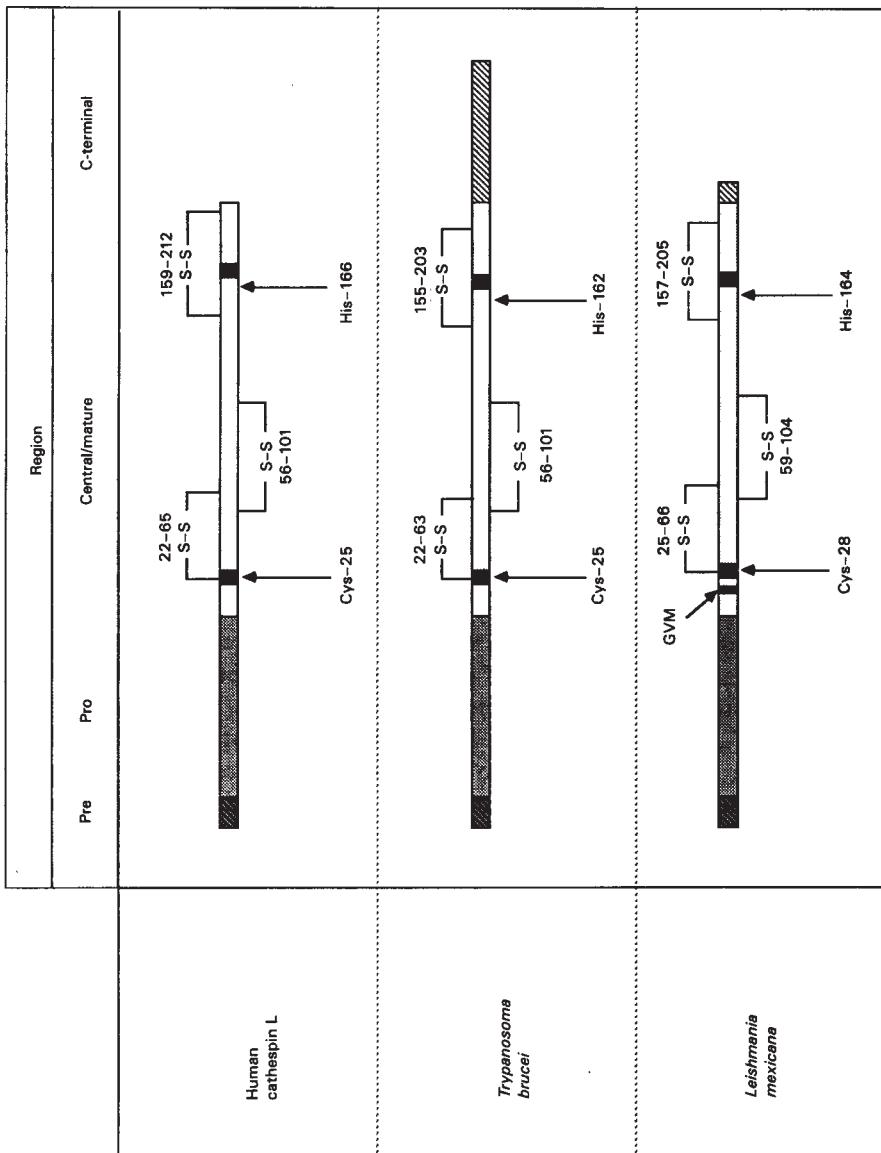


Figure 19.4. Comparison of the primary structures of cysteine proteinases from *L. m. mexicana*, *T. brucei* and human cathepsin L. The diagram gives outlines of the structures showing the conserved cysteine and histidine residues (thought to be involved in the active site) and the cysteine residues apparently involved in disulphide bridges. The two highly conserved regions exploited in the design of oligonucleotides used to isolate the parasite genes are shown as black boxes. The unusual three amino acid insertion (Gly-Val-Met) near the N-terminus of the central region of the leishmania gene is also indicated. Numbering is from the known or predicted N-terminus of the central region in each case.

stringency conditions (Mottram *et al.*, unpublished). The protein sequence encoded by the gene has highest homology (60 per cent over the central domain) to the *T. brucei* enzyme, but also significant homology (43 per cent) to human cathepsin L. The leishmanial protein contains the conserved domains characteristic of all cysteine proteinases but has an unusual three amino acid insertion near the N-terminus of the central domain. It has only a short C-terminal extension of approximately 10 amino acids with little homology to the trypanosome extension. The differences between cathepsin L and these cysteine proteinases of *L. mexicana* and *T. brucei* are shown diagrammatically in Figure 19.4.

A transcriptional analysis using Northern blots on mRNA isolated from different stages of *L. mexicana* revealed that the leishmanial gene is expressed in both early log phase (multiplicative) promastigotes and amastigotes at about the same level, but that the expression is greatest in stationary phase populations of promastigotes (containing metacyclic promastigotes). This is surprising because, as described above, the majority of cysteine proteinases are most abundant in amastigotes, with only low activities being detected in metacyclic promastigotes and the enzymes apparently being absent from multiplicative promastigotes. There is the possibility, therefore, that this gene encodes an enzyme specifically activated in the metacyclic promastigotes. Southern blot analysis using the *L. mexicana* cDNA under relaxed stringency conditions showed the presence of two other related genes, probably also encoding cysteine proteinases. An analysis of how these genes relate to the cysteine proteinase enzymes present in amastigotes should provide insights into how expression of the proteins is regulated, the ways in which they are post-translationally modified and the relationships between the multiple enzymes.

ROLES OF AMASTIGOTE ENZYMES

A number of hypotheses have been postulated for how the amastigote-specific cysteine proteinases of *L. mexicana* aid survival of the parasite in macrophages. Within these cells, the amastigotes reside in a parasitophorous vacuole that appears to be the equivalent of a secondary lysosome, and so the parasite is, at least potentially, exposed to the degradative activities of the lysosomal contents. It has been suggested (Coombs, 1982) that the proteinases may antagonize the host cell's cytotoxic responses, either by hydrolysing and inactivating host proteins such as lysosomal enzymes, or by participating in the production of amines which upon secretion into the parasitophorous vacuole would raise the vacuolar pH and thus inactivate the host hydrolases. Current evidence, although somewhat fragmentary, does not conform fully with either suggestion. Amastigotes do not appear to take up proteins from the parasitophorous vacuole (either for nutritional purposes or as a source material for amine production) and amastigote cysteine proteinases do not appear to be secreted into the parasitophorous vacuole (Pupkis *et al.*, 1986; Prina *et al.*, 1990). It has also been reported that the parasitophorous vacuole is acidic (Rivas and Chang, 1983; Antoine *et al.*, 1990), although it is difficult to rule out (or indeed prove) that the amastigote causes a small, but nevertheless significant, change. The

parasite may also modulate the microenvironment in the vacuole in some other beneficial but as yet undiscovered way. An interesting but unexplained observation is that the parasitophorous vacuoles in which amastigotes of *L. mexicana* multiply are large, whereas other species of *Leishmania* live in small vacuoles. From the above it should be clear that there is at present little firm evidence for any particular role of the amastigote-specific proteinases, although their very existence at high activity and the finding that cysteine proteinase inhibitors hinder amastigote survival (Coombs and Baxter, 1984) are strongly suggestive that they have one. This is one of the most challenging topics currently under investigation.

CYSTEINE PROTEINASES OF OTHER *LEISHMANIA* SPECIES

Proteolytic activity towards a variety of substrates has been described for the promastigotes of many species of *Leishmania* and the amastigotes of a few (for examples, see Camargo *et al.* (1978), Simon and Mukkada (1983) and references quoted above). Although none contains comparable activities to those in amastigotes of *L. mexicana*, proteolytic activities with some of the properties of cysteine proteinases have been described in other *Leishmania* species and it seems very likely that all species contain enzymes of this type. For example, when extracts of the reptile parasite *L. tarentolae* were analysed by electrophoresis in gels containing haemoglobin as a substrate, two clear bands indicative of digested haemoglobin were produced and these activities were stimulated by dithiothreitol and inhibited by a series of proteinase inhibitors that characteristically inhibit cysteine proteinases (North *et al.*, 1983). The apparent slower mobility of these two enzymes compared to the *L. mexicana* proteinases in the same electrophoretic system, as well as activity differences, suggest that there are differences between the cysteine proteinases of species infecting mammalian or reptilian hosts. The discovery of a cysteine proteinase gene in *L. major* (Sakanari and Bouvier, personal communication; and see Chapter 16) suggests that this species contains at least one such enzyme and the authors have preliminary evidence for two.

A high-molecular-weight enzyme with some characteristics of a cysteine proteinase has been described recently in extracts of *L. donovani* promastigotes (Ghoshal *et al.*, 1989), although cysteine proteinase activity was not detected in promastigotes or amastigotes of *L. donovani* in a previous study (Lockwood *et al.*, 1987). Interestingly, the activity was shown to be thermostable *in vitro* to 70°C, but was lost when promastigotes were heat shocked to 37°C. This suggests that either the enzyme undergoes rapid turnover at this temperature or it is lost during transformation to amastigotes. In either case, it would appear unlikely that the enzyme plays a major role in maintaining the parasite's viability in the mammalian host.

Leishmania promastigotes also contain a high molecular weight enzyme characterized by its high activity towards *N*-benzoylarginyl-*p*-nitroanilide (Bz-Arg-Nan) at alkaline pH (Camargo *et al.*, 1978; Pupkis and Coombs, 1984; Ashall, 1990). A similar activity has also been reported in other trypanosomatids (North *et al.*, 1983;

Ashall, 1990). Although the enzyme is not strictly a proteinase, being unable to hydrolyse protein substrates significantly, it is capable of hydrolysing small peptides such as those used routinely to assay cysteine proteinase activity and so its activity cannot be ignored when proteinase assays are performed with unpurified enzymes. The alkaline peptidase is not particularly sensitive to the cysteine proteinase inhibitor E-64, yet is inhibited by TLCK, leupeptin and various peptidyldiazomethanes and peptidylfluoromethanes which are considered to be specific cysteine proteinase inhibitors (Green and Shaw, 1981; North *et al.*, 1990). Its substrate preferences overlap with those of other *Leishmania* cysteine proteinases (Pupkis and Coombs, 1984; Robertson and Coombs, 1990), but are subtly different; for instance, this enzyme has high activity towards Bz-Arg-Nan which is hydrolysed only slowly by the amastigote-specific enzymes. The current view is that this enzyme is probably an unusual peptidase with some characteristics of a serine proteinase, but the low sensitivity of this alkaline peptidase to E-64 may prove useful in distinguishing its contribution to proteinase activity measurements in crude proteinase mixtures.

Promastigotes of all species of *Leishmania* investigated, including *L. m. mexicana*, contain a surface metalloproteinase (see Chapter 20) and there are also some indications of a membrane-bound enzyme with features characteristic of cysteine proteinases. It is an intriguing idea that the occurrence of a cysteine proteinase with such an unusual location could explain at least some of the peculiarities already detected in the genes encoding trypanosomatid cysteine proteinases.

CYSTEINE PROTEINASES AS DRUG TARGETS

The amastigote-specific cysteine proteinases of *L. m. mexicana* appear to provide particularly attractive targets for drug attack. Their abundance and stage specificity indicate that the enzymes are central to the survival and growth of the parasite in macrophages, a conclusion supported by the finding that enzyme inhibitors interfered with the growth of the parasite (Coombs and Baxter, 1984). Two main approaches for exploiting the enzymes have been forwarded (Coombs, 1986, 1989). Inhibitors of the enzymes could be effective, providing they are sufficiently specific. We have shown already that the leishmanial enzymes are susceptible to a range of peptidyldiazomethanes and greater information on the substrate specificities of the enzymes should allow more specific analogues to be designed.

Inhibitors of cysteine proteinases have been shown to be active against leishmanias *in vitro*. Some peptidyldiazomethanes also inhibit the growth of promastigotes (unpublished). In addition, antipain has been shown to inhibit *L. m. mexicana* promastigote growth and also to have an effect on the transformation *in vitro* of amastigotes to promastigotes (Coombs *et al.*, 1982). Both antipain and leupeptin inhibited growth of amastigotes in macrophages *in vitro* (Coombs and Baxter, 1984). It is not known, however, if all these effects were due to specific inhibition of cysteine proteinases. Neither antipain nor leupeptin are totally specific and, of course, cysteine proteinases have not been detected in multiplicative promastigotes of *L. m. mexicana*, even though they have been reported for other

species. A drug must have specificity *in vivo* and it is yet to be discovered whether or not any of these compounds have antileishmanial activity against *L. mexicana* in animals. The possibility that currently used antileishmanial drugs exert their primary action on cysteine proteinases has been studied, but the evidence suggests that it is not the case with antimoniais or pentamidine (Coombs *et al.*, 1982).

A second approach is to design compounds (prodrugs) that are non-toxic themselves but would be specifically activated by the enzymes. In this case, the specificity could be achieved on the basis of differences in activity (the presence of exceptionally high activities of cysteine proteinases in the amastigotes) or specificity (designing prodrugs that would act as substrates for the parasite enzymes but not those of the host) between parasite and host enzymes. A range of amino acid esters have been shown to kill *L. mexicana* amastigotes in macrophages and have some antileishmanial activity *in vivo* against *L. m. amazonensis* (Rabinovitch, 1989). There is strong evidence that these act as prodrugs, being activated by the amastigote cysteine proteinases. It is thought that the particular sensitivity of the amastigotes is due to their abundant cysteine proteinases and that the hydrolysis of the esters results in the accumulation of amino acids in the megasomes and their eventual rupture through osmotic stress (Rabinovitch, 1989). Electron microscopic studies have shown that toxic amino acid esters cause megasome fusion and, ultimately, rupture with release of destructive cysteine proteinases into the cytosol. Inhibitors of cysteine proteinases antagonize the toxic effect of the esters (Alfieri *et al.*, 1988). At the same time they prevent megasome fusion, and the use of radiolabelled esters has shown that the inhibitors act not by preventing ester uptake but by blocking their hydrolysis (Antoine *et al.*, 1989). Ester hydrolysis by cysteine proteinases has also been demonstrated directly, using cysteine proteinases purified from amastigotes (Hunter *et al.*, 1991). It seems unlikely that amino acid esters themselves will prove to have sufficient specificity to be clinically useful, but these observations have provided great encouragement that the use of prodrugs is a worthwhile approach.

Overall, the findings suggest that the cysteine proteinases present in amastigotes of *L. mexicana* are good targets for chemotherapeutic attack. The absence of similar enzymes in other *Leishmania* species, however, suggests that any drug developed would have a rather limited spectrum of activity. It remains to be seen whether the enzymes that occur more widely can be exploited by such proteinase-directed drugs.

PROSPECTS

At present, more is known about the biochemistry of the *L. mexicana* cysteine proteinases than about their biological role. There is still much to learn about these enzymes. Future biochemical and molecular biological studies should help to discern the similarities and differences between the multiple cysteine proteinases, and further studies on the enzymes' substrate preferences will allow the design of inhibitors that are more specific for particular cysteine proteinases. In addition to having potential pharmacological roles, such inhibitors may allow insights into the

proteinases' function by observing how specific proteinase inhibition affects cell behaviour and properties. Much of this research is already under way, and the next few years should see answers being provided to some of the questions.

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20. The promastigote surface proteinase of *Leishmania*

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IDENTIFICATION OF THE PROMASTIGOTE SURFACE PROTEINASE (PSP) AS THE 'MAJOR SURFACE PROTEIN' OF *LEISHMANIA*

A membrane-bound proteinase is expressed at the surface of *Leishmania* promastigotes, and has been called the promastigote surface proteinase (PSP). Prior to its identification as an active proteolytic enzyme (a zinc metalloproteinase, see below), the 'major surface antigen' of the promastigote was called p63 or gp63. PSP has been shown to occur at the surface of all species of *Leishmania* that have been examined (Bouvier *et al.*, 1987; Etges, unpublished) and, although it appears to be expressed at low levels by the amastigote from an abundant, constitutively transcribed mRNA (Button *et al.*, 1989; Wilson and Hardin, 1990), the results of efforts to demonstrate the presence of the active protease at the surface of amastigotes have been negative or inconclusive. Independent of its proteolytic activity, PSP has been identified as the major complement component 3 (C3) acceptor of *Leishmania mexicana* promastigotes (Russell, 1987) and to be involved, either directly or via bound C3, in the attachment of the promastigote to the host macrophage (Russell and Wilhelm, 1986). The proteolytic shedding of C3 fragments from opsonized *L. donovani* promastigotes could, however, involve the activity of PSP (Puentes *et al.*, 1989).

Surface labelling of *Leishmania* promastigotes with ^{125}I revealed an abundant protein of about 63 kDa (Lepay *et al.*, 1983; Ramasamy *et al.*, 1983; Bouvier *et al.*, 1985; Colomer-Gould *et al.*, 1985; Etges *et al.*, 1985). Peptide-mapping analyses showed that these structurally related proteins are conserved among the recognized species of *Leishmania* (Colomer-Gould *et al.*, 1985; Etges *et al.*, 1985). Reactivity with polyclonal antibodies raised against chemically deglycosylated *L. major* PSP, as well as surface location and surface-oriented proteolytic activity, was demonstrated for PSP in seven species of *Leishmania* (Bouvier *et al.*, 1987). More recently, promastigotes of 15 species of *Leishmania* have been shown to possess surface metalloproteinase activity (Etges, unpublished). In *L. major*, PSP accounts for 70 per cent of surface iodinatable proteins, 15 per cent of [^{35}S] methionine

labelled membrane proteins, and over 1 per cent of the total cellular protein of the promastigote (Bouvier *et al.*, 1985), but only 0.1 per cent of *L. mexicana* amastigote cellular protein (Medina-Acosta *et al.*, 1989). The amount of PSP, however, depends on the strain and species considered, and the enzyme may not necessarily represent the major surface protein accessible to surface iodination.

The relative abundance and ease of purification of PSP in some species of *Leishmania* has led to considerable effort to use the surface metalloproteinase as a molecularly defined vaccine. Indeed, intraperitoneal vaccination of inbred mice with purified *L. mexicana* PSP with Freund's complete adjuvant confers significant protection to CBA mice upon challenge infection (Russell and Alexander, 1988); however, attempts to protect BALB/c mice with recombinant *L. major* PSP were unsuccessful (Handman *et al.*, 1990). More recently, synthetic peptides of predicted T-cell epitopes of *L. major* PSP were shown to protect mice from subsequent low-dose challenge infection with both *L. major* and *L. mexicana* promastigotes (Jardim *et al.*, 1990). However, the incapacity of T-cells from human cutaneous leishmaniasis patients to recognize purified PSP *in vitro* suggests that PSP or PSP-derived peptides alone are inadequate to vaccinate human populations (Jaffe *et al.*, 1990).

ACTIVITY AND EXPRESSION OF PSP IN THE AMASTIGOTE

Little is known concerning the activity of the metalloproteinase in amastigotes, but its role is likely to be limited by the acid pH within the phagolysosome of the infected macrophage (Antoine *et al.*, 1990): purified *L. major* PSP exhibits less than 10 per cent of its maximal activity at pH 5 as compared to its activity at pH 8.5 (Etges *et al.*, 1986b, 1989; Ip *et al.*, 1990; Bouvier *et al.*, 1990). Although PSP has been reported to be expressed (albeit in reduced quantities) by the amastigotes of *L. amazonensis* (Chaudhuri *et al.*, 1989), *L. mexicana* (Medina-Acosta *et al.*, 1989) and *L. major* (Frommel *et al.*, 1990), its presence at the surface of the cell in an enzymatically active form remains to be demonstrated.

GENOMIC ORGANIZATION AND EXPRESSION OF PSP

Genes encoding PSP are linked at a single chromosome locus in *L. major* and occur in five repeats of 3.1 kb (with 1.8 kb of open reading frame) plus an additional gene 8 kb away, all of which display conserved restriction maps (Button *et al.*, 1989). Similar tandemly linked genes occur in *L. chagasi* (Miller *et al.*, 1990). One of the PSP genes was sequenced in both organisms. Both *L. major* and *L. chagasi* PSP are, like human fibroblast collagenase, synthesized as pre-pro-proteins with conventional signal sequences followed by pro-sequences of 100 amino acids. Messenger RNA for PSP is present at comparable, high levels in both promastigotes and amastigotes (Button *et al.*, 1989; Wilson and Hardin, 1990). The fact that some monoclonal antibodies directed to promastigote PSP fail to recognize amastigote

PSP (Fong and Chang, 1982) suggests that PSP is differentially processed by amastigotes. Polyclonal antibodies against recombinant (thus not glycosylated) PSP recognize two polypeptides of slightly higher molecular weight in *L. major* amastigote extracts (Frommel *et al.*, 1990). The amastigote protein was shown to be structurally related to PSP by peptide map analysis, and the increase in molecular weight was suggested to be the result of quantitative and qualitative differences in glycosylation, and perhaps by the mode of anchoring (Medina-Acosta *et al.*, 1989).

GLYCOSYLATION OF PSP

The glycosylation of PSP has been studied in some detail. Chemical deglycosylation of the purified enzyme with trifluoromethanesulphonic acid results in a molecular weight decrease from 63 to 57 kDa with a concomitant loss of concanavalin A binding capacity (Bouvier *et al.*, 1985). Immunoaffinity-purified PSP from *L. amazonensis* treated with endoglycosidase H, or synthesized by promastigotes treated with the glycosylation inhibitor tunicamycin, shows a decreased molecular weight of 54 kDa (Chang *et al.*, 1986). These findings are in agreement with the molecular weight of 53 kDa predicted from the genes encoding the mature polypeptides of *L. major* and *L. chagasi* PSP (Button *et al.*, 1988; Button and McMaster, 1990; Miller *et al.*, 1990). The predicted amino acid sequence contains two or three potential *N*-glycosylation sites. Structural analysis of the *N*-linked oligosaccharides of *L. mexicana* PSP revealed glycans consisting of four related biantennary oligomannoses with a unique terminal glucopyranosyl residue on the *a*(1, 3)-arm in one case (Olafson *et al.*, 1990), representing a rare occurrence of glucose in the *N*-linked glycans of a mature surface glycoprotein. In contrast, *N*-linked glycans of *Trypanosoma brucei* variant surface glycoprotein (VSG) are very similar to mammalian high-mannose structures (Holder, 1985).

PSP IS ANCHORED TO THE PROMASTIGOTE MEMBRANE BY A GLYCOPHOSPHATIDYLINOSITOL LIPID

PSP is anchored to the promastigote membrane by a glycosylphosphatidylinositol (GPI) anchor (see Chapter 27), and can be solubilized in detergent solution by the action of phosphatidylinositol specific phospholipases C (PI-PLC) from bacterial or trypanosomal origins (Bordier *et al.*, 1986; Etges *et al.*, 1986a). *Leishmania* promastigotes themselves are devoid of PI-PLC activity and, therefore, unable to remove GPI-anchored membrane component. After lipid removal, PSP exposes a new epitope, the cross-reacting determinant (CRD), which is common to many PI-PLC-solubilized GPI-anchored proteins (Bordier *et al.*, 1986; Ferguson and Williams, 1988; and see Chapter 27). An important feature recognized by anti-CRD antibodies consists of the inositol 1, 2-cyclic phosphate that remains after PI-PLC action (Ferguson *et al.*, 1988; Zamze *et al.*, 1988).

All glycoprotein GPI anchors so far characterized have an identical carbohydrate core structure that is completely conserved in *Leishmania* PSP. In *Leishmania*, the anchoring lipid consists of a (1, 0)-alkyl-(2, 0)-acyl glycerol with a marked preference for a fully saturated 24 carbon atom alkyl chain, in contrast to the diacylglycerol of *T. brucei* VSG, which contains uniquely the 14 carbon atom myristic acid. The mature COOH terminal asparagine of PSP to which the GPI anchor is attached is located 25 residues before the predicted COOH terminus of the protein (Schneider *et al.*, 1990).

CHARACTERIZATION OF PSP AS A ZINC METALLOENDOPEPTIDASE

PSP is a zinc containing metalloproteinase that occurs as a dimer of 63 kDa monomers at the surface of the promastigote and in detergent solution. Each monomer of PSP contains one atom of zinc, as shown by atomic emission and atomic absorption spectroscopy, as well as by biosynthetic labelling with [^{65}Zn]Cl₂ (Bouvier *et al.*, 1989). Its proteolytic activity is abolished by dialysis against 1, 10-phenanthroline, and can be restored upon addition of micromolar quantities of zinc (Chaudhuri *et al.*, 1989). In addition, a highly conserved amino acid sequence found in a variety of otherwise unrelated zinc metalloproteinases was identified in PSP (Bouvier *et al.*, 1989; Chaudhuri *et al.*, 1989; Jongeneel *et al.*, 1989a) This 'zinc signature' contains a glutamic acid and two histidine residues, which in the small, soluble bacterial metalloproteinase thermolysin were shown to be involved in catalysis and zinc coordination, respectively (Monzingo and Matthews, 1984). However, the secondary structure of PSP contains approximately 50 per cent antiparallel β -sheet, which is in striking contrast to the predominantly α -helical thermolysin (Jähnig and Etges, 1988; Bouvier *et al.*, 1989).

PSP is proteolytically active at the surface of live and fixed promastigotes (Etges *et al.*, 1986b), and is able to hydrolyse a wide range of denatured polypeptides. The optimum pH for PSP activity was found to be neutral to basic for *L. major* PSP (Etges *et al.*, 1986b, 1987, 1989; Bouvier *et al.*, 1990) and *L. mexicana* PSP (Ip *et al.*, 1990), but was reported to be acidic for *L. amazonensis* PSP (Chaudhuri and Chang, 1988; Chaudhuri *et al.*, 1989). This striking difference may reflect the use of different assays which are not applicable over the extended pH range in which they were used. Specifically, the 'acid' proteinase of *L. amazonensis*, which is reported to cleave iodinated BSA in solution at pH 4, does so with an insignificant specific activity. Curiously, the same investigators showed that the enzyme was able to digest fibrinogen after SDS-PAGE separation only at neutral pH (Chaudhuri *et al.*, 1989), in accord with their earlier observation of neutral-to-alkaline (pH 7.5–10) 43.68 kDa proteolytic activity detected by both fibrinogen and gelatin-SDS-PAGE analysis of *L. amazonensis* extracts (Fong and Chang, 1981). In assays with defined synthetic peptide substrates (see below), where conformational constraints of the substrates are unlikely to interfere with access to susceptible peptide bonds, both the *L. mexicana* and *L. major* enzymes show a neutral optimum pH (Ip *et al.*, 1990; Bouvier *et al.*, 1990). The reported acidic proteolytic

activity of *L. amazonensis* PSP might be due to partial denaturation of otherwise resistant substrates that become sensitive to the residual proteolytic activity of PSP, or contaminating acid proteinases, at low pH. In addition, the alkaline elution of *L. amazonensis* PSP from a monoclonal antibody affinity column (20 mM lysine at pH 11 (Chang and Chang, 1986)) may remove the zinc from the enzyme, as was shown in the case of the mammalian membrane metalloproteinase enkephalinase (Jongeneel *et al.*, 1989b). In the pH stability experiments conducted by Bouvier *et al.* (1990), the activity of PSP was shown not to be irreversibly affected by a 30-min incubation at pH values between 4 and 11, but that at pH 11.5 and higher values, enzymatic activity was not recovered upon neutralization.

Little is known concerning the relevant substrates of PSP *in vivo*; that is, within the sandfly vector. A plausible role for the promastigote ectoproteinase could be in the acquisition of nutrients from the blood meals of its insect vector. *L. major* promastigotes cultivated *in vitro* utilize amino acids, especially proline, rather than glucose as a source of carbon (Mukkada, 1985), and all species of *Leishmania* require an exogenous source of preformed haem for growth (Chang and Chang, 1985). Clearly, the proteolytic degradation of haemoglobin, which is readily available to the promastigote in the midgut of the haematophagous sandfly vector, would provide an abundant supply of both nutrients. The presence of the surface metalloproteinase on promastigotes of *L. infantum* in the midgut of experimentally infected *Phlebotomus perniciosus* (Grimm *et al.*, 1987), and the identification of surface-oriented amphiphilic metalloproteinases on the monoxenous insect trypanosomatids *Crithidia fasciculata* and *Herpetomonas samuelpessoai* (Etges, unpublished) suggest that PSP is likely to play its role in the insect, rather than in the mammalian host of *Leishmania*.

Conservation of this proteolytic activity among *Leishmania* species, even in many non-infective laboratory strains, suggests that PSP plays an important role in the metabolism of the promastigote. Although the increased expression of PSP has been suggested to be a determinant of virulence in *L. chagasi* and *L. braziliensis* (Kweider *et al.*, 1987; Wilson *et al.*, 1989), it is clearly not the only factor affecting promastigote infectivity. Indeed, the lipophosphoglycan (LPG) deficient strain of *L. major*, LRC-L119, which produces significantly more PSP compared to several virulent stains of the same species, is completely non-infective (Murray *et al.*, 1989; R. Etges, unpublished; and see Chapter 28 for details on LPG). On the other hand, a non-infective tunicamycin-resistant variant of *L. chagasi* has been described which does not express immunoreactive PSP, despite the fact that the promastigotes transcribe as much PSP mRNA as the parental population (Wilson and Hardin, 1990).

PEPTIDE BOND PREFERENCE OF PSP

In an elegant series of experiments to determine the peptide bond specificity of *L. major* PSP using synthetic peptide substrates, the results of which are shown in Figure 20.1, Bouvier *et al.* (1990) demonstrated that PSP, like many metalloendopeptidases, shows a clear, but not absolute preference for hydrophobic

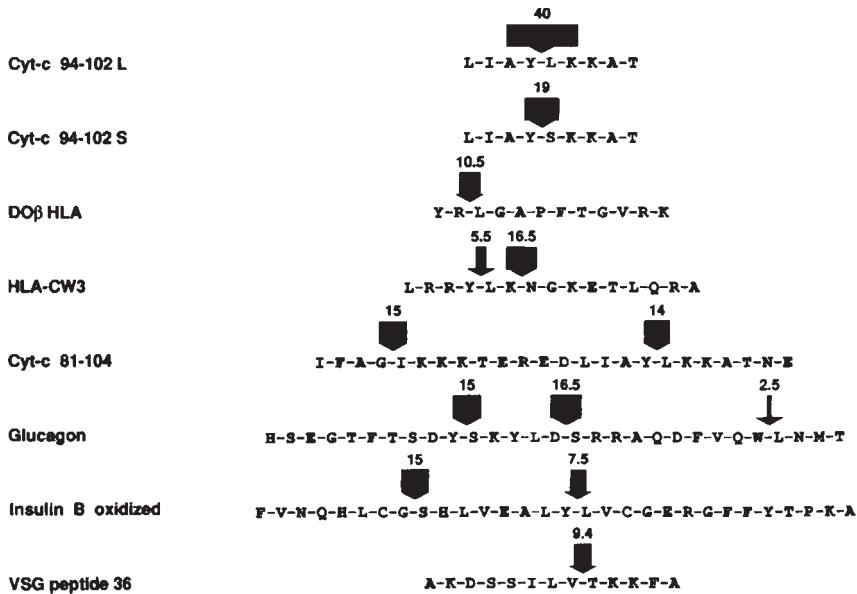


Figure 20.1. Proteolytic activity of purified *L. major* and *L. mexicana* PSP on peptides. The first seven peptides were digested as described by Bouvier *et al.* (1990); briefly, 250 μ M of peptide was digested with 8 DM of *L. major* PSP at 37°C in TBS at pH 7.5. The digestion of VSG peptide 36 with *L. mexicana* PSP was conducted as described in Ip *et al.* (1990). The sites of hydrolysis are indicated by arrows whose width is proportional to the initial rate of hydrolysis. The numbers indicate the specific activity of PSP on each peptide in moles of peptide bond cleaved per second per mole of PSP.

amino acids at the P_1' site; indeed, its peptide bond specificity seems to be defined essentially by the P' subsites of the substrate. PSP fails to cleave synthetic chromogenic or fluorogenic substrates designed for thermolysin, and its requirement for a hydrophobic residue at the P_1' site is as strict as that of other metalloproteinases, as it can accommodate polar residues like serine (and threonine in VSG peptide 36, shown in Figure 20.1 (Ip *et al.*, 1990)). Single residues of the substrate polypeptide do not alone define proteinease specificity; rather, extended substrate binding regions have been described for most endopeptidases. In thermolysin, for example, three residues on the amino side and two residues on the carboxyl side of the scissile bond of the substrate have been shown to affect catalysis (Pozsgay *et al.*, 1986; Mäkinen *et al.*, 1989; Kester and Matthews, 1977). The presence of an extended substrate binding site for PSP is suggested by the strong sequence similarity between the putative active site of PSP and the active sites of several metalloendopeptidases (McKerrow, 1987; Bouvier *et al.*, 1989; Jongeneel *et al.*, 1989a). In one instance, when the DO β -HLA peptide was hydrolysed, PSP exhibits an aminodipeptidase activity (Figure 20.1). Furthermore, the fact that the peptide bond Tyr-Leu, which is often cleaved within several of the peptides analysed, is not cleaved at the same velocity, and in the case of glucagon, not at all (Figure 20.1), clearly indicates the importance of the amino acids flanking the scissile bond in determining the susceptibility of that bond to attack by PSP. The

frequent presence of tyrosine at the P₁ site and of basic amino acids at the P_{2'} and P_{3'} sites of susceptible peptides suggests some specificity exists at these subsites (Bouvier *et al.*, 1990). In an independent study, Ip *et al.*, (1990) described the specific cleavage by *L. mexicana* PSP of a synthetic peptide with Val in the P₁ site, Thr in the P_{1'} site, and lysines in both the P_{2'} and P_{3'} sites (Figure 20.1). These findings strongly suggest that the latter two sites may contribute more to the actual peptide bond specificity of PSP than those forming the scissile peptide bond in the P₁ and P_{1'} sites. The synthetic peptide cyt.C 94–102L, which has a tyrosine at the P₁ site, a leucine at the P_{1'} site, and the basic amino acid lysine in both the P_{2'} and P_{3'} sites, was used as a model substrate to determine the K_{cat}/K_m ratio of $1.8 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$.

This synthetic substrate for PSP was, however, not hydrolysed at all by thermolysin, indicating that the enzymes differ with respect to substrate specificity in spite of their great similarity in primary structure in the region of their active sites.

INHIBITORS OF PSP

PSP is inhibited by metal chelating agents like 1, 10-phenanthroline (but not its non-chelating analogue 1, 7-phenanthroline), peptide hydroxamate derivatives, and divalent metal ions like zinc, but not inhibitors of serine, cysteine, or aspartic proteinases (Etges *et al.*, 1986b, 1987, 1989; Chaudhuri and Chang, 1988; Chaudhuri *et al.*, 1989; Bouvier *et al.*, 1990). Purified PSP was also shown to be inhibited in solution by human α_2 -macroglobulin. This inhibition does not occur at the surface of promastigotes, however, indicating that the active site of the membrane bound PSP is inaccessible to the relatively large α_2 -macroglobulin *in vivo* (Heumann *et al.*, 1989). This could be due either to its orientation relative to the membrane or to steric interference caused by the abundant surface glycoconjugate of the promastigote, lipophosphoglycan (LPG) (Turco, 1990; and see Chapter 28).

The identification of a chelating peptide hydroxamate derivative as a potent inhibitor for PSP represents an important step in the characterisation of the enzyme, both biochemically and biologically. Z-Tyr-Leu-hydroxamate (Z, N-benzyloxycarbonyl) inhibits PSP in the high micromolar range, which is much lower than the millimolar concentrations needed for inhibition with 1, 10-phenanthroline, and exhibits somewhat lower toxicity to mammalian cells *in vitro* compared to 1, 10-phenanthroline. This inhibitor does not appear to be a strict substrate analogue; rather, it appears to fit into the active site of PSP with leucine and tyrosine occupying the P_{1'} and P_{2'} sites, respectively, allowing the hydroxamate to chelate the zinc atom or to displace the zinc coordinated water molecule essential for catalysis. The observation that hydroxamate derivatives of leucine or tyrosine alone do not inhibit PSP, in contrast to the derivative of Z-Tyr-Leu, indicates either that at least two residues are required to target the inhibitor to the enzyme's active site, or only that the N-terminus of the peptide must be blocked. The identification of an inhibitor of PSP effective in the micromolar range suggests that less toxic compounds might be developed that will allow basic biological experiments to be conducted to establish the role of PSP on the life cycle of *Leishmania*.

LATENCY AND AUTOCATALYTIC ACTIVATION OF PRO-PSP

Cleavage of the propeptide of human fibroblast collagenase between Gln₉₉–Phe₁₀₀, Phe₁₀₀–Val₁₀₁, or Val₁₀₁–Leu₁₀₂ has been shown to proceed by an autocatalytic mechanism (discussed in Fields *et al.*, 1990). To evaluate the capacity of *L. major* PSP to self-activate by cleavage of its propeptide, a synthetic peptide covering the V₁₀₀–V₁₀₁ cleavage site (H₂N-A⁹⁷-R-S-V-V-R-D-V-N¹⁰⁵-COOH; residues numbered according to Button and McMaster (1988), was prepared and evaluated as a substrate. PSP hydrolyses this synthetic peptide uniquely at the Val-Val peptide bond with a V of only 0.46 mol s⁻¹, or about 90 times less than that found with cyt.C 94–102L (V=40 mol s⁻¹; Bouvier *et al.*, 1990). The possibility that PSP cleaves its own propeptide opens the interesting possibility of designing an inhibitor to block this process, in order better to understand both the biosynthesis of the enzyme, and its contribution to the survival of the parasite in the mammal and vector. Since the endosomal compartment of *Leishmania*, and presumably its flagellar pocket, are acidic, we postulate that newly synthesized PSP would be inactive until exposed at the surface of the promastigote, where it could either autocatalytically active itself, or be activated by previously activated PSP by cleavage of the propeptide from the inactive newly exposed molecules. The low initial velocity of cleavage of the synthetic peptide spanning the cleavage site, compared to cyt.C 94–102L would ensure minimal activation of PSP intracellularly, and given the high density of the enzyme at the surface of the promastigote (5×10^5 per cell), would still permit efficient activation at the surface. PSP-pro-PSP interactions at the promastigote surface would occur in two dimensions in the fluid membrane of the promastigote, while synthetic peptide-H-PSP interactions in solution are constrained by diffusion of both enzyme and substrate in three dimensions. Furthermore, the secondary structure of PSP, predicted from the primary structure by Button *et al.* (1988), shows that the scissile Val-Val bond occurs in a β -turn at the end of an extended α -helix on the propeptide, where it might be more susceptible than the same conformationally unconstrained sequence in a short synthetic peptide.

A more intriguing hypothesis postulates that residues within the propeptide itself interact directly with the active-site zinc to ensure latency of PSP during its biosynthesis and transport to the promastigote surface. The latency of human fibroblast collagenase (HFC), like human neutrophil collagenase and stromelysin (Hasty *et al.*, 1990) is mediated by the interaction of cysteine⁷³ (in HFC) in the enzyme's pro-sequence with the active-site zinc (Springman *et al.*, 1990). Interestingly, the pre-pro-peptide of *L. major* PSP has two Arg-Cys sites, R-C¹³ in the putative presequence, and R-C⁴⁸ in the prosequence, both of which are similar to the R-C⁷³ of human fibroblast collagenase, whereas the PSP of *L. chagasi* contains only the second prosequence R-C⁷³ (Button and McMaster, 1988, 1990; Miller *et al.*, 1990). This conserved, unpaired cysteine could complex the active-site zinc of the *Leishmania* enzyme, preventing its premature activation prior to expression at the surface of the promastigote. Mutations introduced into this portion of the prosequence of the metalloendopeptidase transin, the rat homologue of human

stromelysin, results in the spontaneous activation of the enzyme (Sanchez-Lopez *et al.*, 1988). These hypotheses can be tested with the *Leishmania* enzyme only when sufficient quantities of pro-PSP can be isolated, or enzymatically active, genetically engineered proteinase can be expressed to permit site-directed mutagenesis experiments to be performed.

FUTURE DIRECTIONS

Although metalloproteinases of bacterial and mammalian origins share important structural features and some similarities in substrate specificity, they differ widely, and presumably represent the products of convergent evolution. There is little, if any, structural similarity between the metalloproteinases outside of the highly conserved zinc binding domain. All metalloproteinases, including the exopeptidases, use the same basic mechanism for peptide bond hydrolysis (Vallee and Galdes, 1984; Vallee and Auld, 1990). Virtually every inhibitor of metalloproteinases contain a functional group that is able to interact with the active-site zinc. In general, the inhibitor either coordinates or chelates the zinc, removing it from the polypeptide or merely rendering it unable to participate in catalysis, or displaces the molecule of water coordinated by the zinc. In addition to the absolutely conserved residues that coordinate the zinc, the active site contains several amino acids thought to be responsible for substrate recognition and binding. These residues are more variable, but still show some degree of functional conservation (Bouvier *et al.*, 1989), leading to both the observed similarities and differences in peptide bond cleavage specificities manifested by the metalloproteinases. These less-conserved substrate binding residues must receive careful consideration in the design of the substrate analogue to which the inhibitory zinc ligand will be attached (Chu and Orlowski, 1984; Vencill *et al.*, 1985; Orlowski *et al.*, 1988; Mookhtiar *et al.*, 1988). In this regard, in the absence of precise three-dimensional coordinates for the active-site residues of PSP, the characterization of its substrate specificity still requires more information. Still, these preliminary results represent an important starting point for the synthesis of more specific inhibitors, and perhaps more convenient fluorogenic or chromogenic substrates (Vencill *et al.*, 1985, Stack and Gray, 1989; Ng and Auld, 1989) which could be used to investigate the role of PSP in the life cycle of *Leishmania*.

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21. Proteinases of trichomonads and *Giardia*

M.J.North

INTRODUCTION

This chapter is concerned with the proteinases of two groups of anaerobic flagellates. *Giardia* and many trichomonads are inhabitants of the gastrointestinal tract, although the major interest among the trichomonads is in two species which infect the urogenital tract and cause sexually transmitted diseases. These are the human parasite *Trichomonas vaginalis* and the cattle parasite *Tritrichomonas foetus*. The two groups have been included in the same chapter for convenience and this does not imply that their proteinases have been found to share features not encountered among other parasitic protozoa. The trichomonad proteinases have been investigated in greater detail than those of *Giardia*, but in both groups it has been found that cysteine proteinases predominate.

PROTEOLYTIC ENZYMES OF TRICHOMONADS

Proteinases

In comparison with many other protozoa, trichomonads have particularly high levels of proteinases. These can cause problems during the isolation and analysis of trichomonad proteins (Alderete and Neale, 1989; North, 1989). The proteinases are active over a wide range of pH, the highest activity towards protein substrates occurring between pH 4 and pH 8. With peptide derivatives as substrates, the optimum pH is normally between 6 and 8. Gelatin-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has revealed that all the species examined to date have multiple proteinases, the majority of which are of the cysteine type (Table 21.1). Recently, however, it has been shown that at least three species have multiple metalloproteinases as well. The latter are optimally active at higher pHs (8–9) than the cysteine proteinases.

Table 21.1 Proteinases of trichomonads detected by gelatin-SDS-PAGE.

Species	Apparent molecular weight (kDa)		Reference
	Cysteine	Metallo-	
<i>Pentatrichomonas hominis</i>	32*, 45*, 52*, 60		Lockwood <i>et al.</i> (1987)
<i>Trichomonas tenax</i>	35, 45*, 56*	76, 87, 102, 270	Bózner and Demeš (1991)
<i>Trichomonas vaginalis</i>	20, 28, 38*, 54*, 68*, 140, 270 76, 86*, 96 (+ up to 15 others)		Lockwood <i>et al.</i> (1987), Neale and Alderete (1990), Bózner and Demeš (1991a), North <i>et al.</i> (1990)
<i>Tritrichomonas augusta</i>	32*, 34, 40*, 43, 45, 48, 60, 84		Lockwood <i>et al.</i> (1987)
<i>Tritrichomonas foetus</i>	18, 20*, 25, 27, 32*, 34, 64, 70, 86, 120		Lockwood <i>et al.</i> (1987), North <i>et al.</i> (1990)
<i>Tritrichomonas mobilensis</i>	18*, 23*, 30	67, 86, 104, 120	Bózner and Demeš (1991), Bózner <i>et al.</i> (1990)

* The most active cysteine proteinases.

Trichomonas vaginalis

This is the species of trichomonad which has been studied in most detail. Coombs (1982) first described high levels of activity which was then shown by electrophoresis in non-denaturing polyacrylamide gels containing haemoglobin to be due to multiple cysteine proteinases (Coombs and North, 1983). Subsequently Lockwood *et al.* (1987), using gelatin-SDS-PAGE, showed that there are at least 11 different cysteine proteinases. All of these enzymes are inhibited by the cysteine proteinase specific reagents E-64 and cystatin (North *et al.*, 1990). Many, including those most active towards gelatin, have apparent molecular weights significantly larger than those of the well characterized cysteine proteinases of plants and animals (see Chapter 14). An even greater complexity has now been demonstrated using two-dimensional gelatin-SDS-PAGE which has resolved as many as 23 distinct proteinases having isoelectric points in the range 5.7–7.0 (Neale and Alderete, 1990). Proteinases with lower isoelectric points have been detected by one-dimensional gelatin-SDS-PAGE following preparative isoelectric focussing using the Bio-Rad Rotofor system (North and Buchan, unpublished). In general, the smaller proteinases are more acidic. Bózner and Demeš (1991) have recently reported two high-molecular-weight enzymes which are inhibited by EDTA but not by cysteine proteinase inhibitors and are thus considered to be metalloproteinases.

The pattern of proteinases detected by substrate-SDS-PAGE shows some variation between isolates (Lockwood *et al.*, 1987; Neale and Alderete, 1990). Comparisons will be helpful in indicating which of the individual enzymes play a role in pathogenicity, for example. Interestingly, a change in proteinase pattern during *in vitro* passage has been observed with some isolates (Neale and Alderete, 1990).

Lockwood *et al.* (1985, 1986) described the purification of two intracellular cysteine proteinases, proteinase D and proteinase H, which differed in their size (M_r 18000 and 64000, respectively, by gel filtration) and isoelectric point (5.2 and 5.9, respectively). Two extracellular cysteine proteinases (see below) have also been purified (Garber and Lemchuk-Favel, 1989). The first of these enzymes had a molecular weight of 60 kDa and could be separated into 23 and 43 kDa subunits, while the second enzyme had a molecular weight of 30 kDa. Antibody raised against the M_r =23000 subunit recognized the M_r =60000 enzyme and both of its subunits but not the M_r =30000 enzyme. Thus it is unlikely that proteinase multiplicity is the result only of differential modification of a single gene product. This also suggested by differences in substrate specificity.

Lysates of *T. vaginalis* are capable of hydrolysing a number of proteins and peptide derivatives. The purified proteinases D and H showed little difference in specificity from one another or from the activity in crude lysates. Among the peptide nitroanilides tested, the best substrate was Bz-Pro-Phe-Arg-Nan (Bz=benzoyl; Nan=nitroanilide) and the preferred substrates were those which combined an arginine at the P_1 position with a bulky residue at the P_2 position (Lockwood *et al.*, 1984, 1986). The extracellular enzymes also preferred Bz-Pro-Phe-Arg-Nan (Garber and Lemchuk-Favel, 1989). However, the recent use of fluorogenic substrates (peptidylamidomethylcoumarins) in combination with gelatin-SDS-PAGE (North *et al.*, 1990) has allowed details of the substrate specificity of individual enzymes to be determined without the need for purification. Based on differences in specificity three groups of *T. vaginalis* proteinase have been defined:

1. an M_r =86000 enzyme which only hydrolysed Z-Arg-Arg-NHMec (Z, *N*-benzyloxycarbonyl; NHMec, 7-amido-(4-methylcoumarin);
2. a M_r =54000 enzyme with preferential activity towards Z-Phe-Arg-NHMec; and
3. the remaining enzymes which all preferred substrates with bulky residues at both the P_2 and P_5 positions and accommodated arginine, lysine or tyrosine at the P_1 position.

The best substrates for the third group were Boc-Val-Leu-Lys-NHMec (Boc, *N*-tertbutyloxycarbonyl) and Leu-Val-Tyr-NHMec. Differences between these three groups of enzyme were also apparent from their sensitivity to cysteine proteinase inhibitors such as E-64, 2-Phe-Phe-CHN₂ (CHN₂, diazomethane) and Z-Phe-Ala-CHN₂ (North *et al.*, 1990). For example, the M_r =86000 Z-Arg-Arg-NHMec specific enzyme was much less sensitive to E-64 than the other enzymes.

Tritrichomonas foetus

Proteinase activity in *T. foetus* was first reported by Müller (1973) in a study of lysosomal hydrolases. Lockwood *et al.* (1984, 1987) showed that the activity is due to multiple cysteine proteinases. Those with highest activity towards gelatin were of low apparent molecular weight which distinguishes them from the *T. vaginalis* proteinases. All of the enzymes are inhibited by E-64 and cystatin, although there

are differences in their sensitivity to E-64 (North *et al.*, 1990). The enzymes have isoelectric points in the range 4.1–5.2 (North and Buchan, unpublished).

McLaughlin and Müller (1979) purified a cysteine proteinase with a molecular weight of about 20 kDa which hydrolysed a number of proteins and arginine derivatives. *T. foetus* lysates can hydrolyse a range of peptide nitroanilides (Lockwood *et al.*, 1984), the specificity being similar to that of *T. vaginalis*. More detailed analysis of the specificity of individual enzymes (North *et al.*, 1990) has shown that the two most active enzymes (M_r 20000 and 32000) have similar specificities to one another, preferring substrates with bulky residues at P₂ and P₃. In this respect they resemble many of the *T. vaginalis* proteinases. Three enzymes (M_r of 25000, 27000 and 34000) which are predominantly extracellular (see below) have a very narrow specificity hydrolysing only Z-Arg-Arg-NHMec.

Trichomonas tenax

T. tenax is common inhabitant of the human mouth which may be associated with periodontal disease and pulmonary infections. Collagenolytic activity was reported by Ribaux *et al.* (1980, 1981). Bózner and Demeš (1991b) have now demonstrated multiple metallo- and cysteine proteinases.

Pentatrichomonas hominis

Like all other trichomonads, this non-pathogenic inhabitant of the human gut contains multiple cysteine proteinases (Lockwood *et al.*, 1987).

Tritrichomonas augusta

In the original studies this species was incorrectly described as being *Trichomitus batrachorum* which, like *T. augusta*, inhabits the intestine of the leopard frog (Lockwood *et al.*, 1984, 1987). Although its activity towards some proteins and peptide nitroanilides is similar to that of *T. vaginalis* and *T. foetus*, its gelatin-SDS-PAGE proteinase pattern is less complex and the enzymes are less active towards gelatin.

Tritrichomonas mobilensis

This species was first discovered in the intestine of the squirrel monkey and is of interest because it is invasive in its natural host as well as in experimental animals. Three cysteine proteinases have been demonstrated (Bózner *et al.*, 1990) and four higher molecular weight metalloproteinases (Bózner and Demeš, 1991a).

Monocercomonas sp.

Proteinase activity has been detected in this primitive trichomonad (Lindmark and Müller, 1974), but it has not been characterized.

Exopeptidases

There have been few reports on exopeptidase activity in trichomonads. Lockwood (1987) described activity towards Z-Gly-Leu, Z-Glu-Tyr and Z-Leu-Tyr in lysates of *T. vaginalis*, *T. foetus* and *T. augusta* which was probably due to carboxypeptidases. For each species the best substrate was Z-Gly-Leu and activity was optimal at about pH 6.0.

Aminopeptidase activity has been detected in *T. vaginalis*, *T. foetus*, *T. augusta* and *P. hominis* using nitroanilide derivatives of alanine, S-benzylcysteine, glycine, leucine and proline (Lockwood, 1987). In *T. vaginalis*, activity with L-alanine-4-nitroaniline was significantly higher than with any of the other substrates and was also higher than the equivalent activity in any of the other three species. In these other species, similar activities on each of the five substrates were found. McLaughlin and Müller (1979) had previously reported an aminopeptidase activity which hydrolysed Gly-Phe-2Nap (2Nap=2-naphthylamide) in *T. foetus*. Its subcellular distribution was identical to that of *N*-acetyl- β -D-glucosaminidase, a marker for the large hydrolase-containing organelles.

Nadler and Honigberg (1988) used a Leu-Gly-Gly peptidase in a study of biochemical polymorphism among trichomonads. Peptidases were detected in *T. vaginalis*, *T. gallinae*, *Tetratrichomonas gallinarium*, *T. foetus* (in only one of five strains tested) and *P. hominis*.

PROTEINASE SUBCELLULAR LOCALIZATION AND RELEASE

Subcellular localization

Most of the proteinase activity in *T. vaginalis*, *T. foetus* and *Monocercomonas* sp. has been shown to be located in lysosome-like particles which contain other hydrolases (Müller, 1973; Landmark and Müller, 1974; Lockwood *et al.*, 1988). Separation of the particle fractions of *T. vaginalis* and *T. foetus* by isopycnic centrifugation on Percoll gradients has revealed heterogeneity among the lysosomal population and differences in the distribution of proteinase activity. In *T. vaginalis* activity towards azocasein is present in both light (density=1.035 g cm⁻³) and heavy (density=1.05 g cm⁻³) fractions, whereas activity towards Hide Powder Azure is present only in the lighter fraction. However, no differential distribution of the proteinase detected by gelatin-SDS-PAGE has been demonstrated.

Neale and Alderete (1990) have recently presented evidence that a M_r =43000 proteinase of *T. vaginalis* resides on the cell surface. Its activity can be removed by treatment of live trichomonads with pronase or proteinase K.

Release of proteinase activity

During axenic growth *in vitro*, large quantities of proteinases are released into the growth medium by *T. vaginalis* and *T. foetus* (Lockwood *et al.*, 1987, 1988), and in

late exponential cultures extracellular activity can account for at least 50 per cent of the total activity in a culture. Much less proteinase activity is secreted by *T. augusta*, which initially suggested that the process may be of especial importance in the urogenital parasites. Proteinase release has, however, now been demonstrated in the gastrointestinal trichomonads *T. mobilensis* (Bózner *et al.*, 1990) and *T. tenax* (Bózner and Demes, 1991a). In both *T. vaginalis* and *T. foetus*, the process occurs continuously throughout growth and is accompanied by the release of other hydrolytic enzymes such as *N*-acetyl- β -D-glucosaminidase, acid phosphatase and *a*-mannosidase (Lockwood *et al.*, 1988; North and Buchan, 1990). Cells transferred to fresh medium continue to release proteinases (North *et al.*, 1989), and the process is not affected by the omission of serum from the medium. Transferring cells to serumfree medium provides a convenient system for investigating the factors affecting enzyme release and is also useful for obtaining material for the purification of extracellular enzymes. Indeed, Garber and Lemchuk-Favel (1989) have described the purification of two extracellular cysteine proteinases of *T. vaginalis* from serumfree medium (see above).

All of the extracellular proteinases detected to date are cysteine proteinases, as indicated by their inhibitor sensitivity. In *T. tenax*, which has both cysteine and metalloproteinases, only cysteine proteinases can be detected in the medium (Bózner and Demes, 1991b). The most likely explanation is that there is differential release of proteinase types, although the possibility that metalloproteinases are released but are then inactivated cannot be ruled out at present. Differences are also apparent between the intracellular and extracellular cysteine proteinases indicating that the various forms are differentially released (Lockwood *et al.*, 1987; North *et al.*, 1990). A comparison of *T. vaginalis* proteinases separated by isoelectric focussing has revealed a lower M_r form which has a high isoelectric point and is found only in extracellular samples (North and Buchan, unpublished observations). In *T. foetus*, a M_r 34000 enzyme appears to be almost exclusive to the medium, while two others, of M_r 25000 and 27000, are at significantly higher levels outside than inside the cells. These three *T. foetus* enzymes have a narrow specificity towards peptidyl amidomethylcoumarins, hydrolysing only Z-Arg-Arg-NHMec (North *et al.*, 1990). The extracellular *T. foetus* proteinases apparently have higher isoelectric points than their intracellular counterparts (North and Buchan, unpublished).

The release of hydrolases may be linked to a specific subpopulation of lysosomes. In *T. vaginalis*, there is some correlation between the extent to which some hydrolases, including proteinases are released and their presence in the higher density lysosomal fraction (Lockwood *et al.*, 1988).

TOWARDS AN UNDERSTANDING OF PROTEINASE FUNCTION IN TRICHOMONADS

The especially high levels of proteinase activity in trichomonads and the continuous release from the cells suggests that proteolysis is likely to be important in these organisms. Possible roles include the utilization of host proteins for nutrition (for

example the degradation of human low density lipoprotein by *T. vaginalis* has been demonstrated (Peterson and Alderete, 1984), the destruction of components of the host immune system (lysates of *T. vaginalis* will degrade IgA1 (Parenti, 1989)), the release of amino acids for amine production to counter the low pH of the vagina, and direct effects on the host cells to assist cytadherence. As *T. mobilensis* is invasive, there is a possible role for proteinases in tissue penetration. Evidence which supports the view that proteinases are of importance has largely come from studies of the effects of proteinase inhibitors on *T. vaginalis*.

In axenic medium *in vitro*, the cysteine proteinase inhibitors Z-Phe-Ala-CHN₂ and Z-Phe-Phe-CHN₂ inhibit growth, reducing the number of parasites of both *T. vaginalis* and *T. foetus*. The latter species is the more sensitive, all parasites being killed by Z-Phe-Phe-CHN₂ at a concentration of 100 µg ml⁻¹ (North *et al.*, 1990). However, neither antipain nor leupeptin at concentrations up to 100 µg ml⁻¹ inhibited the growth of *T. vaginalis* (Coombs and North, 1983; Bremner *et al.*, 1986), although both are inhibitors of endogenous cysteine proteinases. These differences may be related to variations in the ability of the inhibitors to enter the cells. The lack of effect of leupeptin and antipain would appear to rule out extracellular proteolysis as being important during axenic growth.

Tests using a number of model systems have shown that proteinase inhibitors do affect the interaction of *T. vaginalis* with mammalian cells (Bremner *et al.*, 1986; Silva Filho and de Souza, 1988; Arroyo and Alderete, 1989; Honigberg, 1989). Recently, similar effects have been reported for *T. foetus* (Burgess *et al.*, 1990). Most of the inhibitors, which included leupeptin, TLCK and TPCK, do inactivate the endogenous cysteine proteinases. However, none of them is absolutely specific for cysteine proteinases and effects on as yet unknown serine proteinases cannot be ruled out entirely. Tests with more specific cysteine proteinase inhibitors will be required. The results certainly support a role for proteinases in the host-parasite interaction. Arroyo and Alderete (1989) have suggested that a surface proteinase is necessary for parasite adherence to epithelial cells.

A number of workers have described diffusible factors released by *T. vaginalis* which have cytopathic effects on mammalian cells, although a soluble cytotoxin has not been found in all studies (see Garber *et al.*, 1989; Honigberg, 1989). The possibility that such a factor might be a proteinase has been considered (Gentry *et al.*, 1985; Honigberg, 1989; Lushbaugh *et al.*, 1989) but not demonstrated. Garber *et al.*, (1989) found a glycoprotein cell-detaching factor which had an effect resembling that of trypsin. Its molecular weight (200 kDa), however, was higher than that of any of the extracellular proteinases and neither of the purified extracellular proteinases was similar to the factor (Garber and Lemchuk-Favel, 1989). There is evidence, however, that proteinases are released *in vivo*. Trichomonad proteinases have been detected in vaginal washouts of mice infected intravaginally with *T. vaginalis* (Lockwood *et al.*, 1987). As yet antibodies against trichomonad proteinases have not been found in antisera from trichomoniasis patients (Garber and Lemchuk-Favel, 1989), although the enzymes are antigenic (Garber and Lemchuk-Favel, 1989; Neale and Alderete, 1990; Lockwood and North, unpublished).

A need for proteinases during infection has been indicated in a mouse model

system. The appearance of subcutaneous lesions in mice infected with *T. vaginalis* was delayed if leupeptin was administered (Bremner *et al.*, 1986). The most effective dose was 50 mg per kg body weight administered intravenously. This is the only demonstration to date of an effect of proteinase inhibitor on a protozoa *in vivo*.

PROTEINASES OF TRICHOMONADS—CONCLUSIONS

Although it is clear from the inhibitor studies that proteinases do have a role in the interaction between the trichomonads and their hosts, the role of individual enzymes remains to be determined. Comparison of proteinase patterns of different species (see Table 21.1) does not immediately indicate which proteinases might be required for adaptation to a given environment or which are involved in pathogenicity. The patterns of the urogenital trichomonad *T. foetus* and the gastrointestinal parasite *T. mobilensis* appear similar. There are also similarities between the proteinase patterns of the oral trichomonad *T. tenax*, which may be pathogenic, and the intestinal parasite *P. hominis* which is not. The one pattern which is significantly different is that of *T. vaginalis*; this has a greater number of cysteine proteinases, many of which are apparently atypically large. Further analyses of the different proteinase forms using both biochemical and molecular biology approaches will provide valuable insights into proteinase structure-function relationships, the basis of the multiplicity and the features required for enzyme release. With regard to the molecular biology approach, cysteine proteinase gene fragments have already been prepared from *T. vaginalis* and *T. foetus* by PCR amplification, and the initial analyses indicate that multiple genes are present in both species (North and Ferguson, unpublished observations). This supports the view that the multiple enzyme forms are not all the result of post-translational modifications. Nothing is known about the mechanism of enzyme secretion in trichomonads and further studies of the proteinases will be important in establishing details of the pathway involved and in the targeting mechanism.

The results of testing proteinase inhibitors both *in vitro* and *in vivo* have been promising and show that there are prospects of being able to use proteinases as targets for drugs or prodrugs. While the multiplicity of trichomonad proteinases provides a major challenge with respect to establishing which particular enzyme has which function, the large number of enzymes increases the possibility of finding proteinases which are sufficiently different from the host enzymes to be exploited.

PROTEINASES OF GIARDIAS

Relatively little is known about the proteinases of giardias. Lindmark (1988) demonstrated proteinase activity in *Giardia lamblia* using urea-denatured haemoglobin and Z-Arg-2Nap as substrates. After differential and isopycnic centrifugation in sucrose gradients, the activities were located in particle fractions which contained other hydrolases. The enzyme displayed latency and were tightly

bound to the organelle membrane. Parenti (1989) has also demonstrated proteinase activity that is membrane-bound or associated with subcellular particles. The enzyme, which has a molecular weight of 38 kDa by gel filtration, cleaves IgA1 between the CH₂ and CH₃ domains and so differs from bacterial IgA proteinases which are metalloproteinases and cleave IgA1 only at the hinge region. The proteinases of *G. lamblia* trophozoites are primarily of the cysteine type (Hare *et al.*, 1989; Parenti, 1989), being activated by DTT and inhibited significantly by iodoacetamide, TPCK, TLCK, leupeptin and chymostatin. In general, inhibitors specific to other classes of proteinases have no effect, although PMSF does decrease the activity towards some substrates.

Gelatin-SDS-PAGE analysis has revealed two proteinases in trophozoites of *G. lamblia* (Portland 1 strain). These have apparent molecular weights of 40 and 105 kDa (Hare *et al.*, 1989). The former is most active in the pH range 6–7, the latter at slightly lower pHs. It is not known whether the $M_r=38000$ enzyme described by Parenti (1989) is the same as the $M_r=40000$ enzyme. A group of lower activity proteinases with apparent molecular weights in the range 80–95 kDa were also described. All have properties expected of cysteine proteinases. All strains examined have a major $M_r=105000$ proteinase (Jarroll *et al.*, 1989).

Proteinases with molecular weights of 90 and 61 kDa have been detected in *G. muris* cysts (Jarroll *et al.*, 1989).

Some release of proteinase activity has been reported (Parenti, 1989), but the evidence for proteinase secretion is not considered to be definitive.

CONCLUSIONS

Insufficient data are available to draw any firm conclusions about the role of proteinases in *Giardia*. It is likely that proteolysis will be required during the transformation from cyst to trophozoite and during encystment. It is possible that proteinases contribute to pathogenesis, while the ability to degrade IgA1 may reflect an involvement in countering host defences. This may only be likely, however, if the proteinases are secreted and more work is clearly required to establish whether or not this is the case.

Further analysis of the cysteine proteinases themselves should prove very revealing. Those cysteine proteinases in other flagellates whose sequences are known (see Chapters 17 and 19) are, in spite of some unique features, fairly closely related to mammalian cathepsin L. On the basis of small subunit ribosomal RNA sequencing, *G. lamblia* appears to have diverged early in the evolutionary history of eukaryotes and it will be interesting to discover the extent to which the *Giardia* cysteine proteinases reflect this early divergence. It is possible that divergence occurred before the evolution of the papain superfamily of cysteine proteinases, members of which have only been found in eukaryotes. Large differences between the parasite and host proteinases should make it easier for the enzymes to be exploited as targets for antigiardial drugs.

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22. The cysteine proteinase of *Entamoeba histolytica*: cloning of the gene and its role as a virulence factor

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INTRODUCTION

One of the major questions in the pathogenesis of amoebiasis has been what determines whether a specific strain or isolate of *Entamoeba histolytica* will produce a pathogenic or non-pathogenic infection. The biological basis of virulence appears to be multifactorial, and four parasite-derived factors have been proposed as virulence factors.

Adhesion molecules, such as the amoeba lectin, mediate attachment of trophozoites to target cells or matrix (Talamas-Rohana and Meza, 1988; Petri *et al.*, 1989). Cell attachment appears to be a necessary first step in cytolysis (Petri *et al.*, 1989). An ion channel or 'amoebapore' has been identified (Young and Cohn, 1986; Rosenberg *et al.*, 1989), but as yet no sequence data are available to shed light on exactly how it functions or whether it is related to other eukaryotic ion channels. Two types of proteinases have also been identified in virulent strains of *E. histolytica*. A membrane-associated metallocollagenase has been proposed to play a role in degradation of extracellular matrix molecules during invasion of *E. histolytica* into the wall of the intestine (Muñoz *et al.*, 1982). A secreted cysteine proteinase has been shown to produce the cytopathic effect (Lushbaugh *et al.*, 1984; Keene *et al.*, 1986; Luaces and Barren, 1988), degrade important host extracellular matrix molecules (Lushbaugh *et al.*, 1984; Keene *et al.*, 1986; Scholze and Schulte, 1988), and activate the alternative pathway of complement (Reed *et al.*, 1989a). It is this latter molecule which is the focus of this review.

ASSOCIATION OF CYTOPATHIC ACTIVITY AND EXPRESSION OF THE CYSTEINE PROTEINASE

It has been known for many years that there is an association between expression of the cysteine proteinase activity by virulent strains of *E. histolytica* and some aspects of the cytotoxic or cytopathic effect of virulent trophozoites. Lushbaugh *et al.* (1984) identified a 'cytotoxin' in virulent *E. histolytica* strains that produced destruction of tissue culture monolayers. During the course of purification of this cytotoxin, it was found that cysteine proteinase activity co-purified with it. Keene *et al.* (1986) purified the major secreted proteinase species, a 56 kDa cysteine proteinase, from HM-1 trophozoites. The purified enzyme could mimic the cytopathic effect on monolayers of BHK cells. The potential role of this enzyme in producing destructive lesions in the intestinal wall was suggested by its ability to degrade important extracellular matrix macromolecules such as fibronectin and collagen. Its ability to degrade anchoring proteins such as fibronectin and laminin suggested the basis for its action on tissue culture cell monolayers (Keene *et al.*, 1986).

Recently, the association between expression of cysteine proteinase activity and the cytopathic effect was confirmed by two different types of studies. In the first, a series of mutant clones derived from HM-1 parent strain were assayed for both production of the cytopathic effect and proteinase activity (Keene *et al.*, 1990). It was observed that deficiency in expression of cysteine proteinase activity correlated with deficiency of the production of the cytopathic effect. While these results were in keeping with the hypothesis that the proteinase plays an important role in the cytopathic effect of *E. histolytica*, they do not rule out the possibility that other factors were also deficient in these mutants. To study the role of the cysteine proteinase in the cytopathic effect more directly, live HM-1 trophozoites were incubated with a tissue culture monolayer in the presence and absence of a specific inhibitor of the cysteine proteinase, Z-Phe-Ala-CH₂F (Z, N-benzyloxycarbonyl; CH₂F, fluoromethyl ketone). This is a potent, irreversible inhibitor of cathepsin B like enzymes which has minimal toxicity to cells or animals (Smith *et al.*, 1988). It inhibited the cytopathic effect of HM-1 trophozoites in a dose-dependent manner. At an inhibitor concentration of 40μM, the cell monolayer was essentially intact (Keene *et al.*, 1990). This latter study indicates that the cysteine proteinase is a necessary, if not sufficient, factor for production of the cytopathic effect. There are two models of its action that are compatible with these data. In the first, the cysteine proteinase is itself the factor destroying tissue culture monolayers. In the second, it activates other factors, such as the amoebapore, which then produce cytolysis.

CORRELATION OF EXPRESSION OF CYSTEINE PROTEINASE AND PATHOGENICITY OF *E. HISTOLYTICA*

Several investigators have noted the correlation between expression of the cysteine proteinase activity and virulence of laboratory strains of *E. histolytica* (reviewed in Keene *et al.*, 1986). As a more direct test of the importance of this enzyme in clinical

infections, we examined cysteine proteinase expression in 20 patients with varying clinical severity of amoebiasis (Reed *et al.*, 1989b). All 10 patients with severe amoebic disease (colitis or liver abscess) showed high level expression and secretion of the 56 kDa proteinase. Nine of the 10 patients with mild or asymptomatic disease did not secrete the 56 kDa thiol proteinase detectable by gelatin gel electrophoresis, but some proteinase activity was detected by a more sensitive assay. This is the first demonstration of the correlation of a virulence factor, identified in laboratory strains, with pathogenicity of clinical isolates. To substantiate this association, we examined the serologic response of patients with varying clinical severity of amoebiasis to purified cysteine proteinase by ELISA (Reed *et al.*, 1989b). If the cysteine proteinase is secreted by *E. histolytica*, and if it is immunogenic, one would expect patients with severe disease to have an antibody response, while none would be detected in those patients with mild or asymptomatic infections. We found that 83 per cent of patients with colitis or extraintestinal disease had a significant antibody response, whereas those with asymptomatic infections or mild diarrhoea were similar to controls (normal healthy individuals or patients with ulcerative colitis, Crohn's disease, or hepatocellular carcinoma) (Reed *et al.*, 1989b). Correlation of an antibody response to the purified proteinase with clinical severity of disease supports the hypothesis that this enzyme is an important virulence factor in amoebiasis and provides a means of assessing whether a patient is infected with a potentially pathogenic strain. Our success at cloning the gene for this enzyme (see below) also opens up the possibility for future use of recombinant antigen as a serodiagnostic test for pathogenicity.

SECRESSION OF THE CYSTEINE PROTEINASE AND ADHESION

Another intriguing question is whether expression and/or secretion of the cysteine proteinase is related to adherence of trophozoites to target cells. It is conceivable that the cysteine proteinase is part of an armamentarium of virulence factors involved in cytolysis, and that the trigger for secretion is adherence via surface molecules like the amoeba lectin. This hypothesis is in part supported by the observation that concanavalin A binds to virulent trophozoites and causes increased secretion of the cysteine proteinase (M.Brown, unpublished data). However, more work needs to be done to unravel the exact connection between adherence and cysteine proteinase release. In one mutant strain of HM-1, L6, cysteine proteinase expression is deficient while adherence is not affected (Keene *et al.*, 1990). This suggests that if a signal exists between adherence and cysteine proteinase release, the signal has been uncoupled in this mutant.

CLONING OF THE GENE FOR THE CYSTEINE PROTEINASE

Purification and characterization of the major cysteine proteinase of virulent *E. histolytica* by Keene *et al.* (1986), Luaces and Barrett (1988) and Scholze and

Schulte (1988; and see Chapter 23) confirmed the enzyme has a similar substrate specificity and inhibition profile to mammalian cathepsin B. To analyse further the structure of this enzyme and to provide a tool for studying the regulation of its expression in virulent and non-virulent strains, we have isolated a gene fragment using generic molecular primers based upon conserved eukaryotic cysteine proteinase sequences (Eakin *et al.*, 1990). This 450 base pair fragment represents approximately 70 per cent of the coding region of the proteinase. The predicted amino acid sequence shows 45 per cent homology to chicken cathepsin L and 30–40 per cent homology to other eukaryotic cathepsin B or L proteinases including those from *Dictyostelium discoideum*, *Trypanosoma cruzi* and rat (Eakin *et al.*, 1990). Preliminary studies by Southern blot suggest that a 2–2.3 kb restriction fragment carrying this gene is present only in pathogenic strains (Bouvier *et al.*, in press). This restriction fragment linked polymorphism may correlate with the enhanced expression and secretion of the proteinase by pathogenic strains. Based on biochemical assays of clinical isolates (Reed *et al.*, 1989b), we hypothesized that another gene must be present in non-pathogenic strains. Utilizing sequence information from the amino terminus of the first cysteine proteinase gene, we used a second set of PCR primers to isolate a 550 base pair fragment representing a second cysteine proteinase gene. Southern blot analysis with this fragment showed a restriction pattern quite distinct from that of the first gene, and there is hybridization of this fragment to DNA from both pathogenic and non-pathogenic strains. This gene shows near identity to that isolated by Henning Scholze (see Chapter 23). Taken together, the results in both laboratories suggest that there is one cysteine proteinase gene, present in both pathogenic and non-pathogenic amoebae, that most likely functions as a basic metabolic enzyme. A second gene, present in a 2.2 kb *EcoR*1 fragment, is found only in pathogenic amoeba and, therefore, correlates with the enhanced proteinase expression and secretion in pathogenic amoeba.

E. HISTOLYTICA CYSTEINE PROTEINASE AS TARGET FOR DRUG DESIGN

Our observation that a fluoromethyl ketone derivated peptide inhibitor arrested the cytopathic effect of live trophozoites (Keene *et al.*, 1990), suggested that the cysteine proteinase may be an attractive target for novel chemotherapy because of its key role in pathogenesis. Furthermore, we hypothesized that the enzyme must play another vital role for the organism because invasion is not an adaptive behaviour for *E. histolytica*.

What is the adaptive advantage of enhanced proteinase expression for pathogenic strains? We reasoned it must revolve around another role the enzyme plays in amoeba metabolism. There is no evidence that trophozoites which invade ever encyst and leave the host again. In other words, invasion is a ‘dead-end street’. Cysteine proteinases of some bacteria are secreted into the periplasmic space and are thought to play a role in digestion of protein in the environment for enhanced intracellular transport (Baker and Drenth, 1987). In *E. histolytica* we have localized

the proteinase of both pathogenic and non-pathogenic strains to endosome-like vesicles found in abundance in the amoeba cytoplasm. In a model akin to lysosomal targetting of cathepsin B and L, we feel the enzyme is 'normally' transported to the endosomes for intracellular protein degradation. With expression of both of the cysteine proteinase genes in pathogenic amoebae, this endosomal targetting system may be overloaded, resulting in secretion by the default pathway. Alternatively, there may be a biochemical difference in the pathogenic enzyme that makes secretion more likely, or it may be released by exocytosis because of the enhanced phagocytosis noted in pathogenic strains (Orozco *et al.*, 1988). In either case, we have observed that culture of laboratory strains of *Entamoeba* in the presence of micromolar quantities of Z-Phe-Ala-CH₂F ultimately results in decreased replication. The effect is seen after 48 h of culture in a rich medium (M.Brown and J.McKerrow, unpublished results). We interpret this as a slow 'starvation' of the amoeba, due to inhibition of the endosomal cysteine proteinase.

In future studies, we wish to test the effect of inhibitor on animal models of disease. The combination of inhibiting the cytopathic and histolytic effects with inhibiting a key metabolic enzyme may significantly decrease morbidity.

MULTIPLE FORMS OF THE CYSTEINE PROTEINASE

The final issue currently under study at our laboratory is the relationship between the different cysteine proteinase species identified by different investigators in HM-1 trophozoites (Lushbaugh *et al.*, 1984; Keene *et al.*, 1986; Luaces and Barrett, 1988; Scholze and Schulte, 1988; and see Chapter 23). Keene *et al* (1986) showed by gelatin substrate gel electrophoresis that the major secreted species is 56 kDa under non-reducing conditions. Luaces and Barrett (1988) purified a 27 kDa species from extracts of *E. histolytica*. Other investigators have identified cysteine proteinase species ranging in molecular weight from 16 to 70 kDa (Lushbaugh *et al.*, 1984; Keene *et al.*, 1986; Luaces and Barrett, 1988; Scholze and Schulte, 1988). Our working hypothesis is that these multiple species represent different forms of the products of the two cysteine proteinase genes. In our hands, trophozoite conditioned medium, as well as extracts of *E. histolytica*, show three major species of 56, 45, and 27–30 kDa. We have shown, by amino terminal sequence analysis, that the 27–30 kDa species is identical to that purified by Luaces and Barrett (1988). We are currently analysing the higher molecular weight species to determine if these represent multimers of a 27-kDa subunit or active proenzyme species like those identified in secretions of invasive tumour cells (Sloane *et al.*, 1987; Troen *et al.*, 1988).

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23. Amoebapain, the major proteinase of pathogenic *Entamoeba histolytica*

H.Scholze

INTRODUCTION

The major proteolytic enzyme isolated from soluble fractions of the pathogenic strain HM1: IMSS of *Entamoeba histolytica* exhibits the properties of a typical cysteine proteinase (Scholze and Schulte, 1988). It has a molecular weight of 27 kDa, an isoelectric point of about 5, an optimal activity against azocasein in a mildly acidic range with maximum activity at pH 4.5. The N-terminal amino acid sequence determined by automated Edman degradation (Schulte and Scholze, 1989) shows homology to papain and cathepsins B, H and L, as well as to another cysteine proteinase isolated from *E. histolytica*, histolysin (Luaces and Barrett, 1988). It is not yet clear whether the two amoebic proteinases are identical; however, in view of the homology of the enzyme described here to the papain-like cysteine proteinases we propose to name it amoebapain. Since amoebapain is active against native type I collagen from human skin as well as against the extracellular matrix proteins fibronectin and laminin, it has properties consistent with it being a cytotoxin.

At least 85 per cent of the overall proteolytic activity of cell homogenates of *E. histolytica* can be abolished by polyclonal antibodies against purified amoebapain. Since the existence of proteinases of other classes, i.e. a pepsin-like enzyme (McLaughlin and Faubert, 1977) or a specific collagenase localized on the surface of the cells (Muñoz *et al.*, 1982), have not been confirmed, it has to be concluded that amoebapain or other closely related cysteine proteinases represent the cytotoxin with proteolytic activity reported by other workers (Lushbaugh *et al.*, 1979).

LOCALIZATION AND PROCESSING OF AMOEBApAIN

When living amoebae are incubated in a solution containing the synthetic peptide analogue Arg-Arg-M β NA (M β NA, 4-methoxy- β -naphthylamide) and the fluorogenic coupling reagent 5-nitrosalicylaldehyde and viewed under a fluorescence microscope, a granular fluorescence distributed over the whole cell is observed (De Meester *et al.*, 1990). Immunostaining of semi-thin sections of fixed

cells with antiproteinase antibodies and FITC-labelled secondary antibodies gave fluorescence exclusively within vesicles. An electronoptical analysis of ultrathin sections after antiproteinase coupling and immunogold staining confirmed this finding and also suggested that the proteinase was not randomly distributed: rather it appeared to be associated with some subvesicular, matrix-like structures (Löhden-Bendiger *et al.*, in press). Considering that amoebapain appears to be a typical lysosomal enzyme, its biosynthesis and transport to vesicles might be expected to proceed in a similar fashion to that in higher eukaryotes (Figura and Hasilik, 1986). The amoebic proteinase does, however, lack the typical characteristics of a glycoprotein. In pulse-chase experiments, a radioactive band of 38 kDa can be visualized in SDS-PAGE after immunoprecipitation with antiproteinase antibodies. This putative proform of amoebapain is associated with the membrane fraction and is substituted by the putative mature form after a 60-min chase. It remains to be established whether this processing requires a specific peptidase such as cathepsin D, as suggested to occur in mammals by Nishimura *et al.* (1988), or is a consequence of autoproteolysis. Preliminary investigations have shown that the presence during biosynthesis of pepstatin, a specific inhibitor of aspartic proteinases like cathepsin D, apparently does not influence processing.

ANALYSIS OF AMOEBApAIN GENES

Screening of a λ gt11 cDNA library from the pathogenic strain HM1: IMSS with antiproteinase antibodies has allowed the identification of several clones coding for the pre-pro-sequence of the cysteine proteinase. These reveal a hydrophobic leader sequence and a stretch of about 80 amino acids N-terminal to the mature enzyme (Tannich *et al.*, 1991). A homologous clone was found in non-pathogenic *E. histolytica* whose predicted amino acid sequence deviated by 16 per cent from that of the pathogenic isolate. Genomic DNA of pathogenic and non-pathogenic *E. histolytica*, digested with different restriction enzymes, showed characteristic hybridization patterns with a cDNA probe derived from the pathogenic strain. Multiple hybridizing fragments were found with the DNA of the pathogenic isolate, but only one or two fragments hybridized with DNA from the non-pathogenic isolates. The hybridization pattern was conserved within each group of isolates tested, and neither of the genes was found in both pathogenic and non-pathogenic isolates. In addition, Northern blot experiments showed that there was a significantly higher amount of mRNA encoding the proteinase in pathogenic strains compared to non-pathogenic ones. These findings not only argue for an important role of the proteinase in the cytopathogenic process, but also confirm that pathogenic and non-pathogenic *E. histolytica* are genetically distinct (Tannich *et al.*, 1991).

STRUCTURAL AND FUNCTIONAL CONCLUSIONS FROM THE PRIMARY SEQUENCE OF AMOEBApAIN

From the sequence data, amoebapain has a predicted molecular mass of 24.1 kDa. It is 34 per cent homologous to papain (Drenth *et al.*, 1968), 40 per cent to rat cathepsin L (Ishidoh *et al.*, 1987), 42 per cent to an amoebic sequence corresponding to a cysteine proteinase that was obtained by the polymerase chain reaction (Eakin *et al.*, 1990), and 45 per cent to cysteine proteinase 2 from *Dictyostelium discoideum* (Pears *et al.*, 1985). Considering this and the other molecular and enzymological properties the proteinase can, therefore, be classified as a member of the papain superfamily. The deduced amino acid sequence for the proteinase from the non-pathogenic strains shows that it deviates at positions 3 and 4 from amoebapain, but that the residues at these positions are identical with those of histolysin (Luaces and Barrett, 1988) and of a 27 kDa *E. histolytica* proteinase which is only 47 per cent homologous to amoebapain (McKerrow, personal communication; and see Chapter 22).

The substrate specificity of amoebapain has been determined in detail, using many synthetic substrates, and oxidized insulin B chain and the $\alpha 1$ -CB2-peptide of human type I collagen (Scholze *et al.*, 1986, Schulte *et al.*, 1987, Scholze and Schulte, 1988, Otte and Werries, 1989, Otte, 1990) (Figure 23.1). Whereas the insulin chain is cleaved at multiple sites with a strong preference for the Gly-Phe bond that is preceded by an arginine, the collagen peptide contains only one susceptible peptide bond. The bond is between Gly and Leu and also contains an arginine in the P₂ position. More strikingly, with small synthetic substrates the preference for arginine in the P₂ position becomes absolute, and with these substrates the proteinase is able to act both as dipeptidyl aminopeptidase and as peptidyl carboxydiptidase.

For papain-like cysteine proteinases the primary substrate specificity is determined by the nature of their S₂ subsite (Baker and Drenth, 1987). Based on the assumption that the cysteine proteinases all have similar extended active sites, the behaviour of amoebapain can be interpreted in light of the amino acid sequence of the active site. According to the molecular model of papain determined by Drenth *et al.* (1968) the molecule consists of two domains, R and L, which are divided by a deep cleft that builds up the active site. The active site Cys25 is contributed by the L

Insulin B-chain	...-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-...
Insulin B-chain (21-25)	Glu-Arg-Gly-Phe-Phe
Insulin B-chain (22-26)	Arg-Gly-Phe-Phe-Tyr
Insulin B-chain (22-25)	Arg-Gly-Phe-Phe
Type I collagen, $\alpha 1$-CB2-peptide (9-12)	Arg-Gly-Leu-Hyp
Type I collagen, $\alpha 1$-CB2-peptide	...-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-...

Figure 23.1. Substrate specificity of amoebapain.

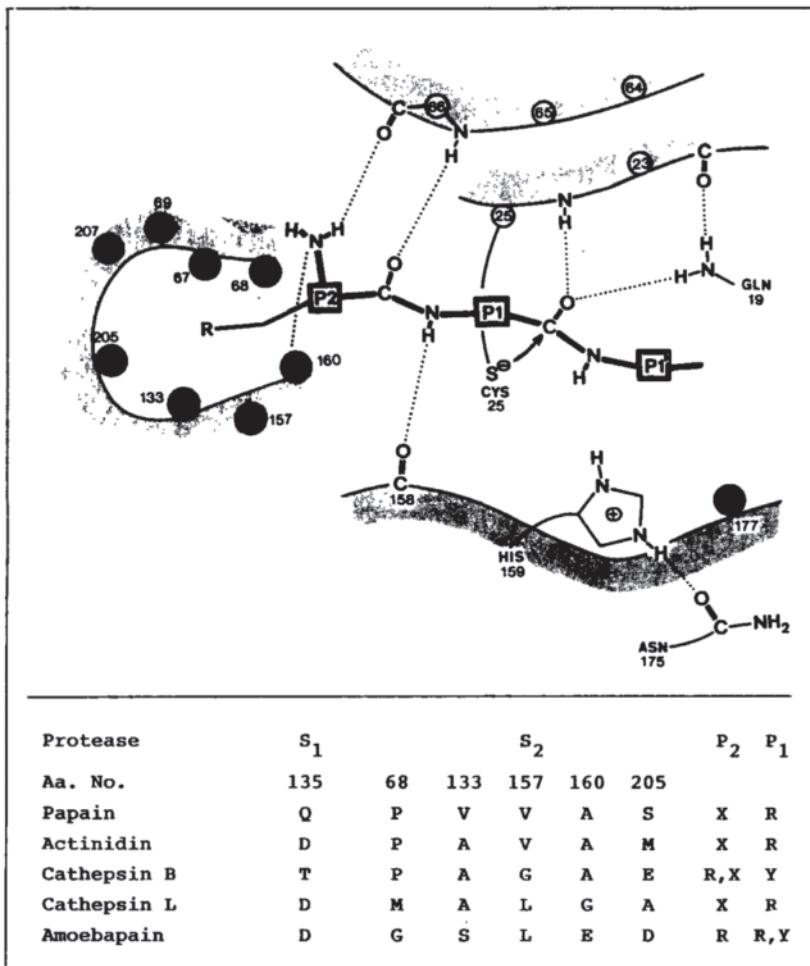


Figure 23.2 Active site residues and probable catalytic centre of amoebapain.
(Modified from Baker and Drenth (1987).)

domain, whereas His 159 is contributed by the R-domain. His159 is hydrogen bonded to Asn175 thus forming a catalytical triad Cys...His...Asn resembling the triad Ser... His...Asp found in the serine proteinases. In this structural model the S₂ hydrophobic binding pocket of papain is formed by the residues Pro58, Val133 and 157, Ala160 and Ser205. If a common polypeptide fold is assumed for all cysteine proteinases, with the three-dimensional structures solved for papain, actininidin and calotropin DI this appears to be so, and if the key positions in the primary structure are strictly conserved, one can speculate about reasons for the differences in specificity between individual cysteine proteinases. Considering the sequence data of amoebapain in conjunction with the structure constructed according to the coordinates of papain the positions 160 (Ala) and 205 (Ser) are replaced by the

acidic residues glutamate and aspartate (Figure 23.2). These would be able to bind the basic arginine from the P₂ position. Although residue 157 remains hydrophobic, this non-polar group could interact with the non-polar side-chain of arginine, and Asp205 at the end of the pocket could form an ion pair with the guanidinium group. The Glu 160 residue at the entrance of the binding pocket should be able to neutralize free primary amino groups and thus explain the dipeptidylaminopeptidase activity of amoebapain. The S₁ subsite includes position 135, according to the alignment of Kamphius *et al.* (1985), which is occupied by Gin in papain, and by Glu and Asp in cathepsin L and amoebapain, respectively. This conforms with the enhanced rate of hydrolysis of substrates with arginine in the P₁ position that is observed with both cathepsin L and amoebapain. Remarkably, the deduced amino acid sequence predicted from the cysteine proteinase gene of non-pathogenic amoebae is absolutely conserved in all those positions that are probably crucial for their specificity. This suggests that over-expression of the proteinase is a more important factor for cytopathogenicity than the precise specificity of the enzyme.

The sequence data also allow some speculation concerning the biosynthesis, post-translational processing and transport of amoebapain. The initial product seems to be a pre-pro-enzyme, which undergoes a series of maturation steps. It could be that, as in higher eukaryotes, the hydrophobic signal peptide is cleaved off during the translational process so that only the proform can be detected at early stages of biosynthesis (Hanewinkel *et al.*, 1987). A putative cleavage site for an ER membrane-associated peptidase lies between Gly-81 and Ile-80. After its transport to vesicles, the enzyme might then be activated by release of the propeptide through cleavage between Ile-1 and Ala1, either by autoproteolysis or with the aid of another peptidase. The sorting mechanisms of higher eukaryotes involve the attachment and then partial release of carbohydrates at different stages of the polypeptide processing. Since amoebapain lacks typical glycosylation sites, two distinct and more rudimentary ways can be considered. First, the non-polar leader sequence remains attached and the whole complex is transported to the digestive vesicles where the hydrophobic leader, together with the propeptide, is cleaved off. Alternatively, the enzyme is synthesized directly into its target vesicle, in which case a complicated transport process would be superfluous. According to our pulse-chase experiments, the proform of the enzyme is predominantly found in the putative ER fraction, so the former model is favoured.

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24. Proteinases of malaria parasites

P.J.Rosenthal

INTRODUCTION

Proteolytic activity appears to be required for malaria parasites to carry out a number of functions, particularly during the asexual or erythrocytic stage of infection. The erythrocytic malarial life cycle is initiated by the invasion of host erythrocytes by free merozoites (Garnham, 1988). The intraerythrocytic parasites then develop from small ring stage organisms to larger, more metabolically active trophozoites, and then to multinucleated schizonts. The erythrocytic cycle is completed when mature schizonts rupture the erythrocytes, releasing numerous invasive merozoites. Asexual-stage parasites require proteolytic activity for the hydrolysis of host and parasite proteins during erythrocyte invasion and rupture, the degradation of host haemoglobin into free amino acids by trophozoites, and routine intracellular housekeeping. While some of these functions might be accomplished by host proteinases, there is evidence that a number of malarial proteinases are involved.

Numerous reports of proteinases of malaria parasites have appeared since the first one in 1946 (Moulder and Evans, 1946; Cook *et al.*, 1961, 1969; Levy and Chou, 1973, 1974, 1975; Chan and Lee, 1974; Levy *et al.*, 1974; Charet *et al.*, 1980; Hempelmann and Wilson, 1980; Banyal *et al.*, 1981a, b; Sherman and Tanigoshi, 1981, 1983; Gyang *et al.*, 1982; Aissi *et al.*, 1983; Slomianny *et al.*, 1983; Schrével *et al.*, 1984, 1988; Vanderjagt *et al.*, 1984, 1986, 1987; Hempelmann *et al.*, 1986; Bernard and Schrevel, 1987; Bernard *et al.*, 1987; Rosenthal *et al.*, 1987, 1988, 1989; Sato *et al.*, 1987; Braun-Breton and Pereira da Silva, 1988; Braun-Breton *et al.*, 1988; Grellier *et al.*, 1989; Deguercy *et al.*, 1990) (Table 24.1), but many of these results are difficult to interpret in the light of inconsistencies with older assays of proteolytic activity and the difficulty of distinguishing host and parasite enzyme activities. All studies of asexual erythrocytic stages of malaria parasites have involved either parasites obtained directly from the blood of an infected animal or, with *Plasmodium falciparum*, parasites grown in culture with human erythrocytes and serum (Trager and Jensen, 1976). In any study in which leukocytes and platelets were not removed from the parasite cultures before study, one must be concerned regarding the possibility that reported activities are actually those of leukocytes or platelets, both of which have significant proteolytic activity (Weiss, 1989;

Table 24.1. Properties of malarial proteinases.^a

Proteinase class	Species	Size (M_r)	pH Optimum	Life-cycle stage ^b	Reference
Serine	<i>falciparum</i>	75 000	7.0	M	Rosenthal <i>et al.</i> (1987)
	<i>falciparum</i>	76 000	7.4–8.3	S/M	Braun-Bretton <i>et al.</i> (1988); Braun-Bretton and Pereira da Silva (1988)
Cysteine	<i>berghei, chabaudi</i>	68 000	7.4	S/M	Schrével <i>et al.</i> (1984); Bernard and Schrével (1987); Bernard <i>et al.</i> (1987)
	<i>falciparum</i>	68 000	7.4	S/M	Schrével <i>et al.</i> (1988); Grellier <i>et al.</i> (1989)
	<i>falciparum</i>	28 000	5.5–6.0	T	Rosenthal <i>et al.</i> (1987, 1988, 1989)
	<i>falciparum</i>	35 000–40 000	7.0	S	Rosenthal <i>et al.</i> (1987)
Aspartic	<i>lophurae</i>	37 000	3.5	NR	Sherman and Tanigoshi (1981, 1983)
	<i>falciparum</i>	148 000	3.5	NR	Gyang <i>et al.</i> (1982)
	<i>yoelii</i>	50 000	3.0	NR	Aissi <i>et al.</i> (1983)
	<i>falciparum</i>	<10 000	4.5	T/S	Vander Jagt <i>et al.</i> (1986)
	<i>berghei</i>	18 000–20 000	3.2	NR	Sato <i>et al.</i> (1987)
Aminopeptidase	<i>yoelii, chabaudi</i>	90 000	7.0	NR	Charet <i>et al.</i> (1980)
	<i>falciparum</i>	186 000	7.5	NR	Gyang <i>et al.</i> (1982)
	<i>falciparum</i>	63 000	7.5	T/S	Vander Jagt <i>et al.</i> (1984, 1987)
Unknown	<i>knowlesi</i>	Multiple	3.2	NR	Hempelmann and Wilson (1980)
	<i>falciparum, berghei</i>	37 000	5.0	NR	Deguercy <i>et al.</i> (1990)

^a Malarial proteinases reported since 1980. Values for M_r and pH optimum may not always be equivalent, as assay conditions have varied among the different studies. When the pH optimum for proteolytic activity was not determined, the pH at which the enzyme was studied is reported.

^b T, trophozoite; S, schizont; M, merozoite; NR, not reported.

Kambayashi and Sakon, 1989). In addition, controls are required to rule out contributions of erythrocyte proteinases (Pontremoli *et al.*, 1979). Most studies reported since 1980 have made efforts to control for the activities of host enzymes, and this review will concentrate on these more recent studies. The older studies of malarial proteinases have also been reviewed previously (Sherman and Tanigoshi, 1981).

In this review I discuss proposed functions of malarial proteinases in asexual erythrocytic stages of malaria parasites, particularly regarding the processes of erythrocyte invasion, host haemoglobin degradation, and erythrocyte rupture (Table 24.2). Reported malarial proteinases are classified, when possible, into the four standard categories based on the amino acid at the catalytic site (serine, cysteine and aspartic proteinases) or metal requirements (metalloproteinases) and the two exopeptidase categories (aminopeptidases and carboxypeptidases) are also

Table 24.2. Functions of malaria parasite proteinases.^a

Function	Proteinase class	Life-cycle stage	Candidate proteinases	References
Erythrocyte invasion/ erythrocyte rupture	Serine	Schizont/merozoite	75 000 76 000	Rosenthal <i>et al.</i> (1987) Braun-Bretton <i>et al.</i> (1988); Braun-Bretton and Pereira da Silva (1988)
	Cysteine	Schizont/merozoite	68 000 35 000–40 000	Schrével <i>et al.</i> (1984, 1988); Bernard and Schrével (1987); Bernard <i>et al.</i> (1987); Grellier <i>et al.</i> (1989) Rosenthal <i>et al.</i> (1987)
Haemoglobin degradation	Aspartic	Trophozoite	37 000 148 000 50 000 <10 000 18 000–20 000	Sherman and Tanigoshi (1981, 1983) Gyang <i>et al.</i> (1982) Aissi <i>et al.</i> (1983) Vander Jagt <i>et al.</i> (1986, 1987) Sato <i>et al.</i> (1987)
	Cysteine	Trophozoite	28 000	Rosenthal <i>et al.</i> (1987, 1988, 1989)

^a Malarial proteinases, with potential functions suggested, by their catalytic class and the life cycle stage at which they are active.

considered (Beynon and Bond, 1989). Characterization of each enzyme as to the asexual stage at which it is active, its relative molecular mass (M_r), and its pH optimum is also helpful in comparing the results of different investigators and hypothesizing biological roles for individual enzymes. At the end of the review, current data supporting specific enzymes as potential chemotherapeutic targets are presented.

ROLE OF PROTEINASES IN ERYTHROCYTE INVASION AND RUPTURE

The processes of erythrocyte invasion by free merozoites and erythrocyte rupture by mature schizonts will be discussed briefly; a more detailed discussion is given in Chapter 25. A number of investigators have studied the effects of peptide proteinase inhibitors on the rupture and invasion of erythrocytes by malaria parasites. In *P. Knowlesi*, both chymostatin, an inhibitor of serine proteinases, and leupeptin, an inhibitor of cysteine proteinases and trypsin-like serine proteinases, blocked erythrocyte rupture, but only chymostatin specifically blocked the invasion of erythrocytes by isolated merozoites (Hadley *et al.*, 1983). Other investigators also showed that in *P. knowlesi* (Banyal *et al.*, 1981 b) and *P. falciparum*

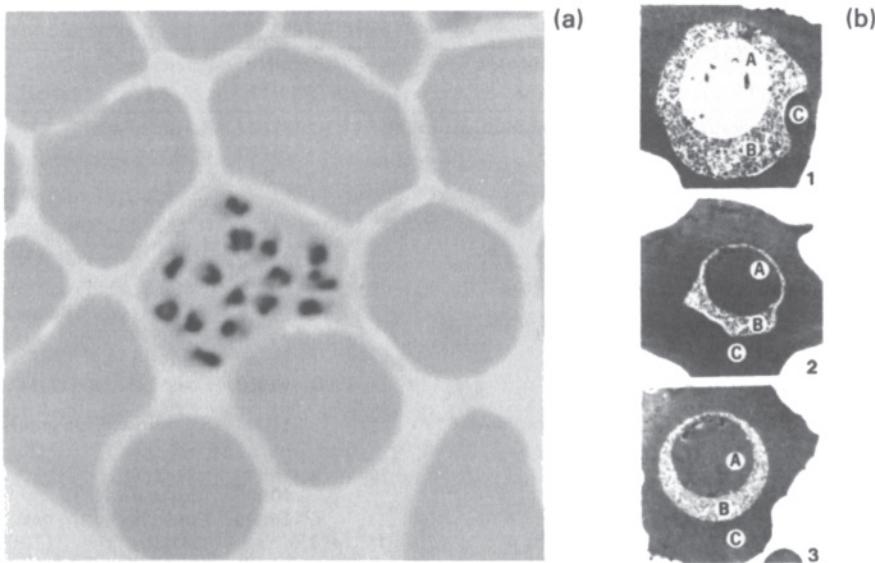


Figure 24.1 Effects of proteinase inhibitors on *P. falciparum* (a) Effects of chymostatin on erythrocyte rupture. Schizont-stage parasites were incubated with chymostatin ($82 \mu\text{M}$) for 12 h. Smears of parasite cultures were then stained with Giemsa and examined by light microscopy. Numerous erythrocytes containing unreleased merozoites (shown in figure) were seen, although some parasites appeared to have had their development halted earlier in the schizont stage and some erythrocyte rupture and new ring formation did occur, (b) Effects of leupeptin and E-64 on trophozoites. Trophozoites were incubated for 12 h with no inhibitor (1), with $100 \mu\text{M}$ leupeptin (2), or with $140 \mu\text{M}$ E-64 (3). In each electron micrograph the food vacuole (A), parasite cytoplasm (B), and erythrocyte cytoplasm (C) are labelled. In parasites incubated with leupeptin or E-64, the trophozoite food vacuole became filled with material that appears to be identical to erythrocyte cytoplasm and that in additional studies was shown to contain undegraded globin (Reproduced with permission from Rosenthal *et al.*, (1988)).

(Dejkriengkraikhul and Walairat, 1983; Lyon and Haynes, 1986; Dluzewski *et al.*, 1986) chymostatin and leupeptin blocked erythrocyte rupture and/or invasion. In some studies, however, reported effects on erythrocyte invasion may actually have represented an effect on the preceding cycle of erythrocyte rupture. Distinguishing these two processes is particularly difficult with *P. falciparum*, as invasive merozoites have not been successfully isolated from this species. In both *P. knowlesi* (Hadley *et al.*, 1983) and *P. falciparum* (Lyon and Haynes, 1986), the incubation of parasites with either chymostatin or leupeptin was followed by the accumulation of erythrocytes containing unreleased merozoites (Figure 24.1 (a)). In these erythrocytes schizonts apparently completed development into merozoites, but the rupture of the erythrocyte cytoskeleton and/or membrane required to free the merozoites was blocked.

Many proteins of mature schizonts and merozoites are proteolytically processed immediately before or during erythrocyte rupture and invasion (reviewed in

Braun-Breton and Pereira da Silva, 1988), suggesting that proteolytic products may have roles in these processes. The proteinases responsible for the processing of schizont and merozoite proteins are unknown. However, the processing of the 230 kDa major surface antigen of *P. knowlesi* schizonts was partially inhibited by the serine proteinase inhibitor diisopropylfluorophosphate (David *et al.*, 1984), and the processing of p 126, a parasitophorous vacuole protein of *P. falciparum* schizonts (Delplace *et al.*, 1987), was altered by leupeptin (Debrabant and Delplace, 1989).

The above results suggest that malarial serine and possibly cysteine proteinases are required for the processing of schizont/merozoite proteins and for erythrocyte rupture and invasion. A number of proteinases have been identified as potentially involved in these processes. An $M_r=68000$ cysteine proteinase was identified in schizonts and merozoites of the rodent parasites *P. berghei* and *P. chabaudi* (Bernard *et al.*, 1987; Schrével *et al.*, 1988) and of *P. falciparum* (Schrevel *et al.*, 1988; Grellier *et al.*, 1989). In immunofluorescence experiments, antisera directed against this proteinase localized primarily to the merozoite apex (Bernard and Schrevel, 1987), suggesting that the enzyme might be released from the rhoptry organelle during invasion. An $M_r=35000-40000$ cysteine proteinase of mature schizonts and an $M_r=75\ 000$ serine proteinase of merozoites were identified in highly synchronized *P. falciparum* parasites (Rosenthal *et al.*, 1987). The stage-specific activities of these proteinases suggest that they may also be involved in erythrocyte rupture or invasion. An $M_r=76000$ serine proteinase of *P. falciparum* schizonts and merozoites was shown to be bound in an inactive form to the schizont/merozoite membrane by a glycosylphosphatidylinositol anchor, and to be activated and solubilized by phosphatidylinositol-specific phospholipase C during the merozoite stage (Braun-Breton *et al.*, 1988), suggesting a role in erythrocyte invasion. An $M_r=37000$ proteinase of *P. falciparum* and *P. berghei* that was inhibited by both chymostatin and leupeptin, but was not easily categorized as to catalytic class, was shown to hydrolyse the erythrocyte cytoskeletal proteins spectrin and band 4.1 (Deguercy *et al.*, 1990), suggesting potential roles in both erythrocyte rupture and erythrocyte invasion. The gene encoding a serine-rich *P. falciparum* schizont antigen of unknown function has recently been sequenced (Bzik *et al.*, 1988; Knapp *et al.*, 1989) and shown to have some sequence homology to cysteine proteinases (Higgins *et al.*, 1989), though the predicted active site cysteine has been replaced by a serine residue (Eakin *et al.*, 1989; Mottram *et al.*, 1989). Whether the gene encodes a malarial proteinase is unknown.

ROLE OF PROTEINASES IN HAEMOGLOBIN DEGRADATION

Extensive evidence suggests that the degradation of host erythrocyte haemoglobin is necessary for the growth of the asexual erythrocytic stages of malaria parasites. The intraerythrocytic parasites have apparently evolved to require haemoglobin as a source of free amino acids, as the parasites have a limited capacity for synthesizing amino acids (Sherman, 1977, 1979), and the quantity of free amino acids within

erythrocytes is not sufficient for parasite needs (Sherman, 1979). Evidence that malaria parasites degrade haemoglobin into free amino acids includes:

1. the haemoglobin content of infected erythrocytes decreases by 25–75 per cent during the life cycle of erythrocytic parasites (Ball *et al.*, 1948; Groman, 1951);
2. the concentration of free amino acids is greater in infected than in uninfected erythrocytes (Sherman and Mudd, 1966);
3. the composition of the amino acid pool of infected erythrocytes is similar to the amino acid composition of haemoglobin (Cenedella *et al.*, 1968; Barry, 1982; Zarchin *et al.*, 1986) and
4. the infection of erythrocytes containing radiolabelled haemoglobin is followed by the appearance of labelled amino acids in parasite proteins (Fulton and Grant, 1956; Sherman and Tanigoshi, 1970; Theakston *et al.*, 1970).

In *P. falciparum*, haemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most metabolically active (McColm *et al.*, 1980; Yayon *et al.*, 1983). Trophozoites ingest erythrocyte cytoplasm and transport it within vesicles to a large central organelle known as the food vacuole (Aikawa, 1971; Yayon *et al.*, 1984b). In the food vacuole haemoglobin is broken down into haem, which is a major component of malarial pigment (Yamada and Sherman, 1979), and globin, which is hydrolysed to its constituent free amino acids. The food vacuole of *P. falciparum* is an acidic (Yayon *et al.*, 1984a; Krogstad *et al.*, 1985), membrane bound (Aikawa, 1971) compartment where proteins are degraded and, therefore, it appears to be analogous to the secondary lysosomes of mammalian cells (Krogstad and Schlesinger, 1987; Bond and Butler, 1987). A number of lysosomal proteinases are well characterized, including cysteine (cathepsins B, H, and L) and aspartic (cathepsin D) proteinases (Bond and Butler, 1987), and it seems plausible that malaria parasites contain analogous food vacuole proteinases that degrade haemoglobin.

Aspartic proteases of $M_r=148000$ (Gyang *et al.*, 1982) and $M_r<10000$ (Vander Jagt *et al.*, 1986, 1987) of *P. falciparum*, $M_r=37000$ of the avian parasite *P. lophurae* (Sherman and Tanigoshi, 1983), $M_r=50000$ of the rodent parasite *P. yoelii* (Aissi *et al.*, 1983) and $M_r=18000–20000$ of *P. berghei* (Sato *et al.*, 1987) have been identified. Both soluble and membrane-bound forms of aspartic proteinases have been reported (Vander Jagt *et al.*, 1987). The proteinases all had acid pH optima and were strongly inhibited by the aspartic proteinase inhibitor pepstatin, suggesting that, despite the differences in their reported sizes, they may all represent the same or closely related enzymes. The acid aspartic proteinases also all degraded denatured haemoglobin. Though denatured haemoglobin is a non-specific proteolytic substrate, the above results suggest that a trophozoite aspartic proteinase may have a role in the degradation of haemoglobin in the malarial food vacuole.

In a number of studies the incubation of trophozoites of *P. falciparum* with leupeptin caused the food vacuole to fill with apparently undegraded erythrocyte cytoplasm (Dluzewski *et al.*, 1986; Rosenthal *et al.*, 1988; Vander Jagt *et al.*, 1989) (Figure 24.1 (b)), suggesting that the cytoplasm was transported normally to the

food vacuole, but that the degradation of cytoplasmic components was blocked. Leupeptin inhibits both cysteine and some serine proteinases, but the highly specific cysteine proteinase inhibitor E-64 also caused the food vacuole abnormality (Rosenthal *et al.*, 1988) (Figure 24.1 (b)), which was not seen after incubation with inhibitors of other classes of proteinases including chymostatin and pepstatin (Dluzewski *et al.*, 1986; Rosenthal *et al.*, 1988; Vanderjagt *et al.*, 1989). Parasites that had been treated with leupeptin or E-64 contained large quantities of apparently undegraded globin, while globin was undetectable in control parasites (Rosenthal *et al.*, 1988). Based on these results it was hypothesized that a cysteine proteinase is required for an initial step in haemoglobin degradation by *P. falciparum* (Rosenthal *et al.*, 1988).

An $M_r=28000$ cysteine proteinase of *P. falciparum* that was active only at the trophozoite stage (Rosenthal *et al.*, 1987) appears to be a good candidate for a food vacuole haemoglobinase. The proteinase was strongly inhibited by leupeptin and E-64, had an acid pH optimum, and degraded haemoglobin *in vitro* (Rosenthal *et al.*, 1988). In addition, the size, pH optimum, substrate specificity, and inhibitor sensitivity of the enzyme were all very similar to those of the mammalian lysosomal cysteine proteinase cathepsin L (Rosenthal *et al.*, 1989). At nanomolar to micromolar concentrations, specific inhibitors of cathepsin L also inhibited the $M_r=28000$ proteinase, blocked haemoglobin degradation, and prevented the development of malaria parasites (Rosenthal *et al.*, 1989; and unpublished observations), supporting the hypothesis that the enzyme is the cysteine proteinase required for haemoglobin degradation. A *P. falciparum* gene encoding a cysteine proteinase typical of the papain superfamily has also recently been identified and sequenced (P.J.Rosenthal, unpublished observations). The deduced gene product predicted to represent the active enzyme (after cleavage of a proform) has >40 per cent amino acid identity to cathepsin L, and so this gene could encode the $M_r=28000$ trophozoite cysteine proteinase.

Two groups have reported the purification of food vacuoles of *P. falciparum* by density gradient centrifugation (Choi and Mego, 1988; Goldberg *et al.*, 1990). The food vacuoles had ATPase activity, as expected for an acidic organelle (Choi and Mego, 1988). Extracts of purified food vacuoles contained acid haemoglobinase activity that was inhibited by both aspartic and cysteine proteinase inhibitors (Goldberg *et al.*, 1990). The degradation of haemoglobin by food vacuole extracts was blocked 67 per cent by the aspartic proteinase inhibitor pepstatin and 32 per cent by the cysteine proteinase inhibitor E-64. These results were similar to those obtained when the degradation of haemoglobin by soluble trophozoite extracts was studied (71 per cent inhibition by pepstatin and 50 per cent inhibition by E-64 (Rosenthal *et al.*, 1989). With food vacuole extracts, however, pepstatin most effectively blocked initial cleavages of haemoglobin, suggesting that an aspartic proteinase, and not a cysteine proteinase (as suggested by the above studies), is responsible for the initial cleavage of haemoglobin. The conflicting conclusions might be explained by the requirement for a cysteine haemoglobinase to be processed by a food vacuole aspartic proteinase for activation. The activation of the lysosomal cysteine proteinases cathepsin B and cathepsin L has, in fact, been shown

to be blocked by pepstatin (Nishimura *et al.*, 1988). In any event, although the precise steps involved in haemoglobin degradation in the food vacuole remain unclear, it seems likely that, as in lysosomes, both aspartic and cysteine proteinases are active and that both classes of enzymes are required for the hydrolysis of haemoglobin to free amino acids.

HOUSEKEEPING FUNCTIONS

Intracellular proteolytic activity appears to be required by most eukaryotic cells both for the processing of polypeptides into mature proteins (Bond and Butler, 1987) and for the turnover of cellular proteins (Beynon and Bond, 1986; Mayer and Doherty, 1986). It seems likely that some of the malarial proteinases that have been discussed above may be responsible for activities that are not unique to malaria parasites. Since intracellular proteolysis occurs in both lysosomal and cytosolic compartments, both acidic and neutral proteases may be involved. The acidic aspartic and cysteine proteinases that have been suggested as potential haemoglobinases might alternatively (or in addition) take part in the lysosomal processing of proteins other than haemoglobin. Of interest in this regard, true lysosomes have not been seen in electron micrographs of malaria parasites (Aikawa, 1971), suggesting that the food vacuole may act as a single large lysosome, analogous to the vacuole of yeast (Wiemken *et al.*, 1979). Intracellular proteolysis also occurs in the cytosol, and this process may be carried out by some of the neutral cysteine and serine proteinases that have been described. Protein catabolism also requires exopeptidase activity, and the malarial exopeptidases that have been described may have this function. It is likely that certain malarial proteinases with important roles in intracellular proteolytic processing or protein catabolism have not yet been described. For example, malarial analogues to calcium-dependent cysteine proteinases (Bond and Butler, 1987) and ATP-dependent proteinases (Bond and Butler, 1987) have not been reported.

MALARIAL PROTEINASES AS POTENTIAL CHEMOTHERAPEUTIC TARGETS

Any enzyme with a necessary function in the malarial life cycle may be an appropriate target for antimarial chemotherapy. The serine and cysteine proteinases that appear to be required for erythrocyte rupture and invasion and the aspartic and cysteine proteinases that appear to be involved in haemoglobin degradation by trophozoites are thus attractive chemotherapeutic targets. A number of investigators have recently evaluated the effects of highly specific peptide proteinase inhibitors on malaria parasites. Peptide ethylamide inhibitors of the *P. falciparum* $M_r=68000$ cysteine proteinase were incubated with schizonts, and they inhibited erythrocyte rupture and/or invasion, though millimolar concentrations of the peptides were required for inhibition (Schrével *et al.*, 1988). Peptide

fluoromethyl ketone inhibitors of cysteine proteinases inhibited *P. falciparum* growth at nanomolar to micromolar concentrations (Rosenthal *et al.*, 1989; Rockett *et al.*, 1990). We have recently evaluated the effects of a panel of fluoromethyl ketone inhibitors on the activity of the $M_r=28000$ trophozoite cysteine proteinase and on haemoglobin degradation by and growth of cultured *P. falciparum* parasites. Z-Phe-Arg-CH₂F (Z, *N*-benzyloxycarbonyl; CH₂F, fluoromethyl ketone) was the most effective inhibitor of the $M_r=28000$ proteinase (50 per cent inhibition at 0.36 nM), most effectively blocked haemoglobin degradation by trophozoites (blocked at 100 nM), and most effectively killed cultured parasites ($IC_{50}=64$ nM) (P.J.Rosenthal, unpublished observations). In preliminary studies, Z-Phe-Arg-CH₂F was also non-toxic to cultured mammalian cells at concentrations up to 100 μ M (P.J.Rosenthal, unpublished observations). Thus the peptide fluoromethyl ketone inhibitors are promising candidate antimalarial drugs. Ultimately, a better understanding of the biochemical properties and biological roles of the malarial proteinases will foster the development of proteinase inhibitors which specifically inhibit only parasite and not host proteinases, and thus are the most suitable candidates for chemotherapy.

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25. *Plasmodium* proteinases during the erythrocytic phase of infection

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INTRODUCTION

In malaria parasites, as in higher eukaryotic cells, proteinases are probably involved in numerous physiological events, including the interactions that occur upon invasion of the parasite into a host cell, that is between sporozoite and hepatocyte and between merozoite and erythrocyte. The latter process, in particular, represents potential for novel chemotherapy. The specific recognition of an erythrocyte by a merozoite (Hadley *et al.*, 1986; Schrével *et al.*, 1986), the subsequent reorientation of the merozoite with its apex in contact with the erythrocyte membrane (Bannister *et al.*, 1975; Dvorak *et al.*, 1975) and the internalization of the merozoite *via* a moving junction (Aikawa *et al.*, 1979), imply the involvement of very specific molecules such as receptors of glycoconjugates, translocator molecules and selective proteinases.

While the first *Plasmodium* proteinases from erythrocytic stages were described more than 40 years ago in studies dealing with haemoglobin digestion at acid pH (Moulder and Evans, 1946), evidence for the presence of proteinases acting at neutral pH during the reinvasion of erythrocytes by merozoites has only been obtained recently (for reviews see Schrével *et al.*, 1988, 1990). There are a number of reasons for this: firstly, neutral proteinases act only for a short period; secondly, proteinases acting outside the acidic parasitophorous vacuole are partially or totally lost during parasite isolation; and, thirdly, proteinases in low amounts in the parasite extracts are difficult to detect by standard enzyme assays.

The activation of parasite or host proteinases during reinvasion could depend on the pH and other physico-chemical conditions within cell compartments. The process may also require special proteinases acting during the release of merozoites from mature schizont-infected erythrocytes (segmenters), during merozoite motion in plasma or after the recognition step between merozoite and erythrocyte, i.e. the merozoite reorientation and internalization process.

In this review we focus on the *Plasmodium* proteinases known to be involved in

different steps of the erythrocytic phase of malaria infections, on the strategy for studying proteinases and on proteinase inhibitor effects during reinvasion of erythrocytes by merozoites.

PROTEINASES INVOLVED DURING THE ERYTHROCYTIC PHASE OF MALARIA INFECTIONS

The erythrocytic maturation phase of *Plasmodium* can be defined as the phase of the life cycle when parasite development needs exogenous supplies from serum. The definition is based on the following considerations. Merozoite release from mature schizonts (segmenters), erythrocyte invasion, the differentiation from merozoite to the ring stage and the trophozoite stage (corresponding to the first 24 h of the erythrocytic phase), are unaffected by the absence of serum, but schizogony does not occur (Grellier *et al.*, 1990). However, the addition of serum or human high density lipoproteins to young trophozoites maintained during the first 24 h in basal culture medium (RPMI), induces a normal schizogony (Grellier *et al.*, 1990). This transition between the trophozoite and schizont stage is characterized by an extensive haemoglobin breakdown which mainly involves proteinases active at acid pHs (see Chapter 24). During schizogony, the parasite undergoes a series of nuclear divisions followed by the differentiation of new merozoites. During this phase there are complex antigen maturation processes and numerous alterations to the erythrocyte membrane (Hommel and Semoff, 1988).

Several *Plasmodium* proteinases have been characterized (see also Chapter 24), but their localization, expression during the life cycle and their cellular or molecular targets are not yet known. Only a few of the proteinases have been purified and are well characterized.

Proteinases from the trophozoite growth phase: acid proteinases and haemoglobin breakdown

Plasmodium-parasitized erythrocytes are characterized by a decrease in the haemoglobin content and an accumulation of a pigment named haemozoin as the parasite matures. Depending upon the species, between 25 and 75 per cent of haemoglobin may be degraded (Groman, 1951). The involvement of parasite proteinases in this process is discussed in detail in Chapter 24.

Maturation phase, schizont and free merozoite

The presence of proteinases which are active at alkaline pH has been demonstrated using globin and acetylalanine *p*-nitroanilide as substrates (for a review see Schrével *et al.*, 1990). Several cysteine and serine proteinases are now being characterized.

Cysteine proteinases

Using fluorogenic GlcA-Val-Leu-Gly-Lys(or Arg)-3-amido-9-ethylcarbazole (AEC) as substrates, we have demonstrated the presence of proteinases active at

neutral pH in 100000 g extracts of *P. berghei*- and *P. chabaudi*-infected erythrocytes (Schrével *et al.*, 1984). These enzymes are different from host proteinases. For example, upon Sephadryl S-200 gel filtration the parasite enzymes have an apparent M_r of about 130000, while the host enzymes have apparent M_r values above 200000. Differences are also apparent from the pH optima (7.4 and 8.2 for the parasite and host enzymes, respectively) and the substrate specificities of the enzymes (Bernard *et al.*, 1987). The *P. berghei* proteinase, which has been purified by HPLC, has a pI of about 4.35 and an M_r of 68000 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Bernard and Schrével, 1987). A similar 68-kDa proteinase has been purified from *P. falciparum* 100000 g extracts of both trophozoite- and schizont-infected erythrocytes obtained from highly synchronized *in vitro* cultures (Grellier *et al.*, 1989). From the effects of inhibitors these enzymes may be classed as cysteine proteinases. Using the system of nomenclature adopted in our laboratory these proteinases are referred to as *Pb68* and *Pf68*.

The *Pb68* and *Pf68* proteinases are found in both schizonts and free merozoites. A specific mouse antiserum against *Pb68* has shown immunofluorescent labelling at the apex of merozoites in both segmented schizonts and free merozoites. No cross-reaction was observed with the *Pf68* proteinase (Bernard and Schrével, 1987).

Gelatin-SDS-PAGE analysis shows the presence of a proteolytic band in the M_r range 96000–110000 in 100000 g extracts from schizonts of *P. berghei* and *P. falciparum* and also in the culture medium of *P. falciparum* after invasion (see below). This enzyme activity is totally inhibited by *N*-ethylmaleimide and partially by phenylmethylsulphonyl fluoride (PMSF) (Schrével *et al.*, 1988).

Deguercy *et al.* (1990) have identified proteinases active at acid pH which act on human spectrin, namely *Pf37* and *Pb37*. These enzymes were strongly inhibited by E-64, aprotinin, chymostatin and leupeptin but weakly by *N*-ethylmaleimide, PMSF and diisopropylfluorophosphate (DFP). Since these enzymes have characteristics of both cysteine and serine proteinases it is not yet certain to which class they belong.

Serine proteinases

Braun-Breton *et al.* (1988) have demonstrated that treatment of membranes from free merozoites with phosphatidylinositol specific phospholipase C (PI-PLC) induces the activation of a membrane-anchored serine proteinase (*Pf76*) and its release in an active soluble form. However, the authors were unable to conclude whether solubilization from the membrane by PI-PLC is an obligatory step prior to activation of the proteinase by another enzyme or if solubilization allowed the protein to adopt an active conformation (Braun-Breton and Pereira da Silva, 1988).

Aminopeptidases

A series of aminopeptidases active at neutral pH was identified in rodent *P. yoelii nigeriensis* and *P. chabaudi* using aminoacyl *p*-nitroanilides (Charet *et al.*, 1980). Inhibitor studies revealed that at least one sulphhydryl group is essential for activity.

A similar aminopeptidase was purified from sonicated *P. falciparum* supernatants (Vander Jagt *et al.*, 1984). On the basis of its pH profile the enzyme was considered to be localized in the cytosol rather than in the food vacuole.

POTENTIAL TARGETS OF THE PLASMODIUM PROTEINASES

Protein processing

Numerous soluble antigens are processed in free merozoites or in the late schizont stage. The nature of the parasite proteinases involved is still unknown.

The 190 kDa precursor of the major merozoite surface antigen (PMMSA) from *P. falciparum* is processed into a series of fragments with a considerable polymorphism (Holder and Freeman, 1984; McBride *et al.*, 1985; McBride and Heidrich, 1987). Heidrich *et al.* (1989) noted from the amino terminal sequences of the PMMSA processing products that the bond cleavages were similar to those obtained with the *Staphylococcus aureus* V8 proteinase. It is of interest that the V8 proteinase is not inhibited by commercially available proteinase inhibitors suggesting a very narrow specificity. If this narrow specificity is shared by the *Plasmodium* enzyme responsible for PMMSA processing, this may be very useful when designing prodrugs which are activated by parasite but not host enzymes.

The *Pf*126 parasitophorous vacuole antigen, which has also been referred to as *Pf*140 (Perrin *et al.*, 1984) and *Pf*110 (Knapp *et al.*, 1989) antigen, is processed into fragments of 50, 47 and 18 kDa (Delplace *et al.*, 1987, 1988; Debrabant and Delplace, 1989). Chymostatin, antipain and pepstatin had no effect on this processing. Leupeptin, however, does inhibit the conversion of a 56 kDa fragment to the 50 kDa fragment (Debrabant and Delplace, 1989).

Neither the proteinases involved in the processing of the glycophorin binding protein (GBP 130) into 100 and 80 kDa fragments (Bonnefoy *et al.*, 1988; Perkins, 1988), nor those involved in the rhoptry antigen processing (Roger *et al.*, 1987; Braun-Breton and Pereira da Silva, 1988) have yet been characterized.

Plasmodium proteinases and erythrocyte cytoskeleton depletion

Proteolytic activity against the erythrocyte cytoskeleton could interfere with the properties of the erythrocyte membrane such as stability and fluidity (Sikorski and Jezierski, 1986). It is well known that malaria induces an enhancement of membrane fluidity (Deguercy *et al.*, 1986; and see Chapter 2).

There have been numerous reports of a decrease in spectrin and other erythrocyte cytoskeletal components during late trophozoite and schizont stages of *Plasmodium* infection. A decrease of spectrin subunits, band 4.1 and PAS1 has been reported in membranes of erythrocytes infected with *P. berghei* (Weidekamm *et al.*, 1973), *P. chabaudi* (Königk and Mirtsch, 1977) or *P. lophuriae* (Sherman and Jones, 1979). The mechanism of the decrease in cytoskeletal components is not yet clearly

understood. When tested on erythrocyte membranes, a *P. lophurae* 37 kDa proteinase caused the proteolysis of spectrin band 2.1–2.6 and band 3 at pH 7.4 and, to a greater degree, at pH 3.5 (Sherman and Tanigoshi, 1983). Recently, Deguercey *et al.* (1990) demonstrated that the *Pf37* and *Pb37* proteinases were also able to degrade spectrin components, especially the β -subunit and the band 4.1.

STRATEGY FOR STUDYING PROTEINASES AND PROTEINASE INHIBITOR EFFECTS DURING THE REINVASION OF ERYTHROCYTES BY MEROZOITES

The advantages of serum-free medium for the identification of *P. falciparum* proteinases

One of the major difficulties in the identification of *Plasmodium* proteinases involved during the reinvasion step is that the probable location of the enzymes means that they may be partially lost during the saponin treatment of infected erythrocytes or during the purification of free merozoites. Among the proteinases concerned would be:

1. proteinases acting inside the parasitophorous vacuole, for example for *Pf126* antigen processing;
2. proteinases acting on the erythrocyte cytoskeleton or on the membrane proteins; and
3. proteinases located in secretory organelles of the merozoites, that is the rhoptries, micronemes and dense granules.

Futhermore, the identification of parasite proteinases can be complicated by processing which leads to their activation during a short period of the reinvasion process, as exemplified by the activation of the serine proteinase *Pf76* (Braun-Breton *et al.*, 1988).

These proteinases cannot easily be distinguished in culture medium containing serum because serum serine proteinases, such as kallikrein and those of the complement system, as well as proteinase inhibitors like α_2 -macroglobulin and α_1 -antitrypsin, can interfere with the identification of the parasite proteinases. For example, Delplace *et al.* (1988) reported that during *Pf126* processing, the cleavage of the 56 kDa to the 50 kDa subfragment was inhibited by α_1 -antitrypsin.

Gelatin-SDS-PAGE analysis of the culture medium (containing 5 per cent serum) obtained during the *in vitro* reinvasion process of erythrocytes by *P. falciparum* merozoites in highly synchronized cultures has shown strong proteolytic activities in the 60–120 kDa range. The activities were, however, also observed in medium from control cultures of uninfected erythrocytes. These serum serine proteinases can be partially eliminated from the cultures by using decomplemented serum (heated at 56°C for 30 min) and are completely absent if serum-free medium based on human lipoproteins is used (Grellier *et al.*, 1990, 1991).

The absence of serum proteinases and proteinase inhibitors in the serum-free medium makes its use ideal for the identification of the parasite proteinases released during the *in vitro* reinvasion step. Serum-free medium from cultures of highly synchronized *P. falciparum* has been collected during the reinvasion step, centrifuged at 100000g and then filtered through a 0.2- μm filter. Gelatin-SDS-PAGE analysis shows a major parasite proteolytic activity in the culture supernatant with an M_r of 96000. Occasionally proteolytic activities in the lower M_r range have also been observed. With lysates of healthy erythrocytes no proteolytic activity is observed. The M_r =96000 proteinase appeared similar to an enzyme of the same size detected in the 100000 g soluble extracts from *P. falciparum* schizonts (*Pf*96–110, see above). Since this M_r =96000 proteinase hydrolyses GlcA-Val-Leu-Gly-Lys-AEC, there is a possible relationship between it and *Pf*68 (unpublished data).

Effects of proteinase inhibitors on the reinvasion step

Many effects of proteinase inhibitors *in vitro* on *P. falciparum* have been reported, but the targets of the inhibited proteinases are still unclear. Some reports have indicated selective effects on merozoite release from segmenters, while others have indicated effects on erythrocyte invasion by merozoites. It is important to note, however, that the inhibitors have usually been employed in mixtures and at high concentrations.

A mixture of leupeptin, chymostatin, antipain and pepstatin, each at 10 $\mu\text{g ml}^{-1}$ (23.4, 16.5, 16.5 and 14.6 μM , respectively) inhibited the release of *P. falciparum* merozoites which were then clustered around pigment granules and surrounded by a permeable erythrocyte membrane (Lyon and Haynes, 1986). A similar observation was made with *P. knowlesi* schizonts incubated with leupeptin or chymostatin at 50 $\mu\text{g ml}^{-1}$ (117, 82.5 μM , respectively) (Hadley *et al.*, 1983). However, such results are questionable since Dluzewski *et al.* (1986) showed that with leupeptin at 50 $\mu\text{g ml}^{-1}$ (117 μM), only 15 per cent of *P. falciparum* failed to rupture, and that with the same concentration of chymostatin this was reduced to 5 per cent.

To gain a better understanding of the sites of action of some proteinase inhibitors, Vander Jagt *et al.* (1989) determined inhibitor effects on the development of *P. falciparum*. Proteinase inhibitors were added to the trophozoite stage and parasite growth was then followed on Giemsa-stained slides. E-64 ($\text{IC}_{50}=8 \mu\text{M}$), a specific cysteine proteinase inhibitor, or leupeptin ($\text{IC}_{50}=30 \mu\text{M}$) produced large vacuolated trophozoites, probably reflecting the presence of undigested haemoglobin, and affected the final steps of the parasite maturation. Bestatin ($\text{IC}_{50}=35 \mu\text{M}$) and chymostatin ($\text{IC}_{50}>50 \mu\text{M}$) also inhibited the maturation of the parasite. For further details of inhibitor effects see Chapter 24.

During the invasion of erythrocytes by merozoites, proteinases could act on erythrocyte and on parasite membranes to ensure the internalization of the parasite. By using invasive *P. knowlesi* merozoites and rhesus erythrocytes, Hadley *et al.* (1983) were able to distinguish the effects of proteinase inhibitors on schizont maturation and on invasion of erythrocytes by merozoites. Chymostatin, TLCK and TPCK caused a marked inhibition of invasion. In contrast, leupeptin, antipain, pepstatin and PMSF had little or no effect. Furthermore, chymostatin did not inhibit

the attachment of the merozoite to the erythrocyte, indicating that chymostatin might act during or after junction formation (Hadley *et al.*, 1983). In contrast to the situation with *P. knowlesi* the invasion of human erythrocytes by *P. falciparum* merozoites was inhibited by leupeptin or chymostatin (Banyal *et al.*, 1981). Furthermore, the inhibitory effects were also observed when inhibitors were introduced in resealed erythrocyte ghosts, indicating a possible action of the inhibitors on the cytoplasmic side of the erythrocyte membrane (Dluzewski *et al.*, 1986). However, by using a different procedure, Vander Jagt *et al.* (1989) were able to show that leupeptin (100 µM) or chymostatin (100 µM) had no effect on the cytoplasmic face of the erythrocyte during the reinvasion process, whereas both bestatin (50 µM) and E-64 (20 µM) were effective.

Thus the effects of proteinase inhibitors during reinvasion appear to be variable. This variability could be related to species differences or to experimental conditions. As leupeptin is a membrane permeant drug, its inhibitory effects cannot discriminate between the merozoite release and the erythrocyte invasion steps. Specific inhibitors of well characterized proteinases such as *Pf*68, could be useful for such a discrimination.

Synthetic peptide derivatives specific for the *Pf*68 proteinase and the inhibition of the *P. falciparum* reinvasion process

As the *Pb*68 and *Pf*68 proteinases are present in free merozoites, enzyme-specific peptide derivatives were synthesized and tested on the *in vitro* reinvasion of human erythrocytes by *P. falciparum* merozoites. The rationale for this approach is based on the design of compounds which, owing to their high affinities for the active site, can compete preferentially with natural substrates for the enzymes and impede the proteolytic process.

In order to avoid the deleterious effects of released AEC on erythrocytes, peptidylalkylamide derivatives were synthesized by replacing the AEC group of GlcA-Val-Leu-Gly-Lys-AEC by ethylamine, diethylamine, (*R*)-2-amino-1-butanol, 2-aminopropanediol or 2-amino-2-methyl-1, 3-propanediol and *N*-methylglycine or proline to create a secondary amide bond. The inhibitory activity of these peptide derivatives was evaluated on highly synchronized *P. falciparum* cultures by incubating schizonts and segmenters with fresh erythrocytes for 6 h (Mayer *et al.*, 1991). At 0.1–1 mM concentrations, a very good inhibitory effect was observed with GlcA-Val-Leu-Gly-Lys-NHC₂H₅ and GlcA-Val-Leu-Gly-Lys-2-amino-1-butanol. The peptide sequence was important in determining the specificity and the extent of the inhibitory effect: GlcA-Val-Leu-Gly-Ala-NHC₂H₅, for example, had no inhibitory effect. Amino alcohols are known to have toxic effects by interfering with phospholipid synthesis (Vial *et al.*, 1984). Under the experimental conditions, however, the toxic effect of the amino alcohols released did not account for the inhibition of reinvasion since GlcA-Val-Leu-Gly-Lys-2-aminopropanediol gave more than 80 per cent inhibition at 1 mM while free 2-aminopropanediol at the same concentration had no effect.

The peptide inhibitors of *Pf*68 act on the invasion step and not on merozoite release from segmenters (Mayer *et al.*, 1991). Discrimination was achieved by using

mature schizont-infected red blood cells (44–48 h) which were incubated in normal medium, with leupeptin (1 mM), with GlcA-Val-Leu-Gly-Lys-NHC₂H₅ (1 mM) (the inhibitor of the *Pf*68 proteinase) or with Ac-Asp-Phe-Arg-Gly-NHC₂H₅ (1 mM) (an unrelated peptide)). Similar inhibition of ring formation was observed with leupeptin and GlcA-Val-Leu-Gly-Lys-NHC₂H₅, but the origin of this inhibition was different. Leupeptin blocked merozoite release while in the presence of GlcA-Val-Leu-Gly-Lys-NHC₂H₅ the release of merozoites was normal, indicating that the inhibitory effect of the latter must be related to the invasion step.

To increase the resistance of these reversible inhibitors to cleavage, the amide bond was modified to create a non-hydrolysable pseudopeptide ψ [CH₂NH] bond. Using this strategy, it was possible to demonstrate that at 0.1 mM GlcA-Val-Leu-Gly-Lys- ψ [CH₂NH]C₂H₅ inhibits erythrocyte invasion by *P. falciparum* but not parasite maturation (trophozoite and schizont stage) or merozoite release. In contrast, 0.1 mM leupeptin inhibits both maturation and merozoite release (unpublished data).

CONCLUSION

Several *Plasmodium* proteinases acting on the erythrocyte phase of malaria have recently been characterized by using sensitive identification methods. The *M_r* 28000 cysteine proteinase hydrolyses haemoglobin and corresponds to a cathepsinL like proteinase (see Chapter 24). The neutral proteinases specific for GlcA-Val-Leu-Gly-Lys-AEC, *Pb*68 and *Pf*68, are mainly located in schizonts and free merozoites and the *Pf*68 is involved in the invasion of erythrocytes by merozoites (Bernard and Schrével, 1987; Grellier *et al.*, 1989; Mayer *et al.*, 1991). The serine proteinase *Pf*76 is associated with the schizont and merozoite, and its selective activation requires a PI-PLC treatment (Braun-Bretton *et al.*, 1988; Braun-Bretton and Pereira da Silva, 1988). Finally, the acid proteinases *Pb*37 and *Pf*37 seem to hydrolyse spectrin selectively (Deguercy *et al.*, 1990).

Some of the questions currently being addressed include:

1. the significance of the stage-specific expression of the proteinase genes;
2. the identification of the natural targets; and
3. the identification of parasite proteinase domains which selectively bind reversible or irreversible inhibitors in order to discriminate between parasite and host proteinases.

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26. Proteinases of *Babesia*

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INTRODUCTION

Relatively little is known about proteinases of *Babesia* species, despite the fact that the pathophysiological changes characteristic of infection by *Babesia* share many common features with those caused by other haemoprotozoa such as *Plasmodium* (Wright *et al.*, 1988). Interest has been enhanced, however, by the discovery that certain purified *Babesia bovis* proteinases act as protective antigens (Wright *et al.*, 1983; Commins *et al.*, 1985).

Proteinases in crude extracts of *B. bovis* were first reported by Wright and Goodger (1973) in an investigation of the causes of increased osmotic fragility of uninfected bovine erythrocytes in babesiosis. Subsequent work led to partial purification and characterization from *B. bovis* of two proteinases, herein referred to as the 72-kDa proteinase and the 20-kDa proteinase. Some of the properties and possible roles of these two enzymes are summarized below.

AMINOPEPTIDASES

Aissi and Charet (1981) described an enzyme from *B. hylomysci* which had been isolated from mouse erythrocytes. Extracts of *Babesia* cells contained an aminopeptidase (90-kDa on the basis of gel filtration) with an isoelectric point of 5.35. This enzyme had activity against the *p*-nitroanilides of L-alanine, L-lysine and L-leucine, but not against the corresponding N-acetyl derivatives. The aminopeptidase appears to be present throughout the *Babesia* cytoplasm (Slomianny *et al.*, 1983). A similar enzyme has been identified in *Plasmodium yoelii nigeriensis* and from *Eimeria* sp. Moss *et al.* (1986) reported the presence of a 161-kDa leucine aminopeptidase in extracts of a variety of strains of *B. microti*. The enzymes were detected using polyacrylamide gel electrophoresis (PAGE) followed by specific development of enzyme activity.

PROTEINASES OF *B. BOVIS*

72-kDa proteinase

This proteinase has been partially purified (Commins *et al.*, 1985) and shown to catalyse the hydrolysis of denatured haemoglobin and gelatin, although its substrate specificity has not been determined further. Recent results indicate that the 72-kDa proteinase is inhibited by the chelating agents EDTA and EGTA, and that the inhibition can be overcome by subsequent incubation with calcium ions (M.A.Commins and P.W.Riddles, unpublished). The purified proteinase has been used for preparation of monoclonal antibodies and for vaccination trials in cattle. As an antigen, this proteinase provides significant protection against challenge by an homologous strain of *Babesia* (Goodger *et al.*, 1989). Immunogold electron microscopy has shown that the monoclonal antibody appears to interact with both the rhoptry and the erythrocyte cytoplasm in thin sections of merozoite-infected erythrocytes taken from bovine brain capillaries (Waltisbuhl, 1990). These observations suggest that the 72-kDa proteinase is secreted from the rhoptry, thereby accounting for its exposure to the host's immune system. It is possible that the 72-kDa proteinase is involved in invasion and/or egress of the merozoite into/from the erythrocyte, or in provision of nutrients for the parasite.

20-kDa proteinase

A 20-kDa proteinase has been partially purified using affinity chromatography on soybean trypsin inhibitor linked to Sepharose, with elution by 8 M urea (Wright *et al.*, 1983) or a pH 3.5 buffer (Wright, 1975). The purified enzyme is active against collagen and denatured haemoglobin as well as against *N*-*a*-tosyl-L-arginine methyl ester. Because of its activity towards the latter substrate, this proteinase has often been referred to as the 'esterase'.

The affinity-purified enzyme was about 20 per cent pure, with several lower molecular weight proteins evident as well. Western transfer analysis using ¹²⁵Ilabelled protein A showed that serum of cattle vaccinated with this preparation contained antibodies directed against only one of the major protein components—the 20 kDa proteinase. This enzyme is located largely in the erythrocyte cytoplasm and on the erythrocyte surface, as judged by studies with fluorescent antibodies on infected bovine erythrocytes. As a vaccine, the proteinase provides weak protection against infection by the same strain of *B. bovis* (Wright *et al.*, 1983). This proteinase tends to aggregate or to bind to other proteins, and it has not yet been purified sufficiently for sequencing.

Virulence of *B. bovis* is attenuated by multiple passages through cattle or by ? irradiation, apparently by selection of strains deficient in the 20-kDa proteinase. In avirulent strains, activity of the enzyme is markedly decreased (Wright *et al.*, 1981), and this enzyme appears to be a 'marker protein' for virulence (Wright and Goodger, 1988). The effect it has is unclear, although it is known to catalyse the release of kinin from its serum precursor.

Infected erythrocytes lodged in brain capillaries during virulent *B. bovis*

infections have a 'stellate' angular shape with external 'knobs'. These knobs may be involved in lodgement by virtue of interactions with fibrinogen, fibronectin and/or thrombospondin (Igarashi *et al.*, 1988; Wright *et al.*, 1988). Loss of these knobs upon multiple passage of *B. bovis* through splenectomized calves appears to correlate with loss of virulence (Ristic and Montenegro-James, 1988). Thus, it is possible that the 20-kDa proteinase has a role in alterations to the erythrocyte membrane which result in knob formation.

PROTEINASES IN OTHER *BABESIA* spp.

Because of the likelihood that 20- and 72-kDa proteinase play a functional role in *B. bovis* infections, the possibility has been investigated that similar proteinases may be present in other species of *Babesia* (Commins, Kung'u, Wright and Goodger, unpublished). Analysis using substrate-sodium dodecyl sulphate (SDS)-PAGE showed that *B. bigemina*, *B. equi* and *B. canis* each gave several bands of proteolytic activity which were distinct from those in normal erythrocytes. The *Babesia* proteinases detected in this study varied greatly in size between the species. However, five size groupings were generally apparent, *viz.* 200–125, 90–85, 72–58, 55–50 and 24–19 kDa.

The smallest proteinases (24–19 kDa) probably correspond to the 20-kDa proteinase that appears to be associated with virulence in *B. bovis*. It is interesting that *B. bigemina*, which often causes infections of low virulence, does not contain this enzyme.

Immunofluorescent antibody tests both with bovine polyclonal antibodies and with monoclonal antibodies raised against the 72-kDa *B. bovis* proteinase, revealed activity in all four species. This suggests that there is a reasonable degree of conservation of this proteinase in different species of *Babesia*.

ROLE OF PROTEINASES IN PATHOPHYSIOLOGY

Wright *et al.* (1988) and Wright and Goodger (1988) have summarized the substantial effects of *Babesia* infections on the complement, coagulation and kallikrein systems of the host animal. The 20-kDa *B. bovis* proteinase described above activates kallikrein and possibly complement. Considering the multitude of pathophysiological effects in acute babesiosis, it is possible that other *Babesia* proteolytic enzymes will also interact with components of these host defence systems.

FEEDING MECHANISMS: A ROLE FOR PROTEINASES?

Ultrastructural studies have led to several suggestions about mechanisms of *Babesia* feeding (Igarashi *et al.*, 1988). Rudzinska and Trager (1962) proposed that *B. rodhaini*, like *P. lophurae*, takes up haemoglobin by phagotrophy, but this was later shown to be very unlikely (Langreth, 1976; Rudzinska, 1976). Simpson *et al.* (1967)

studied the fine structure of *B. equi* and described food vacuoles formed by pinocytotic vesicles and tubular structures in the cytoplasm of the parasite. Molyneux and Bafort (1970) reported the presence of a cytostome in *B. microti*. Frerichs and Holbrook (1974) observed two structures possibly involved in the ingestion of nutrients in *B. equi*: a cytostome that takes in haemoglobin from the host cell and a tubule that extends from the main body of the parasite through the erythrocyte to the blood plasma and appears to ingest plasma during periods of rapid growth and development. Langreth (1976) reported that *B. microti* has no cytostome, and suggested on the basis of ultrastructure studies on the uptake of ferritin that the parasite can pinocytose haemoglobin *in vivo*. Langreth (1976) and Rudzinska (1976) both observed in *B. microti* a special organelle composed of coiled multiple membranes located partly inside and partly outside the parasite. It was suggested that this organelle is involved in feeding via extracellular proteolytic digestion of the host cytoplasm, and in excretion of waste products. However, the details of transport of protein and amino acid nutrients into *Babesia* cells still appear to be largely conjectural.

Three of these ultrastructural studies involving two different species have described *Babesia* organelles or structures external to the parasite cell, which extend through the erythrocyte membrane into the host's plasma (Simpson *et al.*, 1967; Frerichs and Holbrook, 1974; Rudzinska, 1976). While artifacts of preparation and interpretation are not without precedent in ultrastructural studies, there is a clear possibility that proteinases as well as membrane proteins of *Babesia* may have significant exposure to antibodies and drugs in the plasma, even while the parasite is in the intraerythrocytic stage. This concept may be of major significance in strategies for the design and development of antiparasite vaccines and drugs.

FUNCTIONAL PROTECTIVE ANTIGENS AS POTENTIAL DRUG TARGETS

Several proteins isolated from *B. bovis* have the remarkable property of inducing in cattle a protective immune response to varying degrees against challenge by virulent strains of the parasite. These proteins include the 72-kDa and the 20-kDa proteinases described above (Wright *et al.*, 1983; Goodger *et al.*, 1989). Evidence to date leads to the concept, composed of two elements, of a *functional protective antigen*:

1. These protein protective antigens, present in small amounts, possess functions or activities that are critical for the parasite in some aspect of invasion and establishment in the host cells; and
2. The crucial biological activity of these proteins may be diminished by specific antibody binding in the first instance, thereby providing the basis of immune protection as observed in vaccination trials.

Such functional protective antigens should also be excellent targets for drugs, and information on the structure and function of the *Babesia* proteinases described

above should provide a sound basis for design of specific proteinase inhibitors as anti-*Babesia* drugs.

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27. Glycosylated phosphatidylinositols of the trypanosomatidae

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INTRODUCTION

Glycosylphosphatidylinositols (GPIs) are functionally important constituents in the outer leaflet of the plasma membrane of many eukaryotic cells (reviewed by Low, 1989; Cross, 1990; Thomas *et al.*, 1990). These glycolipids can be defined as having the structure glycan-inositolphospholipid where the glycan moiety contains the core sequence Man_α1-4GlcN_α1-6myoinositol and where the inositolphospholipid may be either a glycerolipid or a sphingolipid. GPIs are minor membrane components in higher eukaryotes and generally function as membrane anchors for a diverse group of surface proteins. Free GPIs have been reported, but not characterized, in some mammalian cells and are proposed to act as mediators of some of the actions of insulin (reviewed by Low, 1989). By contrast, GPI glycolipids are often major components in lower eukaryotes. In parasitic protozoa belonging to the Trypanosomatidae, which include the African trypanosomes, *Trypanosoma cruzi* and *Leishmania* spp., they may constitute the major glycolipid class. Interest in these glycolipids has been stimulated by the finding that GPI-anchored molecules and related structures are involved in parasite infectivity and survival. They are also important antigens on the parasite surface and may be involved in host immunity. Three types of GPI glycolipid have been identified in these parasites:

1. those that are covalently linked to protein;
2. those that are covalently linked to polysaccharide (the ‘lipophosphoglycans’); and
3. free GPI glycolipids that are not linked to either protein or polysaccharide.

The latter group encompasses the putative biosynthetic intermediates of the protein anchors, the glycoinositolphospholipids (GIPLs) of *Leishmania* spp. and the lipopeptidophosphoglycan (LPPG) of *T. cruzi*. In this chapter I review recent work on the primary and three-dimensional structures of these diverse lipoglycoconjugates. These studies have been important in understanding the interaction between the parasite and the host. In addition, many of these structures

are unique to the parasite and predict the presence of novel biosynthetic pathways that could be exploited for the development of new antiparasite drugs. Finally, from the three-dimensional studies it may be possible to produce synthetic immunogens that mimic these structures and can be used as vaccines.

PROTEIN-LINKED GPIs

The cell surfaces of these parasites are frequently coated by a few dominant proteins. In all cases where the mode of membrane attachment has been studied, these proteins have been found to contain a GPI anchor (Table 27.1). Detection of a GPI anchor is frequently based on the ability of PI-specific phospholipase C (PI-PLC) to convert these proteins from an amphiphilic to a hydrophilic form, as judged by their interaction with membranes or with detergents such as Triton X-114 (see Low (1989) and references therein). In addition, PI-PLC treatment exposes a carbohydrate epitope known as the cross-reactive epitope (CRD) which can be detected with anti-CRD antibodies after Western blotting or immunoprecipitation. It is important to note, however, that some GPI-anchored proteins are partially or completely resistant to PI-PLC (see below). Biosynthetic labelling with inositol, glucosamine, ethanolamine or fatty acids, or chemical detection of these diagnostic components, provide independent criteria for the presence of a GPI anchor. The reason for the extensive usage of GPI as membrane anchors in the protozoa is unclear. As many of these proteins are present on the cell surface in high copy number, the GPI anchor may allow more efficient packing than if they contained a transmembrane polypeptide domain and/or prevent disruption of the lipid bilayer. The presence of a

Table 27.1. GPI-anchored proteins in the Trypanosomatidae.

Organism	Protein	Location	Reference
<i>T. brucei</i>	Variant surface glycoprotein (VSG)	Major surface glycoprotein of bloodstream trypomastigotes	Ferguson <i>et al.</i> (1985b), Lamont <i>et al.</i> (1987), Ross <i>et al.</i> (1987)
<i>T. congolense</i>			
<i>T. equiperdum</i>			
<i>T. brucei</i>	Procyclin	Major surface glycoprotein of procyclic forms	Clayton and Mowatt (1989)
<i>T. cruzi</i>	Ssp-4	Major surface glycoprotein of amastigotes	Andrews <i>et al.</i> (1988)
	90kDA	Major surface glycoprotein of trypomastigotes	Schenkman <i>et al.</i> (1988)
<i>Leishmania</i> spp.	Promastigote surface proteinase (PSP)	Major surface glycoprotein of promastigotes. Also present on amastigotes	Etges <i>et al.</i> (1986)
	Promastigote surface antigen-2 (PSA-2)	Abundant surface glycoprotein of promastigotes	Murray <i>et al.</i> (1989)

GPI anchor may also allow surface antigens to be selectively released by endogenous anchor-specific phospholipases. While lipase-mediated release of Ssp-4 may occur in *T. cruzi* (Andrews *et al.*, 1988) there is no evidence that these enzymes are responsible for VSG release during antigenic variation or developmental transformation in *T. brucei*.

STRUCTURES OF PARASITE PROTEIN ANCHORS

The complete primary structures of only two parasite GPI anchors have been determined for the VSG of *T. brucei brucei* (Ferguson *et al.*, 1988) and the promastigote surface proteinase (PSP, also referred to as gp63) of *Leishmania* (Schneider *et al.*, 1990; and see Chapter 20). The C-terminal domains of these proteins are linked via ethanolamine phosphate to a linear tetrasaccharide with the sequence $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{GlcNH}_2$ which in turn is linked to

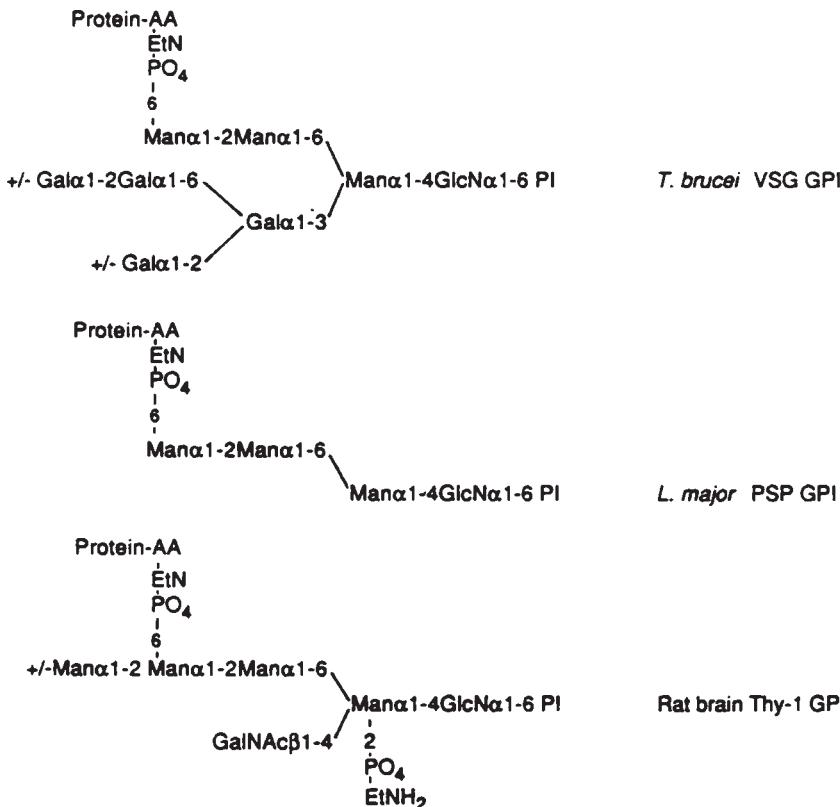


Figure 27.1. Comparison of the primary structures of the protein-linked GPI anchors of *T. brucei* VSG (Ferguson *et al.*, 1988), *L. major* PSP (Schneider *et al.*, 1990) and rat brain Thy-1 (Homans *et al.*, 1988). PI, phosphatidylinositol; EtN, ethanolamine.

phosphatidylinositol (Figure 27.1). The two anchors differ notably in that while the tetrasaccharide sequence is unsubstituted in the PSP anchor, the VSG anchor may be substituted with α Gal (0 to 8 residues) that branch from the 2-position of the inner mannose (Figure 27.1). There are also differences in the lipid moieties of these anchors; while the VSG anchor contains exclusively dimyristylglycerol (Ferguson *et al.*, 1985a), the PSP anchor contains 1-*O*-alkyl-2-*O*-acylglycerols with 12:0, 14:0 and 16:0 fatty acids and 26:0 alkyl chains (Schneider *et al.*, 1990). It is likely that other parasite protein anchors will show additional heterogeneity. In this regard, it is notable that the anchors of the procyclic acidic repetitive protein (PARP) of *T. brucei* (Clayton and Mowatt, 1989) and the 90 kDa antigen of *T. cruzi* trypomastigotes (Schenkman *et al.*, 1988) are resistant to PI-PLC cleavage. PI-PLC resistance has been observed for the anchor of human erythrocyte acetylcholinesterase and is associated with acylation of the inositol with palmitate (Roberts *et al.*, 1989a). It is likely that a similar modification also occurs on these parasite anchors.

Information on the three-dimensional structure of the VSG anchor has been obtained using a combination of high resolution proton NMR measurements and molecular mechanical methods. The glycan moiety is proposed to lie plate-like along the membrane and have a cross-sectional area of 6 nm², which is similar to the cross-sectional area of the crystalline N-terminal domain of VSG (Homans *et al.*, 1989). The galactose side-chain accounts for approximately half of the GPI area, raising the intriguing possibility that it may have a space-filling role which could be important in the dimerization of VSG molecules and/or maintaining the integrity of the VSG coat on the trypanosome surface as a macromolecular diffusion barrier (Homans *et al.*, 1989). This assertion is supported by the finding that GPI galactosylation varies between different VSG subgroups (Homans *et al.*, 1989) and the lack of any similar galactosylation on the *Leishmania* PSP anchor.

Comparison with host protein anchors

There are now more than 100 examples of proteins with GPI anchors in mammalian cells (Low, 1989), although detailed structural information is only available for rat brain Thy-1 (Homans *et al.*, 1989) and human erythrocyte acetylcholinesterase (huAChE) (Roberts *et al.*, 1989a, b). Comparison of the protozoan and mammalian anchors suggests that the ethanolamine-PO₄-6Man α 1-2Man α 1-6Man α 1-4GlcNal-6*myoinositol* sequence has been conserved during eukaryotic evolution and that the glycan moieties of these anchors only differ in the nature and extent of side-chains. Thus the mammalian anchors differ from the protozoan anchors in containing α -linked Man and β -linked GalNAc (Thy-1) or additional ethanolamine phosphate (both Thy-1 and hu AChE) residues. Furthermore, compositional analyses of several mammalian and invertebrate anchors (DAF, ATPase, Scrapie Prion protein and squid glycoprotein-2) support the notion that aGal side-chains are unique to VSG (Low, 1989, and references therein). The *sn*-1, 2-dimyristylglycerol moiety of VSG also appears to be unique, as the mammalian anchors are more like the *Leishmania* PSP anchor in containing 1-*O*-alkyl-2-*O*-acylglycerols (Roberts *et al.*, 1989a; Low, 1989, and references therein).

POLYSACCHARIDE-LINKED GPI: THE LIPOPHOSPHOGLYCANS

The major macromolecule on the surface of *Leishmania* promastigotes is a heterogeneous glycoconjugate, termed lipophosphoglycan (LPG), which consists of a phosphoglycan linked to a GPI glycolipid. LPG epitopes have also been detected on the surface of *L. major* amastigotes and in cutaneous lesions (Handman *et al.*, 1984, 1987). The presence of LPG appears to be critical for successful attachment to and invasion of the macrophage by the promastigote (Handman and Goding, 1985; Elhay *et al.*, 1990; McNeely and Turco, 1990). LPG is thought to mediate attachment to the macrophage in two ways. It may bind directly to macrophage receptors with lectin-like binding sites, such as CR3, p150, 95 and the mannose-fucose receptor (Cooper *et al.*, 1988; Wilson and Pearson, 1988; Russell and Talamas-Rohana, 1989; and references therein). Alternatively, LPG may bind indirectly to the complement receptors CR1 and CR3 after it has been opsonized with C3 complement components (Cooper *et al.*, 1988; da Silva *et al.*, 1989). Complement binds to LPG predominantly as C3b, consistent with the finding that the C3b receptor, CR1, is important in promastigote uptake (Puentes *et al.*, 1988; da Silva *et al.*, 1989). Once inside the macrophage, LPG may protect the parasite from hydrolytic enzymes (El-On *et al.*, 1980; McConville *et al.*, 1990b), or the effects of the oxidative burst (McNeely *et al.*, 1989; Chan *et al.*, 1989; McNeely and Turco, 1990).

Structure of lipophosphoglycan (LPG)

Studies on the LPG from *L. donovani* (Orlandi and Turco, 1987; Turco *et al.*, 1987, 1989; Thomas *et al.*, 1991), *L. major* (McConville *et al.*, 1987, 1990b) and *L. mexicana* (Ilg *et al.*, 1991) suggest that these molecules have the generic structure shown in Figure 27.2, consisting of a phosphoglycan ($M_r=4000$ to 40000), a phosphorylated hexasaccharide core and a *lyso* alkyl-PI lipid moiety. The phosphoglycan moieties consist of repeating units of $\text{PO}_4-6\text{Gal}\beta 1-4\text{Man}\alpha 1-$, where the 3-position of the Gal residue can either be unsubstituted (as in the *L. donovani* LPG), partially substituted with βGlc residues (as in *L. mexicana* LPG), or almost completely substituted with a variety of side-chains containing βGal , βGlc or αAra_p residues (as in the *L. major* LPG) (Figure 27.2). Some of these structures, such as terminal $\alpha\text{-D-arabinopyranose}$ have not been identified before in any glycoconjugate. The presence of a common backbone sequence which is variably elaborated with species-specific side-chains is consistent with serological studies which indicate the presence of both conserved and species-specific epitopes (Tolsen *et al.*, 1989). The non-reducing termini of the phosphoglycan chains appear to be capped with neutral oligosaccharides, containing the sequence $\text{Man}\alpha 1-2\text{Man}\alpha 1-$ (McConville *et al.*, 1990b) (Figure 27.2) which may constitute another epitope common to all the phosphoglycan moieties. The oligosaccharide repeat units are linked, via a phosphodiester bridge, to the hexasaccharide core (Figure 27.2). This core is highly conserved in the LPGs of different species and diverges completely from the protein-linked GPI anchors beyond the core sequence $\text{Man}\alpha 1-4\text{GlcNH}_2\alpha 1-6\text{ myoinositol}$. A

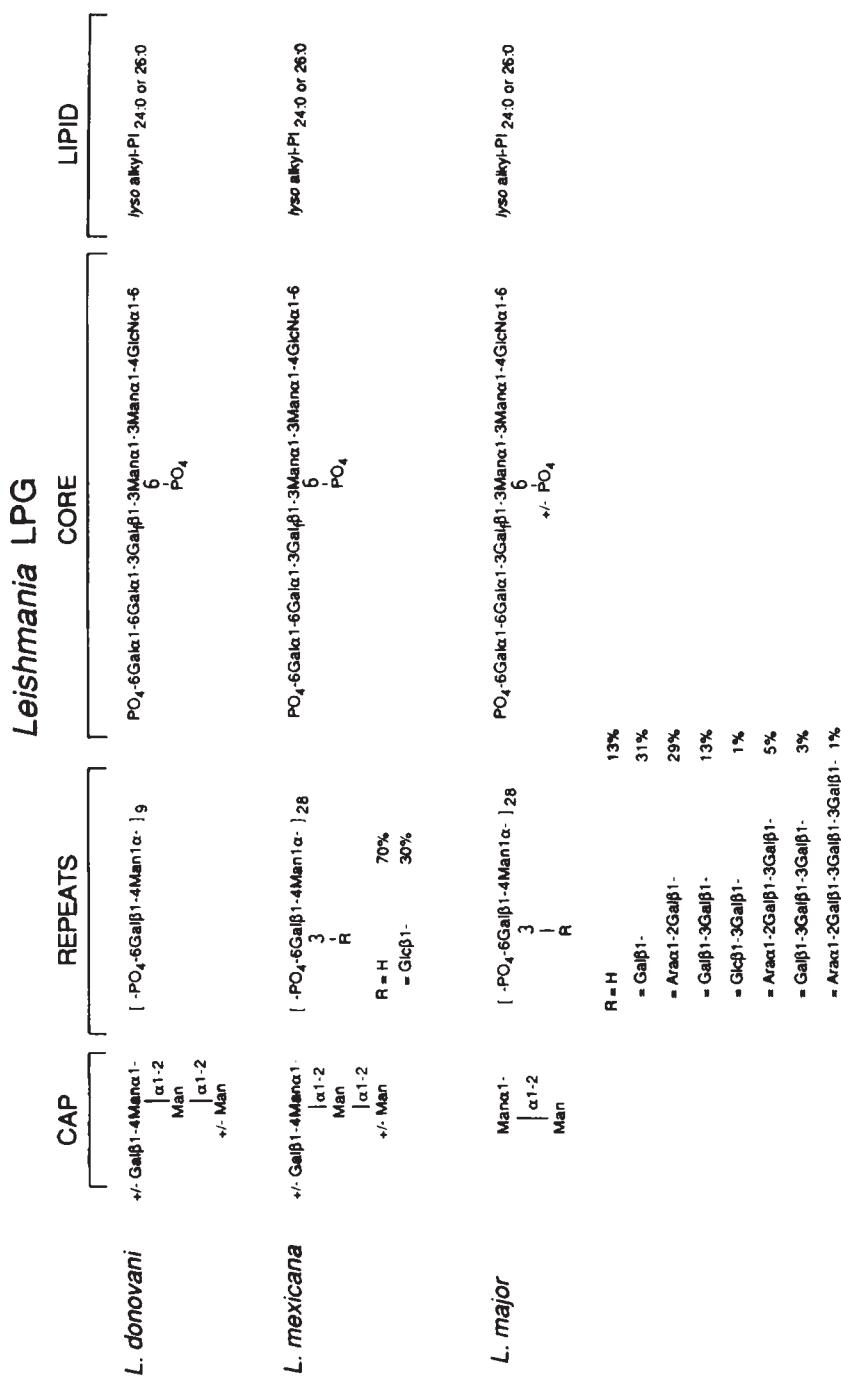


Figure 27.2. Structures of the LPG from *L. donovani* (Orlandi and Turco, 1937; Turco *et al.*, 1987, 1989; Thomas *et al.*, 1991), *L. mexicana* (Ilg *et al.*, 1990) and *L. major* (McConville *et al.*, 1987, 1990b).

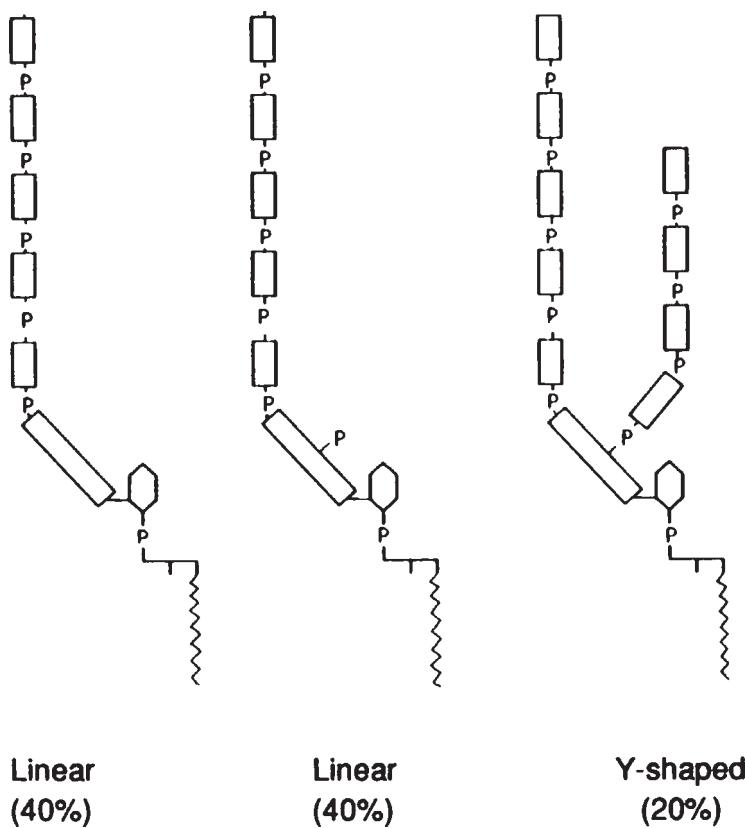


Figure 27.3. Site of attachment of the phosphorylated oligosaccharides to the core of *L. major* LPG. Based on the susceptibility of the core phosphates in the intact LPG to alkaline phosphatase, most of the LPG has a linear structure in which the oligosaccharide repeats are linked to the core via the terminal galactose 6-phosphate. However, in some molecules, both core phosphates may be substituted with repeat units to form a Y-shaped molecule (McConville *et al.*, 1990b).

distinctive feature of the LPG core is the presence of an internal galactofuranose residue. This residue was previously identified as α Gal_f (Turco *et al.*, 1989) but has recently been shown to be in the β -configuration (McConville *et al.*, 1990b; Thomas *et al.*, 1991). One of the inner mannose residues in the core may also be variably phosphorylated. While most of the repeat units are probably attached to the core via the terminal galactose 6-phosphate some of the repeats may also be attached to the core via the inner mannose 6-phosphate residue to form a Y-shaped LPG molecule (Figure 27.3) (McConville *et al.*, 1990b).

The *lyso*-alkyl-PI lipid moiety of LPG, containing predominantly 24:0 and 26:0 alkyl chains is also highly conserved in different species. The lack of substitution on the *sn*-2 position of the glycerol lipid is notable and has not been found in any other GPI anchor, including the anchor of *Leishmania* PSP (see above). This type of lipid

anchor would only provide weak attachment to the surface membrane and may ensure that LPG is passively released from the parasite surface. Consistent with this proposal, is the finding that surface-labelled LPG is continuously released from cultured promastigotes; the half-life for surface LPG on *L. major* promastigotes was found to be approximately 8 h (Handman and Goding, 1985). Most of the released LPG apparently retains the lipid moiety as it interacts specifically with proteins such as serum albumin that contain a hydrophobic pocket (King *et al.*, 1987). Furthermore, the amphiphilic, released form of *L. mexicana* LPG contains predominantly 24:0 alkyl chains, while the cellular form contains mainly 26:0 alkyl chains, suggesting that LPG species with shorter chains are preferentially lost from the parasite membrane (Ilg *et al.*, 1990).

Developmental modification of LPG

As promastigotes move from the midgut to the pharynx and the proboscis of the sandfly vector they undergo both morphological and physiological changes to become metacyclic promastigotes. The metacyclic promastigotes are more resistant to complement-mediated lysis than midgut forms suggesting that they are preadapted for survival in the mammalian host (reviewed by Sacks, 1989). These changes can be mimicked in culture as promastigotes go from logarithmic growth to stationary phase growth. Sacks and colleagues have shown that *L. major* LPG is developmentally modified during metacyclogenesis (Sacks *et al.*, 1984). In particular, the LPG of metacyclic promastigotes has a higher apparent molecular weight on sodium dodecylsulphate (SDS) polyacrylamide gels, it no longer binds the lectin, peanut agglutinin (PNA) and it contains a new carbohydrate epitope (Sacks and da Silva, 1987). Recent studies suggest that the lack of PNA binding is due to capping of the galactose-containing side-chains with α -arabinopyranose residues (McConville *et al.*, 1990b; McConville, Turco and Sacks, unpublished results). Loss of PNA binding also occurs during metacyclogenesis in *L. donovani* (Cooper *et al.*, 1988) even though its LPG does not contain arabinose. It is possible that PNA binds to the neutral cap structure in log phase cells (see Figure 27.2) and that this epitope becomes cryptic in metacyclic promastigotes. Structural modification of LPG, with loss of the PNA binding site, may be physiologically important in regulating the interaction of promastigotes with lectin-like molecules in the sandfly gut or in the mammalian host. See also Chapter 28.

Three-dimensional structure of LPG

Molecular modelling studies of *Leishmania* LPG indicate that the common backbone of phosphorylated disaccharide repeat units exists in several extended configurations which all have a helical pitch (Homans, 1990). The cross-sectional area of the glycan chain shown in Figure 27.4 is around 6 nm^2 (Homans, unpublished results), which is comparable to that of a protein such as VSG. Interestingly, in all the configurations the mannose and phosphate residues are buried within the helix while the galactose residues exist on the outside of the helix.

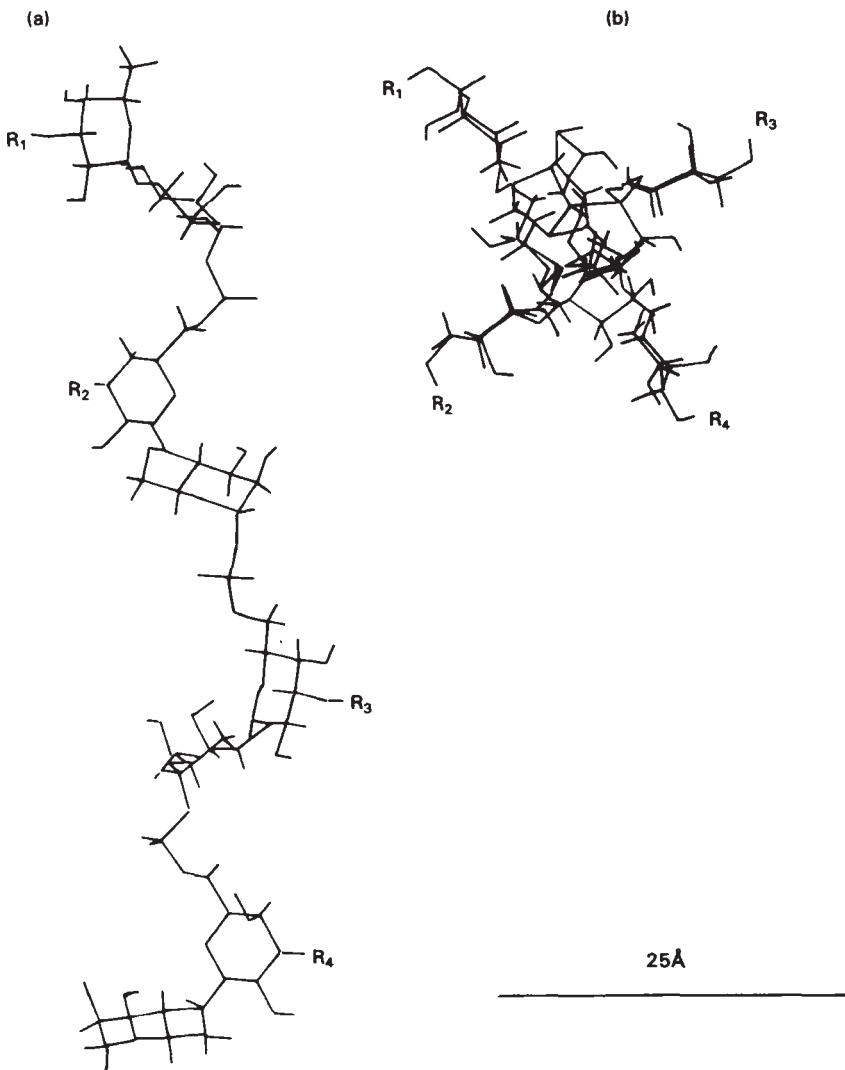


Figure 27.4. Predicted three-dimensional structure of the phosphorylated disaccharide backbone structure that is common to the three *Leishmania* LPGs (see Figure 27.3, data adapted from Homans (1990; unpublished results).) (a) Side view; (b) top view of a sequence of four disaccharide repeat units. R refers to the 3-hydroxyl of the galactose residues which may be substituted with sugar residues in the *L. mexicana* or *L. major* LPGs.

The peripheral disposition of the galactose residues is important as it would allow the side chains that branch off these residues in the *L. major* and *L. mexicana* LPGs to be arranged perpendicular to the axis of the main chain and thus greatly increase the cross-sectional area of the LPG molecule. The core glycan also exists in an extended configuration and has regions of mobility around the Gal α 1–6Gal linkage and within the β -galactofuranose residue (Homans, 1990). These studies predict that

the LPG will extend away from the plasma membrane for some distance and that the LPGs from *L. major* and, to a lesser extent, *L. mexicana*, will have a bottlebrush appearance. It has been estimated that there are $1-5 \times 10^6$ molecules of LPG on the cell surface (Orlandi and Turco, 1987; McConville and Bacic, 1990), which would effectively coat the entire promastigote surface. This coat may form a macromolecular diffusion barrier, as antibodies are unable to bind to epitopes near the plasma membrane (Tolsen *et al.*, 1989) and can be visualized by electron microscopy (Pimenta *et al.*, 1989). While this surface network of LPG is important in activating complement, it may also contribute to the complement resistance of metacyclic promastigotes. Sacks (1989) has proposed that the high molecular weight LPG of metacyclic promastigotes may activate complement further from the cell surface, preventing stable insertion of the membrane attack complex (C5–C9) into the parasite membrane.

FREE GPI GLYCOLIPIDS

Free GPI glycolipids, not linked to either protein or polysaccharide are abundant components in all the Trypanosomatidae that have been investigated. However, there is considerable variation in the structures of these glycolipids in the different species.

T. brucei

Investigations on the biosynthetic intermediates of the VSG anchor identified two major glycolipid species; P2 (also referred to as glycolipid A) and P3 (also referred to as glycolipid C), which have now been characterized (Figure 27.5). P2 has a glycan head group with the sequence ethanolamine-P-Man α 1–2Man α 1–6Man α 1–4GlcNH $_2$ –?inositol and a dimyristylphosphatidylinositol lipid moiety (Menon *et al.*, 1988; Masterson *et al.*, 1989; Mayor *et al.*, 1990a, b), suggesting that it may be the species that is added directly to the C-terminus of newly synthesized VSG in the endoplasmic reticulum (reviewed by Doering *et al.*, 1990). P3 has the same glycan head group and only differs from P2 in containing an acyl chain, probably palmitate, on the inositol ring (Krakow *et al.*, 1989; Mayor *et al.*, 1990a). Labelling experiments in a cell free system have identified several other GPI species which have been partially characterized and are thought to be part of a biosynthetic series (Masterson *et al.*, 1989, Menon *et al.*, 1990). From these studies a pathway of GPI biosynthesis has been proposed whereby P2 is built up by the sequential addition to PI of GlcN (derived from GlcNAc which is donated by UDP-GlcNAc and subsequently deacetylated), three mannose residues and finally ethanolamine phosphate (reviewed by Doering *et al.*, 1990). Masterson and colleagues have also obtained evidence that lipid remodelling occurs at the end of this sequence with sequential replacement of the more hydrophobic fatty acids on *sn*-2 and *sn*-1 with myristate (Masterson *et al.*, 1990). While the significance of acylation of the inositol in some of the GPI species (e.g. P3) is unclear, it may be involved in stabilizing membrane attachment while the acyl groups on the glycerol are being removed

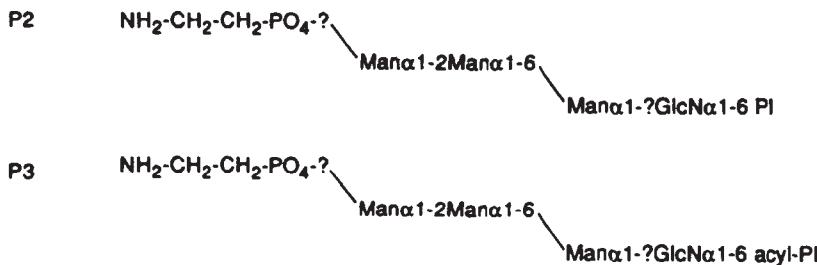
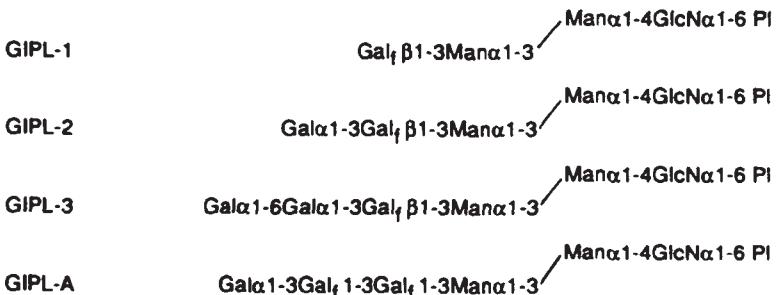
T. brucei candidate precursor glycolipids*Leishmania major* GIPLs*Trypanosoma cruzi*

Figure 27.5. Structures of free GPIs in *T. brucei*, *L. major* and *T. cruzi*. For the structures of P2 and P3 see Mayor *et al.* (1990a, b). The acyl chain on the inositol of P3 is probably palmitate. For the structures of the GIPLs see McConville *et al.* (1990a, b). For structures of LPPG see Previato *et al.* (1990). PI, phosphatidylinositol; Cer, ceramide.

(Masterson *et al.*, 1990). Alternatively, P3 may serve as the precursor for protein anchors in procyclic trypanomastigotes which are known to be PI-PLC resistant (see above).

L. major

The major glycolipids of *L. major* promastigotes belong to a novel class of GPIs termed the glycoinositolphospholipids (GIPLs) (McConville and Bacic, 1989, 1990; McConville *et al.*, 1990a) (Figure 27.5). The glycan moieties of the GIPLs

contain the core sequence $\text{Gal}\beta 1\text{-}3\text{Man}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}4\text{GlcNH}_2\alpha 1\text{-}6\text{myoinositol}$ and are linked to either alkylacyl-PI or *lyso*alkyl-PI (McConville *et al.*, 1990a,b). Significantly different structures were reported by Rosen *et al.*, (1989) from another strain of *L. major*, although reanalysis has revealed that they are in fact the same as described above (Rosen *et al.*, 1990). Some of the GIPLs appear to be mature products (McConville, unpublished observations) and are expressed on the cell surface in high copy number (McConville and Bacic, 1990). In particular, GIPL-2 is the major cell surface glycolipid of promastigotes and is also recognized by antibodies from patients with cutaneous leishmaniasis (Rosen *et al.*, 1988, McConville *et al.*, 1990a). By contrast, GIPL-3 is poorly labelled on the cell surface and is rapidly metabolized *in vivo*, suggesting that it may function as a precursor to LPG (McConville and Bacic, 1990; McConville, unpublished results). Consistent with this role is the finding that GIPL-3 has the same glycan structure as LPG and that it appears to be selectively deacylated *in vivo* (McConville *et al.*, 1990a, b). Other lipid remodelling reactions may occur as there is an increase in the proportion of long chain alkyl groups in the GIPLs with elongation of the glycan head group (McConville and Bacic, 1989; McConville *et al.*, 1990a). The possible pathways of GPI metabolism in *L. major* promastigotes is shown in Figure 27.6. In addition to the GIPLs, the presence of another series of GPI glycolipids is predicted from the structural studies of the PSP anchor (see above).

T. cruzi

T. cruzi contains a third class of free GPI glycolipid, termed the lipopeptidophosphoglycans (LPPGs), which are major cell surface components of epimastigotes (de Lederkremer *et al.*, 1976). These glycoconjugates appear to be stage specific as they are not present in either the trypomastigote (infective) or amastigote stages (Zingales *et al.*, 1982). They were initially identified by Lederkremer and colleagues in 1976 and have since been the subject of several investigations (de Lederkremer *et al.*, 1977, 1985; Medonca-Previato *et al.*, 1983). Recently, the primary structure of LPPG has been reported (Figure 27.5) (Previato *et al.*, 1990). A notable feature of the glycan moiety is that it contains the same tetrasaccharide core sequence as the protein-linked GPI anchors. However, it differs from these structures in containing 2-aminoethylphosphonic acid on the glucosamine and additional αMan residues and terminal β -galactofuranose residues (Lederkremer *et al.*, 1985; Previato *et al.*, 1990). The LPPG also differ from the other parasite GPIs in containing a sphingolipid instead of a glycerolipid. The ceramide portion of the sphingolipid is predominantly lignoceric acid (24:0) and sphinganine (Lederkremer *et al.*, 1977; Previato *et al.*, 1990). In addition, all preparations contain non-stoichiometric amounts of peptide (approximately 0.2 per cent) which can be released by mild acid treatment. At present the site of attachment of this peptide is unknown.

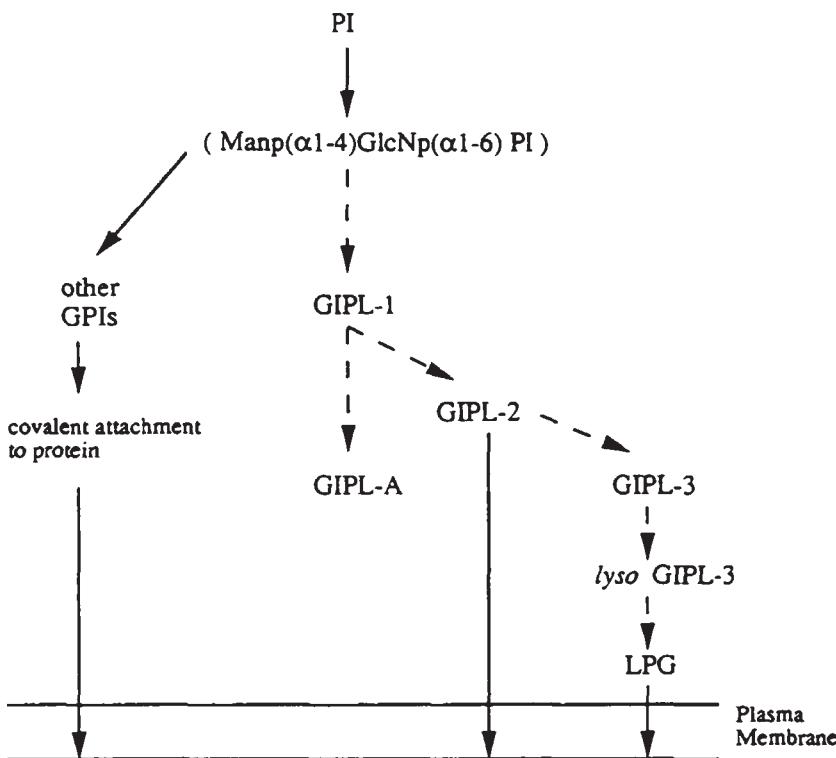


Figure 27.6. Possible pathways of metabolism of GPI glycolipids in *Leishmania*. (Adapted from McConville *et al.* (1990a).)

CONCLUSIONS

While glycosylinositol (GPI) are ubiquitous amongst the eukaryotes, they are most abundant in the protozoa where they appear to be the major glycolipid class. The most extensively characterized of these glycolipids come from parasitic protozoa belonging to the Trypanosomatidae, such as the African trypanosomes, the *Leishmania* species and *T. cruzi*. In all these organisms, GPI glycolipids are the predominant form of membrane anchor for cell surface proteins. While the structures of the parasite anchors are very similar to those that have been characterized from higher eukaryotes, some of the anchors contain additional features that may be important for parasite survival. *Leishmania* and *T. cruzi* contain additional GPI glycolipids which are not linked to protein and are major cell surface molecules. While the function of the *T. cruzi* LPPG is unknown, there is evidence that the GIPLs and LPGs of *Leishmania* are important for parasite infectivity and survival. Studies on the structures of LPG from different species have revealed the presence of both common architectural elements and species-specific features. There is also evidence that these structures are developmentally modified. LPPG, the GIPLs and LPG have no analogues in higher eukaryotes and may represent

relatively recent evolutionary adaptations to a parasitic life-style that have evolved from the protein anchors. Future studies need to be directed towards understanding the biosynthesis of these novel, parasite-specific molecules which may form the basis for the development of antiparasite drugs.

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28. Structural variations of the leishmanial lipophosphoglycan

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INTRODUCTION

The cell surface of *Leishmania* parasites has been a subject of intensive investigation in recent years. The adroitness with which the parasites are able to survive in hostile environments throughout their life cycle suggests that their surface macromolecules undoubtedly have unusual structures and important properties. One of these molecules in the promastigote form of the parasite is its prevalent, polydisperse glycoconjugate called lipophosphoglycan (LPG). The relative abundance (minimally 1 million copies of LPG per promastigote) and novel structural features of LPG indicate that it plays one or more important functions in the parasite's life cycle. In this review, emphasis is placed on the structural variations that have been reported with LPG. The relationships between the intriguing structure of LPG and its possible functions for the parasite have been summarized elsewhere (Turco, 1990; and see Chapter 27).

PURIFICATION OF LPG

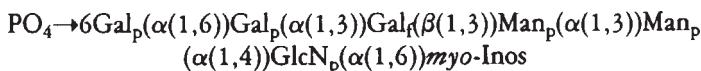
LPG can be extracted from *Leishmania* promastigotes using procedures that employ organic solvent extraction. Further purification can be achieved by gel filtration and hydrophobic chromatography on phenyl-Sepharose (Orlandi and Turco, 1987; Turco *et al.*, 1989) or octyl-Sepharose (McConville *et al.*, 1987). Handman and co-workers have reported the isolation of LPG from *L. major* promastigotes (Handman and Mitchell, 1985) and culture supernatants (Handman and Goding, 1985) using buffered 1 per cent (v/v) Triton X-100 and affinity chromatography on a column containing anti-LPG monoclonal antibody WIC-79.3 bound to Sepharose. A typical yield of LPG is approximately 1 mg of LPG per 10^{10} promastigotes. So far, no LPG has been reported that is unequivocally free of peptide. Whether or not these LPG-associated peptides are contaminants remains an open question.

STRUCTURE OF LPG

The structure of LPG has been extensively examined in *L. donovani*, *L. major* and *L. mexicana* promastigotes. In all these species, LPG has been found to be a polymer of repeating oligosaccharide units linked in linear array via phosphodiester bonds. This section of the glycoconjugate is attached to a phosphosaccharide core region and the entire carbohydrate is anchored into membranes by a 1-*O*-alkyl-2-*lysophosphatidylinositol* lipid. Interestingly, interspecies variability resides in the repeating units while the phosphosaccharide core and the phosphatidylinositol anchor appear to be structurally conserved.

The alkyl group of lipid of LPG consists of a saturated, unbranched C₂₄ or C₂₆ aliphatic chain (Orlandi and Turco, 1987; McConville *et al.*, 1987). The relative portions of the hydrocarbons are approximately 3–4 to 1 with the smaller version predominating. Similar to many glycosyl-phosphatidylinositol (GPI) anchored proteins (reviewed by Ferguson and Williams, 1988; Low, 1989; and see Chapter 27), LPG can be hydrolysed by bacterial phosphatidylinositol-specific phospholipase C yielding the 1-*O*-alkylglycerol and the entire polysaccharide chain (termed phosphoglycan, PG) as products. While the C2 hydroxyl group of the glycerolipid backbone of LPG is unsubstituted, small glycolipids that are believed to be precursors to LPG have been shown to be acylated on that particular carbon (McConville and Bacic, 1989; Rosen *et al.*, 1989; McConville *et al.*, 1990). This would suggest that a deacylation step would have to occur in the biosynthetic pathway of LPG.

The other conserved region of LPG is the phosphosaccharide core (Turco *et al.*, 1989; M.Ferguson and M.McConville, personal communication). The phosphosaccharide-inositol core of the LPG of *L. donovani* was purified and examined by one—and two-dimensional ¹H-NMR and by methylation analysis. From the results, the carbohydrate core was elucidated as a phosphohexasaccharide attached to the inositol residue of the *lysoph*-alkylphosphatidylinositol anchor of LPG as follows:



The anomeric configuration of the galactosylfuranosylmannose bond was originally reported as a. However, recent analysis of much larger preparations of LPG indicated that the bond is in the β -configuration (S.Homans and S.Turco, unpublished observations; and see Chapter 27). The presence of an internal galactofuranose residue is highly unusual in a eukaryotic glycoconjugate. It is interesting to note that the phosphosaccharide core portion of LPG contains the sequence of Gal(α (1, 3)) Gal which is believed to be the epitope for circulating antibodies in patients with leishmaniasis (Towbin *et al.*, 1987; Avila *et al.*, 1988a,b). The Man_p(α (1, 4)) GlcN_p(α (1, 6)) myo-Inos sequence is homologous to the respective portion of the glycosyl-phosphatidylinositol anchors reported for both the *T. brucei* variant surface glycoprotein, the rat brain Thy-1 glycoprotein, and the major surface glycoprotein of *Leishmania* (see Chapter 27).

The variable structural portion of LPG are the repeating units. In *L. donovani*, the repeating phosphorylated saccharide units are $\text{PO}_4 \rightarrow 6\text{Gal}(\beta(1, 4))\text{Man}$ al disaccharides (Turco *et al.*, 1987). There is an average of 16 of these repeat units linked together in a linear array by α -glycosidic linkages between the mannose residue of one unit and the phosphate group of another. One of the characteristic properties of LPG is its extreme liability to dilute acid; conditions of 0.02 MHC1, 15min, 60°C hydrolyses LPG into low-molecular-weight fragments. It is the mannosyl-1 α -phosphate bonds in the repeating units that hydrolyse with the mild acid (Turco *et al.*, 1984, 1989). Consequently, the mild acid generated, low molecular weight fragments are phosphorylated disaccharide units with phosphate groups at the non-reducing ends. Another interesting feature of the repeating units of the *L. donovani* LPG is the presence of the 4-substituted mannose residue. Since this type of sugar substitution is not found in any other known eukaryotic glycoconjugate, its presence in LPG may have important implications for the parasite. For example, it is possible that, during intracellular parasitism in macrophages, the phagolysosome of host cells may not have the appropriate glycosidase to cleave the unique galactosyl($\beta(1, 4)$)mannose linkages of LPG on the surface of the parasite. In future investigations, it will be important to determine whether these repeating phosphorylated saccharide units constitute any secondary or tertiary structure in the glycoconjugate. Such conformations may have prime significance in host cell-parasite interactions.

The repeating phosphorylated saccharide units of the *L. major* LPG are more complex than the *L. donovani* LPG. The units are a series of small phosphorylated oligosaccharides (2, 6-sugars) composed of the hexoses galactose, mannose, glucose, and the pentose arabinose (McConville *et al.*, 1987). In *L. mexicana* and *L. tropica*, the LPG molecule is more similar to the LPG from *L. donovani* in that the repeating units are mostly phosphorylated disaccharide units of $\text{PO}_4 \rightarrow 6\text{Gal}(\beta 1, 4)\text{Man}\alpha 1$ with lesser amounts of phosphorylated trisaccharide units containing an additional glucose residue (S.Turco and D.Sacks, unpublished observations; and see Chapter 27).

DEVELOPMENTAL STRUCTURAL MODIFICATIONS OF LPG DURING METACYCLOGENESIS

Following ingestion of a *Leishmania*-infected bloodmeal by the parasite's insect vector (the plebotomine sandfly), the parasite attaches to the midgut epithelial cells of the insect. Davies and coworkers (1990) recently implicated LPG to be important for this promastigote attachment to the sandfly's epithelial cells. Host lectins on the surface of the epithelial cells probably mediate the attachment. The attached promastigotes multiply and are avirulent.

Eventually, the parasites cease multiplying, detach from the epithelial cells, and migrate to the proboscis of the sandfly. In the latter, the promastigotes await inoculation into the host. During the detachment and migration event in the sandfly's alimentary tract, the promastigote differentiates into a free-swimming, infectious form in a process called *metacyclogenesis* (reviewed by Sacks, 1989) and

the infective forms of the parasite are therefore named metacyclic promastigotes. Differentiation of *Leishmania* parasites from a non-infective to an infective stage has been demonstrated for promastigotes growing within axenic culture and within the sandfly (Giannini, 1974; Keithly, 1976; Sacks and Perkins, 1984; Sacks *et al.*, 1985; Kweider *et al.*, 1987; Howard *et al.*, 1987).

Differentiation of non-infective *L. major* promastigotes into a metacyclic stage is accompanied by changes in cell surface carbohydrates which can be detected by the lectin peanut agglutinin or by stage-specific monoclonal antibodies (Sacks and Perkins, 1984; Sacks *et al.*, 1985). These antibodies were found to recognize a major surface glycolipid which is expressed in two developmental forms, one found exclusively on logarithmically growing promastigotes and the other on metacyclic promastigotes purified from stationary phase cultures. The glycolipid was later shown to be LPG (Sacks *et al.*, 1990). Comparison of the *L. major* LPG derived from these stages revealed that the two characteristic structural features of this molecule, known to be conserved between species, are also developmentally conserved. Both LPGs isolated from non-infective and infective promastigotes contain the *lys*-1-O-alkylphosphatidylinositol lipid anchor, and the polysaccharide fragments of each contain a polymer of repeating acid labile, phosphorylated saccharide units. However, two major developmental distinctions were identified. First, compositional differences were observed in at least some of the repeating phosphorylated saccharide units in the two versions of LPG. Although the structures are not yet known, the changes in structure may account for the expression of a novel epitope on metacyclic LPG (Sacks and da Silva, 1987). The second difference, and the more striking, was an approximate doubling in size displayed by the metacyclic version of LPG, due to an increase in the number of phosphorylated saccharide units in the molecule (Sacks *et al.*, 1990). The relative increase in size of metacyclic LPG is consistent with recent freeze fracture electron microscopic studies of the metacyclic promastigote cell surface which have revealed the presence of densely packed filamentous structures not present on non-infectious promastigotes (Pimenta *et al.*, 1989). These studies also showed a greater than two-fold thickening (17 nm versus 7 nm) of a surface coat which could be specifically labelled with the monoclonal antibody against the metacyclic form of the LPG. In another recent finding, Pimenta *et al.* (1990) showed that the well-characterized, surface glycoprotein (Gp63) appears to be masked by the presence of the densely packed LPG coat. They suggest that this would be analogous to the coat formed by the variant surface glycoprotein in African trypanosomes and the circumsporozoite protein in *Plasmodium* sporozoites. A finding similar to that demonstrated with *L. major* has also been obtained with the metacyclic promastigotes of *L. donovani* in axenic culture (S.Turco and D.Sacks, unpublished observations). Interestingly, the LPG from axenically grown *L. mexicana* was not found to differ in size in various phases of growth. However, a change in proportion of the repeating phosphorylated saccharide units was detected (S.Turco and D.Sacks, unpublished observations).

The significance of these developmentally regulated changes in LPG structure could be important in mediating attachment and release of the parasite from the midgut cells in the sandfly and also its subsequent migration. Following inoculation

of the metacyclic promastigote into a host, the developmentally altered LPG might also pre-adapt the promastigote for survival. As reviewed by Sacks (1989), metacyclic promastigotes are resistant to complement mediated killing, yet they activate complement as efficiently as serum-sensitive non-infectious promastigotes. In each case, C3 deposition on the parasite surface can be found covalently associated with the respective forms of LPG (Puentes *et al.*, 1988). The elongated version of LPG on metacyclic promastigotes (at least with *L. donovani* and *L. major*) may protect the promastigotes from complement-mediated damage by sterically hindering access of macromolecules to the cell membrane, thereby preventing channel formation and lysis by the C5–9 membrane attack complex. Puentes *et al.* (1990) have recently demonstrated the formation and release of C5–C9 from the surface of *L. major*. Thus, metacyclic *Leishmania* spp. that express elongated LPG appear to have evolved a mechanism of complement-resistance similar to that reported in Gram-negative bacteria, for which the presence of smooth lipopolysaccharide containing long polysaccharide chains correlates with serum resistance and virulence (Joiner *et al.*, 1986). It is not known how *Leishmania* spp. that do not express elongated LPG (eg. *L. mexicana*) avoid complement-mediated destruction. Furthermore, whether these developmental modifications of LPG during metacyclogenesis also account for the diverse tissue tropisms of the various *Leishmania* species and consequent pathogenesis is unknown. Clearly, elucidation of the structures of the metacyclic version of LPG and the enzymology that accounts for this process will be an important area of research.

EXTRACELLULAR LPG

LPG can be found in the culture medium of axenically grown promastigotes in two structurally distinct forms (Slutzky *et al.*, 1979; El-On *et al.*, 1979; King *et al.*, 1987). One form of LPG binds very tightly to albumin in the medium, and analysis of this form indicates that the LPG retained its lipid moiety. The most likely explanation is that the lipid portion of LPG interacts with a hydrophobic binding pocket of albumin facilitating its release from the surface of the parasite. The other form of LPG found in conditioned medium is a hydrophilic form (believed to be the 'excreted factor') in which the lipid is not present on the glycoconjugate. The structure of the hydrophilic form and the mechanism of its release are not known. A phospholipase cleavage to initiate its release has been suggested (Handman and Goding, 1985; Elhay *et al.*, 1988). However, there has been no convincing proof as yet. It is just as possible that the hydrophilic form may be a secreted polysaccharide analogous to secreted polysaccharides in bacteria (Braatz and Heath, 1974).

Interestingly, the prominent repeating phosphorylated saccharides of LPG have been shown to constitute a carbohydrate component of an acid phosphatase secreted by *L. donovani* (Bates *et al.*, 1990) and *L. tropica* (Jaffe *et al.*, 1989, 1990). Since LPG, PG and acid phosphatase (and possibly other glycoconjugates) can be released into extracellular medium and share the repeating phosphorylated saccharide epitope, interpretations of immunological results concerning the originally described excreted factor may have to be re-evaluated.

LPG FROM AMASTIGOTES

All of the structural information known about LPG has been obtained from LPG isolated from the promastigote form of the *Leishmania* parasite. There has been no information reported regarding the structure of LPG from amastigotes, and the little definitive evidence that amastigotes even synthesize any LPG. There has been several reports which suggest that this intracellular form of the parasite expresses LPG since epitopes on the surface of infected macrophages could be detected using antipromastigote LPG antibodies (Handman and Hocking, 1982; de Ibarra *et al.*, 1982; Handman, 1990). In these studies, however, promastigotes were used to infect macrophages, and the possibility cannot be excluded that the antigen detected was residual amounts of promastigote derived LPG. In this regard, we have shown that the repeating phosphorylated saccharide epitope of promastigote LPG can be visualized by immunofluorescence on the surface of infected macrophages maximally 1–2 days post-infection with residual detection lasting 5–6 days post-infection (Tolson *et al.*, 1990). Furthermore, even when direct, albeit minimal reaction of antipromastigote LPG monoclonal antibodies with purified amastigotes has been described (Handman *et al.*, 1987), the identity of the detected antigen was not necessarily LPG in view of recent evidence that LPG shares common epitopes (the repeating phosphorylated saccharide units) with other glycoconjugates in promastigotes (Bates *et al.*, 1990; Jaffe *et al.*, 1990).

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29. Lipid biochemistry of trypanosomatids

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The lipids of the trypanosomatids are increasingly being viewed as targets for exploitation, both in respect of chemotaxonomy and chemotherapy. They present a diverse and plentiful system which may easily be analysed and manipulated towards those ends. The following account is a class by class review of the lipids of these flagellates, with emphasis on the sterols, as these are becoming a major focus of interest. In many cases, data are available for one form of a parasite only. For instance, most data for *Leishmania* species come from analyses of multiplicative promastigotes; little is known about amastigotes and metacyclines. The extent of variation of lipids during the life cycle is yet to be determined for most protozoa.

TRIACYLGLYCEROLS

A high incorporation of [¹⁴C]acetate into triacylglycerols (TAGs) is observed in *Leishmania tropica* (Harvey, 1962) and in *L. tarentolae*, where triacylglycerols account for 74.4 per cent of the [¹⁴C]acetate taken into the neutral lipid fraction but only amount to 43.0 per cent by mol % of that lipid fraction (Beach *et al.*, 1979). TAGs are also one of the major lipid forms of African trypanosomes (Dixon and Williamson, 1970; Venkatesan and Omerod, 1976; Oliveira *et al.*, 1977) and are found in procyclic forms in considerably higher amounts than in bloodstream forms (Dixon and Williamson, 1970). Analysis of the fatty acid chains associated with leishmanial TAGs reveals highly unsaturated, long-chain molecules with 18, 20 or 22 carbon atoms and 1 to 6 double bonds (Gercken *et al.*, 1976).

The triacylglycerols are thought to be used by protozoal parasites as energy depots and as storage of fatty acyl groups. Triacylglycerol pools are decreased selectively and continuously as trypanosome cultures age in fatty acid depleted media (Dixon and Williamson, 1970). This indicates their possible function as a source of storage for fatty acids. It is possible that bloodstream forms of trypanosomes contain

Dedication: this review is dedicated to the memory of the late Professor George G. Holz Jr, a valued friend and mentor, who contributed so much to our knowledge of the lipid biochemistry of protozoa.

trypanosomes contain lower amounts than procyclic forms as they have a ready supply of TAGs from the host body and so have no need to synthesize or store these compounds.

PHOSPHOLIPIDS

The phospholipids constitute a major proportion of the lipids of trypanosomatids. In blood and culture forms of *Trypanosoma lewisi* and *T. rhodesiense* they account for 73–77 per cent of the total lipid (Dixon and Williamson, 1970). In *L. donovani* promastigotes, phospholipids comprise 58 per cent of total lipid (Ghosh, 1963) and in other flagellates this figure ranges from 65 to 79 per cent (Von Brand, 1962; Korn *et al.*, 1965; Meyer and Holz, 1966; Hunt and Ellar, 1974; Venkatesan and Omerod, 1976). Generally, the amount of phospholipid is 2–3 times that of neutral lipid. Beach *et al.* (1979) analysed the lipids from promastigotes of a range of *Leishmania* species in which polar lipids accounted for 45–86 per cent of total lipid and showed that the more vigorous the growth of a culture, the higher this proportion became. Their subsequent work (Beach *et al.*, 1982) shows a decrease in the neutral lipid: phospholipid ratio from 1.63 to 0.59 when culture temperature was increased from 25°C to 37°C. This temperature change is typical of that experienced as a parasite leaves the sandfly gut and enters the tissues of a vertebrate host. The increase in phospholipid and the decrease in neutral lipid reflects temperature compensation by the parasite, involving incorporation of extra sterol in addition to impairment of fatty acid desaturation. This results in greater stability of the parasite membranes at the higher temperature.

The predominant phospholipids of *Leishmania* species are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). In addition, there are significant amounts of phosphatidylserine (PS), cardiolipin (DPG), lysophospholipids, *N*, *N*-dimethylphosphatidylethanolamine and phosphatidic acid (Hack *et al.*, 1962; Gercken *et al.*, 1976; Beach *et al.*, 1979). Similar results have been recorded for African trypanosomes (Williamson and Brown, 1964; Godfrey, 1967; Dixon and Williams, 1970; Venkatesan and Omerod, 1976). In cultured procyclic trypomastigotes and bloodstream forms of *T. brucei*, PC, PE, and PI all represent a significant percentage of the total phospholipid, whereas isolated glycosomes from both forms contain PC and PE, but only glycosomes of procyclic stages also contain PI and PS (Hart *et al.*, 1984). It has been frequently observed, however, that there are no major differences in the phospholipid composition between the various strains of these flagellates. An exchange of fatty acids occurs between phospholipid and internal and external fatty acid pools in bloodstream forms of *T. rhodesiense* (Dixon *et al.*, 1971).

Phosphatidylcholine

In *T. cruzi*, phosphatidylcholine accounts for 44 per cent of the polar lipid (Oliviera *et al.*, 1977). Similar proportions have been found in several other species (Gercken

et al., 1976; Beach et al., 1979). This is thought to be almost entirely in the diacyl form, i.e. that with two fatty acid chains attached, although Beach et al. (1979) found 5 per cent of the PC in *Leishmania* in the alkenylacyl and alkylacyl forms. Generally, the fatty acids are long chain molecules with several double bonds. In *Leishmania* species, the major fatty acid of PC is 18:3 (n-3), with 18:2 (n-6) and 22:6 (n-3) making significant contributions. An exception is *L. braziliensis*, in which 18:2 (n-6) is the predominant fatty acid, accounting for 45 per cent of the total. Other fatty acids found in significant quantities are 18:4 (n-3), 18:1 (n-9) and 18:2 (n-6).

Incubation with [2^3H]glycerol results in immediate incorporation into PC but $^{32}\text{P}_i$ incorporation has a lag period (Herrman and Gercken, 1980). This leads to the assumption that biosynthesis of phosphatidylcholine in *Leishmania* occurs via the 1, 2-DAG-CDP base pathway.

Phosphatidylethanolamine

Ethanolamine containing phospholipids account for 25–30 mol% of the leishmanial and trypanosomal polar lipids (Gercken et al., 1976; Beach et al., 1979). Similar forms of ethanolamine containing phospholipids have been identified in *Crithidia fasciculata* (Meyer and Holz, 1966), blood and culture forms of *T. lewisi*, in which they account for 28 per cent of total phospholipid (Godfrey, 1967; Dixon and Williamson, 1970), and also in *T. rhodesiense* (Dixon and Williamson, 1970).

These compounds can be in the alk-1-enylacyl, alkylacyl or phosphatidyl forms. Gercken et al. (1976) suggested that the majority is in the alk-1-enylacyl form, with the aliphatic chains being either 16 or 18 carbons in length. In contrast, however, in *L. tarentolae* the major compound is phosphatidylethanolamine, with the alkenyl acyl and alkyl acyl forms representing only 20 and 12 per cent, respectively. The alkylacyl forms of all species studied contain the 1-hexadec-9-enyl and 1-octadec-9-enyl chains most abundantly (Beach et al., 1979).

The diacyl form of phosphatidylethanolamine in *L. tarentolae*, *L. enrietti*, *L. hertigi* and *L. tropica* contains 18:1 (n-9) as the major fatty acid. In *L. donovani*, *L. braziliensis* and *L. alderi* it is 18:2 (n-6) and in *L. mexicana* 20:3 (n-3). There are also significant amounts of 18:0, 16:0, 18:3 (n-3) and a C19 cyclopropane fatty acid (Beach et al., 1979). The occurrence of this cyclic fatty acid is unusual and is confined strictly to ethanolamine containing phospholipids of the *Leishmania* species. This is discussed further in a later part of this chapter.

In a similar manner to PC, synthesis of PE occurs through the 1, 2-DAG-CDP base pathway (Herrman and Gercken, 1980). Alkyl and alkenyl forms, however, are biosynthesized directly from dihydroxyacetone phosphate (DHAP). DHAP is acylated, the acyl group is then replaced by an ether moiety and the DHAP backbone converted to glycerol phosphate. The alkyl chain of the ether may then be desaturated.

Diphosphatidylglycerol

Diphosphatidylglycerol (DPG), or cardiolipin as it is more commonly called, is widely regarded as a mitochondrial phospholipid. It has been identified in *C.*

fasciculata (Meyer and Holz, 1966), *T. lewisi* and *T. rhodesiense* (Dixon and Williamson, 1970) and in a variety of strains of *Leishmania* (Beach *et al.*, 1979). In the *Trypanosoma* it is present in only trace amounts, but in *Leishmania* it accounts for 3–5 per cent of total phospholipid (Gercken *et al.*, 1976; Hack *et al.*, 1962; Venkatesan and Omerod, 1976; Beach *et al.*, 1979). It is mainly in the diacyl form, the majority of the fatty acyl chains being 18:1 (*n*-9), 18:2 (*n*-6) and 18:3 (*n*-3) (Beach *et al.*, 1979).

Phosphatidylglycerol has also been detected in the phospholipids of *Leishmania* (Herrman and Gercken, 1980). This suggests that biosynthesis of DPG occurs in a manner similar to other eukaryotes (Thompson, 1980), such that PG is formed via the CDP-DAG pathway and the additional phosphatidyl group of DPG originates from CDP-DAG.

Phosphatidylinositol

In 1967, Godfrey made a tentative identification of phosphatidylinositols in some trypanosomes. They were subsequently found in blood and procyclic forms of *T. rhodesiense* (Dixon and Williamson, 1970) and in epimastigotes of *T. cruzi* (Oliveira *et al.*, 1977). Phosphatidylinositols account for 10–20 mol % of leishmanial phospholipids (Gercken *et al.*, 1976; Beach *et al.*, 1979; Kaneshiro, 1982). Of this, around 84 per cent is in the diacyl form with 18:1 (*n*-9), 18:0, and 18:3 (*n*-3) as the major fatty acyl chains, and 8 per cent is in each of the alkenylacyl and alkylacyl forms (Beach *et al.*, 1979). Phosphatidylinositol 4-phosphate and phosphatidylinositol 4, 5-bisphosphate are also found (Kaneshiro, 1982). PI amounts to 16 per cent of the total phospholipid in bloodstream forms of *T. brucei* (Hart *et al.*, 1984).

The functions of PI have not yet been fully elucidated. It is known to form part of the surface glycoproteins in trypanosomes, as is discussed later in this chapter. The diacyl forms of PI are involved in signal transduction (Thompson, 1980). Lyso-1-alkylphosphatidylinositols with 17:0 and 18:0 alkyl substituents, found in *L. mexicana mexicana*, are thought to be degradation products of 1-alkyl-2-acylphosphatidylinositols (Singh *et al.*, 1988). As there is no difference in lag-time for either $^{32}\text{P}_i$ or 2- ^3H labelling of glycerol, it is thought that PI is biosynthesized via the CDP-DAG pathway (Herrman and Gercken, 1980).

FATTY ACIDS

The fatty acids of the trypanosomatids have been frequently studied, and may exist as free acids or conjugated to sterols, phospholipids and TAGs.

In 1970, Dixon and Williamson showed that there was a great similarity between the total fatty acids of *T. lewisi* and the composition of those in its culture medium. They concluded that there is a limited biosynthetic capacity for fatty acids in *Trypanosoma* and that instead they could be freely absorbed from the culture medium. *T. lewisi* and *T. rhodesiense* absorb both saturated and unsaturated long-chain fatty acids from their environment. The extent of this absorption varies over

the stages of the life cycle, different fatty acids being absorbed at different points (Dixon *et al.*, 1971). Available non-esterified fatty acids are used 'exhaustively' as a source of nutrition by amastigotes and promastigotes of *L. mexicana*. They undergo high rates of catabolism to CO₂ and the parasites apparently show little preference for chain length or degree of unsaturation (Hart and Coombs, 1982). Trypanosomatids can incorporate labelled acetate, and also carbon derived from glucose or glycerol, into fatty acids (Korn *et al.*, 1965; Meyer and Holz, 1966; Dixon *et al.*, 1971). There is little or no *de novo* synthesis of fatty acids, the acetate is used only for elongation of existing long-chain fatty acids; such as in *T. lewisi* where [1-¹⁴C] acetate labels only the C₂₀ and C₂₂ polyunsaturated fatty acids (Korn *et al.*, 1965). In *L. tarentolae* 20:3, 22:5, 22:5 and 22:6 may be biosynthesized from 18:3 and 20:4 (*n*-6), (Korn *et al.*, 1965). The flagellates are also able to synthesize monounsaturated fatty acids from exogenously supplied palmitate and stearate and desaturate them further through linoleate and linolenate (Meyer and Holz, 1966).

The fatty acids of the trypanosomatids are mostly C₁₆, C₁₈, C₂₀ or C₂₂ with 1-6 double bonds. Saturated, *iso* branched and cyclopropane molecules are also found (Korn *et al.*, 1965; Gercken *et al.*, 1976; Beach *et al.*, 1979). The saturated and unsaturated C₁₈ fatty acids are most prominent in the leishmanias (Korn and Greenblatt, 1963; Gercken *et al.*, 1976). The identification of the 18:3 isomeric molecules is not yet clear. Meyer and Holz (1966) found -γ-linolenic acid [18:3 (*n*-6)] as the major fatty acid in *L. tarentolae* grown in defined media. More recent findings and those of Korn and Greenblatt (1963) and Korn *et al.* (1965) have shown that this (*n*-6) form, which is typical of the 'animal pathway' of fatty acid biosynthesis, is only present in trace amounts. The majority of 18:3 fatty acids are instead in the (*n*-3) form, as are the longer chain molecules, 20:3 and 22:6 (Beach *et al.*, 1979). The presence of these (*n*-3) molecules suggests that fatty acid desaturation occurs via the 'plant pathway'. The presence of traces of (*n*-6), however, suggest that in *Leishmania*, as in *Euglena* (Erwin, 1973), the animal and plant pathways of desaturation co-exist (Holz, 1985). Whatever the pathway involved, the presence of so many highly unsaturated fatty acyl chains endows leishmanial membranes with a high degree of fluidity. On exposure of culture forms of *L. donovani* to 37°C the C₁₆ and C₁₈ saturated and monounsaturated acids increase whilst the C₁₈, C₂₀ and C₂₂ polyunsaturates decrease (Beach *et al.*, 1982). These changes occur to maintain constant fluidity, over the disorganizing increase in temperature, by shortening chain length and lowering unsaturation and so allowing closer packing of phospholipid fatty acyl chains.

The occurrence of the cyclopropane fatty acid *cis*-9, 10-methyleneoctadecanoic acid is limited to certain species of *Leishmania* and is the only fatty acid to be taxonomically restricted in this way. It is found exclusively within the diacyl, alkylacyl and alk-1-enylacyl forms of phosphatidylethanolamine (Beach *et al.*, 1979) and is biosynthesized by the methylenation of oleic acid (Holz, 1985). This biosynthesis requires the expenditure of three ATP molecules per cyclopropane ring and, because of this high energy requirement, the molecule is assumed to have some essential function. Although this has not yet been elucidated, it is possible that it is a virulence or interaction factor. Cyclic fatty acid synthetase is a typically prokaryote

enzyme (Fish *et al.*, 1981) and it has been suggested that its presence in leishmanias could be an adaptation to life inside an insect gut (Holz, 1985).

A tentative identification of the cyclopropane fatty acid was first made by Meyer and Holz (1966), but other studies on *L. tarentolae* failed to identify it (Gercken *et al.*, 1976; Korn *et al.*, 1965), although it is possible that in these analyses it was degraded during methylation procedures. It has since been shown to occur in *L. tarentolae*, *L. alderi*, *L. donovani*, *L. braziliensis*, *L. chagasi* and *L. infantum*, accounting for 2–11 per cent of the total fatty acids of phosphatidylethanolamine (Beach *et al.*, 1979; Fish *et al.*, 1981). There is no evidence for its occurrence in the *Trypanosoma*, but very high levels have been found in some *Cryptosporidium*, accounting for up to 50 per cent of the phosphatidylethanolamine derived fatty acids (Fish *et al.*, 1981).

As the occurrence and amounts of the cyclic fatty acid vary greatly over the various species of *Leishmania*, it is utilized as a useful phenotypic character. The highest levels are found in the *L. tropica* complex (Holz and Beach, 1982), followed by *L. donovani*. Variable amounts are found in the *L. aethiopica* species and none in *L. major*. This phenotypic characterization has been discussed fully in previous reviews (Holz, 1985).

GLYCOLIPIDS

Glycolipids generally only occur in small amounts in the parasitic protozoa. Sphingolipids have been identified in *Cryptosporidium fasciculata* (Meyer and Holz, 1966), *T. brucei* (Williamson and Brown, 1964), in bloodstream forms, and to a lesser extent procyclic forms of *T. rhodesiense* (Dixon and Williamson, 1970) and, in trace amounts if at all, in bloodstream and culture forms of *T. lewisi* (Godfrey, 1967; Dixon and Williamson, 1970; Hibbard and Dusanic, 1970). Sphingomyelin accounts for 4 per cent of phospholipids in epimastigotes of *T. cruzi* (Oliveira *et al.*, 1977) and 24 per cent in bloodstream forms of *T. brucei* (Hart *et al.*, 1984), but is not found at all in cells grown in lipid-free media suggesting uptake of this molecule from the environment, (Beach *et al.*, 1979; Herrman and Gercken, 1980). Inositol phosphosphingolipid and lyso-1-alkyl PI-phosphoglycan (lipophosphoglycan) are also found in *Leishmania* (Kaneshiro *et al.*, 1986; Orlandi and Turco, 1987; and see Chapters 27 and 28). Inositol phosphosphingolipids are part of an antigen complex in *L. donovani* (Kaneshiro *et al.*, 1986) and lyso-1-alkyl-phosphatidylinositols are anchors of the lipophosphoglycans of *L. donovani* and *L. major* (McConvile *et al.*, 1987; Turco, 1989). Inositolphosphosphingolipids have also been identified in promastigotes of *L. mexicana mexicana* (Singh *et al.*, 1988). Trypanosomes contain relatively high concentrations of glycolipid and ganglioside (Carroll and McCrorie, 1986). The presence of large amounts of lipid bound sialic acid suggests the presence of gangliosides which are important in cell surface phenomena. Trypanosomatid flagellates are known to have surface coats of glycoproteins and glycolipids (Holz, 1985). The variant surface glycoproteins (VSG) of *T. brucei* are anchored to the cell surface via a glycosylphosphatidylinositol structure (Ferguson and Williams, 1988; and see Chapter 27). The lipopeptidophosphoglycan of *T. cruzi*

has mannose, galactose, glucosamine and inositol phosphate containing oligosaccharides in addition to sphingoid bases (De Lederkremer *et al.*, 1977, 1978, 1980). Other compounds that have been identified in *Leishmania* (Slutzky and Greenblatt, 1977; Dwyer, 1980) contain various sugars including inositol, peptides, fatty acids, sphingoid bases and phosphorus (Semprevivo and MacLeod, 1981; Kaneshiro, 1982). A single surface glycoprotein of *Leishmania*, which has proteinase activity and is known as promastigote surface proteinase (PSP or gp63) has a hydrophobic phosphatidylinositol containing membrane anchor (Bordier, 1987; and see Chapters 20 and 27). The glycolipids of the leishmanias are interesting compounds whose biochemistry has yet to be fully elucidated but which may prove useful as chemotherapeutic tools.

ALKOXYDIACYLGLYCEROLS

Alkoxydiacylglycerols are found in small amounts in African trypanosomes (Dixon and Williamson, 1970) and in *L. donovani* (Gercken *et al.*, 1976). In *L. tarentolae* they account for 1.7 per cent of the neutral lipids and acquire 5.1 per cent of added [^{14}C]acetate (Beach *et al.*, 1979). Generally, they occur as a mixture of ether and vinyl ether analogues of the observed triacylglycerols within a species. Saturated alkoxy moieties, of 16 or 18 carbon atoms in length, are attached at position 1 of the glycerol backbone, whilst the fatty acyl groups found at position 2 and 3 are mostly unsaturated (Gercken *et al.*, 1976). Alkoxydiacylglycerols are thought to be biosynthesized in *Leishmania* by the substitution of a fatty acid chain of an existing diacylglycerol with a fatty alcohol which may then be desaturated. This is further substantiated by the observation that incubation with [$1-^{14}\text{C}$]octadecanol labels alkenyl and alkyl groups of alkoxydiacylglycerols (Herrman and Gercken, 1980).

STEROLS¹

Early work on the sterols of the flagellates identified only a small proportion as anything other than cholesterol (e.g. von Brand *et al.*, 1959). In retrospect, this may be because of the labile nature of the sterols found within these cells. The presence of a $\Delta^{5,7}$ conjugated diene ring system renders the molecules susceptible to light, heat and oxygen.

Growth of *Leishmania* cultures in serum supplemented, or cholesterol containing, media results in uptake of sterol from that media (Williamson and Ginger, 1965;

¹ Steroid numbering used in this section is that described by the IUPAC-IUB Joint Commission 1989 (*European Journal of Biochemistry*, 186, 429–58). In other respects, sterol names are those used by the author of each respective paper and so may differ in the definition of the carbon skeleton, especially if the stereochemistry of the side-chain is uncertain. For example, 24-methylcholesterol may be used instead of ergost-5-en-3 β -ol if the stereochemistry of the 24-methyl group is unknown. Trivial names are also used in some instances and, where possible, a reference to the structure is given. Numbers in brackets refer to molecules present in one of the figures.

Pieragostini *et al.*, 1974) and this cholesterol content is superimposed on to the pattern of sterols originating from the parasite itself (Williamson and Ginger, 1965; Korn *et al.*, 1969). Bloodstream and culture forms of *T. lewisi* and procyclic forms of *T. rhodesiense* biosynthesize 24-alkyl sterols *de novo*. In addition exogenous cholesterol is incorporated and esterified in the parasite, but is not metabolized (Dixon *et al.*, 1972). This is unlike the situation in *Tetrahymena* where exogenous cholesterol is metabolized to cholesta-5, 7, 22-trien-3 β -ol (Conner *et al.*, 1969). The 24-alkyl sterols synthesized by the parasites incorporate both [^{14}C] acetate and [^{14}C]methionine (Dixon *et al.*, 1972). In serum-free media, ergosterol (**14**), 22, 23-dihydroergosterol, 7-dehydroporiferasterol (**15**) and 7-dehydroclionasterol (or their C24 isomers), 24-methylene-7-dehydrocholesterol (**12**), 24-ethylidine-7-dehydrocholesterol (**13**) are all biosynthesized by *T. cruzi*. The relative proportions depend on the age of the culture (Korn *et al.*, 1969). These results are mirrored by findings in *L. tarentolae* (Halevy and Avivi, 1966), *T. ranarum* (Halevy and Gisry, 1964), *T. lewisi* and *T. rhodesiense* (Dixon *et al.*, 1972), and *T. mega* (Williams *et al.*, 1966).

Further reports of synthesis of $\Delta^{5,7}$ sterols by parasites have included the use of more sophisticated techniques, such as gas chromatography-mass spectrometry (GC-MS), to confirm these identifications. In *L. donovani* and *L. tropica*, various $\Delta^{5,7}$ C₂₈ and C₂₉ sterols have been identified, including ergosta-5, 7, 24(24 1)-trien-3 β -ol (**13**) (Pieragostini *et al.*, 1974; Gercken *et al.*, 1976). The sterol patterns are used to distinguish between strains of parasites. For example, free sterols of *T. platydactyli* and *Leishmania* contain large proportions of ergosta-5, 7, 24(24 1)-trien-3 β -ol (**12**), but this sterol is completely absent from *T. cyclops*, *T. lewisi* and *T. conorrhini* (Gomez-Eichelmann *et al.*, 1988).

Goad *et al.* (1984) identified squalene, lanosterol (**1**) and 4 α , 14 α -dimethylcholest-8, 24-dien-3 β -ol (**2**) in various species of *Leishmania*. The discovery of these compounds suggests that sterol biosynthesis occurs in a similar vein to that occurring in the fungi (Mercer, 1984). The absence of 24-methyl derivatives of 4, 4-dimethyl sterols suggests that 24-methylation occurs relatively late in the pathway and it appears that zymosterol (cholest-8, 24-dien-3 β -ol) (**6**) may be the major substrate for this reaction. Further modifications of the sterol molecule include the isomerization of the Δ^8 double bond to a Δ^7 double bond to form the major sterol of *L. major*, ergosta-7, 24(24 1)-dien-3 β -ol (**9**) and the further addition of a Δ^5 double bond to form the ergosta-5, 7, 24(24 1)-trien-3 β -ol (**12**) found in predominance in *L. tropica*, *L. donovani* and various *L. mexicana* strains. The presence of 24-methyl-5, 7, 22-cholestatrien-3 β -ol (**14**), 24-ethylcholest-5, 7, 22-trien-3 β -ol (**15**) and ergosta-5, 7, 22, 24(24 1)-tetraen-3 β -ol indicates that a second side-chain methylation and Δ^{22} desaturation also occur late on in the pathway (Goad *et al.*, 1984).

More recent studies on the sterol biosynthetic pathway of *Leishmania* have used inhibitors, which block various steps within the pathway and, in some cases, are already used in a chemotherapeutic role. These compounds are exploited to cause accumulation of intermediates, normally only transiently present, to allow further elucidation of the pathway.

Ketoconazole is an antimycotic imidazole which has chemotherapeutic value in a number of parasitic systems. It inhibits multiplication of *L. mexicana mexicana* promastigotes (Berman *et al.*, 1984) and *L. mexicana mexicana* and *L. donovani* amastigotes in mouse peritoneal macrophages (Berman, 1981). In animal systems it is active against visceral strains (*L. donovani*) but not against cutaneous *L. major* or *L. mexicana amazonensis* (Raether and Seidenath, 1984; Weinrauch and El-On, 1984). The antimycotic imidazoles act in fungi by disruption of sterol biosynthesis. They inhibit the 14α -demethylation step and cause the accumulation of 4α , 4β , 14α -trimethylsterols, 4α , 14α -dimethylsterols and 14α -methylsterols. The imidazole molecule is thought to bind to the haem group of cytochrome P-450, preventing the binding of molecular oxygen and so inhibiting demethylation of the sterol nucleus. The mode of action of these inhibitors has been extensively reviewed (Van den Bossche *et al.*, 1980; Weete, 1987).

Inhibition of *L. mexicana mexicana* promastigote proliferation by ketoconazole is accompanied by disruption of sterol biosynthesis. This allows further elucidation of the biosynthetic pathway. On treatment, the major sterol which accumulates is 4α , 14α -dimethylcholesta-8, 24-dien- 3β -ol (**2**) (Goad *et al.*, 1985). This suggests that the C14 methyl group is removed after one of the C4 methyl groups of lanosterol (**1**), but before C24 methylation occurs. The presence of 14α -methylcholesta-8, 24-dien- 3β -ol (**4**), 4α , 14α -dimethylergosta-8, 24(24^1)-dien- 3β -ol (**3**) and 14α -methylergosta-8, 24(24^1)-dien- 3β -ol (**5**) indicates that the second 4α -demethylation and the 24-methylation may occur even if the 14α -demethylation does not. In all imidazole treated cells, the pool of ergosta-7, 24(24^1)-dien- 3β -ol (**9**) is depleted to allow production of ergosta-5, 7, 24(24^1)-trien- 3β -ol (**12**), suggesting a possible essential role for this sterol. All these conclusions have served to produce the scheme for sterol biosynthesis in *Leishmania* shown in Figure 29.1.

Having considered the promastigote system, it is important to consider the situation in amastigotes. Treatment with ketoconazole results in decreased incorporation of radiolabel into amastigote sterols (Goad *et al.*, 1985; Berman *et al.*, 1986). The sterols of *L. mexicana mexicana* grown in a cloned cell line of a murine macrophage tumour (J-774) are different to the promastigote sterols. The major sterol is ergosta-7, 24(24^1)-dien- 3β -ol (**9**) rather than ergosta-5, 7, 24(24^1)-trien- 3β -ol (**12**) while desmosterol (**16**), 24-methylenecholesterol (**17**) and stigmasta-5, 7, 24(24^1)-trien- 3β -ol (**13**) and stigmasta-7, 24(24^1)-dien- 3β -ol (**10**) are all additional to the promastigote sterol profile (Berman *et al.*, 1986; Hart *et al.*, 1989; Goad *et al.*, 1989b).

The presence of 24-methylenecholesterol (**17**) suggests that desmosterol (**16**) has been taken up from the macrophage and metabolized to this sterol within the cell as it is unlikely to be biosynthesized *de novo*. The same sterol has also been identified in *L. braziliensis guyanensis* amastigotes (Holz *et al.*, 1986). Treatment with ketoconazole in these systems has similar effects to those previously observed in promastigotes, a decrease in ergosterol type sterols and an accumulation of 14α -methylsterols. The amounts of 24-ethylsterols, however, appear unchanged, possibly because of methylation of existing 24-methylsterols. The cholesterol, desmosterol and 24-methylenecholesterol are also still present and this may be

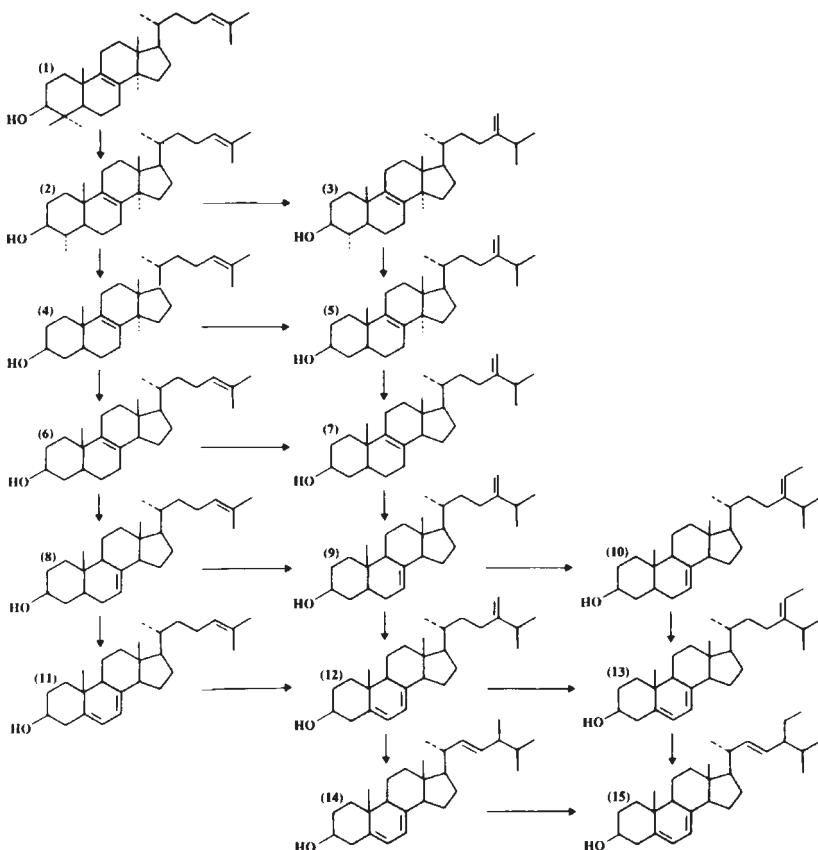


Figure 29.1. Postulated pathway of sterol biosynthesis in *Leishmania*. (1) Lanosta-8, 24-dien-3 β -ol (lanosterol); (2) 4 α , 14 α -dimethylcholesta-8, 24-dien-3 β -ol; (3) 4 α , 14 α -dimethylergosta-8, 24(24 1)-dien-3 β -ol; (4) 14 α -methylcholesta-8, 24-dien-3 β -ol; (5) 14 α -methylergosta-8, 24 (24 1)-dien-3 β -ol; (6) cholesta-8, 24-dien-3 β -ol (zymosterol); (7) ergosta-8, 24(24 1)-dien-3 β -ol (fecosterol); (8) cholesta-7, 24-dien-3 β -ol; (9) ergosta-7, 24(24 1)-dien-3 β -ol (episterol); (10) stigmasta-7, 24(24 1)-dien-3 β -ol; (11) cholesta-5, 7, 24-trien-3 β -ol; (12) ergosta-5, 7, 24(24 1)-trien-3 β -ol (5-dehydroepisterol); (13) stigmasta-5, 7, 24(24 1)-trien-3 β -ol; (14) ergosta-5, 7, 22-trien-3 β -ol (ergosterol); (15) stigmasta-5, 7, 22-trien-3 β -ol (7-dehydroporiferasterol).

viewed as a further indication of uptake and utilization of host sterol which is relatively unaffected by the presence of ketoconazole (Berman *et al.*, 1986). The occurrence of the Δ^5 sterols which are alkylated at C24 is very interesting. These forms of sterol are not seen in promastigotes, possibly because the flagellated forms lack the ability to desaturate the Δ^7 bond, only $\Delta^{5,7}$ and Δ^7 forms are seen in the promastigotes as in the fungi (Mercer, 1984). It is suggested that the Δ^5 sterols are produced in the amastigote from desmosterol (16), absorbed from the host macrophage cell (Weinrauch *et al.*, 1983). *L. braziliensis guyanensis* promastigotes also produce ergosta-5, 7, 22-trien-3 β -ol (14) and amastigotes can apparently

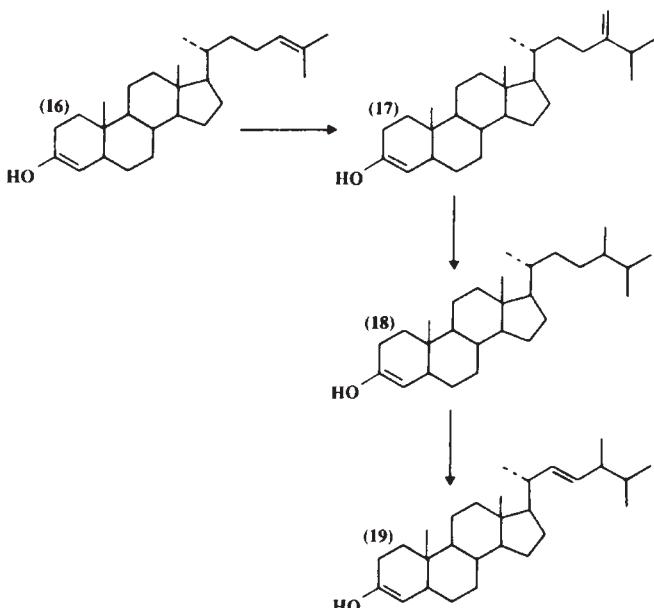


Figure 29.2. Proposed pathway for the conversion of host desmosterol to Δ^5 -methylsterols.
 (16) Cholesta-5, 24-dien- β -ol (desmosterol); (17) 24-methylenecholest-5-en-3 β -ol;
 (18) 24-methylcholest-5-en-3 β -ol; (19) 24-methylcholesta-5, 22-dien-3 β -ol.

convert desmosterol to ergosta-5, 22-dien-3 β -ol (19), using the same enzyme (Holz *et al.*, 1986). In a similar light, *L. mexicana mexicana* promastigotes produce ergosta-5, 7, 24(24¹)-trien-3 β -ol (12) as their major sterol (Goad *et al.*, 1985). This indicates that there is little $\Delta^{24(24^1)}$ -reductase or Δ^{22} -desaturase activity in promastigotes and explains why it is that in amastigotes only 24-methylenecholesterol (17) is produced from desmosterol (16) (Berman *et al.*, 1986) and not Δ^{22} or 24-methylcholesterol. The proposed scheme for utilization of host sterol is shown in Figure 29.2 and is more fully discussed by Goad *et al.*, (1989b).

Work on *T. cruzi* has produced a similar picture with respect to its sterol biochemistry. Treatment with the antimycotic imidazole miconazole causes a decrease in the $\Delta^{5,7}$ sterols of *T. cruzi* epimastigotes (Docampo *et al.*, 1981). The sterol biosynthesis pathway for both epimastigotes and tryptomastigotes has been elucidated in a similar manner to that already described for *Leishmania*, using sterol structures, knowledge of fungal sterol biosynthesis and the structures of sterols accumulating on addition of inhibitors. The pattern of biosynthesis appears to be different from *Leishmania* as the initial step in some strains is the 24-methylation of lanosterol. This methylation occurs much later in *Leishmania*. The major sterols in *T. cruzi* are ergosta-5, 7-dien-3 β -ol and ergosterol. In a similar manner to that already described for *L. mexicana mexicana*, Peru-strain tryptomastigotes of *T. cruzi* may be able to convert host desmosterol to ergost-5-en-3 β -ol and ergosta-5, 24(24¹)-dien-3 β -ol (Goad *et al.*, 1989a).

Sterols are thought to have two roles in normally growing cells. Firstly, a 'bulk' role in maintenance of membrane architecture, the free sterols interacting with the fatty acid chains of the phospholipids to control fluidity of the membrane and so control continuity of its functions. Secondly, sterols are thought to have a 'metabolic' role, involved in the regulation and continuation of normal growth and development. It has been shown that, whereas cholesterol and 5α -cholestane may act as the bulk components for membrane structure (Rodriguez and Parks, 1983), traces of 24β -methylsterols are required for growth in yeast and other fungi (Pinto and Nes, 1983; Rodriguez *et al.*, 1985; Nes and Heupel, 1986; Dahl *et al.*, 1987). The function of this 'metabolic' sterol is thought, in various organisms such as yeast and *Paramecium*, to be involved with aspects of phospholipid metabolism (Guyer and Bloch, 1983; Ramgopal and Bloch, 1983; Rodriguez *et al.*, 1985; Haughan *et al.*, 1987; Whitaker and Nelson, 1987) with possible involvement of a protein kinase antigenically related to pp60 (Dahl *et al.*, 1987).

In *Leishmania* and *Trypanosoma* it is possible that ergosterol type molecules fulfil the 'metabolic' sterol role and that the production of Δ^5 C₂₈ sterols from host desmosterol in the presence of sterol biosynthesis-inhibiting imidazoles is an attempt by the parasite to produce a molecule adequately similar to the metabolic sterol to perform its normal function. Initial studies on this possibility have already been performed (Goad *et al.*, 1989b). The system lays open the possibility of combining normal chemotherapeutic imidazoles such as ketoconazole, which decrease ergosterol biosynthesis in *Leishmania*, with inhibitors of 24-methylation, to prevent formation of 'metabolic' sterol from host desmosterol, to produce a potentially highly effective antileishmanial drug combination. In addition, as the C24 methylation and the Δ^{22} desaturation steps have no parallel in the mammalian sterol biosynthesis pathway (Schroepfer, 1982) there actually seem to be three steps which may be valuable with respect to chemotherapeutic intervention:

1. the SAM-dependent C24 side-chain alkylation;
2. the reduction of the C24 methylene group formed by the alkylation; and
3. the introduction of the Δ^{22} double bond

In yeast (Pierce *et al.*, 1978) and higher plants (Rahier *et al.*, 1984) the sterol 24-methyltransferase is inhibited by heteroatom-substituted sterols. In *C. fasciculata*, certain of these compounds cause a change of the major sterol from ergosterol, present with significant amounts of ergost-7-enol, ergosta-7, 24 (24¹)-dien-3 β -ol and ergosta-5, 7, 22, 24 (24¹)-tetraen-3 β -ol, to cholesta-5, 7, 24-trien-3 β -ol. This demonstrates the inhibition of the SAM-dependent C24 methyltransferase, but shows that general ring processing is still continuing (Rahman and Pascal, 1990). Structurally different heteroatom-substituted sterols cause accumulation of ergosta-5, 7, 22, 24 (24¹)-tetraen-3 β -ol and ergosta-7, 24 (24¹)-dien-3 β -ol, indicating an inhibition of the 24-methylene reductase enzyme. The use of these compounds, or possibly other inhibitors of such steps, in combination with compounds such as ketoconazole which inhibit *de novo* biosynthesis of sterols within the promastigotes, seems a potential site for chemotherapeutic action. In addition, many other

compounds exist which inhibit sterol biosynthesis at different stages, such as the allylamines at squalene epoxidase and the morpholines at the sterol Δ^8 - Δ^7 isomerase. The use of these compounds, in a similar manner to the imidazoles, to reduce the synthesis of C24 alkyl sterols by the parasite, may also be significant in the treatment of leishmaniasis.

SUMMARY

The lipids of the trypanosomatids are of a diverse nature with respect to both structure and distribution. The occurrence of certain molecules such as the cyclopropane fatty acid and various specific sterols, however, allows them to be viewed as valuable targets for exploitation with respect to both chemotherapy and chemotaxonomy.

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30. Lipids and lipid metabolism of trichomonads (*Tritrichomonas foetus* and *Trichomonas vaginalis*)

D.G.Lindmark, D.H.Beach, B.N.Singh and G.G.Holz Jr.

INTRODUCTION

Lipid metabolism in trichomonads has been little studied, but is clearly defective, the organisms being viewed as fatty acid and sterol auxotrophs. These beliefs date from the nutritional experiments of Cailleau (1936, 1938) who demonstrated that *Tritrichomonas foetus* required cholesterol when cultured in a solvent-extracted peptone and serum medium. This association of the cholesterol requirement with serum then led to determined efforts by Shorb and Lund (1959) to culture a variety of trichomonads in chemically defined media, by substituting lipids for serum. The fatty acids (palmitic and oleic), cholesterol, tocopherol and a solvent-extracted tryptic digest of casein were effective serum substitutes for the growth of *Trichomonas gallinae* (Shorb and Lund, 1959). It was found that C₁₄ to C₁₈ saturated fatty acids were nutritionally active when coupled with oleic acid, and C₁₈ to C₂₂ unsaturated fatty acids were active when coupled with palmitic acid. Purified cholesterol, cholestanol and zymosterol were nutritionally active (Lund and Shorb, 1962). In 1981, Linstead succeeded in growing *Trichomonas vaginalis* in a basal medium patterned after those used for vertebrate tissue cultures. Good growth was obtained when the medium was supplemented with palmitic, stearic and oleic acid (supplied as such or in a synthetic triacylglycerol) and cholesterol all complexed to bovine serum albumin. Peterson and Alderete (1984) presented evidence that the lipid requirements of *T. vaginalis* can be met by receptor-mediated endocytosis of human lipoproteins, supplied in Diamond's medium (1968) or in a semi-defined medium containing trypticase, vitamins, nucleic acid precursors and maltose.

The lipid composition of trichomonads has been little studied. Halevy (1963), Etinger and Havley (1964) and Shorb (1964) noted that the fatty acids and sterols of cultured organisms reflected the lipid composition of the culture medium. Paltauf and Meingassner (1982) identified phosphatidylcholine and phosphatidylethanolamine as major components of hydrogenosomes of *T. foetus* and *T. vaginalis*.

All nutritional and compositional studies suggested that trichomonads were unable to carry out *de novo* synthesis of fatty acids and sterols. In addition, enzymatic studies by Holz *et al.* (1986) demonstrated the absence of enzymes of β -oxidation of fatty acids, suggesting that trichomonads lack the ability to interconvert fatty acids.

Cultured *T. foetus* (Halevy, 1963) and *T. vaginalis* (Ettinger and Halvey, 1964) failed to incorporate [^{14}C]acetate into cholesterol, nor were [U^{14}C]acetate, glucose or threonine incorporated into cholesterol or into fatty acids (Roitman *et al.*, 1978). This information, and the apparent absence of sterols, other than cholesterol, in *T. vaginalis* appeared to indicate that the culture-medium cholesterol was incorporated unaltered.

With the knowledge that trichomonads are fatty acid and sterol auxotrophs it became of interest to determine (1) the lipid composition of trichomonads, and (2) whether they took up and incorporated directly fatty acids, fatty acid precursors, sterols and their precursors, and phospholipids and their precursors from their environment, and if those fatty acids, sterols and phospholipids could be recovered unaltered.

Only a limited number of eukaryotic cells require exogenous fatty acids and sterols. They have been exploited with considerable success in the study of lipid roles in membrane structure-function relationships in vertebrate tissue cells, anaerobic yeasts and ciliate protozoa. In addition, since trichomonads are dependent on environmental lipids it also became of interest to examine the metabolism of the most dynamic of the lipids, the phospholipids. Accordingly, we have grown trichomonads in the presence of radiolabelled fatty acids and cholesterol and their precursors, and various phospholipids and their precursors. We present our data and summarize our conclusions based on results obtained after extraction and characterization of the major trichomonad lipids, after examination of the distribution of radioactivity among the lipid subfractions, and after analysis of the radiolabelled fatty acyl groups and cholesterol for evidence of metabolic alterations (for experimental details see: Holz *et al.*, 1986; and Beach *et al.*, 1990). A similar approach has been taken with phospholipid and phospholipid precursors.

LIPID COMPOSITION OF TRICHOMONADS AND THEIR CULTURE MEDIA

The characterization and quantitation of the major lipid components of the trichomonads and their culture medium yielded the information summarized in Table 30.1. In a representative analysis of the trichomonads, neutral lipids comprised approximately 33 per cent of the total lipids; cholesteryl esters, triacylglycerols and cholesterol were the major components. Phospholipids constituted approximately 66 per cent of the total lipids. The major components were phosphatidylcholine and phosphatidylethanolamine, together making up approximately 50 per cent of the total phospholipids. In *T. vaginalis*, a third component, sphingomyelin, was approximately 13 per cent of the phospholipids,

Table 30.1. Major lipids of *T. vaginalis* (*T.v.*) and *T. foetus* (*T.f.*), and Diamond's medium with 10 per cent fetal calf serum (DM).

Identity	Percentage of total lipid					
	By weight			By phosphorus		
	<i>T.v.</i>	<i>T.f.</i>	DM	<i>T.v.</i>	<i>T.f.</i>	DM
Neutral lipids	32.2	32.8	18.3			
Cholesteryl esters	5.4	3.3	4.8			
Triacylglycerols	4.8	3.6	0.6			
Unesterified fatty acids	2.2	0.8	0.8			
Cholesterol	19.8	25.1	12.1			
Monoacylglycerols	> 1	> 1	1.3			
Neutral glycolipids	0.6	0.9	8.4			
Phospholipids	67.2	66.3	73.3			
<i>O</i> -Acylphosphatidylglycerol				6.2	6.3	
Phosphatidylglycerol				8.5	10.0	
Phosphatidic acid				4.9	< 1	
Phosphatidylethanolamine				21.9	18.4	10.2
<i>N,N</i> -Dimethylphosphatidylethanolamine				2.7	< 1	< 1
Phosphatidylserine				8.7	4.7	< 1
Phosphatidylinositol				6.6	7.8	7.5
Phosphatidylcholine				19.3	28.8	43.3
Sphingomyelin				13.3	2.2	24.8
Lysophosphatidylcholine				< 1	2.2	14.2
Glycophosphosphingolipids				5.3	17.5	< 1
Other				2.6	2.1	< 1

while in *T. foetus* glycophosphosphingolipids totalled approximately 18 percent. In contrast, the lipids in the culture medium comprised neutral lipids (20 per cent), glycolipids (8 per cent) and phospholipids (72 per cent). Choline containing lipids (phosphatidylcholine, sphingomyelin and lysophosphatidylcholine) accounted for 82 per cent of the phospholipids.

The kinds and amounts of the neutral lipids and phospholipids in the two trichomonad species were much the same, except that *T. vaginalis* was found to contain a large amount of sphingomyelin, while the sphingolipids of *T. foetus* were mainly glycophosphosphingolipids.

Analysis of the fatty acyl groups of these lipids revealed that, with a few exceptions, the composition (qualitative and quantitative) of a given lipid isolated from trichomonads was quite similar to the same lipid isolated from the culture medium. For example, two-thirds of the cholesteryl esters from both sources were unsaturated, the unesterified fatty acids were mostly C₁₀ to C₂₂ saturated and unsaturated compounds and phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol demonstrated similar patterns of 16:0, 16:1, 18:0, 18:1, 18:2 and 20:4. The natures of the fatty acyl groups of the trichomonad lipids and Diamond's medium were similar. All the lipid composition results presented in Table 30.1 strongly suggest that many trichomonad lipids have their origins in the culture medium.

INCORPORATION OF FATTY ACIDS AND FATTY ACID PRECURSORS

When trichomonads were grown to stationary phase in the presence of ^{14}C labelled glucose, acetate or threonine, incorporation into the chloroform/methanol-soluble lipids was poor. Saponification and further separation of the saponifiables demonstrated that over 87 per cent of the incorporation was in the water soluble fraction.

When trichomonads were grown with ^{14}C labelled, C_{12} to C_{22} , straight-chain saturated and unsaturated fatty acids, they actively incorporated all the fatty acids into their chloroform/methanol soluble lipids. The trichomonads did not significantly alter the fatty acids. Preparative radio-gas/liquid chromatography (GLC) demonstrated that 95 per cent of the radioactivity of a fraction was associated with the identical fatty acid which had been supplied during growth.

Further separation of the phospholipids revealed that phosphatidylcholine and phosphatidylethanolamine were the most heavily labelled by C_{12} to C_{22} saturated and unsaturated fatty acids. Acidic phosphoglycerides (phosphatidylinositol, phosphatidyl glycerol, phosphatidic acid and *O*-acylphosphatidylglycerol) were well labelled and were preferentially labelled with unsaturated fatty acids. The sphingolipids were specifically labelled by saturated fatty acids. The radioactivity of the isolated fractions containing the more polar neutral lipids was associated mainly with the neutral glycolipids. The fatty acids failed to label cholesteryl esters or triacylglycerols.

Organism-oriented features of the distributions of the radioactivity of the ^{14}C fatty acids were observed. For *T. vaginalis* labelling of the sphingomyelin by $[1-^{14}\text{C}]16:0$ (20 per cent) and labelling of phosphatidic acid by unsaturated fatty acids was conspicuous, while the modest amount of $[\text{U}-^{14}\text{C}]22:6$ incorporated remained mostly among the unesterified fatty acids. For *T. foetus*, a variety of glycophosphosphingolipids, but not sphingomyelin, were labelled by saturated fatty acids. Phosphatidic acid labelling was not generally evident, whereas phosphatidylcholine was preferentially and heavily labelled by $[\text{U}-^{14}\text{C}]22:6$ (95 per cent).

The results of fatty acid precursor experiments demonstrated that trichomonads were unable to synthesize fatty acids from acetate or acetate precursors such as threonine and glucose. No chromatographic or spectrophotometric evidence was found for changes in the structure of the fatty acids such as chain elongation, desaturation, retroconversion or the introduction of branches and rings. Earlier work by Holz *et al.* (1986) failed to demonstrate enzymatic activities associated with the β -oxidation of fatty acids (crotonase, β -hydroxybutyryl-CoA dehydrogenase and thiolase). In addition, trichomonads were able to introduce fatty acids into phosphoglycerides and sphingolipids but not into cholesteryl esters or triacylglycerols. These data also demonstrated that trichomonads incorporated the fatty acids 12:0, 14:0, 16:0, 18:0, 18:w9, 18:2w6, 20:4w6, and 22:6w6 unaltered into chloroform/methanol soluble lipids.

INCORPORATION OF STEROLS, CHOLESTEROL, CHOLESTERYL ESTERS AND THEIR PRECURSORS

Growth of trichomonads with radiolabelled [2-¹⁴C]mevalonate, [4, 8, 12, 13, 17, 21-³H]squalene, [4-¹⁴C]cholesterol, cholesteryl [1-¹⁴C]oleate and [1, 2-³H]cholesteryl oleate resulted in excellent incorporation of the radioactivity of squalene (32 per cent), cholesterol (21 per cent) and cholesteryl oleate (27 per cent) into chloroform/methanol soluble lipids. Mevalonic acid, a precursor of squalene and cholesterol, and other polyisoprenoids were poorly incorporated. In the case of *T. vaginalis*, 87 per cent of the cholesteryl [1-¹⁴C]oleate was found in the neutral lipid fraction. The remainder labelled a variety of phosphoglyceride fractions. In the case of *T. foetus*, 13 per cent was found with the neutral lipid fraction and 85 per cent with the phosphoglyceride fractions. For both trichomonads, the radioactivity of squalene, [1, 2-³H]cholesterol oleate and cholesterol were found exclusively in the neutral lipid fraction. Examination of the neutral lipid fraction revealed that [1, 2-³H]cholesteryl oleate labelled the free cholesterol fraction but [4-¹⁴C]cholesterol did not label the cholesteryl ester fraction. Collection of the free sterol fraction from trichomonads grown in [1-¹⁴C]cholesterol and analysed by preparative radio-GLC and GC/MS indicated that the structure of the cholesterol incorporated from the medium was unaltered.

The absence of incorporation of the radioactivity of exogenous acetate, glucose, threonine, mevalonate and squalene into cholesterol demonstrated the inability of trichomonad to synthesize cholesterol. The failure to detect sterols other than cholesterol suggests that trichomonads lack the ability to alter the structure of exogenous cholesterol. Labelling of the free sterol fraction by [4-¹⁴C]cholesterol was not accompanied by labelling of the steryl ester fraction, implying that the trichomonads were unable to esterify cholesterol. This interpretation is supported by the fact that labelled fatty acids did not label the steryl ester fraction. Both trichomonads actively incorporated cholesteryl [1-¹⁴C]oleate, but the ester was more efficiently hydrolysed by *T. foetus* than by *T. vaginalis*—85 per cent of the radioactivity of the chloroform/methanol soluble lipids of *T. foetus* but only 13 per cent of that of *T. vaginalis* were found associated with the phosphoglycerides.

INCORPORATION OF TRIACYLGLYCEROL AND PHOSPHOLIPIDS

Other lipids containing fatty acyl groups, glycerol tri [1-¹⁴C]palmitate, *L*-3-phosphatidylcholine, 1, 2-[dipalmitoyl-1-¹⁴C], 1-3-phosphatidylethanolamine, 1, 2-[dipalmitoyl-1-¹⁴C] and [N-methyl¹⁴C]sphingomyelin, were well incorporated by the trichomonads. 51 per cent of the radioactivity associated with the incorporation of phosphatidylcholine by *T. vaginalis* was found in phosphatidylcholine and 61 per cent of the phosphatidylethanolamine was found in the phosphatidylethanolamine fraction. The remaining amounts of radioactivity were among other phospholipids, notably in the sphingomyelin fraction. In a comparable experiment with *T. foetus*, 50

per cent of the phosphatidylcholine was found in phosphatidylcholine, while 70 per cent of the radioactivity of the phosphatidylethanolamine was found in phosphatidylethanolamine. The balance of the radioactivity in each case was distributed among the phosphoglyceride and glycophosphosphingolipid fractions. More than 90 per cent of the radioactivity from sphingomyelin was found in sphingomyelin. Choline, phosphorylcholine, ethanolamine, serine, inositol, glycerol and methionine were incorporated poorly or failed to label the phosphoglycerides in both of the trichomonads demonstrating an impairment in synthesis. *De novo* synthesis of triacylglycerols was not evident. Trichomonads do not appear to use triacylglycerols as fatty acid reservoirs. Our results suggest extensive deacylation and reacylation of phospholipids; however, *de novo* synthesis of complex phosphoglycerides is not evident, hence the trichomonads must rely on environmental sources.

SUMMARY

The trichomonads do not synthesize fatty acids *de novo* from acetate or acetate sources. Instead, they take up unesterified and esterified, medium and long chain, saturated and unsaturated fatty acids from the medium. Demonstrated sources of fatty acids are phosphatidylethanolamine, lysophosphatidyl choline, phosphatidylethanolamine, phosphatidylinositol, triacylglycerols and cholestryl esters. The fatty acids are not employed as energy sources, nor are they elongated, shortened, saturated or desaturated. Rather they are used directly in the formation and in the turnover of phosphoglycerides and sphingolipids. They are not, however, incorporated into triacylglycerols or cholestryl esters. Trichomonads are unable to form cholesterol or other sterols from sources of acetate, from mevalonic acid or from squalene. They do, however, incorporate environmental free cholesterol and cholestryl esters, probably by non-specific lipoprotein binding and endocytosis. Cholestryl esters are deacylated and the fatty acyl groups are used to acylate other lipids, e.g. phosphoglycerides. Trichomonads do not use internal or external cholesterol to form cholestryl esters; nor do they metabolize cholesterol to form other sterols. Deacylation and reacylation of phospholipids occurs in trichomonads, but *de novo* biosynthesis of phosphoglycerides does not occur.

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31. Lipid composition of the membranes of malaria-infected erythrocytes and the role of drug-lipid interactions in the mechanism of action of chloroquine and other antimalarials

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INTRODUCTION

The aminoquinoline antimalarials, which include quinine, quinacrine, chloroquine and mefloquine have been the most widely used antimalarials from the beginning of this century until the present. Quinine was isolated from the bark of the *Cinchona* tree by Caventou and Pelleitier in 1820, and the correct structure was proposed by Rabe (1908). Effective clinically, its use declined between the 1940s and the 1960s owing to the introduction of new, effective synthetic antimalarial drugs such as the 4-aminoquinoline, chloroquine.

Chloroquine, introduced as an antimalarial agent in the 1930s (Magidson and Grigorovskii, 1936; Magidson and Rubtsov, 1937) is less toxic than quinine (Loeb *et al.*, 1946). It has been used clinically to treat malaria, rheumatoid arthritis and collagen vascular disease (Rollo, 1980). However, chloroquine can cause toxic side-effects, such as myopathy (Whisnant *et al.*, 1963) and retinopathy (Hobbs *et al.*, 1959). Biochemically, the mechanism of antimalarial action by chloroquine is not understood. Some contend that chloroquine by its accumulation in the parasite food vacuoles binds to ferriprotoporphyrin, and it is the resultant product that is toxic to the parasite (Fitch, 1983), whereas others suggest that the drug acts directly on enzymes present in the food vacuole to inhibit their action (Yayon *et al.*, 1984). Since the early 1960s there has been a worldwide resurgence of malaria infections owing in part to the appearance of chloroquine-resistant parasite strains. Chloroquine, which is accumulated to high levels in erythrocytes infected with drug-sensitive strains, is reduced in accumulation in resistant strains (Fitch *et al.*, 1979; and see Chapter 39).

Quinacrine was discovered in 1932 as a result of the search for a quinine substitute (Kikuth, 1932) and was widely used as an antimalarial in the decades following its discovery. Subsequently, however, its use as an antimalarial agent declined owing to the effectiveness of chloroquine. Quinacrine cannot be used for treatment of chloroquine-resistant malaria because of a high degree of cross-resistance. At present quinacrine is widely used as an inhibitor of phospholipase A₂ derived from a variety of sources (see below).

Mefloquine is a new antimalarial drug developed to provide therapy against chloroquine-resistant *Plasmodium falciparum* (Peters *et al.*, 1977; Schmidt *et al.*, 1978). Mefloquine binds with high affinity to erythrocyte membranes (San George *et al.*, 1984) and to phospholipids extracted from erythrocytes, whereas chloroquine does not (Chevli and Fitch, 1982). This high affinity binding can be explained by lipophilicity of mefloquine and probably accounts for its reported bactericidal effect (Brown *et al.*, 1979). The membrane activity of mefloquine has been suggested to be responsible for its chemotherapeutic effectiveness (San George *et al.*, 1984).

Taking into account the fact that aminoquinoline drugs are the most widely used antimarialials, it is surprising that the precise mode of action of these drugs is still unknown. However, few converging lines of evidence point out the importance of drug-lipid interactions in the mechanism of actions of these antimalarials. The aminoquinoline antimalarials are cationic amphiphilic drugs and, like many such drugs, exhibit high lipophilicity. Furthermore, the four antimalarials considered in this review (chloroquine, quinacrine, mefloquine and quinine) are weak bases at physiological pH and could be expected to accumulate in acidic intracellular compartments, such as lysosomes (for a review see Krogstad and Schlessinger, 1986). Since such antimalarial drugs must cross four phospholipid containing membranes (plasma membrane of the erythrocyte, parasitophorous vacuolar membrane, parasite plasma membrane and the membrane of the food vacuole) in order to arrive in the food vacuole, the interactions of the antimalarials with lipids may, at least to some degree, determine their capacity to accumulate and may also affect their susceptibility to be pumped out of the infected erythrocyte by the action of a P-glycoprotein, associated with multidrug resistance (see Chapter 39). It is also possible that resistance to these antimalarials could be achieved by parasites having an altered phospholipid composition which restricts their uptake. Moreover, lipid composition of normal and malaria-infected erythrocytes may affect the efficiency of inhibition of lysosomal phospholipases by an antimalarial, which is a possible mode of action of these compounds. It is therefore important to obtain answers to the following questions:

1. What is the lipid composition of malaria-infected erythrocytes?
2. How do the antimalarials interact with phospholipid membranes of various composition?
3. How do the changes in lipid composition and the resultant changes in drug-lipid interactions affect the activity of phospholipases and their inhibition by these drugs?

LIPID COMPOSITION OF ERYTHROCYTE MEMBRANES DURING MALARIAL INFECTION

We characterized the lipids of the red cell membrane during the intraerythrocytic development of *P. falciparum* (Maguire and Sherman, 1990). In addition, we have made determinations of the phospholipid organization in the red cell membrane (Maguire, Prudhomme and Sherman, unpublished).

No differences upon infection of the red cell were found in the content of phosphatidylethanolamine (PE) and only small changes were observed for phosphatidylcholine (PC) and phosphatidylserine (PS). The sphingomyelin (SM) content in red cell membranes of both trophozoite and schizont-infected cells was up to 47 per cent less than that of uninfected cells, and the cholesterol/phospholipid ratio was decreased by 55 per cent. Trophozoite—and schizont-infected cells exchanged 29 and 33 per cent less cholesterol, respectively, than uninfected cells (Maguire and Sherman, 1990).

The decrease in sphingomyelin content and the decline in the cholesterol/phospholipid ratio in the host membrane of trophozoite- and schizont-infected cells could have a marked effect on the physical properties and physiological function of the infected red cell membrane. A reduction in SM would be expected to decrease the order within the membrane usually resulting in increased fluidity, and it has been shown that artificial membranes composed entirely of SM are much less fluid than those which are pure PC. It is also interesting to note that SM was shown to inhibit intracellular phospholipases C, A₁, and A₂, (Dawson *et al.*, 1985).

The reduced exchange rate for cholesterol in trophozoite- and schizont-infected cells may be explained simply by the change in SM and cholesterol content of the red cell membrane of infected cells. Although cholesterol exchange was found to be independent of cholesterol concentration in membranes containing SM, it is important to note that membranes which were depleted of SM did not incorporate labelled cholesterol as well as those with higher SM levels. Furthermore, cholesterol has a higher 'affinity' for SM, so a depletion of SM from the membrane could result in the movement of cholesterol out of the membrane, leading to an impaired capacity of the membrane to solubilize cholesterol. Another possible explanation for the reduced cholesterol exchange rate in the *P. falciparum*-infected cell is the cross-linking of aminophospholipids, a consequence of lipid peroxidation. This occurs in irreversibly sickled cells and in normal red cells treated with malonyldialdehyde (MDA), a product of lipid peroxidation.

Lipid asymmetry during malaria infection

The published data on phospholipid (PL) asymmetry in the erythrocyte membrane of the malaria-infected red cell are both perplexing and contradictory (see Table 31.1). Therefore we studied the change in PL distribution during the *P. falciparum* infection.

The application of bee venom phospholipase A₂ to freshly collected human red cells resulted in the hydrolysis of c. 70 per cent of the PC and c. 10 per cent of the PE; treatment of cells with sphingomyelinase C resulted in the hydrolysis of c. 75 per cent

Table 31.1. Changes in phospholipid accessibility in red cell membranes upon infection with malaria parasites.

Species	Parasitemia (%)	Stage ^a	PL accessibility (infected vs. uninfected red cells) ^b				Reference
			SM	PC	PE	PS	
<i>P. knowlesi</i>	90	Rings	0	—	+	0	Gupta and Mishra (1981)
<i>P. knowlesi</i>	70–90	Trophs/Schiz	N.D.	+	+	+	Joshi <i>et al.</i> (1987)
<i>P. knowlesi</i>	95	?	0	0	0	0	Moll <i>et al.</i> (1990)
<i>P. knowlesi</i>	39	Trophs	0	0	0	0	Van der Schaft <i>et al.</i> (1987)
<i>P. falciparum</i>	11	?	0	0	0	0	Moll <i>et al.</i> (1990)
<i>P. falciparum</i>	80–90	Trophs/Schiz	0	0	+	+	Joshi and Gupta (1988)
<i>P. falciparum</i>	~20	Trophs/Schiz	0	—	+	+	Schwartz <i>et al.</i> (1987)

^a Trophs, trophozoites; Schiz, schizonts;

^b PL, phospholipid; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ND, not determined.

of the SM. The PS-sensitive prothrombinase assay (Connor *et al.*, 1989), as well as the hydrolytic action of the phospholipases, indicated that there was no PS exposed in uninfected red cells. In contrast to the findings for fresh red cells, a decline in the exposure of PC was seen for malaria-infected cells; in addition, the red cell membranes derived from heavily infected cells demonstrated an increased exposure of PE and PS, as well as a decline in exposure of SM. The changes in PE and PC were directly related to the percentage of infected red cells (parasitemia), but not to the developmental stage of the parasite. The observed changes for SM and PS appeared to be correlated with both parasitemia and degree of maturation of the intracellular parasite: the higher the parasitemia and the more mature the parasite, the more severe the decline in SM accessibility, and the greater the surface exposure of PS.

Our data for *P. falciparum*-infected red cells are similar to the findings for erythrocytes oxidatively stressed by exposure to phenylhydrazine, that is a marked exposure of PS (Arduini *et al.*, 1989). In addition, when red cells were treated with other oxidants, such as hydrogen peroxide or malonyldialdehyde, or cross-linkers such as tetrathionate, it was possible to produce exposure of both PS and PE.

What might account for the decreased exposure of PC in the erythrocyte membrane of the malaria-infected cell? Gupta (1987) suggested that the migration of PE from the outer monolayer was compensated for by a reverse movement of PC, but did not provide for a mechanism. It is possible that by an increase in the flip-flop rate of PC in infected cells (Van der Schaft *et al.*, 1987; Beaumelle *et al.*, 1988), as well as endocytotic internalization and transfer to the plasmodium (Haldar *et al.*, 1989), the outer monolayer of the membrane contains less exposable PC. In this context, it is worth noting that in sickled cells an increase in transbilayer movement of PC corresponded to the uncoupling of the bilayer to the cytoskeleton (Op den Kamp *et al.*, 1985) and that PC was less accessible to phospholipase action in spectrin-free microvesicles (Raval and Allan, 1984).

We found a decline in the levels of SM and cholesterol in the red cell membrane from *P. falciparum*-infected erythrocytes (Maguire and Sherman, 1990); therefore, it was not entirely unexpected to find a reduced exposure or accessibility of SM to sphingomyelinase C. When the plasma membrane of a cell loses its SM there is a reduction in the cholesterol-solubilizing capacity of that membrane, and cholesterol can be moved from the surface to the intracellular cholesterol pool (Slotte and Beirman, 1988; Slotte *et al.*, 1989). Were this to happen in a malaria-infected red cell, cholesterol would then be made available to the intracellular parasite. Such a source of cholesterol may be critical to parasite growth, since all species of *Plasmodium* are incapable of *de novo* synthesis of cholesterol.

It is now generally accepted that an ATP-dependent red cell membrane pump is responsible for the translocation of PE and PS from the outer layer to the inner layer (Devaux, 1988) and when pump (or translocase) activity is impaired, there is increased exposure of PE and PS. Elevated levels of Ca^{2+} and alterations in the cytoskeletal proteins may play ancillary roles in the maintenance of phospholipid asymmetry. Since ATP depletion does occur in the malaria-infected red cell, and there are cytoskeletal alterations as well as increased levels of calcium, the appearance of PE and PS in the outer monolayer may be a natural consequence of changes in the intracellular milieu. The presence of these aminophospholipids in the outer monolayer could influence the surface properties of the infected red cell. Indeed, PS exposure in red cells has been shown to be a determinant in reticuloendothelial cell clearance (Allen *et al.*, 1988), enhanced phagocytosis (Tanaka and Schroit, 1983), increased monocyte and endothelial adherence (Wali *et al.*, 1987) and a greater susceptibility to fusion with either Sendai virus or vesicular stomatitis virus (VSV) (Herrmann *et al.*, 1990) or polyethyleneglycol (PEG) (Tullius *et al.*, 1989). Increased fusibility (Tanabe *et al.*, 1982; Nakornchai *et al.*, 1983), endothelial adherence (Sherman and Valdez, 1989) and phagocytosis (see Zuckerman, 1945) are characteristics of malaria-infected cells, and these surfaclerelated properties may result, in part, from the parasite induced increases of PE and PS in the outer monolayer of the red cell membrane.

We can thus conclude, on the basis of this and earlier studies, that infection of the red cell by *P. falciparum* effects dramatic changes in the composition and structure of the erythrocyte membrane (see also Chapter 2).

ANTIMALARIAL-LIPID INTERACTIONS

It was pointed out by Lüllmann *et al.* (1980) that a general correlation exists between the potency of cationic amphiphilic drugs to induce lysosomal accumulation of polar lipids and cardiodepressive effects and the drug's affinity to bind to phospholipids. This is the case for such diverse drugs as local anaesthetics, (β -blockers, psychotropic and antimalarial drugs (including quinine, chloroquine and quinacrine). Similar ideas about amphiphilic drug effects have also been advanced in earlier studies (reviewed in Lüllmann *et al.*, 1975).

Chevli and Fitch (1982) studied binding of antimalarials to mouse erythrocytes and demonstrated that mefloquine, but not chloroquine, binds to the extracted

phospholipids. The binding of mefloquine to phospholipid accounted for most, if not all, of the mefloquine bound to the erythrocyte membrane.

We have studied the interaction of antimalarials with dipalmitoylphosphatidylcholine (DPPC) bilayers using nuclear magnetic resonance (NMR) spectroscopy (Zidovetzki *et al.*, 1989). The results showed no significant perturbation of lipid bilayer structure by the presence of chloroquine up to a molar ratio of 1:2 (drug: lipid). Addition of quinacrine to DPPC at the same molar ratio resulted in a 2.5°C decrease in the gel to liquid crystalline phase transition temperature (T_c) of the lipids, with only a small perturbation of the order parameters of the lipid side chains. ^{31}P NMR spectra of quinacrine-DPPC mixtures indicated a quinacrine induced change of head group conformation of DPPC above the T_c . These findings are consistent with quinacrine interacting only with the surface of DPPC bilayers. In contrast, both mefloquine and quinine exhibited stronger interactions with DPPC, decreasing the T_c of the lipids by 10°C and 9°C, respectively, and causing significant disordering of the lipid side-chains. The basic bilayer structure of DPPC was, however, maintained even at the highest tested molar ratio of drug to lipid (1:2). Such behaviour is consistent with penetration of both mefloquine and quinine into the interior of the bilayers.

We have also used ^2H —and ^{31}P -NMR to investigate the interaction of these antimalarials with membranes reformed from lipid extracts of normal human erythrocytes (Zidovetzki *et al.*, 1990). Inclusion of small amounts of chain-perdeuterated DPPC or dipalmitoylphosphatidylethanolamine (DPPE) as an ^2H -NMR probe allowed us to study separately the effects of drugs on PC or PE components of the membranes. Only a very small decrease of the order parameters of the DPPE, but not the DPPC probe, was observed in the presence of chloroquine at a molar ratio of 1:5 of drug to lipid. Addition of quinacrine at the same molar ratio resulted in a small but significant decrease of the order parameters of the lipid side-chains; identical effects were obtained with DPPC or DPPE perdeuterated probes, which contradicts the results of Dise *et al.* (1982), who reported formation of PE-quinacrine complexes. Our results, however, agree with those of Sterin-Speziale *et al.*, (1989), who did not observe the formation of such complexes. The presence of quinacrine did not induce non-bilayer lipid phases. Our results are compatible with the interaction of quinacrine with the surface of phospholipid bilayers. Such surface interaction would be sufficient to account for quinacrine induced inhibition of lysosomal phospholipases (see below).

Using the same methodology, mefloquine and quinine produced a significant disordering of the lipid side-chains; the effect was considerably larger with the DPPE probe. In addition, both mefloquine and quinine induced non-bilayer phases of the lipids; mefloquine induced formation of hexagonal and micellar lipid conformation, whereas addition of quinine resulted in the formation of lipid micelles only. The lipid polymorphism induced by these drugs was more pronounced when the DPPE component was observed, indicating that the non-bilayer phases were enriched in PE. The results suggest the presence of strong interactions between mefloquine and quinine with lipid bilayers, especially with the PE component. The concentration dependence of these interactions was biphasic with the first phase consisting of the drug molecule intercalating into the bilayer, and

the second phase involving the formation of drug-induced non-bilayer lipid phases. Our results agree with an earlier report on the binding of mefloquine, but not chloroquine, to mouse erythrocytes and lipid extracts (Chevli and Fitch, 1982). The intercalation of mefloquine and quinine, but not chloroquine, into the bilayers may result in the higher accumulation of these drugs in erythrocytes, and at least partially account for the lower incidence of resistance to mefloquine and quinine.

In both lipid systems (DPPC and erythrocyte lipid extracts) we observed correlation of the effects of the antimalarials on lipid structure with their belonging to group I or group II of weak bases. Group I, to which chloroquine and quinacrine belong, have high pK_a values and, therefore, exist largely in a charged form at physiological pH. Therefore, under physiological conditions, the interaction of chloroquine and quinacrine with the interior of the bilayers would be expected to be very limited, and indeed this was found by us. It is important to point out, however, that these results do not contradict accumulation of chloroquine or quinacrine in acidic organelles by a weak base effect: only a very small uncharged fraction of a drug would be present in the cell membrane at any given time and thus the perturbation of the bulk phospholipids would be minimal and not observable by our methods. This weak base-driven accumulation of chloroquine was reported recently (Ferrari and Cutler, 1990). Mefloquine and quinine belong to weak base group II, that is compounds with a low pK_a and a significant amount in the uncharged form at physiological pH. The uncharged forms are highly lipophilic which correlates with our findings of strong interactions of mefloquine and quinine with DPPC and the erythrocyte lipids.

The observed strong perturbation of bilayer lipid structure by mefloquine and quinine raises the question as to whether these drugs affect the activity of lysosomal phospholipases, because phospholipase activity is known to strongly depend on the structure of phospholipid substrate (reviewed by Jain and Berg, 1989).

EFFECT OF ANTIMALARIALS ON LYSOSOMAL PHOSPHOLIPASES

One of the steps in the digestion of the host cell cytoplasm by the parasite is the digestion of the endocytic vesicle (Krugliak *et al.*, 1987) which presumably involves acidic phospholipases; this process may also be necessary for the transfer of haemoglobin from the endocytic vesicles to the food vacuole of the parasite (Yayon, *et al.*, 1983, 1984; Zarchin *et al.*, 1986; Ginsburg and Geary, 1987).

We are unaware of any study which addresses the effects of quinine or mefloquine on the activity of phospholipase A₂. Numerous studies (Mallorga *et al.*, 1980; Matsuzawa and Hostetler, 1980a,b; Naor and Catt, 1981; Dise *et al.*, 1982; Jain *et al.*, 1984, Kubo and Hostetler, 1985; Waite, 1985), however, have demonstrated inhibition of phospholipase A₂ and other lysosomal phospholipases by quinacrine or chloroquine. Thus, quinacrine was shown to inhibit phospholipase A₂ from platelets (Vargaftig and Dao Hai, 1972; Derksen and Cohen, 1975; Flower and Blackwell, 1976; Blackwell *et al.*, 1977; Jesse and Franson, 1979; Franson *et al.*, 1980; Billah *et*

al., 1981; Winocour *et al.*, 1981; Lapetina *et al.*, 1981), toad bladder (Yorio and Bentley, 1978), leukocytes (Hirata *et al.*, 1979), astrocytoma (Mallorga *et al.*, 1980), colonic mucosa (Craven and De Rubertis, 1980) and pituitary (Naor and Catt, 1981). It has been suggested that this inhibition is probably due to drug-phospholipid interactions (Lüllmann *et al.*, 1978) and may provide the main mechanism of the pharmacological action of these drugs (Brown *et al.*, 1979; Chevli and Fitch, 1982). It was pointed out by Ginsburg and Geary (1987), as additional indirect evidence for the involvement of phospholipases in the mechanism of chloroquine action, that the stage of the parasite cycle which was most sensitive to chloroquine (Yayon *et al.*, 1983) was also that characterized by a high rate of phospholipid synthesis (Vial *et al.*, 1982). Generally, drug concentrations in the order of 0.1 to 1mM are required to inhibit phospholipases: such concentrations were shown to be attainable in the food vacuoles of the parasite (Geary *et al.*, 1986).

Many cationic amphiphilic drugs bind to phospholipids and it has been suggested that the resulting complex is resistant to phospholipase A₂ (Lüllmann *et al.*, 1978). This mechanism was proposed to be responsible for chloroquine induced phospholipidosis (Hostetler, 1986) and retinopathy, the main toxic side-effects of chloroquine. Detailed studies on the mechanism of action of phospholipase A₂, (for a review see Jain and Berg, 1989) emphasize the importance of the physical state of the substrate (i.e. lipid bilayer). In general, any agent that disturbs the architecture of the lipid-water interface could physically prevent phospholipase A₂ from hydrolysing the phospholipid substrate. Among the endogeneous regulators of phospholipase A₂ is a whole class of substances whose mode of action is interference with the substrate-enzyme interface. The recent work of Jain *et al.* (1984) emphasizes that many phospholipase A₂ inhibitors, including quinacrine, act by intercalating into bilayer, changing its physical properties and promoting desorption of phospholipase A₂ from the surface of the lipid substrate.

Based on an understanding of the importance of the physical state of the lipid for phospholipase A₂ activity, it follows that changes in lipid composition may modulate enzyme activity. Indeed, it was shown that SM caused a marked inhibition of total lipid hydrolysis by phospholipase A₂ (Dawson *et al.*, 1985) and platelet-derived phospholipase A₂ was inhibited by 20 mol% cholesterol (Kannagi and Koizumi, 1979). It is interesting to note that cholesterol decreases the interaction of chloroquine with phospholipids (Seydel and Wassermann, 1976; Harder *et al.*, 1980, 1983). Moreover, as indicated above, both the SM and the cholesterol content of the erythrocyte membrane undergo dramatic changes during the intracellular development of the malaria parasite.

Conversely, phospholipases may modify membrane phospholipid composition *in situ*, by:

1. generating hydrolysis products such as lysophospholipid, free fatty acid, and diacylglycerol, which have different physical properties from intact phospholipids; and
2. allowing modification of membrane phospholipid fatty acid composition via an endogenous deacylation-reacylation pathway.

It was also shown that accumulation of chloroquine in lysosomes can modify their lipid composition: the ratio of acidic to neutral lipids increases, mostly due to a decrease in PE and an increase in phosphatidylinositol (PI) (Harder and Debuch, 1982; Hosteller *et al.*, 1985).

Clearly, the interaction between the antimalarial drug, phospholipid membrane and phospholipase is complex, with each element affecting the others. Only by a systematic study of the inter-relationships will the information necessary for understanding the mode of action of these antimalarial agents be provided.

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32. Adaptation of *Leishmania* species to an acidic environment

D.Zilberstein

INTRODUCTION

Leishmania are parasitic protozoa with a digenetic life cycle, proliferating as extracellular flagellated promastigotes in the alimentary tract of the insect vector and as obligate intracellular amastigotes in the phagolysosomal vacuoles of mammalian macrophages (Chang and Dwyer, 1978; Berman *et al.*, 1979; Dwyer, 1979). During their life cycle, these organisms are subjected to marked changes in environmental pH, from relatively alkaline (pH 7–7.5) of the sandfly vector's gut, to the neutral pH of the bloodstream, to the acid pH of the lysosomes of macrophages (pH 4.5–5). Furthermore, the transformation of promastigotes to amastigotes occurs during phagocytosis by host macrophages with exposure of the parasites to an acidic environment (Chang and Dwyer, 1976; Chang *et al.*, 1985). Both promastigotes and amastigotes appear to be highly adapted to their corresponding environmental pHs. For example, *L. donovani* promastigotes metabolize glucose, proline and nucleosides most rapidly at pH 7–7.5. Amastigotes, on the other hand, catabolize these substrates with an optimal pH of 4.5–5 (Mukkada *et al.*, 1985). Similarly, transport of proline is optimal at pH 7–7.5 with promastigotes and at pH 5.5 with amastigotes (Zilberstein *et al.*, 1989; Mukkada *et al.*, 1989).

The pH is likely to play an important role in triggering transformation of promastigotes to amastigotes, since there exists a difference of approximately 2 pH units between the environments of the extracellular and intracellular stages of the parasites. In this review I summarize the work that has been done to study the mechanism of adaptation of *Leishmania* parasites to their environmental pH values, and the possible role of pH in the development of these organisms.

PROTON PUMPS IN LEISHMANIA

In various micro-organisms, intracellular pH is maintained by means of proton pumps. Such pumps are able to move protons in and out of cells efficiently, thus controlling the cellular concentration of protons (Padan *et al.*, 1981; Padan and

Schuldiner, 1987). The presence of proton pumps in *L. donovani* was first demonstrated by Zilberstein and Dwyer (1985). They showed that the active transport of L-proline and D-glucose was driven by proton motive force. A proton electrochemical gradient is created across the promastigote plasma membrane and this is coupled to transport by maintaining symport translocation of the specific substrates with protons. Electrochemical gradients across plasma membranes are created by primary proton pumps such as the proton-translocating ATPase (H^+ -ATPase) (Perlin *et al.*, 1984; Pederson and Carafoli, 1987). In a variety of cells and organelles, membrane bound H^+ -ATPases have been demonstrated to be energy transducers which utilize the energy of ATP hydrolysis to generate a proton electrochemical gradient (Goffeau and Slayman, 1981; Pederson and Carafoli, 1987). It was therefore anticipated that a H^+ -ATPase should be present in the plasma membrane of *Leishmania* cells.

Zilberstein and co-workers (Zilberstein *et al.*, 1987; Zilberstein and Dwyer, 1988) have identified an H^+ -ATPase activity in the plasma membrane of *L. donovani* promastigotes. This enzyme is Mg^{2+} dependent, has optimal activity at pH 6.5 and is inhibited by o-vanadate ($IC_{50}=7.5\ \mu M$). The H^+ -ATPase possesses high affinity ($K_m=1\ mM$, $V_{max}=225\ nmol\ min^{-1}\ protein$) and specificity for ATP. More importantly, this enzyme is a proton pump as was demonstrated using membrane vesicles derived from promastigote plasma membrane. This H^+ -ATPase is the primary proton pump in *L. donovani*, responsible for the creation of a proton electrochemical gradient across the parasite's plasma membrane (Zilberstein and Dwyer, 1985; Zilberstein *et al.*, 1987). This gradient drives energy transduction processes such as active transport of sugars and amino acids across the leishmanial plasma membrane. The H^+ -ATPase also has a role in regulating intracellular pH and in maintaining chemiosmotic energy for energy-requiring processes in promastigotes and amastigotes of *Leishmania*. This enzyme is essential for parasite survival (Zilberstein and Dwyer, 1984; Zilberstein *et al.*, 1990).

The enzymatic activity of the *L. donovani* H^+ -ATPase resembles that of the H^+ -ATPase of yeast and fungi (Goffeau and Slayman, 1981). Furthermore, antibodies raised against the H^+ -ATPase from *Saccharomyces cerevisiae* reacted with a 66 kDa membrane protein of *L. donovani* promastigotes (Liveanu *et al.*, 1991) and, when immunoprecipitated, this protein possesses an ATPase activity similar to that of the parasite's H^+ -ATPase. Indirect immunofluorescence assays, antibody labelling of cryosections and fine structure cytochemistry assays localized the H^+ -ATPase to the plasma membrane of promastigotes, including the cell and flagellar surface and the flagellar pocket (Zilberstein and Dwyer, 1988; Liveanu *et al.*, 1991).

The molecular weight of the leishmania H^+ -ATPase (66 kDa) appears to be different from those of yeast and fungi (100 kDa) (Serrano *et al.*, 1986; Bowman and Bowman, 1986; Nelson and Taiz, 1989; Liveanu *et al.*, 1991). It is possible, however, that the 66 kDa enzyme might represent a subunit of a larger protein or one that had been enzymatically cleaved by endogenous proteinase activity, even though proteinase inhibitors were present throughout the experiments.

Meade *et al.* (1987a) used an oligonucleotide probe containing the sequence of the phosphate binding site that is highly conserved between all P type cationtranslocating

ATPases (Serrano *et al.*, 1986) to clone a gene that encodes a 107.4 kDa protein. The putative protein has all the characteristics of a plasma membrane cation-translocating ATPase of the P type family (Pederson and Carafoli, 1987). It displays about 45 per cent homology to *S. cerevisiae* H⁺-ATPase but much less to mammalian cation-translocating ATPases (< 27 per cent). This putative H⁺-ATPase is developmentally regulated, being expressed more abundantly in amastigotes than in promastigotes (Meade *et al.*, 1987b). However, no direct evidence has been presented to link the cloned gene and the *L. donovani* H⁺-ATPase described above.

MAINTENANCE OF CYTOPLASMIC pH IN PROMASTIGOTES AND AMASTIGOTES

In order to overcome the extreme changes in environmental pH, parasites must regulate their cytoplasmic proton concentration at a level which allows enzymatic activities in both developmental stages. Intracellular pH (pH_i) was determined using various methods, most of which were carried out in *L. donovani* (Glaser *et al.*, 1988; Zilberstein *et al.*, 1989). Zilberstein *et al.* (1989) used the fluorescence pH indicator 2', 7'-bis(carboxyethyl)-5, 6-carboxyfluorescein (BCECF) to measure the pH_i of *L. donovani* promastigotes. This dye was introduced into promastigotes as a tetraacetoxymethyl ester, which permeates the cells and is subsequently hydrolysed by cytoplasmic esterases, thus entrapping the dye in the cytosol (Rink *et al.*, 1982). Using this method it was found that *L. donovani* promastigotes regulate their pH; at 6.4–6.7 throughout a wide extracellular pH (pH_o) range of 5–7.5 (Zilberstein *et al.*, 1989). Similar results were also obtained using two other independent methods: pH null-point assays, and the determination of the distribution across the parasite plasma membrane of the fluorescence amine acridine orange and the weak acid 5, 5-dimethyl-2–4-oxazolidinedione (DMO). pH_i was also estimated by Glaser *et al.* (1988) using phosphate NMR and by using the DMO method. They measured pH_i values of 6.8–7.4 throughout a pH_o range of 5–7.4. These values are higher than those determined using BCECF. This is most probably owing to accumulation of DMO in the mitochondrion of promastigotes, which might lead to an overestimation of the pH_i values (Zilberstein *et al.*, 1989). Nevertheless, Glaser *et al.* (1988) showed that amastigotes of *L. donovani* maintain a pH_i value similar to that of promastigotes, but throughout the wider pH_o range of 4–7.3.

The pH_i value of *L. donovani* is lower than that measured in mammalian cells (Rink *et al.*, 1982; Madshus, 1988). However, the values are similar to those found in other protists such as *Dictyostelium discoideum* (Jentoft and Town, 1985), *S. cerevisiae* (Toh-e *et al.*, 1978) and *Aspergillus nidulans* (Caddick *et al.* 1986). These organisms live in a wide range of environmental pH ranging from acidic to neutral. All three organisms maintain a constant pH_i of 6.5 throughout a wide environmental pH range of 4–8. A pH_i around 6.5 might therefore be a general feature common to all lower eukaryotic micro-organisms that grow in a wide range of environmental pH.

The foregoing observations imply that when residing in the vector's midgut, promastigotes have a cytosolic pH which is more acidic than in their environment, whereas in the acid environment of the lysosome the pH_i of amastigotes is more alkaline. This means that as pH_o increases, the chemical gradient of protons across the parasite plasma membrane (ΔpH) decreases. This raises the question of how active transport processes that are driven by proton motive force are able to function at the various environmental pHs. Both L-proline and D-glucose are actively accumulated by promastigotes throughout a pH_o range of 4.5–8.0 (Zilberstein *et al.*, 1989; Zilberstein, 1991; Zilberstein and Gepstein, 1991). L-Proline is also actively transported in amastigotes throughout the same pH_o range (Mukkada *et al.*, 1989). This indicates that a proton electrochemical gradient (directed inward) exists across the parasite's plasma membrane throughout the pH range mentioned above. How is it possible for parasites to maintain both pH_i and the proton motive force?

The proton electrochemical gradient (Δp) is composed of two components, the pH gradient (ΔpH) and membrane potential ($\Delta \psi$) according to

$$\Delta p = \Delta \psi + (2.3RT/F)\Delta pH$$

where R is the gas constant, T is the temperature in Kelvin and F is the Faraday constant.

In order to keep both Δp and pH_i constant, the membrane potential has to compensate for the change in ΔpH at the various external pHs. We have shown that such compensation occurs in *L. donovani* promastigotes (Zilberstein, *et al.*, 1989). Hence, proton pumps are capable of maintaining both pH_i and the chemiosmotic energy required to drive active transport of nutrients in environments of widely different pH.

THE ROLE OF pH IN LEISHMANIAL DEVELOPMENT

The effect of growth-medium pH on phenotypic expression

The transformation of promastigotes to amastigotes occurs after phagocytosis of promastigotes by host macrophages and exposure of the parasites to an acidic environment (Chang and Dwyer, 1976; Chang *et al.*, 1985). There is a difference of 2 pH units between the environments of the extracellular and intracellular stage of *Leishmania* parasites. It is therefore likely that pH plays an important role in triggering the transformation of promastigotes to amastigotes. We have tested this hypothesis by determining the effect of the growth medium pH on phenotypic expression in promastigotes (Zilberstein *et al.*, 1991; Zilberstein and Gepstein, 1991; Levi and Zilberstein, unpublished).

As outlined above, promastigotes and amastigotes maintain a similar pH_i throughout the same range of pH_o . It was therefore expected that promastigotes can grow at pHs in which they are capable of maintaining pH_i homeostasis. Based on this idea, we have adapted promastigotes of *L. donovani*, *L. major* and *L. mexicana*

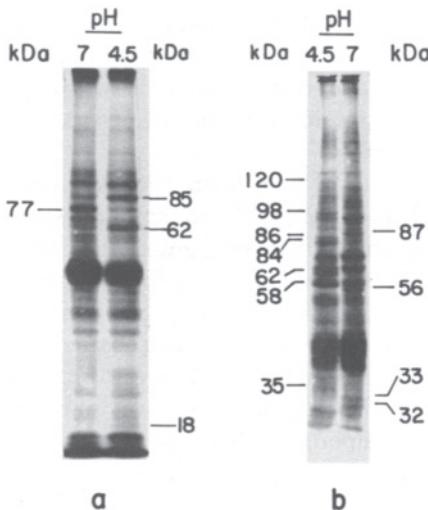


Figure 32.1. Effect of growth medium pH on metabolic labelling of *L. major* (a) and *L. mexicana* (b) promastigotes with [^{35}S] methionine. Promastigotes of *L. major* (strain WR309) and *L. mexicana amazonensis* (strain LTB0016) grown in medium 199 at either pH 7 or pH 4.5 to 1×10^7 cells ml $^{-1}$ were harvested and washed twice in Earl's salt solution containing 10 mM Tris/succinate at the pH of the corresponding growth medium. The cells were resuspended to 5×10^7 cells ml $^{-1}$ MEM plus 10 per cent dialysed fetal calf serum. L-[^{35}S] methionine was added to give 5 μCi ml $^{-1}$ and the cells were incubated at 26°C for 2 h. The cells were washed twice in Earl's salt solution and resuspended to 5×10^8 cells ml $^{-1}$ in Earl's salt solution containing 10 μg ml $^{-1}$ leupeptin and 1.6 mM phenylmethylsulfonyl fluoride. The suspension was solubilized in Laemmli sample buffer and analysed by SDS-PAGE using 9 per cent gels. Fluorography was performed following incubation of fixed gels for 30 min in Amplify (Amersham Inc.).

amazonensis to long-term culture at pH 4.5, 5 and 6. The growth rate of promastigotes at pH 6 was similar to that of cells grown at pH 7, whereas the growth rate decreased below pH 6 (Zilberstein and Gepstein, 1991). At pH 4.5, promastigotes maintained a doubling time which was 1.6 times that of promastigotes grown at pH 7. Nevertheless, these cells were viable and maintained normal levels of cellular ATP, cell volume and protein synthesis (Zilberstein *et al.*, 1991; Zilberstein and Gepstein, 1991). When promastigotes grown at pH 4.5 were switched back to pH 7, the growth rate of the cells returned within a few hours to that of cells grown at pH 7. This reversibility of the pH-dependent characteristics of promastigotes indicated that promastigotes adapted to the acidic pH and that no pH-dependent mutants developed (or at least were selected) while growing at that pH. These cells were then used to study the effect of physiological changes in pH on the expression of proteins in *Leishmania* promastigotes.

Growth of leishmanial promastigotes at a pH similar to that of macrophage lysosomes (pH 4.5) induced changes in phenotypic expression; such cells expressed an amastigote stage-specific protein (Zilberstein *et al.*, 1991). The level of expression of this protein appears to correlate with the morphology of the cells: rounded amastigote-like cells express high levels, while cells that retain the

elongated shape of promastigotes at pH 7 do not express the stage-specific protein. The rate of appearance and disappearance of this protein upon switching the medium pH from alkaline to acid and vice versa is similar to the rate of parasite transformation *in vivo* (Jaffe and Rachamim, 1989).

Using metabolic labelling of cells with [³⁵S]methionine, other pH-specific proteins were identified. In *L. major*, for example, the synthesis of proteins with molecular mass of 18, 72 and 85kDa was significantly enhanced in promastigotes grown at pH 4.5 (Figure 32.1 (a)). However, in *L. major* promastigotes grown at pH 7, a 77 kDa protein was labelled more strongly than in promastigotes grown at pH 4.5. Many more changes in protein synthesis were observed in the new world species *L. mexicana amazonensis* (Figure 32.1.(b)). A few proteins (35, 62, 84, 98 and 120 kDa) were specifically labelled in acid-grown promastigotes. These results indicate that pH plays an important role in the development of *Leishmania*. The role of the pH-specific proteins in leishmanial development is, however, still an open question.

pH regulation of L-proline transport

L-Proline is an important energy source for the insect stages (promastigotes) of *Leishmania* (Krassner and Flory, 1972; Mukkada *et al.*, 1974; Krassner and Flory, 1977). This reflects an adaptation of promastigotes to the proline-rich environment in its insect vector (Brusell, 1970). L-proline is actively accumulated by promastigotes of *L. donovani* and *L. major* via a specific carrier and the transport is driven by proton motive force across the parasite plasma membrane as described above (Law and Mukkada, 1979; Bonay and Cohen, 1983; Zilberstein and Dwyer, 1985). In promastigotes, the transport is optimal at pH 7.5 and remains active throughout a wide range of extracellular pH (Zilberstein *et al.*, 1989). In amastigotes, on the other hand, proline transport is optimal at pH 5.5 and its rate is lower than that in promastigotes (Mukkad *et al.*, 1989). Since the pH optimum and rate of proline transport differs between the two main developmental stages of *Leishmania*s spp., it was of interest to determine the role of pH on the function of this system.

Zilberstein and Gepstein (1991) found that the characteristics of L-proline transport in *L. donovani* were strongly influenced by culture pH. In promastigotes grown at pH 4.5, the V_{max} of transport is one-tenth that and the affinity for proline is twice that found in cells grown at pH 7. Furthermore, while the optimum pH for transport in pH 7 grown promastigotes was 7–7.5, it decreased as the culture pH was lowered, reaching an optimal pH of 5.5 in the pH 4.5 grown cells. Steady-state levels of transport also decreased with the pH of the growth medium. Proline transport in promastigotes grown at pH 4.5 was at least three times lower than in the pH 7 cells. Moreover, these characteristics of proline transport in promastigotes reversibly responded to changes in culture pH. It took 48–72 h after shifting promastigotes to acid pH for the proline transport system to switch back to the level of the acid-grown cells. This is about the same time it takes promastigotes to transform to amastigotes *in vivo* after invading the macrophage (Chang and Dwyer, 1976; Jaffe and

Rachamim, 1989). Upon switching the culture pH from 4.5 to 7, proline transport resumed the activity of pH 7 cells in less than 24 h. This process was inhibited by the protein synthesis inhibitor cycloheximide, indicating that new proline transporters were synthesized during the adaptation process of pH 4.5 cells to growth at pH 7.

The foregoing observations suggested that *L. donovani* possesses two distinct L-proline transporters, the expression of which is regulated by culture pH. Moreover, the characteristics of the L-proline transport system in promastigotes grown at pH 4.5 were found to be similar to those described for proline transport in amastigotes of *L. donovani*; that is, transport was optimal at pH 5.5 and about four times lower than in promastigotes (Law and Mukkada, 1979; Zilberstein *et al.*, 1989; Mukkada *et al.*, 1989).

In contrast to proline transport, the kinetic parameters and pH optimum of the *L. donovani* glucose transport system are independent of growth medium pH. Cairns *et al.* (1989) have recently cloned a gene from *L. enriettii* that encodes a protein of 61.4 kDa that has all the characteristics of a glucose transporter. This leishmanial gene displays about 44 per cent homology to the human erythrocyte glucose transporter and to a lower extent to various hexose transporters in *Escherichia coli*. In addition, an equivalent gene was also found in promastigotes of *L. major* and *L. donovani* (Stein *et al.*, 1990). The putative glucose transporter gene is developmentally regulated, being expressed more abundantly in promastigotes than in amastigotes. However, there is no direct evidence linking the cloned gene and the leishmanial glucose transporter described above (Zilberstein and Dwyer, 1988; Liveanu *et al.*, 1991). According to the results of Zilberstein and Gepstein (1991), pH does not trigger changes in the expression of the *L. donovani* glucose transport system. See also Chapter 33.

OTHER TRIGGERS FOR LEISHMANIAL TRANSFORMATION

Two major environmental changes are encountered by promastigotes upon invasion of the host: an increase in temperature and a dramatic decrease in pH. In the New World *Leishmania* species, temperature elevation appears to be a major signal in the induction of morphological change to amastigote shaped cells (Shapira *et al.*, 1978; Hunter *et al.*, 1982; Eperon and McMahon-Pratt, 1989a), some of which express amastigote stage-specific proteins (Eperon and McMahon-Pratt, 1989b). Heat shock proteins such as Hsp70 and Hsp83 do not seem to play a role in the regulation of this response (Shapira *et al.*, 1978). Elevation of the incubation temperature of promastigotes of either *L. donovani* or *L. major* (Old World species) did not induce morphological changes or expression of amastigote stage-specific proteins, even though the heat shock response was present (Shapira *et al.*, 1978; Zilberstein *et al.*, 1991). It was therefore suggested that with Old World *Leishmania* the external pH may act as the main trigger of parasite development. Recent preliminary studies have indeed suggested that combining high temperature with low pH induces morphological change of promastigotes to amastigote-shaped cells.

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33. Glucose uptake mechanisms as potential targets for drugs against trypanosomatids

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INTRODUCTION

To maximize the chances of success, the development of drugs against parasitic protozoa should be aimed at those steps in a metabolic pathway that either are absent from the host or differ from analogous steps in the host. Ideally, such steps should catalyse a rate-limiting reaction somewhere in a metabolic sequence that is directly involved in, or of vital importance to, the parasite's growth or generation of energy. The ability of such a drug to reach its target without having to cross the parasite's plasma membrane would mean that there are fewer restrictions on its structure and properties. The aim of this chapter is to examine the possibility that the glucose carriers present in the various members of the family Trypanosomatidae meet these requirements and so may serve as suitable targets for future drug development. Evidence that the uptake of glucose may be the overall rate-limiting step in the catabolism of carbohydrates is discussed. The glucose uptake mechanisms in the Trypanosomatidae are compared with those of other eukaryotic cells, especially erythrocytes, to establish whether there are sufficient differences between the two groups to warrant expectations of a successful chemotherapy with drugs that interfere with glucose uptake by the parasites.

UPTAKE AS THE RATE-LIMITING STEP FOR GLUCOSE METABOLISM

Trypanosoma brucei bloodstream form

The rate-limiting step for energy supply of *T. brucei* is glycolysis, which takes place in the glycosome (see e.g. Opperdoes and Borst, 1977; Opperdoes, 1987). Glycolysis itself is thought to be unregulated (Opperdoes *et al.*, 1976; Oduro *et al.*, 1980; Misset *et al.*, 1986) and hence the rate-limiting step is either phosphorylation

of glucose by hexokinase, or one of the transport steps preceding phosphorylation (Visser and Opperdoes, 1980; Nwagwu and Opperdoes, 1982). In fact, transport of glucose has been proposed to be the overall rate-limiting step in glycolysis in *T. brucei* (Gmenbeig *et al.*, 1978; Game *et al.*, 1986; Eisenthal *et al.*, 1989). These studies used glucose analogues as probes for uptake, eliminating the effect of later metabolic steps. A study using D-glucose demonstrated that at external glucose concentrations (glc_{out}) below 5 mM the metabolism of glucose is limited by the uptake step, while at higher concentrations a later step in the glycolytic sequence, probably phosphorylation by hexokinase, becomes rate limiting (Ter Kuile and Opperdoes, 1991a). Generally, transport has been suggested to be the rate-limiting step for the overall glucose metabolism in a wide variety of organisms (for a review see D'Amore and Lo, 1986). Therefore it seems probable that inhibition of glucose uptake would be an effective way to inhibit growth of *T. brucei* bloodstream form.

***Leishmania* species**

The situation with *Leishmania* species is different. *L. donovani* promastigotes concentrate glucose against a gradient by means of a proton-motive-force driven mechanism (Zilberstein and Dwyer, 1984, 1985; and see Chapter 32). High free internal concentrations of glucose would suggest that the uptake step is not rate-limiting. On the other hand, *L. donovani* promastigotes in chemostat cultures were found to regulate the internal glucose level (glc_{in}) strictly at approximately 50mM, independent of the external concentration (Ter Kuile and Opperdoes, 1991 b). Only at severe energy stress is glc_{in} reduced, concurrent with a large reduction of the internal ATP concentration. Hence reduction of glucose uptake may reduce ATP levels and, therefore, growth.

Less clear but more relevant for drug development is the situation in the amastigote form, living in the macrophages of the mammalian host. Whereas high levels of mRNA encoding a protein homologous to the human erythrocyte glucose transporter are found in the promastigote form, much lower levels occur in amastigotes (Cairns *et al.*, 1989). Nevertheless, this does suggest that amastigotes also utilize active uptake as the primary glucose uptake mechanism, in contrast to the *T. brucei* bloodstream form. So the evidence to date suggests that glucose uptake is not the rate-limiting step of glucose metabolism in *L. donovani* but it remains possible that inhibition of glucose uptake would be damaging to *L. donovani*.

GLUCOSE UPTAKE MECHANISMS

A comparison of the glucose uptake mechanisms within the family Trypanosomatidae is complicated by the fact that most studies using African trypanosomes have been performed using the dividing bloodstream form, while the insect stages (promastigotes) of *Leishmania* species have been used. The suggested induction of a facilitated diffusion carrier upon transformation from amastigote to promastigote of *L. enrietti* (Cairns *et al.*, 1989) makes the validity of promastigotes as models for amastigotes doubtful at best.

The *T. brucei* group

Glucose uptake in *T. brucei* is specific for D-glucose; only traces of L-glucose are taken up. Uptake of D-glucose can therefore not be explained by simple diffusion, even though active uptake does not occur (Gruenberg *et al.*, 1978; Game *et al.*, 1986; Eisenthal *et al.*, 1989; Ter Kuile and Opperdoes, 1991a). Mathematical analysis showed that facilitated diffusion, as defined by Stein (1986), can explain the observed data (Eisenthal *et al.*, 1989; Ter Kuile and Opperdoes, 1991a). The facilitated diffusion carrier has an asymmetry factor of about 1.8, with higher affinity for the uptake than for the efflux reaction. Glucose analogues such as 1-, 2- or 6-deoxy-D-glucose are readily taken up and competitively inhibit glucose uptake (Gruenberg *et al.*, 1978; Game *et al.*, 1986; Eisenthal *et al.*, 1989). D-Fructose is accepted, and so is a variety of glucose analogues, except those that do not allow hydrogen bond formation with the C3 or C4 hydroxyl groups (Eisenthal *et al.*, 1989). Involvement of a glucose carrier, similar to *T. brucei*, was proposed for *T. gambiense* as well, except that fructose was suggested to be taken up by a different carrier (Southworth and Read, 1969, 1970).

Glucose uptake in the procyclic tryponastigote of *T. brucei* has also been studied, but much less extensively. One report suggests the active concentration of 2-deoxy-D-glucose (Parsons and Nielsen, 1990). This compound is reversibly phosphorylated by hexokinase (Blum, personal communication). More than three-quarters of the 2-deoxy-D-glucose taken up was recovered in phosphorylated form. Phosphorylation may well account for all uptake measured because the reaction is reversible. Indeed, our own findings suggest that glucose uptake depends on facilitated diffusion (Ter Kuile and Opperdoes, 1991 b).

Leishmania species

Unlike *T. brucei*, *Leishmania* species do actively concentrate glucose against a gradient (Schaefer *et al.*, 1974; Schaefer and Mukkada, 1976; Zilberstein and Dwyer, 1984, 1985). In a series of elegant experiments, Zilberstein and Dwyer (1984, 1985) demonstrated that *L. donovani* concentrates glucose 79-fold above the external concentration by means of a proton-motive-force dependent mechanism. Because cellular ATP could not replace the proton-motive force and sodium was not required, these authors suggested the occurrence of a glucose/H⁺ symporter.

Earlier studies had shown that the glucose carrier of *L. tropica* promastigotes is less specific than the one of *T. brucei*; changes at the C2 and C4 positions of the sugar did not affect the affinity, whereas changes at C1 and C3 did (Schaefer *et al.*, 1974). This carrier did not transport glycerol. The transporter is specific for the pyranose form and has low affinity for the furanose forms (Schaefer and Mukkada, 1976). L-Glucose is not taken up (Schaefer and Mukkada, 1976; Zilberstein and Dwyer, 1984, 1985).

Current evidence suggests that glucose uptake varies with the form of *Leishmania* species and so is regulated. The exact mechanisms for this regulation remain to be elucidated, hence a parallel to yeasts may be instructive. In the yeast

Kluyveromyces maxianus two mechanisms for glucose exist (Postma and Van Den Broek, 1990). A high affinity proton-glucose symporter and a low affinity facilitated diffusion carrier. Three different systems were found in *Candida utilis* (Postma *et al.*, 1988); a high, an intermediate and a low affinity transporter. These different systems were induced by removal of catabolite repression (Postma and Van Den Broek, 1990). The regulation of transport activity in *L. donovani* promastigotes, however, appears to be aimed at internal homeostasis, rather than the most efficient adaptation (Ter Kuile and Opperdoes, 1991b).

Nothing is known at present about the potential regulation and adaptation of glucose transport in amastigotes. Conditions within the macrophage are fairly constant, certainly when compared with the environmental changes to which promastigotes and yeasts are exposed. The great extent of adaptability encountered in yeasts is, therefore, unlikely to be found in those life cycle stages of the parasites that reside in the mammalian host.

Human erythrocytes

Glucose transport into most eukaryotic cells occurs by facilitated diffusion (for reviews see e.g. Baldwin and Lienard, 1981; Wheeler and Hinkle, 1985; Stein, 1986; Walmsley, 1988; Gould and Bell, 1990). A great diversity of glucose transporters exists and more than one system may be present in one cell (reviewed by D'Amore and Lo, 1986). The most extensively studied glucose transporter is that of human erythrocytes which, because it competes directly with *T. brucei* bloodstream forms, is probably more relevant for comparison with the transporter of parasitic protozoa than transporters located in tissue cells.

Even though a vast number of data have been accumulated over the years, a strong controversy still exists with respect to the mechanisms involved in glucose uptake into erythrocytes (e.g. Stein, 1986; Walmsley, 1988; Naftalin, 1988; Carruthers, 1988; Wheeler and Whelan, 1988). A simple carrier model cannot explain all observations (Stein, 1986). The glucose transporter of the human erythrocyte seems to be less specific than its counterparts in the Trypanosomatidae because no single hydroxyl group is essential for binding (Barnett *et al.*, 1973). The closed pyranose form is bound. On the other hand, all facilitative glucose transporters in eukaryotes seem to be structurally related (Gould and Bell, 1990). The human carriers all have 50 per cent or more identity in their amino acid sequence (Kayano *et al.*, 1988). There is even a 30 per cent homology between sugar transporters from eukaryotes and prokaryotes (Baldwin and Henderson, 1989). Hence it has been proposed that all facilitated diffusion glucose carriers have a common ancestor (Kayano *et al.*, 1988; Baldwin and Henderson, 1989; Gould and Bell, 1990). The sodium/glucose co-transporter from the intestinal brush border and other mammalian sodium driven sugar transporters have no homology in their DNA sequence and, therefore, no evolutionary relationship with mammalian facilitated glucose carriers (Hediger *et al.*, 1987). The proton/sugar symporter of *L. donovani* is probably again greatly different from the intestinal brush border sodium/glucose co-transporter. The former has a molecular weight of 20kDa (Zilberstein *et al.*, 1986),

while the latter has a molecular mass of 75 kDa (Hediger *et al.*, 1987). This indicates that the active transport systems have much less homology with each other than the facilitated diffusion sugar carriers and so perhaps provide greater opportunity for selective inhibition by drugs.

INTERACTION OF THE TRANSPORTER WITH LATER METABOLIC STEPS

Three observations on the interaction of the transporter with the whole cell and its regulation in human erythrocytes are particularly relevant for the situation in the bloodstream form of *T. brucei*.

1. Under physiological conditions the carrier is strongly asymmetric with respect to V_{\max} and K_m for the influx and efflux of glucose (Naftalin *et al.*, 1985; Carruthers, 1986a,b); the V_{\max} and K_m for influx are considerably lower than those for efflux. This asymmetry is dependent on the ATP concentration (Carruthers, 1986a); in the absence of ATP the system becomes symmetric. The activation is at the level of the transporter itself, no additional transporters are synthesized, and the effect is maximal at the physiological ATP concentration (Jacquez, 1983). A similar asymmetry was found in *T. brucei* (Eisenthal *et al.*, 1989).
2. The K_m for zero-trans entry (entry into depleted cells) is 1.8mM (see Stein, 1986), which is approximately the same as the observed K_m for glucose in *T. brucei* (2.0 mM) (Eisenthal *et al.*, 1989; Ter Kuile and Opperdoes, 1991a). This suggests that *T. brucei* does not compete for glucose with erythrocytes as long as the normal glucose level in blood is maintained.
3. The net transport of glucose over the erythrocyte membrane is probably rate limited by the exchange with other compartments (Naftalin *et al.*, 1985). Similarly, net glucose uptake by *T. brucei* has been implicated to have two rate-limiting steps: transport over the plasma membrane and another step, probably phosphorylation by hexokinase but possibly transport over the glycosomal membrane (Ter Kuile and Opperdoes, 1991a). The promastigotes of *L. donovani* have a much more complex interaction, aimed at homeostasis of the internal glucose concentration (Ter Kuile and Opperdoes, 1991b).

IMPLICATIONS FOR DRUG DESIGN AIMED AT GLUCOSE UPTAKE BY AFRICAN TRYPARASITES

The glucose transporter of African trypanosomes is a suitable target for chemotherapy as judged on two criteria: it is easily accessible and it is likely to be the rate-limiting step in overall glucose metabolism. It is too early to tell whether there is sufficient difference between this sugar transporter and those in the host. The properties of the glucose carrier in *T. brucei* strongly resemble those of its counterpart in erythrocytes. There are, however, a few differences: the parasite

transporter has a much higher specificity, not transporting glucose analogues unable to form hydrogen bonds to the C3 and C4 hydroxyl oxygen atoms (Southworth and Read, 1970; Eisenthal *et al.*, 1989), whereas changing any single hydroxyl group does not hamper uptake in human erythrocytes (Barnett *et al.*, 1973). Of the few effective transport inhibitors tested in *T. brucei*, such as phloridzin and cytochalasin B, none is without effect on human erythrocytes. Structural homologies occur between all facilitated diffusion sugar-transporters, whether of prokaryotic or eukaryotic origin (Baldwin and Henderson, 1989; Gould and Bell, 1990). The above considerations indicate that it may not be easy to develop a specific antitrypanosome chemotherapy which is based on glucose carriers. The best chance of developing inhibitors with sufficient specificity probably is to adopt the approach of 'rational drug design' (Opperdoes *et al.*, 1990; Hol *et al.*, 1989). This should involve the isolation and crystallization of the parasite transporter protein and the subsequent resolution of its three-dimensional structure. Detailed structural knowledge of the subtle differences between host and parasite proteins could then be exploited for the synthesis of highly selective inhibitors that block the parasite carrier and leave the host carrier unharmed.

The possibilities of developing a vaccine based on the glucose carrier are worth investigating. Monoclonal antibodies specific for the C-terminus of the human erythrocyte glucose transporter have been made using hydrophobic segments (Andersson and Lundahl, 1988). Peptide-specific antibodies recognized the intact erythrocyte glucose transporter, while binding only at one location (Davies *et al.*, 1987). This indicates that it may be possible to vaccinate against the glucose carrier of parasites, utilizing isolated segments that are selected for having as little homology as possible with the host transporter. Because sugar transporters are by necessity exposed to the surroundings, antibodies aimed against glucose carriers have a high chance of reaching their targets.

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34. Transport and compartmentation in *Entamoeba histolytica*

T.Bakker-Grunwald

INTRODUCTION

In *Entamoeba histolytica*, transport in its widest sense, i.e. both transmembrane transport of substrates or ions and vesicular transport of endocytized or secretory material, has been a relatively neglected field of investigation. There are two good reasons for intensifying the efforts along these lines: firstly, transport systems are potential targets for antiparasite chemotherapy (see for instance, Chapter 33); and, secondly, *E. histolytica* may represent a model of a very primitive eukaryote (Reeves, 1984; Cavalier-Smith, 1987; Avron and Chayen, 1988) and, as such, should provide insight into the evolution of transport systems in higher eukaryotic cells.

Because of the vacuolization and the high pinocytic activity of *E. histolytica*, the interpretation of transport phenomena in this organism is not always straightforward. In this chapter, I present two models: a qualitative model for the functional compartmentation of the amoeba and a quantitative model for endocytic turnover of its plasma membrane. I then briefly review the data available on transmembrane substrate and ion transport in *E. histolytica*; these data are interpreted in the light of the two models described.

TRANSPORT AND COMPARTMENTATION: BASIC CONCEPTS

Transport

E. histolytica takes up close to 30 per cent of its own volume per hour by fluid-phase pinocytosis (Serrano and Reeves, 1975; Aley *et al.*, 1984; Bakker-Grunwald *et al.*, 1985). This process is constitutive, i.e. it proceeds without any apparent need for an external trigger. As a consequence, all solutes present in the medium will be taken up through the pinocytic route. The rate by which this occurs will (at least in the absence of binding) depend linearly on the solute concentration; thus, without additional data this mode of transport is kinetically indistinguishable from free

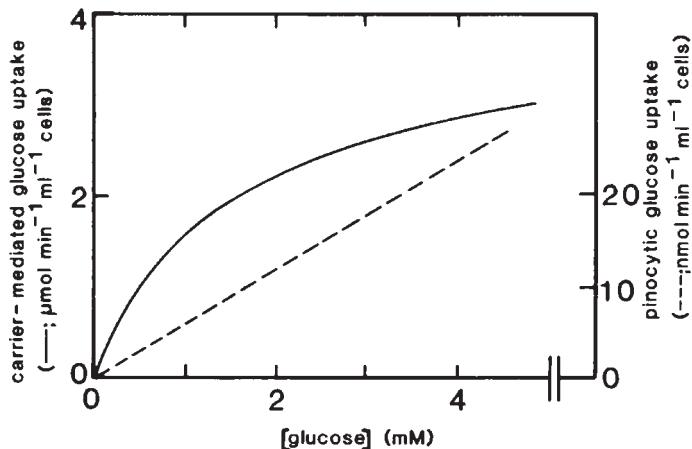


Figure 34.1. Glucose uptake as a function of glucose concentration in *E.histolytica*. The curves are calculated from the data reported by Serrano and Reeves (1974, 1975). (—) Carrier-mediated uptake; (---) pinocytic uptake. Note that, at physiological glucose concentrations (up to 5 mM), pinocytic uptake contributes less than 1 per cent of total (carrier-mediated plus pinocytic) uptake.

diffusion across the plasma membrane. It should be kept in mind that, after pinocytic uptake, substrates are not yet available to the cytoplasmic enzymes: for this, they would still have to cross a membrane barrier.¹ Pinocytic uptake depends on acidification of the pinocytic compartment (Löhden-Bendinger and Bakker-Grunwald, 1990) and, presumably, on active cytoskeletal rearrangements; in this sense it is an energy-requiring process. As in higher eukaryotic cells, both pinocytic uptake and exocytic secretion are inhibited by cytochalasin B and by weak permeant bases (Bakker-Grunwald *et al.*, 1985, 1986).

In addition to the pinocytic route, some substrates are taken up by a transport system ('carrier') in the plasma membrane. This type of uptake is saturable; as a consequence, the ratio of carrier-mediated to total (carrier-mediated plus pinocytic) uptake decreases with increasing substrate concentration (see Figure 34.1). Total uptake can be corrected for the pinocytic component by measuring the rate of uptake of an impermeant marker such as FITC-dextran (Aley *et al.*, 1984) or [¹²⁵I] polyvinylpyrrolidone (Bakker-Grunwald *et al.*, 1986); alternatively, pinocytic uptake can be suppressed by cytochalasin B.

From an energetic point of view, transmembrane transport may be passive (equilibrative, facilitated diffusion) or active; the latter term implies that the substrate is transported against its electrochemical gradient. Active transport requires the input of energy; this input may be either in chemical form (e.g. ATP;

¹ In higher eukaryotic cells, a substrate for which the plasma membrane (and the primary pinocytic vesicles) lacks a carrier can theoretically pass into the cytoplasm only after (i) fusion of the primary vesicles with membranes that do contain a carrier (e.g. lysosomes), or (ii) aspecific leak associated with fusional events or with autophagy of the vesicles. According to model I, only the second of these possibilities applies to *Entamoeba*.

primary-active transport) or in chemiosmotic form (e.g. co-transport with H⁺ or Na⁺; secondary-active transport). A lucid introduction to all aspects of transmembrane transport is given by Stein (1986).

As described below *E. histolytica* is highly vacuolated. As a consequence, any substrate, particularly if it is charged, may be subjected to additional, ill-defined gradients across the subcellular membranes. This implies that intact amoebae are inherently unsuitable for the evaluation of transport energetics; at any rate, it would seem good practice to supplement data obtained on intact cells with measurements on membrane vesicles (e.g. Serrano *et al.*, 1977).

Compartmentation

E. histolytica lacks mitochondria, a rough endoplasmic reticulum (ER), and a well-defined Golgi apparatus (Martínez-Palomo, 1982; Lushbaugh and Miller, 1988). Most conspicuous is the presence of numerous vesicles and vacuoles, which may be rather large (up to 2 µm); these were originally described as 'food vacuoles', and later distinguished into phagocytic, macro—and micro-pinocytic vacuoles, primary and secondary lysosomes, residual bodies, and authophagic vacuoles (Martínez-Palomo, 1982). The existence of real, 'classical' lysosomes² in *E. histolytica* is controversial (Schlesinger, 1988); I return to this below.

Less disputed is the fact that the protein composition of the pinocytic and phagocytic vesicles on the one hand (Warren *et al.* 1982) and that of plasma membrane and pinocytic vesicles on the other hand (Serrano *et al.*, 1977; Aley *et al.*, 1984) is very similar. This is not surprising, as plasma membrane and pinocytic membranes communicate extensively by endocytic/exocytic processes; there is no evidence that this communication is restricted in any way by specialized structures, such as the coated pits found in higher eukaryotic cells.

In addition to the vacuoles, *E. histolytica* contains a tubular membranous reticulum thought to correspond to the smooth ER of higher eukaryotic cells (Martínez-Palomo, 1982; Lushbaugh and Miller, 1988).

TWO MODELS USEFUL IN EVALUATING TRANSPORT DATA

The interpretation of transmembrane transport data in *E. histolytica* tends to be confused by the vacuolization and the high pinocytic activity of the cells. Below I present two models which we find useful in dealing conceptually with transport

² Functional evidence for the existence of a separate lysosomal compartment in *E. histolytica* is limited to the observation by Aley *et al.* (1984) that after many hours FITC-dextran ended up in a slowly exchanging compartment consisting of small, acidic vesicles. We have confirmed this observation. However, these vesicles were present in rather divergent copy numbers, and some cells appeared to lack them completely. This, and the observation that virtually all digestive activity was localized within the pinocytic compartment (see model I), makes it improbable that these vesicles are 'classical' lysosomes. Rather, they may correspond to residual bodies.

phenomena in these amoebae. The models apply to the kind of basal state one would impose for transmembrane transport studies: the cells are suspended in a defined, fluid medium, which limits their endocytic activity to the constitutive pinocytosis mentioned above.

Model I: *E. histolytica* has two functional membrane compartments

This model states that, on a functional level, there are only two membrane compartments in *E. histolytica*: a pinocytically active compartment that is in continuous exchange with the extracellular phase and a biosynthetically active, ER-like compartment that is permanently sequestered within the cells. The pinocytic compartment consists of a single population of pinocytic vesicles together with the plasma membrane; it constitutes a multifunctional 'ur' compartment, performing functions that in higher eukaryotic cells are taken over by lysosomes² and cytotoxic vesicles.

Experimental support for this model (which, in less explicit form, has already been proposed by Serrano *et al.*, (1977)) includes the findings that acid phosphatase, a typically lysosomal enzyme, as well as the cysteine proteinase amebapain (Scholze and Werries, 1984; see Chapters 22 and 23), which in addition to its digestive function also plays a role in tissue destruction, appear to be localized both within the pinocytic vesicles and on the outer face of the plasma membrane (Martínez-Palomo, 1982; Löhden-Bendinger *et al.*, unpublished). Measurements on magnetically separated (Löhden and Bakker-Grunwald, 1989) pinocytic vesicles suggest that this localization is quantitative (i.e. the pinocytically active compartment accounts for virtually all the activity of those proteins).

In this model, pinocytic vesicles and plasma membrane are transient, interconvertible manifestations of one and the same membrane compartment. Support for this notion comes from the observation that the two membrane fractions have a very similar protein composition on sodium dodecyl sulphate (SDS) gels (Serrano *et al.*, 1977), and that they share at least two transport systems; that for glucose (Serrano *et al.*, 1977) and that for leucine (Bakker-Grunwald, unpublished observation). Extrapolating from these data, the model predicts that: (i) any transport system present in the plasma membrane will also be present in the pinocytic vesicles and vice versa; and (ii) any substance for which the plasma membrane lacks a transport system is impermeable: in principle, it will be excluded from the cytoplasm even after it has been taken up pinocytically.¹

The model states that the pinocytic compartment is responsible for both cellular digestion and the secretion of cytotoxic compounds; in this sense it is multifunctional. However, it is clearly not the site of membrane protein biosynthesis and glycosylation: we found that, after a short pulse, [³⁵S] methionine and [³H] mannose were incorporated into a population of small vesicles that had a sedimentation behaviour different from that of the pinocytic compartment (Bakker-Grunwald and Löhden-Bendinger, 1989). This biosynthetically active membrane fraction may be derived at least in part from the tubular 'smooth ER' observed in electron microscopy (EM) pictures (Martínez-Palomo, 1982; Lushbaugh and Miller, 1988).

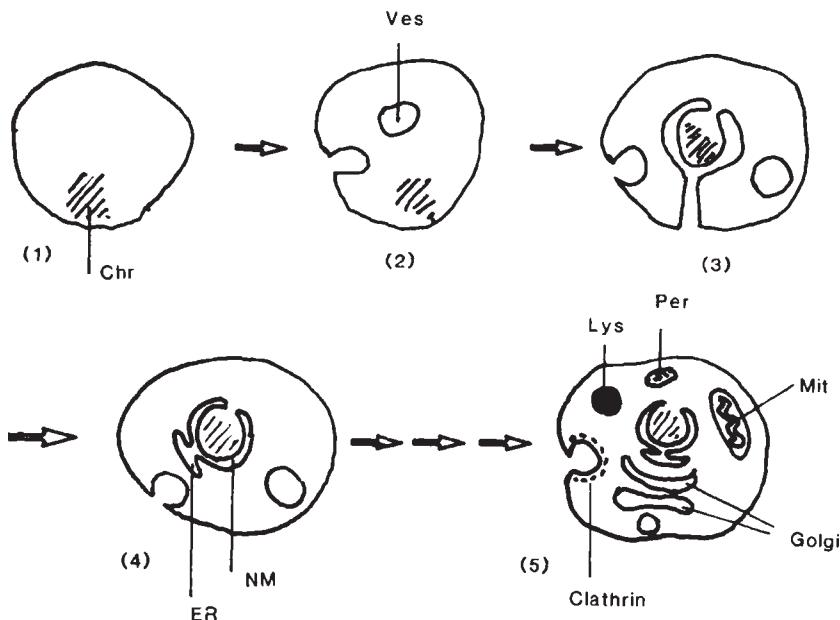


Figure 34.2. Evolutionary development of the eukaryotic cell. Chr, chromosome(s); Ves, endocytic vesicle; NM, nuclear membrane; Per, peroxisome; Mit, mitochondrion. For further explanation, see text.

In an evolutionary context, higher eukaryotes may have developed according to the scenario given in Figure 34.2. With the acquisition of cytoskeletal elements the cells developed the capacity to endocytize and exocytize (stage 2). This was a prerequisite for the invagination of their chromosome(s) (stage 3) and for the subsequent development of a discrete nucleus surrounded by an ER-like structure (stage 4). The sequence of events that finally led to the form depicted in stage 5 is unclear; however, in our model the amoebae would most closely correspond to stage 4.

Model II: turnover of the pinocytic compartment is a stochastic process

In this model, *E. histolytica* is a sphere (diameter 20 μm) stuffed with 400 pinocytic vesicles (diameter 2 μm). Every 12s, one of these vesicles fuses with the plasma membrane, exocytizing its soluble contents; with the same time course, another vesicle is generated somewhere else along the surface by invagination of the plasma membrane. The model states that these two events are independent and stochastic: i.e. the newly invaginated vesicle immediately mixes with the existing pool of 399 ‘older’ vesicles,³ and ends up with the same probability as any of those to be exocytized within the next 12s. At any moment, 80 per cent of the pinocytically

³ The ‘age’ of the pinocytic vesicles is defined as the time after pinocytic membrane invagination (Löhden and Bakker-Grunwald, 1989).

active compartment is sequestered inside, against 20 per cent facing the outside medium.

In higher eukaryotic cells, pinocytic vesicles are subjected to an ordered sequence of fusional events and sorting that eventually leads to the formation of secondary lysosomes (Helenius *et al.*, 1983). The main implication of model II is that this does not apply to the amoebal vesicles: after swimming around within the cell for a statistically determined period of time ($t_{1/2}=55$ min), each of them fuses again with the plasma membrane. Apart from some obvious simplifications (e.g. healthy *Entamoeba* are not spherical (Martínez-Palomo, 1982) and the numbers given are approximations based on macroscopic observations⁴), two aspects of the model need some comment: the stochastic behaviour of the vesicles and their inertness. Of these, only the former is fundamental to the model. It has been deduced from the observation (Aley *et al.*, 1984; Löhdén and Bakker-Grunwald, 1989) that pinocytic tracers are taken up and released with first-order kinetics, implying that the pinocytic compartment is homogeneous and that newly invaginated vesicles mix indiscriminately with the pre-existing intracellular pool. Evidence for the inertness of the vesicles is less compelling: it boils down to the observation (Löhdén and Bakker-Grunwald, 1989) that, at least for cells incubated in a synthetic saline, the exchange of contents between vesicles of different 'age' was negligible. This may not always be true, as ultrastructural studies (Lushbaugh and Miller, 1988) suggest that fusion between vacuoles does occur. In this case the model may conceptually be adapted by assuming some form of communication within the intracellular pool. At any rate, the model implies that amoebal digestion (at least of solutes) may be inherently inefficient: governed by statistics, the cells will continuously regurgitate partly digested foodstuffs.⁵

From a transport point of view, it is important to remember that, within the vesicles, only those products of digestion for which there is a transmembrane carrier present will benefit amoebal metabolism.¹

SUBSTRATE TRANSPORT SYSTEMS IN *E. HISTOLYTICA*

In this section, I summarize what is known on substrate transport in *E. histolytica*. As far as I am aware, the only previous review explicitly covering this topic is that by Reeves (1984); significantly, the literature on the subject has not even doubled since then.

⁴ The calculation is based on a rate of pinocytosis of $0.3\text{ml h}^{-1}\text{ ml}^{-1}$ cells and a pinocytic compartment occupying 40 per cent of the cell volume (Löhdén and Bakker-Grunwald, 1989), assuming that the radius of the pinocytic vesicles is one-tenth that of the cells.

⁵ In this context it is interesting to note that cysteine proteinase activity is secreted into the medium at a much lower rate (<10 per cent per hour) than that expected (>50 per cent per hour) for a freely diffusible enzyme; immunocyto logically, the proteinase appears to be associated with electron-dense intravesicular structures (Löhdén-Bendinger *et al.*, unpublished). This indicates that the vesicles may contain a (proteoglycan?) matrix that helps to retain the proteinase (and possibly other digestive and cytotoxic factors) upon exocytic fusion of the vesicles with the plasma membrane.

The glucose carrier

Of the few transport systems investigated in *E. histolytica*, that for glucose has been characterized most extensively. Serrano and Reeves (1974, 1975) showed that at an extracellular glucose concentration of a few millimolar virtually all glucose metabolized by the cells is taken up by a saturable, equilibrative transport system in the plasma membrane; in contrast to the situation in free-living amoebae, pinocytosis contributes only a minor part to cellular glucose uptake (Figure 34.1). Under these same conditions (where the carrier is close to saturated: $K_m=1.6\text{mM}$), transport is apparently the rate-limiting step in glucose utilization: any glucose taken up is immediately phosphorylated by the amoebal glucokinase. In a later paper on cell fractionation, Serrano *et al.* (1977) demonstrated that the same plasma membrane glucose transport system is present in a subcellular vesicle preparation; this is one of the observations in favour of model I (see above).

Pyrimidine, purine and nucleoside uptake

Booden *et al.* (1978) and Boolayangoor *et al.* (1978) demonstrated that uptake of certain purine and pyrimidine bases and nucleosides is carrier mediated. Based on mutual inhibition studies, they postulated at least four transmembrane transport systems for adenine/adenosine, adenosine/guanosine, uridine/cytidine and uridine/adenosine. The data presented (initial rates of uptake into intact cells) do not allow any conclusion as to the energetics of transport, i.e. whether these systems are equilibrative (as is the glucose carrier) or whether they can accumulate substrate (e.g. through a H^+ /substrate co-transport mechanism). In contrast to the compounds mentioned, inosine, guanine and hypoxanthine were taken up by a non-saturable process, denoted by the authors as diffusion. This latter term is somewhat confusing, as probably most or all of this uptake occurs through pinocytosis.

Amino acid transport

Cerbón-Solórzano and Noriega-Martínez (1986) have investigated amino acid transport both in *E. histolytica* and in the reptilian parasite, *E. invadens*. They reported evidence for the existence of two transport systems, one for leucine and one for lysine/arginine. We have characterized the leucine carrier more extensively (unpublished observations). It has a rather broad specificity towards neutral amino acids, accepting (in order of decreasing affinity) Leu, Ile > Val > Met, Cys > Phe, Thr. Also, similar to the glucose carrier (see above), it turned out to be present in both the plasma membrane and a subcellular vesicle preparation. Cerbón-Solórzano and Noriega-Martínez (1986) suggested that uptake of both arginine and leucine may be driven by an electrochemical proton gradient. At least for leucine, we could not confirm this: in the subcellular vesicles leucine transport was purely equilibrative (unpublished observation).

ION TRANSPORT IN *E. HISTOLYTICA*

Cells need ion pumps to offset ongoing passive influx with an active efflux (Maloney and Wilson, 1985). Most cells have as their main primary active ion pump either a H^+ ATPase (e.g. see Chapter 32) or a Na^+ -ATPase (Stein, 1986). According to their structure and catalytic mechanism, ion-pumping ATPases are classified into types F (or F_0F_1), P (or E_1E_2) and V (vacuolar) (Pedersen and Carafoli, 1987). Below, I list what is known on ion transport in *E. histolytica*.

Proton pumping

As monitored by the fluorescence of FITC-dextran, vesicles acidify to pH 5.2 within 1 min of pinocytic invagination of the plasma membrane; this acidification is inhibited very effectively by baflomycin A₁, a specific inhibitor of vacuolar type (V) H^+ -ATPases in higher eukaryotic cells (Löhden-Bendinger and Bakker-Grunwald, 1990). Thus, pinocytic vesicles appear to contain a V-H⁺-ATPase; according to model I, this same H^+ -ATPase should also be present in the plasma membrane.

Na^+ circulation

Similar to other cells, *E. histolytica* extrudes Na^+ against its electrochemical gradient. Most of the unidirectional Na^+ uptake into the cells is by pinocytosis; upon inhibition of vesicular traffic by cytochalasin B, cells lose NaCl and osmotically associated water. This Na^+ release occurs through a transmembrane pathway; the latter involves a Na^+ pump in the plasma membrane in series with a Na^+ leak in the vesicular membrane (Bakker-Grunwald *et al.*, 1985, 1986).

Other ions

Of the ions not dealt with here, K^+ may be close to electrochemical equilibrium. Ca^{2+} is probably pumped out of the cytoplasm (McLaughlin and Aley, 1985); however, the nature of the Ca^{2+} pump is still unclear.⁶

CONCLUSION

Clearly, our knowledge of transport processes in *E. histolytica* is still extremely fragmentary. So far, only a few carriers for substrates have been kinetically defined; of these, none has been purified yet, nor has any corresponding gene been cloned. The same applies to ion transport systems. In addition, it has not been investigated whether *E. histolytica* possesses pumps (such as the P glycoprotein (see Foote *et al.*,

⁶ It has been suggested (McLaughlin and Müller, 1981) that intracellular levels of Ca^{2+} are controlled by a membrane-bound Ca^{2+} -ATPase with rather complex kinetic properties. However, we have obtained evidence (unpublished) that this particular ATPase is an ectoenzyme, which would discredit it as a candidate for the Ca^{2+} pump.

1989; and see Chapter 39)) for the removal of cytotoxic substances. Finally, vesicular transport in *E. histolytica* is very pronounced; yet next to nothing is known on the cytoplasmic factors involved. Intensified research along these lines should be rewarding from both a therapeutic and theoretical point of view.

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35. Substrate specificities for malaria-inducible red cell transport in relation to parasite metabolism

B.C.Elford, R.A.Pinches, C.Ellory and C.I.Newbold

During the asexual intraerythrocytic stage of malaria and babesia parasites, membrane transport pathways are induced in the host erythrocyte that have substrate selectivities dependent upon the parasite species. In the present paper we review some recent findings which show that, in addition to the wide range of solutes described previously, two positively charged substrates, choline and L-carnitine (but not the basic amino acid L-lysine), and the anionic substrate 2-oxoglutarate (a-ketoglutarate) have enhanced transport into *Plasmodium falciparum*-infected human erythrocytes. In contrast to the parasite-induced influx of L-carnitine, acetyl-L-carnitine remains almost excluded from the parasitized human red cell. Normal human red cells seem to lack constitutive transport systems for both L-carnitine and the acetylated derivative, whereas a saturable endogenous transport system for choline influx has been well characterized. The cinchona alkaloids (quinine and quinidine) selectively inhibit both the *P. falciparum*-induced choline influx and the constitutive choline transport systems, but have no inhibitory effects on the parasite-induced influx of 2-oxoglutarate (or of L-glutamine). These data indicate that heterogeneous and functionally separate transport systems for anionic and cationic substrates (and possibly amino acids) are induced during the maturation of *P. falciparum* trophozoites.

INTRODUCTION

In addition to the parasites utilizing haemoglobin as a source of nutrients during the asexual red cell stage of development (Charet *et al.*, 1983; Goldberg *et al.*, 1990), malaria- and babesia-infected erythrocytes also take up and metabolize a variety of exogenously supplied substrates such as amino acids (Sherman, 1979; Elford *et al.*, 1985), sugars (Homewood and Neame, 1974; Allison *et al.*, 1979; Lambros and Vanderberg, 1979; Ginsberg *et al.*, 1983), purine bases (Webster and Whaun, 1981;

Queen *et al.*, 1988) and nucleosides (Gero *et al.*, 1988). For some substrates and certain species of malaria, the transport characteristics of the normal red cell are rate limiting for the demands of the trophozoite during parasite maturation. For example, the normal human erythrocyte seems to lack constitutive transport systems for the acidic amino acid L-glutamate (Elford *et al.*, 1985) and, as we describe below, for L-carnitine. As the influx rate for L-glutamate also remains low in the infected red cell, Elford *et al.* (1985) have argued that the synthesis of L-glutamate by trophozoites of *P. falciparum* may be regulated in part by the enhanced influx of exogenously supplied L-glutamine.

It has been suggested, largely on the basis of data obtained from the selective lysis of infected red cells exposed to isosmolar non-physiological media (Ginsburg *et al.*, 1983, 1985), that in human erythrocytes parasitized by *P. falciparum* the functional basis of parasite-mediated transport is a small number of uniform aqueous channels induced in the host erythrocyte during the late-ring to early pigmented trophozoite stage of maturation (Kutner *et al.*, 1982; Ginsburg and Stein, 1987a; Cabantchik, 1990). It has also been argued for several years that, although parasite-induced pores accommodate a wide range of anions, zwitterions and neutral solutes, they exclude cations selectively (Ginsburg and Stein, 1987a). More recently, Cabantchik (1990) has shown that the Renkin equation adequately describes the relationship between solute size and parasite-enhanced flux rate through induced pores of about 3.7 Å limiting radius. However, as Cabantchik (1990) and others (Elford *et al.*, 1990a, b) have pointed out, the parasite-induced permeability properties for many amino acids predicted by the Renkin equation are far from those determined experimentally.

Our recent studies have focussed on the substrate selectivity of malaria-mediated transformations in the transport properties of the human erythrocyte and, in particular, on the inhibition by cinchona alkaloids of parasite-induced choline transport in the *P. falciparum*-infected human red cell. These findings strongly suggest that the transport pathways developmentally regulated by malaria parasites have well-defined molecular exclusion limits (selectivity on the basis of size), are functionally discrete for anionic and cationic substrates, and also have independent control mechanisms for different types of substrate. In this review we present some representative results and discuss their relevance in the context of previous findings.

ENDOGENOUS VERSUS PARASITE-MEDIATED TRANSPORT PROCESSES

In general, the induced rates of influx of various radiolabelled substrates are found to be linearly related to the parasitaemia of parasitized suspensions of red cells. These relationships hold true for the *P. falciparum*-induced influx of L-carnitine (Elford *et al.*, unpublished), choline, L-glutamine (Elford *et al.*, 1985) and certain other parasite-mediated transport systems (Allison *et al.*, 1979).

For solutes where the normal erythrocyte membrane has little or no transport capacity, parasite-mediated uptake mechanisms assume special significance in

Table 35.1. Substrate selectivity for parasite-enhanced influx in *P. falciparum*-infected human erythrocytes.

Amino acids	Carbohydrates	Cations	Anions
<i>Key substrates with significantly increased influx^a</i>			
Glutamine	Sorbitol	K ⁺ , Na ⁺ , Rb ⁺	2-Oxoglutarate
Glycine	myo-Inositol	Ca ²⁺	Phosphate
Proline	Monosaccharides	Choline	
Alanine		Carnitine	
Asparagine			
<i>Substrates exhibiting little or no increased influx</i>			
Glutamate	Disaccharides	Acetyl carnitine	Citrate
Lysine			
Arginine			

^a Enhanced transport of nucleosides has also been reported (Gero *et al.*, 1988; and see Chapter 36) and some other solutes have been listed by Ginsburg and Stein (1987a).

terms of the control of growth of the intraerythrocytic parasite. However, it must be stressed that, for certain solutes, parasite-induced modifications to the transport characteristics of the host red cell are dependent not only upon the parasite species but also the permeability properties of the host red cell. For example, the transport of L-glucose and *myo*-inositol into murine and human red cells is enhanced by infection with malaria or babesia (Homewood and Neame, 1974; Allison *et al.*, 1979; Elford *et al.*, 1985), whereas red cell permeability to D-sorbitol is transformed only in the malaria-infected human (but not murine) red cell (Lambros and Vanderberg, 1979; B.C. Elford, unpublished). Similarly, L-glutamine influx is enhanced markedly only in human and not in murine red cells following infection with malaria parasites (Elford *et al.*, 1985).

Within a detection limit of about 10⁻¹³ mol/10⁸ cells/minute, we have recently found there was no significant influx of ¹⁴C-labelled L-carnitine in normal human erythrocytes, but this substrate entered malaria-infected human red cells with a half-life of 2–5 min. The rate of acetyl-L-carnitine influx into erythrocytes parasitized with pigmented (but non-segmenting) trophozoites of *P. falciparum* was 40-to 60-fold less than that of L-carnitine. There was no significant influx of acetyl-L-carnitine into normal human red cells. Hence, as with parasite-induced transport of sugars and anions, we have some evidence that selectivity on the basis of size is a characteristic of the induced uptake of organic cations. Table 35.1 summarizes the selective nature of the enhanced permeability to some key substrates mediated by the maturation of *P. falciparum*.

Martin (1977) established the characteristics of choline transport into normal human red cells and we have confirmed that it occurs via a saturable influx mechanism with relatively low values for K_m and V_{max} (Elford *et al.*, 1990a,b). By contrast, the enhanced influx of choline into the malaria-infected human red cell was non-saturable and, at an external choline concentration of 0.5 mM, the influx rate was about 40-fold greater than the constitutive maximal rate for choline influx

Table 33.2. Comparison of constitutive and parasite-induced rates of influx of two organic cationic substrates, L-carnitine and choline, into uninfected and *P. falciparum*-infected erythrocytes.

Substrate	Influx (10^{-13} mol/ 10^8 cells/min)	
	uninfected	<i>P. falciparum</i> infected ^a
L-Methyl [14 C] carnitine	Negligible over 10–20 min	363 ± 37 ($n = 7$)
Methyl [14 C] choline	36 ± 2 ($n = 3$) ^b	224 ± 10 ($n = 3$)

^a Initial influx rates were normalized to 100% parasitized red cells but no attempt was made to differentiate between singly and multiply infected red cells. Initial influx rates were computed from numerical fits to the time course for the influx curves using a reciprocal function (Elford *et al.*, 1983).

^b Influxes were linear for 10–20 min. Rates of influx were determined by linear regression. Each substrate (50 μ M) were presented to washed red cells suspended in Dulbecco's phosphate-buffered saline with HEPES (25 mM). The influx rate for choline is effectively the maximal rate for uninfected erythrocytes.

into uninfected erythrocytes (Elford *et al.*, 1990a). The enhanced rates of influx of the basic (i.e. cationic) substrates choline and L-carnitine into normal parasitized human erythrocytes are summarized in Table 35.2. It should be stressed, however, that under the conditions in which we found marked parasite-induced enhancement of choline and L-carnitine influx, there was no significant increase in the influx of the basic amino acid L-lysine.

PARASITE-INDUCED INFLUX RATES ARE NON-SATURABLE

For a wide range of solutes, including anionic and cationic substrates, amino acids, myo-inositol and sugars, parasite-induced influx rates showed no tendency to saturate as a function of increasing extracellular concentration. For instance, this was true for L-glutamine up to 20.0mM (Ellory *et al.*, 1988), 2-oxoglutarate up to 1.2mM, L-carnitine up to 1.0mM and choline up to 0.5mM (Elford *et al.*, unpublished). There was a constant ratio of 100 between the influx rates of 2-oxoglutarate in infected and normal erythrocytes since neither type of cell exhibited saturable influx kinetics for this anion.

EXOGENOUSLY SUPPLIED PROMOTERS OF PARASITE GROWTH

L-Glutamine is essential for the optimal growth of *P. falciparum* in culture (Elford *et al.*, 1985; Divo *et al.*, 1985). We have extended our earlier findings to show that L-glutamine, (but not L-glutamate) and 2-oxoglutarate (but not certain other exogenously supplied TCA-cycle intermediates, e.g. succinate or citrate) promote parasite growth under a range of conditions *in vitro* (Elford *et al.*, unpublished). Under similar conditions, however, we found that neither L-carnitine nor choline

had any significant effect on the promotion of parasite growth. Flux experiments with [¹⁴C]citrate indicated that this anion was excluded from both normal and parasitized human erythrocytes.

INHIBITORS OF PARASITE-INDUCIBLE TRANSPORT AS ANTIMALARIAL AGENTS

Out of a range of established antimalarial compounds, including chloroquine and artemisinine, we have found (Elford, 1986) that only the cinchona alkaloids inhibited *P. falciparum*-induced transport systems at concentrations approaching established therapeutic levels. Quinine and, to a greater extent, quinidine inhibited choline fluxes both in normal and in parasitized red cells (Elford *et al.*, 1990b), but these antimalarials had little or no significant inhibitory effects on either the constitutive or the parasite-inducible fluxes of L-glutamine or 2-oxoglutarate. *P. falciparum*-induced L-carnitine influx was also inhibited by the cinchona alkaloids (Elford *et al.*, unpublished). Ancelin and Vial (1986) have inferred previously that the antimalarial activity of quaternary ammonium compounds might relate to their capacity to inhibit choline transport in the infected red cell, but their conclusions were based on enzyme assays and not directly on membrane fluxes.

In contrast to the selective inhibitory effects of quinine and quinidine, the alkaloid piperine was found to be a potent reversible but non-selective inhibitor of the entire range of parasite-mediated permeability pathways examined so far in both human and murine malaria.

CHARACTERISTICS OF ENDOGENOUS AND PARASITE-INDUCED TRANSPORT SYSTEMS

It is now clear that parasite-mediated transport mechanisms have properties that are markedly different from the constitutive transport systems in the normal red cell. For example, endogenous glutamine influx mechanisms in human red cells are Na⁺ dependent and saturable, and there is strong competition from other amino acids (Elford *et al.*, 1988; Ellory *et al.*, 1988), whereas the parasite-induced glutamine influx system is non-saturable and is unaffected by the replacement of extracellular Na⁺ with K⁺, or by the presence of amino acids known to compete with the endogenous transport mechanisms. Furthermore, the parasite-induced and endogenous influx mechanisms have markedly differing sensitivities to various inhibitors (Elford, 1986) and to changes in pH (unpublished results). However, whether the enhanced transport properties of infected red cells are regulated directly by parasite proteins synthesized *de novo*, or are associated with the unmasking of cryptic sites in the mature erythrocyte membrane during parasite development is not yet known. Affinity labelling with inhibitors or covalently binding substrate analogues (e.g. L-DON (6-diazo-5-oxo-L-norleucine) for L-glutamine (Elford *et al.*, 1990a,b)) might help to answer these questions.

Although much emphasis has been placed on the enhanced uptake of solutes

induced by infection with haemoprotozoa (and viruses (Carrasco, 1978)), it should also be borne in mind that parasite-mediated transport pathways might be induced to facilitate the release of metabolites, especially the osmotically active digestion products of haemoglobin as recently suggested by Ginsburg (1990).

DIVERSITY OF INDUCED TRANSPORT SYSTEMS

An obvious question concerns the number and variety of induced transport systems in the parasitized red cell. Until our recent work with choline and L-carnitine, it was considered that cations were excluded from malaria-induced transport systems (Ginsburg and Stein, 1987a). Our recent studies have shown that L-carnitine does not penetrate significantly beyond the extracellular space defined by inulin, indicating that the normal human red cell has no constitutive transport system for L-carnitine. However, it has been reported that L-carnitine is present in erythrocytes at concentrations comparable to those in plasma (Borum *et al.*, 1985), hence an influx rate lower than our level of detectability cannot be excluded.

Originally the idea of a single class of transporter with broad specificity was favoured to account for the characteristics of parasite-mediated transport processes (Ginsburg and Stein, 1987a, b; Cabantchik, 1990). Subsequently, the use of differential inhibitors has suggested that discrete systems for nucleosides, certain amino acids, alkali metal cations and cationic organic substrates are induced. Our work on choline and L-carnitine has shown the *P. falciparum*-induced systems to be highly selective, as emphasized by the indication of molecular exclusion between L-carnitine and acetyl-L-carnitine. If acetylcarnitine is effectively excluded from the parasitized red cell, it indicates that, as with uncharged and anionic substrates, the parasitized erythrocyte membrane can act as a molecular sieve and, therefore, shares some features with the bacterial cell surface. Bacterial membranes are known to possess various porins with cation and anion selectivity as well as sugar selectivity (Hancock, 1987; Benz, 1988). It should be stressed, however, that, although organic cationic substrates are transported across the infected red cell surface, intracellular concentrations of Na^+ and K^+ are altered only slightly at the young trophozoite stage of *P. falciparum* infection *in vitro* (Lee *et al.*, 1988), and *P. Knowlesi*-infection in monkeys (Dunn, 1969; Boehm and Dunn, 1970). However, Lee *et al.* (1988) showed that some loss of cytoplasmic K^+ occurs at a more mature stage of development.

A second important point arising from studies of choline and L-carnitine transport in infected cells is the action of quinine and piperine as differential inhibitors of induced transport. The fact that piperine inhibits uptake of all substrates so far studied, whilst quinine is an effective inhibitor for cationic substrates only, provides further evidence for separate induced pathways for cations and anions.

The effective inhibition of transport also identifies a possible strategy for chemotherapy in malaria. It is possible that drugs targetted at inducible transport systems could adventitiously share the induced influx pathways or, alternatively, could interact at the infected cell surface and block transport, thereby avoiding any requirement for uptake and accumulation by the parasite *per se*.

PARASITE GROWTH IN RELATION TO TRANSPORT

The metabolic importance of choline (Ancelin and Vial, 1986) and L-carnitine to the parasite is obvious, but the latter has not been investigated in detail. In man, L-carnitine is derived from *e-N*-trimethyl-L-lysine arising from post-translationally modified proteins, but in some micro-organisms carnitine can be synthesized from lysine directly (Broquist, 1980). Red cell membranes have endogenous carnitine acyltransferase activity (Wittels and Hochstein, 1967) that could conceivably constitute the basis of a 'metabolic sink' in the malaria-infected erythrocyte. Since lysine influx is not increased in malaria-infected red cells, it seems possible that the parasite may rely on host-derived carnitine for mitochondrial function. However, unlike 2-oxoglutarate and L-glutamine, neither choline nor L-carnitine promoted parasite growth *in vitro*. It is possible that the single-cycle assay used was not a definitive test of growth enhancement in long-term culture. Furthermore, the parasite isolate in these studies (ITO4) (Berendt *et al.*, 1989) was phenotypically selected and adapted to culture in a medium at low concentrations of choline and L-carnitine. It would be of interest to extend the studies to primary field isolates. It should also be pointed out that, at the pigmented-trophozoite stage of maturation, *P. falciparum*-infected red cells *in vivo* would be attached to capillary endothelium; how this process of sequestration might affect induced transport or parasite development is unknown at present.

Another important metabolic question relates to the transport and turnover of TCA-cycle intermediates in the parasitized cell. We have screened a variety of TCA intermediates for enhanced transport and have shown greatly enhanced uptake of 2-oxoglutarate into the infected cell. L-Glutamine, but not L-glutamate uptake is also elevated in infected cells. Parasite growth was also enhanced when media were supplemented with L-glutamine and 2-oxoglutarate, but not when supplemented with L-glutamate. The role of host—versus parasite-derived TCA-cycle intermediates in parasite metabolism requires further investigation.

CONCLUSIONS

There are many questions still to be answered concerning the nature of the inducible pathways:

1. Are parasite proteins regulating parasite-augmented influx, and if so where are they located?
2. If induced transport is controlled by parasite gene products, how are these exported from the parasite, transported through the parasitophorous vacuole and assembled?
3. Are components of endogenous red cell transporters modified during parasite maturation either by parasite-specific or constitutive red cell enzymes?
4. Where are the rate-limiting steps in the overall process of influx into the infected cell and how do these alter with parasite maturation?

To summarize, we feel that the uniform pore model needs to be modified to explain the data on the diverse nature of the parasite-induced systems in different species of malaria and babesia. Furthermore, when the selective inhibitory activity of the cinchona alkaloids is taken into consideration, we feel that the model developed by Ginsburg and Stein (1987a,b) and Cabantchik (1990) is insufficient to support our concept of heterogeneous classes of induced transport systems with selectivity for anions, cations, zwitterions and uncharged solutes. It seems possible that as yet uncharacterized plasmoidal porins and transporters, akin to the diverse bacterial systems of porins, periplasmic binding proteins and inner membrane transport systems, will constitute some of the components that regulate transport into the malaria-infected red cell and allow the parasite to regulate its own micro-environment for optimal growth. The characterization of the components of the malarial transport systems, their developmental control or expression, and their localization within the multicompartment series membrane system that constitutes the infected erythrocyte remain an interesting and demanding challenge.

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36. Nucleoside transport in malaria- and *Babesia*-infected cells

A.M.Gero

INTRODUCTION

One of the logical approaches to providing effective chemoprophylaxis and treatment of malaria is to develop new drugs that will specifically inhibit parasite growth and reproduction. One such approach involves taking advantage of changes in the permeability properties of the membrane of the infected red cell which are caused by the growth and reproduction of the intraerythrocytic malarial parasite. Previous studies have established that permeability to a variety of compounds such as glucose, nucleosides, amino acids, sodium, potassium, calcium, zinc, iron, and several antimalarial drugs is altered in the infected red cell compared to the normal erythrocyte (for a review see Sherman, 1988). Several of these permeability changes and their implications are discussed in other chapters in this book (see Chapters 2, 35 and 39). This article is principally concerned with the changes in the transport of nucleosides in the malaria-infected erythrocyte, and the related intraerythrocytic parasite *Babesia*, and its applicability to chemotherapy.

As an approach to chemotherapy, analogues of purine nucleosides and possibly pyrimidine nucleosides as antiprotozoal agents have advantages on two fronts.

1. Nucleic acid precursors are required by the intraerythrocytic parasite for its growth and reproduction and, as the majority of these needs cannot be supplied by the host red cell, many of the compounds, in particular purine nucleosides, are salvaged from the serum. In addition, the metabolic pathways involving purines and pyrimidines in malaria-infected cells are markedly different from those in the normal red cell. The uninfected human erythrocyte has a relatively limited network of purine salvage enzymes and no capacity for pyrimidine bio-synthesis. Infection with the malarial parasite leads to elaboration of the purine nucleotide pathways and to the expression of the enzymes for pyrimidine bio-synthesis *de novo* (for reviews see Sherman, 1984; Gero and O'Sullivan, 1990; and Chapter

- 2). Thus these differences may allow the introduction of purines or pyrimidine analogues which are toxic to the parasite but relatively harmless to the host.
2. As there are qualitative differences between the mode of nucleoside transport in the malaria-infected erythrocyte and the normal cell, it may be possible to achieve selective permeability of toxic nucleosides into the infected cell whilst the normal host cell remains unaffected.

BACKGROUND

The general features of purine nucleoside transport with particular attention to the changes in the malaria-infected erythrocyte are described below. The alteration of transport in the *Babesia*-infected cell, although similar in some aspects, will be discussed in a later section of this article.

Essential requirement for purine salvage by the intraerythrocytic malarial parasites

Plasmodium falciparum and other malarial parasites lack the pathway of purine bio-synthesis *de novo* and thus are dependent for their metabolic requirements on salvage (and hence transport) of exogenous purine bases and nucleosides from either the host cell or the serum (for reviews see Sherman, 1979, 1984). Notably, Trigg and Gutteridge (1971) and Tracy and Sherman (1972), demonstrated that *P. knowlesi* and *P. lophurae*, respectively, required only exogenous adenosine to sustain growth. Incorporation studies in *P. lophurae* indicated that adenosine was converted to both adenine and guanine nucleotides. Thus adenylosuccinate synthetase, adenylosuccinate lyase, IMP dehydrogenase and GMP synthetase, which are absent in the normal erythrocyte, have all been detected in *P. falciparum* extracts (Reyes *et al.*, 1982; Webster *et al.*, 1984). Therefore, the dependence of the parasite on adenosine and the importance of adenosine salvage is apparent (Figure 36.1).

A vast amount of work has been done in this area but, in summary, the nucleosides adenosine, deoxyadenosine, guanosine and inosine (and the purine bases, hypoxanthine and guanine), but not the pyrimidine nucleosides, are transported into the parasite-infected cell and utilized by the intraerythrocytic stages of *Plasmodium*. The malaria-infected cell is also able to transport pyrimidine nucleosides (thymidine, uridine, cytidine and deoxycytidine), but these are not converted to nucleotides in the parasite because it lacks the appropriate enzymes (Neame *et al.*, 1974; Reyes *et al.*, 1982; Gero *et al.*, 1984). Thus interference or alteration of purine nucleoside transport represents a logical approach to the development of new antimarial agents.

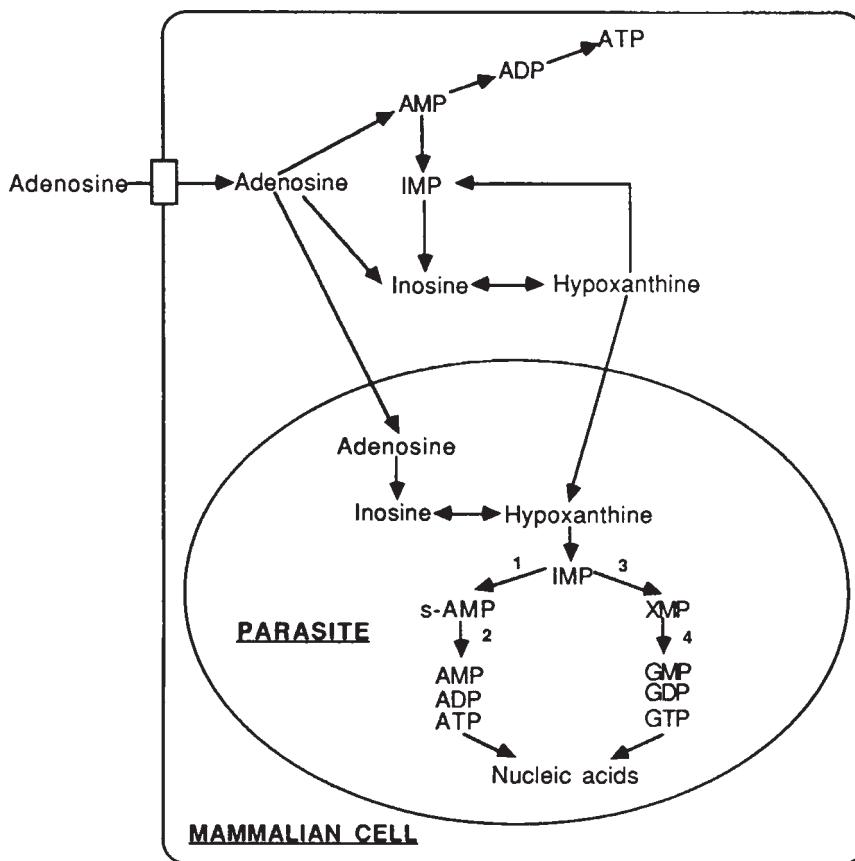


Figure 36.1. Summary of the purine metabolism following transport of adenosine in *P. falciparum* infected cells. Enzymes: 1, adenylosuccinate synthetase; 2, adenylosuccinate lyase; 3, IMP dehydrogenase; 4, GMP synthetase. The small box represents the adenosine transporter. AMP and GMP in the parasite are converted via the diphosphates to ATP and GTP, respectively, in the normal way. Arrows have been omitted due to lack of space.

Nucleoside transport

In mammalian cells

What is the definition of ‘transport’? It is important to discriminate between ‘transport’ and ‘uptake’. Transport, which occurs within seconds, is referred to as the transfer of an unaltered molecule across the cell membrane. By comparison ‘uptake’ is the incorporation of the molecule into the cell over periods of many minutes or hours and refers to the combination of the transport of the unaltered molecule as well as its metabolism within the cell.

In normal human erythrocytes, nucleosides are transported into the cell by a transport protein in the erythrocyte plasma membrane which mediates the entry and

exit of nucleoside molecules (for reviews see Paterson and Cass, 1986; Jarvis, 1987). The transport mediated entry of nucleosides is a reversible, non-concentrative process and the substrate specificity of the nucleoside transporter is broad in that both ribosides and deoxyribosides of both purines and pyrimidines are transported as well as synthetic nucleosides of a remarkable structural diversity (Plagemann and Wohlheuter, 1980; Paterson *et al.*, 1981).

In other types of mammalian cells, nucleosides have been shown to permeate the cell membrane by utilization of one or more of at least three different types of carrier mediated transport mechanisms:

- (a) the broadly specific bidirectional carrier, mentioned above, which is energy independent and subject to potent inhibition by 6S derivatives of 6-thiopurine nucleosides (see next section on nucleoside transport inhibitors) occurs in many mammalian cells including human erythrocytes and leukocytes (Jarvis, 1987);
- (b) a second type of non-concentrative nucleoside transporter occurs in some lines of cultured tumour cells; it can be distinguished by its relative insensitivity to nucleoside transport inhibitors (Belt, 1983; Plagemann and Wohlhueter, 1985).
- (c) in tissues such as hepatocytes, splenocytes, intestinal epithelial cells and some cultured cell lines, a concentrative, sodium and energy dependent nucleoside transporter has been identified; it has been reported to be insensitive to nucleoside transport inhibitors (Jakobs and Paterson, 1986; Vijayalakshmi and Belt, 1988; Plagemann and Woffendin, 1989).

All three of the above nucleoside transporters appear to be saturable and subject to competition by other nucleosides. Although some differences in permeation specificity can exist, virtually all nucleosides which have been investigated (both physiological or synthetic analogues) have been shown to rely directly on one or more of these transporters for their entry into cells.

Inhibitors of nucleoside transport in mammalian cells

In erythrocytes from several species, the equilibrative, non-concentrative energy-independent transport system (type (a) above) is subject to potent inhibition by 6S derivatives of 6-thiopurine nucleosides, such as nitrobenzylthioinosine (NBMPR) or nitrobenzylthioguanosine (NBTGR) (for reviews see Jarvis *et al.*, 1982; Paterson and Cass, 1986; Jarvis and Young, 1986) as well as a variety of vasodilators such as dipyridamole, dilazep and hexabendine (Paterson *et al.*, 1983; Jarvis, 1986) (see Figure 36.2 for structures). In human erythrocytes the inhibition of nucleoside transport by NBMPR is related to the tight ($K_D=0.3\text{--}1\text{ nM}$) but reversible binding of NBMPR to specific plasma membrane binding sites which are either on, or are part of, functional, equilibrative nucleoside transporters (Paterson and Cass, 1986).

Some lines of cultured tumour cells (e.g. mouse L1210) express the second type (type (b)) of nucleoside transporter which can be distinguished by its relative insensitivity to NBMPR and other nucleoside inhibitors (Belt, 1983; Plagemann and

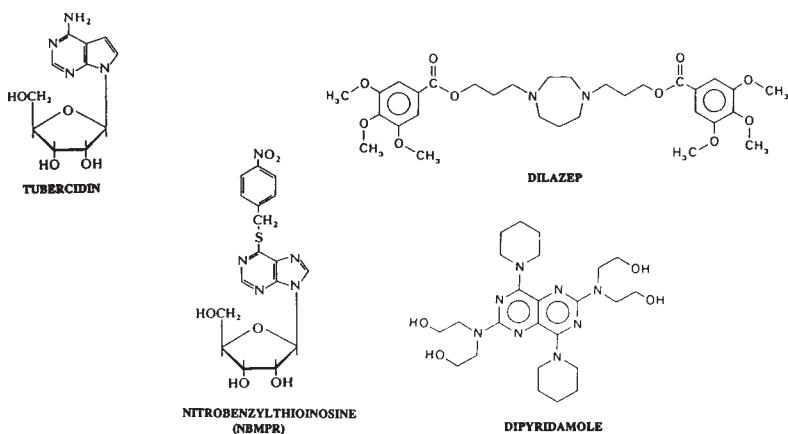


Figure 36.2. Chemical structures of the adenosine analogue, tubercidin, and the nucleoside transport inhibitors NBMPR, dilazep and dipyridamole. The representation of aromatic ring structures in different ways is simply for convenience and is not significant.

Wohlheuter, 1985). These insensitive permeation mechanisms require high (micromolar) concentrations of NBMPR for inhibition. The sodium-dependent nucleoside transporter (type (c)) has also been reported to be partly insensitive to NBMPR inhibition (Jakobs and Paterson, 1986; Vijayalakshmi and Belt, 1988; Plagemann and Woffendin, 1989).

Characterization of the mammalian nucleoside transporter has involved utilizing the tight binding capacity of radiolabelled NBMPR and ABA (*6N-(p*-azidobenzyl)adenosine), a less potent inhibitor of nucleoside transport than NBMPR which competitively inhibits NBMPR binding to the high affinity nucleoside transport sites (Young *et al.*, 1983). Both these compounds can also be covalently attached to the transporter polypeptides by photoactivation of the bound ligands with ultraviolet light. They have been used to measure the number of binding sites on the surface of human erythrocytes, which are equivalent to the number of nucleoside transporters (*c.* 11,000 sites cell⁻¹) (Young *et al.*, 1983; Gati *et al.*, 1986). Membrane nucleoside transport proteins prepared from erythrocytes and photolabelled, migrate on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as a discrete band (band 4.5) with a molecular weight of 45–60kDa (Young *et al.*, 1983). In human erythrocytes, the nucleoside transporter is also part of band 4.5. Both transporters have been reported to exist in the membrane as dimers (Jarvis *et al.*, 1986). Purification of the erythrocyte nucleoside transporter has been hampered by the presence of the glucose transporter in band 4.5, which consists of about 95 per cent glucose transporter and only about 5 per cent of the nucleoside transporter. However, the erythrocyte nucleoside transporter has recently been purified to near homogeneity by the passage of band 4.5 proteins through a column of antibodies specific for the glucose transporter (Kwong *et al.*, 1988).

ALTERATION OF NUCLEOSIDE TRANSPORT IN *PLASMODIUM*

Although a vast amount of work has been carried out on nucleoside transport in normal mammalian cells, little information is available on the transport of nucleosides or bases into parasites or into parasite-infected erythrocytes. In a *Plasmodium*-infected cell, the purine must cross possibly three membranes to penetrate into the parasite and the nature of these transport systems still remains to be elucidated. Recently, the major consideration has been the characterization of the altered permeability of the infected erythrocyte membrane to purine nucleosides.

Much of the early information came from studies on species of *Plasmodium* other than *P. falciparum*, notably *P. lophurae* and *P. berghei*. Incorporation of labelled purines into *P. lophurae*- and *P. berghei*-infected erythrocytes suggested fundamental changes in the 'transport' mechanism of purine nucleosides and bases when compared with uninfected cells (Tracy and Sherman, 1972; Manandhar and Van Dyke, 1975; Hansen *et al.*, 1980). (However, these experiments were carried out over minutes, rather than seconds, i.e. 'uptake' was measured rather than 'transport'.) In the uninfected erythrocytes, three transport systems were identified; one system transported the nucleosides, adenosine and inosine, the second, the bases, hypoxanthine and adenine, and the third adenine. However, in infected cells, adenosine, hypoxanthine and inosine appeared to enter the cells through a common transporter (Tracy and Sherman, 1972; Hansen *et al.*, 1980), whilst a second system was present for adenine.

Results from experiments carried out using saponin 'freed' *P. berghei* or *P. lophurae* parasites have also suggested similar transport systems to those in the membrane of the infected erythrocyte, *viz.* one with specificity for adenine and the other for adenosine, inosine and hypoxanthine (Tracy and Sherman, 1972; Hansen *et al.*, 1980). Other workers, using saponin 'freed' *P. berghei*, obtained evidence that radiolabel from adenosine or ATP could be incorporated into the parasite probably via adenosine being sequentially deaminated to inosine and deribosylated to hypoxanthine (Lantz *et al.*, 1971; Carter and Van Dyke, 1972; Manandhar and Van Dyke, 1975). Nucleoside transport inhibitors failed to block the uptake and/or incorporation of adenosine into nucleic acids of the free parasites (Van Dyke *et al.*, 1977). However 'free' parasites may contain residual erythrocyte plasma membrane, as it is difficult to separate the parasite completely from the host cell membrane. In addition, it has been suggested that parasites freed by saponin lysis of the host cell may leak cytosolic enzymes into the medium and it is quite possible that, although they are metabolically active, the membrane or transport sites may be altered owing to the method of preparation (Sherman, 1988).

Recent work from this laboratory (Gero *et al.*, 1988, 1989) has determined a marked change in the permeation characteristics of adenosine and the cytotoxic adenosine analogue, tubercidin, in *P. falciparum*-infected erythrocytes. Although the rates of transport were not increased over those in uninfected red blood cells, it was shown that at concentrations five orders of magnitude higher than those required to block transport completely in normal erythrocytes, NBMPR, NBTGR,

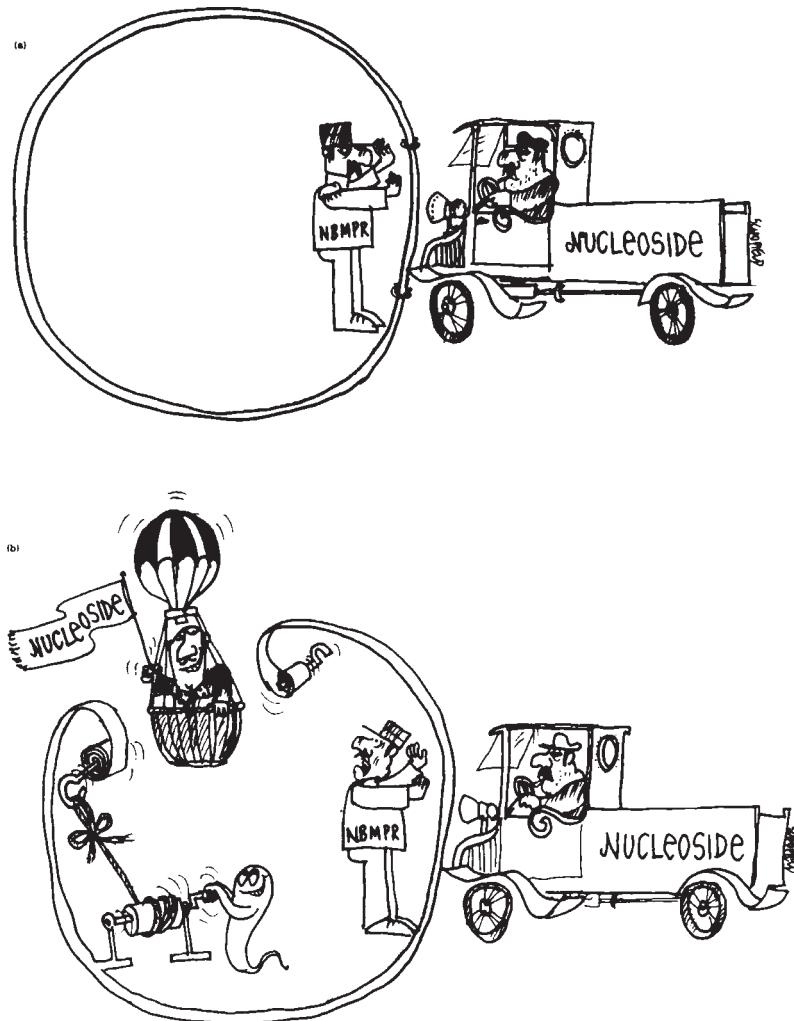


Figure 36.3. (a) Diagrammatic representation of the inhibition of nucleoside transport by NBMPr in the human erythrocyte. (b) Diagrammatic representation of the second component of nucleoside transport induced in the membrane of the malaria-infected erythrocytes.

dipyridamole and dilazep were ineffective in blocking a component (representing about 30–40 per cent of the total) of the transport of these nucleosides into infected cells. The pyrimidine nucleosides, uridine and thymidine, were also transported into infected cells in the presence of NBMPr (Gero, unpublished data). The ineffectiveness of nucleoside transport inhibitors was related to the stage of development of the intraerythrocytic parasite and insensitivity was greatest at the trophozoite or schizont stage for both chloroquine sensitive and resistant strains.

Binding studies of radiolabelled NBMPR to both normal erythrocytes and those harbouring parasites at each morphological stage indicated that fewer high affinity NBMPR binding sites were present on cells containing mature parasites than on the uninfected cells (Gero *et al.*, 1988). These changes which increased during parasite maturation to the trophozoite or schizont stage, suggested the presence in the infected cells of an altered or new nucleoside permeation mechanism of low sensitivity to NBMPR which increased as the parasite matured. However, the high affinity NBMPR binding sites present on cells containing mature parasites indicated that the host red cell transporter was still present in addition to the new transporter (Figure 36.3).

This phenomenon has also been studied in mouse erythrocytes infected with the rodent malarial parasite, *P. yoelii* (Gati *et al.*, 1987). The NBMPR-insensitive transport component was determined for both adenosine and tubercidin influx, at the trophozoite and schizont stages. The permeation of adenosine in normal mouse erythrocytes was at least 10^4 times more sensitive to NBMPR than the infected red cells. However, the NBMPR binding sites appeared to be more numerous on *P. yoelii*-infected cells than on uninfected cells, and a proportion of the sites showed low affinity for NBMPR. Further work is required to discriminate between the differences in the number of binding sites in *P. falciparum*- and *P. yoelii*-infected cells.

Photoaffinity labelling of the crude membrane from normal mouse erythrocytes and *P. yoelii*-infected erythrocytes with radiolabelled NBMPR demonstrated NBMPR binding polypeptides which migrated on SDS-PAGE as a single labelled peak in each preparation. The peak M_r values were similar for normal and parasite-infected cells and were of the order of 54000–56000 (Gati *et al.*, 1987). These values are similar to those reported for human erythrocytes (Young *et al.*, 1983). However, these experiments do not indicate whether the new or induced transporter had been photolabelled with the NBMPR and hence the polypeptide peak from the infected membranes may only reflect the original host transporters on the infected cell.

The fact that NBMPR may not be able to bind to the new or induced transporter has been further substantiated by experiments which demonstrate that NBMPR is permeable to the infected cell (Gero *et al.*, 1989). Both NBMPR and NBTGR have been found to exhibit antimalarial activity in their own right against cultures of a *P. falciparum* multidrug resistant strain. Their toxicity is only slight with ID₅₀ values 10-to 20-fold higher than tubercidin. However, the finding that NBMPR has antimalarial activity suggested that it may enter the infected cell. High performance liquid chromatography (HPLC) analysis of the NBMPR-treated infected cells suggested that NBMPR crossed the membrane of the infected cell and was catabolized to mercaptopurine ribonucleoside (analogous to the liberation of 6-mercaptopurine from azathiopurine), which would have the potential for interfering with a number of purine interconversions (Gero *et al.*, 1989). Internalization of transport inhibitors by trophozoite-infected cells has also been reported for other transport systems (Sherman, 1988).

Characterization of the induced transport component in the infected cells has been hampered by the presence of the normal erythrocyte transporter and also by the fact that, as yet, no inhibitors or compounds that bind tightly to the induced

transporter have been found. Gati *et al.* (1987) have suggested that the NBMPR-insensitive transport may be carrier mediated in *P. yoelii* infected cells. Unpublished results from our laboratory have suggested that adenosine transport in *P. falciparum*-infected cells was not saturable in the presence of NBMPR, at concentrations of adenosine up to at least 100 μM , and this compares with a K_m of about 10 μM for adenosine transport in uninfected cells.

Verapamil (10 μM), an inhibitor of the multidrug resistance channel in infected cells (Martin *et al.*, 1987), had no inhibitory effect on the transport of adenosine through the new or induced transporter in *P. falciparum*-infected cells (Gero, unpublished data), nor did diamide, a highly specific sulphhydryl (-SH) group oxidant (Gero *et al.*, 1991). The reaction of diamide with normal human erythrocytes has been shown to inhibit completely the normal transport mechanism of adenosine. The inhibition was found to be dependent on the concentration of diamide and also appeared to be effectively instantaneous upon exposure of the cells to diamide. Adenosine transport into *P. falciparum*-infected human erythrocytes was only partially inhibited by diamide treatment, suggesting that diamide did not affect the parasite-induced component of nucleoside transport (Gero *et al.*, 1991).

Preliminary experiments done in our laboratory to investigate the sodium dependence of the *P. falciparum*-induced nucleoside transporter using a sodium free buffer for transport studies showed no difference in its transport properties to normal erythrocytes. These results differ from those with mouse leukemia cells where nucleoside transport is decreased in the absence of sodium (Dagnino *et al.*, 1987).

NUCLEOSIDE TRANSPORT IN BABESIA-INFECTED ERYTHROCYTES

To circumvent the difficulty in working with two transporters on an infected cell membrane, nucleoside transport in *Babesia*-infected cells has been investigated (Gero, 1989). Many similarities exist between the two diseases malaria and babesiosis in both their clinical manifestations, their intraerythrocytic development in the mammalian host and their antigenic properties (James *et al.*, 1987; Wright *et al.*, 1988). Interestingly, however, normal bovine erythrocytes, unlike human red cells, do not have the ability to transport nucleosides and hence have no nucleoside transporters in their membranes (Young and Jarvis, 1983). Infection of these cells with *Babesia bovis* has been found to induce nucleoside permeation sites into the bovine host cell membrane (Gero, 1989). The transport rate of adenosine into the infected cell was shown to be three-fold higher than for normal human erythrocytes and malaria-infected cells and the induced transport rate of thymidine and uridine was equivalent to one-tenth of the adenosine rate (Matias *et al.*, 1990). The mammalian nucleoside transport inhibitors, NBMPR, NBTGR, dilazep and dipyridamole were found to inhibit the induced nucleoside transport mechanism in *Babesia*-infected erythrocytes, but at higher concentrations than those required to inhibit normal human erythrocyte transport. However, binding studies with

[³H]NBMPR indicated that no high-affinity NBMPR binding sites could be detected in either normal or *B. bovis*-infected bovine erythrocytes.

Preliminary data suggest that the permeation into *Babesia*-infected cells may occur via a mediated nucleoside pathway with a very high K_m for adenosine of 1.8 mM. In addition, thymidine, uridine and tubercidin were shown to be inhibitors of the adenosine transport (Gero, 1989; Matias *et al.*, 1990). Phloretin, which has been reported to inhibit transport through pores in *P. falciparum*-infected cells (Kutner *et al.*, 1987), and the adenosine analogue, 5'-*p*-fluorosulphonylbenzoyladenosine (5FSBA), were also shown to be inhibitory, while verapamil had no effect (Gero, 1989).

Under the same conditions in which diamide had no effect on the induced transport in *P. falciparum*-infected cells, the effect of diamide on the induced nucleoside permeation in *B. bovis*-infected bovine erythrocytes resulted in complete inhibition of adenosine transport (Gero *et al.*, 1991).

These results, in addition to those described above, suggest that the induced nucleoside permeation site(s) in *B. bovis*-infected erythrocytes differs in its characteristics from both the induced nucleoside transport sites in erythrocytes infected with the malarial parasites *P. falciparum* or *P. yoelii* and from normal human and mouse erythrocytes.

APPLICABILITY TO CHEMOTHERAPY

A prerequisite for a new antimalarial compound is that it should affect the parasite with minimal effect on the host. Although cytotoxic nucleosides are unlikely to be toxic to normal erythrocytes (due to their lack of nucleic acid synthesis), other nucleated host cells are likely to be affected. The altered transporter in *Plasmodium*-infected cell membranes does offer opportunities for chemotherapeutic attack. Two approaches are currently being investigated. Firstly, cytotoxic nucleosides which do not normally penetrate the host cell can be tailor-made to meet the selectivity properties of the new permeability pathway in the parasitized erythrocyte and, therefore, be targeted specifically into the parasitized erythrocyte. Secondly, a regime of simultaneous administration of two compounds would be feasible, in which the first, a toxic nucleoside, destroys the viability of the intraerythrocytic malarial parasite, while a second compound, a nucleoside transport inhibitor, protects normal host cells from the toxicity of the first compound by blocking its transport into them (Figure 36.3).

Studies of cytotoxic nucleosides in parasite cultures *in vitro*

Although many nucleoside analogues have been examined over the years as potential antiparasitic agents (Jaffe, 1975), few have been tested against *P. falciparum*. Tubercidin has been shown to have antimalarial activity against the intraerythrocytic stages of *P. knowlesi* (McCormick *et al.*, 1974) and cordycepin (3'-deoxyadenosine) is effective against *P. knowlesi* and *P. berghei* (Trigg *et al.*, 1971).

More recently, we have re-investigated the antimalarial activity of tubercidin (Scott *et al.*, 1987; Gero *et al.*, 1989) and a wide range of other purine nucleoside analogues, many of which have been shown to be at least as potent as tubercidin against *P. falciparum* in *in vitro* cultures. Many of these will be re-examined for their activity when administered together with a nucleoside transport inhibitor. Further results have indicated that the permeation of cytotoxic analogues in the presence of a nucleoside transport inhibitor *in vitro* is equally applicable to both normal and chloroquine-resistant strains of *P. falciparum* (Gero *et al.*, 1989). In addition, nucleoside transport inhibitors other than NBMPR such as NBGTR, dipyridamole and dilazep (the latter two used clinically as vasodilators) have been shown to be equally effective in protecting the host cell against transport of the toxic nucleoside. However, it appears that dipyridamole and dilazep are not able to interfere with purine metabolism in the infected cell in the same manner as NBMPR (Gero *et al.*, 1989).

Cytotoxic nucleosides in combination with nucleoside transport inhibitors *in vivo*

The feasibility of a two-drug regime has already been established in mice bearing transplanted neoplasms that have an altered nucleoside transport system. Thus the nucleoside transport inhibitor NBMPR-P (nitrobenzylthioinosine-5'-monophosphate, a prodrug form of NBMPR) was shown to protect mice from the otherwise lethal doses of certain cytotoxic nucleosides (tubercidin, nebularine or toyocamycin). When combinations of NBMPR-P and high doses of tubercidin or nebularine were used to treat mice bearing implants of neoplastic cells, selective toxicity toward the neoplastic cells was achieved, with some long-term survivors among the experimental animals (Lynch *et al.*, 1981; Kolassa *et al.*, 1982).

Similar results have been obtained by co-administration of tubercidin and NBMPR-P in mice infected with the rodent malaria *P. yoelii*. A lethal concentration of tubercidin was used with NBMPR-P. The drug combination administered over four consecutive days was shown to decrease the percentage of parasitemia and to increase the survival time of the treated animals (Gati *et al.*, 1987). It is promising that similar results have been obtained by co-administration of tubercidin and NBMPR-P in mice infected with either *Schistosoma mansoni* or *S. japonicum* (El Kouni *et al.*, 1983, 1985) and with mice infected with *Trypanosoma gambiense* (Ogbunde and Ikediobi, 1982a). In each case eradication of the parasites was obtained.

As NBMPR-sensitive nucleoside transport sites have also been demonstrated in the membranes of several extracellular parasitic protozoa, *Leishmania donovani* promastigotes (Aronow *et al.*, 1987), *T. gambiense* (Ogbunde and Ikediobi, 1982b), *T. cruzi* (Finley *et al.*, 1988) and *Trichomonas vaginalis* (Harris *et al.*, 1988), a two-drug regime as treatment of these parasitic diseases may also be feasible.

WHAT IS THIS ALTERED PERMEABILITY?

It is now apparent that the nucleoside transporters induced in the host cell membrane by the intraerythrocytic parasite are related to the development of the parasite in the host cell and appear to be most pronounced at the trophozoite stage. This is particularly obvious with the development of *B. bovis* in the bovine host cell, as no nucleoside transporters are present in the non-parasitized bovine red cell membrane. It also appears, in the case of the malaria-infected cell, that the original host nucleoside transporters are still operative in the infected cell.

Altered permeability in parasitized erythrocytes towards a number of other small molecules, such as glucose, amino acids, anions, cations and hexitols, has also been demonstrated (for review see Ginsburg and Stein, 1987a; Sherman, 1988). It has not yet been resolved whether there is more than one induced transporter involved in the altered permeation of all these compounds. The way in which these sites are induced has been open to several interpretations. Sherman (1988) has suggested several possibilities: they are caused by parasite-encoded proteins inserted into the host cell membrane: alteration of the membrane such that it becomes 'leaky' or non-specific pores are formed; or there is alteration of the lipid component.

Initially, Ginsburg *et al.* (1985) characterized a permeation pathway in *P. falciparum*-infected human red blood cells to small electrolytes and ions. It was suggested that membrane pores could be detected in the infected cell membrane with up to 16 pores per cell at the trophozoite stage. However, more recently, Ginsburg and Stein (1987a,b) have questioned whether the characteristics of these induced permeability sites fit the model for a pore and have suggested another model based on hydrocarbon partitioning of solutes across the membrane where a parasite protein was inserted with loose seals into the lipid bilayer of the host membrane.

It is well established that the malarial parasite alters the structure of the host cell erythrocyte including the insertion of polypeptides of parasitic origin into the host cell membrane and the alteration of the lipid components (for reviews see Howard, 1982; Sherman, 1985). The intraerythrocytic parasite is able to export malarial proteins beyond its plasma membrane, to become membrane bound to the host erythrocyte membrane either on the cytoplasmic face or the extracellular surface (Howard, 1988). Furthermore, parasite proteins can affect the cytoskeleton of the host erythrocyte (Wiser *et al.*, 1988, 1990). Consequently, protein insertion into the host cell membrane could represent a parasite-encoded transport protein, or a misadjustment between phospholipids and proteins which can cause leakiness in the membrane (Ginsburg and Stein, 1987a,b). Several recent studies have demonstrated that parasite development is also accompanied by alterations in the distribution of membrane phospholipids leading to the suggestion that such changes might be the basis of altered plasma membrane permeability (Taraschi *et al.*, 1986; Schwartz *et al.*, 1987; Joshi *et al.*, 1987). Maguire and Sherman (1990) have suggested that the alteration of lipid composition and cholesterol exchange in *P. falciparum*-infected cells may, in part, be responsible for the increased fluidity and altered permeability (see also Chapter 2).

One further suggestion has involved the parasite creating oxidative stress within the host cell which may increase permeability (Sherman, 1985). Aqueous membrane leaks can occur in erythrocytes by biochemical modifications such as treatment of cells with the oxidizing agent diamide (Deuticke *et al.*, 1983; Haest *et al.*, 1977). However, as discussed above, our work demonstrated that erythrocytes treated with diamide were incapable of nucleoside transport. Thus it seems likely that these leaks are essentially different from those created in the membrane by the intraerythrocytic parasite.

It is not yet evident how the observed changes in nucleoside permeability during the maturation of the intraerythrocytic parasite are caused and whether they are attributable to the insertion of parasite-specified components into the host membrane or to structural modification of the host cell membrane. This is a challenge for future research.

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37. Approaches to investigating Cation metabolism in malaria-infected erythrocytes

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INTRODUCTION

We are utilizing molecular technology to isolate a family of parasite-encoded genes for proteins likely to differ functionally and, by inference, structurally from host cells. Such proteins are likely to be attractive targets for chemotherapeutic attack. The majority of malarial genes characterized to date code for 'antigens' that have been isolated by screening expression libraries with hyperimmune sera in the pursuit of candidates for vaccines (Kemp *et al.*, 1986). None of these gene products has clearly established functions within the parasite. Of the 'housekeeping' genes that have been isolated from *Plasmodium falciparum* and sequenced, most share at least 50 per cent of their derived amino acid sequence with the equivalent mammalian protein (see Table 37.1) (Weber, 1988). The one parasite protein known to be a useful drug target, the product of the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene complex, has much less similarity in the susceptible moiety (DHFR, <30 per cent), which is the site of action of pyrimethamine and proguanil (Walter, 1986). Resistance to these drugs has emerged and elegant work comparing the derived amino acid sequences of the DHFR-TS gene complex between sensitive and resistant strains has demonstrated altered amino acid residues at the substrate binding site (Bzik *et al.*, 1987; Peterson *et al.*, 1988; Cowman *et al.*, 1988; Snewin *et al.*, 1989; Foote *et al.*, 1990; Peterson *et al.*, 1990).

We sought to study a novel family of parasite-encoded genes, the cation-motive ATPases, for the following reasons:

1. the data outlined below support the notion that the intraerythrocytic parasite is able to regulate its own ionic composition;
2. cation-motive ATPases are an essential and phylogenetically ubiquitous class of proteins central to ion homeostasis (see chapter 38 for a summary of the

Table 37.1. Comparison of derived amino acid sequences from parasite genes to those from other organisms.

Gene	Degree of amino acid conservation (%) (organisms compared) ^a	Repeats ^b	Reference
Calmodulin	89 (human)	No	Robson and Jennings (1991)
Tubulin α	83 (human)	No	Holloway <i>et al.</i> (1989)
Tubulin β	87 (human)	No	Delves <i>et al.</i> (1989), Sen and Godson (1990)
Pf Actin 1	81–82 α & β (vertebrate)	No	Wesseling <i>et al.</i> (1988)
Pf Actin 2	76 α & β (vertebrate)	No	
HSP-70	59 (human)	Yes (GGMP) ₄	Bianco <i>et al.</i> (1986)
	70 (<i>Drosophila</i>)	No	
Aldolase	> 60 (vertebrates chicken, rat, rabbit)	No	Knapp <i>et al.</i> (1990)
TS	56 (human)	No	Snewin <i>et al.</i> (1989)
RNA pol II	54 (in conserved regions, mouse)	Yes (YSPTPSP) ₁₇	Li <i>et al.</i> (1989)
Pf mdr ^c	54 (human)	No	Foote <i>et al.</i> (1989)
HGPRT	48 (mouse)	No	King and Melton (1987)
DHFR	27 (human)	No	Snewin <i>et al.</i> (1989)

^a The amino acid comparisons have been made by the authors of the papers cited, and do not include semiconservative changes.

^b G, Gly; M, Met; P, Pro; Y, Tyr, S, Ser, T, Thr. For other abbreviations, see text.

^c Pf mdr is the malarial homologue of the multidrug resistance gene.

^d Other RNA polymerase II enzymes (RNA pol II) also have repeated amino acid motifs which are important for function in some organisms (see Suzuki, 1990). The comparison involved conserved regions only, which were 53 per cent of amino acids.

- properties of the three major classes of cation-transporting ATPases). The physiological properties of malarial plasma-membrane ATPases (at least in the case of Ca^{2+} , Na^+/K^+ or K^+) are likely to differ from those of the host cell, because the ionic microenvironment of the parasite approximates to an intracellular not an extracellular one, and the two are very different. This is true in the early stages of infection, and may also be true later in the cell cycle. Thus we expect the structural properties of parasite-derived plasma-membrane ATPases to reflect these functional differences;
3. considerable divergence exists at the amino acid level between ATPases from different organisms, in those regions that lie outside conserved cytoplasmic domains of functional importance (see Table 37.2) (Green and MacLennan, 1989). Thus parasite encoded ATPases may be expected to differ from the host proteins for both phylogenetic and functional reasons;
 4. there is experience with isoform selective ATPase inhibitors in man (e.g. the cardiac selective Na^+/K^+ pump inhibitor, digitalis (Allen *et al.*, 1985), and the H^+ pump inhibitor, omeprazole (Sachs *et al.*, 1988)), suggesting that a similar approach to develop antimarial ATPase inhibitors is feasible.

We have chosen the erythrocytic phase of the life cycle of the parasite to study because very little is known of the biochemical changes induced by the liver stage of infection and the symptomatic phase of infection is associated with multiplication of parasites within erythrocytes. This stage can conveniently be studied *in vitro*, although results should be interpreted cautiously as conditions only approximate those *in vivo*. In the remainder of this chapter we outline the perturbation of cation homeostasis in erythrocytes by infection. Subsequently, some approaches to the isolation of functionally important proteins are discussed and illustrated by their application to the isolation of cation motive ATPases from the malarial parasite. More information is given in Chapter 38. A putative proton pump has also been isolated from another intracellular parasite, *Leishmania donovani*, and other members of the family are being characterized (Meade *et al.*, 1987, 1991), and there is biochemical evidence that *Trypanosoma rhodesiensi* possesses a plasma membrane Ca^{2+} -ATPase (McLaughlin, 1985). No plasma-membrane ATPase from *P. falciparum* have yet been isolated.

NORMAL ERYTHROCYTES

The normal erythrocyte has elaborate enabling mechanisms geared to the execution of relatively simple functions, such as the transport of oxygen and carbon dioxide. These include the Ca^{2+} -and Na^+/K^+ -ATPases and secondarily active transporters (those dependent on electrochemical gradients generated by cation-ATPases). It must therefore maintain its cation composition within close limits, and the means whereby this has been achieved have been studied in considerable detail, initially by physiological and biochemical studies and more recently at the molecular level (see Agre and Parker, 1989).

Table 37.2 Ion pumps of known sequence.^a

Source and type ^c	Reference	Ions transported			Identity within type (%)			Identity, cytoplasmic or TM between groups ^d (%)		
		Out	In ^b	No sequenced	Length	Mammals	Plants and lower eukaryotes	Bacteria		
<i>Mammals</i>										
PM	Shull and Greef, 1988; Verma <i>et al.</i> (1988)	Ca ²⁺ (1)	—	2	1220	88 (96)				
SR, ER	MacLennan <i>et al.</i> , (1985)	Ca ²⁺ (1)	—	3	1000	90 (97)				
PM	Shull <i>et al.</i> , 1986	Na ⁺ (3)	K ⁺ (2)	7	1016	91 (92)				
PM	Shull and Lingrel (1986)	H ⁺ (1)	K ⁺ (1)	1	1033					
<i>Lower eukaryotes and plants</i>										
<i>Leishmania donovani</i> ^e	Meade <i>et al.</i> (1987)	?	?	1	974					
<i>Saccharomyces</i>	Serrano (1988)	H ⁺	—	4	918	83 (87)				
<i>Neurospora crassa</i>	Hager <i>et al.</i> (1986)	H ⁺	—	1	920					
<i>Nicotiana</i> ^f		H ⁺	—	1	957					
<i>Bacteria</i>										
<i>Escherichia coli</i>	Hesse <i>et al.</i> (1984)	?	K ⁺	1	680					
<i>Staphylococcus aureus</i>	Silver <i>et al.</i> (1989)	Cd ²⁺	?	1	727					
<i>Rhizobium</i>	Kahn <i>et al.</i> (1989)	?	?	1	734					
<i>Streptococcus faecalis</i>	Solioz <i>et al.</i> (1987)	H ⁺	K ⁺	1	580					

^a Modified, with permission, from Green and MacLennan (1989).^b —, indicates counterions have not proved detectable; ?, indicates they have not been investigated.^c PM, plasma membrane; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum.^d The percentage identities were calculated for the conserved segments of the cytoplasmic regions or for the first six transmembrane (TM) segments. When comparisons were made between groups of ATPases the mean of pairwise comparisons was made.^e Although protozoal, the sequence belongs clearly to the fungal family.^f M. Boutry, B. Michelet and A. Goffeau, unpublished work.

CALCIUM METABOLISM IN INFECTED CELLS

A recent review has addressed some questions about calcium metabolism in the infected erythrocyte (Krishna and Squire-Pollard, 1990; see also Chapter 38). Intraparasitic calcium levels increase 10- to 20-fold over levels observed in uninfected cells as the parasite matures (Tanabe *et al.*, 1982). The mechanism of this increase, the sites of localization of calcium and the possible functions of calcium within parasites are all subject to speculation. Evidence from Wasserman *et al.* (1982) suggests that early in the cell cycle (<26h post-invasion), calcium in the extracellular medium is required for parasites to develop normally, although this may not be the case for the later stages. The process of invasion of red cells is impaired by the calcium chelator (ethylene glycol-bis- β -aminoethyl ether)-N, N'-tetraacetic acid; EGTA) in the culture medium sufficient to reduce free calcium levels in the medium to around 100 μ M. Some evidence in the rodent parasite *P. chabaudi* suggests that a H⁺-ATPase may play a part in calcium uptake, although alterations in the homeostatic mechanisms of the host cell, or the participation of other parasite derived ion pumps have not been analysed (Tanabe *et al.*, 1983).

SODIUM AND POTASSIUM METABOLISM IN INFECTED CELLS

Intracellular changes in the normal ratio of Na⁺/K⁺ from 1:12 to about 1:8 for the later stages of the cell cycle demonstrate that infected red cells gain Na⁺ and lose K⁺ ions (reviewed by Krishna and Ng, 1989). The mechanisms that may contribute to these changes have been studied, particularly with respect to the functioning of the host membrane Na⁺/K⁺-ATPase. Dunn (1969) and others concluded that there may be some inhibition of this pump. Regardless of the changes induced by this impairment of host pump function, it is clear that the parasite can tolerate both the depletion of intraerythrocytic K⁺ and loading with Na⁺ (Ginsburg *et al.*, 1986a; Tanabe *et al.*, 1986). This suggests that the location of parasite-derived pumps may be either within the parasitophorous vacuolar membrane, or in the parasite plasma membrane (or both). This suggestion is also consistent with the data for calcium metabolism. It is difficult to see how intraerythrocytic ion gradients could be maintained if the pumps were to be exported to and function within the red cell membrane alone.

PARASITE MEMBRANE POTENTIAL AND pH REGULATION

Evidence from the use of ionophores on parasites freed from the host cell membrane suggests that parasites may regulate their intracellular pH by a combination of a H⁺-ATPase and a K⁺/H⁺ exchanger (present perhaps at the parasite plasma membrane).

The parasite food vacuoles may, in addition, possess specialized proton pumps which maintain their low pH (reviewed in Krishna and Ng, 1989). The metabolism of two other cations has been studied in infected erythrocytes. Iron metabolism has been reviewed by Peto and Hershko (1989). Zinc transport has been investigated by Ginsburg *et al.*, (1986b).

APPROACHES TO THE ISOLATION OF CATION ATPASES FROM *P. FALCIPARUM*

The classical route to isolate a parasite-encoded protein would be to establish its presence in the infected erythrocyte by biochemical, physiological or functional studies. Subsequently, assays which define its characteristics can be used for enrichment by standard biochemical techniques. If sufficiently pure, the protein can be used to generate specific antibodies or partially sequenced to enable oligonucleotide probes to be synthesized (see below). The gene encoding the protein can then be identified by the application of molecular technology. This route is fraught with difficulties that afflict any protein biochemist working with small quantities of parasite material present in a proteinase rich milieu.

The advent of recombinant DNA technology has allowed the assignment of function to some genes as well as answering fundamental questions about their structure and expression. Optimizing the available strategies to work on the genome of *P. falciparum* should take into account the following considerations:

1. the stage specificity of expression of the gene product;
2. the expected size of the gene product and the corresponding mRNA;
3. the possibility of selection based on the biological activity of the gene product; and
4. the availability of amino acid sequences for similar gene products, in other organisms.

The stage specificity of expression is an important consideration for the cloning of genes under tight developmental regulation, such as the cyclin homologue (Murray and Kirschner, 1989). The expected size of the gene product may make it useful to enrich for this cDNA by size fractionation of the starting mRNA, but this presupposes a conservation of mRNA size which is not always applicable. Another disadvantage of this procedure is the requirement for large quantities of RNA also not always easy to obtain. Malarial genes do not always contain introns, and when present they are usually small (<0.5kB; Weber, 1988). Therefore, an alternative approach which we have utilized to identify a malarial cation ATPase like molecule is to screen a genomic library. Selection of sequences based on their biological activity has been used in other systems, for example, the cloning of interferon genes (Nagata *et al.*, 1980), the Na⁺/glucose antiporter of mammalian cells in *Xenopus oocytes* (Hediger *et al.*, 1987), and many surface glycoproteins from lymphocytes in Cos cells (Seed and Aruffo, 1987). Complementation of biological activity in

deficient cells (bacteria, yeast or mammalian systems) is an alternative method of selection which has been applied successfully to isolate a hitherto uncharacterized gene from the malarial parasite (see Kaslow and Hill, 1990; Hall *et al.*, 1990).

Heterologous probes have been used to identify 'house-keeping' genes from *P. falciparum* (e.g. for the hypoxanthine guanine phosphoribosyltransferase-HGPRT, and calmodulin genes, see Table 37.1), but the almost unique AT bias in the genome of the parasite (70 per cent for coding regions, 85 per cent for sequences flanking genes, the highest value for any eukaryote), means that conservation at the amino acid level is unlikely to be reflected in the DNA sequence of most probes. This was found to be the case for initial attempts to isolate an ATPase from *P. yoelii* (see Chapter 38). Minimizing the amount of flanking (non-coding) sequence in probes may improve the chances of accurately identifying homologues in the parasite.

A more general approach is to use oligonucleotide probes to screen gene libraries made from *P. falciparum*. Success using this approach will be determined by many factors, including what is already known about the gene and derived amino acid sequences of interest in other organisms. Comparison of sequences from all the organisms for which these have been determined should highlight the least variable regions, and these may in turn reflect functional constraints. The example we have chosen is based on the phosphorylation site of cation-ATPases (see Table 37.3 and compare with Figure 38.2 in Chapter 38) which is a functionally important and invariant feature of this group of ion pumps. Independently, Tanabe *et al.* (see Chapter 38) have employed a very similar approach to isolate sequences coding for a putative organellar-type ATPase from *P. yoelii*. The longer the conserved region, the more likely that a corresponding oligonucleotide will be both specific and tolerant of some mismatch. Clearly those amino acids that have the least number of codons (e.g. one for Met and Trp) are the least ambiguous at the nucleotide level.

The other consideration which has been discussed in detail elsewhere is the preference for an 'A' or a 'T' nucleotide in the third position of a codon in *P. falciparum* (Hyde and Sims, 1987; Saul and Battista, 1988; Hyde *et al.*, 1989). This means that for approximately 90 per cent of the time, where there is a choice of two codons for amino acids (Asp, Asn, Cys, Lys, Glu, Gln, His, Tyr and Phe), this bias prefers the use of the one with the higher 'A' or 'T' content. Other amino acids (Gly, Ala and Pro) have a G or a C in both the first and second positions of their codons, and their use may increase specificity. Arginine is a less useful amino acid on which to base oligonucleotide design (as although AGA may be a preferred codon and CGG is rarely used, there are a total of six possibilities). Serine is probably one of the least useful because the two most commonly used codons (AGT and TCA again out of a total six possibilities) share no nucleotides. Having designed an oligonucleotide probe, its potential usefulness can be checked on a Southern blot of parasite DNA to confirm the conditions of hybridization and washing that gives discrete bands. The probe can then be applied under similar conditions to screen malarial libraries. More recently, the polymerase chain reaction (PCR) has been successfully used to isolate partial sequences of a putative multidrug resistance gene from *P. falciparum* (Wilson *et al.*, 1989). The oligonucleotide primers used were

Table 37.3. Comparison of the sequence of an oligonucleotide probe used to screen a *malarial gene* library, with the sequence from which it was derived.

	*	Asp ^a	Lys	Thr	Gly	Thr	Leu	Thr
Amino acid sequence			GAC	AAA	ACT	GGA	ACT	CTG
Sheep nucleotide sequence			AAA	TTT	TGN ^c	CCT	TGN	ACC
Malarial oligonucleotide probe (anti-sense) ^b						AAT	TGN ^c	
Mismatch ^d			+				+	+

^a The aspartic acid forms a phosphorylated intermediate in the enzyme.

^b Anti-sense oligonucleotide probes can be useful in detecting RNA on Northern blots.

^c 'N' stands for all four nucleotides, incorporated into this degenerate probe in approximately equimolar ratios.

^d A comparison with the oligonucleotide used to isolate the ATPase from *P.yoelii* reveals that the degeneracy in the codons for the first and last threonines was useful because the *P.yoelii* probe was mismatched at these nucleotides with the coding strand.

designed to hybridize to nucleotide sequences corresponding to conserved amino acid motifs in homologues.

In this chapter we have put forward a case for studying the cation transporting ATPases of the malarial parasite. We have also discussed some general approaches that are available to isolate genes coding for proteins with relatively well defined functions from the malarial parasite, in the hope that they may prove useful targets for chemotherapy. The proof of this particular pudding will only come when novel drugs, tested on parasite-derived targets which have been identified by rational approaches *in vitro*, are finally applied in the field.

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38. Cation-transporting ATPases of *Plasmodium*

K.Tanabe

INTRODUCTION

Maintenance of specific intracellular concentrations of cations is of vital importance to living organisms. Cells maintain these equilibria via the actions of transport systems which operate in the face of ion gradients across cell membranes. Transmembrane gradients of cations such as Na^+ and H^+ created in this way drive the transport of nutrients and ions into cells (secondary transport).

The intracellular parasitism of malaria parasites, the genus *Plasmodium*, presents an intriguing problem with respect to cation transport because, in vertebrate hosts, the parasites spend much of their cell cycle inside erythrocytes. Concentrations of cations such as Na^+ , K^+ and Ca^{2+} differ greatly between the cytoplasm of the host erythrocyte and the extracellular fluids. Therefore, it seems likely that malaria parasites have unique mechanisms for transporting cations within the host erythrocytes. The possibly unique nature of malarial systems for the cation transport suggests that it may be worthwhile to investigate the structure and function of cation-transporting ATPases (cation pumps) of *Plasmodium*.

CATION-TRANSPORTING ATPASES

The cation-transporting ATPases found to date (Figure 38.1) can be categorized into three major groups: the P, V and F types. The P-type ATPases share common features: a molecular weight of 100 to 140 kDa, the formation of a covalently phosphorylated intermediate in the course of ATP hydrolysis and high sensitivity to vanadate (Pederson and Carafoli, 1987). The P-type ATPases include H^+ -transporting ATPases, Ca^{2+} -transporting ATPases, Na^+ , K^+ -transporting ATPases and H^+ , K^+ -transporting ATPases of the plasma membrane and Ca^{2+} -transporting ATPases of the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER). The

Cation - transporting ATPases

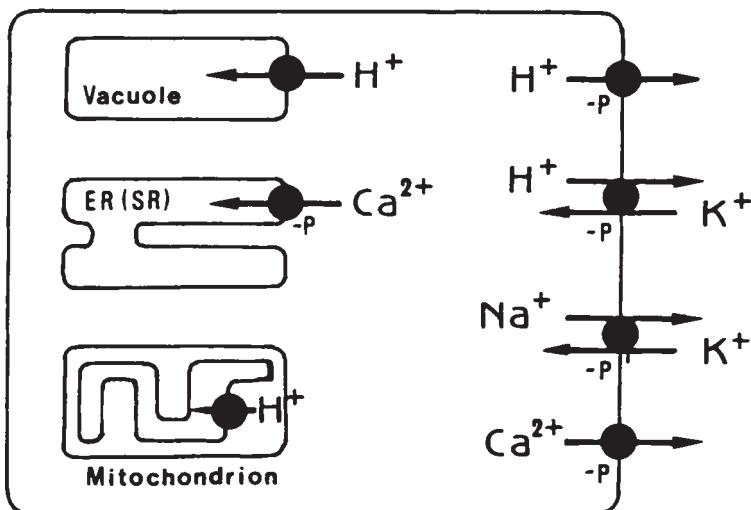


Figure 38.1. Localization of eukaryotic cation-transporting ATPases. Those ATPases marked with P are P-type cation-transporting ATPases. ER, endoplasmic reticulum; SR, sarcoplasmic reticulum.

K^+ -transporting ATPases of *Escherichia coli* and *Streptococcus faecalis* are also of the P-type.

The V-type ATPases are defined as those associated with membranes of organelles other than the mitochondria and the SR/ER and they are composed of several subunits with high sensitivity to nitrate and *N*-ethylmaleimide (MEM) (Nelson and Taiz, 1989). Proton-transporting ATPases of vacuoles, the Golgi vesicles, endosomes, coated vesicles and lysosomes are commonly found in eukaryotic cells. Archaeobacterial species also contain H^+ -ATPases which are structurally very similar to the V-type ATPases (Nelson and Taiz, 1989).

The F-type ATPases are the F_0F_1 -type H^+ -ATPases found in bacteria, chloroplasts and mitochondria and they are very sensitive to oligomycin and azide (Pederson and Carafoli, 1987; Nelson and Taiz, 1989). They consist of a water-soluble F_1 moiety required for the synthesis or hydrolysis of ATP and a membrane-spanning F_0 moiety for translocation of protons.

CATION-TRANSPORTING ATPASES OF *PLASMODIUM*

Little is known about the structure and function of cation-transporting ATPases of malaria parasites. However, limited data obtained from physiological studies suggest the presence of cation-transporting ATPases in *Plasmodium*. An Na^+ , K^+ -ATPase is probably present at the plasma membrane of *P. falciparum* since the

parasite maintains high levels of K^+ and low levels of Na^+ in spite of substantial changes in the levels of alkali cations within the host erythrocytes, i.e. the elevation of Na^+ levels and concomitant reduction in K^+ levels in the cytoplasm of *P. falciparum*-infected human erythrocytes (Lee *et al.*, 1988). Alternatively, an Na^+ , K^+ -ATPase may be present in the membrane of the parasitophorous vacuole (PV), which is originally derived from the host erythrocyte's membrane upon entry by the merozoite. Such a possibility is suggested by early histochemical electron-microscopic studies which showed the activity of Na^+ , K^+ -ATPase in the PV membrane of *P. lophurae*-infected duck erythrocytes but not in the parasite plasma membrane (Langreth, 1977). The Na^+ , K^+ -ATPase would pump Na^+ out (i.e. into the erythrocyte cytoplasm) and move K^+ in (i.e. into the PV) if its orientation were reversed with reference to the standard orientation in the erythrocyte membrane. In this case, the parasite plasma membrane is presumed to be permeable to Na^+ and K^+ so that, as a consequence, a high level of K^+ and a low level of Na^+ are maintained passively in the parasite cytoplasm.

The plasma membrane of intraerythrocytic *Plasmodium* has also been suggested to have an H^+ -ATPase that functions to extrude protons from the parasite (Mikkelsen *et al.*, 1982; Izumo *et al.*, 1988). The H^+ -ATPase is sensitive to both vanadate and dicyclohexylcarbodiimide (DCCD), inhibitors of the P-type of cationtransporting ATPases (Mikkelsen *et al.*, 1986). A gradient of protons across the plasma membrane, generated by the operation of the H^+ -ATPase, accompanied with a potential difference, seems to provide energy for the uptake of Ca^{2+} and glucose by *Plasmodium* (Tanabe *et al.*, 1982; Tanabe, 1990).

Calcium ions accumulate to a greater extent in malaria-infected erythrocytes than in uninfected erythrocytes (Tanabe *et al.*, 1982). The accumulated Ca^{2+} is exclusively localized in the parasite compartment but not in the cytoplasm of the host erythrocyte. Since it is unlikely that levels of Ca^{2+} elevate evenly intracellularly, Ca^{2+} is assumed to be sequestered into Ca^{2+} pools such as the ER or calciosomes, although the latter has not been identified to date in *Plasmodium*. A gene that encodes an organeller Ca^{2+} -ATPase from *P. yoelii* has recently been cloned (see below).

Like many eukaryotic cells, malaria parasites contain mitochondria. Failures to demonstrate the presence of enzymes of the TCA cycle and of a mitochondrial electron transport system, as well as acristate morphology of the parasite mitochondria, have long been considered to indicate that the parasite mitochondria are non-functional (see Sherman, 1979). However it has been demonstrated that plasmodial mitochondria do maintain a high internal negative membrane potential (Divo *et al.*, 1985; Izumo *et al.*, 1988); and synthesize ATP (Kanaai and Ginsburg, 1989). Thus, it seems that an H^+ -ATPase of the parasite mitochondrion is operative in the synthesis of ATP via translocation of protons across the inner membrane (see also Chapter 13).

The presence of the V-type ATPase has been suggested in the digestive vacuole membranes of *P. falciparum* (Choi and Mego, 1988). The ATPase is sensitive to NEM but not to vanadate, ouabain or oligomycin. Interestingly, the antimalarial drugs quinine and quinacrine, inhibited considerably the ATPase activity (Choi and Mego, 1988).

CLONING OF MALARIAL CATION-TRANSPORTING ATPASES

Recent progress in the elucidation of the structure and function of cationtransporting ATPases in eukaryotic cells has been brought by application of molecular cloning techniques. The conventional approach to the identification of malarial genes for ATPases is to use a DNA probe derived from another organism that encodes a cation-transporting ATPase. However, experiments using both the H⁺-ATPase from the yeast plasma membrane and the Ca²⁺-ATPase of the SR of rabbit skeletal muscle have been unsuccessful (unpublished data), probably as a result of insufficient hybridization between probes derived from genes for heterologous ATPases and malarial DNA which has an extremely high A+T content (Weber, 1987).

An alternative approach to the cloning of malarial genes for cation-transporting ATPases is to design oligonucleotides derived from amino acid sequences that are conserved among the ATPases from a variety of organisms. Recent sequencing studies revealed that the P-type cation-transporting ATPases, including Ca²⁺-ATPases from the SR of rabbit skeletal muscle and from human teratoma plasma membrane, fungal plasma membrane H⁺-ATPases, rat stomach H⁺, K⁺-ATPase, sheep kidney Na⁺, K⁺-ATPase and cation-transporting ATPase from *Leishmania donovani*, contain highly conserved amino acid sequences for phosphorylation region, ATP binding region and regions with unidentified functions (Serrano, 1988). The most highly conserved sequence is a stretch of seven amino acids, Asp-Lys-Thr-Gly-Thr-Leu-Thr, the Asp residue being the site of phosphorylation. Therefore, a 21-mer oligonucleotide corresponding to this amino acid sequence was designed by taking account of malarial codon usage with a high frequency of A+T, of which A is more frequent than T in coding regions (Figure 38.2), and this probe was used in screening experiments.

Using this probe, we identified a clone that encompasses the entire gene for a cation-transporting ATPase from *P. yoelii*, which is expressed at the asexual stage (Murakami *et al.*, 1990). The parasite ATPase showed a high degree of conservation of amino acid sequences for the phosphorylation region, the fluorescein isothiocyanate (FITC) binding region and the 5' p-fluorosulfonylbenzoyladenosine (FSBA) binding region (Figure 38.3). The binding regions form the intrinsic ATP-binding site of the P-type ATPases. Some residues (Ile-355, Asp-358, Lys-359, Leu-363 and Lys-613 in the *P. yoelii* protein) in these regions have recently been demonstrated to be necessary for the formation of the phosphorylated intermediate of the SR Ca²⁺-ATPase of rabbit skeletal muscle by substitution of these residues by non-conservative amino acids (Maruyama and MacLennan, 1988; Maruyama *et al.*, 1989). These residues are perfectly conserved in the P-type ATPases sequenced to date and in the ATPase from *P. yoelii*.

Among the P-type ATPases, the *P. yoelii* ATPase exhibits the highest overall homology in terms of amino acid sequence to the SR Ca²⁺-ATPase of rabbit muscle (42.3 per cent) and it differs to a greater extent from other P-type ATPases, including a plasma membrane Ca²⁺-ATPase, the degree of homology ranging between 12.9 and 23.9 per cent (Murakami *et al.*, 1990). The hydropathy profile of the *P. yoelii*

Asp Lys Thr Gly Thr Leu Thr

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GAT AAA ACT GGT ACT TTA ACT
GAC AAG ACC GGC ACC TTG ACC
ACA GGA ACA CTT ACA
ACG GGG ACG CTC AGG
CTA
CTG

```

Figure 38.2. Design of an oligonucleotide probe that corresponds to a sequence for the phosphorylation region of P-type cation-transporting ATPases. Codons used are underlined.

	Phosphory- lation	FITC	FSBA
	355	614	795
<u>P. yoelii</u> ATPase	ICSDKTGTLT	KGAPE	VAMTGDGVNDAPALKSAD
SR Ca ²⁺ -ATPase	ICSDKTGTLT	KGAPE	TAMTGDGVNDAPALKKAE
PM Ca ²⁺ -ATPase	ICSDKTGTLT	KGASE	VAVTGDGTNDGPALKKAD
SC H ⁺ -ATPase	LCSDKTGTLT	KGAPL	VAMTGDGVNDAPSLKKAD
RS H ⁺ ,K ⁺ -ATPase	ICSDKTGTLT	KGAPE	VAVTGDGVNDSPALKKAD
SK Na ⁺ ,K ⁺ -ATPase	ICSDKTGTLT	KGAPE	VAVTGDGVNDSPALKKAD
<u>Leishmania</u> ATPase	LCSDKTGTLT	KGAPH	CAMTGDGVNDAPALKRAD

Figure 38.3. Conservation of amino acid sequences for the phosphorylation region, FITC-binding region and FSBA-binding region among the cation-transporting ATPase from *P. yoelii* and the P-type ATPases from a variety of organisms: rabbit skeletal muscle sarcoplasmic reticulum (SR) Ca²⁺-ATPase (MacLennan *et al.*, 1985), human teratoma plasma membrane (PM) Ca²⁺-ATPase (Verma *et al.*, 1988), *S. cerevisiae* (SC) H⁺-ATPase (Serrano *et al.*, 1986), rat stomach (RS) H⁺,K⁺-ATPase (Shull and Lingrel, 1986), sheep kidney (SK) Na⁺,K⁺-ATPase (Shull *et al.*, 1985) and *L. donovani* cationtransporting ATPase (Meade *et al.*, 1987). Positions of residues are indicated for the sequence of the polypeptide from *P. yoelii* (Murakami *et al.*, 1990).

ATPase is very similar to that of the SR Ca²⁺-ATPase. Furthermore, the *P. yoelii* protein contains all six residues (Glu, Glu, Asn, Thr, Asp and Glu) in the transmembrane sequences M4, M5, M6 and M8 (Figure 38.4) that have recently been shown to be the high-affinity Ca²⁺-binding site of the SR Ca²⁺-ATPase (Clarke *et al.*, 1989a). By contrast, the *P. yoelii* ATPase does not conserve the six glutamate residues in the sequences that correspond to the five stalk sectors of the SR Ca²⁺-ATPase (Figure 38.4) which were previously considered to be the sites for binding of Ca²⁺(MacLennan *et al.*, 1985). However, a recent mutagenesis study has

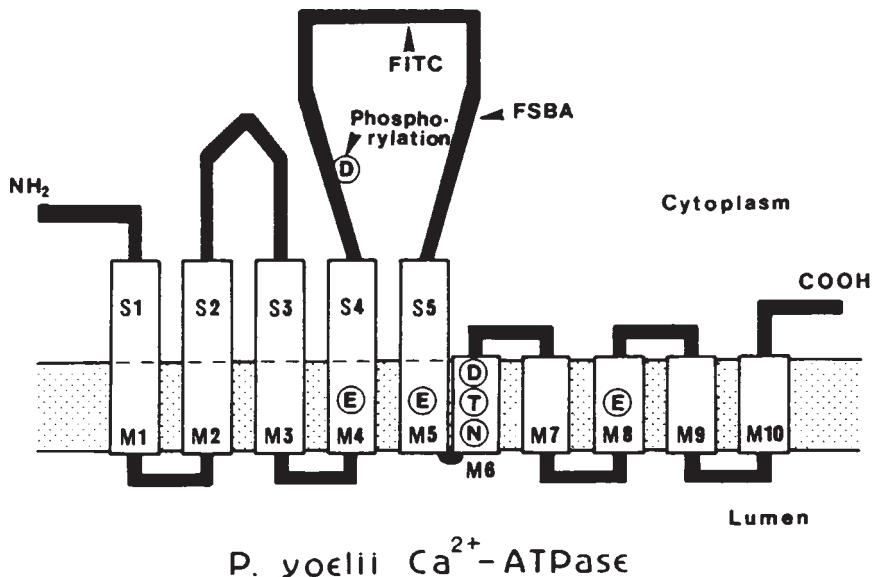


Figure 38.4. Topography of the Ca^{2+} -ATPase from *P. yoelii*, as inferred from its structural similarity to the Ca^{2+} -ATPase of the sarcoplasmic reticulum of rabbit muscle (MacLennan *et al.*, 1985). Stalk sectors and transmembrane domains are indicated by S1 to S5 and M1 to M10, respectively. Residues within circles in M4, M5, M6 and M8 form the high-affinity Ca^{2+} -binding site. E, glu; D, asp; T, thr; N, asn. The site of phosphorylation is indicated by D within a circle above S4.

demonstrated that these residues do not affect either the transport of Ca^{2+} or the formation of the phosphorylated intermediate (Clarke *et al.*, 1989b). The *P. yoelii* protein also does not contain sequences for the ouabain binding site of Na^+ , K^+ -ATPases or the calmodulin binding site of plasma membrane Ca^{2+} -ATPases. From these observations, it appears that the *P. yoelii* protein is an organellar type of Ca^{2+} -ATPase.

FUNCTION OF THE Ca^{2+} -ATPASE in *P. YOELII*

Eukaryotic cells control the cytoplasmic concentrations of Ca^{2+} via operation of a Ca^{2+} -ATPase located either at the plasma membrane or at membranes of organelles such as the ER and calciosomes. Cytoplasmic levels of Ca^{2+} are mainly controlled by release of Ca^{2+} from and sequestration into the SR in skeletal muscle cells, whereas they are maintained to a greater extent by the flux of Ca^{2+} across the plasma membrane in non-muscle cells. In the case of malaria parasites, it seems unlikely that a Ca^{2+} -ATPase is present at the plasma membrane because the parasite resides in an environment in which concentrations of Ca^{2+} are extremely low, i.e. cytoplasm of the host erythrocyte. Cytoplasmic Ca^{2+} is also accumulated in mitochondria by a transport process that is dependent on a high potential difference (internal negative)

across the inner membrane of the organelle (Carafoli, 1987). However, since uptake of Ca^{2+} by intraerythrocytic *P. chabaudi* is only slightly reduced by mitochondrial inhibitors (Tanabe *et al.*, 1982), the mitochondrion of *Plasmodium* does not seem to be involved in the regulation of cytoplasmic levels of Ca^{2+} in the parasite. Therefore, the Ca^{2+} -ATPase of *P. yoelii* appears to participate actively in the fine-tuning of the concentrations of Ca^{2+} in the parasite cytoplasm, thereby controlling Ca^{2+} -dependent metabolic processes. It remains to be determined whether or not the *P. yoelii* Ca^{2+} -ATPase is associated with the ER or with calciosomes.

Cytoplasmic concentrations of Ca^{2+} and cellular levels of ATP are variables that affect the activity of Ca^{2+} -ATPases. In addition, the enzyme activity is also regulated by binding proteins. Thus, calmodulin activates a plasma membrane Ca^{2+} -ATPase by binding to the enzyme (Carafoli, 1987). Phospholamban (PLN) binds to the SR Ca^{2+} -ATPase of rabbit skeletal muscle to regulate the enzyme activity (Tada and Katz, 1982). PLN has recently been shown to bind to a sequence shortly after the phosphorylation site of the SR Ca^{2+} -ATPase (James *et al.*, 1989). It seems noteworthy that the sequence in this region varies greatly among isoforms of organellar Ca^{2+} -ATPases (Burk *et al.*, 1989). The *P. yoelii* Ca^{2+} -ATPase does not contain a PLN-binding sequence. Instead, the parasite ATPase shows sequences rich in charged amino acids (Murakami *et al.*, 1990), as observed in the case of the calmodulin binding site of a plasma membrane Ca^{2+} -ATPase. This observation suggests that the parasite may have its own regulatory protein specific for its Ca^{2+} -ATPase. Since *P. yoelii* does not possess isoforms of the Ca^{2+} -ATPase (unpublished data), such a regulatory protein, if present and specific to the parasite, could be a future target for new antimalarial drugs.

SUMMARY

Cells maintain specific intracellular concentrations of cations via the actions of cation-transporting ATPases which operate in the face of an ion gradient across cell membranes. Studies of cation transport in malaria-infected erythrocytes suggest the presence and, possibly, the unique nature of transport systems for cations in *Plasmodium*. Investigations of the structure of cation-transporting ATPases of the parasite by molecular cloning have identified a gene for a P-type ATPase in *P. yoelii*. Comparisons with other P-type ATPases from a variety of organisms indicate that the parasite protein is an organellar type of Ca^{2+} -ATPase.

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39. Is the multidrug resistant protein of the malaria parasite involved in drug resistance?

H.Ginsburg

INTRODUCTION

Quinine and its congener, chloroquine, have been used as antimalarial drugs for many years, yet their definitive mode of action is not completely understood (Ginsburg and Geary, 1987). The quest for the molecular mode of antimalarial action has followed the natural dialectic of scientific research, yielding periodically novel putative mechanisms that have subsequently been rejected after experimental testing. At the present time it seems that the lysosomotropic hypothesis is reigning (Krogstad and Schlesinger, 1987; Schlesinger *et al.*, 1988). Since this hypothesis must also be compatible with drug resistance and its reversal by various agents, I first discuss its merits, then deal with its compatibility with the proposed active drug efflux pump acting in drug resistant parasites. Finally, I discuss the latter in detail to evaluate whether the currently accepted mode of this pump in cancer cells can be extrapolated to drug resistance and its reversal in malaria parasites.

THE LYSOSOMOTROPIC HYPOTHESIS OF DRUG ACTION

Lysosomotropic compounds are weak bases whose unprotonated form can translocate by free diffusion across biological membranes while their protonated form(s) cannot (De Duve *et al.*, 1974). Thus, the free base diffuses down its concentration gradient into the various compartments of the cell. In each of those compartments the free base becomes protonated to an extent that is determined by the compartment's pH and the pK_a of the base according to the Henderson-Hasselbalch equation. Hence, the pH gradient between the extracellular medium and any cellular compartment is the driving force for the accumulation of a lysosomotropic compound in this compartment. Steady-state accumulation is reached when the free base reaches an equal concentration in all the compartments.

The total concentration of the compound in each compartment is the algebraic sum of the free and the protonated base and will obviously be higher in correlation with the acidity of the compartment.

The lysosomotropic nature of quinoline containing antimalarial drugs (QCDs) such as chloroquine, quinine and mefloquine inspired the hypothesis of their lysosomotropic mode of action (Homewood *et al.*, 1972). Indeed, chloroquine accumulation has been observed in the acid food vacuole of intraerythrocytic malaria parasites, the stage of the parasite's life cycle that is most susceptible to the drugs (Aikawa, 1972; Yayon *et al.*, 1984a). That the pH gradient between the extracellular medium and the acidic compartment(s) is the driving force for drug accumulation has received wide experimental support in recent years (Yayon *et al.*, 1984a, 1985; Krogstad *et al.*, 1985). Since drug resistant parasites accumulate less drug, it was further suggested that the pH of the accumulating compartment is more acidic in the chloroquine susceptible parasites than in their chloroquine resistant counterparts (Geary *et al.*, 1986).

The lysosomotropic hypothesis of the mode of action of antimalarial drugs implies that drug accumulation would increase the pH of the acidic compartment (in itself maintained in the acid range by a proton pump), thereby inhibiting resident acidic hydrolases of lysosomal origin. Such enzymes are implicated in the process of digestion of host cytosol by the parasite that takes place in the acid food vacuole. Alkalization of this organelle could be achieved either by titration of the protons by the incoming free base or by using protonated drug to shuttle them out of the acid compartment. In the first case the rise in vacuolar pH should depend only on the buffer capacity of the food vacuole and the pK_a of the weak base. In the second case, one should observe an apparent decrease in the buffer capacity correlated with increased ability of the protonated drug to translocate across the vacuolar membrane. To clarify this point, one should realize that the buffer capacity is the algebraic sum of three elements:

1. the vacuolar sap's inherent capacity;
2. the vacuolar proton pump, which acts to increase the buffer capacity; and
3. the proton leak, which acts to reduce the buffer capacity.

Since QCDs are able to translocate protons across cell membranes (Nissani and Ginsburg, 1989), they could, in principle, alkalinize the parasite's food vacuole by both titration and proton shuttling.

IS THE LYSOSOMOTROPIC HYPOTHESIS VALID?

In an attempt to substantiate the lysosomotropic hypothesis, it has been recently demonstrated that QCDs do alkalinize the parasite food vacuole at concentrations significantly lower than those needed to alkalinize the lysosomes of mammalian cells (Krogstad *et al.*, 1985). It was therefore concluded that the parasite's greater susceptibility to QCDs is due to their lower buffer capacity. However, these

experiments were performed on parasites freed from their host cells by digitonin treatment, a procedure which probably permeabilizes all membranes (Sherman, 1979). Indeed, the parasites in these experiments were described as having an abnormal morphology and a swollen food vacuole, casting serious doubts as to the validity of this experimental system and the results obtained with it. Moreover, there were definite differences between drug concentrations that affected the vacuolar pH and that arrested parasite growth, contrary to the authors' claims. Although in itself this discrepancy casts serious doubts on the lysosomotropic hypothesis, there are several other arguments against it and I shall review them briefly (see also Ginsburg 1990).

- (a) The hypothesis fails to explain the lack of correlation between the pK_a values of different weak bases and their effect on parasite growth. Thus for example, mefloquine has an IC_{50} in the 10^{-9} M range, quinine in the 10^{-6} M range, NH_4^+ in the 10^{-3} M range and methylamine in the 10^{-4} M range, in inhibiting parasite growth, while all four compounds might be expected to accumulate to the same level in acidic organelles (Schuldiner *et al.*, 1972) and cause similar alkalinization by titration—since they all have similar pK_a values. The greater antimalarial efficacy of quinine and mefloquine could be reconciled if their protonated forms were able to translocate across the vacuolar membrane.
- (b) The hypothesis is also inconsistent with the lack of drug cross-resistance (Geary and Jensen, 1983; Oduola *et al.*, 1988). If resistance is a result of reduced drug accumulation capacity, it should be equally operative for all QCDs. It seems altogether that the notion of multiple drug resistance as it is known in cancer cells has no experimental basis in malaria infection.
- (c) Using intact infected cells and a non-invasive method for measuring changes in the vacuolar pH, we have recently demonstrated that the pH of the food vacuole is not altered by therapeutic levels of chloroquine, mefloquine and quinine (Ginsburg *et al.*, 1989). At drug concentrations of 1.5–2 orders of magnitude higher (suprapharmacological), the vacuolar pH did increase, with mefloquine being the most effective, followed by quinine, chloroquine and NH_4^+ , i.e. at variance with their antimalarial efficiency. Hence, alkalinization of the food vacuole probably has nothing to do with the antimalarial action of the drug.
- (d) Chloroquine accumulation in different parasite isolates decreases with their increasing resistance to the drug (Geary *et al.*, 1986). The vacuolar pH of the drug-resistant strains was calculated to be accordingly higher than that of the sensitive strains, reiterating the role of transvacuolar pH gradient in drug accumulation. We have recently devised a model based exclusively on the weak-base properties of chloroquine and its ΔpH -driven accumulation in acid parasite compartments (Geary *et al.*, 1990). The model assumes that the drug translocates across membranes only in its free base form, and that its accumulation does not affect the pH of the accumulating compartment. Using chloroquine sensitive (FCC₁) and chloroquine resistant (FCR₃ and VNS) isolates of *Plasmodium falciparum*, we showed that the experimental results

were in full agreement with the predictions of the model. This implies that therapeutic concentrations of chloroquine do not raise the pH of the food vacuole and that there is no need to invoke an active chloroquine efflux pump to explain drug resistance (see below). Calculations based on the model and the experimental data demonstrated that resistance to chloroquine is correlated with higher pH and/or higher resistance of the intracellular target to the drug concentration in the parasite food vacuole. Hence, lysosomotropism can explain the mode of drug accumulation but reveals nothing about the identity of the drug's target.

- (e) If, in spite of all the reservations mentioned above, QCDs indeed act by raising the vacuolar pH, one would expect to find an identical vacuolar pH in all isolates when they are exposed to chloroquine concentrations causing 50 per cent inhibition of parasite growth. Calculations of the vacuolar pH from chloroquine accumulation, showed that this was not the case (Geary *et al.*, 1986). Alternatively, one could have argued that the pH-dependence of the vacuolar hydrolases of the various isolates is different. This proposition has been refuted experimentally (Vander Jagt *et al.*, 1987).
- (f) The theory also fails to account for the fact that the inhibitory effect of chloroquine is irreversible after a relatively short exposure, while the effect of other lysosomotropic agents such as ammonia and methylamine is reversible (Yayon *et al.*, 1983, 1985; Divo and Geary, personal communication; Ginsburg and Krugliak, unpublished results). Preliminary results obtained in our laboratory show that not all accumulated chloroquine can be washed away from infected cells, suggesting that the irreversible effect could be due to tight binding of chloroquine to putative intracellular targets.

Although QCDs do not raise the vacuolar pH at pharmacologically relevant concentrations, they demonstrably inhibit the digestion of host cell cytosol at these concentrations (Zarchin *et al.*, 1986): Zhang (1987) has corroborated these findings by showing that chloroquine and NH_4Cl treated parasites retain higher levels of undigested haemoglobin. Ultrastructural studies showed that in chloroquine treated *P. falciparum*, undigested endocytic vesicles containing host cell cytosol, accumulate in the food vacuole (Yayon *et al.*, 1984b). Mefloquine and quinine also inhibit host cell digestion, but undigested vesicles cannot be seen in the food vacuole, suggesting that these drugs act in a different mode than chloroquine. The same is true for lysosomotropic detergents (Cabantchik *et al.*, 1989). Nevertheless, the mere inhibition of the feeding process is insufficient to explain drug action:

1. Inhibition of feeding with leupeptin merely arrests parasite development at the trophozoite stage and, upon removal of the inhibitor, growth is resumed (Rosenthal *et al.*, 1988); and
2. temporary alkalinization of the food vacuole with NH_4Cl (Yayon *et al.*, 1985) and lysosomotropic detergents (Cabantchik *et al.*, 1989), which result in inhibition of digestion, does not inhibit parasite growth.

These results stand in contrast to the irreversible effect of chloroquine mentioned above, and call for the elucidation of the specific effect of QCDs and the identification of their target(s).

REVERSERS OF CHLOROQUINE RESISTANCE

Recently, several compounds have been shown to reverse chloroquine resistance in *in vitro* cultures of *P. falciparum*. Thus, in the presence of verapamil (Martin *et al.*, 1987), methoxyverapamil, RO11-2933/001 (a tipamil analogue), chloropromazine and its analogue SKF 21133-A, diltiazem (Kyle *et al.*, 1990), imipramine and several of its analogues (Bitonti *et al.*, 1987; and see Chapter 47) and cycloheptadine (Peters *et al.*, 1989), chloroquine resistant *P. falciparum* becomes sensitive to chloroquine. Subsequently, it was shown that chloroquine sensitive parasites release the preaccumulated drug with a half-life substantially longer than do chloroquine resistant strains (Krogstad *et al.*, 1987). These authors concluded that the failure of the resistant parasites to accumulate the drug may result from enhanced (active) efflux. They have also shown that verapamil, diltiazem, vinblastine and daunomycin increase the ability of chloroquine resistant parasites to accumulate chloroquine and related this observation to the increased half-life of chloroquine efflux in presence of these compounds. The same drugs are known to reverse drug resistance in multidrug resistant (MDR) cancer cells, by increasing cellular drug levels to those found in their drug-sensitive progenitors (Slater *et al.*, 1982; Rogan *et al.*, 1984; Fojo *et al.*, 1985). It was therefore suggested that reversers of chloroquine resistance in malaria parasites may inhibit an active drug efflux pump, as has been suggested for their effect on the release of anticancer drugs from cancer cells with the multidrug resistance phenotype.

While the effect of these compounds on the antimalarial effect of chloroquine seems well founded, the transport experiments seem rather dubious. The reason for the shorter half-life of chloroquine efflux from a resistant strain of *P. falciparum* can be easily explained without invoking active extrusion (Ginsburg, 1988): diffusive efflux of the drug clearly depends on the concentration of the permeating species, i.e. the unprotonated form. When malaria-infected red blood cells are equilibrated with a given concentration of chloroquine, the unprotonated form reaches equal concentrations in all compartments, but the total drug concentration in the accumulating compartment of the chloroquine sensitive parasites is substantially higher than in the chloroquine resistant strain, owing to the higher vacuolar pH of the latter. Thus, although the vacuolar concentration of unprotonated drug and hence the rate of efflux, is equal in both strains, the half-life of drug efflux should correlate directly to the total vacuolar concentration, exactly as was found by Krogstad *et al.* (1987). It seems therefore, that these authors confounded half-life and instantaneous rate of efflux. Consequently, the whole experimental foundation which underlies the supposition for an active drug efflux pump seems specious.

AMPLIFICATION AND TRANSCRIPTION OF THE PARASITE'S MULTIDRUG RESISTANT GENE

The active efflux pumping of drugs in cancer cells has been assigned to a cell membrane protein, the 170kDa or P-glycoprotein. This designation relies on a correlation found between the expression of the protein and drug resistance, as well as on the protein's ability to bind drugs and the competitive displacement of the drug by compounds that reduce drug efflux. The quest for the P-glycoprotein in malarial parasites has been launched only recently, after it was found that verapamil, which reverses drug resistance in cancer cells, also reverses chloroquine resistance in malaria parasites (Martin *et al.*, 1987). It was soon found that *P.falciparum* contains at least two genes that have been shown to be related to the mammalian MDR gene (Wilson *et al.*, 1989). One of these genes is present in higher copy number and is transcribed to higher levels in a parasite strain that is more resistant to mefloquine (but more sensitive to chloroquine). In another study (Foote *et al.*, 1989), several isolates and clones of *P.falciparum* displaying various levels of chloroquine resistance were investigated. An MDR gene (*pfmdr*) showing high homology to both the human and the murine MDR genes was cloned and sequenced. In all the chloroquine sensitive strains the *pfmdr* gene was present at low copy number. In two out of the five chloroquine strains it was present at high copy number, but not in the other three, including the most extensively studied multidrug resistant strain K1, where the gene was absent altogether. It was argued, however, that the MDR genes of these strains may have been mutated and hence been undetectable by the probes used. Here too the RNA levels were higher in those strains displaying amplification of *pfmdr*. Most disturbing is the fact that increased transcription was observed only at the schizont stage which is relatively insensitive to chloroquine, while none could be seen at the trophozoite stage, the stage most sensitive to chloroquine (Geary *et al.*, 1989).

Two more recent reports confuse this issue even further. Wellem *et al.* (1990) performed a genetic cross between chloroquine sensitive and chloroquine resistant clones of *P.falciparum* and inspected the progenies for drug resistant markers and for the chloroquine resistant and chloroquine sensitive phenotypes. They did not find a relationship between the inheritance and amplification of either *pfmdr1* or *pfmdr2* genes and the chloroquine resistant phenotype, and concluded that *pfmdr* is not linked to chloroquine resistance. Foote *et al.* (1990) cloned the *pfmdr1* gene from chloroquine sensitive and chloroquine resistant isolates. While the gene had an identical sequence in two chloroquine sensitive isolates, that of five chloroquine resistant isolates had 1–4 nucleotide differences, resulting in amino acid substitutions. Hence, an apparent relationship between mutated *pfmdr1* and chloroquine resistance seems to have been established. However, some of the chloroquine sensitive genes had the same mutated bases as found in some of the chloroquine resistant genes. To reconcile this contradiction and to account for the fact that chloroquine resistance arises much less frequently than would be predicted by a single-point mutation, Foote and his colleagues concluded that a mutated *pfmdr1* gene is only one of at least two mutated genes required for chloroquine

resistance. The joint conclusion that can be reached from both studies is that *pfdm1r* mutations are insufficient (and possibly unimportant) in conferring the chloroquine resistant phenotype. Most importantly, the phenotypic typing of chloroquine resistance performed by Foote *et al.*, relied on IC₅₀ values determined in *in vitro* cultures. In many cases these values differed only by a factor of 2–3, a difference that can be easily result from inappropriate control of pH or inoculum size in tests (Geary *et al.*, 1990). A variation of only 0.15pH units in the growth medium and/or a factor 2 in the inoculum size, could have resulted in the observed differences in the IC₅₀ values.

In none of the above reports was the level of expression of the MDR glycoprotein assayed, nor was any attempt made to localize it subcellularly. It is rather surprising that, given the high homology found between the human and the malaria MDR genes, antibodies raised against the mammalian P-glycoprotein failed to react with parasite membranes (Kyle, personal communication). However, using rabbit antiserum raised against a fusion protein constructed with a DNA fragment of from the *pfdm1r* gene, Serrano *et al.* (1989) were able to demonstrate over-expression of a 150 kD antigen in a mefloquine resistant strain and to localize it to the parasite membrane. Furthermore, using [³H] azidopine as a label for the MDR protein, it was shown that a doublet of 155–170kDa proteins were labelled in a chloroquine resistant but not in a chloroquine sensitive strain of *P. falciparum* (Ye *et al.*, 1989). Verapamil, chloropromazine, nicardipine, reserpine and trifluoroperazine competitively reduced labelling, but only the two first compounds have been tested for their ability to reverse chloroquine resistance (Martin *et al.*, 1987, Kyle *et al.*, 1990). Interestingly, the antimalarial drugs chloroquine and quinacrine (mepacrine) also reduce labelling by azidopine. It is worth noting that many other parasite proteins were labelled by azidopine, and their labelling was equally reduced by the competitive compounds. This result casts some doubt on the specificity of labelling and on the meaning of the competitive displacement.

THE PHENOMENOLOGY OF DRUG RESISTANCE IN PARASITES AND CANCER CELLS IS FUNDAMENTALLY DIFFERENT

Altogether, it seems that the existing evidence relating the MDR gene to chloroquine resistance is at best circumstantial, and much more work is still needed in order to justify the extrapolation that has been made between the mechanism of MDR in cancer cells and chloroquine resistance in malaria parasites. That such extrapolation is probably unwarranted is underscored by several fundamental differences that exist between the pheonomenology of chloroquine resistance and reversal in malaria parasites and the events observed in MDR cancer cells.

1. Anticancer drugs permeate into cells relatively slowly ($t_{1/2} > 1\text{h}$ (Fojo *et al.*, 1985)) and it is therefore possible to assume that an efflux pump could extrude them at a rate sufficient to keep their cellular levels low. However, the half-time of

equilibration of QCDs in malaria-infected cells (IRBCs) is at least two orders of magnitude shorter (Geary *et al.*, 1986; Vanderkooi *et al.*, 1988; Ginsburg *et al.*, 1989). A pump that would be able to cope with such high flux rates should have either an unusually high turnover number or, alternatively, be present at very high copy number.

2. Both MDR cancer cells and chloroquine resistant parasites accumulate less drug than their sensitive homologues. However, upon metabolic deprivation, the cellular drug concentrations in MDR cancer cells reach those that are found in the drug-susceptible parent cell line (Inaba *et al.*, 1979; Merry *et al.*, 1986). In fact, it was this observation that initiated the concept of an ATP-driven drug efflux pump. Metabolic deprivation of malaria parasites invariably results in inhibition of drug uptake, i.e. in lowered drug levels (Fitch *et al.*, 1974, 1975), probably because no ATP is available for fuelling the vacular H⁺-pump. Inhibition of the H⁺-pump results in a rapid alkalinization of the food vacuole (Ginsburg *et al.*, 1989), thereby reducing the driving force for drug accumulation.
3. Drugs which reverse resistance in MDR cancer cells, restore cellular drug concentrations to levels found in the drug-sensitive parent line. In chloroquine resistant malarial parasites these drugs restore chloroquine sensitivity but increase cellular chloroquine concentrations to levels which are still at least one order of magnitude lower than those found in the susceptible strains (Krogstad *et al.*, 1987). This observation suggests that the reversers may act synergistically with chloroquine on an as yet undefined target of chloroquine, without needing to invoke an active drug efflux pump. However, the absence of the same synergistic effect in chloroquine sensitive parasite strains, may imply a different nature of the drugs' target in these strains.
4. The concept of MDR in cancer cells invokes an efflux pump which mediates the extrusion of a variety of structurally and functionally unrelated drugs, although the resistant cancer cells have been selected under the pressure of one specific drug. In other words, MDR cancer cells display cross-resistance. This is certainly not the case in isolates of drug-resistant malaria parasites (see for example Geary and Jensen, 1983; Geary *et al.*, 1987), nor in cloned strains, where the chloroquine resistant clone W2 is sensitive to mefloquine, while the chloroquine sensitive clone D6 is resistant to mefloquine (Oduola *et al.*, 1988). Thus, multidrug resistance, as it is known in cancer cells, has no analogy in malaria parasites. Most interestingly, penfluridol was found to reverse mefloquine but not chloroquine resistance, while chloropromazine, which reverses chloroquine resistance, had no effect on mefloquine resistance (Kyle *et al.*, 1989). These results clearly suggest different drug resistance phenotypes for these two quinoline-containing analogues.
5. Some calmodulin antagonists which reverse drug resistance in MDR cancer cells are ineffective in increasing the susceptibility to chloroquine in malarial parasites (Scheibel *et al.*, 1987).

Altogether, it seems that the basic differences in the phenomenology of drug resistance in cancer cells and malaria parasites do not warrant a direct mechanistic extrapolation from one type of organism to the other.

THE BOTTOM LINE

It is clear from the above description that some crucial details on the mode of action of QCDs are still missing. While we understand the involvement of the vacuolar pH in drug accumulation, only few clues exist concerning the effect(s) of the drug once it reaches its site(s) of action (essentially, those which could explain the irreversible binding and effect of the drugs and the differential drug susceptibility of the target). The mechanism of reversal of drug resistance needs probing since, in all probability the use of the reversers in the chemotherapy of malaria will be seriously considered in the near future. More specifically, one should elucidate whether the reversers of drug resistance act on the level of drug accumulation or on the action of the drug on its putative target, or on both.

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40. S-Adenosylmethionine metabolism in parasitic protozoa

F.Lawrence and M.Robert-Gero

S-Adenosylmethionine (AdoMet), discovered by J.Cantoni in 1953, is a sulphonium compound found in all living cells. This molecule is a substrate or precursor in at least four classes of enzymatic transfer reactions

1. its adenosyl group is transferred to tripolyphosphate in the reversal of AdoMet synthetase reaction (Mudd and Mann, 1963);
2. its 3-amino-3-carboxyl group is transferred to special uridylate residues of certain bacterial tRNA (Nishimura *et al.*, 1974);
3. its aminopropyl group after decarboxylation is transferred in polyamine biosynthesis (Tabor *et al.*, 1961); and
4. its methyl group is transferred to different classes of compounds including proteins (Paik and Kim, 1974), nucleic acids (Borek and Srinivasan, 1965; Vanyushin *et al.*, 1970), phospholipids (Bremmer and Greenberg, 1961; Hirata and Axelrod, 1980) and small molecules such as biogenic amines (Axelrod and Tomchick, 1958).

The major biosynthetic and catabolic pathways involving AdoMet are shown in Figure 40.1.

The chemistry, enzymology, pharmacology and the biological significance of this very important molecule has been extensively reviewed (see Usdin *et al.*, 1979, 1982; Borchardt *et al.*, 1986). The sulphur atom in AdoMet carries three substituents indicating a possible stereoisomerism around this centre (Cornforth *et al.*, 1977). The molecule is stable only in acidic conditions ($\text{pH} \leq 2.8$), in increasing pH it undergoes slow decomposition and epimerization (Matos and Wong, 1987). AdoMet has been the subject of many clinical investigations. An important feature of the cellular distribution of this molecule is its ability to cross the blood-brain barrier. AdoMet is widely used in Europe under the brand names of Samyr in Italy and Gumbabal in West Germany. It has a number of reported effects in particular for the treatment of osteoarthritis (Caruso *et al.*, 1986), inflammation (Gualano *et al.*,

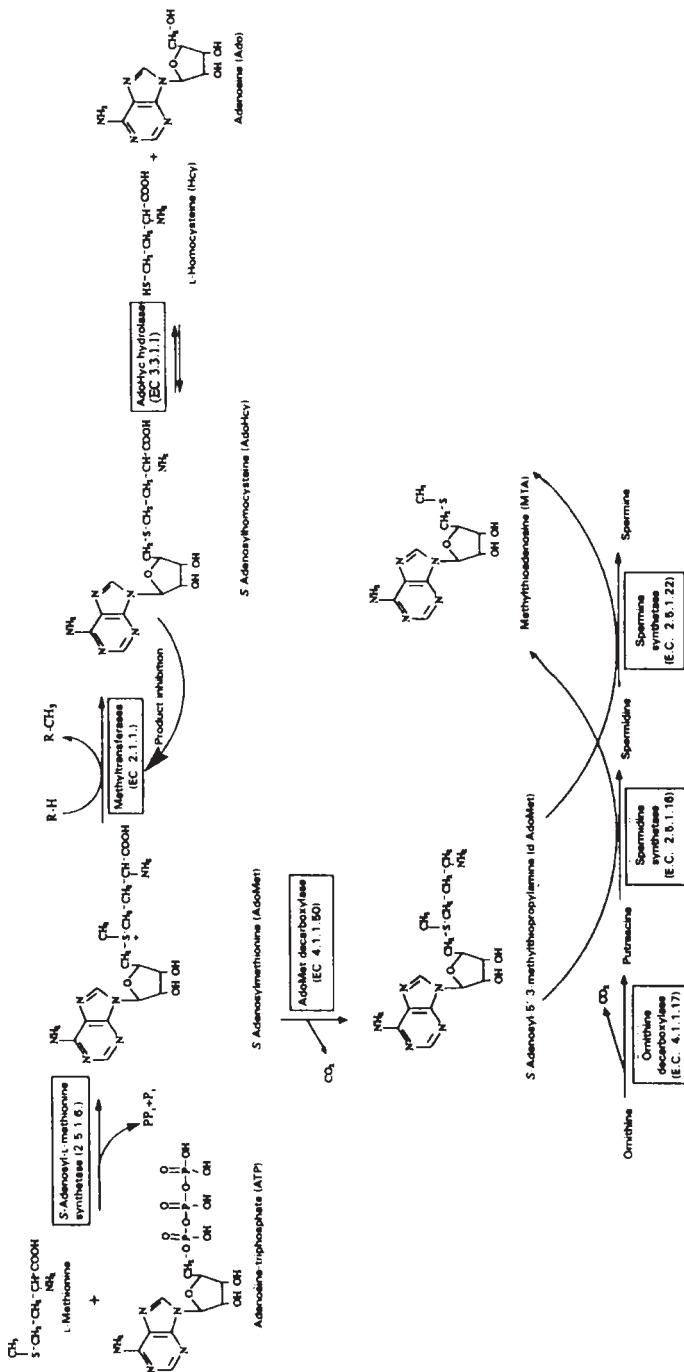


Figure 40.1. AdoMet metabolic pathways.

1983) and mental depression (Fazio *et al.*, 1974). The exact mechanism of action of AdoMet on osteoarthritis is still unknown. However, some preliminary data suggest that it may interfere at some level with prostaglandin synthesis (Caruso *et al.*, 1986). The anti-inflammatory effect of the molecule was shown to be related to the inhibition of the cyclooxygenase metabolic pathway of arachidonic acid (Gualano *et al.*, 1985). AdoMet has been shown to reduce the blood levels of norepinephrine in the standing position. Since increased standing blood norepinephrine levels are found in depression, this effect of AdoMet may be related to its antidepressant activity (Sherer *et al.*, 1986).

Although AdoMet metabolism in bacteria and mammalian cells is well documented, few reports have appeared on its metabolism in protozoa and even less is known about its role in parasitic protozoa. The information available on the enzymatic activities directly involved in AdoMet metabolism in parasitic protozoa is summarized in this chapter. Further details on the enzymes of African trypanosomes are given in Chapter 43.

INTRACELLULAR CONCENTRATION OF AdoMet

The level of AdoMet was found to be between 1.1 and 3.8nmol/mg protein in *Leishmania mexicana* (promastigotes and amastigotes) *L. tarentolae*, *Crithidia fasciculata*, *Herpetomonas muscarum muscarum*, *Trichomonas vaginalis*, *Tririchomitus batrachorum* and *Tritchomonas foetus* (Thong *et al.*, 1987). A similar concentration, 0.74 nmol/mg protein was determined by Yarlett and Bacchi (1988a) for *Trypanosoma brucei brucei*. In *Plasmodium falciparum*-infected red blood cells the intracellular AdoMet concentration was 0.2 nmol/10⁹ cells (Whaun *et al.*, 1986). A much greater concentration (23.5nmol/mg protein was found in *H. m. ingenoplastis* (Thong *et al.*, 1987). Among the natural metabolites of AdoMet, S-adenosylhomocysteine (AdoHcy) was shown to be at a level of 0.11 nmol/mg protein in *T. b. brucei* (Yarlett and Bacchi, 1988a) and 0.3 nmol in 10⁹ cells of *P. falciparum*-infected red blood cells (Whaun *et al.*, 1986). The concentration of decarboxylated AdoMet in *T. b. brucei* was 0.17 nmol/mg protein (Yarlett and Bacchi, 1988a), whereas it was undetectable in *Trichomonas vaginalis* (Yarlett and Bacchi, 1988b).

S-ADENOSYLMETHIONINE SYNTHETASE

The adenosylmethionine synthetase (ATP: L-methionine S-adenosyl transferase; EC 2.5.1.6) catalyses the formation of AdoMet from L-methionine and ATP. The group activation is coupled to the cleavage of ATP at C5' with the formation of enzyme-bound tripolyphosphate which is then hydrolysed to phosphate and pyrophosphate (Mudd and Mann, 1963). The enzyme synthesizes only the (-)-S-AdoMet, one of the two possible diastereoisomers (De La Haba *et al.*, 1959; Cornforth *et al.*, 1977). AdoMet synthetase is a regulatory enzyme under control *in vitro* (Lombardini and Talalay, 1971; Chiang and Cantoni, 1977).

AdoMet synthetase activity has been measured in *T. b. brucei* cell free extracts (Yarlett and Bacchi, 1988a). The activity of the crude *T. b. brucei* AdoMet synthetase was 100 pmol min⁻¹ mg protein⁻¹. This activity was reduced by 45 per cent by 240 nM AdoMet. The enzyme is cytosolic. The reaction was shown to be ATP-dependent, with an apparent K_m for the crude enzyme of 0.2 mM. The apparent K_m for methionine was 0.1 mM. Differences in the substrate kinetics of the trypanosome enzyme from those published for the rat liver enzyme (Sullivan and Huffman, 1983) may be indicative of binding-site differences exploitable in chemotherapy.

Nolan (1987) reported the presence of AdoMet synthetase in promastigotes of *L. braziliensis panamensis*. The enzyme was partially purified but the kinetic constants were not determined. AdoMet synthetase was also detected (0.36 nmol⁻¹ mg protein) in extracts of *Trichomonas vaginalis* by Yarlett and Bacchi (1988b).

S-ADENOSYLMETHIONINE DECARBOXYLASE

AdoMet decarboxylase (EC 4.1.1.50) is the enzyme which provides the aminopropyl groups required for the synthesis of both spermidine and spermine. AdoMet decarboxylase contains a covalently bound pyruvate, essential for the catalytic activity, and forms a Schiff base intermediate with the substrate (Tabor *et al.*, 1961).

AdoMet decarboxylase activity has been detected in lysates of *T. vaginalis* strain G3 (0.04 nmol CO₂ evolved min⁻¹/mg protein). Only trace amounts were found in *T. foetus* and no activity could be detected in *T. batrachorum* (North *et al.*, 1986) and in *T. vaginalis* C1-NIH or IR78 strains (Yarlett and Bacchi, 1988b). AdoMet decarboxylase was shown to be present in *T. b. brucei* (Bacchi *et al.*, 1983). In parasites treated with DFMO for 12 h the enzyme activity was 30–70 per cent below control levels, whereas the concentration of decarboxylated AdoMet was 1000-fold greater than control levels. The typanosomal AdoMet decarboxylase was shown to be inhibited by a structural analogue of decarboxy AdoMet; MDL 73811. The inhibition was time dependent, exhibited pseudo-first-order kinetics (K_i 1.5 μM) and was apparently irreversible. Treatment of *T. b. brucei*-infected mice with 20 mg kg⁻¹ MDL 73811 (given intraperitoneally twice daily for 4 days) cured the infected animals. Furthermore, mice infected with drug-resistant *T. b. rhodesiense* were cured by either a combination of MDL 73811 and relatively low oral doses of DFMO or with MDL 73811 alone administered in implanted miniosmotic pumps. These data suggest that AdoMet decarboxylase is a potential target for drug design against African trypanosomiasis (Bitonti *et al.*, 1990; and see Chapters 43 and 46).

METHYLTRANSFERASES

AdoMet-mediated transmethylation reactions contribute to the metabolism of a number of different classes of compounds including proteins, nucleic acids, lipids

and small molecules. Interest in transmethylation has been heightened in recent years by observations demonstrating or suggesting that such reactions are required for or involved in chemotaxis, neurosecretion, mast cell degranulation, membrane receptor interactions, gene expression, DNA modifications restriction, DNA mismatch repair and cellular differentiation (Springer *et al.*, 1979; Razin and Riggs, 1980; O'Dea *et al.*, 1981; Doerfler, 1981; Burckhardt *et al.*, 1981). In most cases, the methyl group is provided by AdoMet. Transmethylation reactions involving AdoMet result in the formation of AdoHcy which is also an inhibitor of these transmethylases. The ratio of AdoMet to AdoHcy (methylation index) determines the capacity of the cell to methylate various acceptors and as such exerts strong control over AdoMet formation (Ueland, 1982).

To establish whether some of the transmethylases may constitute a target for antiparasitic chemotherapy, specific inhibitors of AdoMet-dependent transmethylation may be used. An example is sinefungin (compound 1 in Figure 40.2), a natural nucleoside produced by *Streptomyces griseolus* (Hamill and Hoehn, 1973) and *S. incarnatus* (Rhone Poulenc Patent no. 7611141, April 1976), related to AdoMet (3) and AdoHcy (2). This molecule exhibits antifungal and antiparasitic activity *in vitro* and *in vivo* (Robert-Gero *et al.*, 1989).

Although methylation and transmethylases are relatively well documented in bacteria and higher eukaryotes (Nur *et al.*, 1985; Antequera *et al.*, 1985; Heitman and Model, 1987; Paik and Kim, 1990), few data have been reported with regard to the methylation and transmethylases in protozoa and especially for parasitic protozoa.

Table 40.1. Activities of some transmethylases in *T. b. brucei* extracts.^a

	Activity (pmol min ⁻¹ /mg protein)
<i>S</i> -Adenosyl-L-methionine: L-homocysteine methyltransferase (EC 2.1.1.10)	163 ± 37
<i>S</i> -Adenosyl-L-methionine: guanido acetic acid methyltransferase (EC 2.1.1.2)	19 ± 6
<i>S</i> -Adenosyl-L-methionine: glycine methyltransferase (EC 2.1.1.20)	60 ± 13
<i>S</i> -Adenosyl-L-methionine: protoporphyrin methyltransferase	not detected

^a Assays were performed at 37°C as described by Yarlett and Bacchi (1988a).

Activities of enzymes involved in transmethylation reactions were determined in blood stream trypomastigotes of *T. b. brucei* infected rats. Several transmethylases were detected and shown to be cytosolic rather than particulate. The activities detected are shown in Table 40.1.

The glycine-AT-methyltransferase catalyses the transfer of methyl group from AdoMet to glycine and results in the formation of sarcosine and AdoHcy. It was also detected in American *Leishmania* species, the specific activity being 86 ± 12 nmol⁻¹ mg protein⁻¹ and the *K_i* for sinefungin was $0.26 \mu\text{M}$ (Avila and Avila, 1987). AdoMet-L-homocysteine methyltransferase activity was also detected in extracts of *Trichomonas vaginalis* at a specific activity of 0.04 nmol min⁻¹ mg protein⁻¹ (Yarlett and Bacchi, 1989b).

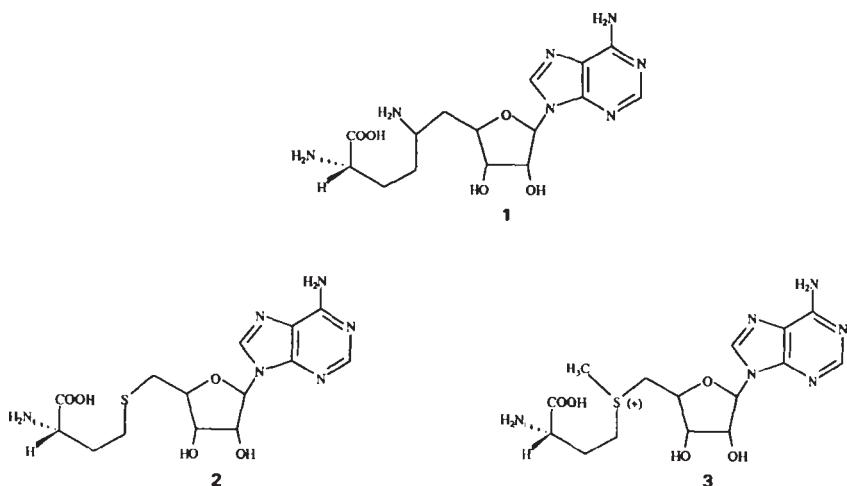


Figure 40.2. Structures: **1**, sinefungin; **2**, adenosylhomocysteine (AdoHcy); **3**, *S*-adenosylmethionine (AdoMet).

Protein methylases

Various amino acid residues are methylated by specific methyltransferases (Paik and Kim, 1974, 1980). Until now three distinct types of protein methylases have been identified and classified according to the amino acid methylated.

S-Adenosylmethionine :protein-arginine-N-methyltransferase (EC 2.1.1.23)

This catalyses the methylation of the guanidino group of arginine residues of proteins. It is currently named protein methylase I since it was the first methylating enzyme identified (Paik and Kim, 1968). The existence of three methylated arginine derivatives in proteins is well established. They are N^G -monomethylarginine, N^G , $N^{G'}$ -dimethylarginine (symmetric) and N^G , $N^{G'}$ -dimethylarginine (asymmetric). They are widely distributed in proteins ranging from those of *Actinomyces* to mammals (Paik and Kim, 1980).

S-Adenosylmethionine :protein-lysine-N-methyltransferase (EC 2.1.1.43)

This catalyses the methylation of the ϵ -amino group of lysine residues of proteins. They are also known as protein methylase III (Paik and Kim, 1970). Three methylated derivatives of lysine are found in proteins ϵ -*N*-monomethyl-, ϵ -*N*-dimethyl—and ϵ -*N*-trimethyl-lysine. They occur in a wide variety of proteins from many species (Paik and Kim, 1980).

AdoMet: protein O-methyltransferase (EC 2.1.1.24)

This is known either as protein methylase II (PM II) or as carboxymethyltransferase (CMT), it catalyses the transfer of a methyl group from AdoMet to the carboxyl

groups of glutamyl and aspartyl moieties of intracellular proteins or polypeptide substrates (Kim and Paik, 1970; Van Waarde, 1987).

Standard conditions described for mammalian protein methylase I and protein methylase III (i.e. at pH 7.2 and at pH 9.0, respectively) were used to measure *in vitro* protein methylase activities in extracts of *L. donovani*, and *L. tropica* promastigotes (Paolantonacci *et al.*, 1985). A 12000g supernatant of these parasites catalysed the incorporation of methyl groups into exogenous histone at both pH values. AdoHcy and sinefungin were competitive inhibitors of protein methylation *in vitro* at both pH values. However, the inhibition constants of sinefungin for the leishmanial enzymes were high compared with the values for enzymes from other sources (Paolantonacci *et al.*, 1986). Amino acid analyses of the *in vitro* methylated histone showed that the percentage of methylated arginine residues was unaffected by the pH change for *L. donovani* while it decreased with pH increase for *L. tropica*. For both species the percentage of methyl lysines decreased with increasing pH.

These results lead to the conclusion that protein methylases of *L. donovani* and *L. tropica* promastigotes behave differently from those of vertebrates. Mammalian protein methylase I (which methylates the arginine residues) is known to be more active at pH 7.2 than at pH 9.0, the contrary being true for protein methylase III (which methylates the lysine residues). In *L. donovani* promastigotes arginine methylation is insensitive to pH variation and in both *Leishmania* species lysine methylation decreases as pH increases.

Protein methylase II has been detected in crude homogenates of American *Leishmania* species (Avila and Avila, 1987) and *L. donovani* and *L. tropica* (Lawrence and Robert-Gero, unpublished). The data are summarized in Table 40.2. The enzymes from Old World and New World *Leishmania* species appear to differ in their affinity for AdoMet and their sensitivity for sinefungin.

Methylated lysine residues were identified at the N-terminal sequences of cytochrome C-557 in *Critchidia oncopelti* and *C. fasciculata*. In the former methylated proline residues were also shown (Hill *et al.*, 1971; Pettigrew and Smith, 1977). Stock *et al.* (1987) postulated the existence of a specific methyltransferase which would be responsible for all N-terminal methylations in eucaryotes. In *C. oncopelti* different fractions of a crude enzyme preparation methylate the N-terminal proline and lysine of cytochrome C-557 with different ratios, suggesting the existence of two specific methyltransferases (Valentine and Pettigrew, 1982). The presence of AdoMet-dependent protein methylation in *Trichomonas vaginalis* was suggested by Thong *et al.* (1987), and confirmed recently by Yarlett and Bacchi (1989). See also Chapter 42.

Δ²⁴ Sterol methylase and fatty acid methylase

Ergosterol is the principal sterol of parasitic trypanosomatid flagellates (Korn *et al.*, 1969; and see Chapter 29), it differs from cholesterol, the predominant mammalian sterol, by the presence of a 24-methyl group and Δ⁷ and Δ²² double bonds. The three enzymatic reactions which introduce the extra methyl group and Δ²² double bond to ergosterol have no counterpart in mammalian sterol biosynthesis and they may be

Table 40.2. Some properties of PM II from various sources.

Source of enzyme	Strain	M_r	Optimum pH	Activity ^a in extracts	K_m (μM)		K_i (μM)
					Protein	AdoMet	
<i>Leishmania</i>							
Promastigotes							
<i>L. mexicana</i> ^b	AMP	32 000	6.0	0.14	100	1.0	0.96
	LMA	32 000	6.0	0.14	100	1.0	1.16
	GML111	32 000	6.0	0.14	100	1.0	1.14
<i>L. m. amazonensis</i> ^b	GML18	32 000	6.0	0.14	100	1.0	0.90
	IRCL52	ND	ND	0.14	ND	48	ND
<i>L. brasiliensis</i> ^b	MHOM Gr81 LA35	ND	ND	0.33	ND	66	ND
<i>L. donovani</i> ^c		30 000	6.9	0.12	ND	5.0	0.2
<i>L. tropicalis</i> ^d		25 000	—	ND	ND	0.87	0.65
<i>B. subtillis</i> ^e	Calf brain ^f	35 000	6.0	—	ND	1.0	0.2
	Chick embryo ^g	ND	ND	—	ND	1.5	0.7
	Murine neuroblastoma ^h	ND	ND	—	ND	3.2	0.3
	Human erythrocyte ⁱ	ND	ND	0.12	ND	ND	ND
	Rabbit brain ^j	ND	—	0.14	ND	ND	ND

ND, not determined.

^a Activity expressed in nmol of methyl groups incorporated per hour per mg protein.^b Avila and Avila (1987).^c F. Lawrence and M. Robert-Gero, unpublished (3- 3 mg ovalbumin as substrate, 37°C incubation).^d Tzora-Shoufou *et al.* (1990).^e Burgess-Cassler *et al.* (1982).^f Kim *et al.* (1978).^g Paik and Kim (1970).^h Pierré and Robert-Gero (1980).ⁱ O'Dea *et al.* (1982).^j Kim (1984).^k Kim *et al.* (1975).

regarded as potential targets for new antiparasitic drugs. An analogous strategy is well established in the area of fungicide development where many inhibitors of ergosterol biosynthesis have been shown to be antifungal agents (Berman *et al.*, 1984; and see Chapter 29).

Alkylation at C24 of the sterol in *Critchidia* is inhibited by heteroatom substituted sterols particularly azasterol (24-aza-5 α , 20 ϵ -cholestane-3 β -ol) and thiasterol (24-thia-5 α , 20 ϵ -cholestane-3 β -ol) (Rhaman and Pascal, 1990). The proposed mechanism of Δ^{24} methyltransferase inhibition by azasterol is that at physiological pH the azasterol, which is positively charged, is an analogue of a carbonium ion intermediate in the enzyme; such an intermediate analogue should bind tightly at the active site of the enzyme. This concept is generally invoked to explain the observed inhibition of many carbonium ion-utilizing enzymes by positively charged substrate analogues. However, thiasterol is not positively charged and yet is also a potent inhibitor of C24 alkylation. The authors hypothesized that the thioether is methylated in the active site of the Δ^{24} -methyltransferase to yield a sulphonium ion which then functions as a cationic reaction intermediate analogue in the usual way. Several thioether bearing fatty acids are potent inhibitors of the biosynthesis of dihydrosterculic acid (a cyclopropane containing fatty acid) in *C. fasciculata* (Pascal *et al.*, 1986; Rhaman *et al.*, 1988). This cyclopropane synthesis is another example of an AdoMet-dependent methyl transfer to an olefin, as it is likely that a similar mechanism of inhibition is involved as well in the methylation of the thioether to give a sulphonium ion at the active site (Pascal *et al.*, 1986).

Nucleic acid methylation

DNA, RNA and tRNA methylation is well documented in eukaryotic cells. The methylated base in DNA is 5-methylcytosine, in mRNA the 5' terminal cap is 7-methylguanine. The tRNAs may contain various methylated bases. These methylations are catalysed by specific AdoMet-dependent transmethylases. In parasitic protozoa only a few studies have concerned nucleic acid methylation. According to Yarlett and Bacchi (1989a, b), DNA methylation occurs and DNA methylases are present in *Trichomonas vaginalis*. In *Leishmania*, kDNA but not nDNA contains methylated bases (J.Keithly, personal communication). Restriction enzyme analysis of *T. b. berucei* DNA indicated the presence of 5-methylcytosine in a sequence of m CCGG (Griffin and Wunderle, 1989). Most of the trypanosomal mRNAs contain a common 35 nucleoside sequence at the 5' end. These 5' terminal sequences which are derived from short mini exon-derived RNA (medRNA) are capped. The cap of both medRNA and mRNA is 7-methylguanine (Sutton and Boothroyd, 1988). The enzyme that catalyses this methylation is not known.

Phospholipid methylation

In various tissues and cells the conversion of phosphatidyl ethanolamine to phosphatidyl choline is mediated either by the CDP-choline pathway or by

AdoMet-dependent phospholipid methyltransferases. Phospholipid methylation was shown to play an important role in certain membrane functions, altering membrane lipid structure and fluidity. Phospholipid methylation is catalysed by two enzymes. The first methylates phosphatidyl ethanolamine to form monomethylphosphatidyl ethanolamine. This enzyme is tightly bound to the membranes. The second enzyme adds two more methyl groups to monomethylphosphatidyl ethanolamine to synthesize phosphatidyl choline. This enzyme could be partially solubilized from the membranes suggesting different topography (Hirata and Axelrod, 1980). Phosphatidyl ethanolamine and phosphatidyl choline have been identified in many parasitic protozoa including *T. b. brucei* (Carrol and McCrorie, 1986), *Trichomonas vaginalis* and *Tritrichomonas foetus* (see Chapter 30). Working with *P. knowlesi*-infected erythrocytes, Vial *et al.*, 1982) showed that [¹⁴C] ethanolamine radioactivity was recovered in phosphatidyl ethanolamine and in phosphatidyl choline, emphasizing the existence of methyltransferase activity in infected cells.

TARGETS FOR CHEMOTHERAPEUTIC ATTACK

In spite of the fact that many parasitic enzymes involved in AdoMet metabolism are not yet characterized some promising leads have already been found. AdoMet synthetase in *Trypanosoma* spp. and *Trichomonas* spp. differ significantly from the enzymes of rat liver. AdoMet decarboxylase is a potential target for drug design against African trypanosomiasis. Protein methylases I and III have different properties in *Leishmania* and in macrophages. Thus, the design of specific inhibitors of the leishmanial enzymes may be of value. Sinefungin, an AdoMet analogue, was shown to be potent antileishmanial agent. One of the primary targets of this compound seems to be the protein carboxymethyl transferase. Finally, the three enzymatic reactions to introduce the extra methyl group and the Δ²² double bond to ergosterol are specific for the parasites and may also be regarded as potential targets for drugs.

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41. Methionine recycling as a target for antiprotozoal drug development

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The development of new and effective antiprotozoal drugs has been difficult because of the close metabolic relationship between protozoa and mammalian cells. In this chapter, we highlight the importance of methionine salvage in the survival of parasitic protozoa and point out possibilities for chemotherapeutic intervention. We discuss in detail the chemotherapeutic exploitation of methylthioribose (MTR) kinase, an enzyme critical to methionine salvage in certain protozoa and other microbes. We show that analogues of MTR, if properly designed, are toxic to organisms that contain MTR kinase but not to mammalian cells which lack this enzyme.

INTRODUCTION

The sulphur containing amino acid methionine plays a critical role in a variety of cellular functions including protein synthesis, transmethylation and polyamine biosynthesis (Cooper, 1983). Methionine is an essential amino acid in humans which is derived primarily from the diet and, therefore, levels of this amino acid may be substantially reduced in malnourished populations. Like humans, most parasitic protozoa require exogenous methionine. For example, *Plasmodium falciparum* requires methionine in addition to that obtained through the proteolysis of red cell haemoglobin in order to sustain growth (Sherman, 1979). This observation underscores the high demand for methionine in proliferating cells.

Cell proliferation is accompanied by a high level of transmethylation and polyamine production, metabolic activities which are dependent upon a steady supply of methionine (Tabor and Tabor, 1976). Because the amount of methionine in the environment is limited, and since in those organisms capable of *de novo* methionine synthesis the process is energetically 'expensive', the ability to salvage

this amino acid is important for parasite survival. One such salvage pathway involves remethylating homocysteine, a product of transmethylation, in a folate-dependent process catalysed by the enzyme methionine synthase (Krungkrai *et al.*, 1989; Banerjee and Matthews, 1990).

In the past decade, research has shown that methionine can also be salvaged from methylthioadenosine (MTA), thereby conserving the amino acid during the synthesis of the polyamines spermidine and spermine (Schlenk, 1983). In the course of studying MTA metabolism, we recognized a subtle but potentially exploitable difference between certain microbes (including members of the protists) and mammalian cells in the mechanism by which they recycle methionine. In these organisms, MTA metabolism involves methylthioribose (MTR) kinase, an enzyme not present in mammalian cells. Based on this observation, we hypothesized that MTR kinase represents a target for the selective killing of organisms that contain the enzyme (Riscoe *et al.*, 1988).

METHIONINE RECYCLING FROM MTA

Methionine is converted to *S*-adenosylmethionine (SAM) in a unique reaction involving ATP as an adenosyl donor (Figure 41.1). The formation of SAM is important for methyl transfer reactions (see Chapter 40) and for the enzymatic production of polyamines (see Chapters 42 and 43). In polyamine biosynthesis, SAM is first decarboxylated and then a propylamine group is transferred to form the polyamines. This leaves methylthioadenosine (MTA).

Early studies on *Enterobacter aerogenes*, *Candida utilis* and the photosynthetic protozoan *Ochromonas malhamensis* showed that the methylthio group of MTA can be incorporated into methionine (Schwartz and Shapiro, 1954; Schlenk *et al.*, 1973; Sugimoto and Fukui, 1974, 1976). In two seminal papers, Shapiro and Barrett (1981) and Backlund and Smith (1981) showed that, in addition to the methylthio group, most of the ribose moiety of MTA is salvaged back into methionine.

The conversion of MTA to methionine is accomplished by one of two mechanisms (Figure 41.1). In mammalian cells and some micro-organisms, MTA is degraded in one step to adenine and methylthioribose 1-phosphate (MTR-1-P) by MTA phosphorylase (Pegg and Williams-Ashman, 1969). In other microbes, however, MTA is catabolized in two steps: first to adenine and MTR via MTA nucleosidase (Duerre, 1962; Della-Ragione *et al.*, 1985), followed by conversion of MTR to MTR-1-P via MTR kinase (Ferro *et al.*, 1978). In both cases, MTR-1-P is subsequently recycled into methionine via a diketo intermediate (Furfine and Abeles, 1988) and 2-ketomethylthiobutyrate (Backlund *et al.*, 1982; Trackman and Abeles, 1983). This metabolic difference is potentially exploitable.

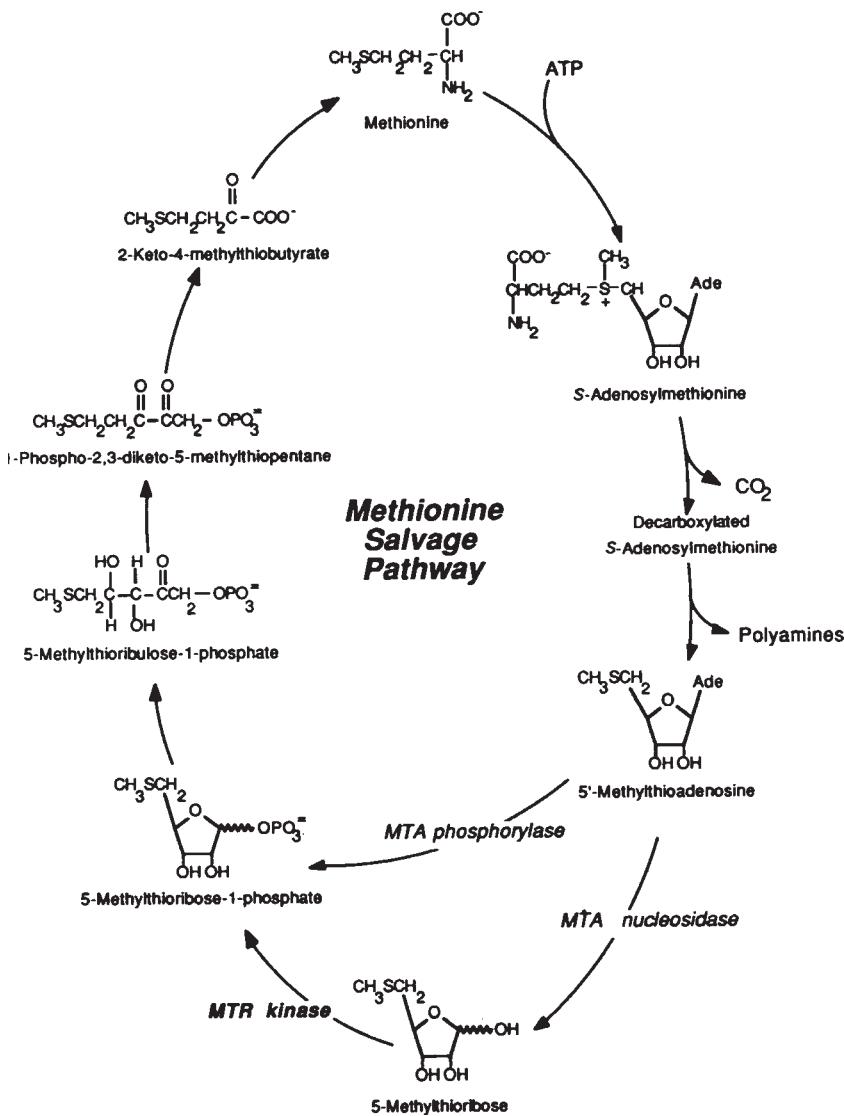


Figure 41.1. The salvage of methionine via 5'-methylthioadenosine (MTA). In mammalian cells, MTA is converted to 5-methylthioribose-1-phosphate (MTR-I-P) in one step. In contrast, certain protozoa and other microbes convert MTA to MTR-I-P in two steps. Note that MTR kinase is unique to the latter system.

TARGETTING OF MTR KINASE

Since MTA nucleosidase and MTR kinase are not found in mammalian cells, both enzymes are potential targets for chemotherapeutic agents. We focussed on MTR kinase because its activity (phosphorylation of MTR) is unique to micro-organisms

unlike microbial MTA nucleosidase which acts on the same substrate (MTA) as human MTA phosphorylase. MTR kinase activity is detectable in cell-free extracts from several protozoa, including *P. falciparum* and *Giardia lamblia* (Riscoe *et al.*, 1988), as well as certain enteric bacteria including *Enterobacter aerogenes* and *Klebsiella pneumoniae* (Gianotti *et al.*, 1990) (Table 41.1). Such organisms should be uniquely susceptible to analogues of MTR.

Table 41.1. Presence (+) or absence (-) of activity for the enzymes MTA phosphorylase, MTA nucleosidase, and MTR kinase in a variety of organisms and tissues.

Organism/tissue	MTA phosphorylase	Enzyme activity ^a	
		MTA nucleosidase	MTR kinase
Human red blood cells	+	-	-
Mouse liver	+	-	-
<i>Trypanosoma brucei</i> ^b	+	NT	NT
<i>Enterobacter aerogenes</i>	-	+	+
<i>Enterobacter cloacae</i> ^c	-	+	+
<i>Klebsiella pneumoniae</i>	-	+	+
<i>Ochromonas malhamensis</i>	-	+	+
<i>Serratia marcescens</i> ^c	-	+	+
<i>Giardia lamblia</i>	-	+	+
<i>Plasmodium falciparum</i>	Trace ^d	+	+
<i>Entamoeba histolytica</i> ^c	-	+	+

^a Enzyme assays were as described in Gianotti *et al.* (1990).

^b Results from Ghoda *et al.* (1988). NT, not tested. See Chapter 43.

^c M.K.Riscoe, unpublished.

^d Residual activity of MTA phosphorylase may be due to contamination with host cell protein.

Depending upon their design, MTR analogues could kill microbes by one of three mechanisms:

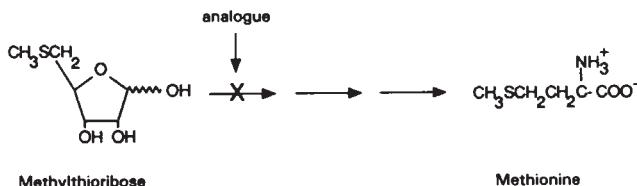
1. inhibition of MTR kinase and prevention of methionine salvage;
2. conversion via MTR kinase and the recycling pathway to toxic analogues of methionine; or
3. conversion via MTR kinase to lethal intermediates (Figure 41.2).

DRUG DESIGN

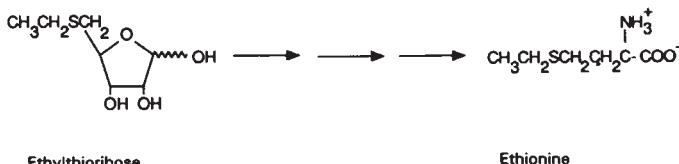
Ethionine and selenomethionine are known toxic analogues of methionine (Colombani *et al.*, 1975). It occurred to us that the ethyl and seleno derivatives of MTR, if they were substrates for MTR kinase and the other enzymes of the methionine recycling pathway, might be converted to ethionine and selenomethionine in MTR kinase containing organisms. Accordingly, we synthesized ethylthioribose (ETR) and methylselenoribose (MSeR) and tested them for antiprotozoal activity. ETR and MSeR were cytocidal to *P. falciparum*, *G. lamblia* and *O. malhamensis* in micromolar concentrations (Riscoe *et al.*, 1988)

Possible Modes of Action of MTR Analogs:

(a) Inhibition of methionine recycling



(b) Conversion to toxic methionine analogues



(c) Conversion to lethal intermediates

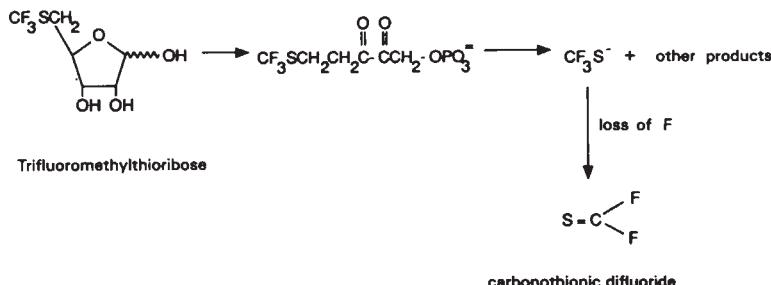


Figure 41.2. The design and proposed modes of action of methylthioribose analogues.

(Table 41.2). As predicted, analysis by high performance liquid chromatography indicated the presence of ethionine in MTR kinase-containing organisms, including *O. malhamensis* (Fitchen *et al.*, 1988) and *K. pneumoniae* (Myers and Abeles, 1989), treated with ETR.

In addition, we have synthesized trifluoromethylthioribose (TFMTR) (Gianotti *et al.*, 1990). We reasoned that if TFMTR is a substrate for MTR kinase and the methionine recycling pathway, it would be converted to the corresponding diketo

Table 41.2. Effect of analogues of MTR on growth of MTR kinase-containing microbes *in vitro*.

Compound	<i>O. malbamiensis</i>	<i>P. falciparum</i> ^b	IC ₅₀ (μ M) ^a
			<i>K. pneumoniae</i>
Methylthioribose	> 5000	> 5000	> 5000
Ethylthioribose	75	100	> 25 000
Methylselenoribose	75	400	25
Trifluoromethylthioribose	< 1	50	0.05

^a Indicated 50 per cent inhibitory concentrations (IC₅₀ values) are the mean of at least three experiments for each organism.

^b Experiments were carried out as described in Riscoe *et al.* (1988) American Type Culture Collection strain No. 30950.

intermediate (l-phospho-2, 3-diketotri fluoromethylthiopentane; see Figure 41.2). In this configuration, it has been shown that a trifluoromethylthio group in the β -position relative to a keto group decomposes to carbonothionic difluoride, a chemical analogue of phosgene (Alston and Bright, 1983). Thus, MTR kinase containing organisms may commit 'suicide' by converting TFMTR into a highly reactive cross-linking agent. In fact, TFMTR has demonstrated greater potency than ETR or MSeR against MTR kinase-containing pathogens (Table 41.2). Preliminary studies employing ¹⁹F NMR spectroscopy have demonstrated the formation of inorganic fluoride ion in cell-free extracts of malaria-infected red blood cells suggesting the decomposition of the trifluoromethylolate ion as proposed.

If analogues of MTR are truly selective for MTR kinase containing organisms, they should have no effect on mammalian cells, which lack the enzyme. In fact, at concentrations up to 1000-fold greater than those required for antiprotozoal activity, ETR is without effect on cultured mammalian cells or on mice *in vivo* (Riscoe *et al.*, 1988, 1989). In preliminary experiments, TFMTR also appears to be non-toxic in mice.

SUMMARY AND CONCLUDING REMARKS

Although little is known about the methionine biosynthetic capability of most parasitic protozoa, it is likely that these organisms are incapable of synthesizing the amino acid *de novo*. As a result, the survival of parasitic protozoa depends upon their ability to scavenge methionine from the environment and recycle this valuable metabolic resource. Two primary methionine salvage pathways are known to exist. The folate dependent conversion of homocysteine to methionine is carried out by methionine synthase. This enzyme is unique in *P. falciparum* (Krungkrai *et al.*, 1989) and, therefore, may be a potential chemotherapeutic target. The other salvage pathway involves MTR kinase and is responsible for conservation of methionine during polyamine biosynthesis. We have exploited MTR kinase and methionine recycling with MTR analogues. Since these analogues are unrelated in chemical structure to existing antiprotozoal agents, they could represent a new generation of drugs with a novel mode of action and, therefore, activity against parasites that are resistant to available drugs.

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42. Polyamine metabolism in anaerobic protozoa

N.Yarlett and C.Bacchi

INTRODUCTION

Polyamines are biologically active low molecular weight diamines and triamines which include putrescine, spermidine and spermine. These are regarded as essential for cell multiplication differentiation, and as co-factors for the synthesis of macromolecules (Marton and Morris, 1987; Pegg and McCann, 1988). They can also contribute to the stabilization of membranes at higher temperature (Tabor and Tabor, 1984). Other diamines such as cadaverine, triamines such as norspermine and norspermidine, tetramines and pentamines are also functional in some micro-organisms (Table 42.1) (Villanueva, 1981). In mammalian cells and the majority of protozoa, synthesis of polyamines occurs by the decarboxylation of ornithine to putrescine by ornithine decarboxylase (ODC) (Linstead and Cranshaw, 1983; Pegg and McCann, 1988; Yarlett, 1988) (Figure 42.1). In plants, fungi, many prokaryotes and possibly *Trypanosoma cruzi* (Pegg and McCann, 1988), agmatine, which is derived from the decarboxylation of arginine, is the precursor of putrescine.

Decarboxylated *S*-adenosylmethionine (*S*-AdoMet) donates aminopropyl groups to putrescine and spermidine forming spermidine and spermine, respectively, and yielding methylthioadenosine from decarboxylated *S*-AdoMet (Figure 42.1). The aminopropyl transferase reactions are irreversible but spermidine and spermine may be converted back into putrescine by the combined actions of spermidine/spermine-1-N-acetyl transferase, and the respective polyamine oxidases (Pegg and McCann, 1988).

Table 42.1. Naturally occurring polyamines present in micro-organisms.

Amine	Structure	Micro-organism	Reference
1,3-Diaminopropane	NH ₂ (CH ₂) ₃ NH ₂	<i>Acanthamoeba castellani</i>	Poulin <i>et al.</i> (1984)
Purescine	NH ₂ (CH ₂) ₄ NH ₂	All eukaryotes	
Cadaverine	NH ₂ (CH ₂) ₃ NH ₂	<i>Trichomonas vaginalis</i>	Yarlett and Bacchi (1988a)
Agmatine	NH ₂ (CH ₂) ₄ NHC(NH)NH ₂	<i>Trypanosoma cruzi</i>	Pegg and MacCann (1988)
<i>sym</i> -Norspermidine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₂ NH ₂	<i>Euglena gracilis</i>	Villanueva (1981)
Spermidine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₄ NH ₂	Majority of eukaryotes	
<i>sym</i> -Homospermidine	NH ₂ (CH ₂) ₄ NH(CH ₂) ₂ NH ₂	<i>Euglena gracilis</i>	Villanueva (1981)
Aminopropylcadaverine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₂ NH ₂	<i>Paracoccus denitrificans</i>	Hammama <i>et al.</i> (1990)
<i>sym</i> -Norspermine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₃ NH(CH ₂) ₃ NH ₂	<i>Euglena gracilis</i>	Villanueva <i>et al.</i> (1981)
Thermospermine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₂ NH(CH ₂) ₂ NH ₂	<i>Paracoccus denitrificans</i>	Hamana <i>et al.</i> (1990)
Spermine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₂ NH(CH ₂) ₃ NH(CH ₂) ₂ NH ₂	<i>Plasmotium falciparum</i>	Assraf <i>et al.</i> , (1984)
Canavalamine	NH ₂ (CH ₂) ₄ NH(CH ₂) ₂ NH(CH ₂) ₄ NH ₂	<i>Rhizobium</i> sp.	Fujihara and Harada (1989)
Aminobutyrylhomospermidine	NH ₂ (CH ₂) ₄ NH(CH ₂) ₄ NH(CH ₂) ₄ NH ₂		Kneifel <i>et al.</i> (1986)
Caldopentamine	NH ₂ (CH ₂) ₃ NH(CH ₂) ₂ NH(CH ₂) ₃ NH(CH ₂) ₃ NH ₂	<i>Thermus thermophilus</i>	
Homocaldopentamine	NH ₂ (CH ₂) ₂ NH(CH ₂) ₃ NH(CH ₂) ₃ NH(CH ₂) ₄ NH ₂		

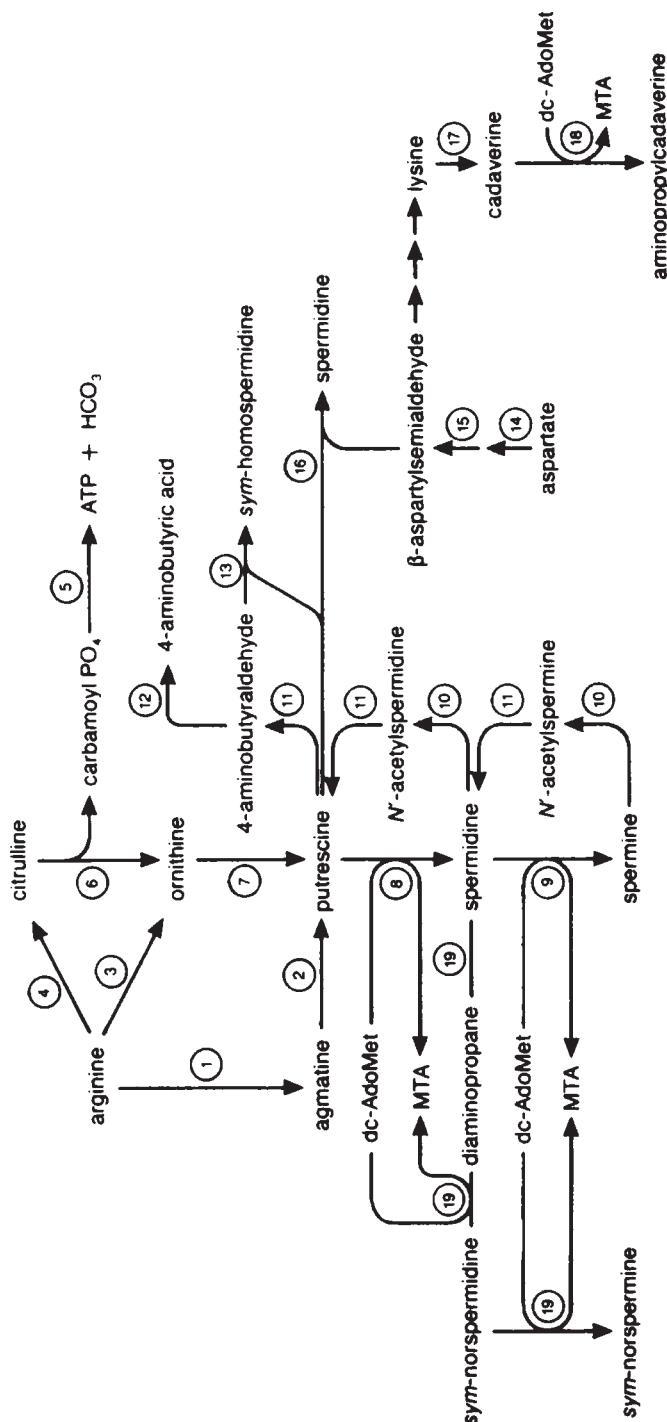


Figure 42.1. Biosynthesis of polyamines and their analogues. 1, Arginine decarboxylase; 2, agmatine deiminase; 3, arginase; 4, aarginine deiminase; 5, carbamoyl phosphate kinase; 6, catabolic ornithine carbamoyl transferase; 7, ornithine decarboxylase; 8, spermidine synthase; 9, spermine synthase; 10, polyamineacyt last; 11, diamine oxidase; 12, aldehyde dehydrogenase; 13, reactions occurring in some archaeabacteria; 14, aspartate kinase; 15, aspartyl-phosphate dehydrogenase; 16, aspartyl semialdehyde dehydrogenase; 17, lysine decarboxylase; 18, aminopropyl cadaverine synthase; 19, reactions occurring in *Euglena gracilis*. MTA, methylthioadenosine; dc-AdoMet, decarboxylated S-adenosylmethionine.

ANAEROBIC PROTOZOA

Anaerobic protozoa are a diverse group of micro-organisms which are represented in almost every ecosystem with a suitably low oxygen tension. Hence they have colonized the rumen of herbivores (Williams, 1986; and Chapter 5), the sulphide deposits of oceans and lakes (Fenchel *et al.*, 1977), and various anaerobic niches of the human body, such as the gastrointestinal and urogenital tracts (Arean and Koppisch, 1956; Meyer and Jarroll, 1980; Hammond *et al.*, 1990). Representative protozoa from various anaerobic environments are deficient in mitochondria and mitochondrial function, but many species have been found to contain a specialized redox organelle, termed the hydrogenosome (Müller, 1980; Yarlett *et al.*, 1984; Zwart *et al.*, 1990; and see Chapter 6). Three genera have been studied with respect to polyamine content and metabolism. The urogenital parasite, *Trichomonas vaginalis*, has received most of the attention because of its unusual content of putrescine (White *et al.*, 1983); *Giardilamblia* and *Entamoeba histolytica* (Gillin *et al.*, 1984) have also been examined, albeit to a lesser degree.

Trichomonas vaginalis, in common with other members of the trichomonad group contains large intracellular pools of putrescine with lower, but detectable, amounts of spermidine and spermine (Table 42.2). As a result, the putrescine spermidine ratio ranges from 6 to 38 (White *et al.*, 1983; Gillin *et al.*, 1984; North *et al.*, 1986; Yarlett and Bacchi; 1988a). With the exception of the intestinal parasite *Entamoeba histolytica*, which also contains high concentrations of putrescine (Table 42.2) (Gillin *et al.*, 1984), this is atypical of other eukaryotes and aerobic protozoa, which usually have putrescine/spermidine ratios of 0.01–1.0 (Table 42.2) (Bacchi, 1981; White *et al.*, 1983). The high intracellular concentration of putrescine in *T. vaginalis* cultured in non-defined and semidefined media leads to its eventual efflux into the medium, reaching a concentration of 40 µM (Yarlett and Bacchi, 1988a). Efflux of putrescine also occurs *in vivo* as judged by the high concentrations present (160–2420 µM) in vaginal fluid during trichomonal vaginitis (Chen *et al.*, 1982; Sanderson *et al.*, 1983). In contrast, polyamines are undetectable in vaginal fluid from healthy volunteers, and in trichomoniasis patients after successful treatment with metronidazole (Sanderson *et al.*, 1983).

Polyamine biosynthesis in *T. vaginalis* originates from arginine via the three enzymes of the dihydrolase pathway (arginine deiminase, catabolic ornithine carbamoyl transferase and ornithine decarboxylase) (Linstead and Cranshaw, 1983; and see Chapter 9). This route of putrescine biosynthesis results in substrate level phosphorylation from the carbamoyl phosphate formed (Figure 42.1). Based upon the following observations there is evidence to suggest that this pathway may be energetically important to this amitochondrial pathogen, especially *in vivo*. The concentration of putrescine detected in vaginal fluid from trichomoniasis patients varies from 160 to 2420 µM compared to about 45 µM detected in culture supernatants. Thus a 4-to 54-fold greater amount of putrescine is found *in vivo* even though the cell density *in vitro* is much greater (4×10^6 cells ml⁻¹ *in vitro* compared with 4×10^4 cells ml⁻¹ *in vivo*). Consistent with these observations is the complete disappearance of free arginine from infected vaginal fluid (Chen *et al.*, 1982) and

Table 42.2. Polyamine content of some parasites.^a

Parasite	Put.	Spd.	Spm.	Put./Spm.	Reference
<i>Trichomonas vaginalis</i> Cl-NIH	38	3.5	1.3	10.9	Yarlett and Bacchi (1988a)
965691	190	5.0	19.0	38.0	White <i>et al.</i> (1983)
Clone 31	67	4.5	4.6	14.9	Gillen <i>et al.</i> (1984)
Clone G3	57	9.7	9.5	5.9	North <i>et al.</i> (1986)
<i>Tririchomonas foetus</i>	F2	79	19.8	9.6	North <i>et al.</i> (1986)
<i>Trichomitus batrachorum</i>	B2	114	17.6	15.0	North <i>et al.</i> (1986)
<i>Tetrahymena thermophila</i> ⁺	10	3.0	0	3.1	Eichler (1989)
<i>Entamoeba histolytica</i>	92	2.6	0.03	35.5	Gillen <i>et al.</i> (1984)
<i>Giardia lamblia</i>	10	9.6	0.8	1.0	Gillen <i>et al.</i> (1984)
<i>Trypanosoma brucei</i>	4	24.5	0	0.17	White <i>et al.</i> (1983)
<i>Trypanosoma rhodesiense</i>	4	21.0	1.2	0.18	White <i>et al.</i> (1983)
<i>Leishmania donovani</i> (promastigotes)	35	37.1	0	0.9	White <i>et al.</i> (1983)
<i>Leishmania donovani</i> (amastigotes)	2	18.8	3.5	0.1	White <i>et al.</i> (1983)
<i>Trypanosoma cruzi</i>	+	+	+	...	Bacchi and Clarkson (unpublished)
<i>Plasmodium falciparum</i> *	9	33.0	8.0	0.3	Assafrof <i>et al.</i> (1984)

^a Values are expressed as: nmol/mg protein; (*) pmol/10⁶ parasitized erythrocytes; (+) millimolar. Put., putrescine; Spd., spermidine; Spm., spermine.

also the depletion by more than 90 per cent of the arginine (1mM) present in a semidefined medium (Linstead and Cranshaw, 1983). However, after successful treatment with metronidazole, levels of arginine are similar to those found in fluid from healthy patients (about 210 μ M) and putrescine returns to undetectable levels.

The reason(s) for the presence of large intracellular concentrations of putrescine is not known, but several possibilities exist. The energy gain to the parasite may be significant. *T. vaginalis* obtains a total of only 4 mol of ATP from the combined action of glycolysis and hydrogenosomal enzymes on 1 mol of glucose (see Chapter 6). The arginine dihydrolase pathway offers one extra ATP gain for every molecule of putrescine produced. *In vivo* the parasite may be glucose-limited, hence arginine may have a major role in energy production. Based upon histochemical staining, the vaginal epithelium is said to contain large amounts of glycogen (Gregoire *et al.*, 1971). Although epithelial cells slough off to some extent from the surface of the human vagina and uterine cervix, the intact vaginal epithelium has little true secretory activity. The transudate that comprises most of the fluid that enters the vagina during human female sexual excitement is derived as a transudate from blood vessels in the vaginal wall during vasocongestion, and may well enter the vagina by passing through interstices between the epithelial cells rather than actually passing through these cells. Vaginal secretions undoubtedly contain varying amounts of glycoprotein and glucosaminoglycans, but in the human subject these substances are almost certainly largely derived from cervical secretions that leak into the vagina (Moghissi, 1979). Fresh clinical isolates of *T. vaginalis* appear morphologically to be amoeboid adherent forms, whereas in laboratory culture they appear as oval motile forms. Growth in a carbohydrate-free medium, however, caused reversion to the amoeboid adherent forms and hence simulates more closely the clinical situation (Hollander and Tysor, 1987). Thus it appears that the arginine dihydrolase pathway contributes significantly to cellular energy requirements of the parasite under limited carbohydrate conditions as is known in some other organisms where this is the sole pathway for arginine catabolism (Broman *et al.*, 1978).

The release of putrescine by *T. vaginalis* may be beneficial to the parasite by inhibiting the natural defence system of vaginal tissue to foreign organisms. It has recently been shown that putrescine was effective in blocking the inflammatory response produced by various model systems (Bird *et al.*, 1983). In these models, putrescine was 10-fold more active than spermidine as an anti-inflammatory agent. Hence a potential role for putrescine in *T. vaginalis* induced vaginitis may be as an anti-inflammatory agent; this view point is bolstered by the fact that the enteric trichomonad, *Tritrichomonas mobilensis*, despite causing severe pathophysiological changes and even necrosis of the intestinal epithelia, does not elicit an inflammatory response by the host (Scimeca *et al.*, 1989).

Cadaverine is also produced by *T. vaginalis*, both *in vivo* and *in vitro* (Chen *et al.*, 1982; Sanderson *et al.*, 1983; Yarlett and Bacchi, 1988a). This polyamine has no known function in mammalian cells, but is found in certain bacteria (Bey *et al.*, 1987) and may be the only polyamine synthesized by *Mycoplasma dispar* (Pöso *et al.*, 1984). Arginine starved cultures of *Tetrahymena* also produced cadaverine at about one-quarter of the concentration of putrescine (5mM) (Eichler, 1989). The

amount of cadaverine produced by *T. vaginalis* appears to be dependent upon the growth media, and is undetectable in modified Bushby's medium (White *et al.*, 1983), but present at high concentrations in other media (Yarlett and Bacchi, 1988a). The relationship of cadaverine synthesis to arginine or lysine concentrations in the trichomonad growth media is not known. The intracellular concentration of cadaverine from *T. vaginalis* grown in a semidefined medium was 2.8nmol/mg protein, which resulted in its eventual efflux into the growth medium attaining a concentration of 6 μ M after 24 h (Yarlett and Bacchi, 1988a). Cadaverine has also been detected in vaginal fluids from trichomoniasis patients, though the concentration varied considerably (55–2020 μ M (Sanderson *et al.*, 1983). A lysine decarboxylase activity has been detected in *T. vaginalis* which had a specific activity five-fold higher than that determined for ornithine decarboxylase. Enzyme activity was unaffected by the addition of ornithine to the incubation, indicating the measured activity was not a function of the ODC (Yarlett, Goldberg, Moharrami and Bacchi, unpublished).

Many potent inhibitors of the polyamine biosynthetic pathway have been synthesized and tested as potential antiparasitic agents. DL- α -Difluoromethylornithine (DFMO) is an enzyme-activated irreversible inhibitor of ODC and an effective antitrypanosomal agent (Schechter *et al.*, 1987; and see Chapter 46). The success of DFMO against African trypanosomes initiated a series of studies with other parasitic protozoa (reviewed by Bacchi and McCann, 1987). Growth of *G. lamblia* was inhibited by 1.25 mM DFMO (Gillin *et al.*, 1984), spermidine was shown to reverse growth inhibition if added within 53 h of incubation with DFMO, but had no effect if added after 73 h, indicating eventual cell death (Gillin *et al.*, 1984). In contrast, DFMO up to 20mM was not growth inhibitory to *Entamoeba histolytica* or *T. vaginalis* in a non-defined medium (Gillin *et al.*, 1984), although the trichomonad ODC is inhibited by DFMO (North *et al.*, 1986). In a semidefined medium however, 5 mM DFMO completely inhibited putrescine and cadaverine production by *T. vaginalis*, resulting in the intracellular and extracellular depletion of these polyamines and cessation of cell division after 48 h (Yarlett and Bacchi, 1988a). Hence failure to observe growth inhibition in the earlier studies was probably due to the presence of exogenous polyamines in the medium. Moreover, *T. vaginalis* is known to accumulate polyamines from the medium, and polyamine uptake increases in trichomonads inhibited in polyamine biosynthesis by DFMO (North *et al.*, 1986; Yarlett and Bacchi, 1988a). DFMO was found to be effective in blocking cytotoxicity of *T. vaginalis* towards mammalian cells in culture, and further delayed the development of subcutaneous abscesses due to model *T. vaginalis* infections (Bremner *et al.*, 1987). Against this background of success, however, DFMO failed to cure mouse intravaginal model infections (Bremner *et al.*, 1987). Since at least healthy human vaginal fluid does not contain significant amounts of polyamines (Chen *et al.*, 1982), it is unlikely that the lack of DFMO efficacy in mouse intravaginal infections is due to the presence of exogenous polyamines. Lack of a cure may thus be a function of the pharmacokinetics of drug transport across the vaginal epithelia.

In all other eukaryotic cells challenged with DFMO, inhibition of spermidine and/or spermine occurs concurrently with inhibition of cell division (Sunkara *et al.*, 1987; Fairlamb *et al.*, 1987). In *T. vaginalis* the relatively low levels of spermidine and spermine present remain unchanged even at the end of eight days of exposure to DFMO (Yarlett and Bacchi, 1988a). Rather in this case, inhibition of cell division seems to be concurrent with depletion of intracellular putrescine. In addition, decarboxylated *S*-AdoMet remains undetectable in DFMO treated *T. vaginalis*, whereas in mammalian cells (Mamont *et al.*, 1982) and *T. brucei brucei* (Yarlett and Bacchi, 1988b), DFMO treatment results in a dramatic increase of this metabolite. *S*-AdoMet decarboxylase a key enzyme in spermidine and spermine synthesis could not be detected in cell-free extracts of *T. vaginalis* C1-NIH (Yarlett and Bacchi, 1988a), or in *Trichomitus batrachorum* (North *et al.*, 1986) although the enzyme was present at low levels in other trichomonads (North *et al.*, 1986). This finding, coupled with the normally low concentrations of polyamines in *Trichomonas* and their failure to change during DFMO treatment makes it likely that the organism fulfils its spermidine and/or spermine requirements from the host. *T. vaginalis* is unaffected by the high concentration of spermine in seminal plasma, and may even rely upon it as a source of this polyamine (Daly *et al.*, 1989). Other physiological evidence points to this conclusion. *T. vaginalis* parasitises the genitourinary tract of males and females; in the female it is found on the squamous epithelial cells lining the anterior fornix of the vagina where the seminal plasma remains for several days after intercourse (Williams-Ashman, 1989). In the male the organism is found in the urethra and the prostate gland in which spermine can be present in concentrations greater than 10 mM (Williams-Ashman, 1989).

DFMO inhibition of *T. vaginalis* growth in semidefined media therefore appears to be the result of putrescine depletion, implicating putrescine as a trichomonad growth factor, possibly as a precursor of 4-aminobutyric acid (GABA: Figure 42.1) or hypusine, as is known in eukaryotic cells and some archaeabacterial species (Pegg and McCann, 1988; Schumann and Klink, 1989; Wolff *et al.*, 1990). In addition putrescine has antioxidant functions, and may function as a defence mechanism against free-radical production due to iron mediated peroxide formation (Tadolini, 1988). Although the functions of polyamines in anaerobic protozoa have barely been explored, they appear to have unique roles not previously attributed to them in eukaryotes: in energy metabolism, in acting as a pH buffer in the environment, and as an aid to parasitism in repressing immune function. A better understanding of polyamine functions in urogenital trichomoniasis as well as other anaerobic infections will enable a more rational design of chemotherapeutic agents.

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43. Polyamine metabolism in African trypanosomes

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The biosynthesis of polyamines and reactions related to this pathway are critical to growth, division and control of the life cycle in African trypanosomes (Bacchi and McCann, 1987; Pegg and McCann, 1988; Giffin and McCann, 1989). Figure 43.1 displays the pathways of polyamine synthesis in trypanosomes, as well as the associated pathways and inhibitors of these reactions. This chapter is intended as an overview of polyamine metabolism and related pathways in African trypanosomes, and to highlight inhibitors now under study as well as potential targets in these pathways. Other chapters in this volume (see Chapters 40, 41 and 44–46) deal in depth with specific enzymes of this pathway and their inhibitors.

POLYAMINE CONTENT

The principle polyamines are spermidine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$) and spermine ($\text{H}_2\text{N}(\text{CH}_2)_2\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}$) and these together with their precursor the diamine putrescine ($\text{H}_2\text{N}(\text{CH}_2)_4\text{NH}_2$), are found nearly universally in eukaryotic cells (see Chapter 42). African trypanosomes contain significant amounts of putrescine and spermidine, but only trace amounts of spermine (<1 nmol/mg protein: Bacchi, 1981; Bacchi *et al.*, 1977, 1983a). Other, less frequently encountered polyamines (e.g. norspermidine and cadaverine) have not been observed in African trypanosomes. Polyamine levels fluctuate during *in vitro* growth of trypanosomes, with putrescine and spermidine peaking during the initial 50 h of growth and decreasing thereafter. The ratio of spermidine to putrescine ranges from 5:1 to 19:1 in blood forms of African trypanosomes and is about 2:1 in nonpathogenic insect trypanosomes and in *Leishmania* spp. (Bacchi *et al.*, 1977, 1983a; Bachrach *et al.*, 1979). In culture at 27°C, *Trypanosoma brucei* procyclic (insect vector) forms have peak levels (1.0–1.5 mol/mg protein) of putrescine early in log phase (6–24 h) and this is followed by a rapid decline in putrescine and a concurrent rise in spermidine to approximately five-fold higher levels (5.0–6.0

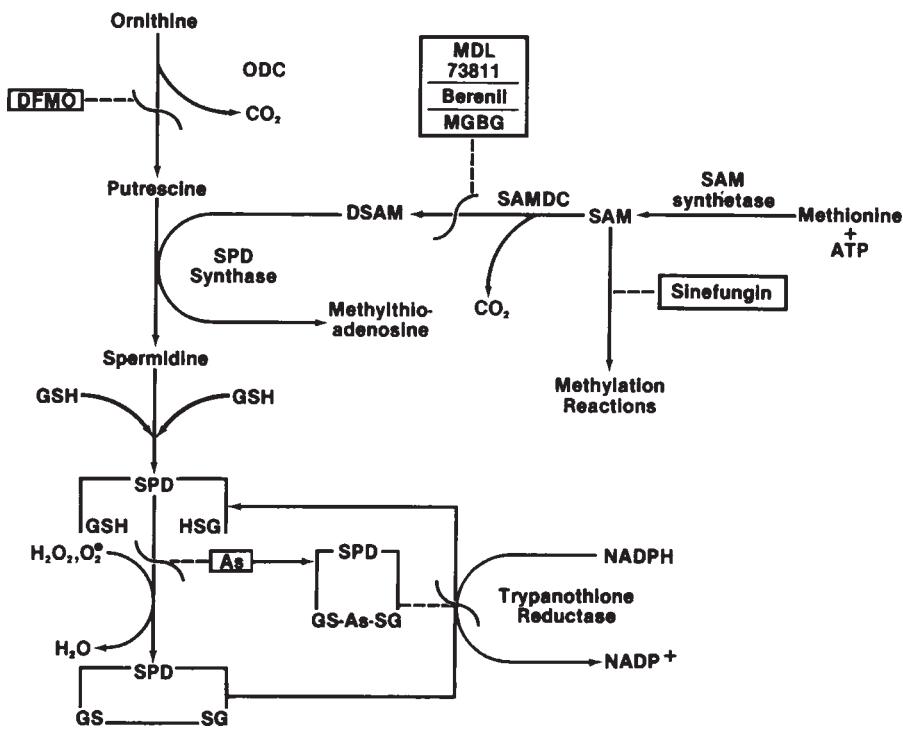


Figure 43.1. Polyamine and *S*-adenosylmethionine metabolism in African trypanosomes. This figure represents biochemical reactions and pathways demonstrated to be present in African trypanosomes. Inhibitors of these reactions are enclosed in boxes; they have been shown to inhibit the trypanosome enzymes. SAM, *S*-Adenosylmethionine; SAMDC, SAM decarboxylase; DCSAM, decarboxylated SAM, MGBG, methylglyoxal-bis (guanylhydrazone); ODC, ornithine decarboxylase; SPD, spermidine; GSH, reduced glutathione; GSH (SPD) GSH, reduced trypanothione; As, arsenical drugs.

nmol/mg protein: Bacchi and Garofalo, unpublished). Blood forms of trypanosomes will accumulate exogenous polyamines, but only at a much lower rate than in mammalian cells (Bacchi and McCann, 1987).

POLYAMINE BIOSYNTHESIS

A key step in polyamine biosynthesis is the decarboxylation of ornithine to putrescine via ornithine decarboxylase (ODC). In eukaryotes, this enzyme is rate-limiting for the pathway: it has a short half-life (<1 h) and is rapidly inducible upon dilution of cells into fresh culture medium or application of other favourable growth conditions (Pegg and McCann, 1988). In trypanosomes, ODC half-life is prolonged (>3 h) (Phillips *et al.*, 1987; Bacchi *et al.*, 1989) apparently due to the absence of a 36 amino acid segment on the C-terminal end of this protein (Phillips *et al.*, 1987).

This sequence, termed a PEST sequence, is an important feature of mammalian enzymes which are subject to rapid turnover (Rogers *et al.*, 1986).

The further utilization of putrescine depends upon a sequence of reactions (Figure 43.1) involving the synthesis of a critical intermediate, *S*-adenosylmethionine (AdoMet), via AdoMet synthetase and its decarboxylation to *S*-adenosyl-5'-deoxy-3, 5'-methylthiopropylamine (decarboxylated AdoMet). The latter intermediate serves as aminopropyl group donor to putrescine via spermidine synthetase, which then generates a 5'-methylthioadenosine (MTA) molecule for every molecule of spermidine synthesized. MTA is metabolized by an MTA phosphorylase which generates adenosine and 5'-methylthioribose 1-phosphate. This is eventually recycled to methionine in a series of reactions which have not been completely characterized (Pegg, 1986; and see Chapter 41).

African trypanosomes do not synthesize spermine (Bacchi *et al.*, 1983a, b; Bitonti *et al.*, 1986a), nor are they able to interconvert exogenously supplied spermine to spermidine via polyamine oxidase and acetyl transferases as in mammalian cells (Pegg, 1988). However, in trypanosomes spermidine is further metabolized to a unique glutathione-containing peptide, trypanothione (*N, N'* bis (glutathionyl) spermidine), through a series of ATP-requiring reactions involving a specific synthetase (Fairlamb *et al.*, 1986 and see Chapter 44). The significance of trypanothione to trypanosomes lies in its key role in detoxifying (reducing) free oxygen radicals and peroxide, and the parasite's reliance on a trypanothione-specific peroxidase and a reductase in these reactions (Fairlamb, 1989; and see Chapter 44).

Reduced trypanothione forms a stable adduct with the arsenic moiety of arsenical drugs in intact cells, and the complex (MeI T) is a potent inhibitor of trypanothione reductase (Fairlamb *et al.*, 1989). Trypanosomes accumulate arsenical drugs by an unknown mechanism: one possibility may be that the formation of the intracellular MeI T complex may block efflux of the arsenical from the cell (Fairlamb *et al.*, 1989). Clinical isolates of arsenic resistant *T. b. rhodesiense* form 50 to 70 per cent less MeI T than drug-sensitive strains when intact trypanosomes are incubated *in vitro* with physiological concentrations of drug, suggesting that lack of drug uptake may be responsible for resistance (Yarlett *et al.*, 1991)

INHIBITION OF POLYAMINE METABOLISM: ORNITHINE DECARBOXYLASE (ODC) AS TARGET

DL- α -difluoromethylornithine (DFMO; Ornidyl[®]), a specific enzyme-activated inhibitor of ODC, was developed as a potential antitumour agent in the late 1970s. DFMO is apparently not metabolized to a great extent in other cellular reactions and this has allowed studies in which polyamines could be selectively depleted from cells without additional, directly cytotoxic effects (for a review see Bey *et al.*, 1987). In mammalian cells, DFMO completely depletes putrescine and reduces spermidine by c.70 per cent but does not affect spermine levels. Cell division proceeds until spermidine is reduced to a critical threshold level, c.30 per cent of the normal control level (Porter and Sufrin, 1986).

Table 43.1. Potential or proven inhibitors of polyamine or AdoMet pathways in trypanosomes.

Enzyme	Inhibitor	References
AdoMet decarboxylase	MDL73811; Berenil, pentamidine	Bitonti <i>et al.</i> (1986a, 1990)
AdoMet synthetase	L-cis-AMB; Se-methionine, cycloleucine	Sufrin <i>et al.</i> (1986)
AdoHcy hydrolase	Neplanocin A, 3-deazadenosines	Ueland (1982), Chiang and Miura (1986)
ODC	DFMO	Bacchi (1981), Bacchi and McCann (1987)
MTA phosphorylase	5'-chloroformycin A; deoxyadenosine analogues (substrates)	Ghoda <i>et al.</i> (1988)
Protein methylase II	Sinefungin	Yarlett <i>et al.</i> (1991)
Trypanothione reductase	Arsenical drugs (Arsobal®, melarsen oxide)	Fairlamb <i>et al.</i> (1989)

The initial finding that DFMO was effective in curing laboratory infections of *T. b. brucei* (Table 43.1) (Bacchi *et al.*, 1980) was followed by limited clinical trials in West Africa in which favourable results were obtained. Trials were then expanded to >400 clinical cases in which a >90 per cent response rate was obtained with only minor, reversible side-effects. Many of these patients had late-stage (central nervous system) infections and had failed one or more treatment regimens with the standard treatment, Arsobal® (Schechter *et al.*, 1987; Schechter and Sjoerdsma, 1989). DFMO therapy has thus become an effective, non-toxic alternative to standard treatment with arsenical drugs in West Africa.

Several events take place in parasites during DFMO treatment of *T. b. brucei* model infections:

1. complete inhibition of ODC activity, along with depletion of putrescine and spermidine (Bacchi *et al.*, 1983a; Bitonti *et al.*, 1985);
2. inhibition of cell division and blockage of DNA, RNA and protein synthesis (Bacchi *et al.*, 1983a; Bitonti *et al.*, 1988);
3. inhibition of variant-specific glycoprotein (VSG) formation, explaining the need for a competent immune response to effect cures with DFMO (Bitonti *et al.*, 1986b, 1988);
4. rapid change in morphology and respiratory metabolism from slender blood forms to intermediate and stumpy forms which are restricted to growth in the insect vector (summarized in Giffin and McCann, 1989);
5. reduction in trypanothione content (Fairlamb *et al.*, 1987); and
6. elevation in AdoMet and decarboxylated AdoMet levels (Yarlett and Bacchi, 1988).

Thus inhibition of ODC causes a series of biochemical and cellular events which collectively block parasite replication, stop antigenic variation and ultimately allow the host immune system to destroy the parasite (Bacchi and McCann, 1987; Bitonti *et al.*, 1988).

POTENTIAL CHEMOTHERAPEUTIC TARGETS WITHIN PARASITE POLYAMINE PATHWAYS

Despite the encouraging clinical results with DFMO in treatment of West African sleeping sickness, alternative measures may be needed to eradicate the East African form of the disease caused by *T. b. rhodesiense*, since recent evidence indicates that a number of clinical isolates are refractory to DFMO as well as standard trypanocides (Bacchi *et al.*, 1990).

An important consideration as an alternative target within the polyamine pathway is the metabolism of AdoMet (Figure 43.1). This metabolite is the critical link between polyamine metabolism, through decarboxylated AdoMet as aminopropyl group donor, and for most cell methylation reactions, for which it is the methyl group donor (Ueland, 1982; Hoffman, 1985; and see Chapter 40). Moreover, the product of transmethylation via AdoMet is *S*-adenosylhomocysteine (AdoHcy), which is a potent inhibitor of methyltransferase reactions if allowed to accumulate in the cell (Ueland, 1982). The ratio of AdoMet to AdoHcy is termed the methylation index, and is normally maintained at a level of about 5. Transmethylation reactions are inhibited as the index falls below this value and hypermethylation occurs at higher levels (Hoffman, 1985; Kramer *et al.*, 1990).

One important consideration in the spectrum of biochemical effects of DFMO is the marked (about 50-fold) elevation of AdoMet in 12 to 36 h treated bloodstream parasites. The increase in AdoMet plus the relatively stable level of AdoHcy results in an increase in the methylation index from 6.5 to 114 (Yarlett and Bacchi, 1988). The dramatic increase in AdoMet levels observed in trypanosomes does not appear to occur in DFMO-treated mammalian cells (Oredsson *et al.*, 1986), and points to the over-production of this metabolite as a strategic difference between host and parasite metabolism.

S-Adenosylmethionine synthetase

This enzyme, owing to its importance in transmethylation and polyamine synthesis, has been extensively studied in tumour cells as a potential target for selective inhibition. At least two isoforms of the enzyme have been found in liver and tumour cells, and these appear to differ in binding site specificity for L-methionine, the K_m value for methionine and inhibition by the reaction products Adomet and pyrophosphate (Sufrin *et al.*, 1986; Cabrero *et al.*, 1987).

AdoMet synthetase in *T. b. brucei* is a cytosolic enzyme with specific activities of 100–400 pmol min⁻¹/mg protein (Yarlett and Bacchi, 1988). The enzyme in trypanosomes is currently under further study and has been partially purified. There appears to be only a single isomer of AdoMet synthetase in *T. b. brucei*, inhibitable by high concentrations (>200 µM) of AdoMet, and pyrophosphate (>1 mM), and unaffected by AdoHcy (>2mM) (C.J.Bacchi, N.Yarlett, J.Garofalo, unpublished). These properties appear to align the trypanosome enzyme with isoform II from rat liver (Cabrero *et al.*, 1987) and indicate that the enzyme in *T. b. brucei* is essentially unregulated under physiological conditions.

Methionine analogues such as L-2-amino-4-methoxy-*cis*-but-3-enoic acid (L-*cis*-AMB) (Sufrin *et al.*, 1986) are being developed as specific inhibitors of AdoMet synthetase. L-*cis*-AMB substantially reduces AdoMet levels prior to blocking growth in tumour cells and may act by altering transmethylation patterns since polyamine levels remain constant during exposure (Sufrin *et al.*, 1986; Porter and Sufrin, 1986). In trypanosomes, L-*cis*-AMB is inhibitory to growth of procylic forms of *T. b. brucei* (50 per cent inhibition at 300 µM) (Bacchi and Sufrin, unpublished).

S-Adenosylmethionine decarboxylase

This enzyme decarboxylates AdoMet and irreversibly commits it to polyamine synthesis, since decarboxylated AdoMet cannot participate in transmethylation reactions (Pegg, 1986). Although AdoMet decarboxylase in mammalian cells is one of the rate-limiting reactions of polyamine synthesis with a short (<1 h) half-life, in bloodstream trypanosomes incubated *in vitro* its half-life is >3 h (Bacchi and Garofalo, unpublished), distinguishing it, like ODC, from its mammalian counterpart. Trypanosome AdoMet decarboxylase is inhibited by the non-competitive inhibitor, methylglyoxal-bis (guanylhydrazone) (MGBG) (Chang *et al.*, 1978; Bitonti *et al.*, 1986a), with a K_i of 32 µM, about 100 times that of the mammalian enzyme (Bitonti *et al.*, 1986a). Although MGBG reduced the parasitemia and prolonged the lives of infected animals (*T. b. brucei* infection), it was not curative in several model infections (Chang *et al.*, 1978; Bacchi *et al.*, 1983b). The diamidine trypanocides Berenil and pentamidine also inhibit AdoMet decarboxylase in trypanosomes (Table 43.1), causing an increase in putrescine content (Bitonti *et al.*, 1986a). Although polyamines antagonize the action of these diamidines in mouse model infections (Bacchi *et al.*, 1983b; Bitonti *et al.*, 1986a), this effect is most likely not due to simply by-passing the enzyme, but may alleviate binding of diamidines to alternate polyamine sites within the trypanosome (Bacchi, 1981) or compete with these agents for uptake sites on the cell surface (Bitonti *et al.*, 1986a).

A novel analogue of AdoMet (5'-[(Z)-4-amino-2-butenyl] methylamino-5'-deoxyadenosine; MDL73811) has recently been synthesized at the Merrell Dow Research Institute (Casara *et al.*, 1989). This analogue rapidly inhibits AdoMet decarboxylase and decreases spermidine content in bloodstream trypanosomes, while curing model infections of *T. b. brucei* and human infective *T. b. rhodesiense* (Bitonti *et al.*, 1990; and see Chapter 46). The dose level of MDL73811 required for cures indicates that it is about 100-fold more potent than DFMO as a trypanocide. MDL73811 acts synergistically with DFMO against drug-resistant *T. b. rhodesiense* and may, therefore, be an effective combination therapy for DFMO-refractory clinical isolates (Bitonti *et al.*, 1990).

Although MDL73811 inactivates trypanosome AdoMet decarboxylase *in vivo* and reduces spermidine content, co-administration of spermidine in model infections does not block drug action. Since the curative effects of DFMO are reversible with putrescine, spermidine or spermine, further studies on the mechanism of action of this promising analogue are warranted (Bitonti *et al.*, 1990).

Methylthioadenosine phosphorylase

This enzyme catalyses the phosphorolytic conversion of Methylthioadenosine (MTA) to adenine and 5'-deoxymethylthioribose-1-phosphate (MTR-1-P). In mammalian cells the enzyme is responsible for recycling adenine through purine salvage and converting the MTR-1-P to methionine via a multistep pathway (Pegg and Williams-Ashman, 1969; Parks *et al.*, 1981; Savarese *et al.*, 1983 and see Chapter 41). Normally, intracellular MTA levels are very low due to removal by MTP-ase. This is important, since accumulation of MTA inhibits growth of mammalian cells and interacts with several enzymes of the polyamine pathway including spermidine synthetase and AdoHcy hydrolase (Williams-Ashman *et al.*, 1982; Porter and Sufrin, 1986). There are two approaches to antitumour chemotherapy using MTP-ase as a target:

1. direct inhibitors of the enzyme, allowing accumulation of the substrate MTA, with ensuing growth inhibition (Chu *et al.*, 1986); and
2. substrate analogues of MTA which would liberate analogues of MTR-1-P and/or adenine upon phosphorolysis (Parks *et al.*, 1981; Savarese *et al.*, 1983).

T. b. brucei has significant MTP-ase activity which is separable from purine nucleoside hydrolysing activity by column chromatofocusing. Parasite MTP-ase activity is phosphate dependent and inhibitible by 5'-deoxy-5'-chloroformycin A (Ghoda *et al.*, 1988), a specific inhibitor of mammalian MTP-ase (Chu *et al.*, 1986). *T. b. brucei* MTP-ase has a broad substrate specificity and is able to cleave adenosine, 2'-deoxy and 2', 3'-dideoxy analogues of adenosine as well as adenosine analogues with a methyl group replacing the 6-amino group of the adenine moiety (Table 43.1) (Ghoda *et al.*, 1988). Adenine nucleoside phosphorylysing enzymes having broad substrate specificities have also been identified in *T. cruzi* (Miller *et al.*, 1987) and *Leishmania donovani* (Koszalka and Krenitsky, 1986). In contrast to the broad range of analogues serving as substrates for the parasite enzymes, mammalian MTP-ase is limited in substrate specificity, and has little activity with 2'- or 2', 3'-dideoxyribose analogues of adenosine (Parks *et al.*, 1981). Since trypanosomes must obtain purines from the environment or from salvage pathways such as MTP-ase, they may be particularly susceptible to substituted purine analogues or those configured to yield a purine analogue and a MTR-1-P analogue upon cleavage. Although conversion of MTR-1-P to methionine has not been demonstrated in the parasite, ketomethylthiobutyric acid, an intermediate in this pathway, fully replaces methionine in growth of *T. b. brucei* promastigotes (C.J. Bacchi, L.Katz and J.Garofolo, unpublished).

S-Adenosylhomocysteine hydrolase

As noted previously, this enzyme acts to generate adenosine and homocysteine from S-adenosylhomocysteine (AdoHcy), maintaining a constant, but low, level of this toxic metabolite and allowing transmethylation reactions to proceed (Ueland,

1982). As a result of the critical balance of AdoMet/AdoHcy needed in metabolism, several approaches to interference with AdoHcy are being pursued (Ueland, 1982; Chiang and Miura, 1986):

1. inhibitors of the enzyme which serve to elevate AdoHcy and decrease methylation activity, and;
2. substrate analogues of AdoHcy which liberate toxic purine analogues,

In practice, analogues which are competitive inhibitors of the enzyme also serve secondarily as substrates (Chiang and Miura, 1986). The competitive inhibitors appear to be the best antiviral agents and these include 3-deaza nucleosides such as 3-deaza (\pm)-aristeromycin and 3-deazaneplanocin; these analogues also markedly elevate AdoHcy levels in mammalian cells (Ueland *et al.*, 1986). Bloodstream forms of *T. b. brucei* have AdoHcy hydrolase activity which is cytosolic in subcellular localization. Enzyme activity in bloodstream forms remained stable during DFMO treatment, while AdoHcy levels also remained relatively constant (Yarlett and Bacchi, 1988). Inhibition of AdoHcy metabolism has not been extensively studied as a potential target in African trypanosomes, although earlier studies with cordycepin (3'-deoxyadenosine) demonstrated that this inhibitor of AdoHcy hydrolase cured *T. congolense* infections (Williamson, 1970). Neoplanocin A is a potent inhibitor of trypanosome AdoHcy hydrolase ($K_i=1.6\mu M$) and inhibits growth of procyclic forms of *T. b. brucei* *in vitro* (95 per cent inhibition at $0.5 \mu M$) (Bacchi, Yarlett and Garofalo, unpublished). A comparative study is needed to determine the spectrum of substrates or inhibitors active against the trypanosome enzyme and their activity against the mammalian enzyme.

Methylation Pathways

The requirement for AdoMet in nearly all transmethylation pathways is well documented (for reviews see Ueland, 1982; Hoffman, 1985; and see Chapter 40); however, there is little information on specific methyltransferase enzymes and the roles of methylation in African trypanosomes. In contrast to tumour cells in which altered methylation potential affects DNA and RNA transmethylase activities (Kramer *et al.*, 1990), neither methylated bases in DNA nor DNA methyltransferases been detected in African trypanosomes (Pays *et al.*, 1984; Crozatier *et al.*, 1988; Yarlett and Bacchi, unpublished).

Bloodstream forms of *T. b. brucei* have three protein methylases acting on distinct amino acid residues (I, II, and III) (Yarlett *et al.*, 1991). Activities of these enzymes were examined during DFMO treatment and protein methylase II, a cytosolic enzyme acting on carboxyl groups of aspartate and glutamate (Kim and Paik, 1970) was found to be six-fold higher than control activity (Yarlett *et al.*, 1991). This enzyme utilized histone VIIIS as preferred substrate and was sensitive to inhibition with AdoHcy ($K_i=13 \mu M$) and its analogue, the antifungal agent sinefungin ($K_i=2 \mu M$). Sinefungin was active against model infections of African trypanosomes (Dube *et al.*, 1983; Bacchi *et al.*, 1987) in *in vitro* growth of *T. cruzi*

(Nadler *et al.*, 1982) as well as against *in vitro* and *in vivo* growth of pathogenic *Leishmania* spp. (Neal *et al.*, 1985; Paolantonacci *et al.*, 1986; Avila and Avila, 1987; and see Chapter 40). The activity of sinefungin in susceptible *Leishmania* spp. correlated with a low K_i (2 µM) for protein methylase II; the enzyme from resistant strains had a 28-fold higher K_i (Avila and Avila, 1987).

While a clear-cut link between changes in polyamine levels, methylation potential and morphological changes in African trypanosomes has yet to be demonstrated, it is interesting to note that the substantial increase in methylation index of the parasite observed during DFMO treatment is accompanied by a six-fold elevation in protein (histone) methylase II activity (Yarlett *et al.*, 1991). The morphological and biochemical changes observed during protracted (12–36 h) DFMO treatment (Giffin and McCann, 1989) can apparently be duplicated by brief (15 min) exposure of blood forms to known methylating agents (streptozotocin, procarbazine, etc.) (Penketh *et al.*, 1990). These findings strongly suggest the participation of AdoMet and methylation events as underlying reasons for the efficacy of DFMO in cytostasis and morphological alterations in African trypanosomes.

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44. Trypanothione metabolism as a target for drug design: molecular modelling of trypanothione reductase

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INTRODUCTION

There is a great need for the development of new and better drugs for the treatment of many parasitic diseases. Rationally based drug design against essential parasitic enzymes or pathways is now widely regarded as a viable approach. Ideally, such targets should be essential for the survival of the parasite and absent from the host. In reality, this is seldom the case (Fairlamb, 1989a). However, trypanothione metabolism meets these criteria and its importance as a target is highlighted by the fact that a number of existing trypanocidal drugs, notably the arsenicals (Fairlamb *et al.*, 1989) and difluoromethylornithine (DFMO) (Fairlamb *et al.*, 1987; Bellofatto *et al.*, 1987), interfere with the metabolism and functions of this important metabolite. In addition, since trypanothione is found in all trypanosomatids examined to date, there is the potential additional bonus of developing a drug with broad-spectrum activity against all disease caused by *Trypanosoma* spp. and *Leishmania* spp. The purpose of this article is to provide an overview of recent developments in this field, with particular reference to the current strategies for the development of new trypanocidal agents based on the selective inhibition of its metabolism. The reader is referred elsewhere for more comprehensive reviews on other aspects of this subject (Fairlamb, 1988, 1989a,b; and see Chapter 45).

FUNCTIONS OF GLUTATHIONE AND TRYPANOThIONE

Glutathione (γ -glutamylcysteinylglycine; GSH) is the principal low-molecular-weight intracellular thiol in most aerobic organisms. Its functions are numerous and varied, involving several areas of metabolism including cellular

protection (against reactive oxygen compounds, free radicals and other toxic compounds of both exogenous and endogenous origin), catalysis (as a co-factor), metabolism (as a substrate) and transport of amino acids via the γ -glutamyl cycle (see review by Meister, 1989). Under physiological conditions, less than 0.5 per cent of the total cellular glutathione is found as glutathione disulphide (GSSG) due to the high activity of glutathione reductase (EC 1.6.4.2; GR), thereby providing the cell with a reducing environment (Meister, 1989).

Although GSH is present in all trypanosomatids, its metabolism is uniquely different from mammalian cells in a number of respects. First, >70 per cent of the total intracellular GSH is found in exponentially growing cells as the polyamine conjugate N^1, N^8 -bis(glutathionyl)spermidine, usually referred to as trypanothione (Fairlamb *et al.*, 1985; Fairlamb, 1988).

Second, these organisms are devoid of classical glutathione reductase activity (Fairlamb and Cerami, 1985). Instead, they contain a unique disulphide oxidoreductase, trypanothione reductase (EC 1.6.4.8; TR) (Shames *et al.*, 1986; Krauth-Siegel *et al.*, 1987). This enzyme utilizes NADPH to maintain trypanothione disulphide ($T[S]_2$) as the dithiol, dihydrotrypanothione ($T[SH]_2$). Since trypanothione has a standard redox potential of -0.242 V, which is slightly more electronegative than that for glutathione (-0.230 V), GSSG can be readily reduced non-enzymatically by thiol-disulphide exchange with $T[SH]_2$ (Fairlamb and Henderson, 1987). Thus, trypanothione and trypanothione reductase may play a pivotal role in the maintenance of the correct intracellular thiol redox potential in these organisms (Figure 44.1).

Third, some of the antioxidant defences of trypanosomatids appear to be dependent on trypanothione rather than glutathione. *Crithidia fasciculata* and *Trypanosoma brucei* both lack classical glutathione peroxidase activity and instead possess an isofunctional trypanothione dependent peroxidase activity (Henderson *et al.*, 1987a). This enzyme has not yet been purified and characterized. The situation may be somewhat different for *T. cruzi* since we have been unable to detect trypanothione peroxidase (Smith and Fairlamb, unpublished). Nonetheless, other workers have presented indirect evidence to show that H_2O_2 metabolism occurs via a trypanothione dependent mechanism in several salivarian and stercorarian trypanosomes and *Leishmania* species (Penketh and Klein, 1986; Penketh *et al.*, 1987). Thus, in some of these parasites, the non-enzymatic removal of H_2O_2 and other peroxides by $T[SH]_2$ may be important (Henderson *et al.*, 1987a). In addition, since GSH acts as a radical scavenger in other organisms, it seems likely that trypanothione and its immediate biosynthetic precursor (N^1 -glutathionylspermidine), which together account for more than 80 per cent of the intracellular GSH (Shim and Fairlamb, 1988), make a major contribution to the trapping of free radicals. Thus, trypanothione and trypanothione peroxidase (when present) play an important role in defence against oxidant and radical damage.

An additional role for trypanothione metabolism may be to regulate free spermidine levels during growth (Shim and Fairlamb, 1988). In stationary phase cells, *C. fasciculata* sequesters about one-third of the total intracellular free spermidine as N^1 -glutathionylspermidine, which, on restoration of favourable

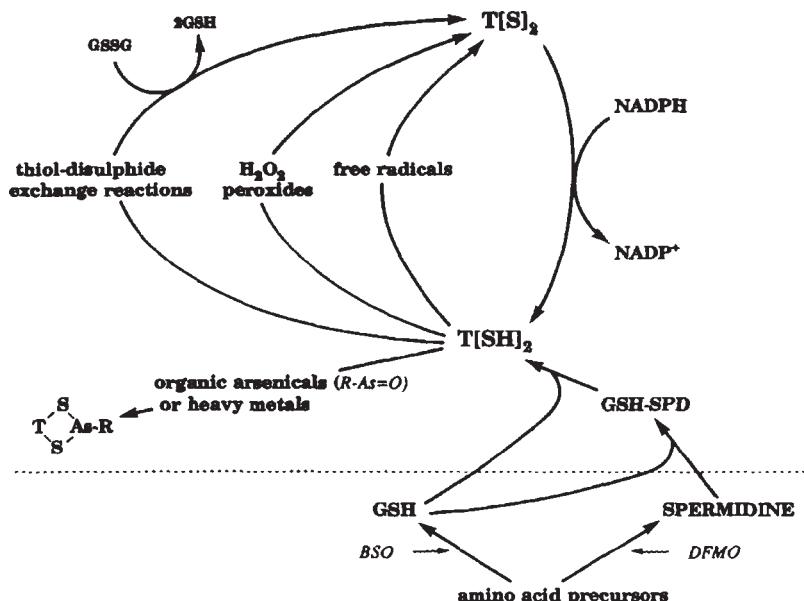


Figure 44.1. Metabolism and functions of trypanothione in trypanosomatids. The pathway below the dashed line is common to host and parasite, whereas the pathway above it is unique to the parasites. T[SH]₂ and T[S]₂ are the dithiol and disulphide forms of trypanothione, respectively. Inhibitors are shown in italics: R-As=O, melarsen oxide; BSO, buthionine sulphoximine; DFMO, difluoromethylornithine. Further details of the metabolic steps have been published elsewhere (Fairlamb *et al.*, 1986; Fairlamb, 1989a).

growth conditions, can be rapidly converted back into free spermidine and trypanothione without the need for *de novo* synthesis of either glutathione or spermidine. Polyamine levels in mammalian cells are highly regulated by rapid turnover of three key enzymes in polyamine biosynthesis, namely ornithine decarboxylase, S-adenosylmethionine decarboxylase and polyamine-*N*¹-acetyltransferase (see reviews by Pegg, 1986; Pegg and McCann, 1988). The rapid degradation of these enzymes ($t_{\frac{1}{2}} < 1$ h) occurs by a mechanism that is not well understood at present, but may involve 'PEST' sequences found so far in two of these enzymes, which have been postulated to be important in the breakdown of rapidly degraded proteins (Rogers *et al.*, 1986). In support of this hypothesis, mouse ornithine decarboxylase (ODC) can be converted to a stable protein by truncating 37 residues at its carboxy terminus (Ghoda *et al.*, 1989). In contrast, ODC from *T.brucei* does not have this 'PEST'-rich carboxy terminal region (Phillips *et al.*, 1987) and apparently turns over much more slowly (Phillips *et al.*, 1987; Bacchi *et al.*, 1989; and see Chapter 43). Perhaps trypanosomatids do not need to rapidly regulate ODC levels by enzyme turnover, since they may be able to modulate spermidine levels using glutathionylspermidine instead.

Trypanothione forms a stable complex with trivalent organic arsenicals such as melarsen oxide and thus may represent a primary target for the toxic action of these

drugs (Fairlamb *et al.*, 1989). Since glutathione has recently been shown to act as the first line of defence in mice against cadmium toxicity (Singhal *et al.*, 1987), we have recently investigated the role of glutathione, glutathionylspermidine and trypanothione in defence against heavy metals. Exposure of *C. fasciculata* to sublethal concentrations of cadmium or mercury leads to a marked increase in dihydrotrypanothione (10-fold), a moderate increase in glutathionylspermidine (4-fold) but only a slight increase in glutathione levels (2.2-fold) (Smith and Fairlamb, unpublished). Moreover, following inhibition of glutathione and trypanothione synthesis by buthionine sulphoximine, the cells become hyper-sensitive to the toxic effects of cadmium. Thus, thiol metabolism, particularly trypanothione, is of paramount importance in the defence against cadmium toxicity in *C. fasciculata*.

PROPERTIES OF TRYPANOThIONE REDUCTASE

Trypanothione reductase (TR) is essential for maintaining trypanothione as T[SH]2 and therefore represents one of our primary targets for the design of new trypanocidal drugs. TR has been purified and characterized from *C. fasciculata* (Shames *et al.*, 1986) and the human pathogen *T. cruzi* (Krauth-Siegel *et al.*, 1987) and found to be structurally similar to human glutathione reductase in several ways: all have similar monomeric molecular weights (50–55 kDa), are active in the dimeric form, contain FAD as co-factor and use NADPH as electron donor (Shames *et al.*, 1986; Krauth-Siegel *et al.*, 1987; and see Chapter 45). The TR gene from the cattle pathogen *T. congolense* has been cloned, sequenced (Shames *et al.*, 1988) and expressed in *Escherichia coli* (Sullivan *et al.*, 1989). The structural gene encodes 492 amino acids and the inferred amino acid sequence shows 41 per cent identity and >50 per cent homology with human GR (Shames *et al.*, 1988).

Table 44.1. Catalytic and substrate-binding residues of glutathione reductase interacting with the glycine carboxylates of glutathione disulphide and their homologues in trypanothione reductase.

Glutathione reductase ^a	Function	Trypanothione reductase ^b
Cys-58	Redox-active disulphide	Cys-52
Cys-63	Redox-active disulphide	Cys-57
Tyr-114	Stacks between GS moieties in GSSG	Tyr-110
His-467'	Active site base	His-461'
Glu-472'	Hydrogen bonds to active site histidine	Glu-466'
Ala-34	Interacts with Gly-1 carboxylate in GSSG	Absent ^c
Arg-37	Interacts with Gly-1 carboxylate in GSSG	Absent ^c
Asn-117	Interacts with Gly-2 carboxylate in GSSG	Absent ^c

^a Data from Karplus *et al.* (1989).

^b Data from Shames *et al.* (1988).

^c The equivalent residues to Ala-34, Arg-37 and Asn-117 in glutathione reductase are Glu-18, Trp-21 and Met-113, respectively.

The catalytic mechanism of human erythrocyte GR is known in great detail (see the review by Ghisla and Massey, 1989; and Chapter 45) and all of the important catalytic residues are apparently conserved in TR (Shames *et al.*, 1988), including the redox-active Cys-52 and Cys-57, the active site base His-461' and Glu-466' which holds the histidine in the correct orientation (see Table 44.1). Sequence data from *C. fasciculata* (T.Aboagye-Kwarteng and A.H.Fairlamb, unpublished) and *Leishmania donovani* (Taylor *et al.*, 1989; unpublished) supports these findings and thus GR and TR appear to have similar reaction mechanisms (Krauth-Siegel *et al.*, 1989). Despite these close similarities, GR and TR show striking differences in their substrate specificities (Henderson *et al.*, 1987b). Most importantly, TR will not reduce GSSG, neither will human GR reduce T[S]₂ to any significant extent (Shames *et al.*, 1986). Likewise, *N*¹-glutathionylspermidine disulphide and the mixed disulphide of *N*¹-glutathionylspermidine and glutathione are substrates for TR but not human GR (Henderson *et al.*, 1987b). Together, these observations suggest that TR is an excellent target for drug design.

MOLECULAR MODELLING OF TRYPANOPTHIONE REDUCTASE

In an attempt to understand the molecular basis for the mutually exclusive specificities of the two enzymes we have used the high resolution crystal structure of GR as a template to model the interaction of trypanothione reductase with its substrate. As an initial step the active site of human glutathione reductase was built using the Brookhaven coordinates (Code P3GRS) for the crystallographic structure (Karplus and Schulz, 1987). A dimer was generated by copying the monomer coordinates (without water molecules) by 180° around the two-fold axis provided in the crystallographic reference, and then adding in the FAD co-enzyme and the GSSG substrate (coordinates kindly provided by G.E.Schulz). The active site was examined on an Evans & Sutherland PS390 graphics terminal using FRODO (Jones,

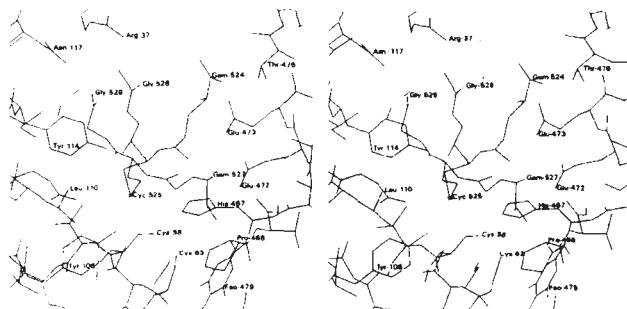


Figure 44.2. Stereo view of glutathione disulphide bound to the active site of human erythrocyte glutathione reductase. Residues Gly-526 and Gly-529 are the glycine residues of GS-I and GS-II, respectively, where GS-I is the half of GSSG which becomes covalently bound to the enzyme during catalysis.

1978). The disposition of GSSG in the active site of GR is shown in Figure 44.2. Particular details of the mode of binding and the residues involved have been discussed elsewhere (Karplus *et al.*, 1989).

The active site of TR from *T. congolense* was then modelled on this structure using the COMPOSER programme suite (Blundell *et al.*, 1989) as follows. The secondary structure and hydrogen bonding pattern of GR were extracted from the Brookhaven coordinates with the DSSP programme (Kabsch and Sander, 1983) and this information was used together with the programme SEQALIGN to establish an alignment of equivalent stretches of residues to be conserved in the core structures of the two molecules. A three-dimensional model of the structurally conserved core of TR was then built using COMCOR and side-chains substituted according to a rule-based procedure. CMPALL was then used to select fragments for the variable loop regions from a data base using constraints from the core model, and these loops were melded onto the core structure. The resultant model was 'dimerized' by rotation as mentioned above. No account was taken of possible bad contacts at the dimer interface and no attempt was made to model water molecules. The GSSG molecule was added into the active site of the TR model and examined under FRODO.

It is striking to notice the extent to which the active site features near to the redox disulphides were conserved (Table 44.1, Figure 44.3). This was encouraging since it implies that the major portion of the substrate molecule, trypanothione, could remain in the same disposition as in the GR active site. The most significant differences between the two active sites seemed to be the presence in TR of a cluster of hydrophobic residues around one edge of the active site opening, somewhat away from the catalytic cysteines (Cys-52 and Cys-57) and more exposed to the solvent. This cluster comprised residues Leu-17, Trp-21, Met-113 and Phe-114 at the contact between helices 1 and 3. At this point in the modelling, the two glycine carboxylates of GSSG could no longer be hydrogen bonded to Arg-37 and Asn-117, but seemed to reach to embrace the hydrophobic side-chains Trp-21 and Met-113 present in TR.

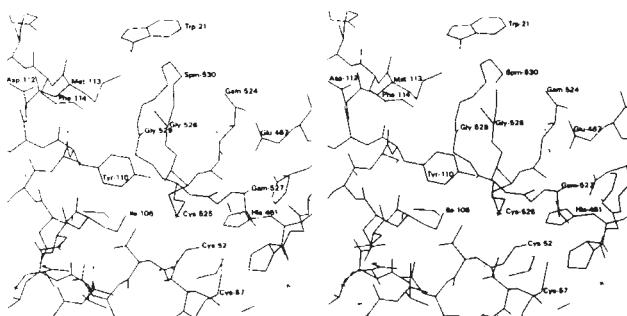


Figure 44.3. Stereo view of a molecular model of trypanothione disulphide bound to the active site of trypanothione reductase. Details of the molecular modelling are given in the text and the catalytically important residues are identified in Table 44.1. Note the close association between the spermidine moiety of the substrate (Spm-530) and Trp-21 and Met-113 of the enzyme.

It was clear that the spermidine to be built joining the two glycines might well be subject to hydrophobic forces in TR, with the methylene groups of the spermidine excluding solvent from around this cluster. Thus this weak bonding force in the case of the TR/T[S]₂ system contrasted with the hydrogen bonding of the glycine carboxylates with Arg-37 and Asn-117 in the GR/GSSG system and may help to explain the differential specificity of the two enzymes for their respective substrates.

Next, spermidine was incorporated into the model substrate thus converting GSSG to T[S]₂ (see Figure 44.3) and the geometry was improved using the REFI option in FRODO to regularize the model locally. The side chain torsion angles of the Trp-21 and Met-113 were altered somewhat to optimize the packing. It was clear that the hydrophobic clustering could only have been avoided by severe disruption of the substrate's disposition; no other conformation appeared as favourable as that allowing the spermidine loop to lie intimately against the methionine and tryptophan residues. In another modelling study of *T. congolense* TR, Murgolo *et al.*, (1989) have suggested that two acidic residues Asp-112 and Asp-116 may interact with the spermidine moiety of trypanothione. This was not apparent from our model as these residues seemed somewhat distant. Crystallographic and site-directed mutagenesis studies should clarify the importance of these residues in conferring substrate specificity.

CRYSTALLIZATION OF TRYPANOTHIONE REDUCTASE

Molecular modelling of both enzyme and its substrate clearly has its limitations and accurate elucidation of the three-dimensional structure of TR is required to assist in the rational design of inhibitors. Recently, we have obtained well-ordered, radiation-stable crystals of TR from *C. fasciculata* (Hunter *et al.* 1990). The yellow crystals are tetragonal rods which grow on occasion to lengths in excess of 1.0mm and thickness of 0.2 mm and have cell dimensions of $a=128.6$, $c=92.5\text{ \AA}$. A partial data set to 2.7 Å resolution has been obtained. Preliminary results suggest a single non-crystallographic two-fold axis which is commensurate with a dimer in the asymmetric unit. The sequence homology between TR and GR indicates that the method of molecular replacement (Rossmann, 1972) may facilitate solution of the structure.

INHIBITORS OF TRYPANOTHIONE REDUCTASE

Glutathione reductases from mammalian sources are inhibited by nitrofuran and naphthoquinone derivatives (Buzard and Kopko, 1963). A series of nitrofuran and naphthoquinone derivatives with basic substituents have been synthesized and screened for inhibition of TR (Henderson *et al.*, 1988). Several of these novel compounds were potent inhibitors of TR; two were subsequently shown to be much better inhibitors of TR than for GR (Jockers-Scherubl *et al.*, 1989). Moreover, TR was shown to catalyse the reduction of these compounds. In the case of nitrofurans

the resulting nitrofuran radical undergoes futile cycling in the presence of molecular O₂ to form the parent compound and reactive oxygen species such as superoxide and H₂O₂. When tested for biological activity, these compounds were trypanocidal against *T. cruzi* trypomastigotes and this activity correlated with their relative ability to be redox cycled by TR. It is envisaged that these compounds subvert the normal antioxidant role of the enzyme within the cell leading to parasite death. The cytotoxic effect of nifurtimox, a nitrofuran used for the treatment of Chagas' disease, is likewise believed to be mediated by the generation of reactive oxygen species (Docampo, 1990). Inhibitors which affect TR and GR alike may serve as leads for the development of more potent and ultimately selective inhibitors. For example, 2, 4, 6-trinitrobenzenesulphonate (TNBS) is a potent reversible inhibitor of human GR (IC₅₀<50 nM) (Carlberg and Mannervick, 1979), whereas TR is three orders of magnitude less sensitive to inhibition (IC₅₀50 μM). However, a simple analogue of this compound (*N*¹-imidazole-2, 4, 6-trinitrobenzene) is a strong uncompetitive inhibitor of TR (*K*_i=2 μM) and indeed GR (*K*_i=0.5 μM). Further refinement of these and other inhibitors will be greatly aided by an analysis of crystalline-enzyme/inhibitor complexes (see also Chapter 45).

BIOSYNTHESIS OF TRYPANOPTHIONE

The biosynthesis of trypanothione represents another excellent target for the rational design of new trypanocidal drugs. Preliminary studies using cell-free extracts of *C. fasciculata* demonstrated that trypanothione was synthesized from GSH and spermidine with the concomitant hydrolysis of ATP to ADP (Fairlamb *et al.*, 1986). Other nucleotide triphosphates will not substitute for ATP. Trypanothione synthetase from *C. fasciculata* has been purified approximately 15000-fold to homogeneity (Henderson *et al.*, 1990). The enzyme, active in the monomeric form, *M*_r=90000, catalyses the synthesis of trypanothione from GSH, spermidine and ATP/Mg²⁺ via either of the intermediates *N*¹-or *N*⁸-glutathionylspermidine. A consideration of the catalytic parameters for the enzyme suggests that the preferred route for trypanothione synthesis is via the *N*⁸ isomer, consistent with the finding that the *N*⁸ isomer does not accumulate substantially within the cell (Fairlamb *et al.*, 1986; Henderson *et al.*, 1990). Moreover, *N*⁸-acetylspermidine, but not *N*¹-acetylspermidine is a substrate for the enzyme (Smith and Fairlamb, unpublished). The reaction mechanism is postulated to involve activation of the glycyl carboxyl of GSH to an acyl phosphate intermediate followed by nucleophilic attack by the *N*¹ or *N*⁸ amino groups of spermidine (or glutathionylspermidine) to form the amide linkage (Henderson *et al.*, 1990). Trypanothione synthetase is thus a new member of the ATP-dependent ligases, such as γ -glutamylcysteine synthetase. As with other members of this class of enzyme, it should be feasible to design suitable sulphoximine (Griffith and Meister, 1979) and alkylphosphinate derivatives (Duncan and Walsh, 1988) as specific inhibitors of trypanothione synthetase.

CONCLUSION

In conclusion, trypanothione metabolism plays at least four important roles in these organisms: in maintenance of intracellular thiol redox; in defences against oxidant stress and heavy-metal toxicity and in regulation of intracellular spermidine levels. Thus, trypanosomatids have evolved a branch of metabolism based on trypanothione that is closely related to that of glutathione in the host cell. It is not clear why this should be so, but it does afford an excellent target for the development of new trypanocidal agents.

ACKNOWLEDGEMENTS

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Note added in proof: Kuriyan *et al.*, 1990, *Journal of Molecular Biology*, **215**, 335–7 have also presented a preliminary Crystallographic Analysis of Trypanothione Reductase from *Crithidia fasciculata*.

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45. The antioxidant enzymes glutathione reductase and trypanothione reductase as drug targets

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R.H.Schirmer*

Many parasites including the causative agents of malaria (*Plasmodium falciparum*) and of Chagas' heart disease (*Trypanosoma cruzi*) appear to be more sensitive to oxidative stress than their mammalian hosts. Consequently, we have chosen thiol generating antioxidant enzymes as potential targets for the chemotherapy of these diseases. In the case of malaria we are attempting to develop inhibitors against a host protein, namely erythrocyte glutathione reductase, ($\text{NADPH}+\text{GSSG}+\text{H}^+ = \text{NADP}^++2\text{GSH}$); a flavoprotein whose stereochemistry of catalysis is known in atomic detail. With Chagas' disease, our target protein is trypanothione reductase of *T. cruzi*. This enzyme, which appears to occur exclusively in trypanosomatids, catalyses the reduction of glutathionylspermidine disulphides by NADPH.

INTRODUCTION

The classical approaches of drug development suffer from the fact that the targets are invisible. Being able to see one's target or opponent is often a big help. This is what the strategy of 'drug design' aims to contribute (Hol, 1986). The cornerstone of this approach is the accurate knowledge of the three-dimensional structure of potential target proteins and other biomacromolecules. Our report deals with two mechanistically and structurally related enzymes, namely red blood cell glutathione reductase (GR) as a potential target of antimalarial drugs and with trypanothione reductase (TR) from pathogenic trypanosomatids (Table 45.1).

Table 45.1. Properties of human glutathione reductase and of trypanothione reductase from *T. cruzi*. (Schirmer *et al.* 1989; Krauth-Siegel *et al.* 1987; Jockers-Scheriibl *et al.* 1989).

	Glutathione reductase (human erythrocytes)	Trypanothione reductase (<i>T. cruzi</i> epimastigotes)
Subunit M_r	52 500	50 000
Co-factor	FAD	FAD
Oligomeric structure	Dimer	Dimer
Intracellular concentration (μM)	0.1	1.25
<i>Substrates at the pyridine nucleotide site</i>		
NADPH, K_m (μM)	8.5	5
Activity with NADH/activity with NADPH	0.05	0.03
<i>Substrates at the disulphide site</i>		
Glutathione disulphide, K_m (μM)	65	No activity
Trypanothione, K_m (μM)	No activity	18
Glutathionylspermidine disulphide, K_m (μM)	No activity	22
Specific activity (U/mg protein)	240	100
<i>Highly reactive cysteine residues per subunit in the native</i>		
Oxidized enzyme species E	0.3 ^a	0.0
Reduced enzyme species EH ₂	1.3 ^a	1.0
Catalytic disulphide	Yes (Cys-58–Cys-63)	Yes
$\lambda_{\text{max}, \text{vis}}$ of E (nm)	463	461
Absorption band at 530 nm in EH ₂	Yes	Yes
$A_{530 \text{ nm}}^{1 \text{ nM}}$ of the EH ₂ ·NADPH complex	4.5	4.9

^a Both E and EH₂ contain the rather reactive Cys-2. The additional highly reactive thiol of EH₂ is Cys-58.

DETOXICATION OF REACTIVE OXYGEN SPECIES IN THE GLUTATHIONE REDOX CYCLE

The intraerythrocytic development of *P. falciparum* depends on an intact thiol metabolism of the host cell (Hunt and Stocker, 1990). The predominant thiol compound of red cells is the tripeptide glutathione (GSH); its concentration is approximately 2 mM whereas the level of oxidized glutathione (glutathione disulphide, GSSG) is 100-fold lower. The high [GSH]:[GSSG] ratio is established by the activity of the flavoenzyme glutathione reductase which catalyses the reaction GSSG+NADPH+H⁺=2GSH+NADP+. Thus GR maintains the reducing milieu of the primordial atmosphere within cells against the extracellular and environmental redox conditions which are dominated by molecular oxygen (Schirmer *et al.*, 1989). Man consumes approximately 1000 g O₂ per day, 50 g (*c.* 40 l) of which are converted to toxic compounds such as , H₂O₂ and OH. As is the case for most aerobically living organisms, human cells contain the glutathione redox cycle (Figure 45.1) and other antioxidant mechanisms for the detoxication of reactive oxygen species. Oxidative stress (Sies, 1986) is the metabolic situation where these mechanisms cannot cope with the challenge by oxidants. However, oxidative stress is not just an unwanted side-effect of aerobic life; leucocytes and macrophages as well as redox-cycling drugs (Figure 45.2) produce reactive oxygen species for combatting bacteria, intracellular parasites and other pathogens

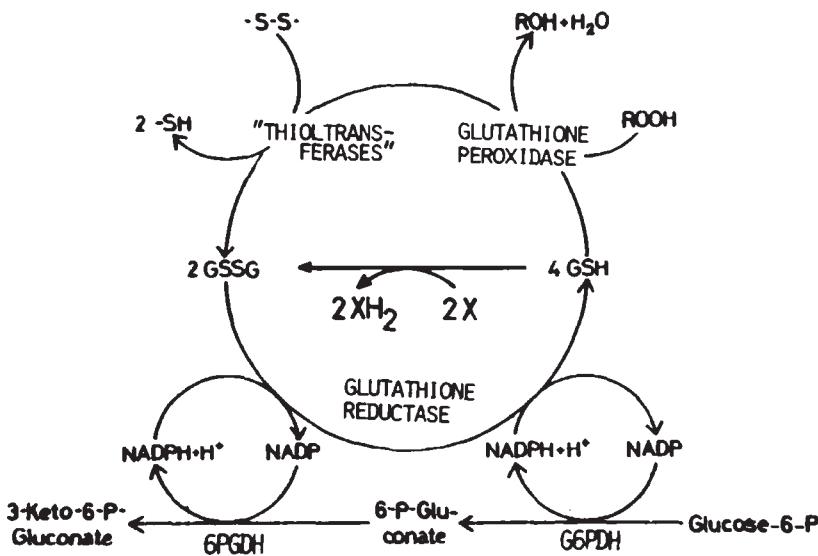


Figure 45.1 The glutathione redox cycle in red blood cells. The cycle provides reducing equivalents for the detoxification of peroxides and of oxygen centred radicals such as O₂[·] and ROO[·]. These radicals can react directly with GSH; the resulting thiyl radicals (GS[·]) combine to give GSSG (Sies, 1989). The small cycles indicate that the reduction of glutathione disulphide is based on the NADPH-regenerating enzymes, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). The X in the centre denotes a redox-cycling agent, which means that XH₂ undergoes autoxidation. A typical example of X is divicine (2, 4-diamino-5, 6-dihydroxypyrimidine), the antimalarial principle of fava beans (Golenser and Chevion, 1989).

(Schirmer *et al.*, 1987). It should be noted that oxidants exert indirect effects as well. For instance, GSSG is a potent inhibitor of glutaredoxin (Holmgren, 1979) which means that DNA synthesis is likely to be affected by oxidative stress.

PROTECTION AGAINST MALARIA BY IMPAIRED ANTIOXIDATIVE CAPACITY

Inherited or acquired conditions which predispose erythrocytes to oxidative stress offer partial protection against *P. falciparum* infections (Hunt and Stocker, 1990). The best-studied example of impaired antioxidant capacity in man is favism or drug-sensitive glucose-6-phosphate dehydrogenase (G6PDH) deficiency (Golenser and Chevion, 1989). A major function of G6PDH is the supply of NADPH as a substrate of the glutathione reductase-catalysed reaction (Figure 45.1). Consequently, inhibitors of erythrocyte GR are expected to mimic G6PDH deficiency.

Experiments on *P. falciparum* cultures support this notion: erythrocytes were treated with the specific inhibitor carmustine (BCNU) and then tested as host cells for *P. falciparum* (Zhang *et al.*, 1988). When the GR-level was below 20 mU/ml

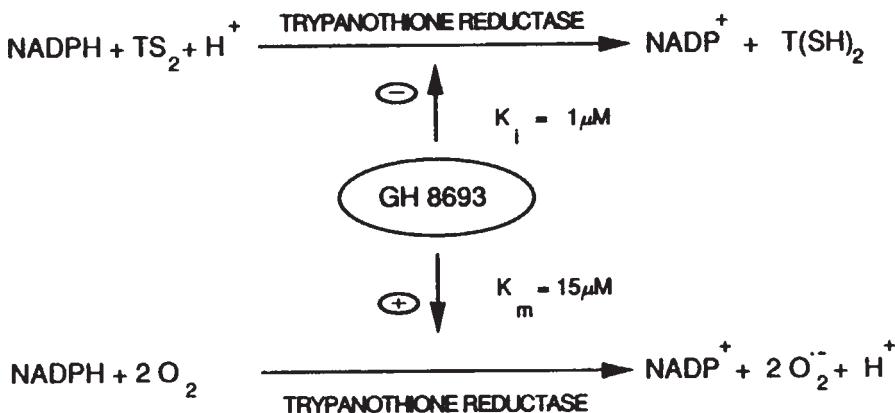


Figure 45.2 GH 8693 as a turncoat inhibitor of *T. cruzi* trypanothione reductase. (2, 3-Bis(3-(2-amidinohydrazono)-butyl)-1, 4-naphthoquinone (GH 8693), a trypanocidal compound synthesized by G.B. Henderson (*Henderson et al.*, 1988), is an effective inhibitor of *T. cruzi* TR ($K_{I^{50\%}}=1 \mu\text{M}$). At higher concentrations it induces an oxidase activity in the enzyme leading to the generation of reduced oxygen species that are toxic; hence the name turncoat inhibitor (*Jockers-Scherübl et al.*, 1989).

viable. This is consistent with the observation that red cells without detectable GR activity can fulfil their physiological functions (*Schirmer et al.*, 1989).

ERYTHROCYTE GLUTATHIONE REDUCTASE AS A DRUG TARGET

These and other findings encouraged us to study erythrocyte GR as a potential target of antimalarial drugs. When choosing a host enzyme as a drug target, one consideration was that drugs directed against parasitic structures also select for drug resistance among the parasites. This is much less the case for pharmacological agents directed against host proteins.

Glutathione reductase fulfils the requirements for the methodology of drug design since the following structural features are known: the amino-acid sequence (*Krauth-Siegel et al.*, 1982); the three-dimensional geometry—now at 154pm resolution (*Karplus and Schulz*, 1987); the substrate-binding sites (Figure 45.3) as well as the stereochemistry of catalysis (*Pai and Schulz*, 1983; *Karplus and Schulz*, 1989). The crystal structure of the FAD-free apoenzyme has also been determined (*Ermler*, 1989). This structure must be taken into consideration in drug-design studies since apoGR can be the predominant form of the protein in malaria-afflicted countries where riboflavin deficiency is often endemic (*Becker et al.*, 1991). The cDNA of human GR has been recently cloned and expressed in *Escherichia coli* (*Bücheler et al.*, 1990; *Tutic et al.*, 1990). Thus the methods of directed mutagenesis can be applied to studying structure-activity relationships in greater detail as well as for predicting the probability of drug resistance. Finally, the structures of a number

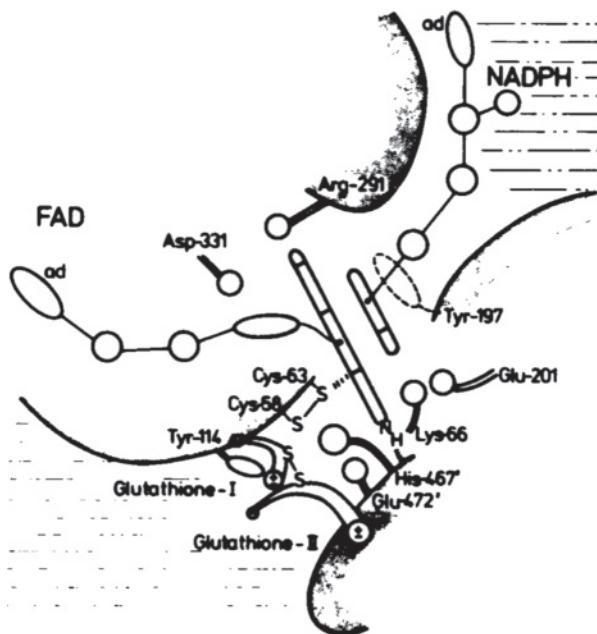


Figure 45.3 Sketch of the active site of glutathione reductase. The dinucleotides FAD and NADPH bind to the protein in elongated conformations; *ad* indicates the adenine moieties of FAD or NADPH. The flavin ring (seen edge-on) forms a chemical and mechanical barrier between the nicotinamide compartment and the glutathione disulphide compartment. GSSG is bound to residues of both subunits. The active site disulphide Cys-58—Cys-63 is located between flavin and GSSG. During catalysis the enzyme E is reduced to EH_2 which is a stable intermediate characterized by an active site dithiol. The nascent thiol of Cys-58 forms a hydrogen bond to His-467' of the other subunit. (Reproduced, with permission, from Pai and Schulz (1983)).

of enzyme-inhibitor complexes have been crystallized and analysed; these inhibitors are expected to serve as models for tailoring drugs (Schirmer *et al.*, 1989; Janes and Schulz, 1990).

Enzyme-inhibitor complexes of known three-dimensional structure

Glutathione reductase is inhibited by a number of compounds which have little chemical similarities among themselves. Of particular pharmacological interest are turncoat inhibitors such as paraquat or trinitrobenzenesulphonate. These compounds convert GR from an antioxidant to a pro-oxidant enzyme by inhibiting its GSSG-reducing activity and promoting its otherwise negligible oxidase activity (Carlberg and Mannervik, 1980; Schirmer *et al.*, 1987). Figure 45.2 shows the action of a turncoat inhibitor of TR. Unfortunately, the mode of binding is not known for any of these subversive compounds. Here we shall restrict ourselves to inhibitors for which the geometry of binding has been established by X-ray crystallography.

Carbamoylation of Cys-58 by carmustine derivatives

As discovered by Frischer and Ahmad (1977) treatment of patients with the cytostatic agent BCNU (1, 3-bis (2'-chloroethyl)-l-nitrosourea; carmustine) leads to complete inhibition of glutathione reductase in red cells. The activities of 19 other tested erythrocytic enzymes were not impaired. The nature of the modification by BCNU could not be established by chemical analyses. Consequently, we modified GR with BCNU *in vitro* and crystallized the inactivated enzyme species (Karplus *et al.*, 1988). X-ray diffraction analysis showed that the modified enzyme contains an S-carbamoyl moiety at the active-site thiol Cys-58 (Figure 45.4). It should be noted how well the carbamoyl group fits into its site making van der Waals contacts with a number of residues and forming hydrogen bonds with His-467' and with one of the numerous fixed water molecules. Structural details of this kind represent the fine tuning in the art of drug design (Hol, 1986).

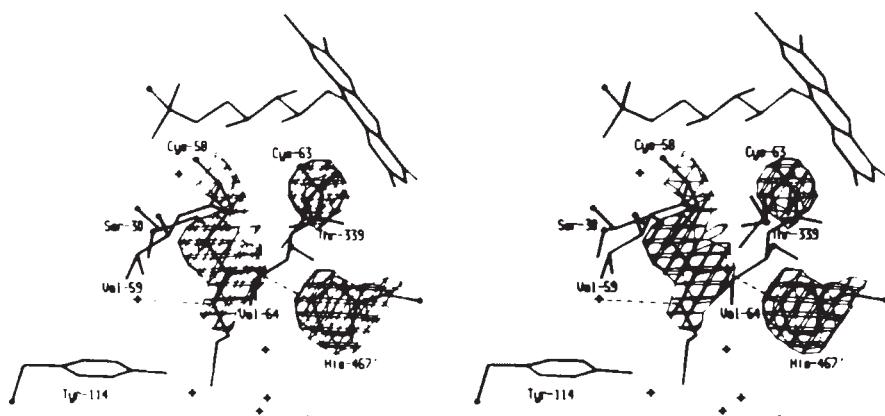


Figure 45.4. Stereo view of the structure of BCNU-modified glutathione reductase as revealed by X-ray diffraction analysis. The structure on top is the FMN moiety of FAD. Tightly bound water molecules are indicated by '+'. The residues Cys-58, Cys-63 and His-467' are marked by their electron densities. The sulphur atom of Cys-58 carries the modifying carbamoyl group which is also fixed by a hydrogen bond to His-467' (Karplus *et al.* 1988).

HeCNU, a 2'-dechloro, 2'-hydroxy analogue of BCNU, inactivates glutathione reductase by hydroxyethylating, i.e. by alkylating rather than by carbamoylating, the enzyme (Schirmer *et al.*, 1987). HeCNU has a curative effect on *P. vinckeii* malaria in rodents (Schirmer *et al.*, 1984).

Substrate analogues

Glutathione derivatives are not necessarily ideal starting points for the design of new GR inhibitors. A point in case is *S*-(2, 4-dinitrophenyl)glutathione (DNPG). The

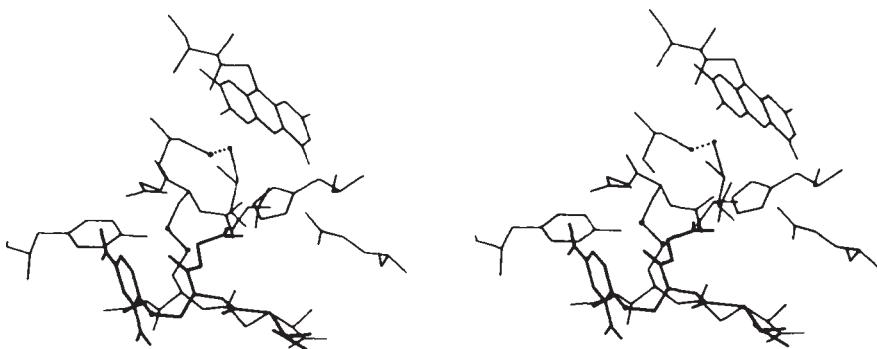


Figure 45.5. Stereo view of a model of S-2, 4, dinitrophenylglutathione (DNPG) as bound to the GSSG-binding site. Thick lines represent DNPG, the γ -glutamyl part being located at the lower right-hand side. GR is represented by Tyr-114, the redox active disulphide bridge Cys-58-Cys-63, His-467', Glu-472', and the riboflavin moiety of FAD. The GSSG molecule, as bound to glutathione reductase, is inserted as a reference. All molecular parts drawn with thin lines can be identified in Figure 45.2, where they are labelled and given in approximately the same view (Reproduced, with permission, from Bilzer *et al.* (1984)).

major binding site of this glutathione conjugate overlaps with the binding site of the substrate GSSG (Figure 45.5). Since GSSG can be regarded as the homoconjugate of glutathione, it is surprising that the glutathione moiety of bound DNPG does not coincide with either of the two glutathione moieties of GSSG. Only the binding modes of the γ -glutamyl moiety of DNPG and one γ -glutamyl moiety of GSSG are identical. Thus the overall orientation of the glutathione portion of this conjugate depends on the non-glutathione moiety (Bilzer *et al.*, 1984). Consequently the binding modes and affinities are expected to vary greatly among glutathione conjugates. As discussed in a recent crystallographic study by Janes and Schulz (1990) the incorrect binding of substrate analogues to GR is a meaningful molecular mechanism which helps avoid the processing of unphysiological ligands.

Flavins with antimalarial activity

A typical representative of this group is 10-(4' chlorophenyl)-3-methylflavin (Hunt and Stocker, 1990). When bound to glutathione reductase, this compound does not compete for the binding site of the flavin ring of FAD (Becker *et al.*, 1990). Rather, the data suggest that the inhibitor is sandwiched between the aromatic side-chains of Phe-78 and Phe-78' in the large intersubunit cavity of GR. The situation would be analogous to that with the structurally similar dye safranin (Figure 45.6), where one inhibitor molecule at this position can influence both catalytic sites and both GSSG-binding sites of the enzyme (Karplus *et al.*, 1989; Becker *et al.*, 1990).

It is planned to develop flavin analogues with extended side-chains in order to weaken the tight parts of the interface structure between the two GR subunits (Karplus and Schulz, 1987). As is clear from inspecting the catalytic site of the

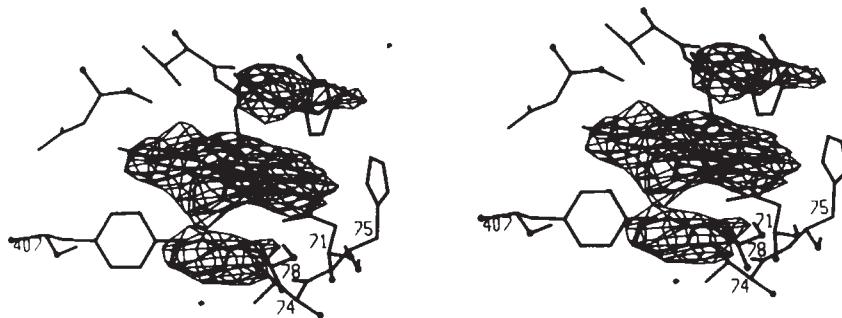
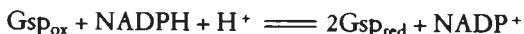
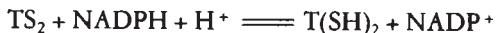


Figure 45.6. Stereo view of the safranin-binding site. Phenosafranin binds between Phe-78, Phe-78', His-75 and His-75'. These residues located in a large cavity between the subunits show high disorder in the absence of the ligand. When the tricyclic ring of safranin binds between the two phenylalanines these residues reorient and become rigid. The mechanism is reminiscent of an induced fit. (Reproduced, with permission, from Karplus *et al.* (1989)).

enzyme, (Figure 45.3) monomeric GR is completely inactive. This geometry exemplifies a general challenge to molecular pharmacology: protein-protein interaction inhibitors (contact peptide mimetics) are highly specific modulators of a given protein function and thus a promising domain for the methods of drug design. Another aspect of the safranin-binding site concerns the predictability of drug resistance. A single mutation, e.g. Phe-78→Gly-78, is expected to render glutathione reductase insensitive to all inhibitors bound at this position. It is unlikely that such an amino acid substitution will affect other biological properties of the enzyme. This point is currently being studied using specifically mutagenized glutathione reductase (U.S.Bücheler, unpublished results).

THIOL METABOLISM OF TRYPANOSOMATIDS

In contrast to nearly all other eukaryotes which have a thiol metabolism based on the glutathione/glutathione reductase system (Schirmer *et al.*, 1989), trypanosomatids do not possess glutathione reductase. The main thiol compounds are conjugates between glutathione and spermidine, namely monoglutathionylspermidine (Gsp_{red}) and bis(glutathionyl)spermidine (trypanothione, $\text{T}(\text{SH})_2$) which are kept in the thiol state by the enzyme trypanothione reductase (TR) (Shames *et al.*, 1986; Krauth-Siegel *et al.*, 1987; and see Chapter 44):



Monoglutathionylspermidine has been found also in *E. coli* which conjugates spermidine nearly quantitatively to glutathione during the stationary phase (Tabor and Tabor, 1975). In contrast, trypanothione has been detected only in trypanosomatids (Fairlamb *et al.*, 1985). The enzyme which synthesizes trypanothione from

glutathione and spermidine has recently been purified from the insect parasite *Crithidia fasciculata* (Henderson *et al.*, 1990).

The detoxication of hydroperoxides in trypanosomatids is probably based on a trypanothione-dependent peroxidase (Penketh and Klein, 1986; Henderson *et al.*, 1987) and not on a glutathione peroxidase as it is the case in other eucaryotes (Figure 45.1). Trypanothione synthetase, trypanothione reductase and trypanothione peroxidase are parasite-specific enzymes and thus regarded as promising targets for a rational drug design against trypanosomatids.

Oxidative stress as a therapeutic strategy against trypanosomes

Trypanosomes and leishmanias are known to be more sensitive towards H_2O_2 and other reduced oxygen species than are their hosts (Penketh and Klein, 1986). Nifurtimox (Lampit[®]) and benznidazole (Radanil[®]) used in the treatment of acute Chagas' disease are redox-cycling compounds which generate reactive oxygen species. Other trypanocidal drugs act indirectly as inducers of oxidative stress by interfering with the trypanothione metabolism of the parasite: buthionine sulphoximine disturbs the synthesis of glutathione by inhibiting the γ -glutamylcysteine synthetase; difluoromethylornithine inhibits ornithine decarboxylase and thus interferes with spermidine synthesis; the trivalent arsenical drug melarsen oxide used in the treatment of sleeping sickness forms a stable 1:1 adduct with the dithiol trypanothione; in addition it is an inhibitor of TR and GR (Fairlamb *et al.*, 1989).

Properties of trypanothione reductase

Trypanothione reductase has been isolated from the insect parasite *Crithidia fasciculata* (Shames *et al.*, 1986) and from *T. cruzi* (Krauth-Siegel *et al.*, 1987). The genes of TR from *T. congolense* (Sullivan *et al.*, 1989) and from *T. cruzi* (Sullivan and Walsh, 1991) have been cloned and expressed in *E. coli*. Trypanothione reductase, an FAD-cystine-oxidoreductase, is closely related to glutathione reductase (Table 45.1), lipoamide dehydrogenase and the bacterial enzyme mercuric ion reductase (Fox and Walsh, 1982; Krauth-Siegel *et al.*, 1989; Williams, 1990). Fitting the amino acid sequence of *T. congolense* TR into the three-dimensional structure of human glutathione reductase indicates that most of the structurally and catalytically essential residues are identical in the two enzymes (Sullivan *et al.*, 1990). The most important difference between TR and GR is their mutually exclusive specificity towards their disulphide substrate (Table 45.1). A crucial amino acid residue for binding glutathione to human glutathione reductase is Arg-37 which makes a close contact with the carboxylate moiety of the glycine residue of glutathione-I (Figure 45.3). In TR this residue is replaced by Trp-21. In contrast to the human enzyme, *E. coli* GR also accepts trypanothione as a disulphide substrate. This agrees well with the finding that Arg-37 is replaced by an asparagine in the bacterial enzyme. Monoglutathionylspermidine, Gsp_{ox}, has not yet been tested as a substrate of *E. coli* GR; since it is an authentic metabolite of *E. coli* it may turn out to be an even better substrate than trypanothione.

Trypanothione reductase inhibitors

A number of drugs have been shown to be inhibitors of *T. cruzi* trypanothione reductase *in vitro*. The cytostatic agent BCNU which is a covalent inhibitor of glutathione reductase and lipoamide dehydrogenase (Ahmad and Frischer, 1985) also inhibits TR. In analogy to the situation with glutathione reductase (Figure 45.4) the drug most probably carbamoylates one of the active-site cysteines in the reduced enzyme species (Jockers-Scherübl *et al.*, 1989).

GH 8693, the turncoat inhibitor of TR shown in Figure 45.2, exhibits a number of desirable features: it is known to be trypanocidal; it does not affect human GR and, when in contact with TR, it would lead to the generation of toxic oxygen species within the parasite.

The antiparasitic drug mepacrine (quinacrine) is known to lyse bloodstream *T. cruzi* at concentrations between 10 and 100 μM (Hammond *et al.*, 1984). We tested whether this effect could be due to the inhibition of trypanothione reductase. The kinetics were measured at 25°C in 40 mM Hepes, 1 mM EDTA, 100 μM NADPH, pH 7.5; the TS₂ concentration was varied between 20 and 200 μM at constant concentrations of 30, 50 and 100 μM mepacrine. The Lineweaver-Burk plot showed a competitive type of inhibition, the K_i being 25 μM . Interestingly, the drug had no effect on glutathione reductase, although a number of other tricyclic compounds have been found to inhibit this enzyme (see for instance, Figure 45.6). The specific binding of mepacrine to TR is probably related to the side-chain which resembles a natural polyamine.

The immunosuppressive agent 15-deoxyspergualin was tested as an inhibitor of *T. cruzi* TR because of its structural similarity with glutathionylspermidine. It competitively inhibits the reduction of 1*N*-glutathionylspermidine with a K_i of 60 μM (Krauth-Siegel *et al.*, 1990). In this context it will be of interest to study if the glutathionylspermidines of *T. cruzi* contribute to the general immunosuppression observed in acute Chagas' disease. For these studies 8*N*-glutathionylspermidine is probably the best candidate since it resembles 15-deoxyspergualin even more closely than the 1*N* isomer.

OUTLOOK

The next major step is the structural analysis of crystalline TR-inhibitor complexes. This information, as well as the geometric comparison of TR with GR, is expected to provide guidelines for the development of new specific inhibitors as drug candidates. Of special interest are:

1. interface peptide mimetics;
2. turncoat inhibitors;
3. carbamoylating agents of lower toxicity than BCNU;
4. compounds with favourable permeation properties; and
5. chimeric inhibitors which contain two ligands covalently linked by a spacer. The length and properties of the spacer have to be designed on the basis of the enzyme

structure. As exemplified for a disubstrate inhibitor of red blood cell adenylate kinase, the association constant of a chimeric inhibitor corresponds to the product of the individual association constants (Feldhaus *et al.*, 1975).

ACKNOWLEDGEMENTS

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46. Polyamine biosynthesis as a target for the chemotherapy of trypanosomatid infections

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AFRICAN TRYPARASITIASIS

Trypanosomiasis (sleeping sickness) remains a major health problem in a large portion of central Africa (*Bulletin of the WHO*, 1986). Until the last decade, no new drugs had been introduced for the treatment of this disease in over 40 years (Sjoerdsma and Schechter, 1984). While the old drugs, including Suramin, pentamidine and melarsoprol, remain remarkably effective in most cases of sleeping sickness, the toxic side-effects of these agents often limit their use (Apted, 1980). Indeed, treatment with melarsoprol, the only drug effective in the treatment of late-stage trypanosomiasis involving the central nervous system, results in 5–10 per cent mortality due to untoward reactions (Haller *et al.*, 1986). Furthermore, as resistance of the organisms to the drug has risen in recent years (Ruppel and Burke, 1977; Ginoux *et al.*, 1984), new, safer chemotherapies are sorely needed.

The polyamines spermidine and spermine and the diamine, putrescine, have been shown to be involved in rapid cell proliferation, although the exact molecular mechanisms of action of these molecules are not yet clearly defined (Pegg and McCann, 1982, 1988; Pegg, 1986). Most of the early work on polyamine metabolism was done with mammalian cells/tissues and bacteria (Pegg and McCann, 1982; Tabor and Tabor, 1985), but in the late 1970s the presence of polyamines and their biosynthetic enzymes were clearly demonstrated in trypanosomatids (Bacchi *et al.*, 1977). In general, polyamine biosynthesis in trypanosomatids is similar to that found in the mammalian hosts (Figure 46.1), but there are differences, as noted below, which seem to be important in determining the antiprotozoal activity of inhibitors of this pathway. See also Chapter 43.

Eflornithine (α -difluoromethylornithine; DFMO; Ornidyl) was synthesized at the Merrell Dow Research Institute as an enzyme-activated, irreversible inhibitor of the first enzyme in polyamine biosynthesis, ornithine decarboxylase (ODC)

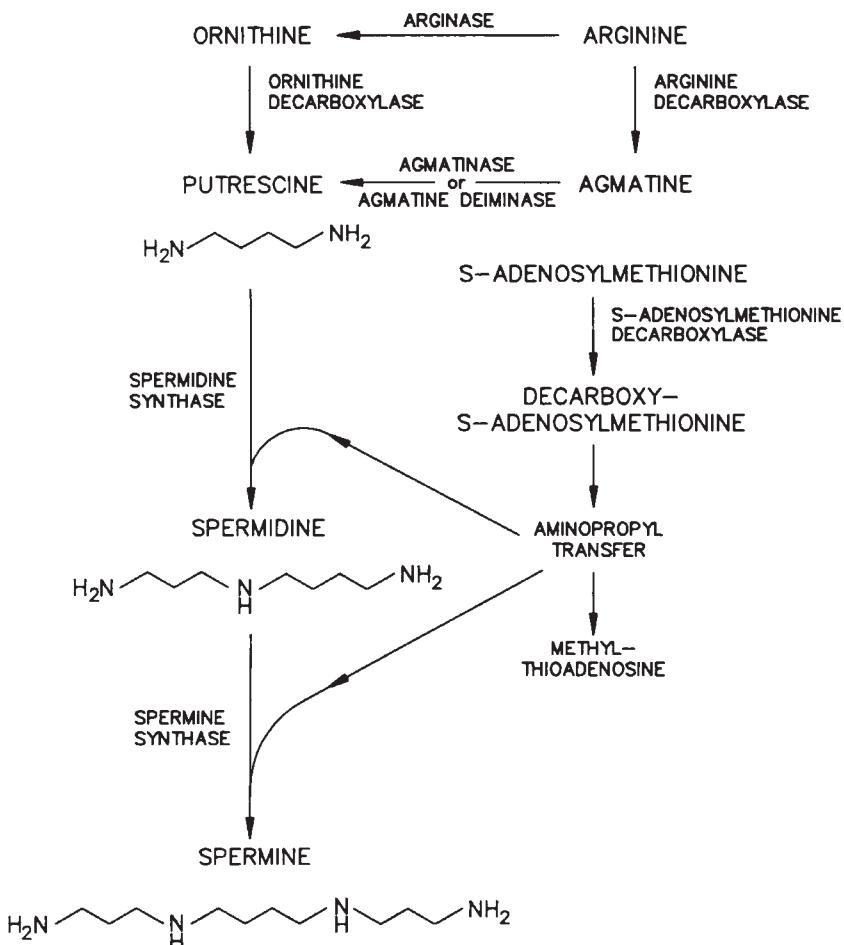


Figure 46.1. Pathways of polyamine biosynthesis.

(Metcalf *et al.*, 1978; Bey *et al.*, 1987). Although originally synthesized as an anti-tumour compound, it was discovered in 1979 that administration of DFMO to mice infected with *Trypanosoma brucei* resulted in complete cures of these infections (Bacchi *et al.*, 1980). Other African trypanosomes, including *T. rhodesiense* (McCann *et al.*, 1981a), *T. gambiense* (McCann *et al.*, 1981b) and *T. congolense* (Karbe *et al.*, 1982) were also found to be susceptible to DFMO. This new drug was remarkable in its ability to cure not only early bloodstream-stage trypanosome infections but also late-stage murine central nervous system infections which were resistant to Berenil (diminazene acetarate) (Bacchi *et al.*, 1987). Because DFMO had already been used extensively in clinical trials for antitumour activity, the laboratory findings with DFMO in various models of animal trypanosome infections led to the rapid trial of DFMO against human trypanosomiasis within 2 years. Treatment of human *T. gambiense* infections was extremely successful, with both

early and late stage disease being susceptible to DFMO (Sjoerdsma and Schechter, 1984; Schechter *et al.*, 1987). Coupled with this huge clinical success, its almost complete lack of serious toxic side-effects makes DFMO the main compound for future treatment of human trypanosomiasis.

Several lines of investigation have been followed in an attempt to explain the selective effects of DFMO against African trypanosomes. The inactivation of *T. brucei* ODC *in vitro* ($K_i=130\text{ }\mu\text{M}$) (Bitonti *et al.*, 1985) was similar to the inactivation of mammalian ODC ($K_i=39\text{ }\mu\text{M}$) (Metcalf *et al.*, 1978), indicating that a marked difference in affinity constant was not responsible for the selective susceptibility. It was also shown that the uptake of DFMO by the trypanosome occurred via passive diffusion (Bitonti *et al.*, 1986a) just as it occurred in mammalian cells (Erwin and Pegg, 1982), thus ruling out the possibility that the drug was concentrated to higher intracellular levels in the parasite. It was shown that, as expected, exposure to DFMO *in vivo* resulted in an almost complete inhibition of trypanosomal ODC and a depletion of trypanosomal putrescine and spermidine of the same magnitude as seen in mammalian cells (Mamont *et al.*, 1978; Bacchi *et al.*, 1983). Therefore, other mechanisms for the DFMO selectivity were sought and several peculiarities, both biological and biochemical, of the trypanosomes were identified which, most likely in combination, could explain the selectivity of DFMO.

The simplest of the possible mechanisms involves the rapid doubling time of trypanosomes of 6–8 h. Because polyamines are required for rapid cell proliferation, the more rapid the rate of replication, the more susceptible a cell would be. The African trypanosome also lacks spermine and may be considered to be somewhat ‘polyamine deficient’. Any disturbance of this potentially compromised condition might lead to death of the organism. It was also found that, upon polyamine depletion, there were changes in the intracellular pools of decarboxylated *S*-adenosyl-L-methionine (dcAdoMet) and *S*-adenosyl-L-methionine (AdoMet), both metabolites increased enormously with >1000-fold increase for dcAdoMet and 50-fold increase for AdoMet (Bacchi *et al.*, 1983; Yarlett and Bacchi, 1988). Changes in AdoMet of this magnitude do not occur in mammalian cells treated with DFMO, although dc AdoMet in mammalian cells is elevated markedly (Haegele *et al.*, 1987). While the cause-effect relationship of these effects of DFMO on AdoMet metabolism is not known, it is interesting that methylating agents have recently been shown to be trypanocidal (Penketh *et al.*, 1990). It is possible that AdoMet and dcAdoMet, at the concentrations attained after DFMO treatment, cause aberrant methylation patterns in trypanosomes leading to cell death.

It is known that a strong host immune response, directed against the variant specific glycoprotein (VSG), which completely covers the plasma membrane of the trypanosome (Vickerman, 1985), is mounted against African trypanosomes leading to cyclical parasitaemia. DFMO has been shown to inhibit the synthesis of the VSG (Bitonti *et al.*, 1988) and thus possibly limits the ability of the trypanosome to shift its antigenicity as it normally does during progression of the infection as a means by which to avoid immune destruction. That the immune system reaction to the

trypanosome is critical to the resolution of an infection during DFMO treatment comes from two studies involving immunosuppressed mice (deGee *et al.*, 1983) and rats (Bitonti *et al.*, 1986b). Both studies showed that in the absence of a full antibody response, clearance of parasitaemia occurred, but complete cure of the infections did not.

Recently, a novel spermidine-containing co-factor, termed trypanothione, was identified in trypanosomatids (Fairlamb *et al.*, 1985; and see Chapters 44 and 45). This co-factor bis(glutathionyl)spermidine, functions to maintain the main intracellular reducing agent, glutathione, in its reduced form and thus protects the organism from oxidative stress. Trypanothione has been shown to be depleted along with spermidine during DFMO treatment (Fairlamb *et al.*, 1987) and this depletion may leave the trypanosome susceptible to oxidative damage.

Another possible mode of action for DFMO lies in its ability to cause differentiation of *T. brucei* into so-called intermediate and short stumpy forms of bloodstream trypomastigotes (Giffin *et al.*, 1986). The intermediate form induced by DFMO can become further differentiated to a procyclic form adapted to life in the tsetse fly midgut, but the short stumpy form is probably a terminal form that dies spontaneously or is cleared from the host bloodstream by the immune response. The relationship of this form to that which occur naturally in infections is not clear. Regardless of its contribution to the curative effects of DFMO, this differentiation process is a naturally occurring phenomenon which appears to be induced and can be studied experimentally with the aid of DFMO.

While DFMO would seem like an ideal drug for the treatment of sleeping sickness, it is currently administered at relatively high doses and has not yet been shown to be effective in *T. rhodesiense* infections. Consequently, the development of a more potent antitrypanosomal agent with broader spectrum of activity became a desirable goal. Recently we have described the antitrypanosomal effects of an inhibitor of S-adenosyl-L-methionine decarboxylase (AdoMet DC) and have shown that this second-generation inhibitor of polyamine biosynthesis is not only markedly more potent than DFMO in the treatment of murine *T. brucei* infections but is also effective against multidrug-resistant *T. rhodesiense* that do not respond fully to DFMO (Bitonti *et al.*, 1990).

AdoMet DC is another obligatory enzyme in the biosynthesis of spermidine (Figure 46.2). It produces dcAdoMet which then functions as a donor of an amino-propyl moiety to one of the amines of putrescine in a reaction catalysed by spermidine synthase. AdoMet DC has been a target of drug development since it was shown that methylglyoxal-bis(guanylhydrazone) (MGBG), a drug with significant antitumour and antitrypanosomal effects (Mihich, 1963; Chang *et al.*, 1978), is also an inhibitor of AdoMet DC (Williams-Ashman and Schenone, 1972; Bitonti *et al.*, 1986a-c). Other well-known trypanocides, pentamidine and Berenil, have also been shown to inhibit trypanosomal AdoMet DC (Bitonti *et al.*, 1986c). Thus, it was logical to test a recently synthesized inhibitor of AdoMet DC, 5'-{[(Z)-4-amino-2-but enyl]methylamino]-5'-deoxyadenosine (MDL 73811) (Casara *et al.*, 1989), for antitrypanosomal activity.

This compound was found to be a potent inhibitor of trypanosomal AdoMet DC ($K=1.5\text{ }\mu\text{M}$) and was apparently enzyme activated and caused irreversible inactivation of the enzyme (Bitonti *et al.*, 1990). Putrescine was increased two- to three-fold and spermidine was decreased by 50 per cent in trypanosomes exposed to MDL 73811 for 4 h *in vivo* as would be expected if, in fact, AdoMet DC was inhibited. The changes in polyamines were only modest and slow to occur considering that trypanosomal AdoMet DC activity was almost completely inhibited within 10min of injection of MDL 73811 to infected rats. The most remarkable biochemical change that occurred after exposure of trypanosomes to MDL 73811 *in vivo* was a rapid (within 1 h) increase in intracellular AdoMet concentration (20- to 50-fold). The increased AdoMet correlated temporally with a reduction in parasitaemia that occurred in rats injected intraperitoneally with 50 mg kg⁻¹ MDL 73811. It is possible that the rapid rise in AdoMet may be connected to disappearance of parasites in treated rats because similar large changes in AdoMet occur after DFMO treatment (see below). The mechanism of the antitrypanosomal effect of MDL 73811 will be of interest for future investigations.

MDL 73811 was found to be remarkably effective against *T. brucei*. Administration of 20–50 mg kg⁻¹ MDL 73811 given intraperitoneally twice daily for 4 days resulted in cures (>30 days survival after infection) of virtually all mice infected with the highly virulent laboratory strain 110/EATRO. A high daily dose (150 mg kg⁻¹) of MDL 73811 given once daily for 4 days was also sufficient to cure these infections. In contrast, DFMO must be administered at a concentration of 2 per cent (w/v) in drinking water to obtain a 100 per cent cure rate in this murine model infection. These data indicate that MDL 73811 is at least 100 times as potent as DFMO against *T. brucei* in mice; a daily dose of 40 mg kg⁻¹ MDL 73811 (intraperitoneal) vs. approximately 5000 mg kg⁻¹ DFMO (per os) being necessary for similar curative activity.

MDL 73811 was tested further against a multidrug-resistant strain of *T. rhodesiense*, KETRI 2538 (recently obtained from the Kenya Trypanosomiasis Research Institute strain bank). Strain KETRI 2538 responded poorly to DFMO with only a 35 per cent cure rate (16 of 46 mice in extended studies) when DFMO was administered as a 2 per cent solution in drinking water for 5 days (Table 46.1). This strain was found also to be apparently somewhat resistant to MDL 73811 (50 mg kg⁻¹ 3 times daily for 5 days), with only 2 of 7 mice being cured using this dosing regimen. However, it was of considerable interest to find that combinations of MDL 73811 and relatively low doses of DFMO (0.25–1 per cent) were almost completely effective in curing *T. rhodesiense* (KETRI 2538) infections.

It was apparent that *T. rhodesiense* was less susceptible than *T. brucei* to MDL 73811. In preliminary experiments it was found that the plasma half-life of MDL 73811 in rats and mice was exceedingly short (about 10min). It is possible that the difference in susceptibility of the two species of trypanosomes was due to differences related to host pharmacokinetics and uptake of the drug by the organism and did not indicate a true drug resistance. Therefore an experiment was performed in which MDL 73811 was administered to *T. rhodesiense*-infected mice using surgically implanted mini-osmotic pumps which slowly release drug over a 7-day period. In these

Table 46.1. Treatment of multidrug-resistant *T. rhodesiense* (strain 2538) with a combination of DFMO and MDL 73811.^a

Treatment (dose)	Mean (range) No. of days to death	No. of mice cured/total
None	14 (11–16)	0/8
DFMO (2%)	24, 27	5/7 (16/46)
MDL 73811 (50 mg kg ⁻¹)	21, 23, 23, 25, 27	2/7
DFMO (2%)–MDL 73811 (50 mg kg ⁻¹)		7/7
DFMO (1%)–MDL 78311 (50 mg kg ⁻¹)		7/7
DFMO (0.5%)–MDL 73811 (50 mg kg ⁻¹)		7/7
DFMO (0.25%)–MDL 73811 (50 mg kg ⁻¹)	31	6/7

^a mice were infected with 10⁵ trypanosomes and drug treatments were begun 72 h later. MDL 73811 was given by intraperitoneal injection 3 times daily for 5 days, and DFMO was given in drinking water for 5 days. Cured mice lived longer than 60 days post-infection. From Bitonti *et al.* (1990)

experiments it was found that infected mice could be cured with either 20 or 50 mg kg⁻¹ MDL 73811 per day. It is possible that MDL 73811 will have a broader spectrum of efficacy than DFMO, being useful in cases of East African trypanosomiasis (Rhodesian sleeping sickness) that are now, in some cases, almost untreatable.

LEISHMANIASIS

Chemotherapy of leishmanial infections is currently limited to the use of two pentavalent antimonials, Glucantime (N-methylglucamine antimonate) and Pentostam (sodium stibogluconate) and two back-up compounds, pentamidine and amphotericin B (Berman, 1988). While generally effective, these drugs suffer from frequent toxic side-effects and their administration is cumbersome because of the need for injection. Therefore, new chemotherapeutic agents are needed.

Work with polyamine biosynthesis in *Leishmania* has not been as extensive as with African trypanosomes but, nonetheless, there has been progress in this area suggesting that the biosynthetic pathway in these organisms may also be amenable to chemotherapeutic intervention. Initial reports suggested that DFMO had no significant effects on *Leishmania*; the drug did not block replication of either *L. mexicana mexicana* promastigotes *in vitro* (Coombs *et al.*, 1983) or *L. donovani* amastigotes *in vivo* (Hanson *et al.*, 1982). However, two later studies showed that addition of DFMO to culture medium at 50–200 µM resulted in inhibition of *L. donovani* promastigote growth (Kaur *et al.*, 1986; Carrera-Ferrer *et al.*, 1987). A recent study examined the effects of DFMO on *L. infantum* in mice and found the drug to be quite effective in comparison to a standard antileishmanial drug, Glucantime (Gradoni *et al.*, 1989). In this study, mice were treated subcutaneously with 100 mg kg⁻¹ DFMO daily for 5 days and 85 per cent suppression of parasite burden was obtained. With longer treatment (40 consecutive days with 200 mg kg⁻¹ DFMO, subcutaneously) 98 per cent suppression of parasite burden was obtained. The latter studies remain unconfirmed, but do suggest that the use of DFMO *in vivo* should be more fully explored.

In recent years we have been investigating other means by which to interfere with polyamine biosynthesis and/or function. It has been shown that analogues of the natural polyamines can repress the enzymes (ODC and AdoMet DC) of polyamine biosynthesis, much as natural polyamines would, but they do not subserve the other physiological functions of the polyamines (Porter and Sufrin, 1986). Thus, a series of polyamine analogues was synthesized and tested for antiprotozoal activity. Out of this series, the bis(benzyl)polyamine analogues were identified initially as possessing potent antimalarial effects (Bitonti *et al.*, 1989; and see Chapter 47). More recently it was found that one of these bis(benzyl)polyamines (MDL 27695; $C_6H_5CH_2NH(CH_2)3NH(CH_2)3NHCH_2C_6H_5$) also had significant activity against *L. donovani* amastigotes both *in vitro* and *in vivo* (Bauman *et al.*, 1990).

Initial studies with *L. donovani*-infected mice treated intraperitoneally with MDL 27695 for 5 days showed that the drug suppressed parasite burdens in a dose-dependent manner with an ED₅₀ of 2.5 mg kg⁻¹ (MDL 27695 given 3 times daily to a total dose of 7.5 mg kg⁻¹ day⁻¹). Maximum suppression was obtained with 15 mg kg⁻¹ MDL 27695. At this dose administered intraperitoneally twice daily for 10 days, MDL 27695 resulted in a parasite suppression of 99.9 per cent and so the drug's efficacy compared favourably with that of Pentostam. Additional experiments in hamsters demonstrated that MDL 27695 was as efficacious as Glucantime against *L. donovani* in this alternate animal model and that MDL 27685 was effective against a strain of parasite with a high level of antimony resistance. Recent experiments have also shown that MDL 27695 has considerable activity when administered orally; 50 mg kg⁻¹ given twice daily for 14 days resulting in >95 per cent suppression of *L. donovani* in mice. Oral administration would be a distinct improvement over the presently available drugs. MDL 27695 is thus a promising lead for future development.

SOUTH AMERICAN TRYPANOSOMIASIS (CHAGAS' DISEASE)

Unlike the African trypanosome, this South American variety of trypanosome has not been well studied with regard to polyamine biosynthesis. Initial attempts to treat *T. cruzi* infections in mice with DFMO failed (Hanson *et al.*, 1982). More recently it was found that inhibitors of arginine decarboxylase (α -difluoromethylarginine and α -fluoromethylagmatine), the first enzyme in an alternate route for polyamine biosynthesis in bacteria and plants (Bitonti *et al.*, 1987; and see Figure 46.1), inhibited the development of *T. cruzi* amastigotes in human macrophages *in vitro* (Kierszenbaum *et al.*, 1987). This finding raises the possibility of a unique target for chemotherapy in *T. cruzi* infections because arginine decarboxylase is not present in the mammalian host cell. Further work should determine whether this approach to chemotherapy of this disease will be useful.

CONCLUSION

Polyamine biosynthesis has been a fruitful target for the development of novel chemotherapeutic agents. DFMO is the most advanced of the compounds, but a new inhibitor of AdoMet DC activity (MDL 73811) and a bis(benzyl)polyamine analogue (MDL 27695) may prove useful for the future treatment of African trypanosomiasis and leishmaniasis, respectively. Polyamine biosynthesis should be explored further with the aim of developing even more powerful chemotherapeutic tools.

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47. New approaches to the chemotherapy of drug-resistant malaria

A.J.Bitonti, P.P.McCann and A.Sjoerdsma

Malaria remains one of the most important infectious diseases in the world. A resurgence of malaria has been accompanied by a disturbing spread of chloroquine resistance to almost all geographic areas where malaria is found (WHO, 1987). Radical departure from the chemical entities of the past is necessary for the development of new chemotherapies to avoid the common problem of cross-resistance amongst various agents. New drugs such as mefloquine and halofantrine, which are effective against chloroquine-resistant *Plasmodium falciparum*, have been introduced and will certainly help this situation for the near future. Unfortunately, malaria parasites resistant to both drugs are already known (Boudreau *et al.*, 1982; Bygbjerg *et al.*, 1983; Robinson *et al.*, 1986). Therefore, it is evident that the research for novel chemotherapeutic agents remains an important endeavour. Over the past 5 years we have tried to apply some new tactics towards obtaining new antimarials. This chapter is a discussion of work on inhibition of polyamine biosynthesis/function with eflornithine alone and in combination with polyamine analogues and also of the potential use of desipramine, a tricyclic antidepressant, for the reversal of chloroquine resistance in *P. falciparum*.

EFLORNITHINE

The biosynthesis of polyamines has been a target for chemotherapeutic intervention in various cancers and, more recently, in African trypanosomiasis (see Chapter 46). Eflornithine (α -difluoromethylornithine; DFMO; Ornidyl®), an enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC), the first enzyme of polyamine biosynthesis, has proven to be remarkably effective for the treatment of human West African sleeping sickness. Because of its success against the latter disease, DFMO was tested against a variety of parasitic protozoa (Bacchi and McCann, 1987) including the malaria parasites *P. falciparum* and *P. berghei*.

Initial studies showed that high concentrations ($10 \mu\text{M}$) of DFMO inhibited *P. falciparum* proliferation *in vitro* by 70–80 per cent (McCann *et al.*, 1981). Most interesting in these studies was the finding that exposure to DFMO resulted in synchronization of the parasites at the late trophozoite stage, suggesting that a major role of the polyamines in plasmodia might be the regulation of DNA synthesis that occurs during the trophozoite to schizont transition. The growth inhibition and synchronization of the parasite was blocked by the addition of putrescine, demonstrating the specificity of the drug's effects. Some investigators have used DFMO synchronization and release from synchronization with putrescine to study changes in the parasite's biochemistry that occur during schizogony (Assaraf *et al.*, 1986, 1987).

Later studies showed that DFMO was also inhibitory to the exoerythrocytic schizogony of *P.berghei* in mice (Gillet *et al.*, 1982, 1986; Hollingdale *et al.*, 1985; Lowa *et al.*, 1986) and to sporogony of *P. berghei* in the mosquito vector (Gillet *et al.*, 1983). These studies suggested that DFMO was inactive against erythrocytic schizogony of *P. berghei* in mice, but it was shown subsequently that DFMO does inhibit the process. A reproducible suppression of parasitaemia of approximately 50 per cent was obtained when infected mice were treated with 2 per cent (w/v) DFMO in their drinking water (Bitonti *et al.*, 1987), although the treated mice did not survive significantly longer than untreated animals. As DFMO was inhibitory to *P. falciparum* *in vitro* and *P. berhegi* *in vivo*, it seemed that polyamine biosynthesis was worthy of consideration as a potential chemotherapeutic target in malaria. Clearly, however, more potent drugs were needed.

POLYAMINE ANALOGUES

Another tactic to the inhibition of polyamine biosynthesis and/or function and ultimately to the inhibition of cell growth was the use of analogues of the natural polyamines which might repress polyamine biosynthesis and either block or displace the natural polyamines at intracellular binding sites (Porter and Sufrin, 1986). Approximately 100 of these polyamine analogues were synthesized at the Merrell Dow Research Institute and tested for their antimalarial activity. A series of bis(benzyl)polyamine analogues was identified which inhibited the erythrocytic schizogony of *P. falciparum* *in vitro* (Bitonti *et al.*, 1989a) (Table 47.1). A marked difference in antimalarial potency was noted between the bis(benzyl)polyamine analogues and their free amine counterparts (Table 47.1). The specificity could be explained by the greater uptake of the bis(benzyl)polyamines into human erythrocytes as compared to the free amine analogues (Bitonti *et al.*, 1989b). In fact, the bis(benzyl)polyamine analogues were concentrated in erythrocytes approximately 20-fold over the concentration in the surrounding medium. Based on these *in vitro* data and preliminary determinations of therapeutic indices in mice, MDL 27,695 was chosen for more thorough study.

MDL 27,695 inhibited *P. falciparum* *in vitro* with an IC_{50} of $3 \mu\text{M}$ and was found to be equally effective against a chloroquine susceptible clone (D6) and a

Table 47.1. Growth inhibition of *P. falciparum* *in vitro* by polyamine analogues.

	Compound Y number	Antimalarial activity	
		IC ₅₀ (μM)	IC ₉₀ (μM)
Spermine ($\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_Y\text{NH}(\text{CH}_2)_3\text{NH}_2$)	4	—	—
bis(Benzyl)analogues			
$\text{C}_6\text{H}_5\text{CH}_2\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_Y\text{NH}(\text{CH}_2)_3\text{NHCH}_2\text{C}_6\text{H}_5$	4 MDL 27 847	14	22
	5 MDL 27 957	6.7	10
	6 MDL 27 693	5.3	9.0
	7 MDL 27 695	3.0	4.4
	8 MDL 27 391	0.83	1.4
	9 MDL 27 701	0.69	1.1
	10 MDL 27 700	0.48	0.90
	12 MDL 27 994	0.21	0.35
Free amine analogues			
$\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_Y\text{NH}(\text{CH}_2)_3\text{NH}_2$	7 MDL 26 752	4300	11 000
	8 MDL 26 547	1800	4100

^a The antimalarial activity of the polyamine analogues was measured by following the incorporation of [³H]hypoxanthine into *P. falciparum* done D6 *in vitro*. Y denotes the length of the central methylene chain. From Bitonti *et al.* (1989a).

chloroquine resistant strain (FCR-3) (Figure 47.1). Administration of MDL 27,695 in combination with DFMO to *P. berghei*-infected mice resulted in complete cure of the infections (Table 47.2). Cured mice which were challenged with the same strain of *P. berghei* four months after the original infection were found to have developed

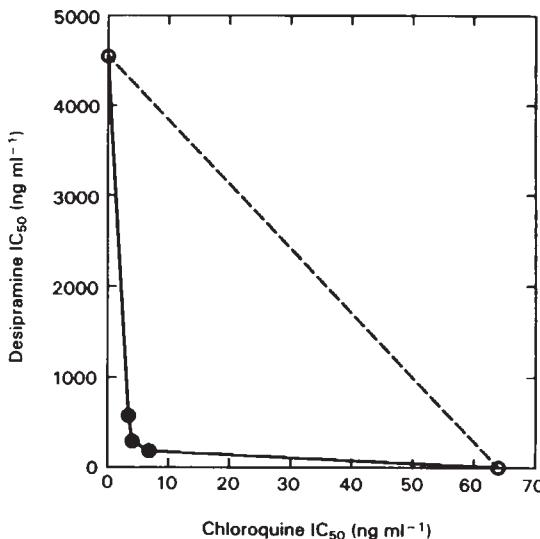


Figure 47.1. Desipramine and chloroquine are synergistic against chloroquine resistant *P. falciparum* (FCR-3) *in vitro*. IC₅₀ values were determined for the drugs alone and in combination by measuring the incorporation of [³H]hypoxanthine into infected erythrocytes.

Table 47.2. Effects of MDL 27 695 plus DFMO on *P. berghei* in mice.

Group	Treatment		Parasitaemia on day 4 (%)	Average day of death	N	Number of animals, cured/total
	MDL 27,695 (mg kg ⁻¹)	DFMO (%)				
Experiment 1						
1	0	0	12.5 ± 3.5	16.7 ± 4.7	14	0/14
2	15	0	3.7 ± 1.2	23.2 ± 5.1	10	3/14
3	15	2	0.13 ± 0.07	23.2 ± 5.1		15/14
Experiment 2						
4	0	0	14.1 ± 4.7	15.8 ± 2.5	20	0/20
5	10	2	1.17 ± 0.61	34.0 ± 2.0	5	15/20
6	15	2	0.77 ± 0.31	22	2	18/20

^a Mice were considered cured when they were aparasitaemic on periodic examination and lived for > 4 months after infection. MDL 27 695 was administered intraperitoneally, 3 times daily, whereas DFMO was given in drinking water. From Bitonti *et al.* (1989a).

Although no direct evidence was obtained with either *P. falciparum* or *P. berghei* to suggest that MDL 27,695 was an inhibitor of polyamine biosynthesis, studies with the drug in rat hepatoma (HTC) cells *in vitro* showed that exposure to MDL 27,695 caused repression of both ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase activities (Bitonti *et al.*, 1989c). It is reasonable to believe that similar changes in the polyamine biosynthetic enzymes would occur in the *Plasmodia*. It was also noted that in the studies with HTC cells that the free amine analogue of MDL 27,695 (see Table 47.2 for structure) was more potent than MDL 27,695 at repressing the two biosynthetic enzymes. This is especially relevant because an enzyme of polyamine catabolism, polyamine oxidase, causes debenzylation of the bis(benzyl)polyamine analogue to its free amine counterpart in both HTC cells (Bitonti *et al.*, 1989c) and mouse erythrocytes (Bitonti *et al.*, 1990). Debenzylation of MDL 27,695 was shown to be a contributing factor in determining the effects of the drug on *P. berghei* in mice (Bitonti *et al.*, 1990). This finding may be explained by the necessity for metabolism to the free amine analogue which is capable of repressing polyamine biosynthesis. MDL 27,695 was also found to be a potent agent for the treatment of *Leishmania donovani* infections in mice (Baumann *et al.*, 1990) and, therefore, may be of substantial interest for further development.

REVERSAL OF CHLOROQUINE RESISTANCE

The spread of chloroquine resistance to virtually every malarious area of the globe is a disturbing trend. Rather than only identifying novel agents which have the inherent requisite potency to cure malaria as single agents, it has become popular recently to identify compounds which may act not as primary antimalarial agents but to modify chloroquine resistance by interfering with biochemical mechanisms responsible for the resistance. It has been known for some time that resistant malaria

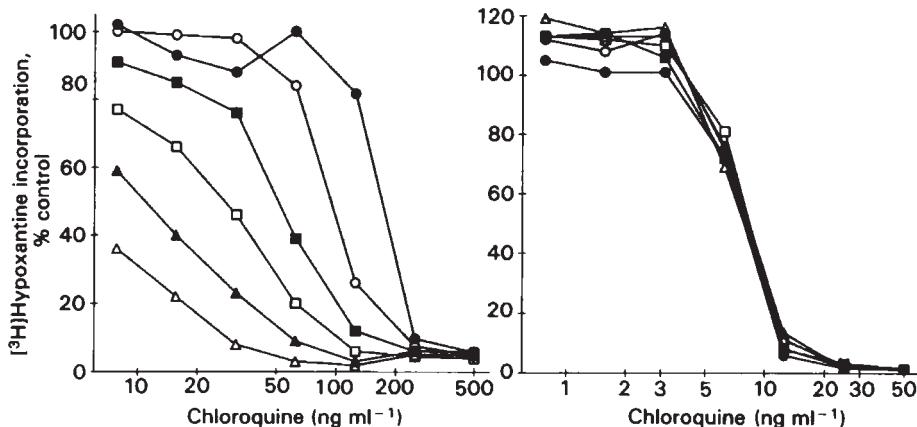


Figure 47.2. Desipramine increases sensitivity to chloroquine *in vitro* in resistant *P. falciparum* done W-2 (left) but not in susceptible *P. falciparum* done D-6 (right). Dose-response curves for chloroquine were done in the absence (●) or presence of 20 (○), 50 (■), 100 (□), 200 (▲), or 500 (△) ng ml⁻¹ desipramine.

(Reproduced, with permission, from Bitonti *et al.*, (1988).)

parasites accumulate less chloroquine than do susceptible parasites (Macomber *et al.*, 1966; Fitch, 1969, 1970). The reason for this decreased accumulation was unexplained until recently when a series of papers shed some light on this matter. Martin *et al.* (1987) found that chloroquine resistance in *P. falciparum* could be reversed by verapamil, a calcium channel blocker, much like the multidrug resistance in cancer cells could be reversed by this drug. In the cancer cells it was known that verapamil bound to, and interfered with the function of, a plasma membrane glycoprotein (P-glycoprotein) involved in the active efflux of cytotoxic drugs from the cells (Gottesman and Pastan, 1988). Verapamil and other drugs which reversed chloroquine resistance were also found to inhibit the rapid efflux of chloroquine from erythrocytes infected with resistant parasites (Krogstad *et al.*, 1987), thus suggesting similar mechanisms in the cancer cells and the plasmodia (but see Chapter 39).

Although the foregoing biochemical studies were of great interest from a mechanistic viewpoint, clinical utility remained elusive because verapamil and other drugs which decreased chloroquine resistance were active at concentrations which were too high to be achieved without toxicity in man. However, using isobologram analysis (Berenbaum, 1978), desipramine, a tricyclic antidepressant, was found recently to be highly synergistic with chloroquine against *P. falciparum* *in vitro* (Bitonti *et al.*, 1988). This reversal of chloroquine resistance differed from reversal of resistance with other drugs in that it occurred at concentrations of desipramine (20–200 ng ml⁻¹; Figure 47.2) which encompassed the range of concentrations found in the serum of patients receiving the drug (Amsterdam *et al.*, 1980) thus making clinical use of a drug combination a more realistic possibility. Reversal of chloroquine resistance with desipramine was correlated with an increased accumulation of chloroquine by the resistant parasites.

The most dramatic evidence that reversal of chloroquine resistance with desipramine has potential for clinical use was obtained in an experiment with *P. falciparum* infections in owl monkeys (*Aotus lemurinus lemurinus*) (Bitonti *et al.*, 1988). Owl monkeys were infected with the highly drug-resistant Vietnam Smith strain of *P. falciparum* and then treated with either chloroquine alone or with a combination of chloroquine and desipramine for three days. Chloroquine alone had little or no effect on the development of parasites, but the combined drug treatment rapidly inhibited parasite growth with an apparent clearing of parasitemias. These data give strong support to the notion that modulators of chloroquine resistance such as desipramine may have clinical utility.

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48. Purine metabolism in parasitic protozoa and its relationship to chemotherapy

J.J.Marr

INTRODUCTION

The metabolism of purines in protozoan parasites is significantly different from that in mammals. In humans, purines are synthesized *de novo* from non-nucleotide precursors (amino acids, carbon dioxide and ammonia) as well as from preformed purine bases via the salvage pathways. The general route for *de novo* biosynthesis is the same in those species of mammals, birds, yeasts, and bacteria that have been studied. This is a successive sequence of reactions by which the purine ring is assembled on C1 of ribose 5'-phosphate leading to the formation of inosinic acid (IMP) (see Figure 48.1). Neither free purines nor nucleosides appear as intermediates in this sequence. IMP is then converted to adenosine 5'-phosphate (AMP) or to guanosine 5'-phosphate (GMP) with subsequent conversion to nucleoside di-and tri-phosphates and nucleic acids. In contrast to mammals, *de novo* purine biosynthesis does not occur in any of the major parasitic protozoa.

Nucleotides can also be formed from free purines and purine nucleosides. These routes are regarded as salvage pathways which permit the reutilization of purines from the degradation of nucleic acid or nucleotides. These pathways are present in both humans and pathogenic protozoa. In the latter they are the only means of synthesizing purine nucleotides. Free purines react with phosphoribosyl-1-pyrophosphate (PRPP) to yield nucleoside 5'-monophosphate. These reversible reactions are catalysed by distinct phosphoribosyltransferases (PRTases) for the activation of particular purine base. Since the pyrophosphate released in these reactions is hydrolysed rapidly, the synthesis of purine nucleosides proceeds irreversibly. Other salvage pathways involve the conversion of free purines to nucleosides and these to nucleotides. The former reactions are catalysed by purine nucleoside phosphorylase, in which ribose-1-phosphate serves as the ribose donor and inorganic phosphate is released along with the purine nucleoside. Conversion of the nucleoside to the nucleotide occurs through the addition of a phosphate group

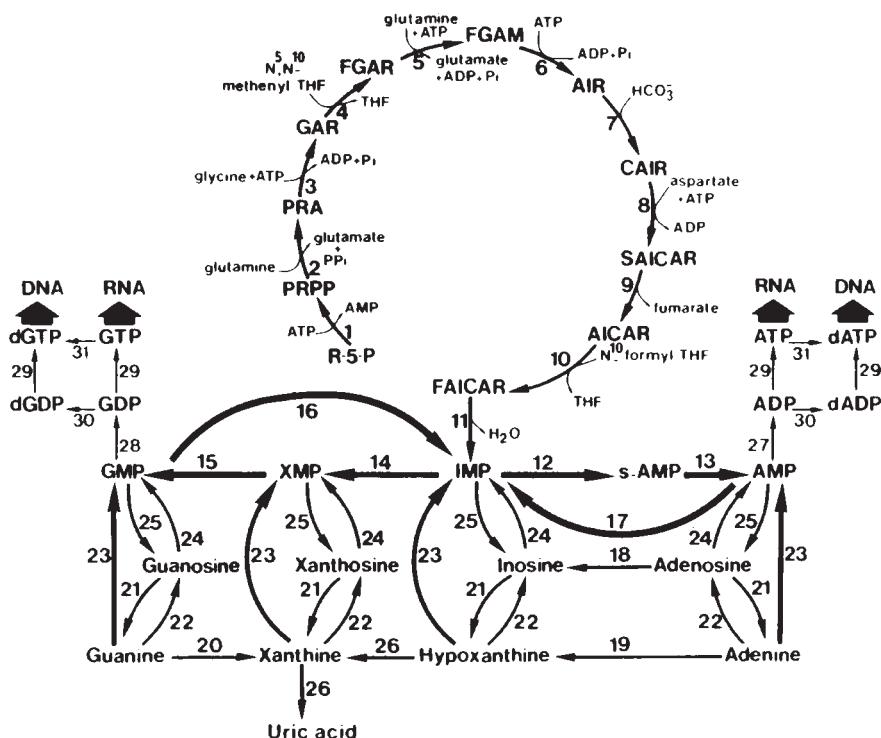


Figure 48.1. *De novo* synthesis of IMP and the salvage and interconversion of purines. Enzymes: 1, phosphoribosylpyrophosphate synthetase (EC 2.7.6.1); 2, amidophosphoribosyltransferase (EC 2.4.2.14); 3, phosphoribosylglycamide synthetase (EC 6.3.4.13); 4, phosphoribosylglycamide formyltransferase (EC 2.1.2.2); 5, phosphoribosylformylglycamide synthetase (EC 6.3.5.3); 6, phosphoribosylaminoimidazole synthetase (EC 6.3.3.1); 7, phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21); 8, phosphoribosylaminoimidazolesuccinocarboxamide synthetase (EC 6.3.2.6); 9, adenylosuccinate lyase (EC 4.3.2.2); 10, phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3); 11, IMP cyclohydrolase (EC 5.5.4.10); 12, adenylosuccinate synthetase (EC 6.3.4.4); 13, adenylosuccinatelyase (EC 4.3.2.2.1); 14, IMP dehydrogenase (EC 1.2.1.14); 15, GMP synthetase (EC 6.3.5.2); 16, GMP reductase (EC 1.6.6.8); 17, AMP deaminase (EC 3.5.4.6); 18, adenosine deaminase (EC 3.5.4.4); 19, adenine deaminase (EC 3.5.4.2); 20, guanine deaminase (EC 3.5.4.3); 21, nucleosidase (EC 3.2.2.1); 22, phosphoribosyltransferase (catabolic) (EC 2.4.2.1); 22, phosphoribosylase (anabolic) (EC 2.4.2.1); 23, phosphoribosyltransferase (EC 2.4.2.7, EC 2.4.2.8, EC 2.4.2.22); 24, nucleoside kinase (EC 2.7.1.15, EC 2.7.1.20, EC 2.7.1.73); 25, nucleotidase (EC 3.1.3.5); 26, xanthine oxidase (EC 1.2.3.2); 27, adenylyl kinase (EC 2.7.4.3); 28, guanylate kinase (EC 2.7.4.8); 29, nucleoside diphosphate kinase (EC 2.7.4.6); 30, ribonucleoside diphosphate reductase (EC 1.17.4.1); 31, ribonucleoside triphosphate reductase (EC 1.17.4.2). AIR, Phosphoribosylaminoimidazole; AICAR, phosphoribosylaminoimidazolecarboxamide; CAIR, phosphoribosylcarboxyaminoimidazole; FGAR, phosphoribosylformylglycaminide; FGAM, phosphoribosylformylglycaminidine; FAICAR, phosphoribosylformamidoimidazolecarboxamide; GAR, phosphoribosylglycaminide; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; PRA, phosphoribosylamine; PRPP, 5-phosphoribosyl-1-pyrophosphate; R-5-P, ribose-5-phosphate; SAICAR, phosphoribosylaminoimidazolesuccinocarboxamide; THF, tetrahydrofolate. (Reproduced, with permission, from Hassan and Coombs (1988).)

to the 5'-position on the ribose moiety by a nucleoside kinase or a nucleoside phosphotransferase. The former is usually relatively specific for a given purine nucleoside while the latter is relatively non-specific with respect to both the donor and recipient of the phosphate group. The salvage pathways that exist are shown in Figure 48.1.

In humans, the first step is the *de novo* synthetic pathway and many steps in the salvage pathways are under metabolic regulation. Unfortunately, little is presently known about regulation of purine salvage or degradation in the parasitic protozoa. There is also little known about the biosynthesis or degradation of nucleic acid in these organisms, except that mRNA degradation is *leishmania* is accelerated at 38°C (Marr and Berens, 1985) and by exposure of promastigotes to pyrazolopyrimidines (Tuttle and Krenitsky, 1980). Purine nucleotides which are released from either RNA or DNA are degraded by non-specific phosphatases or nucleotidases to yield nucleosides and inorganic phosphate. This is true for both parasitic protozoa and humans. This review does not deal with RNA editing (see Chapters 1 and 55 for references) since it does not fall under the strict purview of purine metabolism. The nucleosides are acted upon by nucleoside cleaving enzymes which can be either nucleoside phosphorylases (which require phosphate as a participant in the reaction to yield a purine and ribose 1-phosphate) or nucleoside hydrolysis (which uses water to produce the base and free ribose). Both types of enzyme exist in humans and pathogenic protozoa. The purine base is not known to be degraded further in protozoa and is probably reutilized in the salvage pathways. In humans, the purine ring is usually salvaged as above, but small amounts are partially degraded to urea and allantoin. Most of the bases which are not salvaged are deaminated and then oxidized by xanthine oxidase to uric acid and then excreted. There appears to be no xanthine oxidase in protozoa.

How can this basic biochemical information be related to chemotherapy? To achieve a good therapeutic index, an antimicrobial agent must be directed against a peculiarity in the parasite's metabolism.

Purine metabolism in the pathogenic protozoa appears to offer several opportunities:

1. since there is no *de novo* synthesis of these compounds, interdiction of the salvage pathway has far greater implications than interruption of similar pathways in humans;
2. some of the enzyme systems in the haemoflagellates are capable of accepting purine analogues and metabolizing them to nucleotides; these analogues serve as metabolic inhibitors;
3. in general, the mucosal pathogens have purine salvage pathways which do not cross over between the adenine and the guanine side in either direction. This suggests that inhibition of either adenine or guanine metabolism would be sufficient to prevent growth of the parasite. In humans, ample opportunities for cross-over exist; and
4. with respect to DNA metabolism, some parasitic protozoa lack ribonucleotide

reductase (see below). This means that they are totally dependent upon salvage of preformed deoxynucleosides.

Thus the biochemical differences between parasitic protozoa and humans are significant enough for one to predict that purine analogues should be effective inhibitors of these organisms. Indeed, this prediction has been borne out already with respect to the haemoflagellates *Leishmania donovani*, *L. panamensis*, and *Trypanosoma cruzi*.

Let us now consider the purine metabolism of some parasitic protozoa. The intention is not to review the metabolism in detail but rather to stress differences between the parasites' metabolism and that of the host. For ease of consideration, we group the organisms into the haemoflagellates (leishmanias and trypanosomes) mucosal pathogens (*Entamoeba histolytica*, *Trichomonas vaginalis* and *Giardia lamblia*), and *Toxoplasma gondii*. For details of purine metabolism in other parasitic protozoa see Hassan and Coombs (1988) and Chapters 2, 3, 5 and 36.

HAEMOFLAGELLATES

Leishmania

Promastigotes

Purine metabolism in *Leishmania* can be summarized as being similar to that found in mammalian cells except for the means of entry into the metabolic scheme. The primary means of ingress is through the PRTases. *L. donovani* has three distinct PRTases, one specific for hypoxanthine and guanine, one for xanthine and one for adenine (HGPKTase, XPKTase, APRTase, respectively). The finding of an XPKTase is unusual (Tuttle and Krenitsky, 1980; Marr and Berens, 1985). Mammalian tissues do not contain this enzyme. The major substrates for the PRTases appear to be hypoxanthine and xanthine. There is very active adenase which deaminates xanthine to hypoxanthine. Investigations using radiolabelled substrates have shown that adenine is deaminated to hypoxanthine before entry into the salvage pathways. Similarly, guanine is the deaminated rapidly by a guanase to xanthine. The latter is then activated through the PRTase and enters the salvage pathway. The inosine and guanosine nucleosides can interchange. There is an active GMP reductase and an AMP deaminase (Figure 48.2).

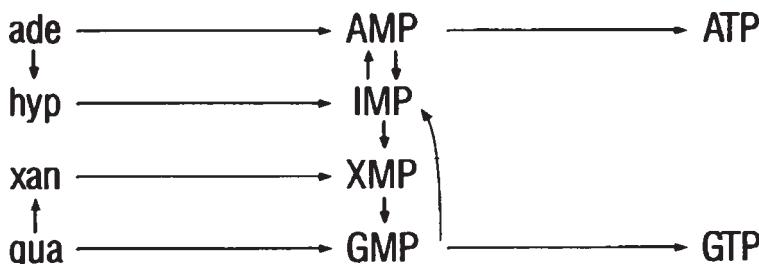


Figure 48.2. Purine metabolism in *Leishmania*.

There are active nucleoside-cleaving activities in leishmanias. There is an adenosine phosphorylase (Konigk and Rasoul, 1978) and an inosine nucleosidase (Konigk, 1978). *L. tropica* contains a nucleotidase which has a broad substrate specificity (Pereira and Konigk, 1981). A study by Kozalka and Krenitsky (1979) has provided detailed information on the nucleosidases of *L. donovani*. One of these is a novel purine 2'-deoxyribonucleosidase, another was specific for the purine nucleosides inosine and guanosine, and the third cleaves both purine and pyrimidine ribonucleosides with a clear preference for the latter.

Amastigotes

Studies by Looker *et al.* (1983) using amastigotes of *L. donovani* isolated from hamster spleen described the purine pathways in these forms. Purine metabolism in the amastigote is similar to that in the promastigote except for adenine and adenosine metabolism. In these forms, there is no adenase. Adenine is activated to AMP by an APRTase. This is a significant departure from purine metabolism in the prosmastigote. Less than 20 per cent of adenine is present in guanine nucleotides compared to 45 per cent when hypoxanthine is precursor. In addition, the relative flow is 55 per cent to AMP and 45 per cent to GMP; this differs from the flow in promastigotes for IMP is converted 75 per cent to AMP and 25 per cent to GMP. The metabolism of guanine, xanthine and their respective nucleosides is similar to that described for the promastigote. Interestingly, the stage-specificity described for *L. donovani* does not appear to occur with other species (see Hassan and Coombs, 1988).

Therapeutic implications

The therapeutic implications of purine metabolism in these organisms are quite substantial (Berens *et al.*, 1980; Marr and Berens, 1983). The HGPRTase is relatively non-specific. It will accept pyrazolopyrimidines as though they are purines. The best studied of these is allopurinol (4-hydroxypyrazolo (3, 4-*d*) pyrimidine). This hypoxanthine analogue is activated to the nucleotide analogue of IMP. The nucleotide is an excellent inhibitor of succinyl AMP synthase (adenylosuccinate synthetase). The net effect is to inhibit this enzyme strongly and prevent the formation of AMP from purine precursors. In addition, this nucleotide analogue inhibits the GMP reductase. This prevents the conversion of GMP to AMP, which might help to overcome the metabolic block. Thus, allopurinol brings about virtually complete inhibition of purine nucleotide biosynthesis in these organisms and halts growth. In addition, the IMP analogue is converted to an AMP analogue which is then phosphorylated to the ATP analogue. This aminopyrazolopyrimidine nucleotide analogue is incorporated into RNA of the organism. Once incorporated into RNA it brings about a net breakdown of mRNA which probably accounts, to a large degree, for the inhibition of protein synthesis. This metabolic sequence is the same for allopurinol and its riboside but does not occur in humans (Figure 48.3).

These biochemical findings have been translated into clinical benefits. There have been several reports of the benefits of allopurinol in visceral leishmaniasis and

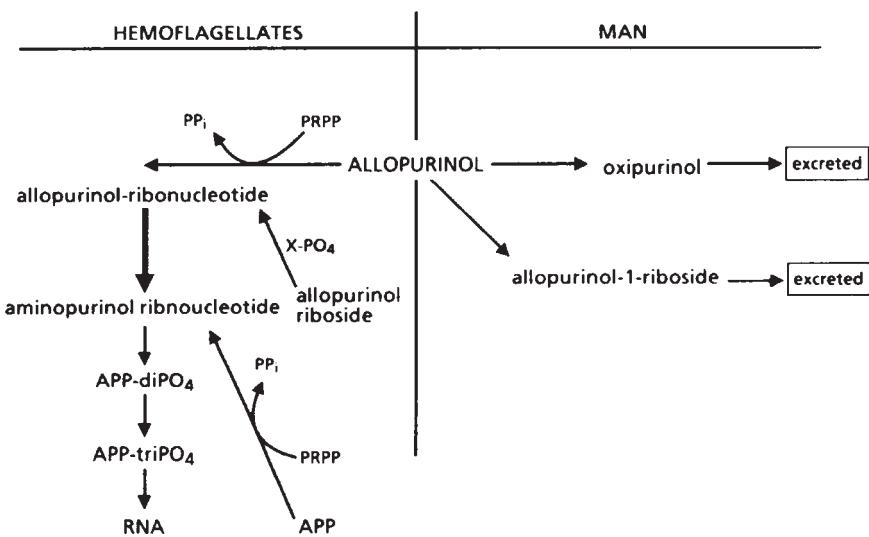


Figure 48.3. Comparative metabolism of allopurinol and allopurinol riboside in protozoa and humans. PP_i, inorganic pyrophosphate; PRPP, phosphoribosylpyrophosphate; X-PO₄, phosphate donor; APP-diPO₄, 4-aminopyrazolo (3, 4-*d*) pyrimidine ribonucleoside diphosphate; APP-triPO₄, 4-aminopyrazolo(3, 4-*d*)pyrimidine ribonucleoside triphosphate; APP, 4-aminopyrazolo(3, 4-*d*)pyrimidinc.

one study using allopurinol riboside in cutaneous leishmaniasis (Saenz *et al.*, 1989). In the latter study, allopurinol riboside was shown to be as effective as glucantime when the total cure rate (cure plus relapse) was considered. Allopurinol currently is being studied by the World Health Organization in a large collaborative trial among four institutions in Columbia, Peru and Bolivia. Preliminary data are very encouraging. The results of this trial should be available within 2 years. The most thoroughly studied purine analogues are shown in Figure 48.4.

Trypanosoma cruzi

Trypanosoma cruzi possesses salvage pathways for adenine, hypoxanthine, guanine and xanthine. The incorporation of purine bases, with the exception of adenine, is similar to that shown by leishmania. There is no adenase. Quantitatively, adenine and hypoxanthine are used more efficiently than guanine or xanthine. There is interconversion among the respective nucleotides. The purine bases are incorporated into ribonucleotides more efficiently than are the ribonucleosides. The major pathway from a purine base to its ribonucleotide is directly through the PKTases.

The ribonucleosides are remarkably stable in these organisms as compared to the leishmania which have very high nucleoside cleaving activities. There is a branch point at IMP and interconversion of the adenine and guanine nucleotides.

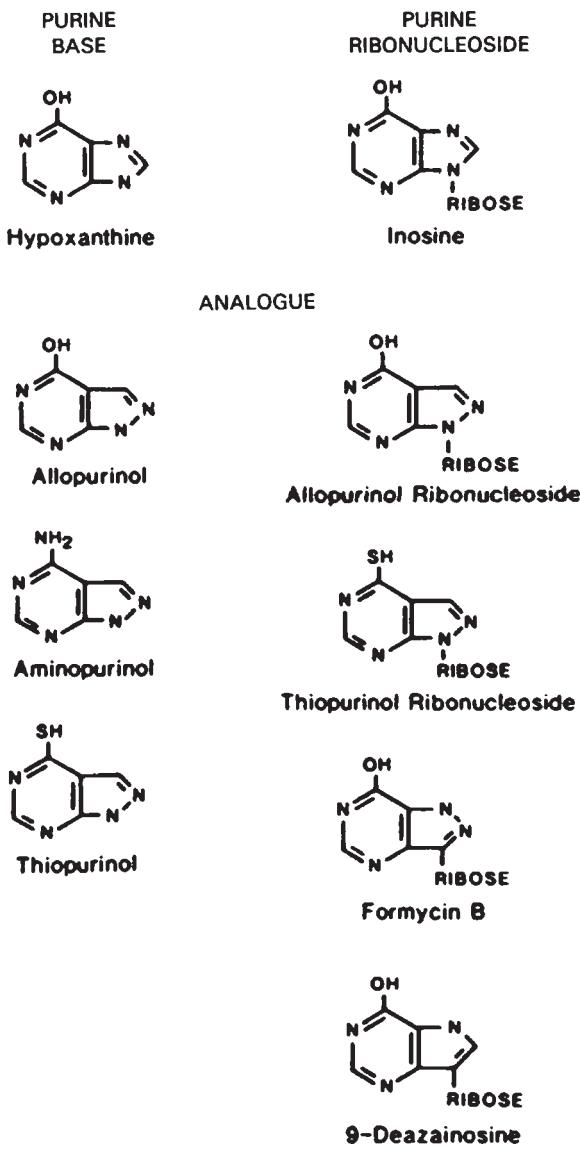


Figure 48.4. Analogues of purine bases and nucleosides.

Therapeutic implications

As metabolic pathways with respect to pyrazolopyrimidines are essentially the same as with leishmanias, so are the therapeutic implications. Inhibition of growth by this compound is rapid and can be shown not only *in vitro*, but also in tissue culture and in animals (Avila and Avila, 1980; Looker *et al.*, 1986).

A recent clinical study has shown that allopurinol in doses of 600 and 900 mg day⁻¹ is equal in efficacy to both nifurtimox and benznidazole. All patients studied had chronic, asymptomatic Chagas' disease. Allopurinol was as efficacious as the nitrofurans as measured by three serologic tests and by xenodiagnosis. Allopurinol had the additional advantage of having little or no toxicity; the nitrofurans showed evidence of significant toxicity in approximately 30 per cent of patients (Gallerano *et al.*, 1990). Two large clinical studies of allopurinol will be undertaken by the World Health Organization during 1990–91 to verify these findings. One study will be undertaken in Argentina and the other in another country in South America.

African trypanosomes

Procyclic forms

Incorporation of free purine bases by *T. brucei gambiense* is qualitatively similar to that found with *T. cruzi* in that both trypanosomes lack adenase and thus differ from leishmanias. Adenase also is absent from *T. b. rhodesiense* (Fish *et al.*, 1982). Otherwise the metabolism is similar to that shown for leishmania. Both adenine and hypoxanthine are readily incorporated into the adenine nucleotides with a rather slow conversion to guanine nucleotides. Adenine is more actively metabolized than hypoxanthine. Guanine and xanthine are more rapidly incorporated into guanine nucleotides. Interconversion of guanine and adenine nucleotides greatly favours formation of the latter. There is an active guanase present.

Purine nucleosides are utilized slightly more rapidly than their corresponding bases. This is similar to a leishmania but differs from *T. cruzi* in that the later shows significantly lower incorporation of ribonucleoside. There is no adenosine deaminase, so deamination probably takes place at the level of AMP. A GMP reductase is present.

Bloodstream forms

The bloodstream forms *T. b. gambiense* and *T. b. rhodesiense* incorporate purine bases in a manner qualitatively similar to the procyclic forms (Fish *et al.*, 1982). As in the latter forms, adenine is incorporated preferentially into adenine ribonucleotides as compared to hypoxanthine. Guanine and xanthine label the guanine ribonucleotides more readily than adenine ribonucleotides. As with the procyclic forms the data suggest that the presence of a PRTase activity for all four purine bases. There is an active guanase as well. All ribonucleosides are hydrolysed rapidly. Interconversion of nucleotides occurs through the AMP deaminase and the GMP reductase.

Therapeutic implications

Pyrazolopyrimidines and their nucleosides are readily incorporated into the corresponding nucleotide analogues in both the bloodstream and procyclic forms of African trypanosomes. Although these compounds inhibit the growth of the organisms they are not as effective as in the leishmania and *T. cruzi*. It is of interest

that DFMO has a striking synergistic activity with purine analogues in animal models of African trypanosomiasis (Bacchi *et al.*, 1987).

MUCOSAL PATHOGENS

Giardia lamblia

This organism has a very simple scheme of purine salvage (Figure 48.5) (Wang, 1983). It possesses only two important enzymes, the adenine and guanine PRTases (Figure 48.4). There is no salvage of hypoxanthine, xanthine, or any purine nucleosides. Most importantly, there is no interconversion between adenine and guanine nucleotides. The parasite has an absolute requirement for both adenine and guanine. The GPRTase does not recognize hypoxanthine or xanthine as a substrate, unlike the corresponding mammalian enzyme which uses hypoxanthine, and a bacterial enzyme which accepts xanthine as a substrate. Nucleosides are hydrolysed very efficiently, and effectively do not undergo direct phosphorylation to nucleotides. The latter pathways do exist, however, and can be demonstrated by the use of nonhydrolysable nucleosides (Figure 48.5).

In addition, these organisms are completely dependent upon deoxynucleoside salvage (Baum *et al.*, 1989), which is extremely unusual among eukaryotic cells (Moore and Hurlbert, 1985). *G. lamblia* lacks the enzyme ribonucleotide reductase and is able to grow in the presence of hydroxyurea, an inhibitor of this enzyme, at concentrations as high as 2 mM. Both deoxyadenosine and deoxyguanosine are phosphorylated by a nucleoside kinase.

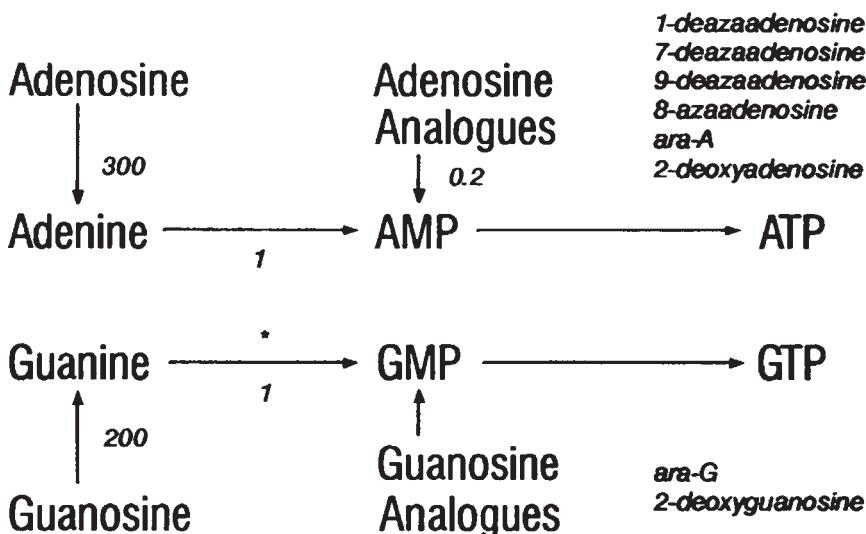


Figure 48.5. Purine metabolism in *Giardia lamblia*. The numbers refer to relative reaction rates. The compounds shown in italics inhibit the growth of the organism, probably by direct phosphorylation to the nucleotide analogue. *PRTase does not accept hypoxanthine or xanthine.

Therapeutic implications

The fact that these organisms require both adenine and guanine suggests than an inhibitor of either pathway will be sufficient to inhibit the organism. This is unlike the situation in the haemoflagellate and malarial parasites where there is cross-over between the adenine and guanine nucleotide pathways. Because of the requirement for deoxynucleosides, it should be possible to inhibit growth with analogue of deoxynucleosides. This has been demonstrated by Miller *et al.* (1987) who demonstrated growth inhibition by guanine arabinoside. This was completely reversed by the addition of deoxyguanosine to the medium. Similar results were obtained by Baum *et al.*

Trichomonas vaginalis

As with *Giardia*, there is no cross-over between the adenine and guanine nucleotide pathways (Figure 48.6). The route to nucleotides is somewhat different; adenine and guanine are ribosylated by a purine nucleoside phosphorylase. The nucleoside is then acted upon by a purine nucleoside kinase. Hypoxanthine is convened to the nucleoside and to IMP but the latter is not convened further to AMP or GMP. A guanase is present in the organisms but PRTases are absent. Interestingly, the pathways operating in the cattle parasite *Tritrichomonas foetus* differ significantly from those in *Trichomonas vaginalis* (for details see Hassan and Coombs, 1988).

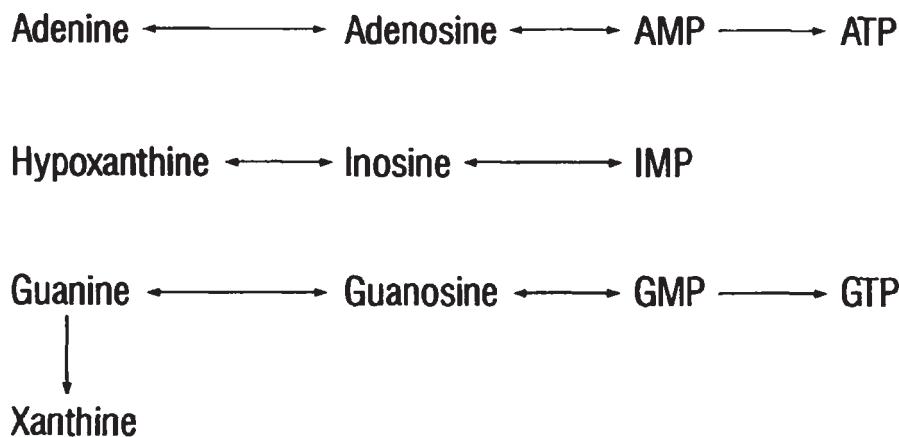


Figure 48.6. Purine metabolism in *Trichomonas vaginalis*. PRTase activities are absent.

Therapeutic implications

Implications are similar to those for *G. lamblia*. As there is no cross-over between the adenine and guanine nucleotide pathways, interdiction of either side should be sufficient to inhibit the growth of the organism.

Entamoeba histolytica

The pathways are similar to those described for the other two mucosal pathogens and thus the therapeutic implications are also similar. There is no cross-over between the adenine and guanine nucleotide pathways. Hypoxanthine is not incorporated. The pathways are based upon metabolic studies, but the enzymes involved are not known with certainty. The major route of entry into nucleotide biosynthesis is from the nucleoside to the nucleotide rather than via a PRTase.

TOXOPLASMA GONDII

This organism can incorporate all four purine bases into the corresponding nucleotides through PRTase activities (Krug, *et al.*, 1989; and see Figure 48.7). Adenine and hypoxanthine have identical distribution of radiolabel into both adenine and guanine nucleotides. This suggests that adenine is deaminated to hypoxanthine prior to incorporation. An interesting feature of this metabolism is that, although adenine and hypoxanthine will label both adenine and guanine nucleotides, the reverse does not occur. There appears to be no GMP reductase activity in these cells. Nucleosides are cleaved readily. Adenosine is incorporated to nucleotides far more rapidly than are other nucleosides. This is largely due to the very high level of adenosine kinase activity present in the cell (Krug *et al.*, 1989). The nucleoside appears to be convened directly to the nucleotide, whereas others are preferentially degraded and then incorporated as bases. There is an active guanase.

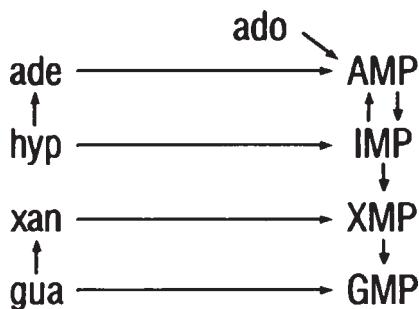


Figure 48.7. Purine metabolism in *Toxoplasma gondii*. No GMP reductase; no nucleoside kinase or phosphotransferase other than adenosine kinase. ade, Adenine; ado, adenosine; hyp, hypoxanthine; xan, xanthine; gua, guanine.

CONCLUSIONS

Purine metabolism of the parasitic organisms is different in many respects from that in mammals. In each instance, there are differences which can be potentially exploited for chemotherapy. The value of this concept already has been shown with

respect to the leishmanias and *T. cruzi*. Allopurinol has developed from a theoretical consideration into a compound which has been shown to be efficacious in the clinic. Further trials continue which will delineate more precisely the role of this compound in human medicine. The prospects for other organisms are attractive as well. This is particularly true for the mucosal pathogens where there is no cross-over between adenine and guanine nucleotide metabolism. Additional attractive considerations are those enzymes which might metabolize xanthine or guanine analogues. The haemoflagellates and *Toxoplasma* metabolize these compounds whereas in the human they are oxidized and excreted.

Purine metabolism in these organisms is one area of basic biochemistry which already has yielded therapeutic benefits for human medicine and is likely to provide us with other important new therapeutic discoveries in the future.

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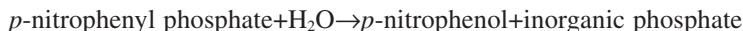
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49. Phosphomonoesterases of parasitic protozoa

P.A.Bates

INTRODUCTION

Phosphomonoesterases are enzymes which are responsible for the hydrolytic cleavage of single phosphate groups from a variety of substrates. They can be distinguished from other enzymes which act on phosphate groups, such as phosphodiesterases, pyrophosphatases and ATPases, in that one of the products of the reaction is always free inorganic phosphate and the other is a dephosphorylated organic molecule. Thermodynamically such reactions are effectively irreversible in biological systems and different enzymes, such as the various kinases, are responsible for the addition of phosphate groups to biomolecules. Phosphomonoesterases are commonly known as phosphatases. An example of a phosphomonoesterase is non-specific acid phosphatase (EC 3.1.3.2.) which can be assayed using the artificial substrate *p*-nitrophenyl phosphate:



The two main roles of phosphomonoesterases are either in removing the phosphate group from the substrate so that the product can then be used in other ways, for example as a biosynthetic precursor, or in providing a source of free phosphate itself. These roles can be broadly characterized as digestive and nutritive. However, other more specialized roles do exist, for example the regulatory effects exerted by specific phosphoprotein phosphatases.

For convenience the phosphomonoesterases described here will be subdivided into three different groups. The first are lysosomal phosphatases which play a direct role in the hydrolysis of internalized substrates. The second are various externally oriented phosphatases present in the surface membranes of parasitic protozoa, which differ in many respects from enzymes of the host and, consequently, have attracted much attention. The third group are secreted phosphatases. With the exception of specialized cell types, secretion of hydrolases is unusual in higher eukaryotes and for this reason the secretory enzymes of parasitic protozoa, including phosphatases, have also been the subject of much research.

Various other phosphatases of parasitic protozoa have been reported. These include phosphoprotein phosphatases, which are likely to be involved in the regulation of cell growth and division, and those responsible for the catabolism of hexose phosphates. The information on these enzymes is either fragmentary or more usefully considered in relation to topics covered in other chapters of the book, and so are not considered here.

LYSOSOMAL ENZYMES

Non-specific acid phosphatase has been employed as a classical cytochemical and biochemical marker for lysosomes. A similar approach has been adopted for many parasitic protozoa, but here the situation is more complex because some of these organisms also secrete acid phosphatase(s). With a few exceptions, it is not known whether the secretory enzymes are related to those of the lysosome or represent discrete enzymes. It is difficult to distinguish between lysosomes and secretory vesicles unless the analysis includes other biochemical or immunochemical markers. Nevertheless, lysosomal acid phosphatase has been reported in many parasitic protozoa (for some of the older literature see Eeckhout, 1973).

There have been a large number of studies on trypanosomatids (see Table 49.1). The results of many of these studies are indeed likely to reflect the presence of lysosomal acid phosphatase. However, despite the number of publications our knowledge remains rather limited: purification of potential lysosomal enzymes has not been performed and the mechanisms by which they are targeted to lysosomes are unknown. Another drawback has been a lack of distinction between multiple enzymes which may be present. Workers have used either fine structure

Table 49.1. Publications relating to lysosomal acid phosphatase in parasitic protozoa.

<i>Critidina</i> spp.	Brooker (1971), Eeckhout (1972), McLaughlin <i>et al.</i> (1976), Steiger <i>et al.</i> (1979), Soares and De Couza (1988)
<i>Trypanosoma theileri</i>	Herbert (1965)
<i>T. melophagium</i>	Herbert (1965)
<i>T. raiiae</i>	Preston (1969)
African trypanosomes	Brooker and Vickerman (1964), Seed <i>et al.</i> (1967), Steiger (1973), Langreth and Balber (1975), Opperdoes <i>et al.</i> (1977), Venkatesan <i>et al.</i> (1977), Steiger <i>et al.</i> (1979, 1980), Rovis and Backkeskov (1980), Opperdoes and Steiger (1981), Williamson and McClaren (1981), McLaughlin (1982, 1986), Grab <i>et al.</i> (1987), Lonsdale-Eccles and Grab (1987)
<i>T. cruzi</i>	Avila <i>et al.</i> (1979), Steiger <i>et al.</i> (1979), Letelier <i>et al.</i> (1985, 1986)
<i>Leishmania</i> spp.	Lewis and Peters (1977), Gottlieb and Dwyer (1981a), Coombs <i>et al.</i> (1982), Barbieri <i>et al.</i> (1985), Pimenta and De Souza (1986), Pukkis <i>et al.</i> (1986), Hassan and Coombs (1987), Avila <i>et al.</i> (1989)
<i>Plasmodium</i> spp.	Aikawa and Thompson (1971), Meis <i>et al.</i> (1984)
Trichomonads	Muller (1973), Lindmark and Muller (1973, 1974), Nielsen (1974), Lindmark <i>et al.</i> (1975), Gradus and Matthews (1985), Lockwood <i>et al.</i> (1988)
<i>Giardia</i> spp.	Feely and Dyer (1987), Lindmark (1988), Jarroll <i>et al.</i> (1989)
<i>Naegleria fowleri</i>	Feldman (1977), Lowrey and McLaughlin (1985), Olomou <i>et al.</i> (1986)
<i>Entamoeba histolytica</i>	Serrano <i>et al.</i> (1977), McLaughlin <i>et al.</i> (1978), Aley <i>et al.</i> (1980), Rosenberg and Gitler (1985), McLaughlin and Aley (1985), Gitler and Mirelman (1986)

cytochemical staining to demonstrate localized intracellular acid phosphatase activity and/or subcellular fractionation techniques to demonstrate particulate activity. Results of both types of approach are often assumed to indicate the presence of lysosomes. However, whilst this may be true, only in a limited number of cases have lysosomes been purified with any certainty and shown to contain acid phosphatase.

In *Crithidia*, soluble and particulate activities of acid phosphatase which differed in their enzymatic properties were described (McLaughlin *et al.*, 1976). The former was ascribed to the flagellar pocket (see below), whilst the latter was probably lysosomal. However, the most extensive subcellular fractionation has been performed with *Trypanosoma brucei*. Here a certain amount of activity co-purified with lysosomes (Grab *et al.*, 1987; Lonsdale-Eccles and Grab, 1987), but in these and other studies most of the latent acid phosphatase activity was found in particulate fractions distinct from lysosomes and identified as microsomal components (Steiger *et al.*, 1980; Opperdoes and Steiger, 1981; Grab *et al.*, 1984). Consequently, other enzymes, such as acid proteinase, appear to be better markers for lysosomes in *T. brucei* (Opperdoes and Van Roy, 1982; Lonsdale-Eccles and Grab, 1987; Opperdoes *et al.*, 1987) and the validity of using acid phosphatase as a lysosomal marker in trypanosomatids remains doubtful.

In *Crithidia*, *Leishmania* and bloodstream *T. brucei* subspecies there is an additional complication in that, as well as the acid phosphatase activity revealed in cytoplasmic vesicles by cytochemistry, reaction product is also detected in the flagellar pocket. This appears to be the main if not exclusive site of endocytosis (Steinert and Novikoff, 1960; Brown *et al.*, 1965; Preston, 1969; Brooker, 1971; Langreth and Balber, 1975; Opperdoes *et al.*, 1987; Coppens *et al.*, 1987, 1988) and exocytosis (Eeckhout, 1972; Bates *et al.*, 1989) in trypanosomatids. Consequently, three possible origins have been suggested for acid phosphatase activity in this location. The first is that such enzymes may be lysosomal in origin and the flagellar pocket represents a 'compartment' in which digestion of macromolecules has begun before internalization. The second possibility is that the activity is due to genuine secretory enzymes which pass through the flagellar pocket en route to the outside world. In either of these two cases the enzyme would be expected to be soluble. The third possibility is that reaction product is generated by membrane bound enzymes, which may be a specialized feature of the flagellar pocket or related to other surfacemembrane enzymes. The available evidence indicates that in the cases of *Crithidia* and *Leishmania* the activity is at least partly due to secretory enzymes, whereas in bloodstream forms of *T. brucei* acid phosphatase is associated with the flagellar pocket membrane (these are discussed fully below). There is no direct evidence for the release of *bona fide* lysosomal acid phosphatase into the flagellar pocket, although this cannot be definitely excluded. The presence of enzyme activity in the flagellar pocket, albeit from different sources, could nevertheless fulfil a similar digestive function for the parasite.

Evidence for lysosomal acid phosphatase has also been obtained in a number of other parasitic protozoa using cytochemistry and/or subcellular fractionation (Table 49.1) As with the trypanosomatids, the degree of proof varies between studies, but

the results are generally consistent with a lysosomal location of enzyme activity. Various claims have been made concerning the presence in *Entamoeba histolytica* of acid phosphatase and other enzymes in lysosomes below the plasma membrane (McLaughlin and Aley, 1985; Gitler and Mirelman, 1986). Putative lysosomal enzymes, including acid phosphatase, have been reported in particulate fractions but have also been found to be membrane bound and not released by cycles of freezing and thawing (Serrano *et al.*, 1977; McLaughlin *et al.*, 1978; Aley *et al.*, 1980; Rosenberg and Gitler, 1985; McLaughlin and Aley, 1985). Since there is other evidence that these enzymes are located in the surface membrane which itself undergoes extensive turnover (Aley *et al.*, 1984; and see Chapter 34), these intracellular activities may not indicate lysosomes in the conventional sense but rather internalized surface membrane activities. Alternatively, the lysosomal enzymes in *Entamoeba* may indeed be membrane bound but also cycle through the surface membrane. Therefore, it is not dear whether acid phosphatase should be regarded as a lysosomal enzyme, a surface membrane enzyme or both simultaneously and indeed whether there are multiple enzymes present. However, support for the idea of membrane bound lysosomal enzymes comes from similar observations in *Giardia* (Lindmark, 1988; Jarroll *et al.*, 1989) and trichomonads (Muller, 1973; Lindmark and Muller, 1974), organisms which do not themselves possess a surface membrane acid phosphatase activity to confuse the situation (Feely and Dyer, 1987).

Soluble, cell-associated acid phosphatase of unknown origin has also been reported in cell homogenates of *Eimeria* species (Farooqui and Hanson, 1988; Hosek *et al.*, 1988) and *Sarcocystis suicanis* (Farooqui *et al.*, 1987).

SURFACE MEMBRANE ENZYMES

Phosphomonoesterases are characteristic components of the surface membranes of higher eukaryotes. Enzymes such as alkaline phosphatase (EC 3.1.3.1) and 5'-nucleotidase (EC 3.1.3.5) are commonly employed as biochemical markers during the isolation of surface membranes. Phosphatases are also common components of surface membranes in parasitic protozoa, but the enzymes present are often different (Table 49.2).

In general, surface membrane alkaline phosphatase activity has not been found in those parasitic protozoa which have been examined to date, although it has been reported in *Entamoeba invadens* (McLaughlin and Meerovitch, 1975) and *Acanthamoeba castellanii* (Ulsamer *et al.*, 1971). If non-specific activity was detected, it usually displayed an acidic pH optimum. Externally oriented surface membrane acid phosphatase has been extensively characterized in *Leishmania* species (Gottlieb and Dwyer 1981a, b; Glew *et al.*, 1982; Dwyer and Gottlieb, 1985; Remaley *et al.*, 1985a; Pimenta and De Souza, 1986; Coombs *et al.*, 1987; Hassan and Coombs, 1987). The enzyme(s) are found on promastigotes of *L. donovani*, *L. mexicana* and *L. amazonensis* cultured *in vitro*. However, there does not appear to be a surface acid phosphatase on *L. major* or *L. tarentolae* promastigotes (Coombs *et al.*, 1987; Mallinson *et al.*, 1988). Demonstration of surface activity in amastigotes is more problematical owing to the potential for adsorption of host enzymes, and

Table 49.2. Surface membrane phosphomonoesterase activities in parasitic protozoa.^a

Organism	AcPase	3'-NTase	5'-NTase
<i>Leishmania donovani</i> promastigotes	+	+	+
<i>L. mexicana</i> promastigotes	+	+	+
<i>L. amazonensis</i> promastigotes	+	+	+
<i>L. major</i> promastigotes	-	+	+
<i>L. tarentolae</i> promastigotes	-	- ^c	-
<i>Leptomonas collosoma</i>	+	ND	-
<i>Crithidia lucilae</i> ^b	+	+	+
<i>Crithidia fasciculata</i>	-	- ^c	-
<i>Herpetomonas muscarum muscarum</i>	+	- ^c	+
<i>H. m. ingenoplastis</i>	-	- ^c	+
<i>Trypanosoma cruzi</i>	+ / - ^d	- ^c	-
<i>T. brucei brucei</i>	- ^e	+	-
<i>T. b. rhodesiense</i>	- ^e	+	-
<i>Entamoeba histolytica</i>	+ ^f	ND	-
<i>Entamoeba invadens</i>	+ ^f	ND	-
<i>Naegleria fowleri</i>	-	ND	+
<i>Acanthamoeba castellani</i>	-	ND	+

^a The table shows the distribution of surface membrane acid phosphatase (AcPase), 3'-nucleotidase (3'-NTase) and 5'-nucleotidase (5'-NTase) amongst a selection of parasitic protozoa. Those where only negative results have been reported or where the data are uncertain have been excluded. +, Present; -, absent; ND, not determined.

^b This organism was originally described as *Crithidia fasciculata* (Gottlieb, 1983), but subsequently corrected to *Crithidia lucilae* (Gottlieb *et al.*, 1988).

^c Effects of purine starvation not examined.

^d Contradictory reports; see text.

^e Flagellar pocket enzyme present in bloodstream trypanosomes; see text.

^f May be a 'lysosomal' enzyme; see text.

cytochemical studies have led to differing conclusions depending on the system studied. Results with *L. mexicana* indicated the presence of acid phosphatase on the surface membrane of isolated amastigotes (Hassan and Coombs, 1987) whereas in *L. amazonensis* activity was not observed (Pimenta and De Souza, 1986). Infected macrophages have also been examined for acid phosphatase activity, although these results are difficult to interpret with respect to surface acid phosphatase, again owing to host enzymes. In some studies reaction product was detected in the parasitophorous vacuole or flagellar pocket which may have resulted from the actions of a surface membrane enzyme, whereas in others there was little or no activity present (Lewis and Peters, 1977; Dwyer, 1979; Ryter *et al.*, 1983; Barbieri *et al.*, 1985; Antoine *et al.*, 1987; Barbieri *et al.*, 1990). Furthermore, there is evidence both for and against the possibility that amastigotes secrete an acid phosphatase (Antoine *et al.*, 1987; Bates *et al.*, 1989). Overall, the evidence that a surface membrane acid phosphatase is a general feature of *Leishmania* amastigotes is rather weak, although it cannot be discounted at present.

An interesting property of the *L. donovani* surface membrane acid phosphatase is

the ability of purified enzyme to block the neutrophil oxidative burst (Remaley *et al.*, 1984, 1985b). This effect could be neutralized by the addition of phosphatase inhibitors. The enzyme activity was also resistant to the effects of various toxic oxygen metabolites suggesting that the enzyme itself can function during phagocytosis if an oxidative burst is triggered (Saha *et al.*, 1985). However, the mode of action remains rather unclear (Das *et al.*, 1986; Glew *et al.*, 1988) as is the significance *in vivo* when macrophages are involved. There are other candidates for the blocking of the oxidative burst, lipophosphoglycan for example (see Chapter 28), and metacyclics of *L. major* can trigger an oxidative burst and survive, despite the lack of surface acid phosphatase (Mallinson *et al.*, 1988, 1989; Mallinson and Coombs, 1989). Additionally, *in vivo*, serum-opsonized parasites may be internalized without triggering an oxidative burst (Da Silva *et al.*, 1989). With the exception of *L. braziliensis*, there is no evidence that metacyclic or stationary phase promastigotes possess higher levels of surface acid phosphatase, as might be expected (Mukhopadhyay *et al.*, 1988), although it has been reported that higher acid phosphatase activity was associated with virulence in *L. donovani* (Katakura, 1986; Katakura and Kobayashi, 1988). More evidence is required before the importance of this potential survival mechanism can be assessed.

Among other trypanosomatids, surface membrane acid phosphatase has also been reported in *Leptomonas collosoma* (Hunt and Ellar, 1974), *Crithidia lucilae* (Gottlieb, 1985) and *Herpetomonas muscarum* (Coombs *et al.*, 1987). It appears to be absent from *H. ingenoplastis* and *C. fasciculata* (Coombs *et al.*, 1987). There are conflicting reports regarding *T. cruzi*, some indicating the presence (Zingales *et al.*, 1979; Nagakura *et al.*, 1985, 1986; Urbina *et al.*, 1988) and others the absence (Meirelles and De Souza 1984, Von Kreuter *et al.*, 1989) of a surface enzyme. Cytochemical and subcellular fractionation techniques have not yet been combined in one study in order to provide a convincing demonstration of surface membrane acid phosphatase in *T. cruzi*. Apart from its actual presence, nothing is known concerning the functional significance of the enzymes in these organisms, with the possible exception of *T. cruzi* where it has been suggested that the possession of a higher activity in amastigotes may be an adaptation to intracellular existence (Nagakura *et al.*, 1985). This, however, remains rather tentative.

Although African trypanosomes do not possess a general surface acid phosphatase, bloodstream forms demonstrated acid phosphatase reaction product in the flagellar pocket (Seed *et al.*, 1967; Langreth and Balber, 1975; Venkatesan *et al.*, 1977; Williamson and McLaren, 1981). In contrast, procyclic trypanosomes were found to lack this activity (Langreth and Balber, 1975; Opperdoes and Steiger, 1981). Cell fractionation studies have indicated that this activity was the major cell-associated acid phosphatase (Steiger *et al.*, 1979, 1980; McLaughlin, 1982), located in the flagellar pocket membrane and not freely soluble (Steiger *et al.*, 1980; Walter and Opperdoes, 1982; McLaughlin, 1986). This was also supported by experiments indicating that the enzyme was separated from its substrate by a diffusion barrier (Langreth and Balber, 1975; Steiger *et al.*, 1980). It has been suggested that there may be a distinct surface membrane acid phosphatase in addition to the flagellar

pocket enzyme (McLaughlin, 1986). The possible relationship of these trypanosomal enzymes to those of other trypanosomatids is unknown.

Surface acid phosphatase has also been reported in *E. invadens* and *E. histolytica* (McLaughlin and Mccrovitch, 1975; Van Vliet *et al.*, 1976; Serrano *et al.*, 1977; Aley *et al.*, 1980; Udezulu and Leitch, 1987). As discussed above, this may be a common activity with a lysosomal membrane bound enzyme. Whatever their origins, functionally such enzyme(s) are likely to play a similar digestive role for the parasite.

Nucleotidases have also been found in the surface membranes of parasitic protozoa (Table 49.2). These include both 5'-nucleotidase and, in certain trypanosomatids, a distinct 3'-nucleotidase (EC 3.1.3.6; reviewed in Gottlieb, 1989). 3'-Nucleotidase is not a host enzyme and, therefore, may have diagnostic or chemotherapeutic value. Amongst parasitic protozoa 3' -nucleotidase appears to be confined to trypanosomatids and has not been detected elsewhere to date, being absent in trichomonads for example (Hassan and Coombs, 1987). Within the trypanosomatids, both 5'- and 3'-nucleotidase are found in *Leishmania* species (Gottlieb and Dwyer, 1981c, 1983; Dwyer and Gottlieb, 1984; Coombs *et al.*, 1987; Hassan and Coombs, 1987; Zlotnick *et al.*, 1987) and *C. lucilae* (Gottlieb, 1985; Gottlieb *et al.*, 1988). These enzymes are externally oriented and have been proposed to play a role in purine salvage from the host by converting exogenous nucleotides into nucleosides which can then be transported across the surface membrane by an appropriate carrier (Gottlieb, 1989). In addition to the activity for which it is named, the 3'-nucleotidase is also active against RNA and, to a lesser extent, single-stranded DNA (Gottlieb and Zlotnick, 1987; Gottlieb *et al.*, 1988; Neubert and Gottlieb, 1990). This 3'-nucleotidase/nuclease and the 5'-nucleotidase acting together may, therefore, be able to satisfy the purine requirements of these organisms from RNA, the former enzyme providing the substrate (5'-nucleotides) for the latter (Figure 49.1). In this regard, it has been reported that high molecular weight RNA is capable of satisfying the purine requirements of *L. donovani* promastigotes *in vitro*, although details were not published (Gottlieb and Zlotnick, 1987). Further support for the importance of the 3'-nucleotidase/nuclease comes

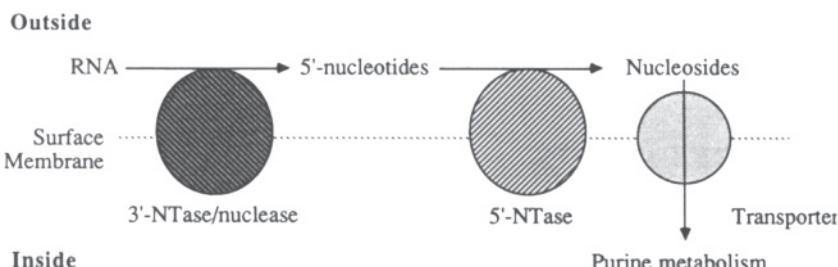


Figure 49.1. A diagram illustrating the possible roles of 3'-nucleotidase (3' -NTase/nuclease) and 5'-nucleotidase (5'-NTase) in purine salvage by *Crithidia* and *Leishmania*. 3'-NTase/nuclease acts on the 3'-phosphate groups in exogenous RNA generating 5'-nucleotides. These in turn are dephosphorylated by 5'-NTase and the resulting nucleosides are internalized via the nucleoside transporters known to be present in the surface membrane (Aronow *et al.*, 1987). (Adapted from Gottlieb (1989).)

from experiments showing that the level of the enzyme was up-regulated in the absence of an exogenous purine or phosphate source (Gottlieb, 1985; Gottlieb *et al.*, 1988; Alleman and Gottlieb, 1990; Sacci *et al.*, 1990). 5'-Nucleotidase and surface acid phosphatase showed small increases in activity under purine starvation conditions using *C. lucilae* (Gottlieb, 1985) but no response was observed with *L. donovani* (Sacci *et al.*, 1990).

3'-Nucleotidase/nuclease activity is also present in *T. brucei* and *T. rhodesiense* (McLaughlin, 1982; Gbenle and Akinrimisi, 1982; Gottlieb *et al.*, 1986; Gbenle *et al.*, 1986) but, in contrast to one report (Voorheis *et al.*, 1979), specific 5'-nucleotidase is absent (Steiger *et al.*, 1980; Coombs *et al.*, 1987; Hassan and Coombs, 1988). Likewise, the low level of 5'-nucleotidase reported in the surface membranes of *Leptomonas collosoma* is probably non-specific (Hunt and Ellar, 1974). The surface membranes of *Herpetomonas* species possess 5'-nucleotidase but lack 3'-nucleotidase activity, even though the activity was detected in cell lysates (Coombs *et al.*, 1987; Hassan and Coombs, 1987, 1988). Similarly, both enzymes were detected in lysates, but are apparently absent from the surface membrane of *L. tarentolae* and *C. fasciculata* (Coombs *et al.*, 1987; Hassan and Coombs, 1987). Neither enzyme was detected in *T. cruzi* (Meirelles and De Souza, 1984; Gottlieb *et al.*, 1986; Urbina *et al.*, 1988), although there is one report documenting surface 5'-nucleotidase in *T. cruzi* (Nagakura *et al.*, 1986). The significance of the presence or absence of the nucleotidases in these other trypanosomatids is unclear, especially if the suggestion that they function as a pair in purine salvage is correct. They may be vestigial enzymes or fulfil some unknown function (Gottlieb, 1989). The location of 3'-nucleotidase undergoes developmental regulation in African trypanosomes, existing as an ectoenzyme in procyclic forms but having a functionally internal location in bloodstream forms, possibly buried within or on the internal face of the plasma-membrane (Gottlieb *et al.*, 1986; Gbenle *et al.*, 1986; Opperdoes *et al.*, 1987).

A 5'-nucleotidase is present in the surface membranes of *Acanthamoeba castellanii* (Ulsamer *et al.*, 1971) and *Naegleria fowleri* (Lowrey and McLaughlin, 1985), but not in *E. invadens* (McLaughlin and Meerovitch, 1975; Van Vliet *et al.*, 1976), *E. histolytica* (McLaughlin and Aley, 1985), *Plasmodium berghei* (Meis *et al.*, 1984) or *Toxoplasma gondii* (De Carvalho and De Souza, 1989).

SECRETORY ENZYMES

Secretory acid phosphatases have been reported in a number of parasitic protozoa. Promastigotes from a wide variety of species of *Leishmania* have been found to release a soluble acid phosphatase activity during *in vitro* cultivation, the only exception being *L. major* (Gottlieb and Dwyer, 1982; Lovelace and Gottlieb, 1986; Lovelace *et al.*, 1986). This glycoprotein has been shown to be the major secretory protein of *L. donovani*, being rapidly synthesized and released into the culture medium (Bates and Dwyer, 1987a, b). Exocytosis of acid phosphatase is mediated by the flagellar pocket membrane rather than through the general surface membrane of *L. donovani* promastigotes (Bates *et al.*, 1989). Aside from this fusion of secretory

vesicles with a specialized region of the plasma membrane, the other characteristics of the process appear to be typical of the constitutive secretion of proteins that occurs with other eukaryotic cells. The secretory acid phosphatase appears to differ enzymatically and antigenically from the surface membrane enzyme of the same organisms (Gottlieb and Dwyer, 1982; Bates *et al.*, 1987, 1989), although it seems likely that there will prove to be some homology at the gene level. Glycosylation is not required for secretion although N-linked oligosaccharides are needed for enzyme activity (Lovelace and Gottlieb, 1987a, Bates and Dwyer, 1987b).

In addition to being the major secretory protein in biosynthetic terms, the secretory acid phosphatase of *L. donovani* promastigotes is also extremely antigenic (Bates *et al.*, 1988). An explanation for this is that secreted acid phosphatase of *L. donovani* is itself phosphorylated (Lovelace and Gottlieb, 1987b) and contains an unusual phosphate-galactose-mannose epitope also present on lipophosphoglycan, the prominent surface and shed antigen of *Leishmania* (Bates *et al.*, 1990; and see Chapters 27 and 28). A similar situation also occurs in *L. tropica* (Jaffe *et al.*, 1990) and may prove to be a general feature of the secreted acid phosphatases of *Leishmania*. These results are intriguing given the variety of potential roles which have been suggested for both lipophosphoglycan (see Chapters 27 and 28) and secretory acid phosphatase. With regard to the latter, one possible role is a nutritional one, dephosphorylating organic phosphates and rendering them suitable for uptake by the parasite, or providing a source of free phosphate itself. This could well be of use to promastigotes inhabiting the gut of the sandfly vector or to intracellular amastigotes within macrophages. Although the evidence is not conclusive, however, the pH optimum of 4.8 (Gottlieb and Dwyer, 1982) and resistance of enzyme activity to toxic oxygen metabolites (Saha *et al.*, 1985) are suggestive of an ability to function in the macrophage phagolysosome. In this environment it is conceivable that the enzyme may be able to dephosphorylate lysosomal hydrolases or phosphoproteins involved in regulation of the oxidative burst with benefits to the parasite. Furthermore, the potential ability of a soluble enzyme to penetrate into other compartments of the host cell could affect macrophage physiology in some unsuspected manner. Whatever the functions of secretory acid phosphatase and lipophosphoglycan may be, one possible explanation for the possession of a common epitope is that it may be a structure resistant to digestion by host enzymes. It could, therefore, confer protection for the molecules concerned against the hydrolytic environments in which *Leishmania* parasites reside, thereby ensuring the integrity and function of these molecules.

As discussed previously, leishmania-infected macrophages have been examined cytochemically for acid phosphatase activity with variable results (Lewis and Peters, 1977; Dwyer, 1979; Ryter *et al.*, 1983; Barbieri *et al.*, 1985, 1990; Antoine *et al.*, 1987). Positive results have been obtained (for example, Dwyer, 1979) which could be interpreted in terms of a parasite secretory enzyme. There is some more direct evidence, both for and against this possibility. In a study of *L. donovani*, indirect immunofluorescence with monoclonal antibodies was used to demonstrate secretory acid phosphatase in infected macrophages (Bates *et al.*, 1989). However, an investigation of *L. mexicana* amastigotes during short-term *in vitro* cultivation failed to reveal any secreted activity (Antoine *et al.*, 1987). As forms of proof, both

these studies have their drawbacks and the question remains open. Those publications which have documented the long-term axenic cultivation of amastigotes (Pan, 1984; Eperon and McMahon-Pratt, 1989) have not addressed the question of secretory products from amastigotes, but this should prove a useful approach to resolve this question in the future.

Amongst other trypanosomatids, only in *Crithidia* was acid phosphatase reaction product both cytochemically localized to the flagellar pocket (Brooker, 1971) and detected extracellularly during growth of organisms *in vitro* (Eeckhout, 1972; Coombs *et al.*, 1987). Soluble cell-associated activity likely to have originated from the flagellar pocket has also been reported (McLaughlin *et al.*, 1976). Whether these enzyme activities have a precursor/product relationship as shown for *L. donovani* promastigotes (Bates *et al.*, 1989) remains to be determined. Cultured procyclic forms of *T. brucei* did not release acid phosphatase (Langreth and Balber, 1975) and a suggestion that acid phosphatase might be released by bloodstream forms of *T. rhodesiense* (Venkatesan *et al.*, 1977) has not been confirmed.

Secretory acid phosphatase has also been reported in *Trichomonas vaginalis*, *Tritrichomonas foetus* (Lockwood *et al.*, 1988; North *et al.*, 1989; Savoia and Martinotti, 1989) and *Entamoeba histolytica* (Muller *et al.*, 1988; Agrawal *et al.*, 1989) and there is evidence for its production by *Naegleria fowleri* (Feldman, 1977). These are all extracellular parasites which are capable of damaging host epithelia and other tissues under appropriate conditions and it has therefore been suggested that secretion of hydrolases, including acid phosphatase, contributes to their pathology (Gitler and Mirelman, 1986; Marciano-Cabral, 1988). In *E. histolytica* it has been reported that virulent strains contained higher levels of acid phosphatase and other hydrolases (Katiyar *et al.*, 1989; and see Chapters 22 and 23), but it is not known whether these are secretory enzymes. The precise role and importance of acid phosphatase secretion in these parasites remains unknown.

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50. Genetic analysis of folate transport and metabolism in *Leishmania donovani*

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INTRODUCTION

The treatment, cure and prevention of parasitic diseases has been hampered by a lack of knowledge about parasite metabolism and nutritional requirements. In large part, this can be attributed to the unavailability of suitable defined growth media in which to cultivate these organisms. One metabolic pathway that has been exploited for the chemotherapeutic manipulation of certain parasitic diseases is that for the synthesis of reduced folate co-enzymes. Folk acid is a vitamin that consists of a pterin ring, 2-amino-4-oxo-6-(hydroxymethyl)pterin, attached to the amino group of *p*-aminobenzoic acid (PABA), which in turn is attached through its carboxylate group to the α -amino group of glutamic acid. The vitamin is converted to its activated co-enzyme derivative, tetrahydrofolate (THF), by a two-step reduction process catalysed by the enzyme dihydrofolate reductase (DHFR). THF participates in a variety of one-carbon-atom transfer reactions in humans, including the synthesis of purine and thymidylate nucleotides, the reformation of methionine from homocysteine, and the interconversion of serine and glycine. Tetrahydrofolate is similar in structure to tetrahydrobiopterin, another reduced pteridine, which participates in a variety of mixed-function oxidase reactions, including the specific hydroxylations of the amino acids phenylalanine, tyrosine and tryptophan. However, unlike tetrahydrobiopterin, which humans can biosynthesize from guanosine triphosphate (GTP), THF cannot be manufactured *de novo* by humans since the enzymatic machinery of human cells is incapable of synthesizing the *p*-aminobenzoic acid moiety. However, plants and protists can synthesize THF by condensing 2-amino-4-oxo-6-(hydroxymethyl)dihydropterin with PABA to form dihydropteroate, a reaction catalysed by dihydropteroate synthetase, followed by conjugation of the dihydropteroate to glutamate (see Figure 51.1 in Chapter 51).

Thus, a spectrum of sulphonamides and sulphones, which are inhibitors of dihydropteroate synthetase, and pyrimethamine and trimethoprim, two inhibitors of DHFR, have been widely used in the treatment of both malaria and toxoplasmosis (Amand, 1983; Rollo, 1983; Scholer *et al.*, 1984; Ferone, 1984; and see Chapter 51). Despite the relevance of the folate pathway to antiprotozoal therapies (Rollo,

1983; Ferone, 1984) and its prominence as a metabolic system to study gene amplification in *Leishmania* (Coderre *et al.*, 1983; Beverley *et al.*, 1984; Washtien *et al.*, 1985), relatively little information is available on the metabolism of folate in *Leishmania*. Several reports have described an assortment of THF-metabolizing enzymes in several *Leishmania* species (Nosei and Avila, 1983; Avila and Nosei, 1985; Scott *et al.*, 1987), and the existence of a bifunctional DHFR/thymidylate synthase activity has been thoroughly established by biochemical (Garrett *et al.*, 1984; Meek *et al.*, 1985), genetic (Coderre *et al.*, 1983), and molecular biological (Beverley *et al.*, 1984; Washtien *et al.*, 1985) methods.

Growth of *Leishmania* under completely defined conditions has established that this genus of parasites is auxotrophic for folate and, therefore, has an absolute requirement for a folate source in the culture medium (Trager, 1969; Scott *et al.* 1987; Petrillo-Peixoto and Beverley, 1988, Kaur *et al.*, 1988). Thymidine can circumvent the toxicity of methotrexate (MTX), another inhibitor of DHFR, toward *L. donovani* (Kaur *et al.*, 1988), although the pyrimidine nucleoside does not allow the parasite to grow in folate-deficient growth medium (fdDME-L) (Beck and Ullman, 1991). These data suggest that folate serves a second function in *L. donovani* other than to provide THF for thymidylate synthesis, it seems likely, that this function is as a source of pterins. Using high performance liquid chromatography (HPLC) we have demonstrated that *L. donovani* can convert folate to a pterin. Replacement of folate in the cell culture medium with biopterin can also support the propagation of *L. donovani* (Kaur *et al.*, 1988; Beck and Ullman, 1991) in folate-deficient medium. In fact, Beck and Ullman (1990) have shown that a variety of pterins can eliminate this folate requirement in *L. donovani*. These data provide nutritional evidence that *L. donovani* is capable of metabolizing pterins to THF. Herein, we have demonstrated by HPLC that wild-type *Leishmania* were indeed capable of metabolizing biopterin to THFs.

Kaur *et al.* (1988) have characterized a MTX-resistant mutant derivative of *L. donovani* in a single step which transports MTX and folate at less than 1 per cent of the rate of wild-type cells. Preliminary characterization of the mutant strain indicated that it could not grow in fdDME-L supplemented with pterins (Kaur *et al.*, 1988; Beck and Ullman, 1991). This could be attributed to a complete inability of the mutant cells to convert biopterin to THFs. The results of these studies with the mutant strain suggest that a single genetic lesion can adversely affect two apparently different biochemical functions, the transport of folate acid and the ability to use pterins as a source of reduced folates.

GROWTH SENSITIVITY TO MTX

The MTXA5 cell line was isolated by virtue of its resistance to 1 mM MTX. In comparative growth-rate experiments, the effective concentration of MTX that inhibited the growth of DI700 wild type cells was 50 µM, whereas the MTXA5 cells were completely insensitive to MTX at a concentration of 4 mM (Kaur *et al.*, 1988). The MTXA5 cells were cross-resistant to aminopterin but were as sensitive as

parental cells to either pyrimethamine or trimethoprim, two inhibitors of prokaryotic DHFRs. Unlike previously isolated MTX-resistant *L. major* (Coderre *et al.*, 1983), no evidence of DNA sequence amplification was visually evident in the MTXA5 cell line, and the DHFR levels in DI700 and MTXA5 cells were equivalent (Kaur *et al.*, 1988).

FOLATE AND MTX INCORPORATION

One mechanism by which cells can become refractory to drugs is by their acquisition of a genetic trait that confers transport deficiency. Comparison of the abilities of DI700 and MTXA5 cells to take up either [³H]MTX or [³H]folate from the culture medium revealed that the MTXA5 cells took up both radiolabels at less than 1 per cent of the rate of DI700 cells. This failure to incorporate [³H]MTX or [³H]folate could account for the growth resistance of MTXA5 cells to MTX and indicated that the transport of folate and MTX by *L. donovani*, unlike that in mammalian cells, requires a common component.

NUTRITIONAL REQUIREMENTS OF WILD TYPE AND MTXA5 CELLS FOR PTERINS AND FOLATES

Whereas wild type *L. donovani* were capable of continuous growth in fdDME-L medium supplemented with biopterin, they could not grow in fdDME-L that was not supplemented with either folate or biopterin (Kaur *et al.*, 1988; Beck and Ullman, 1991). This implied that *L. donovani* are auxotrophic for folate. Surprisingly, MTXA5 cells could not survive in biopterin supplemented fdDME-L, despite the fact that they transport biopterin as efficiently as wild type cells (Kaur *et al.*, 1988; Beck and Ullman, 1991). It has previously been shown that biopterin does not function as a ligand for the folate/MTX transporter of either *L. major* (Ellenberger and Beverley, 1987) or *L. donovani* (Beck and Ullman, 1989). Similar results were obtained with neopterin, 6-hydroxymethylpterin, tetrahydrobiopterin, dihydrobiopterin, sepiapterin, monapterin and dimethylpterin, all of which could promote the propagation of DI700 cells, but not MTXA5 cells, in fdDME-L. Thus, mutant cells failed to grow in fdDME-L supplemented with any one of the eight pterins which permitted wild-type cells to multiply in fdDME-L (Beck and Ullman, 1991). Moreover, the addition of pABA and pABA-glu to the pterin containing fdDME-L did not enhance growth of the MTXA5 cells.

One explanation for the incapacity of MTXA5 cells to grow in pterin supplemented fdDME-L was their inability to convert pterins to tetrahydrofolates. In order to test this hypothesis, 0.1 mM thymidine was added to the cells under various growth conditions. Kaur *et al.* (1988) reported that thymidine at this concentration could completely circumvent the toxicity of MTX to wild-type DI700 *L. donovani*. These data indicated that the sole function of DHFR in *L. donovani* growth in DME-L is to provide a co-factor for thymidylate nucleotide biosynthesis.

Surprisingly, the addition of 0.1 mM thymidine did not permit the growth of either DI700 or MTXA5 cells in fdDME-L (Beck and Ullman, 1991). This demonstrated that although the sole role of DHFR was to provide a vehicle for TMP synthesis, folate served an additional function in *L. donovani*, presumably as a source of pterins. In mammalian cells, GTP, rather than folate, serves as a pterin precursor. Interestingly, however, the addition of 0.1 mM thymidine permitted MTXA5 cells to proliferate in fdDME-L medium supplemented with biopterin (Beck and Ullman, 1991). Thus, the MTXA5 cells could grow in fdDME-L medium containing both biopterin and thymidine, but not in fdDME-L augmented with either the pterin or thymidine alone. These data implied that selection of mutant cells for folate transport deficiency conferred an inability to convert exogenous pterins, which are transported by the MTXA5 cells (Beck and Ullman, 1991), to intracellular THFs.

INCORPORATION OF FOLATE INTO THFs

The capability of wild-type cells to multiply in fdDME-L medium supplemented with either biopterin or neopterin implied that the pterins were serving as a cellular source of reduced folates. When DI700 cells were incubated with 25 nM exogenous [³H]folate for 48 h, extracted and analysed for intracellular THFs by HPLC, three peaks of radioactivity were observed. The first peak eluted in the void volume prior to any folate standard. This peak was collected and rechromatographed on an HPLC system for the separation of pterins. This first radioactive peak co-chromatographed with the dihydrobiopterin standard. Thus, folate can serve as a pterin precursor in *L. donovani*, although GTP and not folate is the biopterin precursor in mammalian cells and tissues. The second and third peaks co-eluted with the two folate standards, 10-formylTHF and 5-methylTHF. No radiolabel was detected as unmetabolized [³H]folate. In order to confirm the identities of the two intracellular THFs, the extracts were also subjected to HPLC analysis on an anion exchange SAX column. Again three peaks were observed, two of which co-eluted with 10-formylTHF and 5-methylTHF. The fact that two of the three radioactive peaks co-chromatographed with known standards in both HPLC systems provided powerful evidence that wild-type *L. donovani* were capable of metabolizing exogenous folate into 5-methylTHF and 10-formylTHF. Scott *et al.* (1987) have previously demonstrated that *L. mexicana* can metabolize folate into 5-methylTHF. As expected by virtue of their folate transport-deficient phenotype, the MTXA5 strain did not incorporate large amounts of [³H]folate into 5-methylTHF or 10-formylTHF.

INCORPORATION OF BIOPTERIN INTO THFs

The ability of DI700 cells to proliferate in fdDME-L supplemented with biopterin or other pterins was consistent with the premise that wild type *L. donovani* could convert pterins to reduced THFs. When DI700 cells were incubated with 25 nM [³H]biopterin for 48 h under conditions similar to those employed with [³H]folate,

three radioactive peaks were again observed, two of which comigrated with the 10-formylTHF and 5-methylTHF standards. The [³H]biopterin employed as the precursor in these experiments did not contain any reduced folate contaminant as determined by HPLC. Both the rate and extent of [³H]biopterin metabolism into THFs was not affected by the addition of *p*-aminobenzoate (pABA) to the culture medium. No radioactivity was associated with 10-formylTHF or 5-methylTHF when the MTXA5 cell line was incubated with [³H]biopterin. As the MTXA5 cell line was selected for its inability to transport MTX and folate, it appears that it expresses a second biochemical lesion that confers an inability to convert pterins to THFs. Thus, a single genetic lesion can adversely affect two seemingly independent, although not altogether unrelated, biochemical functions. Whether the mutation that adversely influences both folate/MTX transport and pterin to THF conversion is in a structural gene for the folate transporter, in a regulatory gene that affects multiple biochemical processes, or even in some gene that affects membrane composition or overall membrane architecture remains to be established.

RELEVANCE TO CHEMOTHERAPY

The ability of *L. donovani* to convert folate to pterins and biopterin to intracellular THFs suggests an obvious potential avenue for the therapy of leishmaniasis. It may be possible to design an analogue of folate that interferes with pterin metabolism or a pterin analogue that obstructs folate metabolism in *L. donovani* without adversely affecting the pterin or folate metabolic pathways of the host cells and tissues. As the pterin and folate pathways in humans are biochemically distinct, whereas these same pathways are intertwined in *L. donovani*, such analogues offer an avenue for chemotherapeutic manipulation of leishmaniasis by selectively inhibiting the growth of the parasite without causing possible toxic side-effects toward the mammalian host.

ACKNOWLEDGEMENT

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51. Folate metabolism as a target for chemotherapy of malaria

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Folate metabolism of malaria parasites has proved to be vulnerable to chemotherapeutic attack. The currently used antimalarial drugs pyrimethamine, sulphonamides and sulphones all act against this area of parasite metabolism. This review is a summary of the present status of knowledge of folate metabolism of malaria parasites, emphasis being given to resistance against antifolates.

BIOSYNTHESIS AND SALVAGE OF DIHYDROFOLATE IN PLASMODIA

The folate metabolism of plasmodia has been reviewed in detail by Ferone (1977) and the present status of knowledge has been briefly summarized by Krungkrai *et al.* (1990). The pathways currently thought to be involved are shown in Figure 51.1.

The discovery that malaria parasites require an exogenous supply of *p*-aminobenzoate (pAB) and the successful therapy of drug-resistant *Plasmodium falciparum* malaria by the combination of quinine or pyrimethamine with sulphonamides indicated that plasmodia have their own biosynthesis of folate cofactors. It was suggested that plasmodia, like bacteria and plants but in contrast to the mammalian host, do not depend on uptake and utilization of exogenous folate. Subsequently, the enzymatic synthesis of dihydropteroate was demonstrated in *P. chabaudi*, *P. berghei*, *P. knowlesi*, *P. lophurae* and *P. gallinaceum* (Walter and Königk, 1971, 1974; Ferone, 1973; McCullough and Maren, 1974). These studies provided direct evidence for the *de novo* synthesis of dihydrofolate and confirmed that the chemotherapeutic action of sulphonamides and sulphones in the therapy of malaria depends on their interaction with dihydropteroate synthase, thereby establishing the key position of this enzymatic step as target for chemotherapy. It was not until 1985, however, that it was confirmed that the pteridine moiety for the synthesis of dihydrofolate is derived from guanosinetriphosphate (GTP); Krungkrai *et al.* (1985) identified and characterized GTP-cyclohydrolase, the initial enzyme in the biosynthetic pathway towards dihydrofolate, in *P. berghei* and *P. knowlesi*.

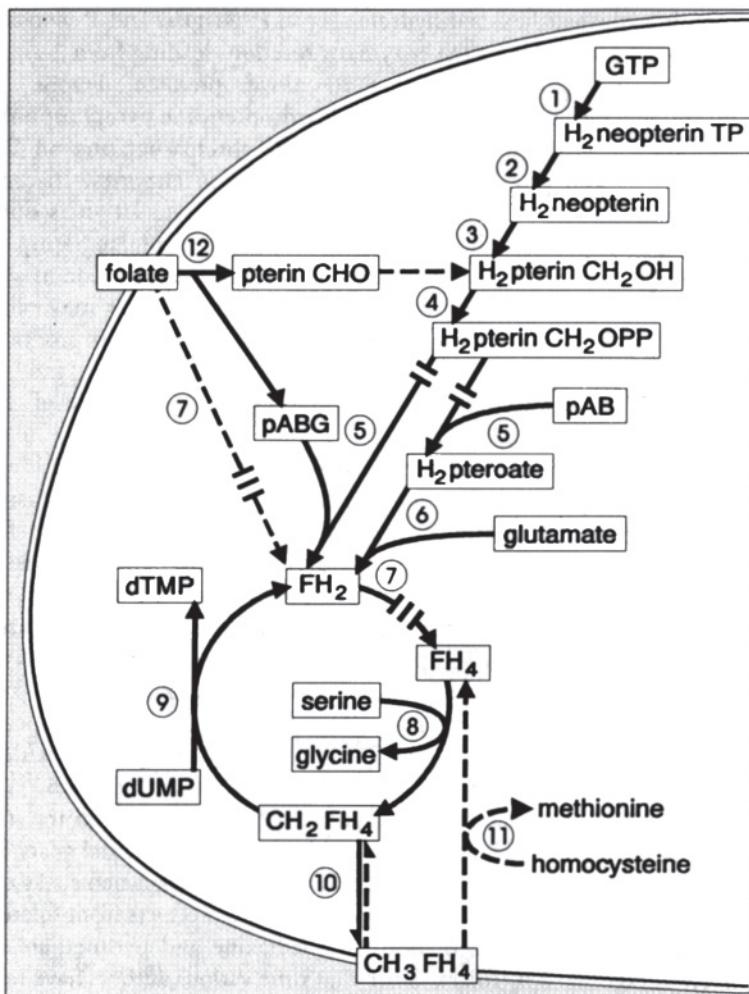


Figure 51.1. Pathway for dihydrofolate biosynthesis in plasmodia and possible utilization of exogenous folates. Enzymes and intermediates involved in the folate metabolism: 1, guanosine triphosphate cyclohydrolase; 2, dihydronoopterin triphosphatepyrophosphohydrolase; 3, dihydronoopterin aldolase; 4, hydroxymethylidihydropteridine pyrophosphokinase; 5, dihydropteroate synthase (the use of *p*-aminobenzoate or *p*-aminobenzoylglutamate as a substrate will yield dihydropteroate or dihydrofolate as product, respectively; for details see text); 6, dihydrofolate synthase; 7, dihydrofolate reductase; 8, serine hydroxymethyltransferase; 9, deoxythymidylate synthase; 10, 5, 10-methylenetetrahydrofolate reductase; 11, methionine synthase; 12, pteridine 6-methylhydrolase. GTP, Guanosine triphosphate; H₂neopterin TP, dihydronoopterin triphosphate; H₂ neopterin, dihydronoopterin; H₂ pterin CH₂OH, 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine; H₂ pterin CH₂OPP, 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphate; pterin CHO, pterinaldehyde; pAB, *p*-aminobcnzoate; pABG, *p*-aminobenzoylglutamate; H₂ pteroate, dihydropteroate; FH₂, dihydrofolate; FH4, tetrahydrofolate; CH₂FH₄, methyltetrahydrofolate; CH₃FH₄, methylenetetrahydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate. (—) Proposed pathways; (-/-) inhibited by sulphones and sulphonamides; (-//-) inhibited by antifolates.

There is still controversy about the enzymatic reactions leading from 2-amino-4-hydroxy-6-hydroxymethylidihydropteridine to dihydropteroate. Ferone (1973) reported on the separation of hydroxymethylidihydropteridine pyrophosphokinase and dihydropteroate synthase from *P. berghei* by chromatography on DEAE-Sephadex, which is also possible for the *Escherichia coli* enzymes. In contrast, Walter and König (1974, 1980) could not separate the two activities during a 950-fold purification which also included DEAE-Sephadex chromatography. These workers concluded that pyrophosphokinase and synthase might reside in a single protein, as found in plants, or alternatively that the two proteins may exist as a complex. The latter idea is supported by the fact that the intermediate products 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridinediphosphate and AMP were liberated and not combined on the enzyme molecule as in case of the isofunctional ligase from pea seedlings.

The last enzymatic step in the sequence towards dihydrofolate, the addition of glutamate to dihydropteroate, has not yet been demonstrated for plasmodia. Although results from utilization experiments provided some evidence for the occurrence of dihydrofolate synthase in plasmodia, a direct demonstration failed and Ferone (1977) questioned its occurrence. Indeed, plasmodia would not necessarily depend on dihydrofolate synthase, because the dihydropteroate synthase from *P. berghei* has been shown to accept *p*-aminobenzoylglutamate (pABG) as an alternate substrate resulting in the production of dihydrofolate directly (Ferone, 1973). This suggestion that dihydrofolate is directly synthesized is not supported by the K_m values reported for pAB and pABG; the latter is a poor substrate with about 100-fold less affinity for the dihydropteroate synthase compared to pAB.

In contrast to the hypothesis that plasmodia depend absolutely on the *de novo* synthesis of dihydrofolate, there has been evidence throughout the years for the utilization of exogenous folate from *in vivo* and *in vitro* experiments. As early as 1955, Rollo (1955) discussed the utilization of cleavage products from folate by *P. gallinaceum*, because the action of both sulphadiazine and pyrimethamine was competitively antagonized by folate. Since that time various authors have reported on antagonizing effects of pAB, folk acid and folinic acid against growth inhibition by sulphonamides and antifolates of *P. falciparum* maintained *in vitro* (McCormick *et al.*, 1971; Chulay *et al.*, 1984; Milhous *et al.*, 1984; Watkins *et al.*, 1985). More recently Krungkrai *et al.* (1989a) investigated the fate of labelled folate added to cultured *P. falciparum*; they found the label incorporated into pterinaldehyde, dihydrofolate and mainly, 5-methyltetrahydropteroylpentaglutamate; the latter was identified as the major folate derivative in plasmodia. These results establish the utilization of preformed folate. On the other hand, the authors demonstrated the synthesis of 5-methyltetrahydropteroylpentaglutamate from precursors such as GTP, pAB and glutamate, thereby also confirming the existence of the dihydrofolate biosynthetic pathway in plasmodia. In the scheme they proposed for folate salvage in plasmodia, they discussed reduction of folate by dihydrofolate reductase as well as degradation of folate by pteridine 6-methylhydrolase to pterinaldehyde and pABG, the latter moieties subsequently being utilized in the synthetic pathway (see Figure 51.1). These possible pathways for reutilization of folate as well as the

speculation about increased salvage synthesis in antifolate-resistant *P. falciparum*-strains need further proof. Previous enzymatic studies on the dihydrofolate reductase from various plasmodia excluded the possibility of the direct reduction and utilization of folate. In contrast to the dihydrofolate reductase from mammals which accepts folate as an alternate substrate, the plasmoidal enzyme is restricted to the reduction of dihydrofolate to tetrahydrofolate (Ferone *et al.*, 1969; Walter *et al.*, 1970; Platzer, 1974). An additional possibility for salvage of folate derivatives could be the uptake and utilization of host provided 5-methyltetrahydrofolate, which has also been shown to antagonize sulphonamide activity against *P. falciparum* *in vitro*. 5-Methyltetrahydrofolate could be used as a co-factor in the synthesis of methionine, thereby providing tetrahydrofolate. Alternatively, 5-methyltetrahydrofolate may be oxidized to methylenetetrahydrofolate as one-carbon-atomdonating co-factor in the synthesis of dTMP as reported from filarial worms (Jaffe, 1980; see also Chapter 50).

DIHYDROFOLATE REDUCTASE AND THE DEOXYTHYMIDYLATE SYNTHESIS CYCLE

Plasmodia differ from the host by their ability for *de novo* synthesis of dihydrofolate, which is subsequently reduced to tetrahydrofolate by the dihydrofolate reductase. Tetrahydrofolate co-factors are used in various pathways including synthesis of purines, deoxythymidylate and methionine. The absence of purine synthesis in plasmodia (Sherman, 1984; and see Chapter 2) restricts the significance of tetrahydrofolate co-factors to the deoxythymidylate synthesis cycle and possibly to the synthesis of methionine. It is generally assumed that the action of antifolates on the growth of plasmodia depends on the depletion of 5, 10-methylenetetrahydrofolate, the necessary co-factor for the synthesis of deoxythymidylate, thereby blocking DNA-synthesis. The existence of the enzymes involved in this 'deoxythymidylate synthesis cycle', dihydrofolate reductase, serine hydroxymethyltransferase and deoxythymidylate synthase, has been demonstrated in various plasmodia (Ferone, 1977). An outstanding feature of plasmodia and all other protozoa studied is the occurrence of dihydrofolate reductase and deoxythymidylate synthase as a bifunctional protein (Ferone and Roland, 1980; Garrett *et al.*, 1984), which additionally points to the unique significance of the folate metabolism for the synthesis of deoxythymidylate. Its significance as a target for chemotherapy is due to the absolute dependence of the parasite on *de novo* synthesis of deoxythymidylate by the deoxythymidylate synthase reaction; salvage of deoxythymidine by the deoxythymidine kinase has been excluded for plasmodia (Krungkrai *et al.*, 1989b). Taken together, the dihydrofolate reductase has a key position in the synthesis of deoxythymidylate and its potential as a chemotherapeutic target is well documented; antifolates used in the treatment of malaria like pyrimethamine, cycloguanil and trimethoprim are potent inhibitors of the dihydrofolate reductase from plasmodia. Results from Ferone *et al.* (1969) have shown that pyrimethamine and trimethoprim are bound more tightly to the *P.*

berghei enzyme than to the mouse erythrocyte enzyme. Knight and Peters (1980) compared dihydrofolate reductases from *P. knowlesi* and rat liver and found that selectivity for pyrimethamine was less than for trimethoprim and cycloguanil.

Combined treatment of malaria with an antifolate and a sulphonamide/sulphone, thereby interacting with sequential steps in the synthesis of tetrahydrofolate, was expected to potentiate the activity of the drugs. Results from Rollo (1955) on combined treatment of *P. gallinaceum* infection with pyrimethamine and sulphadiazine confirmed this hypothesis. Since then, the synergistic effect of such drug combinations has been successfully used in many malaria infections, and has also aided in overcoming antifolate resistance of some degree (Peters, 1987).

ANTIFOLATE RESISTANCE

Antifolates and sulphonamides are among the few antiparasitic drugs for which the mode of action is well known. Biochemical mechanisms of drug resistance have also been investigated. Three possible mechanisms of resistance of *P. falciparum* towards sulphonamides have recently been discussed by Dieckmann and Jung (1986a):

1. the uptake of sulphadoxine was found to be markedly reduced in resistant strains;
2. there was evidence for the *de novo* synthesis of pAB, which would compete and replace both exogenous pAB as well as sulphadoxine as substrates of the dihydropteroate synthase;
3. there was increased specificity of dihydropteroate synthase for pAB and consequently less affinity of the enzyme for sulphadoxine.

A final conclusion about the mechanism of sulphonamide resistance will depend on further experiments.

The mechanism of resistance towards antifolates has been the subject of various studies. Results from Kan and Siddiqui (1979) on *P. falciparum* showed an increased amount of dihydrofolate reductase in resistant strains; the authors suggested that gene amplification was responsible for the pyrimethamine resistance due to the over-production of the target enzyme. This type of mechanism is well known in drug-resistant mammalian cells and in methotrexate resistant *Leishmania tropica* (Coderre *et al.*, 1983; Meek *et al.*, 1985). Other studies on pyrimethamine resistance in *P. berghei* revealed both an increased amount of dihydrofolate reductase and changes in the kinetic properties of the enzyme (Ferone, 1970; Diggens *et al.*, 1970). More recently, studies on *P. chabaudi* and *P. falciparum* have clearly demonstrated that resistance towards pyrimethamine is a result of a modified dihydrofolate reductase that has a decreased affinity for the drug (Sirawaraporn and Yuthavong, 1984; McCutchan *et al.*, 1984; Dieckmann and Jung, 1986b; Walter, 1986; Chen *et al.*, 1987). The inhibition constants for pyrimethamine of enzymes from various resistant strains were shown to be increased up to several-hundred-fold. Slight decreases of the affinity of dihydrofolate reductase for the substrate

dihydrofolate and changes of the inhibition type by pyrimethamine were also reported. The large differences in the inhibition constants for pyrimethamine for the enzymes of drug-sensitive and drug-resistant strains of *P. falciparum* were subsequently shown to result from point mutations in the gene encoding for the dihydrofolate reductase (Inselburg *et al.*, 1988; Cowman *et al.*, 1988; Peterson *et al.*, 1988; Snewin *et al.*, 1989; Zolg *et al.*, 1989, 1990); mutations occurred at positions 59 and 108 of the dihydrofolate reductase gene, these residues are involved in substrate/inhibitor and co-factor binding. In contrast to these results with pyrimethamine resistance in natural isolates, Inselberg and colleagues (Inselberg *et al.*, 1987; Tanaka *et al.*, 1990) found not only point mutations but also gene duplication and over-production of dihydrofolate reductase as the causes of *in vitro* induced pyrimethamine resistance. Results from a recent experiment, however, on induction of methotrexate resistance in cultured *P. falciparum* are in agreement with the mechanism reported for naturally occurring resistance, a structurally altered enzyme with decreased affinity for methotrexate and pyrimethamine was found (Walter *et al.*, 1991).

ANTIFOLATES NOT SIGNIFICANTLY CROSS-RESISTANT WITH PYRIMETHAMINE

Evidence has been provided from the treatment of patients infected with antifolateresistant plasmodia, as well as from *in vitro* experiments, that there is little or no cross-resistance between different antifolates. Enzymatic studies on the dihydrofolate reductase from pyrimethamine resistant strains of *P. falciparum* revealed lack of cross-resistance towards methotrexate, whereas *in vitro* induced methotrexate resistant lines were also resistant towards pyrimethamine (Chen *et al.*, 1987; Walter *et al.*, 1991). The selectivity of methotrexate to inhibit the dihydrofolate reductase from plasmodia rather than the host is much less than for other classes of antifolates, thereby lowering its potential as a lead compound for the design of new antifolates. Potent antifolates are found within the classes of diaminopyrimidines and dihydrotriazines, as shown by pyrimethamine, trimethoprim and cycloguanil (proguanil). Reports on cross-resistance among these classes are contradictory and include a range from not significant to considerable cross-resistance (Mamalis and Werbel, 1984; Peters, 1987). Recently, 2, 4-diamino-5-substituted benzylpyrimidines, derivatives of trimethoprim in which methoxy groups at C3 or C4 of the benzyl ring are replaced by 3(4'-aminophenyl-4-sulphonylphenylamino) propoxy, were reported to be of high potency against pyrimethamine sensitive and pyrimethamine resistant strains of *P. falciparum* (Seydel *et al.*, 1990). The inhibition constant for the trimethoprim derivative K130 of the dihydrofolate reductase from the resistant strain was found to be slightly increased (about three-fold) compared to the sensitive strain, whereas the inhibition constants for pyrimethamine of both strains differed about 100-fold. This low degree of cross-resistance correlates well with results on the *in vitro* activity of various 2, 4-diamino-5-substituted benzylpyrimidines against cultured

pyrimethamine sensitive and pyrimethamine resistant strains. With respect to EC₅₀ values, these newly designed compounds are in the range of that for pyrimethamine and have a marked improvement in activity as compared to trimethoprim. Since they demonstrate lower toxicity in mammals and have a higher selectivity towards the parasite enzyme they have better therapeutic indices compared to pyrimethamine (Seydel *et al.*, 1990). Compounds such as these may have an important role in the treatment of pyrimethamine resistant malaria.

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52. Protein synthesis as a target for antiprotozoal drugs

T.D.Edlind

The protein synthesis inhibitors (PSIs) represent a large, structurally diverse and clinically indispensable group of antibacterial agents (Gale *et al.*, 1981; Kucers and Bennett, 1987). In the chemotherapy of protozoan parasites, however, PSIs have found considerably less use. There are only two, relatively uncommon parasitic infections for which PSIs are the drugs of choice: tetracycline for balantidiasis and clindamycin (plus quinine) for babesiosis (Abramowicz, 1986). Three additional PSIs are recommended as alternative agents: paromomycin, emetine (or its derivative dehydroemetine) and spiramycin. This limited development of anti-protozoal PSIs is in part due to the assumption that the protein synthesis machinery of parasite and host are largely similar, precluding selective toxicity. Recently, however, ribosomal RNA (rRNA) sequence analysis has revealed the protozoa to be a highly diverse group of organisms (Sogin *et al.*, 1989). Studies in our laboratory have focussed on the intestinal parasite *Giardia lamblia*; its rRNA is, overall, no more closely related to mammalian rRNA than is bacterial rRNA (Edlind, 1989a; Sogin *et al.*, 1989). Thus, the potential for selective antiprotozoal toxicity appears to exist. Furthermore, recent clinical studies have rekindled an interest in the anti-parasitic potential of PSIs. These studies include:

1. the use of tetracycline or doxycycline in chloroquine-resistant malaria (Krogstad *et al.*, 1988; Pang *et al.*, 1988);
2. the promising activities of spiramycin and clindamycin against *Cryptosporidium* infection in AIDS patients (Portnoy *et al.*, 1984; Moskovitz *et al.*, 1988); and
3. the treatment of cutaneous leishmaniasis with tropical paromomycin (El-On *et al.*, 1988) or tetracycline derivatives (Khairulin *et al.*, 1989).

These clinical studies of PSIs coincide with a recent rebirth of interest in the mechanism and structural components of protein synthesis (Moore, 1988; Dahlberg, 1989). The intent of this review is to bridge the gap between these diverse areas of research, and thus facilitate further the rational development of antiprotozoal PSIs.

In this chapter *in vitro* studies of PSI activity against protozoan parasites are reviewed and an overview given of the mechanism and components of protein synthesis. For many PSIs, the molecular targets within the protein synthesis machinery have now been defined (typically, in *Escherichia coli*); these data are reviewed. In conjunction with the expanding data base of protozoal rRNA sequences, the PSI target data afford new opportunities for drug development through structure-activity studies and, ultimately, rational drug design. Initial studies towards this goal are reviewed at the end of the chapter.

IN VITRO STUDIES OF PROTOZOAL SENSITIVITY TO PSIs

A comprehensive review of the large body of clinical data relating to antiparasitic activity of various PSIs was published by Steck in 1972; more recent studies are cited in Campbell and Rew (1986). In this review, I focus on the smaller number of *in vitro* studies characterizing parasite sensitivity to PSIs. *In vitro* systems provide greater potential for establishing the molecular basis for activity and selective toxicity.

Table 52.1 lists a selected group of PSIs which have documented *in vitro* activity against various protozoan parasites. Most of these drugs have been used clinically, with varying degrees of success. Clearly, therapeutic success depends on a number of factors, but the most important are activity against the pathogen and host toxicity. The two most active agents, emetine and anisomycin, are also the two most toxic. Emetine is the active alkaloid component of ipecacuanha root, which has been used to treat amoebic dysentery for at least 300 years. It is inactive against bacteria, but appears to have broad spectrum antiprotozoal activity in the range 0.1–10 µg ml⁻¹ (Table 52.1). Oral use, however, induces severe vomiting, and parenteral use must be carefully monitored for cardiac complications (Campbell and Rew, 1986). The derivative dehydroemetine has similar *in vitro* activity against *Entamoeba histolytica*, but has reportedly less toxicity. Anisomycin is a small molecule ($M_r=265$) with similar properties to emetine: antiprotozoal activity below 1 µg ml⁻¹ but high host toxicity. Prior to the development of metronidazole, anisomycin was of clinical interest for *Trichomonas vaginalis* infections.

With the exception of these two agents, the PSIs listed in Table 52.1 were developed as antibacterial agents and subsequently evaluated for their antiprotozoal activity. Not surprisingly, these antibiotics are both less toxic to the host and less active against most parasites. However, as is clear from Table 52.1, this activity can vary for some PSIs from totally lacking to moderately high. For example, chloramphenicol and clindamycin inhibit *Plasmodium falciparum* growth at concentrations as low as 1 µg ml⁻¹. These results are consistent with previous clinical use in malaria (Campbell and Rew, 1986). However, chloramphenicol and clindamycin are at least 100-fold less active *in vitro* against *G. lamblia* and *T. vaginalis*. Clearly, the variation is at the level of the organism under study. Indeed, there may even be significant variation within species. For example, two strains off *E. histolytica* have reported paromomycin and

erythromycin sensitivities that vary about 50-fold (Table 52.1). In one study of different *G. lamblia* isolates from a single hospital, paromomycin sensitivities varied from <1 to >100 µg ml⁻¹ (Gordts *et al.*, 1987).

A second potential source of variation in the *in vitro* antiprotozoal activity of PSIs is illustrated by the tetracyclines (Table 52.1). For all five parasites examined, there is an approximately 10-fold difference in activity between the pharmacologically 'short-acting' derivatives (tetracycline and oxytetracycline) and 'long-acting' derivatives (minocycline and doxycycline). In contrast, most bacteria are equally sensitive to these derivatives (Kucers and Bennett, 1987). There have been many clinical studies evaluating short-acting tetracyclines for various parasitic infections (Steck, 1972); the *in vitro* results clearly suggest that doxycycline or minocycline warrant investigation. Similarly, new macrolides have been developed (again, for bacterial infections) that have clearly enhanced activity against *Toxoplasma gondii* relative to the prototypes erythromycin or spiramycin (Table 52.1). However, when tested for inhibition of *G. lamblia* growth, all macrolides including the new derivatives were inactive.

Variation of antiprotozoal activity within a drug group is perhaps most clearly seen with the aminoglycosides. Eleven different aminoglycosides, along with the aminocyclitol spectinomycin, have negligible activity against *G. lamblia* at 200 µg ml⁻¹ (Edlind, 1989a; unpublished data). Three others, paromomycin, hygromycin and G418, are clearly active at or below 60 µg ml⁻¹. The latter two are toxic to mammalian cells, which leaves only paromomycin as a potentially useful antiparasitic agent (Table 52.1). The low absorption of paromomycin from the gut has focussed its clinical use on intestinal parasites (Abramowicz, 1986). Recently, however, topically applied paromomycin has been used successfully in the treatment of cutaneous leishmaniasis (El-On *et al.*, 1988).

Finally, the PSI most recently demonstrated to have potentially useful antiprotozoal activity is the steroid antibiotic fusidic acid (Table 52.1). This non-toxic, well-absorbed drug is finding increasing use in bacterial infections (Greenwood, 1988). It is moderately active *in vitro* against *G. lamblia* and *P. falciparum* (Table 52.1); the latter is sensitive at concentrations that are clinically achievable in the blood.

MECHANISM AND COMPONENTS OF PROTEIN SYNTHESIS

Coincident with the recent rebirth of interest in the ribosome and protein synthesis, several excellent reviews of this field have been published (Moore, 1988; Dahlberg, 1989). However, it is important to note that the model system for most of these studies remains *E. coli*; there may be significant differences in how proteins are synthesized in protozoa. The purpose here is to review briefly the general mechanism of protein synthesis, with an emphasis on known differences between bacteria and eukaryotes.

The machinery for protein synthesis is outwardly very complex, involving 50–70

Table 52.1. *In vitro* activity of antiprotozoal PSIs.

Drug	Toxicity ^a	Organism	Derivative	IC ₅₀ ^b (μ g ml ⁻¹)	Reference
Emetine	+++	<i>E. histolytica</i>		0.09	Neal (1978)
		<i>G. lamblia</i>		1.5	Gilliland and Diamond (1981)
		<i>L. major</i>		2.4	Cedeno and Krogstad (1983)
Anisomycin	+++	<i>G. lamblia</i>		1.8	Boreham <i>et al.</i> (1985)
		<i>T. vaginalis</i>		7.5	Gilliland and Diamond (1981)
		<i>P. falciparum</i>		0.1	El-On and Greenblatt (1983)
Tetracyclines ^c	+	<i>E. histolytica</i>	<3	Edlind <i>et al.</i> (unpublished)	
		<i>P. falciparum</i>	0.2	Sears and O'Hare (1988)	
		<i>E. histolytica</i>	min	0.014-1.5	Divo <i>et al.</i> (1985)
		<i>P. falciparum</i>	oxy	0.16-19	
		<i>E. histolytica</i>	tet	1.4-4.4	Geary and Jensen (1983)
		<i>G. lamblia</i>	tet	16	Gilliland and Diamond (1981)
		<i>T. vaginalis</i>	min	29	Neal (1978)
		<i>G. lamblia</i>	dox	49	Cedeno and Krogstad (1983)
		<i>L. major</i>	tet	5.2	Edlind (1989b)
		<i>T. vaginalis</i>	oxy	6.4	
		<i>T. vaginalis</i>	dox	36	
		<i>T. vaginalis</i>	tet	71	Katiyar and Edlind (unpublished)
		<i>T. vaginalis</i>	oxy	38	
		<i>T. vaginalis</i>	min	270	
		<i>T. vaginalis</i>	oxy	14	Katiyar and Edlind (unpublished)
		<i>T. vaginalis</i>	dox	20	
		<i>T. vaginalis</i>	tet	80	
		<i>T. vaginalis</i>	oxy	200	
		<i>T. vaginalis</i>	dox	6.4	Chang <i>et al.</i> (1990)
		<i>T. vaginalis</i>	tet	>40	
		<i>P. falciparum</i>		24	Black <i>et al.</i> (1985)
		<i>G. lamblia</i>		110	Farrington and Igge (1986)
Fusidic acid	-	<i>G. lamblia</i>		35	Edlind (unpublished)

Paromomycin	+ / -	<i>E. histolytica</i>	8	Gillin and Diamond (1981)
			0.7	Neal (1978)
		<i>G. lamblia</i>	48-61	Cedeno and Krogstad (1983)
			10	Gillin and Diamond (1981)
		<i>P. falciparum</i>	100	Boreham <i>et al.</i> (1985)
			55	Edlind (1985a)
		<i>L. major</i>	10	El-On and Greenblatt (1983)
			1.0	Geary and Jensen (1983)
		<i>E. histolytica</i>	15-24	Cedeno and Krogstad (1983)
		<i>G. lamblia</i>	40	Gillin and Diamond (1981)
			70	Boreham <i>et al.</i> (1985)
		<i>T. vaginalis</i>	>120	Gillin and Diamond (1981)
			150	Edlind <i>et al.</i> (unpublished)
		<i>P. falciparum</i>	>100	Sears and O'Hare (1988)
			1.4	Geary and Jensen (1983)
		<i>E. histolytica</i>	>250	Gault <i>et al.</i> (1985)
		<i>T. vaginalis</i>	>200	Edlind <i>et al.</i> (unpublished)
			257-360	Cedeno and Krogstad (1983)
		<i>P. falciparum</i>	>250	Gault <i>et al.</i> (1985)
			>100	Sears and O'Hare (1988)
		<i>G. lamblia</i>	rox	Chang and Pechere (1988)
			azi	
		<i>E. histolytica</i>	140	
		<i>T. vaginalis</i>	246	
			ery	Boreham <i>et al.</i> (1985)
		<i>P. falciparum</i>	130	Gault <i>et al.</i> (1985)
			>1250	Edlind <i>et al.</i> (unpublished)
		<i>T. vaginalis</i>	24-34	Cedeno and Krogstad (1983)
			ery, azi	Ravdin and Skilogianis (1989)
		<i>E. histolytica</i>	20	Gault <i>et al.</i> (1985)
			ery	Geary and Jensen (1983)
		<i>P. falciparum</i>	>1250	Sears and O'Hare (1988)
			ery, spi	
		<i>T. vaginalis</i>	7.3-23	
			>100	

^a From Abramowicz (1986). Campbell and Rew (1986) and Kuipers and Bennett (1987).

^b Where multiple strains were tested, the average is given. The range, when shown, represents results of different assays.

^c Abbreviations: tet, tetracycline; oxy, oxytetracycline; min, minocycline; dox, doxycycline.

^d Abbreviations: ery, erythromycin; rox, roxithromycin; azi, azithromycin; spi, spiramycin.

ribosomal proteins, 3–4rRNAs, 10 or more accessory protein ‘factors’, tRNAs and the enzymes required for their aminoacylation and, of course, the individual mRNA templates. Given this complexity, the ‘popularity’ of protein synthesis as a target for inhibition by naturally produced antibiotics and toxins is not surprising (for a review see Gale *et al.*, 1981). In recent years, most of our understanding of the mechanism of protein synthesis has come by focusing on the relatively few rRNA components (Dahlberg, 1989). The validity of this focus on rRNA stems from the current belief that the primordial ribosome was a ‘ribozyme’, composed of RNA alone. The discussion below reflects this perspective.

Protein synthesis initiates with the formation of a complex between the mRNA, small ribosomal subunit and a special initiator met-tRNA_f (in bacteria, the methionine is formylated). This complex is mediated to a considerable degree by standard base-pair interactions. In bacteria, mRNA binding involves pairing of the short ‘Shine-Dalgarno’ sequence on the mRNA with a complementary sequence at the 3'-end of small subunit (SS) rRNA. Eukaryotes employ a fundamentally different mechanism of mRNA binding. Eukaryotic mRNA and SS rRNA lack the Shine-Dalgarna complementarity, and instead use a ‘bind and scan’ mechanism (Kozak, 1981). The small subunit binds to the 5' cap structure common to eukaryotic mRNAs, and then migrates down the 5' leader region to what is generally the first AUG.

For both bacteria and eukaryotes, base pairing is involved in the recognition of the AUG initiation codon by the anticodon (UAC) of met-tRNA_f. To facilitate this codon-anticodon pairing, the met-tRNA_f (and all subsequently bound amino acyl-tRNAs) is specifically positioned on the small subunit, in close contact with the region of SS rRNA centred at nucleotide 1400. This region, termed the ‘decoding’ site, is conserved in bacteria and eukaryotes, although subtle differences correlated with aminoglycoside sensitivity exist (see below).

The formation of the initiation complex between mRNA, met-tRNA_f and the small subunit is followed by joining of the large subunit and the process of peptide elongation. The large subunit contains several functionally and spatially distinct sites for amino acyl-tRNA binding. The peptidyl (P) site is occupied by the tRNA which carries the growing peptide chain (it is also the site into which the met-tRNA_f is placed). The acceptor (A) site is normally occupied by the incoming amino acyl-tRNA, which is selected on the basis of proper codon-anticodon pairing. The central reaction of protein synthesis involves transfer of the peptide chain from the P site tRNA to the A site amino acyl-tRNA. This ‘peptidyl transferase’ reaction forms the new peptide bond. Subsequently, the peptidyl-tRNA returns to the P site in a step termed ‘translocation’, which also involves displacing the deacylated tRNA, and shifting of the mRNA to expose the next codon to the A site. The peptidyl transferase reaction centre, encompassing the A and P sites and the peptidyl transferase and translocase activities, has been localized to a specific secondary structure ‘loop’ within large subunit (LS) rRNA (for a review see Vester and Garrett, 1988).

The incoming amino acyl-tRNA is presented to the ribosome as a complex with an elongation factor plus GTP; in bacteria, this factor is called EF-Tu, and in

eukaryotes eEF1. A second elongation factor, EF-G (eEF2 in eukaryotes), along with a second GTP are required for the translocase reaction.

Additional factors are involved in the termination of protein synthesis, which is signalled by any of three termination codons on the mRNA. However, these codons differ in their efficiency and translational read-through can occur.

MOLECULAR TARGETS FOR PSIs

As mentioned above, recent progress in deciphering ribosome structure and function has been facilitated by focussing on the rRNA component. This applies as well to deciphering the mode of action of most PSIs. However, one PSI listed in Table 52.1 clearly lacks a rRNA target: fusidic acid, which binds to bacterial elongation factor EF-G, preventing its dissociation from the ribosome and thus blocking the elongation cycle. Apparently, fusidic acid demonstrates a gradient of activity from bacteria up to higher eukaryotes (for a review see Gale *et al.*, 1981). Analysis of protozoal eEF-2 sequences from sensitive and resistant organisms may reveal the basis for its moderate but potentially useful activity. Fusidic acid is well

Table 52.2. Ribosomal RNA targets for antiprotozoal PSIs.

rRNA	Drug	Mechanism	Nucleotide ^a	Method ^b	References	
SS	Paromomycin	mRNA misreading	C1409 G1491	Mutant Mutant	Li <i>et al.</i> (1982) Spangler and Blackburn (1985)	
	Tetracycline	Block amino acyl-tRNA	A892	Protection	Moazed and Noller (1987a)	
LS	Chloramphenicol	Peptidyl transferase inhibitor	G2057 G2447 A2451 C2452 A2503 U2504 A2059 A2062 A2451 G2505		Mutant Protection	Vester and Garrett (1988) (review) Moazed and Noller (1987b)
	Lincomycin	Peptidyl transferase inhibitor	A2058	Mutant	Vester and Garrett (1988) (review)	
	Erythromycin	Translocase inhibitor	C2611 G2057 A2058 A2058 A2059 A2505	Mutant Protection	Vester and Garrett (1988) (review) Moazed and Noller (1987b)	
	Spiramycin	Translocase inhibitor	C2611	Mutant	Sor and Fukuhara (1984)	
	Anisomycin	Peptidyl transferase inhibitor	G2447 C2452 A2453	Mutant	Hummel and Bock (1987)	

^a *E. coli* numbering (Dams *et al.*, 1988; Gutell and Fox, 1988).

^b Method used to elucidate mechanism of action.

absorbed and notably lacking in host toxicity, and structurally related fusidanes have been isolated that remain to be tested for antiprotozoal activity.

A second PSI listed in Table 52.1 apparently binds to the small ribosomal subunit and, therefore, presumably to the SS rRNA, but its nucleotide target has not yet been determined. Emetine inhibits protein synthesis in all eukaryotes examined, but not in bacteria (Gale *et al.*, 1981). Emetine resistant mutants have been isolated which have an electrophoretically altered small subunit ribosomal protein. Interestingly, emetine inhibits translocation, similar to the macrolides which bind to the large subunit; perhaps it interferes with mRNA movement required during this step. At this time, the basis for the eukaryote-specific activity of emetine is unclear.

Table 52.2 summarizes current data on PSI targets in rRNA. Two complementary approaches have been used to generate most of these data:

1. Organisms with PSI-resistant mutations in their rRNA can be selected and the mutation identified by DNA sequencing. This can be done directly in systems with single rRNA gene copies (mitochondria, chloroplasts, *Tetrahymena* and some archaebacteria). Alternatively, specific rRNA mutations can be generated by recombinant DNA techniques, introduced into *E. coli* on a multicopy plasmid, and tested for their PSI sensitivity.
2. The PSI binding site can be physically mapped by chemical protection techniques, coupled with rRNA sequencing, as pioneered by Moazed and Noller (1987a, b).

Paromomycin has useful activity against a variety of protozoan parasites (Table 52.1). Paromomycin belongs to a diverse group of PSIs collectively known as the aminoglycosides. No less than six distinct aminoglycoside binding sites have been mapped on SS rRNA (Moazed and Noller, 1987a; for a review see De Stasio *et al.*, 1988). Nevertheless, all but one are located within 140 nucleotides of the 3'-end of SS rRNA, and the exception (A915 for streptomycin) is close to this region when the rRNA is folded into its characteristic secondary structure. Aminoglycosides (with the exception of kasugamycin) have their primary effect on translational fidelity. Recall that the 1400 region of SS rRNA is the 'decoding' site which interacts with amino acyl-tRNA. Aminoglycoside binding adjacent to this site (e.g. gentamicin at G1405) promotes misreading of the mRNA, possibly by stabilising the binding of an incorrect amino acyl-tRNA. The synthesis of aberrant proteins, rather than the inhibition of protein synthesis, may provide these aminoglycosides with their characteristic cidal activity (most other PSIs have static activity). The paromomycin target has been localized by mutational analysis (Table 52.2); the two nucleotides identified form a base pair at the top of a hairpin in the rRNA secondary structure (Spangler and Blackburn, 1985).

Tetracycline is the classic broad-spectrum antibiotic, and its antiprotozoal activity is now also well appreciated (Table 52.1). The only tetracycline derivative whose binding site and mechanism of action have been studied is tetracycline itself (Table 52.2). Tetracycline blocks the amino acyl-tRNA binding site on the small subunit, inhibiting elongation and potentially initiation as well. Its binding site

includes nucleotide A-892 of SS rRNA (Table 52.2), and this nucleotide is conserved in nearly all rRNA molecules (Dams *et al.*, 1988). This provides a rationalization for the broad spectrum of tetracycline activity; cell-free systems from both bacteria and mammalian cells are sensitive to tetracycline (Gale *et al.*, 1981). However, intact bacteria are sensitive because they accumulate the drug by active transport (Franklin and Higginson, 1970); resistant bacteria are often altered in their uptake of the drug (Kucers and Bennett, 1987).

Chloramphenicol and clindamycin are related PSIs which have limited but perhaps clinically useful antiprotozoal activity (Table 52.1). Chloramphenicol and lincomycin (a close structural relative of clindamycin) are both specific inhibitors of the peptidyl transferase reaction in bacteria (Gale *et al.*, 1981). Not surprisingly, the rRNA targets for these agents overlap (Table 52.2). The Chloramphenicol targets fall within three blocks of rRNA which are far apart in the primary sequence, but form a 'loop' within domain V of LS rRNA (Vester and Garrett, 1988). This loop sequence is highly conserved (Gutell and Fox, 1988), although there are distinct differences between eukaryotes and bacteria (Edlind *et al.*, unpublished). The macrolides erythromycin and spiramycin, inhibitors of the translocase reaction, also target nucleotides within this LS rRNA loop (Table 52.2). This loop, of course, corresponds to the peptidyl transferase centre independently identified as the large subunit binding site for amino acyl-tRNA (see above).

MOLECULAR BASIS FOR ANTIPROTOZOAL ACTIVITY AND SELECTIVE TOXICITY

Giardia as a model

Our studies have focussed on the intestinal parasite *G. lamblia* as a model for studies of antiprotozoal PSIs, for a variety of reasons:

1. The organism can be readily adapted to axenic culture.
2. As an anaerobe, it belongs to a small group of mitochondria-lacking eukaryotes (see Chapter 6). The presence of mitochondria in most other protozoa complicates the identification of PSI target, since these organelles have their own protein synthesis machinery. For example, whether or not mitochondrial protein synthesis represents the primary tetracycline target in *P. falciparum* (Kiatfuengfoo *et al.*, 1989) probably depends on the growth conditions employed (Divo *et al.*, 1985).
3. The rRNA of this organism is well characterized (Sogin *et al.*, 1989; Edlind, 1989a; Healey *et al.*, 1990; Edlind *et al.*, 1990), facilitating structure-activity studies.
4. *G. lamblia* possesses possibly the 'simplest' cytoplasmic rRNA of any organism: not only is it unusually short, it is also predominantly (80 per cent) G and C.
5. *G. lamblia* itself is an important pathogen in need of new therapeutic approaches.

Paromomycin

As reviewed above, the aminoglycosides are a diverse group of drugs which primarily induce misreading of the mRNA template, rather than inhibition of protein synthesis. Most are bacteria specific, with the exception of paromomycin whose activity extends to several (if not all) protozoa. The data on aminoglycoside targets in rRNA was recently applied to an analysis of the 3'-end of *G. lamblia* SS rRNA (Edlind, 1989a). As shown in Figure 52.1(a), this region of *G. lamblia* rRNA includes target nucleotides for six different aminoglycosides. The targets for kasugamycin (A1518 and A1519), hygromycin (U1495) and kanamycin/gentamicin (G1405) are conserved in *G. lamblia* and all other organisms. The DNA sequence obtained does not reveal the methylation status of the kasugamycin target, which is required for its activity; therefore, no predictions of sensitivity can be made. However, hygromycin and gentamicin sensitivity would be predicted on the basis of this sequence analysis alone. On the other hand, kanamycin has an additional target (A1408), which it shares with apramycin. *G. lamblia* and all other eukaryotes have G at this position, thus predicting kanamycin/apramycin resistance. Finally, the paromomycin target (base pair C1409-G1491) is present as it is in bacterial rRNA, predicting sensitivity.

When the sensitivity of *G. lamblia* growth *in vitro* to various aminoglycosides was measured (Figure 52.1(b)), the predictions made above were borne out, with one exception. Both paromomycin and hygromycin were active, kasugamycin was weakly active, while kanamycin and apramycin (along with five additional aminoglycosides) were inactive. Contrary to the sequence analysis, gentamicin was inactive, indicating that there are additional, uncharacterized requirements for this aminoglycoside's activity.

The general implication of this initial study is that rRNA sequence analysis can be helpful in understanding the molecular basis for PSI activity. Furthermore, the ability to predict PSI sensitivity on the basis of rRNA sequence alone should be useful in selecting drugs for clinical testing against non-culturable pathogens. For example, the SS rRNA of the AIDS pathogen *Pneumocystis carinii* (Edman *et al.*, 1988) lacks the paromomycin base pair, and sensitivity is thus unlikely. On the other hand, *P. falciparum* (McCutchan *et al.*, 1988), *L. donovani* (Looker *et al.*, 1988), *Trypanosoma brucei*, and *Acanthamoeba castellani* (for compilation see Dams *et al.*, 1988) have this base pair, and should be sensitive. Finally, rRNA sequence analysis can be used to explain the selective toxicity of paromomycin. Both cytoplasmic and mitochondrial SS rRNA in mammalian cells lack the 1409–1491 base pair (Dams *et al.*, 1988), and should be naturally resistant to this aminoglycoside.

Tetracyclines

The molecular basis for the selective toxicity of the tetracyclines appears to be more complex than for the aminoglycosides, since the single rRNA target that has been defined (A892) is conserved in all organisms (Dams *et al.*, 1988). It appears that both cytoplasmic and mitochondrial protein synthesis can be inhibited by these agents. The studies of Divo *et al.* (1985) and Kiatfuengfoo *et al.* (1989) support a

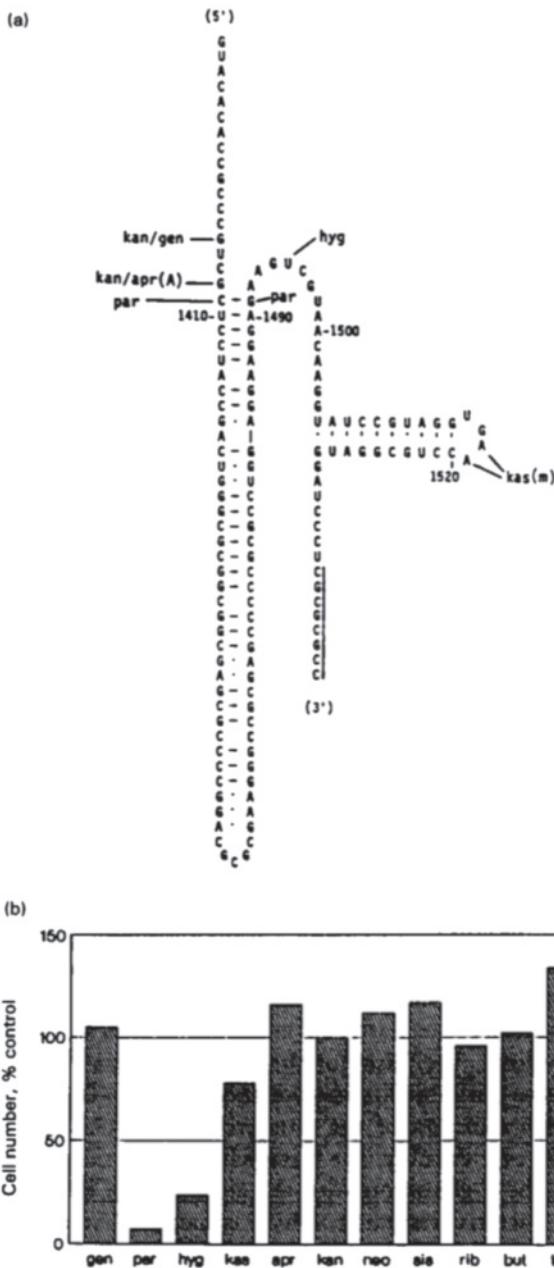


Figure 52.1. (a) Sequence and secondary structure of the 3'-end of *G. lamblia* SS rRNA. The numbering is that of *E. coli* (Dams *et al.*, 1988). Nucleotide targets for six different aminoglycosides are shown, (b) Inhibition of *G. lamblia* growth *in vitro* by various aminoglycosides at 200 µg ml⁻¹. gen, gentamicin; par, paromomycin; hyg, hygromycin; kas, kasugamycin; apr, apramycin; kan, kanamycin; neo, neomycin; sis, sisomycin; rib, ribostamycin; but, butirosin; tob, tobramycin. (Modified from Edlind (1989a).)

Table 52.3. Effects of tetracycline derivatives on *G. lamblia*: correlations between lipophilicity, uptake, inhibition of cell-free protein synthesis and parasite growth.^a

Tetracycline derivative	Lipophilicity ^b	<i>In vitro</i> growth (IC ₅₀ , µg ml ⁻¹)	Cell-free synthesis (IC ₅₀ , µg ml ⁻¹)	Uptake ^c
Oxytetracycline	0.025	71	500	1.7
Tetracycline	0.025	36	340	1.7
Chlortetracycline	0.13	23	NT ^d	7.2
Doxycycline	0.52	6.4	200	47
Thiacycline	2.38	1.8	200	110

^aModified from Edlind (1989b) and Katiyar and Edlind (unpublished).

^b Partition coefficient between octanol and phosphate buffer (pH 7.50) (Rogalski, 1985).

^c Ratio of intracellular to extracellular drug concentration after 1 h incubation.

^d Not tested.

mitochondrial target in *P. falciparum*; on the other hand, three mitochondrial lacking protozoa are sensitive to the tetracyclines (Table 52.1). As reviewed above, *in vitro* studies have revealed a 10-fold difference in antiprotozoal activity of short-and long-acting tetracycline derivatives. Since the differential activity correlates with the lipophilicity of the derivative (Edlind, 1989b), we examined an additional, experimental tetracycline with several-fold higher lipophilicity. This derivative, thiacycline, was indeed three- to four-fold more active than minocycline and doxycycline. (Unfortunately, thiacycline displayed central nervous system toxicity in clinical trials (Rogalski, 1985).)

Given the correlation with lipophilicity, a reasonable hypothesis is that membrane permeability (uptake) determines the relative tetracycline activity. Alternatively, the enhanced antiprotozoal activity of the lipophilic tetracyclines may be mediated at the ribosome level. To test these alternatives, Katiyar and Edlind (unpublished) measured the relative uptake of tetracycline derivatives into *G. lamblia*, and the relative inhibition of *G. lamblia* cell-free protein synthesis. As shown in Table 52.3, there was at most a 2.5-fold difference in the inhibitory activities of lipophilic and non-lipophilic tetracyclines in cell-free protein synthesis. In contrast, there were large differences (up to 65-fold) in uptake which paralleled closely the previously determined growth inhibition activity.

Although these results adequately explain the enhanced antiprotozoal activity of lipophilic tetracyclines, the basis for their selective toxicity remains less clear. Uptake experiments performed in the presence of metabolic poisons provided no evidence for active transport of the tetracyclines into *G. lamblia* (Katiyar and Edlind, unpublished). Possibly, the tetracyclines are actively transported *out* of mammalian cells by P-glycoprotein or the equivalent (Chen *et al.*, 1986). This would resemble bacterial resistance, which is mediated in some cases by active efflux of tetracycline by a plasmid-encoded membrane protein (Kucers and Bennett, 1987).

Peptidyl transferase and translocase inhibitors

Half of the antiprotozoal PSIs listed in Table 52.1 target the peptidyl transferase centre of LS rRNA. Erythromycin and spiramycin, and presumably other

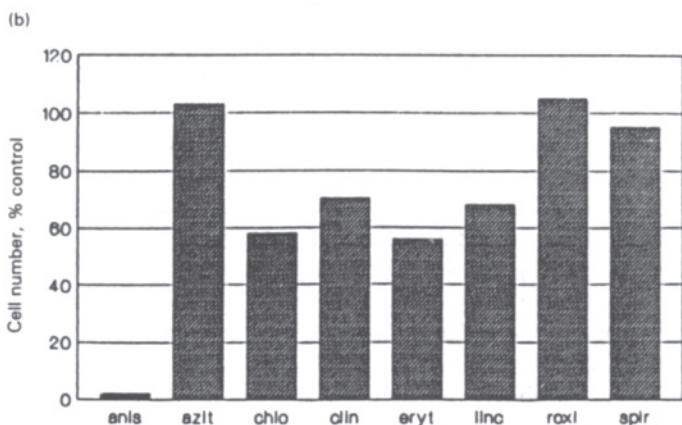
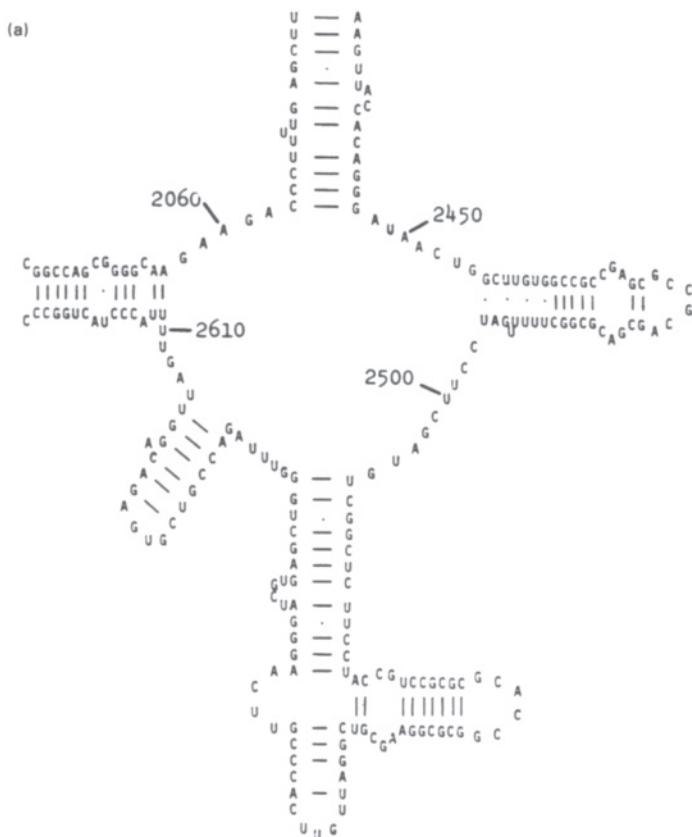


Figure 52.2. (a) Sequence and secondary structure of the peptidyl transferase centre of *G. lamblia* LS rRNA. The numbering is that of *E. coli* (Gutell and Fox, 1988). Refer to Table 52.2 and the text for PSI nucleotide targets. (b) Inhibition of *G. lamblia* growth *in vitro* by macrolides and peptidyl transferase inhibitors at 200 µg ml⁻¹; anis, Anisomycin; azit, azithromycin; chlo, chloramphenicol; din, clindamycin; eryt, erythromycin; line, linomycin; roxi, roxithromycin; spir, spiramycin. (Modified from Edlind *et al.* (unpublished).)

macrolides, block translocation by targetting G2057, A2058 and C2611 in bacteria or mitochondria. When the sequence of the *G. lamblia* peptidyl transferase centre was analysed (Figure 52.2(a)) (Edlind *et al.*, unpublished), it was apparent that all three nucleotide targets were altered. This strongly implied that the macrolides would be inactive against *G. lamblia*, and this was experimentally confirmed (Figure 52.2(b)). Similarly, lincomycin (and presumably the related clindamycin) target A2058, which is altered in *G. lamblia*; both drugs were inactive as predicted from sequence analysis (Figure 52.2). Chloramphenicol may be the most extensively studied PSI, and as a probable consequence more nucleotides within the peptidyl transferase centre have been implicated in its action than for any other drug. However, 9 of the 10 chloramphenicol targets are universally conserved nucleotides and, therefore, uninformative in terms of explaining the selective toxicity of this drug. Between bacteria and mammals, only G2057 is altered. As in mammals, *G. lamblia* has A at this position, and this difference alone may account for the relative inactivity of chloramphenicol against this organism (Figure 52.2). LS rRNA sequences for the other protozoan parasites listed in Table 52.1 are not yet available, but it is reasonable to assume that their peptidyl transferase centres share the eukaryotic consensus sequence. Thus, the low to moderate activity of chloramphenicol, clindamycin, and the macrolides against several other protozoa (Table 52.1) can be understood on a molecular level. A notable exception is *P. falciparum*, which is clearly sensitive to these drugs. As with the tetracyclines, these antibiotics may inhibit growth of this parasite by blocking mitochondrial protein synthesis (Divo *et al.*, 1985; Kiatfuengfoo *et al.*, 1989).

Anisomycin is, like chloramphenicol, a low-molecular-weight inhibitor of the peptidyl transferase reaction. The similarities between these agents extend to their rRNA targets: two of the three anisomycin targets (G2447 and C2452) are universally conserved nucleotides also targetted by chloramphenicol (Table 52.2). However, these two drugs have opposite activities: chloramphenicol is bacteria specific and anisomycin is eukaryote specific (Gale *et al.*, 1981). This is presumably due to the third anisomycin target, U2453, which is conserved in eukaryotes but is an A in all bacteria (for compilation see Gutell and Fox, 1988). *G. lamblia* has U at this position and is, predictably, anisomycin sensitive (Figure 52.2) (Edlind *et al.*, unpublished). This is presumably true as well for other protozoan parasites, although their LS rRNA sequences are not available for analysis. Anisomycin is too toxic to be used clinically. However, it is important to our understanding of the eukaryotic peptidyl transferase centre, and it may provide a starting point for the development of protozoa-specific inhibitors of peptidyl transferase.

The structure-activity relationships examined above for the macrolides and peptidyl transferase inhibitors hinge on the assumption that drug uptake was not variable in the *in vitro* assays. To test this assumption, the sensitivity of *G. lamblia* cell-free protein synthesis to these drugs was evaluated (Edlind *et al.*, unpublished). Anisomycin was inhibitory at levels as low as 3 µg ml⁻¹; the bacteria-specific inhibitors were inactive up to 300 µg ml⁻¹.

CONCLUSIONS AND PROSPECTS

PSIs have a long but less than illustrious history as antiprotozoal agents. There are, however, important reasons for taking a fresh look at PSIs at this time. Primarily, there has been considerable recent progress in identifying the molecular targets for PSIs. When applied to the ever-expanding data base of protozoal rRNA sequences, structure-activity relationships can be deduced. These relationships may have direct clinical relevance; e.g. encouraging (or discouraging, in the case of *Pneumocystis*) the further testing of paromomycin. Similarly, identification of tetracycline uptake as the major variable in its antiprotozoal activity may enhance the use of these agents. For example, substitution of doxycycline for tetracycline may extend activity to the invasive as well as luminal stages of *E. histolytica* and *Balantidium coli* infections.

Structure-activity studies have also argued for a mitochondrial rather than cytoplasmic target for several PSIs. The importance of this organelle and its protein synthesis to parasites such as *P. falciparum* warrants further investigation. Finally, the long term goal is, of course, the rational design of protozoa-specific PSIs. Understanding the molecular basis for the specificity of PSIs such as chloramphenicol and anisomycin, similar in several ways but opposite in their activity spectra, will bring us closer to this goal. In fact, rationally designed PSIs are at hand. Short oligonucleotides complementary to accessible and functionally important regions of rRNA have been used to inhibit protein synthesis in cell-free systems (Hill *et al.*, 1988). In essence, the oligonucleotides mimic the antibiotics, which may do their damage simply by blocking the binding of substrate (i.e. mRNA or amino acyltRNA). Oligonucleotide analogues that are stable and membrane permeable are being actively developed. These and other rationally designed PSIs represent exciting prospects for the next 'generation' of antiprotozoal agents.

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53. The mode of action of benzimidazoles against *Giardia* and their chemotherapeutic potential against *Giardia* and other parasitic protozoa

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INTRODUCTION

Giardia is prevalent in developing countries and is now recognized as one of the ten major parasites of humans, being equal to ascariasis as a cause of death in the developing world (Warren, 1989; Meyer, 1990). In developed countries, *Giardia* has the distinction of being the most commonly reported human intestinal parasite (Wolfe, 1975; Acha and Szarfres, 1987). Chemotherapy remains an important component of giardiasis control, particularly in developing countries (Islam, 1990) and among disadvantaged groups such as Australian Aborigines. However, for effective treatment of nutritional disorders in infants and as a means of interrupting transmission, regular treatment may be necessary. Under these circumstances, the present anti-giardial drugs have limitations with respect to possible side-effects, treatment failures and palatability (Jokipii and Jokipii, 1979; Voogd, 1981; Webster, 1985; Speelman, 1985; Meloni *et al.*, 1990). It is also of concern that there is only a relatively limited range of drugs available for the treatment of giardiasis. These drugs comprise the nitroimidazoles, quinacrine and furazolidone, none of which is considered to be ideal (James and Gilles, 1985).

Benzimidazole drugs (BZs) have been used as anthelmintics for some time and are thought to act by binding to the structural protein tubulin (Borgers and De Nollin, 1975; Lacey, 1985, 1988, 1990). Until recently, however, there had been few reports on the activity of BZs against parasitic protozoa.

MICROTUBULES AS TARGETS FOR ANTIPROTOZOAL DRUGS

The realization of the importance of structural microtubules for motility and as potential drug targets in protozoa has been highlighted by recent biochemical and ultrastructural studies demonstrating the microtubule-based cytoskeleton of trypanosomes and their associated proteins (DeSouza, 1984; Russell and Dubremetz, 1986; Seebeck *et al.*, 1988, 1990; Macrae and Gull, 1990). Similarly, the important role of *Giardia* tubulin in association with giardins for structure and ventral disc function has been well described by Peattie (1990). The integral role of microtubules in the ventral disc along with the presence of actin, myosin, a-actinin and tropomyosin (Feely *et al.*, 1982) suggests that any disruption of microtubules might have dire consequences for the parasite.

The precedent for a chemotherapeutic role for microtubule inhibitors has been set by the extraordinary success of the benzimidazoles (BZs) as anthelmintics (Horton, 1990). There is now much evidence which supports the postulate that the BZs are anthelmintic by virtue of their binding to parasite tubulin (Lacey, 1985, 1988, 1990). Evidence includes the findings that, in some resistant worm populations, tubulin binding is reduced (Sangster *et al.*, 1985; Lacey, 1985; Lacey and Prichard, 1986; Lacey and Snowdon, 1988). Resistance in other species appears to result from mutations affecting β -tubulin (*Aspergillus nidulans*; Sheir-Neiss *et al.* (1978); *Caenorhabditis elegans*. Woods *et al.* (1989).)

There are a number of recent reports of the efficacy of BZs against parasitic protozoa. Mebendazole, which is probably the most widely studied of the BZs, has been reported to be effective in treating a human case of *Trichomonas hominis* (Al Waili, 1987). Subsequently, Al Waili *et al.* (1988) and Cheng-i (1988) reported good success rates with BZs against human infections of *Giardia* and *Entamoeba histolytica*. Gascon *et al.* (1989), however, reported less success.

Support for the potential of the BZs also comes from *in vitro* studies in which several BZs have been shown to reduce viability and growth of *Plasmodium falciparum* (Dieckmann-Schuppert and Franklin, 1989) and *G. duodenalis* (Meloni *et al.*, 1990).

In view of the need for new anti-giardial agents, we have followed up these earlier reports suggesting therapeutic potential for mebendazole and albendazole against *Giardia* by investigating the activity of albendazole against *Giardia* and *Trichomonas* species, both *in vitro* and *in vivo*, and compared its effect with those of the 5-nitroimidazoles, metronidazole and tinidazole, which are currently used in the treatment of giardiasis and trichomoniasis.

EFFECTS OF ALBENDAZOLE ON GIARDIA AND TRICHOMONAS

Albendazole and other BZs are potent *in vitro*, in particular against *Giardia*. Albendazole is active against *Giardia* at markedly lower concentrations than

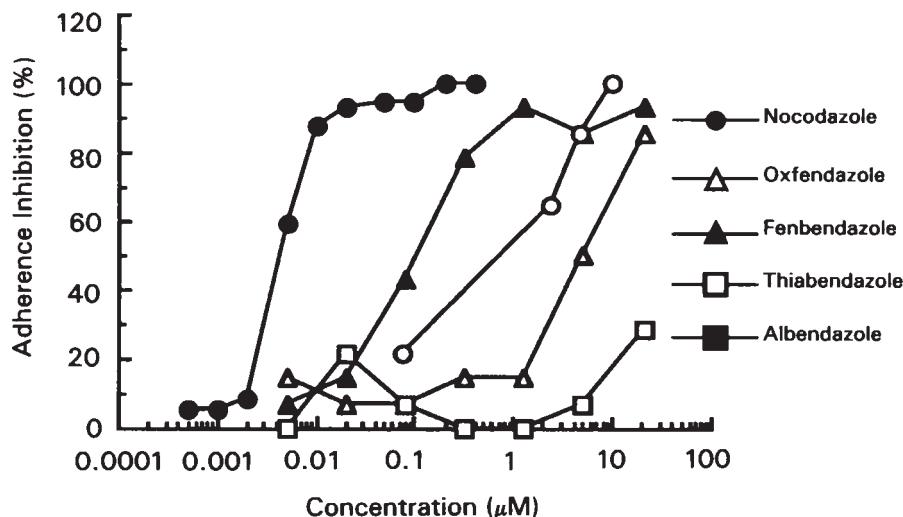


Figure 53.1. Inhibition of adherence of trophozoites to the culture flask by benzimidazole compounds. Incubation with drugs was for 24 h.

metronidazole and the effect persists for 72 h, unlike those of the 5-nitroimidazoles (Meloni *et al.*, 1990). These data suggest that albendazole, if acting on tubulin, may have an 'irreversible' effect. Similarly, the BZs nocodazole, oxfendazole, fenbendazole, thiabendazole and mebendazole, all reduce *Giardia* viability *in vitro* (Figure 53.1). It is interesting that in similar trials albendazole sulphoxide was much less potent than albendazole itself, a finding which differs from those with nematodes. Despite this discrepancy, there is a generally good correlation between the effectiveness of BZs against *Giardia* and against nematode larvae ($r=0.994$; nematode data from Lacey (1988)). In contrast, triclabendazole, a potent benzimidazole antitrematodal agent used in sheep, does not inhibit colchicine binding to helminth tubulin or prevent the embryonation of liver fluke eggs at high concentrations (Fetterer, 1986). Thus triclabendazole appears to act on other, non-tubulin related processes and, interestingly, in our experiments, it did not affect *Giardia* *in vitro* even at a concentration of 112 μM . Similarly, the tubulin inhibitor tubulozole (*cis* and *trans*) was ineffective against *Giardia* at concentrations up to 10 μM .

In vivo studies support the findings that the BZs are effective antigiardial agents. Three to four doses of albendazole (79–100 mg kg⁻¹) are completely effective in clearing the small intestine of trophozoites and eliminating cyst excretion. These data are probably an underestimate of the potential efficacy of albendazole *in vivo* for the gastrointestinal transit in mice is rapid and so contact of drug with the parasite is short.

Both *Trichomonas vaginalis* and *Tritrichomonas foetus* are adversely affected *in vitro* by albendazole, albendazole sulphoxide and mebendazole. However, the BZs are less active against trichomonads than against *Giardia* although the sensitivity of *T. foetus* was greater than that of *T. vaginalis*.

MODE OF ACTION OF BENZIMIDAZOLES AGAINST PROTOZOA

Most authorities consider that the primary mode of nematodal action of BZs, including albendazole, is their selective binding to tubulin which prevents its polymerization (Lacey, 1985, 1988, 1990). Such disruption of the tubulin-microtubule equilibrium and inhibition of the functions of tubulin are considered to lead to a cascade of direct and indirect biochemical and physiological changes resulting in loss of cellular homeostasis (Lacey, 1988, 1990; Criado-Fornelio *et al.*, 1990). A similar primary mode of action for albendazole against *Giardia* tubulin is suggested by our observations. Albendazole was found to slow protein turnover owing to an inhibition of lysosomal-like protein degradation mechanisms (Crowe, Costa and Thompson, unpublished observations). As in nematodes (Prichard *et al.*, 1978), the duration of exposure to albendazole was shown to be important, with inhibition of degradation not becoming apparent until after 12 h of treatment. Microtubules are an essential part of lysosomal degradation (Cleveland and Havercraft, 1983), thus explaining why albendazole causes the retention of proteins within trophozoites without them being degraded. Albendazole also increased DNA levels in trophozoites of *Giardia* (Crowe, Costa and Thompson, unpublished observations). Such an effect can be explained by the occurrence of nuclear division without separation of cytoplasmic membranes. This is supported by the fact that the increase in DNA for cells treated with albendazole did not become significant until 12 h after exposure, which correlates with the mean generation time of 10–12 h.

HUMAN USE OF BENZIMIDAZOLES

Albendazole is rapidly but only relatively poorly absorbed from the gastrointestinal tract and it appears that, once absorbed, it is completely metabolized to the sulphoxide and sulphone (Marriner and Bogan, 1980; Botero, 1986). Albendazole causes relatively few adverse reactions and has now been used at high dose rates extensively throughout developing communities and in patients with hydatidosis (Firth, 1983; Saimot *et al.*, 1983; Morris *et al.*, 1983). Similar lack of severe toxic side-effects has been noted with cambendazole, mebendazole and flubendazole, although side-effects with thiabendazole are common (Botero, 1986). Albendazole and other BZs, however, are known to cause teratogenesis and embryotoxicity in laboratory animals (Botero, 1986). It is possible that flubendazole should be examined more carefully since it has been reported to be free from the teratogenic activity observed with other BZs in rodents (Botero, 1986).

BENZIMIDAZOLES AS ANTIGIARDIAL AGENTS

There are several important features of BZ pharmacology. Most of the recent generation of BZs are relatively insoluble and a reasonable proportion of the

administered dose remains in the gastrointestinal tract (Gottschall *et al.*, 1990), thus optimizing contact with *Giardia*. A number of these agents also have active metabolites (Gyurik *et al.*, 1981; Townsend and Wise, 1990) and these metabolites diffuse across the wall of the gastrointestinal tract or may be secreted in bile. The consequence is that *Giardia* trophozoites can not recover but are expelled and/or digested. This is a similar concept to that of prolonged exposure of nematode parasites to BZs (Prichard *et al.*, 1978).

There have been extensive structure-activity studies completed for the BZs with respect to antinematodal and tubulin-binding activity (Lacey, 1990). There are also many data on the pharmacodynamic properties of the BZs (Gottschall *et al.*, 1990). The results so far indicate that the specificities of BZs for protozoan tubulin are different to those observed for nematodes. Thus the present studies have highlighted the exciting possibilities of BZs for antiprotozoal chemotherapy. More studies on protozoan tubulin are now required.

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54. Biochemical and molecular mechanisms of resistance to nitroheterocyclic drugs in *Giardia intestinalis*

P.F.L.Boreham, J.A.Upcroft and P.Upcroft

INTRODUCTION

Paul Ehrlich first recognized drug resistance in parasites when he noted loss of sensitivity of trypanosomes to arsenicals (Ehrlich, 1910). He called this 'arsenic fastness'. Among the protozoan parasites of humans, the best known example of drug resistance is in the malaria parasite *Plasmodium falciparum*, although it also occurs with a variety of other protozoa including trypanosomes (Apted, 1980), *Leishmania* (Berman *et al.*, 1982), amoebae (Pitman and Pitman, 1974) and trichomonads (Meingassner and Thurner, 1979; Čerkasovová *et al.*, 1986). Two foci of chloroquine-resistant *P. falciparum* malaria were identified almost simultaneously in the late 1950s in Columbia and Thailand. Subsequently, there has been a rapid spread from these foci to cover much of Asia, Oceania, South America and Africa (Bunnag and Harinasuta, 1986). This sudden development and the realization that drug resistance was an important impediment to control of malaria has led to much research (see Chapters 39, 47 and 50). Current reports suggest drug resistance may also now be developing with *P. vivax* (Rombo *et al.*, 1987).

PRINCIPLES OF DRUG RESISTANCE

Drug resistance is defined as 'the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to, or higher than, those usually recommended but within the limits of tolerance of the subject'. It is essential that the form of the drug active against the parasite is present in the cell for the period of time necessary for its normal action (Bruce-Chwatt, 1981). Implicit in the term 'drug resistance' is a gradation in drug sensitivity between isolates; there can be a spectrum of response of the parasite to the drug

ranging from complete sensitivity to complete resistance. It is important to differentiate between resistance to therapy that is attributable to drug failure or innate insensitivity and true selected drug resistance.

The genetics of drug resistance have been discussed by Bryant and Behm (1989). Inheritable drug resistance in an individual or a population may be due to the activity of a particular allele of a single gene or a combination of alleles of different genes (polygenic). The selection pressure of the drug treatment on the parasite population results in an increase in the frequency of the alleles conferring resistance in the treated population. Factors which affect the development of drug resistance include:

1. the uneven distribution of the drug within the environment of the parasite which may result in unequal exposure;
2. different susceptibilities of the various stages of the parasite's life cycle to drugs;
3. rare alleles of certain genes that permit parasites carrying them to survive a drug concentration that destroys a large proportion of the population (i.e. genes conferring drug resistance); and
4. membrane changes affecting transport of drugs into the parasite.

Resistance conferred by a single gene usually occurs when a population is exposed to high persistent concentrations of drug which removes almost all the parasite population. Polygenic resistance is more likely to eventuate when the selection removes a smaller proportion of the parasite population as occurs when the drug is not persistent in the organism's environment.

Many different mechanisms may be involved in the development of drug resistance (Bryant and Behm, 1989). These include:

1. loss of a specific uptake mechanism for a compound;
2. loss of the molecular binding site or target;
3. structural modification of the binding site;
4. compensation for loss of activity of the target;
5. loss of the activation mechanism for a compound;
6. increased protective mechanisms to minimize cell damage;
7. improved drug detoxification mechanisms; and
8. a change in parasite development or behaviour within the host.

Attention has been directed towards the multidrug-resistance phenotype which was originally described in tumour cells resistant to antitumour drugs (Bradley *et al.*, 1988) and more recently described in *P. falciparum* (Krogstad *et al.*, 1987; Foote *et al.*, 1989; Cowman and Foote, 1990; and see Chapter 39 and 47) and *Entamoeba histolytica* (Samuelson *et al.*, 1990). The attribute of the multidrug resistance phenotype is cross-resistance to multiple drugs that are unrelated in structure, mode of action or target site. It is associated with reduced drug accumulation within cells. The most consistent biochemical change in multidrug-resistant tumour cells is overexpression of P-glycoprotein in the plasma membrane. It is also known that

there is sequence homology between P-glycoprotein and a group of bacterial transport proteins and it is suggested that an energy-dependent drugefflux pump ensures reduced drug accumulation within the cell. Calcium channel blocking drugs, such as verapamil and diltiazem, and calmodulin inhibitors reverse this process (Tsuruo *et al.*, 1982). A family of at least three genes (*mdr* genes) code for the 170 kDa P-glycoprotein, an integral cell membrane protein in mammalian cells. At least two *mdr* genes of similar sequence to mammalian *mdr* genes have been reported in *P. falciparum* (Wilson *et al.*, 1989). The presence of such genes does not auger well for the continued use of current drugs or the successful introduction of new, potentially active, chemically unrelated drugs for parasite control.

TREATMENT FAILURES IN GIARDIASIS

Four drugs are commonly used to treat giardiasis; metronidazole and tinidazole (both 5-nitroimidazoles), furazolidone (a nitrofuran) and quinacrine. There is no general agreement among physicians as to which is the drug of choice or what is the most appropriate treatment regimen. The efficacy of these drugs is difficult to appraise since different methods of assessing 'cure' are used by different people. Assessment varies from a single stool examination at the completion of treatment to multiple examinations over several months. Most reports suggest that a smaller number of failures result from nitroimidazole treatment than with furazolidone or quinacrine, but significant failures occur with all four drugs (Levi *et al.*, 1977; Wolfe, 1979; Mendelson, 1980; Craft *et al.*, 1981; Davidson, 1984). All four drugs have side-effects which may discourage the complete course being taken by the patient. The nitroimidazoles can cause nausea, metallic taste, disulphiram-like reactions and are known to be carcinogenic in mice and mutagenic to bacteria. Hypersensitivity reactions, gastrointestinal disturbances and haemolysis in glucose 6-phosphate deficient patients can result from furazolidone treatment. Quinacrine is not well tolerated by children. Side-effects include gastrointestinal irritation, toxic psychoses, exfoliative dermatitis, jaundice, fever, dizziness and headaches as well as a yellow discolouration of the skin (Davidson, 1984; Shepherd and Boreham, 1990).

Multiple reasons for treatment failures have been postulated. These include patient non-compliance, differing pharmacokinetic properties of the drugs in different patients, inaccessibility of the organism to the drug, inactivation of the drug by concomitant bacteria or other agents, and the existence of drug resistant strains of parasite. Certainly patient non-compliance with prescribed therapy is an important reason for treatment failures in giardiasis (Boreham *et al.*, 1986).

EVIDENCE FOR DRUG RESISTANCE IN *GIARDIA*

As a starting point for an investigation into the possibility that drug-resistant strains of *Giardia* are present in patients, we developed an *in vitro* drug assay to measure drug sensitivity (Boreham *et al.*, 1984). The assay utilizes the incorporation of

[³H]thymidine into the DNA of trophozoites, as an indicator of cell viability following drug treatment. From such assays it is possible to measure the dose of drug required to inhibit the uptake of [³H]thymidine by 50 per cent (ID₅₀) and gives a reproducible measure of viability. This offers a quantitative method of comparing drugs or the activity of one drug against multiple isolates. We have compared the drug sensitivity of over 50 *Giardia* isolates and found a 10-fold difference between the sensitivity of isolates to metronidazole and furazolidone. The corresponding figures for tinidazole and quinacrine are 3- and 30-fold (Boreham *et al.*, 1984, McIntyre *et al.*, 1986). While these results do not prove that drug resistance exists, the range of sensitivities observed is consistent with that supposition. Such results could be due to different populations within a single isolate and thus for two of our isolates we obtained 11 and 15 cloned lines, respectively, and tested their sensitivity against the four drugs (Boreham *et al.*, 1987). Again we found considerable variation in the sensitivity of these cloned lines to drugs *in vitro*, but generally less variation than between isolates. An *in vivo* model utilizing the neonatal mouse has also been established (Boreham *et al.*, 1985) and a statistical correlation validated for drug activity in comparison with the *in vitro* assay.

During a study of the clinical features of *Giardia* infections in children and their response to chemotherapy with furazolidone, the sensitivity to drugs *in vitro* of *Giardia* isolates from 10 children was compared with the clinical response of the patient to the treatment. Eight of the 10 patients who received furazolidone alone (8mg kg⁻¹ day⁻¹ for 10 days) showed prompt resolution of their symptoms while the other two patients symptoms remained despite a second 10-day course of treatment. The isolates from the two patients refractory to treatment were the least sensitive to furazolidone *in vitro* of the more than 50 isolates we have tested. Both patients responded satisfactorily to subsequent treatment with nitroimidazoles. In order to prove that this was true drug resistance it is important to show that satisfactory drug levels were present. This is difficult for gut organisms, such as *Giardia*, since there is no way to measure drug concentration at the site of the parasites, i.e. in the jejunum, without subjecting the patient to unnecessary invasive procedures. Thus we cannot be absolutely certain that the active compound was present at a sufficiently high concentration at the desired location. Since furazolidone is largely unabsorbed from the gut (Chamberlain, 1976), unlike the 5-nitroimidazoles which are rapidly absorbed but at different rates in different individuals (Ralph *et al.*, 1974), these results strongly imply the existence of drug resistant populations of *G. intestinalis*.

In the laboratory we have selected a line of *Giardia* that is resistant to metronidazole. We have achieved this by growing a human isolate continuously in a sub-lethal dose of the drug. Approximately 16 weeks after commencing the treatment a decrease in sensitivity was detected which reached a plateau after 60 weeks. This change in sensitivity was not stable and within 22 weeks of removal of the drug the line reverted to its original sensitivity (Boreham *et al.*, 1988). Development of this line, designated BRIS/83/HEPU/106 2ID₁₀, has allowed us to look at the biochemical and molecular mechanisms of resistance and also to investigate cross-resistance.

PROBLEMS OF CROSS-RESISTANCE

Cross-resistance may indicate similarities in structure or mode of action of the drugs. It occurs between the 5-nitroimidazoles in the treatment of *Trichomonas vaginalis* infections but this is not complete. Clinical studies in patients with metronidazole-resistant *Trichomonas*, confirmed by sensitivity testing, have shown that other nitroimidazoles such as tinidazole and ornidazole may be effective treatments especially if the dose is increased (Lossick, 1990). It would be anticipated that cross-resistance between metronidazole and other 5-nitroimidazoles will occur also in *Giardia*, since the mode of action of this group of compounds is believed to be similar. Cross-resistance has not been studied in giardiasis patients but it has been demonstrated using a line selected for resistance to metronidazole which showed an eight-fold decrease in sensitivity to this drug (Boreham *et al.*, 1988). The reduction in sensitivity of other nitroimidazoles was from six-fold for secnidazole to two-fold for nimorazole and fexinidazole. This cross-resistance did not extend to a number of other compounds including nitroheterocyclic compounds such as furazolidone.

MECHANISM OF ACTION OF NITROHETEROCYCLIC COMPOUNDS AGAINST ANAEROBIC PROTOZOA

Few studies have been undertaken on the biochemical mechanisms of action of drugs on *Giardia* and much of our knowledge derives from studies of other organisms. Recently, flow cytometry has been used to begin to investigate the cell cycle in *G. intestinalis*. The use of known mammalian inhibitors of the cell cycle indicate that in *Giardia* there is a different control mechanism from that found in mammalian cells (Hoyne *et al.*, 1989). Twenty-four hour treatment of trophozoites with metronidazole arrested cell cycle progression in the G₂+M phase of susceptible trophozoites, suggesting drug-mediated damage to the DNA which would prevent the normal events of mitosis occurring. Furazolidone arrested trophozoites in the S and G₂+M phase of the cycle indicating that the drug may prevent the progress of DNA synthesis and completion of cell division, again possibly due to DNA damage. These results suggest that in *Giardia*, as in other protozoa, the nitroimidazoles and nitrofurans have different modes of action (Moreno *et al.*, 1983, 1984).

There have been several recent reviews on the mechanisms of action of nitroheterocyclic compounds (Müller, 1983; Lloyd and Pedersen, 1985; Docampo and Moreno, 1986; Edwards, 1986). The mode of action of the 5-nitroimidazoles consists of

1. the passive entry of the drug into the cell;
2. the reductive activation of the drug to form toxic intermediates; and
3. toxic consequences of the reduced intermediates on DNA and other targets (Müller, 1983).

Low redox potential proteins such as ferredoxins rapidly reduce 5-nitroimidazoles. Several intermediates of the reduction process have been described including free radicals and nitroso, hydroxylamine and amine compounds. These could cause strand breakages and cross-linking of DNA resulting in cell death (Edwards, 1986), although they are also likely to interact with other targets in the parasite. Nitrofurans such as furazolidone can be reduced to cytosolic enzymes such as NADH oxidase and NADPH oxidase. Cytotoxicity is effected via the toxic reduced products themselves or by the oxygen metabolites produced as a result of the oxidation of the reduced products by oxygen.

BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE

Based on the knowledge of other parasites and the known mode of action of the nitroheterocyclic drugs we have measured the activity of pyruvate: ferredoxin oxidoreductase (PFOR) the enzyme which oxidizes pyruvate by transferring electrons to ferredoxin, and the thiol cycling enzymes, thiol peroxidase and thiol reductase. The glutathione redox system has been studied in detail in other organisms and provides an important intracellular protection mechanism for removing oxygen derived free radicals (Meister and Anderson, 1983). No free glutathione is present in *Giardia* (Smith *et al.*, 1988), although it does possess abundant quantities of other thiol(s) as well as thiol dependent peroxidase and reductase activities. The thiol(s) present in *Giardia* have not been characterized but do not include trypanothione (Smith and Boreham, unpublished).

Measurement of the PFOR and thiol dependent peroxidase and reductase in isolates of *Giardia* and correlations with *in vitro* drug sensitivities suggested a biochemical mechanism involved in resistance (Smith *et al.*, 1988). The resistance of 17 isolates to metronidazole was inversely correlated with PFOR activity, suggesting that drug activation occurs more slowly. In the case of furazolidone no correlation was found between drug resistance and PFOR activity, but a direct correlation was found between drug resistance and thiol peroxidase and reductase activities (measured as glutathione peroxidase and glutathione reductase). Thiol dependent peroxidase would catalyse the removal of dangerous peroxides produced in the presence of oxygen. The accompanying reductase would ensure that adequate levels of reduced thiol are maintained. From this study these two enzymes appear to be important to furazolidone resistance and their detailed characterization is required.

L-Butathione sulphoximine (BSO), an inhibitor of glutathione synthesis, did not significantly affect the viability of *Giardia* trophozoites, whereas inhibitors of glutathione reductase (1, 3- bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea; BCNU and HeCNU) were effective giardicidal drugs in *in vitro* assays (Smith and Boreham, unpublished). This suggests similarities in the active sites of the different thiol reductases (see Chapters 44 and 45). Other biochemical protection mechanisms, including free-radical removal, may also be important in resistance. Catalase and superoxide dismutase could not be

detected in *Giardia* trophozoites (Smith *et al.*, 1988), but one such mechanism that has been suggested is the abundant cysteine rich protein on the surface of the parasite which may protect against the hostile environment of the human small intestine (Gillin *et al.*, 1990).

MOLECULAR BASIS OF DRUG RESISTANCE

The trait of drug resistance in *Giardia* is not indicated by change in karyotype. Line BRIS/83/HEPU/106 2ID₁₀ and its parent stock both have eight identically sized chromosomes which can be separated by field inversion gel electrophoresis (Upcroft *et al.*, 1989b). Using chromosome-specific probes we have demonstrated that changes have occurred within the chromosomes (Upcroft *et al.*, 1990). Probe G6/1, (Upcroft *et al.*, 1987) shows a marked difference between the sensitive and resistant lines, hybridizing to both the third and fourth chromosomes of the sensitive line and weakly only to the third chromosome of the resistant line (Upcroft *et al.*, 1989a). The change can be seen after the first 30 weeks of selection for resistance and is at the time when there is a rapid increase in resistance of this line. Other probes show no differences in hybridization patterns. Thus it appears that resistance to metronidazole is associated with a chromosome translocation involving the third and fourth chromosomes. Reversion of the drug-resistant line to sensitivity after removal of drug pressure (Boreham *et al.*, 1988a) indicates that there was no major deletion of the genes involved in the metronidazole reduction pathway. It is possible that the change involved the down regulation of these genes.

DETECTION OF DRUG RESISTANCE

The classical way of detecting drug resistance is to isolate the parasite, establish it in axenic culture and carry out drug sensitivity studies. Such a procedure not only has the disadvantage of being time consuming but also may result in the selection of different parasite populations. If one can establish markers for drug resistance, parasites could be tested for that marker immediately after separation from stools and excystation. Such an isolate may consist of populations of trophozoites that have developed resistance via several mechanisms and some (presumably a minority in patients who have been treated with a range of drugs) that are sensitive.

Using laboratory-induced drug-resistant lines, we can establish sets of markers which characterize particular traits. Anonymous DNA probes can be used to generate maps of the *Giardia* genome. These maps can then be used to locate changes in drug-resistant lines which involve DNA rearrangements. Alternatively, specific probes can be synthesized which target the loci involved in resistance once the pathways and mechanisms have been fully elucidated. For example, consensus sequences of ferredoxin, glutathione peroxidase and reductase could provide information on gene amplification and on transcriptional changes in gene

expression. This type of analysis could be extended to the level of enzyme activity and regulation.

CONTROL OF DRUG RESISTANCE

A number of approaches may be taken to prevent the development and spread of drug resistance.

1. The use of anti-giardial drugs should be limited. Currently it is common for physicians to conduct therapeutic trials without a definitive diagnosis. Specific treatment should only be administered once a diagnosis has been obtained. Thus it is essential to develop better diagnostic tests. Our own studies have indicated that, while physicians are more likely to diagnose *Giardia* correctly in children than in adults, based on clinical symptoms alone, accuracy overall is poor (Boreham and Phillips, 1986).
2. Prevention of the spread of resistance by immediate treatment of patients found to be refractory to one drug. In order to determine whether the therapy should be changed, it is essential to differentiate treatment failures due to non-compliance from true drug resistance.
3. Careful surveillance for the presence of resistant strains. Simple tests for detecting drug resistance would aid in monitoring.
4. Management of established drug resistance. In the one resistant strain that has been studied in depth, reversion to a sensitive strain occurred rapidly in the absence of drug pressure. Should this be a consistent feature of drug resistant lines, care with the distribution of the existing drugs may help to reduce the spread.

CONCLUSIONS

Studies of drug resistance are necessary for the rational use of existing drugs and the exploitation of new compounds which may be developed in the future for the treatment of giardiasis. While such studies are still in their infancy and it is likely that multiple mechanisms are involved, the key question to be answered is whether the resistance already detected in *Giardia* is likely to spread. If so, will the spread be sporadic, as occurs with pyrimethamine-resistance in malaria, where it is found in areas with heavy use, or will dissemination result from a single focus as is the situation with chloroquine resistance? Development of resistance is a single point mutation for pyrimethamine, whereas a more complicated mechanism exists for chloroquine resistance and more than one gene may well be involved (Cowman and Foote, 1990). Currently there is no evidence for the rapid spread of drug-resistant strains of *Giardia* but equally there are no data to suggest it will not occur in the future. There is a need for more research on drug resistance in *Giardia* in order to

predict the consequences in terms of pathogenicity and virulence and to highlight possible means of avoiding or overcoming the problem.

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55. Possibilities for new antiprotozoal drugs: the TDR/WHO approach

C.Ginger

PREAMBLE

Thirty years ago I trained as a comparative biochemist in the laboratory of Ernest Baldwin and during my 25 years in research I always worked on parasitic organisms. The varied evolutionary origins of the parasitic protozoa and helminths, and their adaptations to parasitism, make studies of their biochemical structures and metabolism particularly rewarding. There is a great excitement and satisfaction in successfully carrying out a biochemical study which demonstrates a new enzyme, pathway or structure in a parasitic organism: to add one more piece to the jigsaw, which leads to greater understanding of its complex life-style.

But I had another hero from the days of my Ph.D. studies, and that was Paul Ehrlich with his work on the chemotherapy of trypanosomes and spirochaetes. My own studies in comparative biochemistry were always done with a view to finding an exploitable difference for inhibition, and hence chemotherapy, of the parasite. It was therefore almost inevitable that I should spend the latter half of my scientific career, first in the pharmaceutical industry and now in the World Health Organization, developing drugs for use in tropical medicine.

THE ROLE OF TDR IN DRUG DEVELOPMENT

Introduction

In June 1990 the WHO Special Programme for Research and Training in Tropical Diseases (TDR) produced a discussion document (WHO, 1990a) which outlined the role of 'TDR in the 1990s'. This highlighted five key issues for the future work of TDR:

1. priorities among TDR target disease and transdisease areas;
2. the relative emphasis among basic research, product development and operational/health systems research;

3. priorities for research and development/product development;
4. the establishment of a Product Development Unit within the TDR Secretariat; and
5. approaches and priorities for research capability strengthening.

All these issues are obviously of great importance to research workers in the area of drug development against parasitic protozoa. To provide background for such a discussion TDR had also organized in August 1989 a 'Prospective Thematic Review on TDR's Research and Development Related to Drugs' (WHO, 1989). The following is an outline of TDR's current policy which has developed from these discussions, and the reasons for it.

There has been no change in the diseases which will be covered by TDR, and a brief outline of the parasite life cycles, geographical distribution of disease, numbers of persons infected and current research priorities are given in the WHO booklet 'Tropical Diseases 1990' (WHO, 1990b). Three of the six diseases of TDR, malaria, leishmaniasis and the trypanosomiases, are caused by protozoan parasites and the following discussion refers specifically to these three diseases. Malaria is the most widespread of the diseases with about 270 million persons infected by the various species of *Plasmodium*, whereas the leishmaniases have about 12 million infected. The trypanosomiases are subdivided into Chagas' disease in Latin America, where about 16–18 million persons are infected, and African trypanosomiasis, where annual infections are given as 25000, although the disease is under-reported and tends to occur in epidemic form.

The importance accorded to each disease by TDR is reflected in its allocation of the annual budget. During the period 1988–89, the total budget had operated at around US\$32 million per annum. The percentage distribution of funds for 1990–91 (WHO, 1990c) shows that malaria takes about one-third of the research budget, while 7.2 per cent goes to African trypanosomiasis, 6.5 per cent to leishmaniases and 5.6 per cent to Chagas' disease. Each programme supports a wide spectrum of work, and research and development for new drugs represents only a small part of each programme's effort.

Problems in providing new drugs for tropical diseases

For some of the diseases new drugs are urgently required; for others they are highly desirable. For use in the community, often within a relatively unsophisticated medical environment, the ideal drug treatment needs to be not only efficacious but also well tolerated, and preferably active by a single oral dose.

In spite of the proven importance of the tropical diseases, they are badly served in terms of available drug treatments. If one compares the pharmaceutical products available in 1990 for the four major areas of health concern in developed countries with those available for parasitic diseases (Table 55.1), the scale of the problem is immediately apparent.

Pharmaceutical companies are increasingly unwilling to invest resources in the difficult area of tropical medicine when there is an inadequate return on that

Table 55.1. Pharmaceutical products marketed in 1990.

Product type	No.
Gastrointestinal	84
Cardiovascular	328
Central nervous system	283
Anti-infective	180
Antiparasitic	31

investment. Most companies known to us have ceased to carry out fundamental work on tropical diseases and, even if a promising lead is available from previous work, the companies usually do not wish to develop it rather than those potentially more profitable drugs aimed at other disease areas. Thus within industry there is no longer a large commitment to develop drugs for tropical medicine, even if their efficacy and safety appears suitable. As TDR wishes to continue its collaboration with both pharmaceutical companies and academic laboratories, what is the way to proceed? The current position as seen by TDR is as follows:

1. there is a continuing need to control tropical diseases;
2. products that need development are arising from research ('product' includes any tool to combat tropical diseases, but would usually encompass drugs, vaccines and diagnostics); and
3. there is limited industrial support for products in this area.

TDR considers that it must fill this gap.

Drug delivery

The successful delivery of a drug, or other disease-control tool, to the person in need is an aspect of health care which has perhaps received insufficient attention in the past. Apparently useful drugs are available, e.g. praziquantel for schistosomiasis or chloroquine for malaria, but are not always used as widely as anticipated. It is therefore important to ensure, as far as possible, that the right type of drug is being made available through the right channels at the right price. TDR's conclusion is that the needs of the persons or communities receiving the product should influence the nature of the product, and thus the nature of the research needed to develop it. More 'operational research' or 'health systems research' will be undertaken in future to allow feedback into the TDR research areas.

In this context, it is noteworthy that in January 1990 WHO formed a Division of Control of Tropical Diseases (CTD), which unites previously separate control activities for the different diseases into one programme. With a mandate to develop practicable, manageable and sustainable strategies for the control of tropical diseases, this division will collaborate closely with TDR, emphasizing the same diseases, and will help to promote the scientific findings of TDR.

Relationship of TDR to academic laboratories and the pharmaceutical industry

Since it was set up in 1974, TDR has shown that its strengths in the drug discovery process lay in:

1. basic research areas;
2. *in vitro* and *in vivo* drug screening;
3. clinical and field trials in endemic areas; and
4. ability to co-opt scientists and clinicians of international repute to advise in TDR programmes and committees.

However, TDR usually relied on the pharmaceutical industry to provide the resources and expertise in the preclinical phases of drug development and, of course, in the registration, manufacture and distribution of successful drugs. TDR does not have the resources to undertake the complete drug discovery and development process, and must therefore collaborate with others having the same objectives. Collaborators include universities, medical schools, research institutes and, most importantly, the pharmaceutical industry where most of the expertise and resources are to be found.

An important decision of TDR is that in the absence of initial support from industry for the development of a potential drug candidate, it is prepared to fund and carry out any single part of the overall drug development pathway if it is essential for progression of the potential product. At each stage, however, an industrial partner will be sought to continue development to registration, manufacture and distribution.

Management of product development

There are problems in organizing high-throughput screening systems and product development, with the TDR management system based on steering committees formed of non-WHO personnel who meet only once or twice a year. One innovation is the creation of preclinical drug development teams, led by persons in, or preferably recently retired from, the pharmaceutical industry. These teams provide day-to-day assistance to the TDR Secretariat for drug development decisions in the periods between formal steering committee meetings.

Another recent innovation has been the creation of a drug development team to cover both the leishmaniases and the trypanosomiases, with the research emphasis to be put upon drug targets common to all kinetoplastid parasites. A recent document (WHO, 1990d) details some of the plans, and additional funds will be made available by TDR for these activities.

A recent major decision of the Joint Coordinating Board of TDR was to set up a Product Development Unit (PDU) within TDR, whose staff, with industrial experience in drug development and biotechnology, will coordinate product development for all diseases of TDR, in a more professional way than is possible at present.

Financing of product development

TDR currently devotes about 30 per cent of its research and development budget to the development of high-priority products. It is estimated that the percentage of the research and development budget allocated to product development could increase to 45 per cent in the 1990–91 biennium, and 57 per cent in 1992–93. It was also recommended that the PDU would directly manage part of the funding for product development, their share being US\$3 million in 1992. However, it was emphasized that, since basic research is the source and foundation from which future advances will be made, an appreciable percentage of research and development funds would always be reserved for this activity.

Philosophy for research and development in chemotherapy in tropical diseases

TDR will continue to support fundamental research as a basis for developing new ways of preventing or controlling disease. The knowledge of unique drug targets, obtained from basic research projects, needs to be brought to the attention of pharmaceutical companies to find areas of mutual interest with ongoing drug development programmes in industrial laboratories. Tropical medicine needs to 'piggy back' on those research programmes in human and veterinary medicine which are considered profitable by the pharmaceutical industry. There will also be a need to exchange materials, e.g. cloned genes, enzymes, receptors, and to solicit help in sequencing, crystallography, computer graphics and other techniques relating to structure and function of parasitic molecules. It is hoped that industry will provide compounds of current interest to WHO for screening and, if antiparasite activity is found, help in subsequent development. It is obviously advantageous if some preclinical work on toxicology, pharmacology and drug metabolism has already been completed by the company for other purposes.

Drug research and development programmes currently require a multi-million dollar investment, regardless of the disease to be treated. It therefore becomes increasingly difficult for a commercial company, or WHO, to develop a drug for a single disease of the developing world. Malaria, with 267 million persons infected, and an estimated mortality of 1–2 million persons per year, is an exception, particularly with the benefit of a tourist and expatriate market for an efficient prophylactic drug. Nevertheless, drug targets chosen for exploitation should ideally be present in a wide spectrum of parasitic organisms. This potentially allows development of one drug, or a series of analogues, for several diseases.

To summarize, I would advise that to increase the chances of success of a future application for funding to WHO in the chemotherapy area, the project should concern:

1. an area where successful chemotherapy should induce a lethal event;
2. a target enzyme or receptor which is present in more than one parasite and which differs in some way from any isofunctional mammalian component; and
3. either the target or its inhibitors should be of some commercial interest to the pharmaceutical industry.

Recent examples of project areas funded are difluoromethylornithine for African trypanosomiasis, dihydrofolate reductase in malaria, azoles in *Trypanosoma cruzi* and *Leishmania*, mechanism and reversal of multiple drug resistance in malaria, and ivermectin for onchocerciasis and lymphatic filariasis.

CHOICE OF CHEMOTHERAPEUTIC TARGETS IN PARASITIC PROTOZOA

General principles

In a recent paper, a representative of the pharmaceutical industry outlined what is needed to successfully develop an active enzyme inhibitor into a useful drug (Crout, 1990) and, giving the criteria to be met and the obstacles to be overcome, concluded that 'it is not surprising that relatively few of the large number of enzyme inhibitors known to enzymologists have become marketed drugs'.

My own criteria applied to antiparasitic research proposals within industry were relatively simple but difficult to satisfy.

1. There should be some existing evidence that binding of an effector to the chosen target should lead to a lethal event in the parasite;
2. the techniques to carry out the proposed research either existed or could be developed within a reasonable period; and
3. compounds were known or could be synthesized which should theoretically interact with the receptor molecule or pathway proposed for study.

The biochemical work can be initiated in the absence of a programme of chemical synthesis, particularly if the biological area appears unique, but selection of a novel, potentially lethal target is difficult. As a consequence some workers propose, and even carry out, inhibitor studies on enzymes or other targets, whose function can be bypassed using alternative pathways. Often preliminary testing using commercially available compounds can strengthen the proposed concept. At some point, as early as possible, the investigator should try available inhibitors on the biological system under study and, if a lead is found, approach an industrial company or interest a medicinal chemist colleague to make available analogue compounds to follow the initial lead. There is an impression that testing of compounds in the 50s or 100s is an uninteresting exercise for an academic biochemist or molecular biologist, but if one is interacting with an enthusiastic and competent medicinal chemist, the challenge and the achievement can be as great as in any other research field.

Chemotherapeutic targets in parasitic protozoa

Using the titles of papers presented at the meeting in Stirling entitled 'Biochemical Protozoology as a Basis for Drug Design', and knowing something of the authors' previous work, I drew up the list of biochemical areas (shown in Table 55.2) which seem to fulfil most of the criteria for potential chemotherapeutic targets discussed

Table 55.2. Potential targets for chemotherapy in *Trypanosoma*, *Leishmania* and *Plasmodium*.

^a Glycosomes; glycolytic enzymes
Intermediary metabolism: component enzymes
^a Mitochondrial function: electron transport; inhibition by naphthoquinones; NADH-fumarate reductase
^a Membrane transport: nucleosides, folate, glucose
Cation-motive ATP-ases; pH control
^a Glutathione and trypanothione: function and inhibition
^a Polyamines: biosynthesis and metabolism
Methyltransfer reactions; methionine cycling; S-adenosylmethionine
^a Folate metabolism: dihydrofolate reductase-thymidylate synthetase; drug resistance
^a Purines: uptake and metabolism
Pyrimidines: biosynthesis
^a Proteinases: function and inhibition
Ribosomes; protein synthesis
^a Glycoproteins; lipophosphoglycans: structure, function and biosynthesis
^a Lipids: composition and biosynthesis; drug-lipid interactions
^a Microtubules: tubulin; structure and function
^a Drug resistance: chloroquine and MDR mechanisms; naphthoquinones

^a Currently being supported by TDR.

above, either by their unique occurrence in the parasitic protozoa of interest to TDR or, for isofunctional enzymes or receptors, by more subtle differences demonstrated at the molecular level.

Some of these target areas were identified decades ago, but are still worthy of further research effort. Many reviews have been written relating drug design to defined biochemical areas, either as antiparasitic or antiprotozoal agents (Gutteridge and Coombs, 1977; Cohen, 1979; Klein, 1980; Wang, 1981, 1983, 1988; Newton, 1983; Gutteridge, 1987) or for specific disease areas, i.e. malaria (Peters, 1987; Schlesinger *et al.*, 1988; Wernsdorfer and Trigg, 1988; Gutteridge, 1989), trypanosomiasis (Jaffe, 1968; Williamson, 1976; Gutteridge, 1981; Fairlamb, 1982; Opperdoes, 1985) and leishmaniasis (Berman, 1988; Glew *et al.*, 1988).

The TDR programme for malaria chemotherapy supports work on the mode of action of, and mechanisms of resistance to, most antimalarial agents of current interest, i.e. chloroquine, mefloquine, halofantrin, hydroxynaphthoquinones, artemisinin and its ether derivatives, and synthetic trioxanes. There is particular interest in the importance of the P-glycoprotein in the induction of multiple drug resistance in malaria, and any resemblances to or differences from the similar mechanisms demonstrated in tumour cells.

Several biochemical targets have been defined by the malaria programme include:

1. membrane lipids, phospholipid biosynthesis;
2. membrane transport;
3. nuclear and mitochondrial DNAs;
4. purine phosphoribosyltransferases;
5. ribonucleotide reductase;
6. oxygen radicals and redox control;

7. microtubules, microfilaments; and
8. proteinases.

It may be of interest to note that the TDR Drug Development Team for the leishmaniases and trypanosomiases (WHO, 1990d) recently concluded that the most promising areas for immediate action in drug development were allopurinol (purines: uptake and metabolism), difluoromethylornithine (polyamines: biosynthesis and metabolism), and azoles and amphotericin B (lipids: composition and biosynthesis, and drug-lipid interactions). Biochemical work which might give rise to drugs capable of development in the medium term was noted (WHO, 1990d) as:

1. thymidylate synthetase-dihydrofolate reductase;
2. phospholipid metabolism;
3. inhibitors of antigenic variation;
4. trypanothione;
5. intracellular pH regulation and tricyclic antidepressants;
6. microtubules; and
7. inhibitors of DNA polymerases.

Other biochemical areas (e.g. glycosomal enzymes) were considered as long-term investments. Some of these items are worthy of special mention in that work has progressed rapidly in recent years, often in novel areas of biochemistry.

Glycosomes: glycolytic enzymes

The long-term studies on the cloning and structure and organization of the enzymes of glycolysis, originally carried out in African trypanosomiasis by Opperdoes and co-workers, has now been confirmed and extended throughout the kinetoplastidae (Opperdoes, 1983, 1987). However, to date no one has succeeded in exploiting this knowledge in the design of novel drugs as was suggested. See Chapter 11 for more information.

Glutathione and trypanothione

The novel structure of trypanothione and its proven inter-relationships with polyamine metabolism (Henderson and Fairlamb, 1987) make this an interesting area for future chemotherapeutic efforts. See Chapter 44 for more information. In addition, the work has helped to explain the mechanism of action of the arsenical drugs (Fairlamb *et al.*, 1989), nifurtimox (Henderson *et al.*, 1988) and DFMO. See also Chapters 43, 45 and 46.

Polyamines: biosynthesis and metabolism

Perseverence by Bacchi *et al.* (1980) resulted in the successful use of DFMO against *T. gambiense* in man. This drug was one of the early mechanism-based inhibitors, synthesized as an antitumour agent, which was successfully applied in the

antiparasitic area. The dosing regimen of DFMO is far from ideal, and infections of *T. rhodesiense* are not cured by the drug. Further progress will require either the use of combination therapy, e.g. DFMO used with suramin (Clarkson *et al.*, 1984) or arsenicals (Henderson and Fairlamb, 1987), or the development of more effective analogues.

Elegant work by Wang and co-workers, in which the ornithine decarboxylase gene was cloned from *T. brucei* and expressed in *Escherichia coli* (Phillips *et al.*, 1988), allowed them to propose a new mechanism of selective toxicity for irreversible inhibitors based on differences in the half-life of the isofunctional enzymes in man (20min) and parasite (>6h) (Phillips *et al.*, 1987; Wang, 1988). See also Chapters 42, 44 and 46.

Folate metabolism: dihydrofolate reductase—thymidylate synthetase

Following the report of Ferone and Roland (1980) that the high molecular weight of dihydrofolate reductase (DHFR) in protozoa relative to other sources was due to its occurrence as a bifunctional protein with thymidylate synthetase, this enzyme complex has been utilized as a model for drug design and drug resistance in several organisms. The work of Santi and co-workers in cloning and expressing the enzyme from *L. major* (Grumont *et al.*, 1988) and in site-directed mutagenesis of the gene for this enzyme has indicated what can be done with enzymes of parasitic origin. Large quantities of enzymes produced by genetic engineering techniques have allowed three-dimensional structuring and the design of more selective inhibitors of DHFR from *Leishmania*. This will clarify the question of whether *Leishmania* spp. are inherently non-susceptible to DHFR inhibitors, or simply that insufficiently specific inhibitors had previously been tested (Sirawaraporn *et al.*, 1988). Inhibitors of thymidylate synthetase should also be developed from this purified complex.

Molecular biology has also been successfully applied to problems of drug resistance an antifolate drugs in malaria. Recent reports (Hyde, 1989) have shown that point mutations in the DHFR enzyme from *P. falciparum* are responsible for resistance to antifolate drugs such as pyrimethamine and cycloguanil, and that each of these closely related inhibitors induce a unique amino acid change in the resistance enzyme (Foote *et al.*, 1990; Peterson *et al.*, 1990). See also Chapters 50 and 51.

Glycoproteins and lipophosphoglycans

The recent work on the structure and biosynthesis of both the trypanosome variantspecific surface glycoproteins (VSGs), and the lipophosphoglycans of *Leishmania*, whose structure has recently been described (Ferguson and Williams, 1988; Ferguson *et al.*, 1988; Turco, 1988), is impressive and its description has led to the discovery of similar structures in other kinetoplastid flagellates and in other systems (Snary, 1985; Low, 1987). See also Chapters 27 and 28. The role of phospholipase C in the shedding of these surface molecules, and the biological function of such membrane-associated structures, is surely a site for chemotherapeutic attack!

Microtubules: tubulin; structure and function

Mention must be made here of the careful combination of biochemical, immunological and cytological work of Gull and co-workers, who are combining a study of the function of microtubule replication in the cell division mechanism of trypanosomes (Sherwin and Gull, 1989; MacRae and Gull, 1990) with a search for novel inhibitors of the process. The ease of gene cloning for tubulins from all organisms should make a study of their structure and *in vitro* interactions with potential inhibitors a relatively straightforward process. See also Chapter 53.

Drug resistance: chloroquine and multidrug resistance (MDR) mechanisms

The discovery from the tumour chemotherapy area that multiple resistance to several unrelated inhibitor molecules was due to over-production of a transport protein in the membrane of resistant cells, which allowed rapid ejection of the inhibitor (Juranka *et al.*, 1989; Moscow and Cowan, 1990), led to the finding that some chloroquine resistant strains of *P. falciparum* apparently became resistant in the same way (Krogstad *et al.*, 1987). This does not, however, explain all chloroquine resistance (Ward, 1988; and see Chapters 39 and 51). Of especial practical interest was the finding that several compounds (particularly early experiments using verapamil) were able to reverse drug resistance by blocking the function of the MDR transport glycoprotein. The possibility that resistant strains might still be treatable with chloroquine if co-administered with a resistance-reversing agent has stimulated the search for synergizing compounds devoid of adverse pharmacological effects. Similar reversal of MDR by verapamil has been reported in members of the *Kinetoplastidae* (Neal *et al.*, 1989).

Other biochemical areas worthy of further study

Several chemotherapeutic targets in addition to those described above are detailed in other chapters of this book. Additional areas worthy of consideration for chemotherapeutic studies are listed in Table 55.3. The list is not comprehensive and the rapidly growing literature in the field makes it difficult to summarize the work briefly. Consequently, I simply highlight some of the areas noted and give reasons why they are included.

Kinetoplast/mitochondrial DNA

Several antibiotics active against *Plasmodium* would seem to be active against protein synthesis in the mitochondrion (Geary and Jensen, 1983), and the regular changes in mitochondrial function throughout the life cycle of African trypanosomes make this an area of potential inhibition (Feagin and Stuart, 1985; Simpson, 1987). The physical structure and location of kinetoplast DNA make it specifically susceptible to drug binding by compounds such as ethidium, ellipticine and acriflavin (Simpson, 1972), and the more recent inhibitors which complex

Table 55.3. Biochemical areas worthy of further study.

a Kinetoplast/mitochondrial DNA: structure and function
Gene function: trans-splicing; RNA editing
DNA/RNA polymerases
a Topoisomerases
ADP-ribosyl transferase
Dihydroorotate dehydrogenase
a Ribonucleotide reductase
a Antisense oligonucleotides
a Glycoprotein biosynthesis: enzymology
Repetitive proteins
a Inhibitory dipeptide esters
a Oxygen radicals
Haem requirements
Quinone biosynthesis
Membrane structure and function: surface receptors; phospholipid metabolism
Antitumour agents
Intracellular 'second messengers'
a Natural products
Drug targeting; immunomodulators
Mechanism-based inhibitors
a Tricyclic compounds

^a Currently being supported by TDR.

specifically to certain base sequences may also prove to have specificity towards kinetoplast DNA (Hurley *et al.*, 1984).

Gene function: trans-splicing and RNA editing

The unusual mechanisms by which trypanosomes produce messenger RNA coding for variant surface glycoproteins from within the genome by discontinuous transcription (Borst, 1986) with trans-splicing of a common nucleotide leader sequence (Walder *et al.*, 1986; Donelson and Zeng, 1990), and the RNA editing process in the kinetoplast which allows the insertion of additional uridine residues into the messenger RNA (Stuart, 1989), must be sufficiently different to mammalian transcription processes that selective inhibition is possible (Cornelissen *et al.*, 1985). *Plasmodium* is also capable of rapid antigenic variation in the mammalian host, but the mechanism is not as well understood as in trypanosomes.

DNA/RNA polymerases: topoisomerases

In spite of the intense interest in the transcription mechanisms operating in trypanosomes, the actual enzymes involved are not well characterized apart from their sensitivity to a-amanitin. There is also current interest in topoisomerases I and II, both in bacterial cells and in tumours (Douc-Rasy *et al.*, 1988; Liu, 1989; Sutcliffe *et al.*, 1989) as targets for drug therapy. The fluoroquinolones are wide-spectrum antibacterial agents because of their inhibition of topoisomerase II (DNA gyrase) in bacteria, but they also show activity against malaria (Divo *et al.*, 1988; Midgeley *et al.*, 1988), *Leishmania* and *T. cruzi* (Croft and Hogg, 1988; Raether *et al.*, 1989). The

antiprotozoal effects may be due to inhibition of protozoal topoisomerases or there may be an alternative inhibition, and the basis of the inhibition by fluoroquinolones should be examined in more detail and other topoisomerase inhibitors tested.

Dihydroorotate dehydrogenase (oxidase)

The mode of action of antimalarial naphthoquinones, whose interaction with electron transport processes leads to inhibition in the particulate enzyme dihydroorotate dehydrogenase in *Plasmodium* spp., is detailed in Chapters 3 and 13. The isofunctional enzyme of pyrimidine biosynthesis in the *Kinetoplastidae* is a soluble enzyme, dihydroorotate oxidase, with substrate electrons transferred via flavin mononucleotide as a co-factor, and completely unlike the particulate enzyme from mammalian cells (Pascal *et al.*, 1983). It is surprising that work on mechanismbased inhibitors of the soluble dihydroorotate dehydrogenase of the *Kinetoplastidae* appears to have been neglected during recent years.

Glycoprotein biosynthesis: enzymology

The biosynthesis of the phosphatidyl inositol containing phosphoglycans and other glycoproteins within the parasitic protozoa seems generally to follow the mechanisms described from other systems, but with important differences in enzyme specificity (Low, 1987). It may therefore be possible to design specific inhibitors to inhibit the biosynthesis of these key surface proteins, and so allow removal of parasites via the normal host immune mechanisms.

Proteinase-activated prodrugs

The work of Rabinovitch and others on dipeptide esters active against *Leishmania* indicates that they are dependent upon the activity of a cysteine proteinase to exert their toxic effects (Rabinovitch, 1989). Thus even if the dipeptide esters are unable to be developed into effective chemotherapeutic agents, it may be possible to design more specific prodrugs which are cleaved by the unusual cysteine proteinases of the parasite. See Chapter 19 for more details.

Membrane structures and function: surface receptors

It is of great interest that trypanosomes express on their surface receptors very similar to those found on mammalian cells, e.g. receptors for fibronectin (Ouassi *et al.*, 1984), low-density lipoproteins (Coppens *et al.*, 1988) and epidermal growth factor (Hide *et al.*, 1989), which presumably are responsible for retrieving material from the host environment, or in allowing recognition of host cell surfaces prior to penetration. It is suggested that it might be possible to design compounds that block parasite receptors, but do not affect host cell receptors. Malaria parasites have also been shown to have similar homologous proteins on their surface.

Natural products

The success of artemisinin and its derivatives as a treatment for acute malaria, and the activity of several other natural products as antimalarial agents (Phillipson and O'Neill, 1986; Xiao Pei-Gen and Fu Shan-Lin, 1986), has drawn attention to this source of novel antiparasitic agents. The initial activity of a natural product then leads on to further synthetic or semisynthetic work to obtain analogues more suitable for safe therapy.

Tricyclic compounds

Although these compounds were included in Table 55.2 under the heading 'Cation-motive ATP-ases; pH control', it would seem that the wide spectrum of activity of tricyclic compounds against *T. cruzi* and *Leishmania* in the hands of several investigators (Hammond *et al.*, 1984; Hewlett and Pearson, 1985; Doyle and Weinbach, 1989), and the identification of the probable site of action (Zilberstein and Dwyer, 1984), would make a chemical synthesis programme based on these structures a valuable exercise. One would optimize antiprotozoal activity, while avoiding the adverse pharmacological activity exhibited by previous test compounds selected from existing drug libraries.

CONCLUSION

I hope to have convinced you that the essential work on the development of specific inhibitors of parasite metabolism can prove as intellectually rewarding as the initial biological work needed to characterize the enzyme, receptor, gene, etc. The question which is currently being asked of TDR, after a decade of basic research, is: Where is the drug, the vaccine or the diagnostic test which enables a developing country to control its diseases? That is the only impact which ultimately is important and that is why donor countries and organizations provide funding for work in tropical medicine. You need to ask the same question of your own research effort. Where is it leading? Once you find a parasite target which seems exploitable, obtain a few inhibitors and test them in your assays. If there is a lead, contact TDR or a drug company for analogues. As more drug companies withdraw from tropical medicine, collaboration between existing laboratories will be essential for any drug development to proceed. At WHO, with your help, we hope to be able to catalyse that development and provide an infrastructure for laboratory discoveries eventually to become useful products in the field.

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