PURINE AND PYRIMIDINE METABOLISM OF *LEISHMANIA MEXICANA MEXICANA* AND OTHER PARASITIC PROTOZOA

by

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DEDICATION

My main gratitude goes to my wife, daughter Zainab, son Arjan and to my Father and my Mother to whom I dedicate this thesis for their patience and invaluable support.

LIST OF PUBLICATIONS

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7. **Hassan, H.F. and Coombs, G.H. (1986).** Purine-metabolising enzymes in Entamoeba histolytica. Molecular and Biochemical Parasitology, 19, 19-25.

8. **Hassan, H.F. and Coombs, G.H. (1986).** De novo synthesis of purines by Acanthamoeba castellanii and A. astronyxis. IRCS Medical Science, 14, 559-560.

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CONTENTS

SUMMARY

Purine and pyrimidine nucleotides are synthesized either de novo from small precursors or via salvage pathways from preformed purines and pyrimidines. Most parasitic protozoa are incapable of synthesizing purines de novo and so are dependent on salvage pathways. This contrasts markedly with the situation in mammalian host where both de novo synthesis and salvage pathways are significant. In contrast to the situation with purines, most parasitic protozoa are able to synthesize pyrimidines de novo, although salvage pathways are present. I have studied the presence of these pathways in a variety of parasitic protozoa.

The results of this study indicate that Leishmania mexicana mexicana is similar to other pathogenic protozoa in that it lacks the ability to synthesize purines de novo and, therefore, depends upon salvage of preformed purines from its environment for survival. The enzymes involved in the purine salvage pathways of cultured promastigotes and isolated amastigotes of L. m. mexicana were examined. Differences between the enzyme content of the amastigote and promastigote forms of this species were found to be quantitative rather than qualitative. However, significant differences were observed between the enzymes present in L. m. mexicana and those present in L. donovani. Enzymes detected in L. m. mexicana included adenine deaminase, guanine deaminase, adenosine deaminase, two distinct nucleosidases (one active on nucleosides, the other on deoxynucleosides) and nucleotidases (more active on 3'- than 5'-nucleotides). Phosphorylase, phosphoribosyltransferase and nucleoside kinase activities were also detected. Nucleotide interconverting enzymes were present. Cell fractionation studies of

promastigotes revealed the distribution of the enzymes in different cell fractions. This study established that the leishmanial microbody-like organelle, the glycosome, contains xanthine phosphoribosyltransferase, an enzyme not found in mammalian cells. 3'-AMP nucleotidase and 5'-AMP nucleotidase, as well as acid phosphatase activities, were shown to be present on the external surface of both amastigotes and promastigotes. With regard to pyrimidine metabolism, both amastigotes and promastigotes of L. m. mexicana contained cytidine deaminase, uridine nucleosidase, uridine posphorylase and thymidine phosphorylase, whereas only promastigotes contained deoxycytidine deaminase and thymidine nucleosidase. Interestingly, the promastigotes contained 20 times more orotate phosphoribosyltransferase activity than amastigotes.

A survey of purine- and pyrimidine-metabolising enzymes in promastigotes of L. m. amazonensis, L. donovani and L. tarentolae, culture forms of Crithidia fasciculata, Herpetomonas muscarum muscarum and H. m. ingenoplastis and procyclic trypomastigote of Trypanosoma brucei brucei have also been carried out in this study. Several common features between trypanosomatids were observed, including the presence of nucleosidase, catabolic phosphorylase, phosphoribosyltransferases, kinases and cytidine deaminase activities and the apparent absence of AMP deaminase, anabolic phosphorylase and cytosine deaminase. Significant differences between these species were also discovered, notably in adenine and adenosine metabolism.

Extracts of Entamoeba histolytica have also been surveyed for purine salvage enzymes. The results obtained suggest that this pathogen largely relies on nucleoside phosphorylase and nucleoside kinase activities for salvage synthesis of purine nucleotides and so

it differs from Leishmania species which appear to depend more upon phosphoribosyltransferases for synthesis of nucleotides. Entamoeba histolytica also differs from Leishmania in lacking enzymes that are involved in nucleotide interconversions.

The results obtained with Acanthamoeba species indicate that they are capable of de novo synthesis of purine nucleotides. In this respect, they appear to be unique amongst parasitic protozoa studied to date. Acanthamoeba species can also salvage purines and the results suggest that hypoxanthine is of central importance in purine metabolism.

Finally, the survey of enzymes potentially involved in the salvage of purines in extracts of Babesia divergens suggested that phosphoribosyltransferases and nucleoside kinases may be together responsible for the production of both adenine and guanine nucleotides.

My studies on purine and pyrimidine metabolism of parasitic protozoa have revealed more details of the enzymes involved including a variety of differences between these species. In addition, it has been confirmed that there are important differences between these parasites and mammalian cells, differences that may be exploitable by antiprotozoal drugs.

Chapter 1

INTRODUCTION

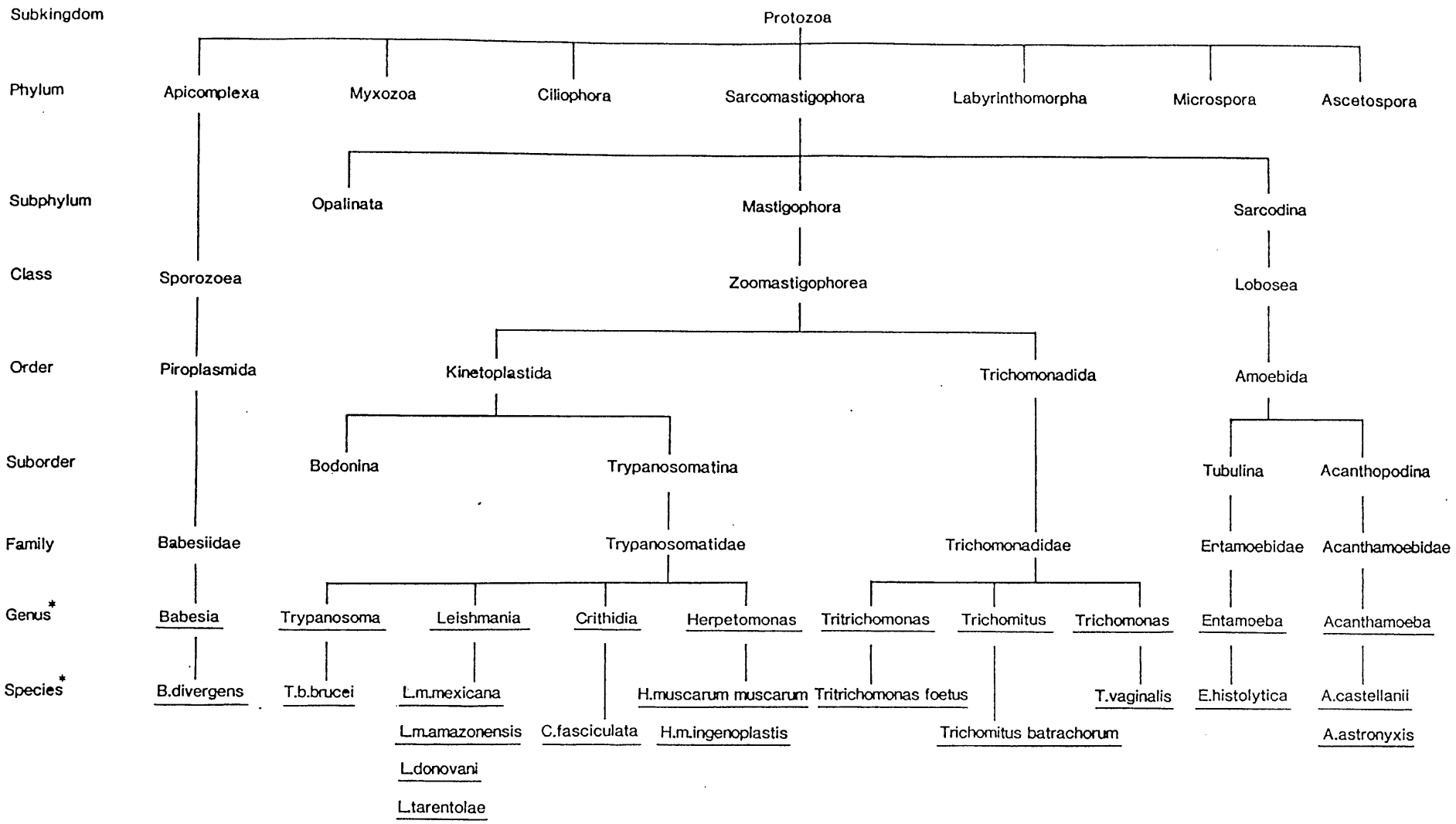
1.1. Classification of Parasitic Protozoa

The taxonomic problems are more difficult to resolve for the protozoa than with most groups of organisms, partly due to their minute size and partly due to the custom of basing relationships on comparative morphology and life cycles. The problem of determining taxonomic relationship is particularly difficult with the parasitic species, since the very unsatisfactory criterion of host specificity is commonly used in assessing the systemic position of the organism. In many instances, morphologically similar types occur in several or many hosts.

In the classical classification of living organisms, the protozoa have been considered as a phylum and were divided into four major classes (Mastigophora, Ciliophora, Sarcodina and Sporozoa) distinguished by their mode of locomotion. In recent years, however, the increasing information in our knowledge concerning the life cycle, ultrastructure, physiology and biochemistry of the protozoa has led protozoologists to designate the protozoa as subkingdom of the kingdom Animalia (Levine et al 1980). A basic taxonomy of the protozoa is outlined in Table 1 with special emphasis being given to those groups that contain species used in my studies.

Parasitic protozoa have always attracted the attention of biologists, physicians and veterinarians because they produce disease in humans and his domestic animals and they remain a major challenge to health authorities in many countries throughout the world. My studies have centred upon one aspect of biochemistry rather than a single protozoan; indeed I have included many parasitic protozoa in my studies.

Table 1. Classification of parasitic protozoa used in this study



* The genus and species names given are those used in this study.

Based on the classification scheme of Levine et al 1980.

There is a wealth of information available of most of these species. Such that it is impractical to review our knowledge of each of them in my introduction. Many of my studies have involved the leishmanias, thus I have dealt with aspects of their biology and biochemistry in detail, whereas the other protozoa have been covered only in brief with references to appropriate reviews on their biochemistry being given. This should provide access to the relevant literature in each area. The purine and pyrimidine metabolism of parasitic protozoa has been reviewed in detail (see chapter 2).

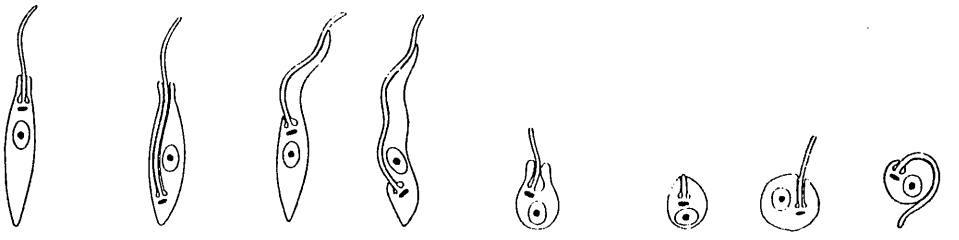
1.2. Biology of kinetoplastid flagellates

The kinetoplastid flagellates are characterised by the presence of an organelle known as the kinetoplast, which contains a complex of DNA and is associated with the single mitochondrion present in the cell. The kinetoplast DNA (k-DNA) is situated near the flagellar basal body and is easily visualised by light microscopy after staining with Giemsa's stain. It represents 10-20% of the total cellular DNA (Englund 1981) and is composed of a single network of thousands of interlocked circular DNA molecules of two size classes, the minicircles and maxicircles (Simpson 1986). The minicircles of DNA are present at 5,000-10,000 copies per network and are heterogeneous in both size and nucleotide sequence; it is unlikely, therefore, that each codes for the same structural RNA or polypeptide chain. The function of minicircles remains to be established. The maxicircles, on the other hand, are present as 20-50 copies, are homogeneous in nucleotide sequence and are transcribed into RNA. Maxicircles contain genetic information similar to that of other

mitochondrial DNAs; they hybridize with mitochondrial gene sequences from other unicellular eukaryotes which encode for cytochrome oxidase, cytochrome b or oligomycin-sensitive ATPase (Stuart 1983).

The most recent classification of kinetoplastid flagellates is given in Table 1. The order Kinetoplastida includes many free-living and symbiotic species (family Bodonidae and family Cryptobiidae) in addition to species parasitic in plants, invertebrates and vertebrates (family Trypanosomatidae). Of the nine genera into which the family Trypanosomatidae is divided, two include organisms seriously pathogenic to man and to his domestic animals (Trypanosoma and Leishmania species) and some Phytomonas species are recognised as pathogens of plants. The life cycle of members of the Trypanosomatidae has generally been considered to be asexual, although there is recent evidence for sexual processes occurring in salivarian trypanosomes (Tait 1980; Borst et al 1982; Vickerman 1986). Trypanosoma, Leishmania, Endotrypanum and Phytomonas are heteroxenous (their life cycle involving two hosts, a definitive host and a vector) whereas all other genera, namely Crithidia, Leptomonas, Herpetomonas, Blastocrithidia and Rhynchoidomonas are monoxenous and are mainly parasites of insects (and a few other invertebrates). During their life cycle, the trypanosomatid parasites may exist in a variety of morphological forms (polymorphism) distinguished by shape, the position of the kinetoplast relative to the nucleus, and the extent of the flagellar apparatus; a convenient nomenclature for these morphological forms is that proposed by Hoare and Wallace (1966). The forms characteristically occurring in the life cycle of each genus are given in Table 2. The promastigote stage has an antenuclear kinetoplast with the flagellum arising nearby and

Table 2. Occurrence of -mastigotes in genera of Trypanosomatidae



	Pro-	Opistho-	Epi-	Trypo-	Choano-	A-	Para-	Sphaero-
Leishmania	+					+	+	
Trypanosoma	+		+	+		+		+
Leptomonas	+					+		
Crithidia					+			
Herpetomonas	+	+					+	
Blastocrithidia	+		+			+		
Phytomonas	+							
Endotrypanum	+		+	+		+		
Rhynchoidomonas				+				

Modified from Vickerman 1976; Molyneux and Ashford 1983.

emerging from the anterior end of the body, whereas the opisthomastigote stage has postnuclear kinetoplast with the flagellum arising near it and passing through the body and directed anteriorly. In the epimastigote (crithidial stage with prenuclear kinetoplast) and the trypomastigote (trypanosome stage with postnuclear kinetoplast) the flagellum is attached to the body by means of an undulating membrane. The choanomastigote has an antenuclear kinetoplast with the flagellum directed anteriorly from funnel shaped reservoir, whereas the paramastigote, which apparently represents a transitional stage between promastigote and opisthomastigote, has the kinetoplast lying close to nucleus with the flagellum emerging from the anterior end. The rounded sphaeromastigote form has a long flagellum and undulating membrane, and the amastigote (rounded immobile leishmanial stage) lacks an emergent flagellum.

I have included many trypanosomatid flagellates in my studies; although in all cases the major emphasis has been on the leishmanias. Consequently I shall deal with the general biology and biochemistry of these parasites in some detail but mention only a few points about the other species.

1.3 Leishmanias and leishmaniasis

Protozoa of the genus Leishmania infect millions of humans and other mammals in the tropical and subtropical regions of the world and are responsible for a spectrum of diseases of man, the leishmaniases, with symptoms ranging from self-healing cutaneous ulcers to disfiguring and progressive mucocutaneous lesions as well as the visceral disease which can be fatal if left untreated (Chance

1981; Chang et al 1985). The importance of leishmaniasis was highlighted by its selection by the World Health Organisation as one of the six major diseases for study in its Special Programme for Research and Training in Tropical Diseases (WHO 1984).

1.3.1. Taxonomy of the leishmanias

The similar morphology of the organisms has hampered the subdivision of the genus Leishmania into species. The definition of species was initially based on the clinical features of infection which was subsequently supported by a variety of factors such as geographical distribution, behaviour in culture, mammalian and sandfly specification together with serological and immunological characteristics (Molyneux and Ashford 1983; Chance 1985). These traditional approaches to speciation are of limited value, since the clinical symptoms may be confused by differing individual host responses to the same parasite (Williams and Coelho 1978). For example, species of Leishmania that produce cutaneous disease in humans can cause visceral disease in rodents. Two biochemical methods, namely isoenzyme electrophoresis and analysis of the buoyant density of nuclear and kinetoplast DNA, have been applied more recently to the study of Leishmania with the aim of providing information on intrinsic parasite characteristics that are not modified or obscured by host or environmental factors (Chance 1985; Barker et al 1985). Other techniques including radiorespirometry, the use of monoclonal antibodies and analysis of antigenic differences in promastigotes or in their excreted factors have also been used successfully to distinguish leishmanial parasites (Chance

and Walton 1982). Using these criteria, clear differences between isolates of apparently the same species in various geographical locations have emerged but it was thought to be premature to decide that these groups represent distinct geographical races, subspecies or species (Chance and Walton 1982; Barker *et al* 1985). The main clinical and epidemiological characteristics of the species of *Leishmania* considered to be valid entities are given in Table 3, which summarises data discussed in recent reviews on the taxonomy of the genus *Leishmania*.

1.3.2. Life cycle

The life cycle of *Leishmania* consists of two main morphologically distinct forms (see Fig. 1): (1) amastigotes, (4-6 μm) the form of the parasite that lives intracellularly in the parasitophorous vacuole of mammalian macrophages and which are non-motile and possess only a residual flagellum; and (2) promastigotes, (20 μm) the extracellular forms that live in the alimentary canal of the sandfly vector and which are highly motile and possess a single prominent flagellum. Several forms of promastigotes have been described (see below). In all forms of leishmaniasis, the organism is transmitted to man by the bite of an insect vector, a phlebotomine sandfly (order:Diptera; family:Psychodidae; subfamily:Phlebotominae). Species of *Phlebotomus* and *Lutzomyia* are the only proven vectors of leishmaniasis in the Old World and the New World, respectively (Ward 1985a). For all species of *Leishmania*, the development in the sandfly begins with the fly taking a bloodmeal from the skin of the infected mammal. As the bloodmeal is digested,

Table 3. Species and subspecies of *Leishmania* and their distribution.

SPECIES	DISTRIBUTION	MAIN DISEASE
L. donovani		visceral leishmaniasis
L. d. donovani	Southern Asia	
L. d. infantum	Mediterranean area and Central Asia	
L. d. sinensis	East Asia	
L. d. archibaldi	East Africa	
L. d. chagasi	Central and South America	
L. tropica	Mediterranean area, South-West and Central Asia	cutaneous leishmaniasis
L. major	South-West and Central Asia, North and West Africa	cutaneous leishmaniasis
L. aethiopica	East Africa	cutaneous leishmaniasis
L. mexicana		cutaneous leishmaniasis
L. m. mexicana	Central America	
L. m. amazonensis	South America	
L. m. venezuelensis	South America	
L. m. pifanoi	Brazil	
L. braziliensis		cutaneous and muco-cutaneous leishmaniasis
L. b. braziliensis	South America	
L. b. guyanensis	South America	
L. b. panamensis	Central America	
L. b. peruviana	South America	

Modified from WHO, 1980, 1984.

Fig. 1. Leishmanial life-cycle in sandfly and in mammalian hosts. 1. Delivery of promastigotes (proboscis form) into human skin by the bite of sandfly vector; 2. attachment and engulfment by phagocytosis of promastigotes by a macrophage; 3. fusion of phagosome containing a promastigote with lysosome in a macrophage; 4. differentiation of promastigote into amastigote in the phagolysosome of the infected macrophage; 5. multiplication of an amastigote in a parasite-containing or parasitophorous vacuole; 6. formation of large parasitophorous vacuole and continuing replication of intravacuolar amastigotes; 7. rupture of heavily parasitized macrophage and release of amastigotes; 8. phagocytosis of released amastigotes by a macrophage; 9. ingestion of parasitized macrophage by sandfly after a blood meal taken from infected person or reservoir animal; 10. rupture of the ingested macrophage and release of amastigotes in the gut of sandfly; 11. replication of amastigotes and their differentiation into promastigotes; 12. replication of promastigotes (termed nectomonads for L.mexicana group) in the abdominal midgut and insertion of their flagella into microvilli of the gut epithelial cells; 13. replication of L.braziliensis group in the pylorus and ileum of the sandfly hindgut as paramastigotes with broadened flagella attached to the chitinous gut wall via hemi-desmosomes; 14. forward movement of promastigotes to thoracic midgut as haptomonads with broad flagella attached to the chitinous gut wall; 15. sessile paramastigotes with broad flagella attached to the chintinous wall of stomadeal valve, pharynx and buccal cavity (cibarium); 16. actively motile promastigotes found in the proboscis or mouth part of sandfly.

From Chang et al., 1985.

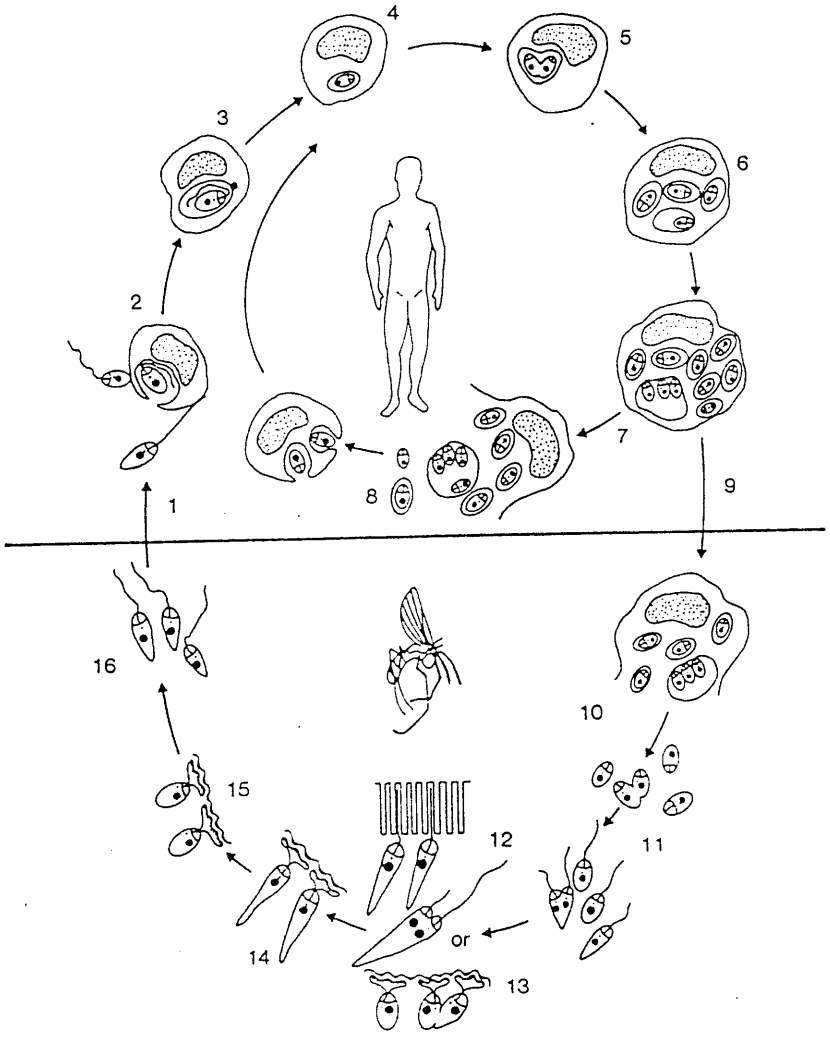


Fig.1 From Chang et.al.1985

most amastigotes are released from their host cell and appear to divide one or more times and then transform into highly active, elongated promastigotes termed nectomonads. In the case of L. mexicana, the nectomonads attach themselves to the abdominal midgut epithelium by inserting their flagellum between the microvilli lining the gut wall and then start a process of active division (Killick-Kendrick 1979). After the bloodmeal is voided, the nectomonads migrate forward into the thoracic midgut where they transform to stumpy, non-dividing haptomonads. Subsequently the parasites attach to the cuticular surface of the stomodaeal valve (cardia) by the disc-like expansion of the flagellar tip. These parasites then migrate forwards into the head of the fly and colonize the pharynx. Subsequently small highly motile forms are produced which move into mouth parts and are deposited in the skin of the next mammalian host upon which the fly feeds. In L. braziliensis, as the peritrophic membrane breaks up, the parasites migrate to the pylorus and ileum of the hindgut where they transform into a short form with broadened flagellum (paramastigote) attached to the chitinous gut wall by means of hemidesmosomes. Subsequently, the parasites migrate forward to the foregut and mouth parts. The significance of these morphological variations throughout the insect stages and the differences in developmental cycle between L. braziliensis and other species remains to be determined, although there is recent evidence suggesting that there is an increase in infectivity concomitant with the morphological changes that occur in the fly. There is similarly sequential development of promastigotes from noninfective to infective (metacyclic) forms during growth in culture (Sacks and Perkins 1984; Mallinson and Coombs 1986).

The transformation of amastigote to promastigote can also be achieved successfully *in vitro*, and has been shown to be dependent on cell division (Brun and Krassner 1976; Hart *et al*. 1981a).

The transmission of *Leishmania* into the mammalian host occurs by the bite of infected sandfly vector during which the extracellular promastigote forms are injected into the skin. Promastigotes are rapidly phagocytized by reticuloendothelial cells, within which they transform into intracellular amastigotes. Inside the macrophages, the amastigotes divide by binary fission (Vickerman 1980) and as they increase in number they burst the cell and reinfect other macrophages (Chang *et al* 1985).

1.3.3. Interaction of leishmanias with macrophages

The nature of the relationship formed between the leishmanial parasite, initially as a promastigote form, and its prospective host cell, a macrophage, is undoubtedly crucial to the establishment of a new infection. Macrophages are numbered amongst the primary line of mammalian cellular defense mechanisms against invading microorganisms, such as protozoa, by virtue of their fulfilling a variety of microbicidal functions. These include the production of toxic oxygen metabolites and lysosomal enzymes and the ability to activate the host's immune system. The means used by *Leishmania* parasites to resist the microbicidal process of macrophages is one of the most intriguing mysteries of leishmanial biology. Our current understanding of the macrophage/*Leishmania* interaction is that it involves three distinct phases: parasite attachment, internalization and the formation of a parasitophorous vacuole.

In vitro studies have indicated that promastigotes of *L. tropica* (Zenian *et al.* 1979), *L. mexicana* (Alexander 1975) and *L. braziliensis* (Merino *et al.* 1977) bind to macrophages primarily via their flagellar end whereas *L. donovani* apparently has no privileged site of interaction with macrophages on its surface (Chang 1979). There appear to be recognition or binding sites that account for the phenomenon of spontaneous binding between leishmanias and macrophages (Pearson *et al.* 1983). It has been proposed, however, that the attachment of *L. donovani* promastigotes to hamster peritoneal macrophages occurs via ligand-receptor interaction and its inhibition by pretreating cells with specific antibodies, glucosidases or monosaccharides further suggests that some of the binding ligands are glycoproteins (Chang 1981). Additional studies on the inhibitory effects of various carbohydrates on the *L. tropica* (Zenian 1981) and *L. mexicana* (Bray 1983) promastigote-macrophage interactions have also implicated a sugar lectin-type binding mechanism. Findings of mannose, mannose/fucose, mannose phosphate and galactose receptors on the macrophage surface (Imamura *et al.* 1984) and the involvement of these receptors in binding to the leishmanias ligands (Handman and Goding 1985; Blackwell *et al.* 1985) underscore the complexity of the binding mechanisms in these systems. More recently, the identification and characterisation of the major surface mannose and N-acetylglucosamine-containing glycoproteins from two subspecies of *L. mexicana* and its involvement in the attachment of promastigote to macrophages provide circumstantial evidence in support of the earlier findings and raises the possibility that uptake via glycoprotein binding may involve more than one macrophage receptor (Chang and Chang 1986; Russell and Wilhelm 1986).

It appears that the entry of Leishmania promastigotes into macrophages, where they will transform into amastigote and multiply, is dependent upon the phagocytic activity of these cells (Alexander 1975; Chang 1980). It has been shown that such internalisation may be accompanied by chemiluminescense and the release of H_2O_2 which are metabolic products of the phagocytic activity of macrophages. Furthermore, internalisation of leishmanias by macrophages was found to be inhibited by cytochalasin B (Alexander 1975; Zenian et al. 1979; Chang 1983) and iodoacetate (Benoliel et al. 1980), providing evidence for the participation of actin filaments and an energy requirement, respectively.

After the parasite has gained entrance into macrophages, it not only survives but also multiplies despite of fusion of secondary lysosomes with the parasitophorous vacuole (Alexander and Vickerman 1975; Chang and Dwyer 1976; Berman et al. 1981; Brazil 1984). In this respect, leishmanias differ from other intracellular parasites such as Toxoplasma gondii and Trypanosoma cruzi, whose interaction with murine macrophages has also been investigated. Toxoplasma gondii survives by preventing lysosomal fusion with the parasitophorous vacuoles and is destroyed if phagolysosomal fusion occurs (Jones and Hirsch 1972), whereas T. cruzi may evade lysosomal enzymes by escaping from the parasitophorous vacuole into the cytosol (Kress et al. 1977). The ability of Leishmania amastigotes to infect and grow within phagolysosomal vacuoles of host cells is an important aspect of pathogenesis in leishmaniasis. The precise mechanisms whereby leishmanias survive and multiply within these cells have not been elucidated. An understanding of these mechanisms might explain the failure of susceptible hosts to develop effective immunity

against the parasites and the ability of the parasite to avoid being destroyed by lysosomal enzymes and other microbicidal toxic metabolites (Chang 1983). Recent findings of the refractory properties of the leishmanial surface glycoproteins to lysosomal hydrolase degradation suggested that these molecule may provide a protective shell for the parasite that is resistant to digestion in the lysosome (Chang and Fong 1983). It has been shown that leishmanias release complex polysaccharides, termed excreted factors, which may enhance the growth of amastigotes in macrophages, possibly by inhibiting lysosomal enzymes (Handman and Greenblatt 1977; Elon et al 1980; Hernandez 1983). It has also been suggested that ammonia produced through the action of a highly active amastigote proteinase and other enzymes may be excreted into the phagolysosome, thus raising the intravacuolar pH and so antagonising the microbicidal actions of the hydrolytic enzymes and enabling the parasite to survive (Coombs and Sanderson 1985). Another feature of importance for intralysosomal survival of leishmanias appears to be the proton pump ATPase which drives the active transport of glucose and amino acids across the membrane of amastigotes, thus maintaining a neutral pH within the parasite despite its location in the acidified phagolysosome (Zilberstein and Dwyer 1985).

The survival of leishmanias within phagolysosomes also probably relates, in part, to their defense mechanisms against oxidant damage by free radicals and other molecules generated by enzymatic reduction of molecular oxygen during the phagocytosis-induced respiratory burst of the macrophage (Klebanoff 1982; Klebanoff et al. 1983). The molecules involved are superoxide, hydrogen peroxide (H_2O_2), hydroxyl free radical ($OH^{\cdot}$) and singlet molecular oxygen ($^1O_2$). Both the promastigote and the amastigote forms of leishmanias have been shown

to trigger an oxidative burst in macrophages; the amastigotes, however, do not elicit a significant respiratory burst response and this has been correlated well with the greater susceptibility of promastigotes to the microbicidal activity of phagocytes (Murray 1982; Murray and Cartelli 1983; Klebanoff et al. 1983; Channon and Blackwell 1985a, 1985b). There is some evidence that amastigotes can inhibit the respiratory burst either via their membrane-bound acid phosphatase activity (Remaley et al. 1984) or via a lipase acting on diacyl glycerol in the macrophage membrane (Blackwell et al. 1986). The levels of endogenous scavengers of oxygen metabolites may also play a part in intracellular survival. Murray (1982) reported that amastigotes of L. donovani contain more superoxide dismutase, catalase and glutathione peroxidase than promastigotes and are four times more resistant to enzymatically-generated toxic oxygen metabolites. In preliminary reports, it was suggested that glutathione peroxidase, superoxide dismutase and catalase of L. m. mexicana do not play a significant role in this parasitic intracellular survival, as the activities of these enzymes were either undetectable or unimpressive as compared to normal aerobic cells (Ghafoor and Coombs 1980). Meshnick and Eaton (1981), however, concluded that L. tropica promastigotes have relatively high activity of an iron-containing superoxide dismutase, which differs from the isofunctional enzymes of their mammalian hosts. The latter usually contain copper or zinc as the active prosthetic group. Although the role of the leishmanial superoxide dismutase in intracellular survival has not been clearly defined, it has been suggested that it has considerable potential as a target for chemotherapeutic attack (Meshnick and Eaton 1981). Recent reports (Penketh and Kennedy 1986) has shown that both amastigotes and promastigotes of L. donovani

contain large reserves of trypanothione which apparently reduces H_2O_2 concentration by a non-enzymatic reaction.

1.3.4. Ultrastructure of *Leishmania*

Most of our knowledge of *Leishmania* ultrastructure in relation to function comes from the study of thin sectioned material by electron microscopy. A transmission electron micrograph of a leishmanial promastigote and amastigote showing the major structural features of the cells are given in Figs. 2 and 3, respectively. The organism is surrounded by a 10-12 nm thick trilaminate membrane beneath which lie longitudinal microtubules that are linked to the inner membrane by fibrils. The microtubules are believed to form the cytoskeleton which is responsible for the cell's rigidity and shape, as with other trypanosomatids (Vickerman and Preston 1976). Our understanding of the *Leishmania* plasma membrane is limited and fragmentary and most of the studies have been concerned mainly with promastigote forms (Dwyer 1986). Using a freeze fracture replica technique, the plasma membrane of *L. m. mexicana* promastigotes was shown to have a denser distribution of intramembranous particles but lower levels of β-hydroxysterols than the membrane of amastigotes (Tetley *et al.* 1986). These studies suggest that the amastigote contains relatively low levels of membrane enzymes but high sterol content. It is of interest to note that surface membrane of *Leishmania* contains phosphomonoesterases (3'- and 5'-nucleotidases and acid phosphatase) (Coombs *et al.* 1986a; Dwyer 1986), phospholipases (Dwyer 1986) and a proteinase (Etges *et al.* 1986). The roles of these enzymes are not fully understood, but they are likely to play parts in the survival of the parasite within the host.

The spherical nucleus is bound by a double membrane with pores and contains peripheral chromatin in addition to the nucleolus. The

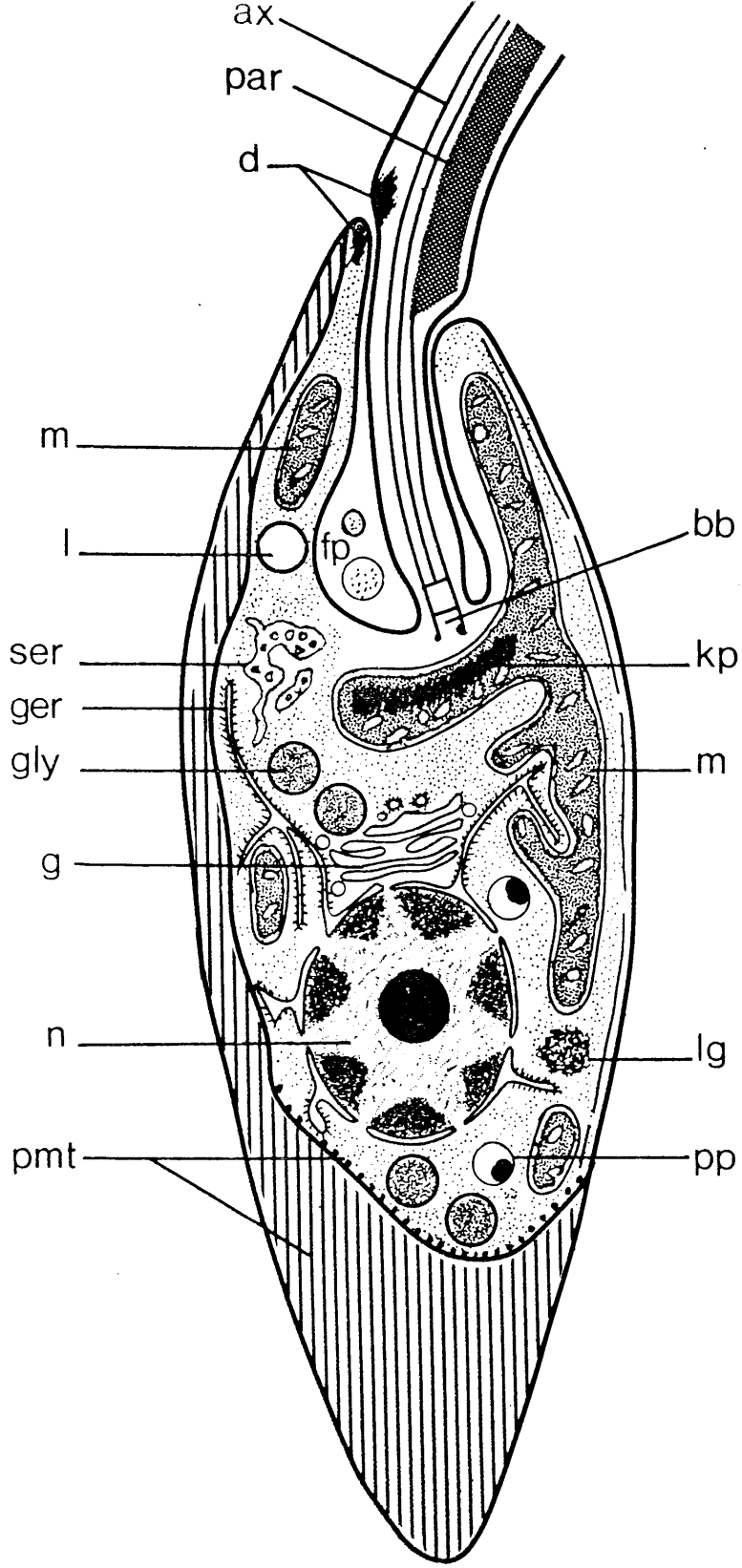
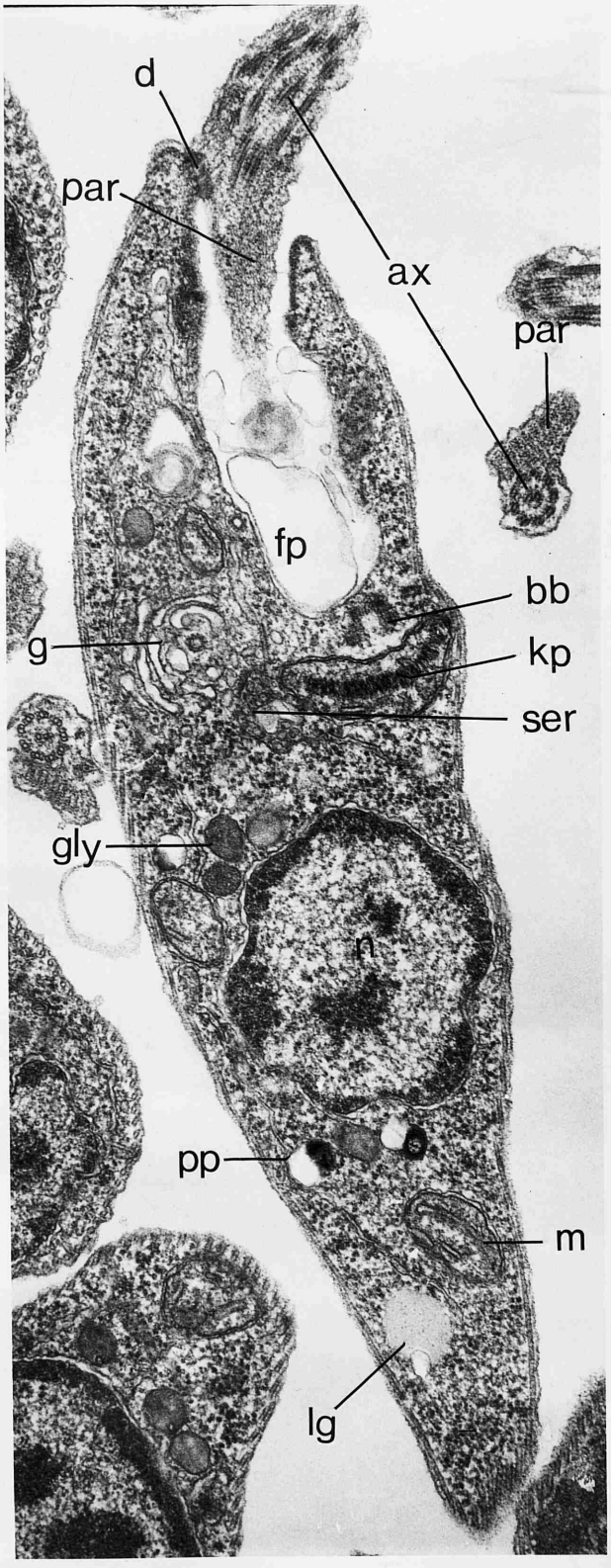


Fig. 2. The ultrastructural features of Leishmania mexicana mexicana promastigotes. The diagram on the left gives the principal structural features shown in the transmission electron micrograph of a sectioned L.m.mexicana promastigote on the right. Scale bar = 0.5µm. Abbreviations: ax, axoneme; bb, basal body; d, desmosome; fp, flagellar pocket; g, golgi apparatus; ger, granular endoplasmic reticulum; gly, glycosome; kp, kinetoplast; l, lysosome; lg, lipid globule; m, mitochondria; n, nucleus; par, paraxial rod; pmt, pellicular microtubule;pp, polyphosphate granule; ser, smooth endoplasmic reticulum. Micrograph courtesy of L. Tetley.


d
par
ax
par
fp
bb
g
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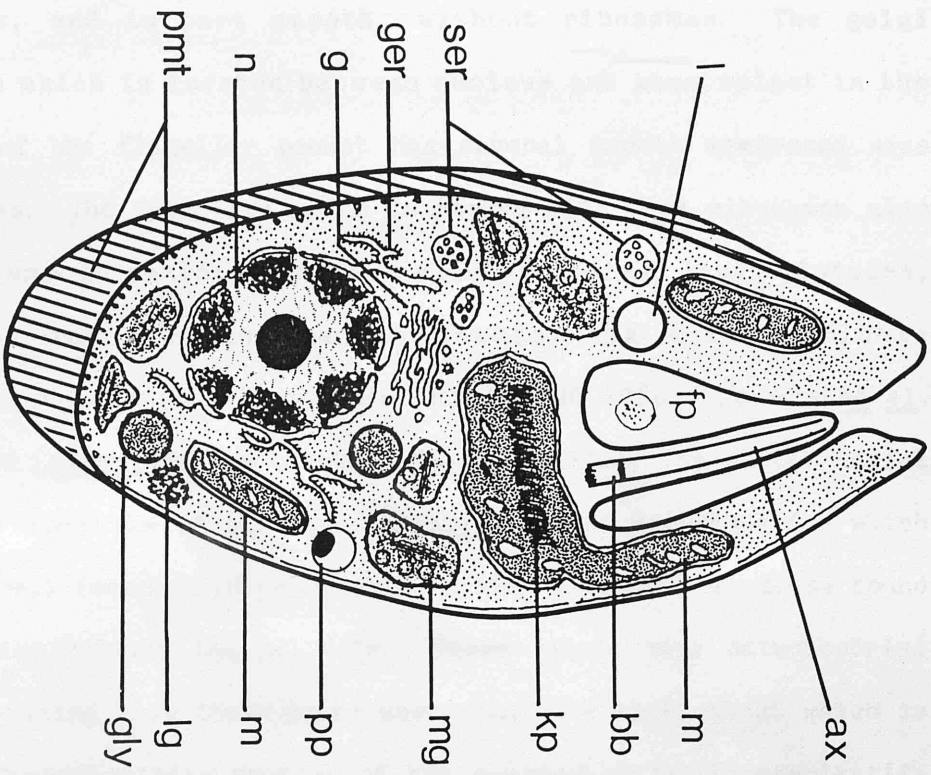
ser
gly
n
pp
m
lg

Fig. 3. The ultrastructural features of Leishmania mexicana mexicana amastigotes. The diagram on the top gives the principal structural features shown in the transmission electron micrograph of a sectioned L.m.mexicana amastigote on the bottom. Scale bar = 1μm. Abbreviations: ax, axoneme; bb, basal body; fp, flagellar pocket; g, golgi apparatus; ger, granular endoplasmic reticulum; gly, glycosome; kp, kinetoplast; l, lysosome; lg, lipid globule; m, mitochondria; mg, megasome; n, nucleus; par, paraxial rod; pmt, pellicular microtubule; pp, polyphosphate granule; ser, smooth endoplasmic reticulum. Micrograph courtesy of L. Tetley.


pmt
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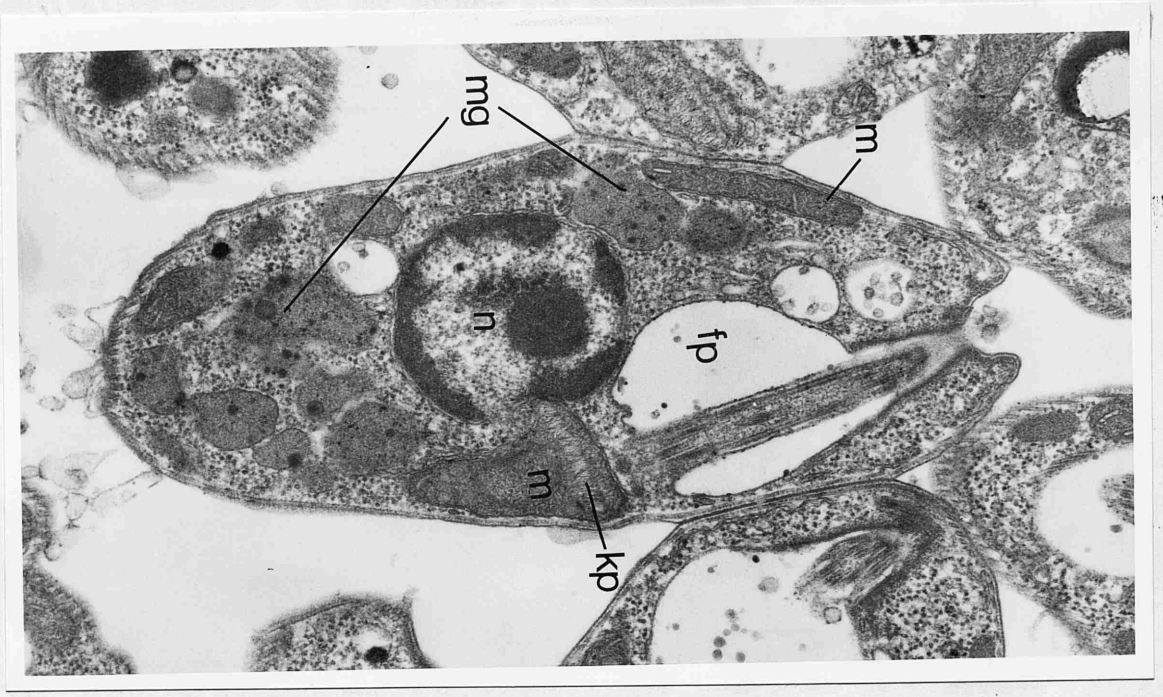
mg
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endoplasmic reticulum consists of flattened, ramifying cisternae and tubules and is in part rough, bearing osmiophilic granules resembling ribosomes, and in part smooth, without ribosomes. The golgi apparatus which is located between nucleus and kinetoplast in the vicinity of the flagellar pocket has several smooth membraned sacs and tubules. The cytoplasm which is packed with free ribosomes also contains smooth vesicles, multivesicular bodies, lipid globules, lysosomes and volutin granules containing acid insoluble polyphosphate and RNA (Vickerman 1974; WHO 1980; Coombs et al. 1986b). In Leishmania, as in other Kinetoplastida, the mitochondrial apparatus consists of a single highly branched mitochondrion which most commonly forms an interconnected series of double-membrane bound canals lying beneath the pellicle. These canals have mitochondrial cristae, arising from their inner membrane. The kinetoplast which is a unique characteristic feature of the members of the Kinetoplastida consists of circular and linear DNA molecules housed in an expansion of the mitochondrial apparatus. It should be pointed out that the quantitative changes in the volume and structure of the mitochondria of leishmanias during amastigote to promastigote transformation (Brun and Krassner 1976) are not as dramatic as those found in the course of transformation of bloodstream forms of Trypanosoma brucei to midgut trypomastigote forms (Vickerman 1985). Relative mitochondrial volume increases significantly during the latter process, a modification which is associated with the development of an active TCA cycle and respiratory chain together with a change in the energy metabolism of the parasite (Vickerman 1985). In Leishmania, however, the transformation was accompanied by a slight decrease in relative mitochondrial volume, but the number of cristae remained unchanged after transformation and respiration was found to be cyanide-sensitive in both forms of the parasite (Simpson 1968; Brun and

Krassner 1976; Hart et al. 1981b). These results suggest that there is a little variation in mitochondrial function between the amastigotes and promastigotes.

The flagellum , which is bounded by a continuation of the cell membrane, arises from a basal body and passes through the flagellar pocket to whose wall it is attached by a series of desmosomes. The cylindrical basal body has the typical centriolar and microtubular-triplet structure. The axoneme has the usual (9d + 2s) microtubular arrangments while a paracrystalline structure, the paraxial rod, runs parallel to the axoneme. In the short transitional zone between the basal body and flagellum proper the axoneme lacks the central singlets and there is no paraxial rod (Hommel 1978).

Glycosome are single membrane-bound organelles seemingly peculiar to the kinetoplastid flagellates. A number of enzymes involved in glycolysis, CO_2 fixation, β-oxidation of fatty acid, de novo pyrimidine biosynthesis and purine salvage have been detected in glycosomes of leishmanial species (Coombs 1986). It has been stated that L. tropica promastigotes may contain 50-100 glycosomes (Hart and Opperdoes 1984) whereas three dimensional reconstruction of L. m. mexicana amastigotes has revealed that there were 9-10 glycosomes per amastigote, representing less than 1% of the total cell volume (Coombs et al. 1986b). This is in contrast to T. brucei which are said to contain 200-300 glycosomes, accounting for 4.3% of the total cell volume (Opperdoes et al. 1984). The ultrastructure of amastigote generally resembles that of promastigotes except for the overall shape and the absence of a free flagellum. The amastigotes of L. m. mexicana also have unusual lysosome-like organelles named megasomes, although these are apparently not found in amastigotes of L. tropica and L. donovani (Pupkis et al. 1986). The megasomes,

which were present mainly at the posterior part of the cells (as many as 34 per cell occupying 15% of the total cell volume), were shown to contain cysteine proteinase and arylsulfatase activities and may play a part in the survival of the amastigotes in the host cell (Pupkis et al. 1986).

1.3.5 The diseases

1.3.5.1. Visceral leishmaniasis

In visceral leishmaniasis (kala-azar), caused by L. donovani, L. d. infantum or L. d. archibaldi in the Old World and L. d. chagasi in the New World, the parasites multiply within phagocytes throughout the reticuloendothelial system of nearly all internal organs, but particularly of the spleen, liver, lymph nodes and bone marrow (Chance 1981). The common symptoms of the disease are intermitent fever, loss of weight, anaemia and pronounced hepatosplenomegaly (Marsden 1984). In general, once the stage of hepatosplenomegaly is reached host defense mechanisms are not able to prevent further parasite multiplication that ultimately leads to death if treatment is not administered (Mauel and Behin 1981). The visceral disease caused by L. donovani may spread to the skin in some individuals after apparently successful drug-induced cure. This form of the disease, called post kala-azar dermal leishmaniasis, occurs in the Indian subcontinent (WHO 1984). It is characterised by the presence of multiple nodular lesions in the skin usually without ulceration (Pearson et al. 1983). Visceral leishmaniasis is anthroponotic in India with man being the sole reservoir but is zoonotic in North Africa, Middle East and Europe (caused by L. d. infantum with dogs acting as reservoir), whereas in the New World rodents may serve as reservoir hosts. The causative agent of visceral leishmaniasis in

Sudan and perhaps Kenya has been named L. d. archibaldi. No definite reservoir host has yet been identified, but most probably the infection has an zoonotic origin (Zukerman and Lainson 1977).

1.3.5.2. Cutaneous leishmaniasis

The cutaneous forms of leishmaniasis occur in both the Old World (L. tropica complex or L. aethiopica) and the New World (L. mexicana complex). Once inoculated by the bite of an infective sandfly into the skin, the parasite multiplies in macrophages which may result in necrosis of these cells. In classic localised cutaneous leishmaniasis, a nodule starts at the site of inoculation. For some species these typically ulcerate and become secondarily infected, whereupon they heal gradually leaving a depressed scar. There is strong immunity to reinfection with the same parasite (Chance 1981). The Old World cutaneous disease, known as oriental sore, comprises an anthroponic and zoonotic type. The former, also named urban type or dry sore, is caused by L. tropica. The latter known as rural or moist type, is caused by L. major, and exists primarily as an enzootic infection among gerbils and jirds in Asia. In both cases, the infection is limited to the development of single or multiple painless lesions that are often ulcerated and heal in 2-8 months.

Diffuse cutaneous leishmaniasis is known in the New World and Ethiopia where L. mexicana and L. aethiopica respectively are the causative agents (WHO 1984). Two subspecies of the L. mexicana complex, namely L. m. pifanoi and L. m. amazonensis, are thought to be the agents of diffuse cutaneous leishmaniasis in Venezuela and Brazil, respectively (Lainson and Shaw 1978). The disease starts as a papule that does not ulcerate. Satellite lesions subsequently

developed around the initial papule and organisms may metastasize to distinct areas of the skin, often to the face and extremities; the disease does not heal spontaneously (Mauel and Behin 1981).

Leishmaniasis recidivans (also known as tuberculoid or lupoid leishmaniasis) is usually associated with L. tropica and is a variant of cutaneous leishmaniasis in which an apparently healed scar is reactivated to give a chronic, non healing ulcer (WHO 1984). There is evidence suggesting that, as in visceral leishmaniasis, cell-mediated immune responses against the parasite are suppressed during diffuse cutaneous leishmaniasis and leishmaniasis recidivans (Marsden 1984).

1.3.5.3. Mucocutaneous leishmaniasis

The horrid mucocutaneous disease, commonly known as espundia, is found predominantly in South America, where members of the L. braziliensis complex (L. b. braziliensis, L. b. panamensis and L. b. guyanensis) are usually implicated as the causative organisms. In the early stage, patients develop a single or multiple cutaneous ulcers which seldom heal spontaneously (Pearson et al. 1983). Depending on the subspecies of parasite, the lesions may spread along the lymphatic system before healing or they may disappear only to reappear in the oronasal/pharyngeal mucosa, often 30 years later, causing the major disfigurement. The disease is characterised by complete destruction of the nasal septum, extensive damage of the pharynx and vocal cords, loss of voice and seriously impeded respiration (WHO 1984). The disease is non-healing but recovery may occur with strong immunity to reinfection. In the absence of appropriate therapy, death may occur through suffocation because of blockage of air passage or through secondarily bacterial infection.

1.3.6. Control of leishmaniasis

Control measures to protect people from the effects of leishmaniasis include destruction of the sandfly vectors and animal reservoirs, treatment of infected people and vaccination. Exposure of individuals to bites of infected sandflies may be minimized by avoiding contact, by insect repellents, by residual spraying in the house and in immediate breeding sites and by sleeping under fine mesh netting (WHO 1984).

1.3.6.1. Control of vectors and reservoir hosts

The only known way of controlling sandflies in or near human dwelling is by periodic spraying of insecticides (Marinkelle 1980). Sandflies are highly susceptible to insecticides but some resistance to DDT and dieldrin has been reported in India and also from Brazil (WHO 1984). However, since breeding sites and larval habitats of sandflies are inadequately known or inaccessible to direct insecticide application, only the adults can be attacked and, therefore, the residual effect of the insecticide is most important (Marsden 1984). In most cases where sandflies have been controlled by insecticides (such as visceral leishmaniasis caused by L. donovani in India or some forms of cutaneous leishmaniasis caused by L. peruviana in Peru and L. tropica in Asia), it was an incidental effect of programmes aimed primarily against malaria (Marsden 1984). Vector control is frequently totally impractical in zoonotic forms of the disease such as South American cutaneous and mucocutaneous leishmaniasis; this could be achieved only by destroying the forest and even this ecologically dangerous process might merely lead the replacement of one parasite by another (Lainson and Shaw 1978).

Control of leishmaniasis through treatment and eradication of reservoir hosts can be possible. Man and dogs are the only known

reservoirs in many parts of the world. Control of human reservoirs has been attempted by active case detection combined with chemotherapy (Marinkelle 1980). Visceral leishmaniasis has been largely eliminated from China by the eradication of infected dogs and treatment of patients, as well as insecticide spraying (Marsden 1984). In the USSR, colonies of rodents have been eradicated from many cities by the use of poisoned bait, deep ploughing of burrows and by the design of irrigation canals to form barriers across which recolonization is impossible (Molyneux and Ashford 1983). In many situations, however, control of the wild animal reservoirs of the disease is impossible.

1.3.6.2. Vaccination

Vaccination (leishmanization) has long been practised against cutaneous leishmaniasis in certain areas where the disease caused by L. major is hyperendemic (Pearson et al. 1983). In order to prevent naturally occurring lesions from developing on the face or in multiple sites, an infective dose of the parasite is administered at a chosen site of the body where the subsequent scar will be hidden by clothing. Following this leishmanization procedure, the disease runs its normal course and, after the lesion has healed, leaves the patient with strong immunity to the parasite (Greenblatt 1985). Using live, virulent and infective promastigotes, vaccination programs have been successful in the Middle East and the USSR whereas attempts to use avirulent or killed parasites has consistently failed (WHO 1984). Attempted leishmanization with attenuated strains of Leishmania has been unsuccessful (Marsden 1984). Furthermore, it has been established that leishmanization with L. major can protect against the urban type of infection (L. tropica) but unfortunately

the reverse does not seem to be true (Marsden 1984). The use of live vaccines is far from ideal and can be complicated by development of large lesions, secondary infections and interference with the immune response to other vaccines. The use of a live vaccine to protect against *L. braziliensis* clearly is impracticable as there may be a dangerous sequel to infection with that organism (Lainson and Shaw 1978).

1.3.6.3. Chemotherapy

Two pentavalent antimonials, namely sodium stibogluconate (pentostam, 28-29.5% Sb^{5+}) and meglumine antimoniate (Glucantime, 33% Sb^{5+}), have been the front-line drugs for treatment of all forms of human leishmaniasis for many years. The therapeutic response to these drugs is very variable (Mahmoud and Warren 1977). They are widely used for the treatment of human kala-azar, for which they usually effective but they must be given parenterally and have common side effects. Pentavalent antimonials are not, unfortunately, always as effective in the treatment of patients with diffuse cutaneous and mucocutaneous disease (Berman 1985). In cases where pentavalent antimonial treatment fails, second line drugs such as pentamidine and amphotericin B can be used (WHO 1984). Frequently, however these drugs fail to eradicate the parasite because of problems relating to toxicity and/or lower efficacy.

It is an interesting but as yet unexplained observation that amastigotes appear to be inherently more susceptible than promastigotes to pentavalent antimonials and pentamidine (Berman 1985), although the differing susceptibilities apparent *in vitro* could be due to the macrophage actively concentrating the drugs within the parasitophorous vacuole. In this regard, it is of interest that incorporation of antimonial drugs into liposomes

increased their effectiveness against the parasite *in vivo* (Alving 1986). Another possible explanation for the differing susceptibilities of amastigotes and promastigotes is that human macrophages may metabolise the drugs to a form with greater leishmanicidal activity.

The basis of sensitivity of *Leishmania* to antimonials is unknown. It has been proposed that some of the pharmacological effects of the antischistosomal antimonials are mediated by inhibition of the glycolytic enzyme phosphofructokinase (Mansour and Bueding 1954). Recent studies on antileishmanial antimonials, however, suggested that these drugs do not act on *L. m. mexicana* through inhibition of a glycolytic enzyme (Mottram and Coombs 1985). Amphotericin B was shown to be toxic to both amastigotes and promastigotes and so it has been suggested that it does not require processing by macrophages to kill amastigotes (Berman 1985). The mode of action of this drug remains uncertain but probably relates to an interaction with parasite membrane sterols which results in the loss of the membrane's functioning as a permeability barrier to small metabolites (Saha *et al* 1986).

It is generally accepted that there is a great need to develop more effective and less toxic drugs for the treatment of leishmaniasis. Recent efforts to find alternative antileishmanial agents have produced several interesting leads including the demonstration that 8-aminoquinolines are active in several models (Berman and Lee 1983). Although the mechanism of action of 8-aminoquinolines has not been proved, it has been suggested that they may interfere with mitochondrial respiration, possibly through interaction with ubiquinones (Peters *et al* 1980). Therefore, further investigation of the ubiquinones and cytochrome systems of *Leishmania*

seems likely to provide a valuable pointer to targets susceptible to drug attack.

Studies on phenothiazine drugs have demonstrated that clomipramine, nitroimipramine and chlorpromazine have lethal effects on L. donovani and L. major promastigotes and amastigotes (within macrophages) (Pearson et al. 1984; Zilberstein and Dwyer 1984); interestingly, amastigotes were more susceptible than promastigotes. More recently, Zilberstein and Dwyer (1986) suggested that the phenothiazine drugs inhibited the plasma membrane H^+-ATPase of L. donovani which resulted in a loss of the ability of the parasite to maintain the proton electrochemical gradient necessary for uptake of nutrients and for maintenance of pH homeostasis and consequently caused rapid cell death.

Another rational approach in the search for new drugs has been the investigation of amino acid esters. Rabinovitch et al. (1986) reported that certain L-amino acid (but not D-amino acid) methyl esters rapidly killed L. m. amazonensis amastigotes (intracellular or isolated) but that promastigotes were less susceptible. The most active esters were found to be those of L-leucine. It has been postulated that the basis of the activity of these esters is that, after entering the parasitophorous vacuole, they are hydrolysed by enzymes, yet to be characterised, to release amino acids which become protonated and so trapped; hence they accumulate in the vacuole. They could be further concentrated in the amastigote lysosomes, leading to damage to the parasites (Rabinovitch et al. 1986).

There are other interesting and exciting areas where the metabolic differences between the leishmanial parasite and its mammalian host offer opportunities for the design of new drugs. These are reviewed in the following section.

1.3.7. Biochemical aspects of leishmanias - potential for drug targetting

The rapid increase there has been in our knowledge of the biochemistry of Leishmania and the diseases they cause and the development of mass isolation methods for amastigotes means that at last it is begining to appear possible to exploit metabolic differences between the parasite and the host cell by designing a new range of antileishmanial agents; the new approaches to chemotherapy may soon bear fruit. In this review, which is not comprehensive, attention will focus on the recent advances made in several important areas of leishmanial metabolism. Purine, pyrimidine and folate metabolism are discussed in detail in chapter 2.

1.3.7.1. Energy metabolism

Many studies have shown that various carbohydrates (glucose, fructose, galactose, maltose, mannose, raffinose, sucrose, glucosamine and glycerol) can be utilised as an energy source by promastigotes of several Leishmania species; glucose, however, has been implicated as a major energy substrate (Mukkada 1985; Coombs 1986). Amino acids, especially proline, also can be used as energy substrates by some species (Krassner and Flory 1972; Wagner and Krassner 1976). Studies on L. m. mexicana showed that both amastigotes and promastigotes catabolise glucose to CO_2 and a mixture of organic acids, the predominant one being succinate (Hart and Coombs 1982). Glycolysis, the TCA cycle and the pentose phosphate shunt are the major pathways of carbohydrate catabolism. In addition, the products of protein and fatty acid degradations can enter into the TCA cycle through amino acid catabolism and β-

oxidation, respectively. All enzymes of these pathways have been found in promastigotes of several leishmanial species (Mukkada 1985). There is great interest in elucidating the differences between the ability of amastigote and promastigote forms to use energy substrates. Studies on L. m. mexicana have shown that non-esterified fatty acids are more used by amastigotes, whereas glucose is the more important substrate of promastigotes (Hart and Coombs 1982). This led to speculation that Leishmania in their natural conditions may encounter adequate hexose supply in the sandfly gut and abundant fatty acids in the parasitophorous vacuole of macrophages (Coombs 1986). It is also interesting to note that the viability of amastigotes was markedly stimulated by high CO_2 concentration whereas promastigotes growing in vitro were unaffected (Hart and Coombs 1982). This correlates with the finding of considerably higher activities of malate dehydrogenase and phosphoenolpyruvate carboxykinase in amastigotes than in promastigotes (Mottram and Coombs 1985). The fact that leishmanial glycosomes are involved in energy metabolism, as well as apparently containing pathways of ether-lipid biosynthesis and CO_2 fixation (Hart and Opperdoes 1984; Coombs 1986), suggest that antiglycolytic drugs currently being sought for trypanosomiasis using a non-empirical drug design approach (Opperdoes 1983; Misset et al. 1986) may also possess antileishmanial activity.

1.3.7.2. Proteolysis

One of the more interesting features of L. m. mexicana is the large activity of highly active soluble cysteine proteinase present in the amastigotes and localised in unusual lysosome-like organelles known as megasomes (Coombs 1986). Similar organelles and enzyme

activity are present in amastigotes of L. m. amazonensis but not L. major or L. donovani (Pupkis et al. 1986). It has been suggested that amastigote proteinases may play a crucial part in parasite survival within host macrophages by producing amines which are secreted into the parasitophorous vacuole where they raise the pH and antagonise the microbicidal activity of potentially destructive hydrolases, thus allowing amastigote to survive (Coombs and Sanderson 1985). Interestingly, the major surface glycoprotein (P63) of leishmanial promastigotes has recently been shown to be a proteinase (Etges et al. 1986). The enzyme is active both in its detergent solubilised form and at the surface of live and fixed promastigotes, and it has been postulated that the surface proteinase may aid promastigote survival in the sandfly gut by degrading microbicidal enzymes.

1.3.7.3. Polyamines

Polyamine metabolism in trypanosomatids looks promising as a potential target for chemotherapy (Bacchi 1981). Among the various species of Leishmania, putrescine and spermidine form the major pool of polyamines. They are synthesized rapidly from ornithine but are taken up much more slowly from extracellular sources (Bachrach et al. 1979a; Morrow et al. 1980; Coombs and Sanderson 1985). As in other types of cells, intracellular levels of the polyamines fluctuate during the growth cycle, being maximal during the log-phase of growth (Bachrach et al. 1979a). Difluoromethylornithine (DFMO) and methylglyoxal bis (guanylhydrazone) (MGBG) have been shown to inhibit leishmanial growth and transformation (Bachrach et al. 1979b; Coombs et al. 1983) and it has been suggested that they exert their inhibitory effect by blocking polyamine biosynthesis and consequently

DNA and protein synthesis (Bachrach et al. 1979b). It has also been suggested that the clinically useful antileishmanial agent pentamidine isethionate may act through interference with polyamine synthesis (Bachrach et al. 1979b). A relatively new compound called sinefungin, which is an analogue of ornithine, although it is yet to be proved that it interferes with polyamine metabolism, has also shown some promise as an antileishmanial (Bachrach et al. 1980; Paolantonacci et al. 1985).

1.3.7.4. Lipid metabolism

The lipids of several Leishmania species have been studied (Holz 1985). Of interest is the finding of high levels of ether-linked forms of phosphatidylethanolamine in the surface membrane. These may play a role in the protection of the parasite from the microbicidal action of hydrolytic enzymes that are present in both its insect and mammalian environments (Wassef et al. 1985). Three distinct lipolytic enzymes (phospholipases A_1, A_2 and C) have also been demonstrated in isolated L. donovani surface membrane; phospholipase C and phospholipase A_2 are capable of hydrolyzing phosphatidylethanolamine (PE) into diglyceride and ethanolamine phosphate and into free fatty acid and lyso PE, respectively (Dwyer and Gottlieb 1985).

Leishmania species have been shown to contain sterols (Goad et al. 1984). The major sterol detected was ergosterol. Lanosterol, its precursor (squalene), and products of its demethylation were also detected, suggesting that sterols are being produced by the pathway characteristic of eukaryotic cells such as fungi but different from that of mammalian cells (Goad et al. 1984).

Ketoconazole has antimycotic properties, apparently due to of preventing the production of ergosterol by inhibiting the demethylation of lanosterol. This leads to the accumulation of 14α -methylsterols and an associated alteration of the permeability properties of sterol-containing membranes (Borgers 1980). The finding that leishmanial infections also respond to ketoconazole correlates well with the similarity of the sterol content and their metabolism in leishmanias and fungi and makes this area of lipid metabolism of especial interest in the search for new antileishmanial drugs (Berman et al. 1984; Goad et al. 1985).

1.4 The biology of Crithidia

Crithidias are exclusively parasites of insects; members of the orders Diptera, Hemiptera and Hymenoptera serve as host for most species. Crithidia species are characterised by the presence of a choanomastigote stage (Wallace 1966; Molyneux and Ashford 1983). They usually parasitize the hindgut and rectum of the host, often attached by the tip of the normally elongate flagella (Vickerman 1976; Molyneux and Ashford 1983). In the hindgut, however, the parasites frequently adhere in clumps around evaginations of the epithelium, giving a rosette appearance. In addition, free swimming stages of C. fasciculata have been observed in water occupied by its host, the mosquito Culex pipiens (Wallace 1979).

Crithidia fasciculata (a parasite of mosquitoes of the genera Anophles and Culex) and other species have been widely used in experimental studies as they are easily cultivated (Kidder and Dutta 1958; Mundim and Roitman 1977; Newton 1976; Roitman et al. 1985). Crithidia oncopelti is unusual as it has no requirement for haemin and can synthesize purines from conventional precursors (Roitman and

Camargo 1985); it can be grown in an extremely simple medium containing a single amino acid (methionine), five vitamins, a mixture of inorganic salts and glucose as a carbon source. It is now known that this flagellate contains a bacterial endosymbiont and it has been suggested that this provides C. oncopelti with a range of metabolites which are mandatory for the growth of other trypanosomatids (Roitman and Camargo 1985). For this reason C. oncopelti should be avoided as a model for other trypanosomatids.

A large number of studies have been carried out on the physiology and biochemistry of Crithidia species; most of the work relates to C. fasciculata but C. oncopelti and C. luciliae have also been examined (see Von Brand 1979; McGhee and Cosgrove 1980; North et al. 1983; Cazzulo et al. 1985).

1.5. The biology of Herpetomonas

The genus Herpetomonas includes monoxenous trypanosomatids with promastigote, paramastigote and opisthomastigote stages in their life cycle (Wallace 1970; Vickerman 1976); the paramastigote probably represents a transitional stage between the pro- and opisthomastigote stages. Opisthomastigotes are not found as homogeneous populations, they are always mixed with promastigotes and paramastigotes (Wallace 1979). The promastigote-opisthomastigote transformation of Herpetomonas species occurs at the end of the growth phase in culture, but interesting studies (Wallace 1979; McGhee and Cosgrove 1980) suggest that the increasing the age of cultures, cultivation at a temperature higher than optimum, raising the pH, addition of urea, and increasing the osmolarity can be used to stimulate the production of, or increase the percentage of opisthomastigotes. Herpetomonas species are found in Diptera (e.g. H. muscarum in the house fly Musca

domestica; H. ampelophilae in Drosophila), Hemiptera (e.g. H. samuelpessoai in the reduviid bugs Zelus leucogrammus) and Hymenoptera (e.g. H. swainei in the sawfly Neodiprion swainei) where they parasitize the mid- and hind gut and less frequently the malpighian tubules and haemocoel (Wallace 1979; Molyneux and Ashford 1983). No true cysts have been described for Herpetomonas and recent evidence indicates that adult Diptera acquire the infection from free-living stages (McGhee and Cosgrove 1980). It has also been found that insects infected in the larval stage retain the infection through the pupal stage to the adult. Herpetomonas muscarum is the only species studied to any extent. It comprises two subspecies: a) H. m. muscarum, (body length of promastigote 10-25 μm) which has a free flagellum twice the body length and has a discoid type A kinetoplast (i.e. DNA nucleoid is separated from its capsule envelope by a varying amount of mitochondrial matrix and is somewhat compact) (Wallace et al. 1973; Vickerman and Preston 1976); b) H. m. ingenoplastis, (body length of biflagellate promastigotes 20-30 μm) which has a very large, pear or tear-drop shaped type B kinetoplast (i.e. the loosely packed DNA nucleoid fills its capsule envelope) (Wallace et al. 1973; Vickerman and Preston 1976). Little is known about their biochemistry.

1.6. The biology of Entamoeba histolytica

The protozoan parasite E. histolytica is the only species of intestinal amoeba known to be pathogenic to humans. In the majority of infected individuals, it lives commensally in the lumen of the colon without producing any symptoms. In a small percentage of cases, however, the parasite, by mechanisms not yet fully elucidated, produces ulceration of the intestinal mucosa resulting in inflamatory

colitis, dysentry or amoeboma (lumenal amoebiasis) and may spread into the blood and invade the liver and other organs such as brain, lungs, skin, bladder, uterus and vagina (invasive amoebiasis) (Martinez-Palomo 1982). Entamoeba histolytica occurs throughout the world but particularly in Africa, South America, Mexico and India and has been estimated that it infects approximately 10% of the worlds population (Albach and Booden 1978), although a much smaller percentage suffer disease. Nevertheless, the morbidity and mortality associated with E. histolytica infection is of sufficient magnitude to make amoebiasis a major public health problem in many areas of the world (WHO 1981).

There are two stages in the life cycle of E. histolytica. (a) Trophozoite, this is a motile feeding form (size 10-60 μm) which has a single nucleus with fine peripheral chromatin and a central karyosome (nucleolus). The cytoplasm has two zones, an outer zone consisting of clear ectoplasm and an inner zone of granular endoplasm containing numerous food vacuoles which, in pathogenic strains, usually contain ingested erythrocytes (Smith et al. 1979; Martinez-Palomo 1982). (b) Cyst, this is a non-motile infective form (size 5-12 μm) which has a cell wall which is resistant to various environmental conditions and, depending on its maturity, contains one to four nuclei with the same morphology as the trophozoite nucleus. In the early stage of formation the cyst may contain a mass of glycogen and densely basophilic chromatoid bodies (Smith et al. 1979; Martinez-Palomo 1982).

Extensive ultrastructure studies of E. histolytica by electron microscopy have revealed that the parasite is devoid of mitochondria and a golgi complex, whereas the endoplasmic reticulum is poorly developed (Martinez-Palomo 1982; McLaughlin and Aley 1985). The

nucleus is bound by a double membrane and is perforated by pores. A number of helical bodies representing an array of ribonucleoprotein have been observed in the cytoplasm. Microfilament-like structures and actin have also been identified in amoebic trophozoites; microtubules, however, have not yet been found. The plasma membrane of E. histolytica is covered by a thin, irregular surface coat. The latter is of particular interest because it probably contains some of the amoebic antigens recognised as foreign by the host. In addition, the pathogenic action of the amoebae may depend upon direct contact with host cells, and the virulence of the amoebae may thus be related to the composition and properties of the surface coat components (McLaughlin and Aley 1985).

The life cycle of E. histolytica has been well characterised (Barker and Swales 1972). The patient becomes infected by ingesting mature cysts which are resistant to the acidic environment of the stomach and pass to the small intestine where they undergo excystation with the final production of eight trophozoites. The trophozoites then pass into the large intestine and after a period of feeding and multiplication by binary fission they form cysts. Some of the trophozoites, however, may become invasive causing ulceration of the intestinal wall. Mature cysts passed with the faeces are the infective form and can survive outside the host for weeks to months in a moist environment (Albach and Booden 1978). The most common symptoms of the disease are abdominal pain and prolonged diarrhoea associated with the loss of blood. Metronidazole has been recommended as the drug of choice for treatment of severe intestinal and hepatic amoebiasis (Neal 1983; Gutteridge 1986). Intestinal amoebiasis can also be treated with emetine or dehydroemetine.

The surface membrane biochemistry, carbohydrate metabolism, lipid metabolism, protein and nucleic acid metabolism of the

Entamoeba have been recently reviewed in detail (Reeves 1984; McLaughlin and Aley 1985).

1.7. The biology of Acanthamoeba

The genus Acanthamoeba includes small amoebae that are ordinarily free-living, but can be opportunistic pathogens of animals and humans (Griffin 1978). They are widely dispersed in fresh water and salt water as well as in soil. The life cycle of Acanthamoeba is relatively simple and involves two forms, the motile trophozoite feeding mostly on bacteria and the sessile cyst often able to resist adverse environmental conditions (Warhurst 1985). The trophozoite has a distinctive nucleus with a large central nucleolus surrounded by a clear zone which separates it from a very thin ring of peripheral nuclear chromatin. The trophozoite is usually covered with characteristic small spiny pseudopodia known as acanthopodia.

Ultrastructure studies of the trophozoite and the cyst of Acanthamoeba by electron microscopy have revealed the presence of mitochondria, rough endoplasmic reticulum, and a centrosphere which consists of a well developed golgi complex and a centriole-like structure from which numerous vesicles and microtubules radiate (Lasman 1982).

Human meningoencephalitis caused by Acanthamoeba species (namely A. culbertsoni and A. castellanii) is commonly a chronic disease which is usually characterised by focal granulomatosis lesions in the brain (Carter et al. 1981). Alternatively, Acanthamoeba may cause granulomatous lesions of the skin, lung, orbit, middle ear or gastric mucos; it may also cause corneal ulcers following direct implantation (Warhurst 1985). At present the only drug known to be

effective against Acanthamoeba in experimental animals is sulphadiazine, although this has not been successful in the treatment of humans with amoebic meningoencephalitis (Martinz 1981). Recently Ferrante et al. (1984) found amphotericin B to be effective against Acanthamoeba growth in vitro. Since Acanthamoeba contain sterols, it seems likely that the action of amphotericin B could be related to its binding to membrane sterols. (Raederstorff and Rohmer 1985).

There have been some studies on the biochemistry of Acanthamoeba species, including those on carbohydrate utilization (Prescott et al. 1973; Ward 1985b), amino acid requirements (Adam 1964), DNA content (Byers 1986) growth and oxygen consumption (Byers et al. 1969; Byers 1979).

1.8 The biology of Babesia

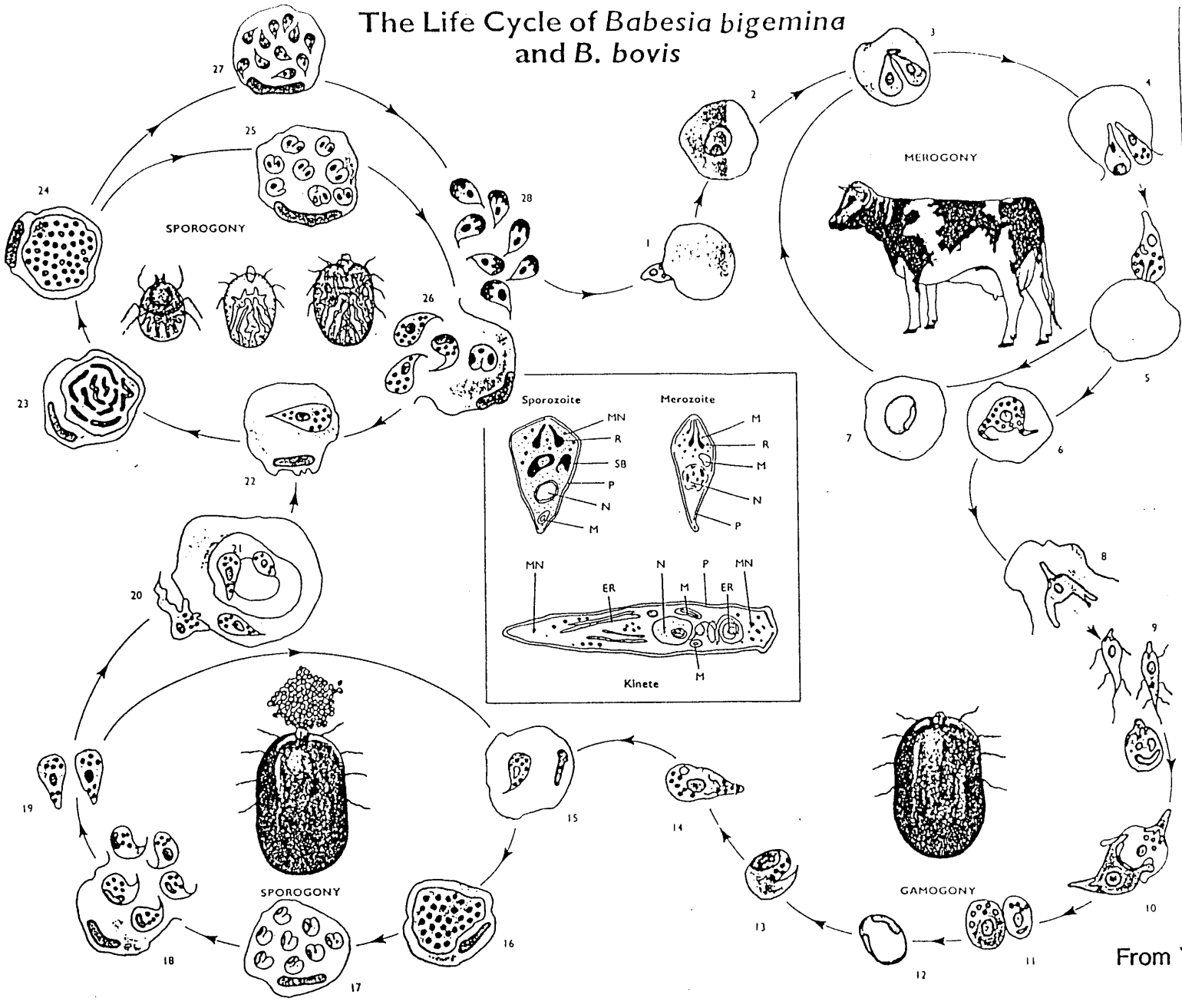
Members of the genus Babesia are protozoan parasites of cattle, dogs, rodents and other animals. Two species are known to infect man: Babesia bovis, a parasite of cattle found all over Europe and Russia, and B. microti, a parasite of rodents in North America and Europe (Ristic and Lewis 1977). The parasites grow intraerythrocytically as ring and rod-shaped bodies which develop to become pear-shaped (piroplasms), resembling malaria parasites except that they contain no pigment and the cytoplasm remains pale (Mahoney 1977). The presence of a complex organelle on the anterior extremity of Babesia, and Plasmodium, is the basis of the phylum, Apicomplexa. This organelle is visible only with the electron microscope, and for this reason its significance was formerly unknown (Young and Morzari 1986).

Important bovine Babesia species include B. bigemina, B. bovis, B. divergens and B. major. The disease caused by B. bovis is more

severe and more difficult to control than that caused by B. bigemina (Irvin 1986). Babesia bovis frequently causes a cerebral form of the infection characterised by formation of thrombi in the brain capillaries (Mahoney 1977). Such animals may develop clinical signs similar to rabies and usually die before any appreciable parasitemia is manifested in the peripheral blood (less than 2% infected erythrocytes). In general and from the economic point of view, infections caused by B. bovis are considered the most important of all bovine babesial infections. The most common symptoms of babesiosis include fever, jaundice, anemia and hemoglobinuria (Cox 1982). All Babesia species are transmitted by ticks (phylum, Arthropoda; class, Acarina; families Ixodidae and Argasidae). Babesia bovis is transmitted by Boophilus species and B. microti by Ixodes dammini. The reservoir mammalian hosts probably include deer mice and meadow voles (Ristic and Lewis 1977).

The life cycle of Babesia has been extensively studied and reviewed (see Mehlhorn and Schein 1984; Young and Morzaria 1986); I shall briefly describe only the main features (Fig. 4). The development in the mammalian host begins when sporozoites are injected into the bloodstream from the salivary gland of the tick and invade erythrocytes. After entry into the red blood cell, the sporozoite initially is situated in a parasitophorous vacuole. This subsequently disappears, however, such that the parasite lies free in the cytopasm of the erythrocyte. This is known as the trophozoite stage. The trophozoite rapidly undergoes asexual reproduction (merogony). This occurs in most species as binary fission leading to the formation of two merozoites inside an erythrocyte. In some species (B. equi and B. microti) four merozoites are often produced which may give rise to tetrad or a 'maltese-cross' arrangement. Some species may also form schizonts in lymphocytes which lead to

The Life Cycle of *Babesia bigemina* and *B. bovis*



1 Sporozoite, inoculated into the cow during tick feeding via the saliva, attaches to and penetrates erythrocytes.
2 The sporozoite becomes an intraerythrocytic trophozoite (feeding stage).
3 The trophozoite undergoes intraerythrocytic division (merogony) to form 2 daughter cells called merozoites.
4 Merozoites escape from the erythrocyte resulting in the destruction of the latter.
5 Free merozoites penetrate new erythrocyte and repeat merogony (7).
6 Some forms stop dividing, these differentiate to form gametocytes.
8 Female *Boophilus* feeds on infected cow and ingests infected erythrocytes. Only the gametocytes continue development in the gut lumen of the tick.
9 Gametes are formed from the Strahlenkörper stage and fuse to form a zygote (10-12).
13 The zygote differentiates to form an ookinete (14) which penetrates the gut epithelium to initiate sporogony (15) in the gut epithelium cells.
16 A multiple fission body is formed with many nuclei which differentiates to form sporokinetes (17) which are liberated and are motile (18).
19 The sporokinetes can initiate further sporogony in various tissues of the tick.
20 Some sporokinetes initiate the infection of the ova before the cuticle is formed and remain dormant (21) until the F_1 ticks start feeding.
22–26 Sporokinetes initiate sporogony in tissues of feeding F_1 ticks resulting in numerous sporokinetes.
27 Sporokinetes invade the salivary glands and initiate sporogony during tick feeding which results in many sporozoites being formed (28). These are released into the salivary ducts of the tick during feeding.

Ultrastructure of 3 invasive stages of *B. bigemina* (shown in the centre of diagram).

R	–	rhoptries
MN	–	micronemes
P	–	pellicle
N	–	nucleus
ER	–	endoplasmic reticulum
SB	–	spherical body
M	–	mitochondrion

Kinetes
These have extensive micronemes and well developed polar-ring. Rhoptries may be present. The pellicle consists of 3 unit membranes and the cytoplasm has several mitochondria.

Sporozoites
They have several prominent rhoptries and several micronemes. The pellicle consists of three membranes, the cytoplasm has a mitochondrion and spherical bodies.

Merozoites
Their morphology is similar to sporozoites. The inner membrane of the pellicle is discontinuous.

Fig. 4.

From Young and Morzaria 1986

formation of several merozoites. The merozoites are released from the host cell and they invade new erythrocytes and repeat merogony. Some of the merozoites formed inside an erythrocyte do not develop further until they are taken up by ticks during feeding. These intraerythrocytic stages are released from their erythrocyte inside the intestine and differentiate to form ray bodies (also called spiky-rayed or Strahlenkorper stages). The uninucleate ray-bodies are spherical, polymorphic or pyrimidal with a diameter of about 4-7 μm. They are bounded by a single cell membrane, have a thorn-like structure and several flagella-like protrusions. The ray-bodies are considered to be the gametes. After 4-6 days, the gametes fuse to form a zygote which differentiates to form a uninucleate elongated motile kinete (7-8 μm). The kinete may then enter epithelial cells of the gut where sporogony takes place. The intracellular kinete is transformed into a polymorphic stage which loses all characteristics of the motile invasive stage and then subdivide into several single-membrane bound, uninucleate cytomers (sporokinetes) that are separated within the host cell cytoplasm. Following the rupture of these cells the released sporokinetes can enter various tissues of the tick (hemocytes, ovaries, peritracheal tissue and muscle) where they initiate further sporogony. In most babesial species, the sporokinetes enter the cells of the salivary gland where many small pyriform infective bodies (sporozites) are formed and subsequently inoculated into a mammalian host.

There have been rather few drugs developed for the chemotherapy of Babesia infections, it has been suggested that the parasite is relatively difficult to treat because it lives in different cell types (Irvin 1986). The currently accepted drugs for the treatment

of babesiasis are diamidine derivatives such as imidocarb and diminazene (Irvin 1986).

Although relatively little is known about the biochemistry of Babesia species, there have been a few studies in recent years. For example, the carbohydrate metabolism (Momen 1979; Mackenzie et al. 1984) and proteinases (Wright et al. 1981; Commins et al. 1985; Coombs 1986) of the erythrocytic forms have been examined in some detail.

1.9. The aims of the project

A relatively large number of parasitic protozoa are of considerable economic, medical and veterinary importance to twentieth century man. For instance, trypanosomiasis constitutes a serious threat to millions of people in many countries of Africa (sleeping sickness) and South America (Chagas disease). The leishmaniases comprise a group of diseases which are widely distributed in tropical and subtropical areas and range in severity from self-healing skin lesions to severe mucocutaneous involvement or visceral infections which are almost always fatal if not treated. Amoebiasis continues to be a serious problem world wide and, by a conservative estimates, approximately 10% of the worlds population are infected with the causative agent Entamoeba histolytica. Malaria is a major killer in the tropics whereas babesiosis is a widespread disease especially of cattle.

These parasites present serious problems in part because of the lack of a reliable, and nontoxic chemotherapeutic compounds. Resistance to the drugs currently available adds to the difficulties. Efforts to develop more effective therapies have often failed due to

our lack of knowledge of the biochemistry of the causative organisms. It is clear that there is an urgent need to increase our understanding of the biochemistry of these pathogens so that new drugs, vaccines and other types of control agents can be developed. It is for these reasons that much interest in recent years in studying the biochemistry of parasitic protozoa.

An area of metabolism that has attracted particular interest has been purine and pyrimidine metabolism as this seems to be an area against which drugs can act selectively. For instance, pyrimethamine, sulphonamides, fluoropyrimidines and allopurinol affect aspects of this area of metabolism. Many parasitic protozoa have been found to contain numerous enzymes, through the action of which they scavenge nucleic acid precursors from the environment and metabolise them, according to their immediate needs, for nucleic acid synthesis. Interestingly, the enzymes differ in detail from their mammalian counterparts. Consequently, there has been an increasing interest in the potential of nucleoside analogues (such as allopurinol riboside and formycin B) in the treatment of parasitic diseases (the leishmaniases and trypanosomiases in these cases). These results emphasise the practical importance of knowledge of the enzymatic reactions of parasites which may be the targets for chemotherapeutic attack. The main objective of the present work has been to present a detailed description of the anabolic and catabolic pathways which may function when nucleic acid precursors (or their analogues) are made available to a range of parasitic protozoa. Special emphasis has been placed on studying the amastigote and promastigote forms of L. m. mexicana in order to evaluate enzymes of the amastigote that provide useful targets at which to aim chemotherapeutic attack against this pathogen.

At the start of my project there was little or no information on this aspect of metabolism of many important parasitic protozoa. It has been my aim to review purine and pyrimidine metabolism in these organisms and so provide the basic information that is required before the studies aimed at drug design and development can proceed.

1.10. References

Adam, K.M. 1964. The amino acid requirements of Acanthamoeba sp. J. Protozool. 11, 98-100.

Albach, R.A. and Booden, T. 1978. Amoebae. In: Parasitic Protozoa, Krier, J.R. ed., Volume 2, pp. 455-506. Academic Press, New York.

Alexander, J. 1975, Effect of the antiphagocytic agent cytochalasin B on macrophage invasion by Leishmania mexicana promastigotes and Trypanosome cruzi epimastigotes. J. Protozool. 22, 237-240.

Alexander, J. and Vickerman, K. 1975. Fusion of host secondary lysosomes with the parasitophorous vacuoles of Leishmania mexicana infected macrophages. J. Protozool. 22, 502-508.

Alving, C.R. 1986. Liposomes as drug carriers in leishmaniasis and malaria. Parasitol. Today, 2, 101-107.

Bacchi, C.J. 1981. Content, synthesis and function of polyamines in trypanosomatids - relationship to chemotherapy. J. Protozool. 28, 20-27.

Bachrach, U., Brem, S., Wertman, S.B., Schnur, L.F. and Greenblatt, C.L. 1979a. Leishmania species: cellular levels and synthesis of polyamines during growth cycles. Exp. Parasitol. 48, 457-463.

Bachrach, U., Brem, S., Wertman, S.B., Schnur, L.F. and Greenblatt, C.L. 1979b. Leishmania species: Effect of inhibitors on growth and on polyamine and macromolecular syntheses. Exp. Parasitol. 48, 464-470.

Bachrach, U., Schnur, L.F., El-On, J., Greenblatt, C.L., Pearlman, E., Robert-Gero, M., and Lederer. E. 1980. Inhibitory activity of Sinefungin and SIBA on the growth of promastigotes and amastigotes of different species of Leishmania. FEBS Lett. 121, 287-291.

Barker, D.C. and Swales, L.S. 1972. Characteristics of ribosomes during differentiation from trophozoite to cyst in axenic Entamoeba sp. Cell Differ. 1, 297-306.

Barker, D.C., Butcher, J., Gibson, L.J. and Williams, R.H. 1985. Characterization of Leishmania sp. by DNA Hybridization Probes. UNDP/WORLD BANK/WHO - Special Programme for Research and Training in Tropical Diseases, Switzerland.

Benoliel, A.M., Capo, C., Bongrand, P., Ryter, A. and Depieds, R. 1980. Non-specific binding by macrophages: Existence of different adhesive mechanisms and modulation by metabolic inhibitors. Immunology. 41, 547-560.

Berman, J.D. 1985. Experimental chemotherapy of leishmaniasis. In: Leishmaniasis, Chang, K-P. and Bray, R.S., eds., pp. 112-138. Elsevier Science Publications, Amsterdam, New York, Oxford.

Berman, J.D. and Lee, L.S. 1983. Activity of 8-aminoquinolines against Leishmania tropica within human macrophages in vitro. Am. J. Trop. Med. Hyg. 32, 753-759.

Berman, J.D., Fioretti, T.B. and Dwyer, D.M. 1981. In vivo and in vitro localisation of Leishmania within macrophage phagolysosomes: use of colloidal gold as a lysosomal label. J. Protozool. 28, 239-242.

Berman, J.D., Holz, G.G. and Beach, D.H. 1984. Effects of ketoconazole on growth and sterol biosynthesis of Leishmania mexicana promastigotes in culture. Mol. Biochem. Parasitol. 12, 1-13.

Blackwell, J.M., Ezekowitz, R.A., Channon, J.Y., Sim, R.B. and Gorden, S. 1985. Macrophage complement and lectin-like receptors bind Leishmania in the absence of serum. J. Exp. Med. 162, 324-333.

Blackwell, J.M., Pratt, D.M. and Shaw, J. 1986. Molecular biology of Leishmania. Parasitol. Today, 2, 45-53.

Borgers, M. 1980. Mechanism of action of antifungal drugs, with special reference to the imidazole derivatives. Rev. Infect. Dis. 2, 520-534.

Borst, P., Van der Ploeg, M., Van Hoek, J., Tas, J. and James, J. 1982. On the DNA content and ploidy of trypanosomes. Mol. Biochem. Parasitol. 6, 12-23.

Bray, R.S. 1983. Leishmania mexicana mexicana: Attachment and uptake of promastigotes to and by macrophages in vitro. J. Protozool. 30, 314-322.

Brazil, R.P. 1984. In vivo fusions of lysosomes with parasitophorous vacuoles of Leishmania-infected macrophages. Ann. Trop. Med. Parasitol. 78, 87-91.

Brun, R. and Krassner, S.M. 1976. Quantitative ultrastructural investigations of mitochondrial development in Leishmania donovani during transformation. J. Protozool. 23, 493-497.

Byers, T.J. 1979. Growth, reproduction and differentiation in Acanthamoeba. Int. Rev. Cytol. 61, 283-338.

Byers, T.J. 1986. Molecular biology of DNA in Acanthamoeba, Amoeba, Entamoeba and Naegleria. Int. Rev. Cytol. 99, 31-341.

Byers, T.J., Rudick, V.L. and Rudick, M.J. 1969. Cell size, macromolecule composition, nuclear number, oxygen consumption and cyst formation during two growth phases in unagitated cultures of Acanthamoeba castellanii. J. Protozool. 16, 698-699

Carter, R.F., Cullity, G., Ojeda V., Silberstein, P. and Willaert, E. 1981. A fatal case of meningoencephalitis due to a free living amoeba of uncertain identity - probably Acanthamoeba sp. Pathology, 13, 51-68.

Cazzulo, J.J., Cazzulo, B.M., Engle, J.C. and Cannata, J.J. 1985. End products and enzyme levels of aerobic glucose fermentation in trypanosomatids. Mol. Biochem. Parasitol. 16, 329-343.

Chance, M.L. 1981. The six diseases of W.H.O. leishmaniasis. Brit. Med. J. 283, 1245-1247.

Chance, M.L. 1985. The biochemical and immunological taxonomy of Leishmania. In: Leishmaniasis, Chang, K-P. and Bray, R.S., eds. pp. 93-110. Elsevier Science Publications, Amsterdam, New York, Oxford.

Chance, M.L. and Walton, B.C. 1982. Biochemical Characterization of Leishmania. UNDP/WORLD BANK/ WHO - Special Programme for Research and Training in Tropical Diseases, Switzerland.

Chang, K-P. 1979. Leishmania donovani: promastigote-macrophage surface interaction in vitro. Exp. Parasitol. 48, 175-189.

Chang, K-P. 1980. Endocytosis of Leishmania-infected macrophages. Fluorometry of pinocytic rate, lysosome-phagosome fusion and intralysosomal pH. In "The Host-Invader Interplay", (Van den Bossche, H. ed.). pp. 231-234. Elsevier, Amsterdam.

Chang, K-P. 1981. Leishmania donovani - macrophage binding mediated by surface glycoproteins/antigens: Characterisation in vitro by a radioisotopic assay. Mol. Biochem. Parasitol. 4, 67-76.

Chang, K-P. 1983. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int. Rev. Cytol. (Suppl. 14), 267-302.

Chang, K-P. and Dwyer, D.M. 1976. Multiplication of human parasite (Leishmania donovani) in phagolysosomes of hamster macrophages in vitro. Science. 193, 678-680.

Chang, K-P. and Fong, D. 1983. Cell biology of host-parasite membrane interactions in leishmaniasis. In "Cytopathology of parasite disease". Ciba Foundation Symposium. 99, 113-137.

Chang, K-P. and Chang C.S. 1986. Monoclonal antibody affinity purification of a Leishmania membrane glycoprotein and its inhibition of Leishmania - macrophage binding. Proc. Natl. Acad. Sci. USA, 83, 100-104.

Chang, K-P, Fong, D. and Bray, R.S. 1985. Biology of Leishmania and leishmaniasis. In: Leishmaniasis, Chang, K-P. and Bray, R.S., eds. pp. 1-30. Elsevier Science Publications, Amsterdam, New York, Oxford.

Channon, J.Y. and Blackwell, J.M. 1985a. A study of the sensitivity of Leishmania donovani promastigotes and amastigotes to hydrogen peroxide. I. Differences in sensitivity correlate with parasite-mediated removal of hydrogen peroxide. Parasitol. 91, 197-206.

Channon, J.Y. and Blackwell, J.M. 1985b. A study of the sensitivity of Leishmania donovani promastigotes and amastigotes to hydrogen peroxide. II. Possible mechanisms involved in protective H_2O_2 scavenging. Parasitol. 1, 207-217.

Commins, M.A., Goodger, B.V. and Wright, I.G. 1985. Proteinases in the lysate of bovine erythrocytes infected with Babesia bovis: initial vaccination studies. Int. J. Parasitol. 15, 491-495.

Coombs, G.H. 1986. Intermediary metabolism in parasitic protozoa. In; Parasitology-Quo Vadit? Proceeding of the 6th International Congress of Parasitology (Howell, M.J. ed.). pp. 97-104. Australian Academy of Science, Canberra.

Coombs, G.H. and Sanderson, B.E. 1985. Amine production by Leishmania mexicana. Ann. Trop. Med. Parasitol. 79, 409-415.

Coombs, G.H., Hart, D.T. and Capaldo, J. 1983. Leishmania mexicana: drug sensitivities of promastigotes and transforming amastigotes. J. Antimicrob. Chemother. 11, 151-162.

Coombs, G.H., Hassan, H.F., Lockwood, B.C., Mallinson, D.J., North, M.J., Alunda, J.M. and Tetley, L. 1986a. Phosphomonoesterases and proteinases of leishmanias. In: Host-Parasite Molecular Recognition and Interaction in Protozoal Infections, Chang, K-P. ed., in press.

Coombs, G.H., Tetley, L., Moss, V.A. and Vickerman, K. 1986b. Three dimensional structure of the Leishmania amastigote as revealed by computer-aided reconstruction from serial sections. Parasitol. 22, 13-23.

Cox, F.E.G. 1982. Modern Parasitology. Blackwell Scientific Publications, Oxford, London.

Dwyer, D.M. 1986. The role of surface membrane enzymes and transporters in the survival of Leishmania. In: Host-Parasite Molecular Recognition and Interaction in Protozoal Infections, Chang, K-P., ed., in press.

Dwyer, D.M. and Gottlieb, M. 1985. The biochemistry of Leishmania surface membrane. In: Leishmaniasis, Chang, K-P. and Bray, R.S. eds., pp. 32-47, Elsevier Science Publications, Amsterdam, New York, Oxford.

El-On, J., Bradley, D.J. and Freeman, J.C. 1980. Leishmania donovani: Action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. Exp. Parasitol. 49, 167-174.

Englund, P.T. 1981. Kinetoplast DNA. In "Biochemistry and Physiology of Protozoa" (Levandowsky, M. and Hunter, S.H. eds.). 2nd. ed., Volume. 4, pp. 334-383. Academic Press, New York.

Etges, R., Bouvier, R. and Bordier, C. 1986. The major surface protein of Leishmania promastigotes is a proteas. J. Biol. Chem. 261, 9098-9101.

Ferrante, A., Kelly, B. and Thong, Y. 1984. In vitro sensitivity of virulent Acanthamoeba culbertsoni to a variety of drugs and antibiotics. Int. J. Parasitol. 14, 53-56.

Ghafoor, M.Y. and Coombs, G.H. 1980. Superoxide dismutase, peroxidase and glutathione metabolism in Leishmania mexicana amastigotes and promastigotes. Parasitol. 81, xxviii-xxxix.

Goad, L.J., Holz, G.G. and Beach, D.H. 1984. Sterols of Leishmania species. Implications for biosynthesis. Mol. Biochem. Parasitol. 10, 161-170.

Goad, L.J., Holz, G.G. and Beach D.H. 1985. Sterols of ketoconazole-inhibited Leishmania mexicana mexicana promastigotes. Mol. Biochem. Parasitol. 15, 257-279.

Greenblatt, C.L. 1985. Vaccination for leishmaniasis. In: Leishmaniasis, Chang, K-P. and Bray, R.S. eds., pp. 163-176. Elsevier Science Publications, Amsterdam, New York, Oxford.

Griffin, J. 1978. The pathogenic free living Amoebae. In: Parasitic Protozoa, Krier, J.P., ed., Volume 2, pp. 508-549. Academic Press, New York.

Gutteridge, W.E. 1986. New antiprotozoal agents. In: Parasitology-Quo Vadit? Proceeding of the 6th International Congress of Parasitology (Howell, M.J. ed.). pp. 121-129. Australian Acadamy of Science, Canberra.

Handman, E. and Greenblatt, C.L. 1977. Promotion of leishmanial infections in non-permissive host macrophages by conditioned medium. Z. Parasitenkd 53, 143-147.

Handman, E. and Goding, J.W. 1985. The Leishmania receptor for macrophages is a lipid-containing glycoconjugate. EMBO J. 4, 329-336.

Hart, D.T. and Coombs, G.H. 1982. Leishmania mexicana: Energy metabolism of amastigotes and promastigotes. Exp. Parasitol. 54, 397-409.

Hart, D.T. and Opperdoes, F.R. 1984. The occurrence of glycosomes (microbodies) in the promastigote stage of four major Leishmania species. Mol. Biochem. Parasitol. 13, 159-172.

Hart, D.T., Vickerman, K. and Coombs, G.H. 1981a. Transformation in vitro of Leishmania mexicana amastigotes to promastigotes: Nutritional requirements and effect of drugs. Parasitol. 83, 529-541.

Hart, D.T., Vickerman, K. and Coombs, G.H. 1981b. Respiration of Leishmania mexicana amastigotes and promastigotes. Mol. Biochem. Parasitol. 4, 39-51

Hernandez, A.G. 1983. Leishmanial excreted factors and their possible biological role. In: Cytopathology of parasite disease. Ciba Foundation Symposium. 99, 138-151.

Hoare, C.A. and Wallace, F.G. 1966. Developmental stages of trypanosomatid flagellates: a new terminology. Nature (London), 212, 1385-1386.

Holz, G.G. 1985. Lipids of leishmanias. In: Leishmaniasis, Chang, K-P. and Bray, R.S., eds., pp. 80-92. Elsevier Science Publications, Amsterdam, New York, Oxford.

Hommel, M. 1978. The genus Leishmania: biology of the parasite and clinical aspects. Bull. Pasteur Inst. 75, 5-102.

Imamura, T., Toyoshima, S. and Osawa, T. 1984. Lectin-like molecules on the murine macrophage cell surface. Biochim. Biophys. Acta 805, 235-244.

Irvin, A.D. 1986. Control of tick-born diseases. In: Parasitology-Quo Vadit? Proceeding of the 6th International Congress of Parasitology (Howell, M.J. ed.) pp. 649-657. Australian Academy of Science, Canberra.

Jones, T.C. and Hirsch, J.G. 1972. The reaction between Toxoplasma and mammalian cells. II. Absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. Exp. Med. 136, 1173-1194.

Kidder G.W. and Dutta, B.M. 1958. The growth and nutrition of Crithidia fasciculata. J. Gen. Microbiol. 18, 621-638.

Killick-Kendrick, R. 1979. Biology of Leishmania in phlebotomine sandflies. In "Biology of the Kinetoplastida" (Lumsden, W.H.R. and Evans, D.A. eds.). Volume 2, pp. 395-460. Academic Press, New York.

Klebanoff, S.J. 1982. Oxygen dependent cytotoxic mechanism of phagocytes. In: Advances in host defense mechanisms, Gallin, J.I. and Fauci, A.S. (eds.). Volume 1, pp. 111-162, Raven Press, New York.

Klebanoff, S.J., Locksley, R.M., Jong, E.C. and Rosen, H. 1983. Oxidative response of phagocytes to parasite invasion. In: "Cytopathology of Parasitic Disease". Ciba Foundation Symposium. 99, 92-112.

Krassner, S.M. and Flory, B. 1972. Proline metabolism in Leishmania donovani promastigotes. J. Protozool. 19, 682-685.

Kress, Y., Tanowitz, B., Bloom, B. and Wittner M. 1977. Trypanosoma cruzi: infection of normal and activated mouse macrophages. Exp. Parasitol. 41, 385-396.

Lainson, R. and Shaw, J.J. 1978. Epidemiology and ecology of leishmaniasis in Latin-America. Nature (London), 273, 595-600.

Lasman, M. 1982. The fine structure of Acanthamoeba astronyxis, with special emphasis on encystment. J. Protozool. 29, 458-464.

Levine, N.D., Corliss, J.O., Cox, F.E., Deroux, G., Grain, J., Honigberg, B.M., Leedale, G.F., Loeblich, A., Lom, J., Lynn, D., Merinfeld, G., Page, F.C., Poljansky, G., Sprague, V., Varra, J. and Wallace, F.G. 1980. A newly revised classification of the protozoa. J. Protozool 27, 37-58.

Mackenzie, N.E., Johnson, J., Burton, G., Wagner, G.G. and Scott, A.I. 1984. ^{13}C NMR studies of glycolysis in intra- and extraerythrocytic Babesia microti. Mol. Biochem. Parasitol. 13, 13-20.

Mahmoud, A.A., and Warren, K.S. 1977. Algorithms in the diagnosis and treatment of exotic diseases. XXIV. Leishmaniasis. J. Infect. Dis. 136, 160-163.

Mahoney, D.F. 1977. Babesia of domestic animals. In: Parasitic Protozoa, Krier, J.P., ed., Volume 4, pp. 1-52. Academic Press, New York, London.

Mallinson, D.J. and Coombs, G.H. 1986. Molecular characterization of the metacyclic forms of Leishmania. IRCS Med. Sci. 14, 557-558.

Mansour, T.E. and Bueding, E. 1954. The actions of antimonials on glycolytic enzymes of Schistosoma mansoni. Brit. J. Pharmacol. 9, 459-462.

Marinkelle, C.J. 1980. The control of leishmaniases. Bull. WHO. 58, 807-818.

Marsden, P.D. 1984. Selective primary health care: strategies for control of disease in the developing world. XIV. Leishmaniasis. Rev. Infect. Dis. 6, 736-744.

Martinez, A.J. 1981. Free living amebas in human infections. In: Current Concepts in a Changing Science, Ball, W. and Iralu, V., ed., pp. 1-57, Philadelphia, USA.

Martinez-Palomo, A. 1982. The Biology of Entamoeba histolytica. Research Studies Press, John Wiley, Sussex, UK.

Mauel, J. and Behin, R. 1981. Immunology of Leishmaniasis. In "Biochemistry and Physiology of Protozoa". (Levandowsky, M. and Hunter, S.H. eds.). 2nd ed. Volume 4, pp. 385-429. Academic Press, New York.

McGhee, R.B. and Cosgrove, W.B. 1980. Biology and physiology of the lower Trypanosomatidae. Microb. Rev. 44, 140-173.

McLaughlin, J. and Aley, S. 1985. The biochemistry and functional morphology of the Entamoeba. J. Protozool. 32, 221-240.

Mehlhorn, H. and Schein, E. 1984. The piroplasms: life cycle and sexual stages. Adv. Parasitol. 23, 37-103.

Merino, F., Ajjam, E., Hernandez, A., Dawidowicz, K. and Merino, E.J. 1977. In vitro infection of murine macrophage by Leishmania braziliensis. Mechanism of penetration. Int. Arch. Allergy. Appl. Immunol. 55, 487-495.

Meshnick, S.R. and Eaton, J.W. 1981. Leishmanial superoxide dismutase: a possible target for chemotherapy. Biochem. Biophys. Res. Commun. 102, 970-976.

Misset, O., Bos, O.J. and Opperdoes, F.R. 1986. Glycolytic enzymes of Trypanosoma brucei. Eur. J. Biochem. 157, 441-453.

Molyneux, D.H. and Ashford, R.W. 1983. The biology of Trypanosoma and Leishmania, parasites of man and domestic animals. Taylor and Francis Ltd., London.

Momen, H. 1979. Biochemistry of intraerythrocytic parasites. II. Comparative studies in carbohydrate metabolism. Ann. Trop. Med. Parasitol. 73, 117-121.

Morrow, C.D., Flory, B. and Krassner, S.M. 1980. Polyamines in the hemoflagellate, Leishmania donovani : evidence for spermine in the amastigote stage. Comp. Biochem. Physiol. 66B, 307-311.

Mottram, J.C. and Coombs, G.H. 1985. Leishmania mexicana mexicana: Enzyme activities of amastigotes and promastigotes and their inhibition by antimonials and arsenicals. Exp. Parasitol. 59, 11-160.

Mukkada, A.J. 1985. Energy metabolism in Leishmania. In: Leishmaniasis, Chang, K-P. and Bray, R.S. eds., pp. 49-64, Elsevier Science Publications, Amsterdam, New York, Oxford.

Mundim, M. and Roitman, I. 1977. Extra nutritional requirements of artificially aposymbiotic Crithidia deanei. J. Protozool. 24, 329-331.

Murray, H.W. 1982. Cell-mediated immune response in experimental visceral leishmaniasis. II: Oxygen-dependent killing of intracellular Leishmania donovani amastigotes. J. Immunol. 129, 351-357.

Murray, H.W. and Cartelli, D.M. 1983. Killing of intracellular Leishmania donovani by human mononuclear phagocytes- evidence of oxygen dependent and oxygen independant leishmanicidal activity. J. Clin. Invest. 72, 32-44.

Neal, R.A. 1983. Experimental amoebiasis and the development of anti-amoebic compounds. Parasitol. 86, 175-194.

Newton, B.A. 1976. Biochemical approaches to the taxonomy of kintoplastid flagellates. In: Biology of the Kinetoplastida, Lumsden, W.H. and Evans, D.A., eds., Volume, 1 pp. 405-434, Academic Press, London.

North, M.J., Coombs, G.H. and Barry, J.D. 1983. A comparative study of the proteolytic enzymes of Trypanosoma brucei, T. equiperdum, T. vivax, Leishmania tarentolae and Crithidia fasciculata. Mol. Biochem. Parasitol. 9, 161-180.

Opperdoes, F.R. 1983 Glycolysis as a target for the development of new trypanocidal drugs. In "Mechanism of drug action" (Singer, T.P., Mansour, T.E. and Undarza, R.N. eds). pp. 121-132. Academic Press, New York.

Opperdoes, F.R., Baudhuin, P., Coppens, I., De Roe, C., Edwards, S.W., Weijers, P.J. and Misset, O. 1984. Purification, morphometric analysis, and characterization of the glycosomes (microbodies) of the protozoan hemoflagellate Trypanosoma brucei. J. Cell. Biol. 98, 1178-1184.

Paolantonacci, P., Lawrence, F. and Gero, M.R. 1985. Differential effect of sinefungin and its analogs on the multiplication of three Leishmania species. Antimicrob. agents Chemother. 28, 528-531.

Pearson, R.D., Wheeler, D.A., Harrison, L.H. and Kay, H. 1983. The immunology of leishmaniasis. Rev. Infect. Dis. 5, 907-927.

Pearson, R.D., Manian, A.A., Hall, D., Harcus, J. and Hewlett, E.L. 1984. Antileishmanial activity of chlorpromazine. Antimicrob. agents Chemother. 25, 571-574.

Penketh, P.G. and Kennedy, W.P. 1986. Hydrogen peroxide metabolism in a Leishmania and Trypanosoma species. In: Parasitology - Quo Vadit? Handbook (Howell, M.J. ed.), pp. 203, Sixth International Congress of Parasitology, Australian Academy of Science, Camberra.

Peters, W., Trotter, E.R. and Robinson, B.L. 1980. The experimental chemotherapy of leishmaniasis, VII. Drug responses of L. major and L. amazonensis, with an analysis of promising chemical leads to new antileishmanial agents. Ann. Trop. Med. Parasitol. 74, 321-335.

Prescott, L., Hoyme, H., Crockett, D. and Hui, E. 1973. Carbohydrate metabolism in Acanthamoeba castellanii. 1. The activity of key enzymes and ^{14}C-glucose metabolism. Can. J. Microbiol. 19, 1131-1136.

Pupkis, M.P., Tetley, L. and Coombs, G.H. 1986. Leishmania mexicana: amastigote hydrolases in unusual lysosomes. Exp. Parasitol. 62, 29-39.

Rabinovitch, M., Zilberfarb, V. and Ramazeilles, C. 1986. Destruction of Leishmania mexicana amazonensis amastigotes within macrophages by lysosomotropic amino acid esters. J. Exp. Med. 163, 520-535.

Raederstorff, D. and Rohmer, M. 1985. Sterol biosynthesis de novo via cycloartenol by the soil amoeba Acanthamoeba polyphaga. Biochem. J. 231, 609-615.

Reeves, R.E. 1984. Metabolism of Entamoeba histolytica. Schaudinn, 1903. Adv. Parasitol. 23, 106-142.

Remaley, A.T., Kuhns, D.B., Basford, R.E., Glew, R.H. and Kaplan, S.S. 1984. Leishmanial phosphatase blocks neutrophil O_2 production. J. Biol. Chem. 259, 11173-11175.

Ristic, M. and Lewis, G.E. 1977. Babesia in man and wild and laboratory-adapted mammals. In: Parasitic Protozoa, Krier, J.P., ed., volume 4, pp. 53-76, Academic Press, New York.

Roitman, I. and Camargo, E.P. 1985. Endosymbionts in Trypanosomatidae. Parasitol. Today, 1, 143-144.

Roitman, I., Angluster, J. and Fiorini, J.E. 1985. Nutrition of the trypanosomatids Crithidia acanthocephali and C. harmosa. Ribose and adenosine: substrates for C. acanthocephali. J. Protozool. 32, 490-492.

Russell, D.G. and Wilhelm, H. 1986. The involvement of the major surface glycoprotein (gp63) of Leishmania promastigotes in attachment to macrophages. J. Immunol. 136, 2613-2620.

Sacks, D.L. and Perkins, P.V. 1984. Identification of an infective stage of Leishmania promastigotes. Science, 223, 1417-1419.

Saha, A.K., Mukherjee, T. and Bhaduri, A. 1986. Mechanism of action of amphotericin B on Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 19, 195-200.

Simpson, L. 1968. The leishmania-leptomonad transformation of Leishmania donovani: Nutritional requirements, respiration changes and antigenic changes. J. Protozool. 15, 201-207.

Simpson, L. 1986. Kinetoplast DNA in trypanosomid flagellates. Int. Rev. Cytol. 99, 120-178.

Smith, J.W., McQuay, R.M., Ash, L.R., Melvin, D.M., Orihel, T.C. and Thompson, J.H. 1979. Diagnostic Medical Parasitology: Intestinal Protozoa. American Society of Clinical Pathologists, Education Products Division, United States.

Stuart, K. 1983. Kinetoplast DNA, mitochondrial DNA with a difference. Mol. Biochem. Parasitol. 9, 93-104.

Tait, A. 1980. Evidence for diploidy and mating in trypanosomes. Nature (London), 287. 536-538.

Tetley, L., Coombs, G.H. and Vickerman, K. 1986. The surface membrane of Leishmania mexicana mexicana: comparison of amastigote and promastigote using freeze-fracture cytochemistry. Z. Parasitenkd. 72, 281-292.

Vickerman, K. 1974. The ultrastructure of pathogenic flagellates. In "Trypanosomiasis and Leishmaniasis with special reference to Chagas' disease". Ciba Foundation Symposium 20 (new series).

Vickerman, K. 1976. The diversity of the kinetoplastid flagellates. In "Biology of the Kinetoplastida". (Lumsden, W.H.R. and Evans, D.A. eds.) Volume 1 pp. 1-34. Academic Press, New York.

Vickerman, K. 1980. Protozoal replication: Effects on the host cell. In "The molecular basis of microbial pathogenicity". (Smith, H., Skehel, J.J. and Turner, M.J. eds.) Life Science Report 16, pp 247-260. Verlag Chemis.

Vickerman, K. 1985. Developmental cycles and biology of pathogenic trypanosomes. Brit. Med. Bull. 41, 105-114.

Vickerman, K. 1986. Clandestine sex in trypanosomes. Nature (London), 322, 113-114.

Vickerman, K. and Preston, T.M. 1976. Comparative cell biology of the kinetoplastid flagellates. In "Biology of the Kinetoplastida". (Lumsden, W.H.R. and Evans, D.A. eds.). Volume, 1, pp. 35-130. Academic Press, New York.

Von Brand, T. 1979. Biochemistry and Physiology of Endoparasites. Elsevier Biochemical Press, Amsterdam, New York, Oxford.

Wagner, K.P. and Krassner, S.M. 1976. Leishmania tarentolae; proline metabolism in promastigotes. Exp. Parasitol. 39, 186-194.

Wallace, F.G. 1966. The trypanosomatid parasites of insects and arachnids. Exp. Parasitol. 18, 124-193.

Wallace, F.G. 1970. Life cycles of trypanosomatid parasites of insects with special reference to Herpetomonas muscarum. J. Parasitol. 56, 358-359.

Wallace, F.G. 1979. Biology of the Kinetoplastida of arthropods. In: Biology of the Kinetoplastida, Lumsden, W.H.R. and Evans, D.A. eds., Volume 2, 213-240. Academic Press, New York.

Wallace, F.G., Wagner, M. and Rogers, W.E. 1973. Varying kinetoplast ultrastructure in two subspecies of Herpetomonas muscarum. J. Protozool. 20, 218-222.

Ward, R.D. 1985a. Vector biology and control. In: Leishmaniasis, Chang, K-P. and Bray, R.S. eds., pp. 199-212, Elsevier Science Publications, Amsterdam, New York, Oxford.

Ward, A.P. 1985b. Genetic encodement of Acanthamoeba lactic dehydrogenase. J. Protozool. 32, 357-359.

Warhurst, D.C. 1985. Pathogenic free-living amoebae. Parasitol. Today, 1, 24-28.

Wassef, M.K., Fioretti, T.B. and Dwyer, D.M. 1985. Lipid analyses of isolated surface membranes of Leishmania donovani promastigotes. Lipids, 20, 108-115.

Williams, P. and Coelho, M. 1978. Taxonomy and transmission of Leishmania. Adv. Parasitol. 16, 1-42.

World Health Organisation 1980. UNDP training seminar on epidemiological methods for the leishmaniases, USSR, 28 May - 19 June. Report. Document TDR/LEISH-SEM/80.3.

World Health Organization 1981. Intestinal protozoan and helminthic infections. WHO Technical Report Series No. 666, pp. 29-45.

World Health Organization 1984. The Leishmaniasis. WHO Technical Report Series No. 701, Switzerland.

Wright, I.G., Goodger, B.V. and Mahoney D.F. 1981. Virulent and avirulent strains of Babesia bovis; the relationship between parasite protease content and pathophysiological effect of the strain, J. Protozool. 28, 118-120.

Young, A.S. and Morzaria, S.P. 1986. Biology of Babesia. Parasitol. Today, 2, 211-219.

Zenian, A. 1981 Leishmania tropica: biochemical aspects of promastigotes attachment to macrophages in vitro. Exp. Parasitol. 51, 175-187.

Zenian, A., Rowles, P. and Gingell, O. 1979. Scanning electron-microscopic study of the uptake of Leishmania parasites by macrophages. J. Cell. Sci. 39, 187-199.

Zilberstein, D. and Dwyer, D.M. 1984. Antidepressants cause lethal disruption of membrane function in the human protozoan parasite Leishmania. Science. 226, 977-979.

Zilberstein, D. and Dwyer, D.M. 1985 Protonmotive force-driven active transport of D-glucose and L-proline in the protozoan parasite Leishmania donovani. Proc. Natl. Acad. Sci. USA, 82, 1716-1720.

Zilberstein, D. and Dwyer, D.M. 1986. Tricyclic drugs cause lethal disruption of membrane function in Leishmania. In: Host-Parasite Molecular Recognition and Interaction in Protozoal Infections, Chang, K-P. ed., in press.

Zuckerman, A. and Lainson, R. 1977. Leishmania. In: Parsasitic Protozoa, Krier, J.P. ed., Volume 1, pp. 58-133. Academic Press, New York.

Chapter 2

PURINE AND PYRIMIDINE METABOLISM IN PARASITIC PROTOZOA

2.1. General nucleic acid metabolism

Nucleic acids are high molecular weight molecules which consist of recurring monomeric units of nucleotides combined one with another through phosphate diester linkages. There are only two main forms of nucleic acid known, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Both have certain constituents in common, namely phosphate groups, the purines adenine and guanine, and the pyrimidine, cytosine. They differ, however, with respect to their pentose and second pyrimidine base. RNA contains D-ribose and uracil, whereas DNA contains 2'-deoxy-D-ribose and thymine.

The synthesis of purine and pyrimidine nucleotides is essential for the growth and functioning of cells, not only because of their roles as the monomeric precursors of RNA and DNA but also because they participate in a wide variety of biochemical processes. For instance, the purine nucleotides serve as high energy currency (e.g. ATP) in biological systems, as regulatory signals (e.g. cyclic AMP and cyclic GMP) in a wide variety of tissues and organisms, and as constituents of the major coenzymes FAD, NAD, coenzyme A and an important methyl donor, S-adenosylmethionine. The pyrimidine nucleotides, on the other hand, serve as activated intermediates, such as UDP-glucose and UDP-galactose in carbohydrate metabolism and CDP-acylglycerol in lipid synthesis.

Purine and pyrimidine nucleotides can by synthesised via de novo pathways from simple precursors and via 'salvage' pathways from preformed bases and nucleosides that originate from exogenous sources or are products of RNA and DNA breakdown. There are a series of pathways known by which purine and pyrimidine nucleotides can be synthesized, interconverted and catabolised; these are summarised in schematic fashion in Figs. 1-4. It should be noted that not all of

the enzymes and pathways are present in all cells. Consequently, it is critical to evaluate the importance of a given pathway in the cell of interest.

Purine and pyrimidine metabolism in parasitic protozoa has been studied extensively in recent years largely with the aim of providing the information required for the rational design of antiparasitic agents (Marr and Berens 1983; Wang 1983). These investigations have resulted in a vast amount of new information on this important aspect of the biochemistry of parasitic protozoa.

The current status of our knowledge is reviewed below with particular attention being given to the possibilities for chemotherapeutic attack.

2.2. Purine metabolism

2.2.1. Introduction

Most organisms are 'prototrophic' for purines, i.e. capable of synthesizing purines de novo and therefore not dependent upon exogenous sources of these compounds (Martin 1981). In contrast, some microorganisms including Mycoplasma (Mitchell and Finch 1977; Hamet et al. 1980) and most parasitic protozoa are incapable of de novo synthesis of purines and therefore are obligate 'auxotrophs' for them (Gutteridge and Coombs 1977). Indeed not all mammalian cells are capable of de novo synthesis of purine nucleotides, mature erythrocytes (Lowy et al. 1962; Fontenelle and Henderson 1969) and lymphocytes (Scholar and Calabresi 1973; Cohen et al. 1983) are totally dependent on salvaging preformed purines. The mammalian liver, however, is a major site of purine nucleotide synthesis and provides purines in the form of bases or nucleosides to be salvaged and utilised by those tissues incapable of synthesizing purines de novo (Murray 1971; Jackson et al. 1980).

2.2.2. *De novo* synthesis of IMP

The purine nucleotides are synthesised *de novo* from simple precursors such as glycine, formate, CO_2, glutamine and aspartate. This biosynthetic route begins with the condensation of glutamine with the phosphorylated sugar 5-phosphoribosyl-1-pyrophosphate (PRPP) and ends, ten enzymatic reactions later, as IMP (Fig. 1). Two step reactions are required for the conversion of IMP to AMP or GMP. It has been confirmed in recent years that most parasitic protozoa are unable to synthesise the purine ring *de novo*. Information on the occurrence or lack of *de novo* purine synthesis in parasitic protozoa has mainly been derived from two types of studies.

1 - Growth requirements

Many parasitic protozoa have been shown to require preformed purines in their growth medium. These include *Trypanosoma ranarum* (Guttman 1966), the insect trypanosomatid *Leptomonas pessoai* (Roitman *et al.* 1972), *T. cruzi* (Azevedo and Roitman 1984), *T. brucei* (Brun and Schonenberger 1979), *T. gambiense* (Fish *et al.* 1982b), *Leishmania tarentolae* (Trager 1974), *L. donovani* and *L. braziliensis* (Steiger and Steiger 1977), *L. tropica* and *L. mexicana* (Melo *et al.* 1985), *Crithidia fasciculata* (Kidder and Dutta 1958), aposymbiotic *C. deanei* (Mundim and Roitman 1977), *C. acanthocephali* and *C. harmosa* (Roitman *et al.* 1985), *Trichomonas vaginalis* (Linstead 1981), *Tritrichomonas foetus* (Wang *et al.* 1984a), *Plasmodium knowlesi* (Trigg and Gutteridge 1971) and *Tetrahymena pyriformis* (Kidder and Dewey 1948). In contrast, the growth of endosymbiont-containing *Crithidia oncopelti* (Newton 1957) and *C. deanei* (Mundim *et al.* 1974) and *Acanthamoeba* species (Adam 1959) were not impaired by deprivation of exogenous purine, which led to postulation that they can synthesise purines *de novo*.

Fig. 1. De novo synthesis of IMP. Enzymes: 1, amidophosphoribosyltransferase (EC 2.4.2.14); 2, phosphoribosylglycinamide synthetase (EC 6.3.4.13); 3, phosphoribosylglycinamide formyltransferase (EC 2.1.2.2); 4, phosphoribosylformylglycinamide synthetase (EC 6.3.5.3); 5, phosphoribosylaminoimidazole synthetase (EC 6.3.3.1); 6, phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21); 7, phosphoribosylaminoimidazole-succinocarboxamide synthetase (EC 6.3.2.6); 8, adenylosuccinate lyase (EC 4.3.2.2); 9, phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3); 10, IMP cyclohydrolase (EC 3.5.4.10).

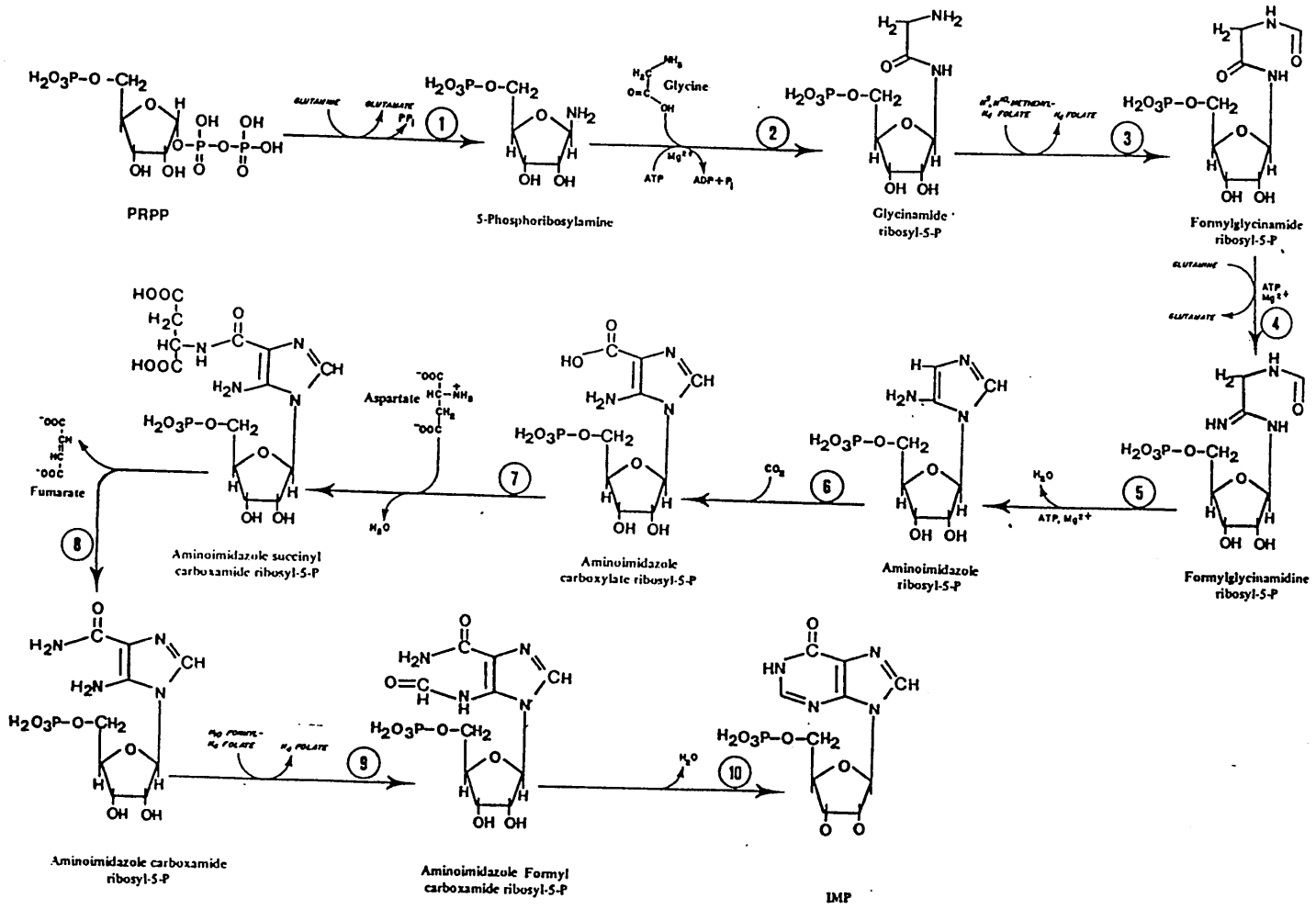

PRPP
5-Phosphoribosylamine
Glycine
Glycinamide ribosyl-5-P
Formylglycinamide ribosyl-5-P
Formylglycinamidine ribosyl-5-P
Aminoimidazole ribosyl-5-P
Aminoimidazole carboxylate ribosyl-5-P
Aminoimidazole succinyl carboxamide ribosyl-5-P
Aspartate
Fumarate
Aminoimidazole carboxamide ribosyl-5-P
Aminoimidazole Formyl carboxamide ribosyl-5-P
IMP

Fig.1

2 - Labeling of nucleic acid purines by glycine and formate

Most parasitic protozoa so far studied have failed to incorporate radiolabeled glycine and formate into their nucleic acid purines. These include Trypanosoma mega (Bone and Steinert 1956), T. lewisi (Pizzi and Taliaferro 1960), T. cruzi (Gutteridge and Gaborak 1979; Berens et al. 1981), T. gambiense, T. rhodesiense and T. brucei gambiense (Fish et al. 1982a, 1982b), Leishmania donovani and L. braziliensis (Marr et al. 1978a), L. m. mexicana (Hansen et al. 1984; Hassan and Coombs 1986a), Trichomonas vaginalis (Heyworth et al. 1982), Tritrichomonas foetus (Wang et al. 1983a), Giardia lamblia (Wang and Aldritt 1983), Entamoeba histolytica (Boonlayangoor et al. 1980; Lo and Wang 1985), Eimeria tenella (Wang and Simashkevich 1981; Lafon and Nelson 1985), Toxoplasma gondii (Perrotto et al. 1971) and Plasmodium lophurae (Walsh and Sherman 1968). This provides strong evidence for their lack of de novo purine synthesis. There are a few exceptions to the general situation. The occasional opportunistic parasites Acanthamoeba castellanii and A. astronyxis were recently found to be capable of de novo purine synthesis as evidenced by progressive incorporation of glycine into nucleic acids (Hassan and Coombs 1986a). A few endosymbiont-containing trypanosomatids including Crithidia oncopelti (Newton 1957) and C. deanei (Ceron et al 1979) also incorporate glycine into nucleic acids. Interestingly, the aposymbiotic strain of C. deanei cannot incorporate glycine into nucleic acids (Ceron et al. 1979) suggesting that de novo synthesis is dependent upon the symbiont. It has been reported that Trypanosoma equiperdum utilises glycine for nucleic acid synthesis (Pizzi and Taliaferro 1960) although the level of incorporation was well below that for preformed purines. It was also claimed that amastigotes of T. cruzi possessed de novo synthetic

pathways (Yoneda 1971; Fernandes and Kimura 1973) although these experiments did not exclude the possibility that the glycine was utilised by the host cell before it was taken up by the parasite. As yet, however, there have been no reported attempts to investigate in detail the enzymes of the purine de novo pathway in any parasitic protozoan. The incorporation of a small amount of labeled formate into nucleotide pool of T. b. gambiense (Fish et al. 1982b) provided evidence for the presence of the last two enzymes of the de novo pathway, namely phosphoribosylaminoimidazolecarboxamide formyltransferase and inosinate cyclohydrolase. This conclusion was supported by the finding that 3-ribosyl-5-aminoimidazolo-4-carboxamide (AICAR) maintained the viability of this organism for over six weeks in vitro (Fish et al. 1982b). The work of Kidder and Nolan (1981) gave indications that T. cruzi also have these two enzymes. They showed that parasite extracts were able to produce the nucleotide 5-phospho-3-ribosyl-5-aminoimidazole-4-carboxamide (AICARP) when incubated with 5-aminoimidazole-4-carboxamide (AICA) and PRPP but not when the extract was heat inactivated or when the PRPP was omitted. The presence of these enzymes could account for the small amount of labeled formate detected in the nucleic acids of amastigotes of T. cruzi (Gutteridge and Gaborak 1979). In contrast to the wealth of knowledge on some parasites, there have not been any reports on the occurrence or the absence of de novo synthetic pathways in piroplasms such as Babesia and Theileria species.

2.2.3. Purine nucleotide interconversions

IMP occupies a central position in purine metabolism, acting as a common precursor for both adenine and guanine nucleotides. The IMP is converted to AMP in most organisms by the combined actions of adenylosuccinate synthetase and adenylosuccinate lyase and to GMP by

IMP dehydrogenase and GMP synthetase (Fig. 2). By subsequent phosphorylations, AMP and GMP are converted to their respective nucleoside diphosphates (ADP and GDP) and nucleoside triphosphates (ATP and GTP) through the actions of nucleoside monophosphate kinase and nucleoside diphosphate kinase, respectively. These reactions are important in adjusting the intracellular supplies of adenine and guanine nucleotides according to the immediate needs of the cells under different physiological conditions. Adenine nucleotide may be converted to IMP by two routes. The first consists of the direct deamination of AMP to IMP catalysed by AMP deaminase, whereas the second route involves cleavage of AMP to adenosine by 5'-nucleotidase with the adenosine thus formed being reutilised for IMP synthesis via the salvage pathway enzymes. The only known route for the conversion of GMP to IMP, however, is via GMP reductase. These conversions are beneficial to most cells because they enable them to synthesize guanine nucleotides from adenine nucleotides and vica versa under conditions of inbalance between the two types of nucleotides.

The adenylosuccinate synthetases of L. donovani (Spector et al. 1979) and T. cruzi (Spector et al. 1982) are similar to the isofunctional enzymes from other sources. In general they show similar pH profiles (optimum: 6.3 - 6.8), similar sensitivity to inhibition by GMP and by GMP analogues, and a similar apparent K_m (10-12 μM) for IMP (Nagy et al. 1973; Muirhead and Bishop 1974; Spector and Miller 1976). Importantly, however, they differ from the enzyme of man (Spector and Miller 1976) in their ability to convert the IMP analogue allopurinol ribotide to succino-4-aminopyrazolo(3,4-d)pyrimidine ribotide. This amination of allopurinol ribotide may be a lethal synthetic step for Leishmania (Nelson et al. 1979a, 1979b), T. cruzi (Avila et al. 1981), T. rhodesiense and T. brucei (Berens et al. 1980a) for the product was found to be rapidly cleaved to 4-

Fig. 2. Salvage and interconversion of purines. Enzymes: 1, adenine deaminase (EC 3.5.4.2); 2, adenosine deaminase (EC 3.5.4.4); 3, AMP deaminase (EC 3.5.4.6); 4, guanine deaminase (EC 3.5.4.3); 5, nucleosidase (EC 3.2.2.1) or phosphorylase (catabolic) (EC 2.4.2.1); 6, phosphorylase (anabolic) (EC 2.4.2.1); 7, nucleoside kinase (EC 2.7.1.15, EC 2.7.1.20, EC 2.7.1.73); 8, nucleotidase (EC 3.1.3.5); 9, phosphoribosyltransferase (EC 2.4.2.7, EC 2.4.2.8, EC 2.4.2.22); 10, adenylosuccinate synthetase (EC 6.3.4.4); 11, adenylosuccinate lyase (EC 4.3.2.2); 12, IMP dehydrogenase (EC 1.2.1.14); 13, GMP synthetase (EC 6.3.5.2); 14, GMP reductase (EC 1.6.6.8); 15, xanthine oxidase (EC 1.2.3.2). Abbreviation: AMP, adenosine-5'-monophosphate; s-AMP, adenylosuccinic acid; IMP, inosine-5'-monophosphate; GMP, guanosine-5'-monophosphate; XMP, xanthosine-5'-monophosphate.

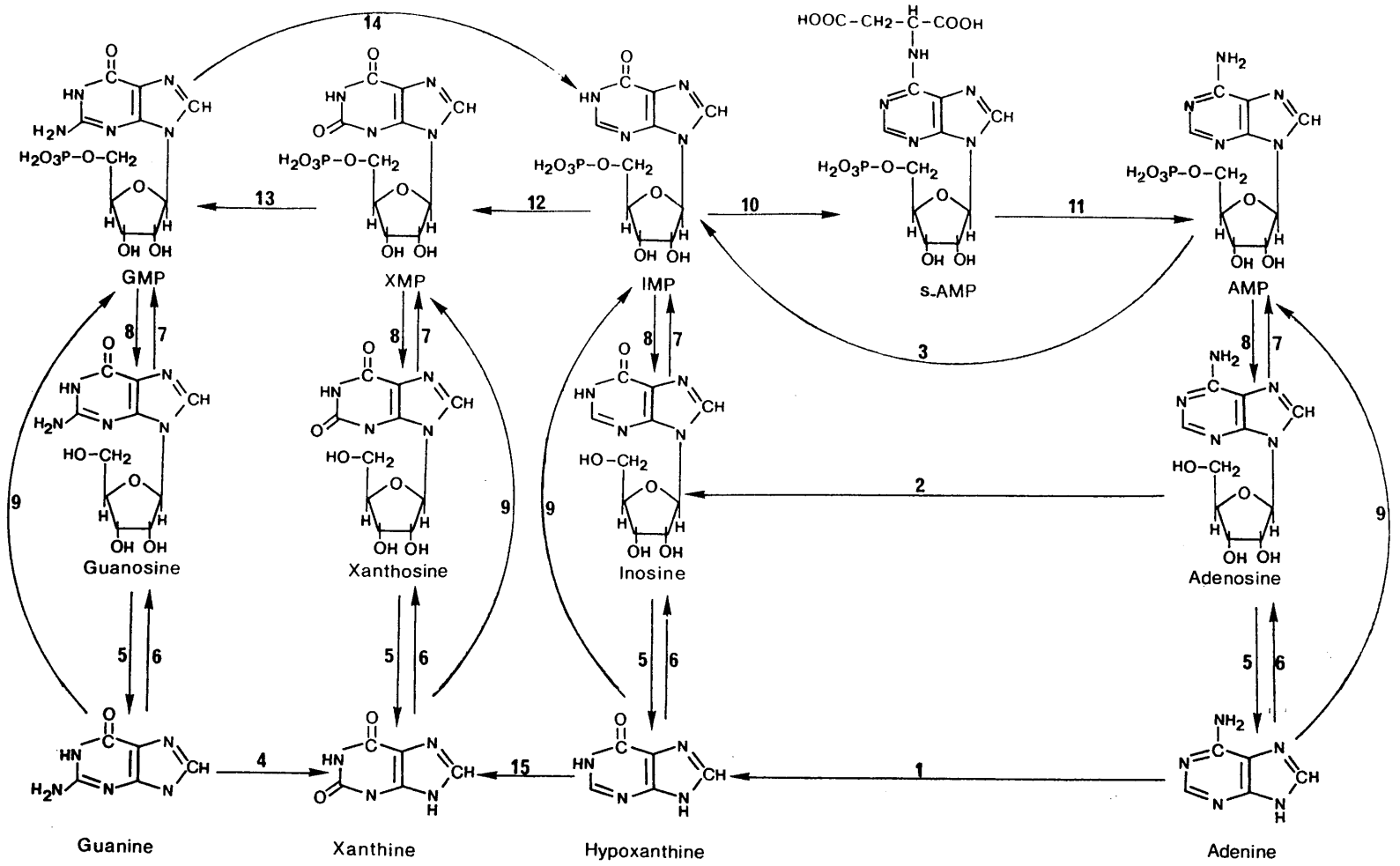

GMP
XMP
IMP
s-AMP
AMP
Guanosine
Xanthosine
Inosine
Adenosine
Guanine
Xanthine
Hypoxanthine
Adenine
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Fig.2

aminopyrazolo(3,4-d)pyrimidine ribotide by the parasite adenylosuccinate lyase. This rogue ribotide is thought to interfere with parasite nucleic acid synthesis and metabolism with lethal consequences for the cell. In contrast, the American trypanosome T. rangeli appears to be unable to aminate allopurinol ribotide to 4-aminopyrazolopyrimidine ribotide and indeed substrate specificity studies carried out on partially purified adenylosuccinate synthetase from T. cruzi and T. rangeli showed the T. cruzi enzyme to be 100-fold more active on allopurinol ribotide than the T. rangeli enzyme (Avila et al. 1981). This probably explains the failure of allopurinol to affect T. rangeli multiplication (Avila et al. 1986). Adenylosuccinate synthetase from L. donovani (Nelson et al. 1982; Rainey and Santi 1983) was also able to use formycin B 5'-monophosphate as an alternate substrate with apparent K_m of 26 μM and a V_m of about 1% the V_m of IMP. In contrast to its low efficiency with the leishmanial enzymes, formycin B 5'-monophosphate was a highly efficient substrate of mammalian adenylosuccinate synthetase with a V_m approaching 40% of the V_m of IMP (Spector et al. 1984). As with Leishmania, formycin B 5'-monophosphate was also aminated by T. cruzi to give 5'-nucleotides of the cytotoxic formycin A which is incorporated into RNA (Rainey et al. 1983). A study of GMP reductase from L. donovani revealed that it is inhibited by allopurinol ribotide, thiopurinol ribotide (Spector and Jones 1982) and by formycin B 5'-monophosphate (Spector et al. 1984). This is of chemotherapeutic interest since these compounds were produced by Leishmania when it was exposed to allopurinol, thiopurinol or their ribosides (Berens et al, 1980b; Marr et al. 1982) and formycin B (Carson and Chang 1981). It has to be remembered, however, that although these compounds are active antileishmanial agents in

experimental models their potential as drugs is still in doubt (Marr and Berens 1983; Marr 1984; Looker et al. 1986). It has recently, been reported that various other trypanosomatids have similar activities of purine nucleotide interconverting enzymes (Hassan and Coombs 1986b). This suggests that these enzymes may be common to all trypanosomatids which helps to explain the sensitivity of this group to allopurinol and other related pyrazolopyrimidine compounds. Interestingly, Berman and Webster (1982) have demonstrated that mycophenolic acid, an inhibitor of IMP dehydrogenase and ultimately guanine nucleotide synthesis, is toxic to L. tropica amastigotes in vitro.

The incorporation of radioactivity from labeled hypoxanthine and inosine equally into adenine and guanine nucleotides and that from labeled xanthine and guanine and their ribosides into adenine nucleotides by L. donovani and L. braziliensis (Marr et al. 1978a) confirms the presence of adenylosuccinate synthetase, adenylosuccinate lyase, IMP dehydrogenase, GMP synthetase and GMP reductase in these leishmanias. Similar results were obtained with various trypanosomes including T. cruzi (Berens et al. 1981), T. gambiense and T. rhodesiense (Fish et al. 1982a) and T. gambiense (Fish et al. 1982b). The presence of adenylosuccinate synthetase in T. gambiense had been established earlier (Schmidt et al. 1975). In contrast, the endosymbiont-containing C. deanei (Ceron et al. 1979) as well as Toxoplasma gondii (Pfefferkorn and Pfefferkorn 1977a) and Eimeria tenella (Lafon and Nelson 1985) appear unable to convert GMP to AMP as evidenced by the failure of radioactivity from guanine to label adenine nucleotides, though conversion of IMP to GMP is possible.

Trichomonas vaginalis (Heyworth et al. 1982), Giardia lamblia (Wang and Aldritt 1983) and E. histolytica (Hassan and Coombs 1986c)

are unusual in apparently lacking the enzymes that interconvert purine nucleotides. In contrast, Tritrichomonas foetus can convert IMP to AMP and GMP (Wang et al. 1983a) suggesting that deficiencies in this aspect of purine metabolism are not a common feature of all anaerobic sarcomastigophora. It is to be noted that mycophenolic acid, an inhibitor of IMP dehydrogenase, and hadacidin, an inhibitor of adenylosuccinate synthetase, inhibited the growth of T. foetus but had no effect on Trichomonas vaginalis (Wang et al. 1984b). These compounds are thought to act by blocking conversion of IMP to GMP and of IMP to AMP, respectively. In contrast, Acanthamoeba species have been shown to possess all the enzymes known to be involved in purine nucleotide interconversions (Hassan and Coombs 1986d).

Studies on the ability of Plasmodium species to interconvert purine nucleotides have indicated that both adenine and guanine nucleotides can be synthesised from hypoxanthine via IMP (Reyes et al. 1982; Webster et al. 1984), although adenylosuccinate synthetase is the only enzyme detected so far (Lukow et al. 1973). In addition, hadacidin, an inhibitor of adenylosuccinate synthetase, selectively blocks the synthesis of adenylate from hypoxanthine (Webster et al. 1984). These and other results suggest that the adenylosuccinate pathway is essential for synthesis of parasite adenosine nucleotides and so offers opportunities for the design of new antimalarial drugs (Webster et al. 1984). It is of interest to note that the selective inhibition of GMP formation from hypoxanthine via IMP by bredinin, an inhibitor of IMP dehydrogenase, in P. falciparum (Webster and Whaun 1982) provides good evidence for the existence of IMP dehydrogenase and a further indication that hypoxanthine is a major source of purines in this parasite. Eimeria tenella grown in tissue culture has been shown to be sensitive to aprinocid, thought to be due to the

compound inhibiting IMP dehydrogenase and so the conversion of IMP to GMP (Wang et al. 1979). Recently Hupe et al. (1986) suggested that the IMP dehydrogenase from E. tenella is a valuable chemotherapeutic target enzyme and that it is specifically inhibited by mycophenolic acid, with the latter being also inhibitory to the growth of this parasite in tissue culture.

The lack of detectable purine nucleotide interconverting enzymes in Babesia divergens (Hassan et al. 1986) is rather surprising, since nucleotide interconversion appear to be significant to piroplasms as indicated by the rapid incorporation of hypoxanthine into parasite nucleic acids (Irvin and Young 1979). It may simply be that the activities of the enzymes are rather low and below the level of detection in the assays used.

The free living ciliate protozoan, Tetrahymena pyriformis has been shown to lack GMP reductase and is unable to convert IMP to GMP. It is also doubtful whether this organism can convert IMP to AMP (Hill 1972).

Thus a reasonable amount of information has been gathered concerning a range of organisms, nevertheless there are still many deficiencies in our knowledge about these interconversions. In most cases more detailed enzymatic analyses are required before the possibilities the enzymes present for chemotherapeutic attack can be fully appreciated.

2.2.4. Purine salvage and nucleotide catabolism

All mammalian cells (Murray 1971), microorganisms (Nygaard 1983) and parasitic protozoa (Gutteridge and Coombs 1977) studied appear to have enzymes for salvaging purine bases and/or nucleosides (Fig. 2). In human and other mammalian tissues, phosphoribosyltransferases (PRTase) are the enzymes mainly responsible for the salvage of purines. They catalyse the condensation of purines and PRPP to form

5'-purine ribonucleotides. Mammals have two distinct enzymes, one active towards adenine (adenine PRTase) (Krenitsky et al. 1969a) and a second specific for hypoxanthine and guanine (hypoxanthine-guanine PRTase) (Krenitsky et al. 1969b). In many microorganisms, however, there are separate PRTases for each of the four bases adenine, hypoxanthine, guanine and xanthine (Miller et al 1972; Nygaard 1983). Another important salvage route for nucleotide formation is via the action of kinases which catalyse the phosphorylation of nucleosides. Adenosine kinase (Miller et al 1979), guanosine kinase (LePage and Junga 1963) and inosine kinase (Pierre and LePage 1968) have been isolated from a number of mammalian sources and they have been shown to play an important role in the control of nucleoside concentrations in vivo.

Nucleotides can be hydrolysed by nucleotidases to nucleosides which can be further degraded by phosphorylases to yield the purine base and ribose-1-phosphate. Adenosine can also be deaminated to inosine by adenosine deaminase. In humans, the purine bases hypoxanthine and guanine can be converted to uric acid by way of xanthine in reactions catalysed by the enzymes guanine deaminase and xanthine oxidase. In contrast, all parasitic protozoa so far studied lack detectable activity of xanthine oxidase and as a result no further degradation of purine bases can occur. In addition, some parasitic protozoa contain adenine deaminase which catalyses the deamination of adenine to hypoxanthine and is an enzyme that has not been found in mammalian cells.

The salvage of preformed purines is essential for most parasitic protozoa, as they lack a functional de novo synthetic pathway. Evidence about purine salvage by parasitic protozoa has been obtained from studying their requirements for purines in minimal defined

growth media, their incorporation of radiolabelled purines into nucleic acids, and from analyses of enzymes in parasite lysates. The purine requirements of many parasitic protozoa can be satisfied by a single purine (one of hypoxanthine, adenine, guanine, or xanthine) or its nucleoside.

2.2.4.a. *Trypanosoma cruzi*

Studies by Gutteridge and Gaborak (1979) demonstrated that epimastigote, trypomastigote and amastigote forms of *T. cruzi* can salvage purine bases and nucleosides but not nucleotides. The preferential incorporation into parasite nucleotides of purine bases rather than nucleosides suggested that there are higher intracellular activities of purine PRTases than nucleoside kinases (Gutteridge and Gaborak 1979; Berens *et al.* 1981). This was later confirmed by enzyme analyses (Gutteridge and Davies 1981) which showed that the parasite has two distinct PRTases, one which is cytosolic and specific for adenine and a second active towards guanine and hypoxanthine and which is found associated with glycosomes. Whether there is a separate xanthine PRTase remains to be established. Interestingly, allopurinol and 4-aminopyrazolopyrimidine which are biologically active against *T. cruzi* (Avila *et al.* 1983; Marr *et al.* 1978b) were readily converted to their ribonucleotides by hypoxanthine-guanine PRTase and adenine PRTase, respectively (Berens *et al.* 1982; Avila *et al.* 1984) and it is likely that these conversions are the basis of the selective toxicity of these drugs towards *T. cruzi*.

Miller *et al.* (1984) purified four nucleoside-cleaving enzymes from *T. cruzi*. Two of the enzymes are nucleoside phosphorylases, one specific for adenosine and the other for uridine. The other two enzymes are nucleoside hydrolases, one specific for inosine and the

other for 2'-deoxyinosine. These nucleoside-metabolising activities are similar to those reported for L. donovani (Koszalka and Krenitsky 1979) with the exception that T. cruzi possesses a uridine phosphorylase rather than a uridine hydrolase. Guanine deaminase has been partially purified from T. cruzi and shown to be inhibited strongly by N^6-methyladenine and 4-amino-5-imidazole carboxamide (Nolan 1984). Surprisingly, the latter compound is not inhibitory to the growth of T. cruzi, although it is against various Leishmania species (Kidder and Nolan 1981). Adenosine kinase of T. cruzi has also been characterised (Kidder 1982). This enzyme resembles that from L. m. mexicana (Hassan and Coombs 1985a) with respect to apparent K_m, Mg^{2+} requirement and ATP activation, but is different from leishmanial enzyme in being optimally active at high pH (8.0). Interestingly, a purine nucleoside phosphotransferase using p-nitrophenylphosphate as phosphate donor and active towards allopurinol riboside and formycin B (Rainey et al. 1983; Berens et al. 1984)but not adenosine (Kidder 1982) has been detected in T. cruzi extracts and it is believed that the conversion of these compounds to their 5'-nucleotides could be responsible for their cytotoxic effects towards trypanosomes (Marr 1983). Neither 3'-AMP nucleotidase nor 5'-AMP nucleotidase have been detected in T. cruzi (Gottlieb et al. 1986). Although there are many common features, it appears that not all strains of T. cruzi are identical in their ability to metabolise purines. For instance, the Sonya strain was found to possess adenosine deaminase and guanosine phosphorylase (Davies et al. 1983) whereas the Peru strain lacks these activities (Berens et al. 1981; Miller et al. 1984).

2.2.4.b. African trypanosomes

The purine salvage pathways of these parasites have been extensively studied by measuring the incorporation of labelled purine bases and nucleosides into nucleotide pools of T. gambiense and T. rhodesiense (Fish et al. 1982b) and analyses of enzymes in cell free extracts of T. gambiense (Schmidt et al. 1975), T. vivax, T. congolense (Ogbunude and Ikediobi 1983; Ogbunude et al. 1985; Emeh et al. 1986) and T. brucei (Davies et al. 1983; Hassan and Coombs 1986b). Adenine deaminase is apparently absent from all of these species except T. vivax, whereas adenosine deaminase has been detected but only in T. brucei and not in the studies by Ogbunude and Ikediobi (1983). Nucleosidase activity towards adenosine seems to be a common feature of all african trypanosomes except T. congolense. Adenosine phosphorylase has also been detected in T. congolense and T. brucei but not in T. vivax and T. gambiense. As with trypanosomes, there are also significant differences between leishmanial species and insect trypanosomatids with respect to adenine and adenosine metabolism (Hassan and Coombs 1986b). The distinct differences between species in the content of adenine deaminase, adenosine deaminase, adenosine nucleosidase and adenosine phosphorylase could form the basis of a means of species identification. All african trypanosomes, however, have some common metabolic activities, notably guanine deaminase, AMP deaminase, purine PRTases and nucleoside kinases. 3'-AMP nucleotidase but not 5'-AMP nucleotidase are also detected in extracts of T. rhodesiense (Gottlieb et al. 1986) and T. brucei (Hassan and Coombs 1986e), the former enzyme being associated with the plasma membrane. Adenylate kinase has been shown to have a dual mitochondrial and glycosomal distribution in both bloodstream and procyclic T. brucei (Opperdoes

et al. 1981) and was also found in glycosomes of bloodstream T. rhodesiense (McLaughlin 1981). Whilst trypanosomal glycosomes contain a number of phosphotransferases, the glycosomal adenylate kinase could play a fundamental regulatory role, possibly in controlling intraglycosomal ATP/ADP levels and preventing complete depletion of glycosomal ATP and hence inhibition of glycolysis. In common with T. cruzi, the preferred pathway of nucleotide formation in african trypanosomes seems to be via PRTases, which also accept pyrazolopyrimidine compounds as substrates (Berens et al. 1980a), and the hypoxanthine PRTase of T. brucei has also been shown to be associated with the glycosomes (Hammond et al. 1985a).

2.2.4.c. Leishmania

There have been many reports on purine salvage pathways in promastigotes of L. donovani (Looker et al. 1983; Marr and Berens 1985), L. braziliensis (Marr et al. 1978a), L. m. amazonensis, L. tarentolae (Davies et al. 1983; Hassan and Coombs 1986b) and amastigotes and promastigotes of L. m. mexicana (Hassan and Coombs 1985a). The apparent lack of xanthine oxidase and the presence of highly active adenine deaminase and guanine deaminase which lead to the rapid production of hypoxanthine and xanthine, respectively, point to hypoxanthine PRTase and xanthine PRTase as being of central importance to leishmanial species. The apparent K_m (adenine) of the partially-purified adenine deaminase from L. tropica (molecular weight 32000daltons (Konigk and Rasoul 1978) was quite similar (0.2 x 10^{-5} M) to those found for L. m. mexicana promastigotes (0.5 x 10^{-5} M) and amastigotes (0.45 x 10^{-5} M) (Hassan and Coombs 1985a) but much lower than those of L. braziliensis, L. donovani and L. tarentolae (0.7-2.2 mM) (Kidder and Nolan 1979). The leishmanial adenine deaminase was found to be inhibited by coformycin, deoxycoformycin

(Kidder and Nolan 1979) and by N^6-methyladenine (Nolan and Kidder 1980). Leishmanial guanine deaminase has also been studied. The apparent K_m value for guanine and the pH optimum of the enzyme from L. m. mexicana amastigotes (K_m, 1 x 10^{-5} M; pH 7.0) were found to be significantly different from those of the promastigote enzyme (K_m, 3 x 10^{-5} M; pH 8.0) (Hassan and Coombs 1985a). The enzymes from other leishmanial species have been reported to have K_ms in the range 1.11 x 10^{-4} - 1.67 x 10^{-4} M and be similar with respect to pH optimum having activity over a broad range (7.5-8.5) and to be inhibited by N^6-methyladenine and 4-aminoimidazole carboxamide (Nolan and Kidder 1980; Kidder and Nolan 1981). Growth is also inhibited by these compounds, thought to be due to their effect on guanine deaminase. In contrast, the mammalian (rabbit liver) enzyme was not inhibited by N^6-methyladenine (Nolan and Kidder 1980). Striking differences have been observed between amastigotes and promastigotes of L. donovani in their content of enzymes converting adenosine to hypoxanthine (Konigk and Putfarken 1980; Looker et al. 1983). Purified amastigotes deaminated adenosine to inosine (adenosine deaminase), an enzyme activity not found in promastigotes, which could be subsequently cleaved to hypoxanthine. With promastigotes extracts, adenosine was cleaved to adenine, which is subsequently deaminated to hypoxanthine by adenine deaminase, neither of these enzymes were found in amastigotes. However, this stage-specific adenosine metabolism apparently does not exist in L. m. mexicana (Hassan and Coombs 1985a); both amastigotes and promastigotes possess adenine deaminase and adenosine deaminase. These species differences have to be confirmed, there is some indication that the adenosine deaminase of L. donovani amastigotes may have been of host origin. In other studies, Iovannisci and Ullman (1984) showed that L. donovani

promastigote extracts possessed a very weak deaminase activity towards formycin A, rather than adenosine, which they referred to as formycin A deaminase. Adenosine deaminase is also apparently absent from L. tarentolae but was detected in extracts of L. m. amazonensis by Hassan and Coombs (1986b) although Davies et al. (1983) had previously been unable to do so.

Koszalka and Krenitsky (1979) purified three distinct nucleoside hydrolases from L. donovani promastigotes, one specific for 2'-deoxynucleosides for which there is no mammalian counterpart. In contrast to the T. cruzi enzyme, which is specific for 2'-deoxyinosine (Miller et al. 1984), this leishmanial enzyme can hydrolyse not only 2'-deoxyinosine but also 2'-deoxyguanosine and 2'-deoxyadenosine. The second nucleoside hydrolase is specific for ribonucleosides whereas the third is active towards both purine and pyrimidine ribonucleosides; it cleaves the latter most effectively and so has been referred to as pyrimidine nucleosidase. None of the nucleoside hydrolases apparently cleaved adenosine to adenine; it was therefore proposed that there was a phosphorylase present to catalyse this conversion. Adenosine phosphorylase, has been detected in L. tropica, and this readily synthesizes adenosine from adenine and ribose-1-phosphate (Konigk 1978). Iovannisci and Ullman (1984), however, suggested that L. donovani promastigotes possess both adenosine hydrolase and adenosine phosphorylase as the cleavage of adenosine to adenine occurred in the absence of inorganic phosphate as well as being stimulated 4 fold by addition of 50 mM phosphate. The presence of catabolic phosphorylase in L. donovani was confirmed by Hassan and Coombs (1986b), although they were not able to detect nucleosidase acting on adenosine in this study. Indeed, the presence of catabolic phosphorylase towards inosine, guanosine and xanthosine was found to be a common feature of leishmanial species (Hassan and

Coombs 1986b). Recently, a nucleoside phosphorylase specific for 6-aminopurine nucleosides has been purified from L. donovani (Koszalka and Krenitsky 1985). The enzyme had a molecular weight of 86,000 daltons, optimal activity in the range pH 5.6-6.5, was found to be capable of synthesizing and cleaving 5'-methylthioadenosine, 2'-deoxyadenosine, 5'-deoxyadenosine and adenosine and was referred to as 5'-methylthioadenosine phosphorylase. Nucleosidase activity towards ribonucleosides and deoxyribonucleosides has also been demonstrated in extracts of L. m. mexicana (Hassan and Coombs 1985a).

Purification of PRTase activities from L. donovani revealed the presence of three distinct enzymes, one active on adenine, another on hypoxanthine and guanine and a third on xanthine (Tuttle and Krenitsky 1980). The adenine PRTase was found to have apparent molecular weights of 54,000 and 25,000 daltons in the presence and absence of PRPP, respectively. It appears that PRPP affects the state of aggregation of the enzyme. The hypoxanthine-guanine PRTase had a molecular weight of 110,000 daltons in the absence of PRPP and addition of the latter caused dissociation of the enzyme into smaller subunits (60,000 daltons) whereas the molecular weight of xanthine PRTase (54,000 daltons) was independent of the presence of PRPP. 4-Aminopyrazolopyrimidine is metabolised by L. donovani adenine PRTase (Tuttle and Krenitsky 1980), but is not deaminated in vitro by adenine deaminase (Nelson et al. 1979a; Marr and Berens 1983). 4-Aminopyrazolopyrimidine-resistant lines of L. donovani were found to be adenine PRTase-deficient; they were found to contain only half the adenine PRTase activity present in wild type cells. This suggests the existence of two gene copies for this enzyme and providing some evidence that these pathogens are diploid (Iovannisci et al. 1984a). Leishmania donovani hypoxanthine-guanine PRTase has

also provoked a lot of interest because it metabolises allopurinol to its ribotide (Tuttle and Krenitsky 1980), which accumulates in large quantities in the parasite and is responsible, at least in part, for the antileishmanial activity of this drug (Nelson et al. 1979b). In contrast, allopurinol is a poor substrate for the human enzyme and its ribotide is produced only in minute quantities within the cells (Nelson et al. 1973). The existence in leishmanial species of xanthine PRTase, an enzyme not found in mammalian cells, makes it another potential target for antileishmanial agents (Wang 1984). Studies by Hassan et al. (1985) using subcellular fractionation techniques, have demonstrated that hypoxanthine and guanine PRTases together with xanthine PRTase are associated with glycosomes in L. m. mexicana. The relevance of the glycosomal location of PRTases is unclear, but the common requirement of the enzymes for PRPP suggests that glycosomes may have a transport system for this compound or that the purine PRTase may be located on the surface of the organelle as has been suggested for orotate PRTase (Hammond and Gutteridge 1983).

Little information is available concerning nucleoside kinases in leishmanial species. A nucleoside kinase from L. m. mexicana, however, has been shown to require Mg^{2+} for activity, to be stabilised by ATP, to be optimally active at pH 6.0 and have an apparent K_m for adenosine of 1.5×10^{-6} M (Hassan and Coombs 1985a). Studies by Looker et al. (1983) on L. donovani show that adenosine and inosine are the only nucleosides phosphorylated by nucleoside kinase and that the activity with adenosine was 50-fold higher in amastigotes than in promastigotes. Nucleoside kinase activity towards adenosine and inosine but not guanosine has also been demonstrated for L. m. amazonensis (Davies et al. 1983). Recently, kinase activity towards all four purine nucleosides has been detected in L. m. amazonensis, L. donovani and L. tarentolae (Hassan and

Coombs 1986b). Leishmanias also contain adenylate kinase which appears to be particulate in L. m. mexicana (Hassan and Coombs 1985a) and associated with glycosomes in L. donovani and L. tropica (Hart and Opperdoes 1984). On the other hand, nucleoside phosphotransferase towards inosine as substrate was not detectable in L. donovani (Looker et al. 1983) or L. m. mexicana (Hassan and Coombs 1986b). Nevertheless, a similar enzyme is known to be present in leishmanias, but whereas allopurinol riboside and formycin B are good substrates, natural nucleosides apparently are not (Krenitsky et al. 1980). Both allopurinol riboside and formycin B have significant antileishmanial activity. The selective toxicity of these agents for Leishmania, as for trypanosomes, is thought to depend upon this conversion such that high levels of allopurinol ribotide and formycin B ribotide are produced. These are converted by a unique amination to 4-aminopyrazolopyrimidine ribotide and formycin A ribotide, respectively, then to their triphosphates and finally incorporated into the parasite's RNA (Carson and Chang 1981; Rainey and Santi 1983; Marr 1984; Nolan et al. 1984; Neal et al. 1985; Looker et al. 1986). Neither allopurinol riboside, nor formycin B is phosphorylated by kinases found in mammalian cells and are remarkably non-toxic to mammals (Umezaw et al. 1967; Ishizuka et al. 1968). 3'-Nucleotidase and 5'-nucleotidase activities have also been found in Leishmania species (Pereira and Konigk 1981; Gottlieb and Dwyer 1983; Hassan and Coombs 1986e) and have been shown to be associated with the plasma membrane of these organisms. The physiological significance of these nucleotidases, however, remains to be established; it seems likely that they play a key role in processing exogenously available nucleotides to a form suitable for transport into the cell.

2.2.4.d. Lower trypanosomatids

The purine salvage pathways of the lower trypanosomatids Crithidia fasciculata. Herpetomonas m. muscarum and H. m. ingenoplastis have been studied in some detail (Hassan and Coombs 1985b, 1986b). It appears that these organisms contain PRTase activities towards adenine, guanine, hypoxanthine and xanthine. The results of earlier studies by Kidder et al. (1979) suggested that C. fasciculata has three separate PRTases, for the synthesis of AMP, IMP and GMP, respectively. It is thought that 4-aminopyrazolo(3,4-d)pyrimidine, which inhibits the growth of C. fasciculata, can be converted to its ribonucleotide by adenine PRTase; adenine antagonises the growth action (Dewey and Kidder 1977). All three species were found to be devoid of adenosine deaminase and AMP deaminase, but they contain highly active adenine deaminase and guanine deaminase; H. m. ingenoplastis lacks the latter activity (Hassan and Coombs 1986b). Furthermore, it appears that deamination of adenine is obligatory for the conversion of adenine to IMP, since C. fasciculata will not grow on adenine in the presence of deoxycoformycin, an inhibitor of adenine deaminase. The growth is unaffected by deoxycoformycin when hypoxanthine is the purine source (Kidder et al. 1977; Kidder and Nolan 1979). Guanosine, inosine and xanthosine but not adenosine are cleaved by extracts of all three species, apparently both phosphorylase and nucleosidase activities were involved (Hassan and Coombs 1986b); adenosine nucleosidase had earlier been found by Dewey and Kidder (1973) to be present in C. fasciculata. Kinases active towards all nucleosides were also detected in these organisms. These parasites, however, seem to differ from leishmanias and trypanosomes in containing nucleoside phosphotransferase towards inosine.

2.2.4.e. *Trichomonas vaginalis*

Studies by Heyworth *et al.* (1982) and Wang (1983) have shown the total lack of incorporation of hypoxanthine, inosine and xanthine into the nucleic acids of *T. vaginalis*. They also found that radioactivity from adenosine and adenine and from guanosine and guanine were only incorporated into adenine and guanine nucleotides, respectively. *Trichomonas vaginalis* appears to be unusual in apparently lacking purine PRTase activities. Instead it possesses purine nucleoside phosphorylase and nucleoside kinase activities (Miller and Linstead 1983). Unlike trypanosomatids (Hammond and Gutteridge 1984), *T. vaginalis* is devoid of adenine deaminase but contains guanine deaminase and adenosine deaminase (Miller and Linstead 1983); the latter appears to be extremely labile to dialysis which explains why it was not found by Heyworth *et al.* (1982). *Trichomonas vaginalis* also lacks nucleoside phosphotransferase towards adenosine, inosine and guanosine (Miller and Linstead 1983) but contains deoxyribonucleoside phosphotransferase activity towards deoxyinosine and deoxyguanosine; this allows the direct salvage of these deoxynucleosides to provide dAMP and dGMP for DNA synthesis (Wang and Cheng 1984a). Purine nucleoside phosphorylase and guanosine kinase have been purified from *T. vaginalis* extracts (Miller and Miller 1985a). The former had a molecular weight of 95,000 daltons and catalysed the synthesis and cleavage of guanosine, adenosine and inosine at maximal velocities of 590,360,240 and 81,16,390 μmol/min/mg protein, respectively. The apparent K_m values ranged from 17-54 μM for the nucleosides and 21-25 μM for the purine bases. On the other hand, the purified guanosine kinase (molecular weight 16,000 daltons) had a specific activity of 34 μmol/min/mg protein at pH 6.8 and an apparent K_m of 1 μM for

guanosine. Inosine, adenosine and 2'-deoxyguanosine can also serve as substrates for the enzymes (Miller and Miller 1985b).

2.2.4.f. *Tritrichomonas foetus*

The extensive studies carried out by Wang *et al.* (1983a) constitute the only reports on purine salvage pathways in *T. foetus*. Adenine, hypoxanthine and inosine were found to be readily incorporated into the nucleotide pool, probably through conversion to IMP. The parasite has hypoxanthine-guanine-xanthine PRTase activities but not adenine PRTase; it is not known yet whether these activities are due to one or several proteins. Kinase activity towards inosine and guanosine as well as phosphotransferase activity towards inosine and adenosine are absent. The presence of adenosine kinase and guanosine phosphotransferase, however, suggests additional routes for obtaining AMP and GMP, respectively, not requiring PRTases. The parasite also contains adenosine deaminase, adenine deaminase, adenosine phosphorylase and inosine phosphorylase activities which is consistent with the suggestion that most purine bases and nucleosides are converted to hypoxanthine before incorporation into nucleotides. Moreover, the results suggests that hypoxanthine could be the major source of purines in *T. foetus*. It is apparent, therefore, that whereas *Trichomonas vaginalis* depends largely on adenosine and guanosine kinases to provide their needs for purine nucleotides, *Tritrichomonas foetus* relies mainly on hypoxanthine PRTase. This explains why 8-azaguanine, an inhibitor of hypoxanthine PRTase, inhibits the growth of *T. foetus* without affecting *Trichomonas vaginalis*, whereas tubercidin, toyocamycin, sangivomycin and arabinosyl adenine, known inhibitors of adenosine kinase, exert significant inhibition on the growth of *T. vaginalis* but not *Tritrichomonas foetus* (Wang *et al.* 1984b). The growth

inhibition with these compounds could also be due to their competing as substrates for the enzymes and eventually being incorporated into RNA.

2.2.4.g. *Giardia lamblia*

There are both similarities and differences between the purine salvage pathways in *G. lamblia* and those of trichomonads. *Giardia lamblia* like *Trichomonas vaginalis* cannot incorporate hypoxanthine, inosine and xanthine into nucleotides, suggesting the absence of hypoxanthine PRTase and xanthine PRTase (Wang and Aldritt 1983). It differs from *T. vaginalis*, however, in that the radioactivity from guanosine cannot be directly incorporated into guanine nucleotide, indicating that guanosine has first to be hydrolysed to guanine before incorporation into GMP (Wang 1983). Of all the purine-metabolising enzymes known, only four have been shown to be present in *G. lamblia*. These are guanine PRTase, adenine PRTase, adenosine hydrolase and guanosine hydrolase (Wang and Aldritt 1983). Purification of guanine PRTase from *G. lamblia* revealed that it has apparent molecular weight of 58,000-63,000 daltons by gel filteration and 29,000 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the native protein is a dimer; hypoxanthine and xanthine are 200-fold less efficient substrates than guanine (Aldritt and Wang 1986). The parasite enzyme is very different from its mammalian counterpart, not cross-reacting immunologically or at the gene level. Recent evidence indicates that *G. lamblia* contains nucleoside phosphotransferase which can phosphorylate 9-deazaadenosine (Berens *et al.* 1985), arabinosyl adenine and arabinosyl guanine (Miller *et al.* 1986) to their respective nucleotides and it is thought that this conversion could

be responsible for the growth inhibitory effect of these compounds on G. lamblia.

2.2.4.h. Entamoeba histolytica

Studies on the ability of E. histolytica to utilize exogenous purine have indicated that adenosine is incorporated at much higher rates than adenine, guanine and guanosine, whereas hypoxanthine, inosine and xanthine were not incorporated into nucleotides (Boonlayangoor et al. 1980; Lo and Wang 1985). Enzyme analyses of E. histolytica extracts revealed the presence of high activities of nucleoside kinases and nucleoside phosphorylases which can function in both anabolic and catabolic directions (Hassan and Coombs 1986c). These findings, together with the apparent absence of guanine PRTase and the very low activities of other PRTases, suggests that nucleotides are synthesised from bases mainly via nucleosides (Hassan and Coombs 1986c). These workers also suggested the presence of two nucleoside phosphorylases, one active on adenosine and the other on inosine, distinguishable by their different responses to pH and their sensitivity towards inhibitors. Adenosine kinase has been purified from E. histolytica (Lobelle-Rich and Reeves 1983) and shown to have activity towards the adenosine analogue tubercidin, but not arabinosyladenine. Both compounds inhibit the growth of E. histolytica (Eubank and Reeves 1981), although the mechanism by which these compounds exert their effect remains to be determined. 3'-Nucleotidase and 5'-nucleotidase activities were also found in extracts of E. histolytica (Hassan and Coombs 1986c), the former being mainly particulate and possibly associated with the plasma membrane. These two nucleotidase activities differ with respect to their pH optimum and sensitivity towards inhibitors.

2.2.4.i. *Acanthamoeba* species

These opportunistic parasites, which have recently been shown to be unusual amongst parasitic protozoa in that they possess *de novo* purine synthesis (Hassan and Coombs 1986a), have also been studied for their ability to salvage purines (Hassan and Coombs 1986d). Hypoxanthine was incorporated into nucleic acids at the highest rate, with the rates of incorporation decreasing in the order guanine, adenosine, adenine, guanosine, inosine and xanthine. Examination of enzyme activities in extracts of *A. castellanii* and *A. astronyxis* revealed the presence of deaminases, phosphorylases, kinases, PRTases and 5'-nucleotidases towards all substrates. AMP deaminase and 3'-AMP nucleotidase were not detected. The results of these studies suggest that hypoxanthine is of central importance in purine metabolism (Hassan and Coombs 1986d).

2.2.4.j. *Eimeria* species

Eimeria tenella appears to be unusual in that its purine PRTase is active towards hypoxanthine and guanine as well as xanthine (Wang and Simashkevich 1981). It has been suggested that this enzyme is of crucial importance for the parasite and that there is a correlation between allopurinol specifically inhibiting both the enzyme (Wang and Simashkevich 1981) and the growth of the parasite in cell culture (Ryley and Wilson 1972). *Eimeria tenella* also contain adenine PRTase and adenosine kinase but not inosine, guanosine or xanthosine kinase or phosphotransferase activities (Wang and Simashkevich 1981). Adenosine kinase has been purified from oocyst extracts of *E. tenella*, *E. acervulina* and *E. brunetti* (Miller *et al.* 1982) and found to accept some pyrazolopyrimidine ribosides as substrates whereas it is inhibited by others. It appears, however, that neither phosphorylation of these compounds by adenosine kinase nor their

inhibition of the enzyme correlates with the drugs' efficacy *in vivo* (Krenitasky *et al.* 1982). Recently it has been suggested that both sporozoites and merozoites of *E. tenella* contain adenine deaminase, adenosine deaminase, guanine deaminase and adenosine and guanosine nucleosidase activities; this explains their ability to interconvert purine bases and nucleosides (Lafon and Nelson 1985).

2.2.4.k. *Toxoplasma gondii*

It is technically difficult to study the incorporation of radioactive purines into actively growing, intracellular *T. gondii*, because the radiolabel becomes extensively incorporated into host cells as well as the parasites. This problem has been largely circumvented by growing the parasite in mutant host cells which lack hypoxanthine-guanine PRTase and so are unable to utilize hypoxanthine and guanine (Pfefferkorn 1981). When these mutant cells were infected with *T. gondii*, they readily incorporated hypoxanthine and guanine into nucleotides probably via hypoxanthine-guanine PRTase (Pfefferkorn 1981). Hypoxanthine PRTase and adenine PRTase activities have been detected in extracts of parasites (O'Sullivan *et al.* 1981). In other studies, adenosine, inosine and hypoxanthine were found to be readily incorporated into the nucleic acids of extracellular *T. gondii*, whereas incorporation of adenosine was decreased by 75% in mutants resistant to adenine arabinoside with defect in adenosine kinase (Schwartzman and Pfefferkorn 1982). These results points to the existence of two routes for adenosine utilisation-direct phosphorylation to AMP by adenosine kinase and hydrolysis by adenosine deaminase to yield inosine, which could be subsequently cleaved to hypoxanthine by purine nucleoside phosphorylase before incorporation into IMP.

2.2.4.1. *Plasmodium* species

The preferred purine for *Plasmodium* species appears to be hypoxanthine, which can be derived intraerythrocytically through the following pathway: ATP ⟶ ADP ⟶ AMP ⟶ IMP ⟶ inosine ⟶ hypoxanthine (Sherman 1979; Yamada and Sherman 1981a). *Plasmodium chabaudi* (Konigk 1977), *P. lophurae* (Yamada and Sherman 1981b) and *P. falciparum* (Reyes *et al.* 1982) have been found to possess the following purine salvage enzymes: adenosine deaminase, purine nucleoside phosphorylase, hypoxanthine-guanine PRTase, adenine PRTase and adenosine kinase. The adenosine deaminase from *P. lophurae* (Schimandle and Sherman 1983) and *P. falciparum* (Daddona *et al.* 1984) are unusual in their lack of sensitivity to the inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), though it is inhibited competitively by coformycin and deoxycoformycin. Unlike the purine nucleoside phosphorylase from human erythrocytes, the *P. lophurae* enzyme (Schimandle *et al.* 1985) consists of five identical subunits, favours inosine and hypoxanthine as substrates and is competitively inhibited by formycin B. Purification of PRTases from *P. chabaudi* revealed two distinct enzymes, one specific for hypoxanthine and guanine and the other for adenine (Walter and Konigk 1974). Only the former was competitively inhibited by the purine analogues 6-mercaptopurine, 2-amino-6-mercaptopurine and thioguanine. In addition, adenosine kinase purified from *P. chabaudi* was found to phosphorylate the adenosine analogues cordycepin, tubercidin and dimethylallyladenosine and enables them to enter the pathway of purine nucleotide synthesis (Schmidt *et al.* 1974). This probably explains the antimalarial activity of cordycepin and tubercidin (Trigg *et al.* 1971).

2.2.4.m. Piroplasms

While a little is known about purine salvage pathways of Babesia species, only limited information is available about those of Theileria species. Studies on the uptake of purines by Babesia-infected erythrocytes indicate that hypoxanthine is likely to be the major purine source in vivo (Irvine et al. 1978; Irvine and Young 1979). It has also been shown that some antibabesial drugs may affect hypoxanthine incorporation (Irvine and Young 1977) and suggested that such inhibition could be predictive of their in vivo activity. Recent evidence indicates that the rates of uptake of adenosine and hypoxanthine by B. bovis were markedly greater than those of adenine and guanosine (Conrad 1986). Studies on extracts of B. divergens (Hassan et al. 1986) confirmed the presence of relatively high activities of adenine deaminase, guanine deaminase, nucleoside phosphorylase, PRTases, kinases and 5'-AMP nucleotidase but failed to detect AMP deaminase and 3'-AMP nucleotidase. The results indicate that B. divergens is capable of synthesizing purine nucleotides by two routes, one involving PRTases and the other employing nucleoside kinases (Hassan et al. 1986).

Irvine and Stagg (1977) studied the uptake of adenine, hypoxanthine and guanine into Theileria parva-infected bovine lymphoid cells and found that adenine was the only one incorporated. Autoradiographic studies also revealed that there was no incorporation of hypoxanthine and guanine into macroschizonts of T. parva, whereas the host cells incorporated small amounts of the former. These findings, together with the failure of macroschizonts to incorporate 8-azaguanine and their rapid death in medium

containing hypoxanthine as the only purine, further indicate the absence of hypoxanthine-guanine PRTase in Theileria (Irvin and Stagg 1977).

2.2.4.n. Tetrahymena species

A survey of purine salvage enzymes in extracts of these free-living protozoa has indicated the presence of highly active adenosine deaminase, guanosine phosphorylase, inosine phosphorylase, adenosine kinase and PRTases wherease adenosine phosphorylase, adenine deaminase, guanine deaminase, AMP deaminase and guanosine kinase were not found (Hill and Chamber 1967; Hill 1972).

2.2.5. Mechanisms of purine uptake

While there have been numerous studies on the mechanisms of purine uptake into various mammalian systems, the systems of parasitic protozoa have been studied little. The bloodstream trypomastigotes of T. brucei and T. congolense have been shown to possess a high affinity uptake system for adenosine; inosine, guanosine, adenine and hypoxanthine were also transported, but less efficiently, although it is not known whether more than one transport system is involved (James and Born 1980). Studies on the mechanism of adenosine uptake into T. vivax revealed that there was a different pattern at higher concentrations (e.g. 10 mM). This suggested that the organism has more than one means of transporting adenosine, although the details of the two systems and their interrelationship remained to be established (Okachi et al. 1983). Hansen et al. (1982) studied adenine, hypoxanthine, adenosine and inosine uptake into promastigotes of L. b. braziliensis and found that they were transported by a mediated process at low concentrations and by diffusion at higher concentrations. Investigations of the

competitive interaction between these purines suggested the presence of at least three transport systems; one for adenine and hypoxanthine, a second for inosine and a third for adenosine. Recently, however, it has been demonstrated from genetic studies that L. donovani promastigotes contain two distinct nucleoside transport sites, one transports inosine, guanosine and formycin B, whereas the other transports pyrimidine nucleosides and the adenosine analogues, formycin A and tubercidin; adenosine itself, however, is transported by both systems (Iovannisci et al. 1984b). The situation in L. donovani contrasts to that of mammalian cells, into which all nucleosides and their analogues are transported by a common function (Cohen et al. 1979; Ullman et al. 1983). There are thought to be two transport systems in C. fasciculata, one of high affinity and low velocity and another of low affinity and high velocity (Kidder et al. 1978). Both will transport all purine bases. The lack of inhibition of transport by respiratory inhibitors such as azide, cyanide and dinitrophenol suggests the uptake of purines by C. fasciculata was not energy-dependent (Kidder et al. 1978).

Studies on the purine uptake into E. histolytica revealed that adenine, adenosine and guanosine can enter the amoeba by a carrier-mediated transport system (Boonlayangoor et al. 1978). This is consistent with the report of Reeves and West (1980) that E. histolytica has nutritional requirements for adenine which can be replaced by adenosine or AMP.

Tracy and Sherman (1972) showed that P. lophurae exhibited a high rate of mediated uptake of adenosine, inosine and hypoxanthine and suggested that these purines may have entered the free parasite through a common transport locus. Similar findings have also been reported for mammalian malaria parasites (Manadhar and Van Dyke

1975). On the other hand, P. berghei has been shown to take up purines both by diffusion and carrier-mediated systems and two transport loci, for adenosine-inosine-hypoxanthine and adenine, respectively, are thought to be present (Hansen et al. 1980). However, to take into account the possible deamination of adenosine at the surface of the parasite membrane, it was suggested that there is a transport locus specific for 6-oxypurines (Hansen et al 1980). Uptake into intracellular parasites such as intraerythrocytic malaria parasites involves entry into the host cell first. The parasite appears to modify this process for several substrates (Tracy and Sherman 1972) in particular both the number and characteristics of the adenosine transporter are changed (Hansen et al. 1980).

2.3. Pyrimidine metabolism

2.3.1. Introduction

Most mammalian cells so far investigated appear to be able to synthesize pyrimidine nucleotides via de novo synthesis that uses CO_2 and glutamine as precursors (Jones 1980; Peter and Veerkamp 1983; Rasenack et al. 1985). In contrast most are unable to convert pyrimidine bases to their respective nucleosides, although they can salvage pyrimidine nucleosides to produce their respective nucleotides (Strauss et al. 1985).

Parasitic protozoa, however, differ considerably in their abilities to synthesize and salvage pyrimidines. For instance, malaria parasites appear to rely almost entirely upon the de novo pathway for synthesis of their nucleotides (Sherman 1979), whereas Trichomonas and Giardia salvage all of their pyrimidine requirements (Wang 1983). Both routes are significant in trypanosomatids (Hammond and Gutteridge 1984) and Toxoplasma gondii (Pfefferkorn 1981).

2.3.2. De novo synthesis of UMP

The biosynthetic pathway leading to pyrimidine nucleotides (Fig. 3) appears to be common to most microorganisms (O'Donovan and Neuhard, 1970), mammals (Levine et al. 1974; Jones, 1980) and parasitic protoza (Gutteridge and Coombs, 1977) that have been studied in detail. The pathway commences with the formation of carbamoylphosphate from glutamine, ATP and CO_2 in a reaction catalysed by carbamoylphosphate synthetase (CPS), an enzyme located in the cytosol. In mammalian systems, an alternative source of carbamoylphosphate for pyrimidine biosynthesis is possibly available from the mitochondrial carbamoylphosphate synthetase, an ammonia-dependent enzyme (Tremblay et al. 1977). The condensation of aspartate and carbamoylphosphate, the first step committed to the pyrimidine pathway, is catalysed by aspartate transcarbamylase (ATCase). In the next step, removal of a water molecule from carbamoylaspartate by the action of dihydroorotase (DHOase), the pyrimidine ring is closed and dihydroorotic acid is produced, which is then oxidised to orotic acid by dihydroorotate dehydrogenase (DHODHase). A ribose-5-phosphate is then added to orotic acid by orotate phosphoribosyltransferase (OPRTase) to form orotidine-5-monophosphate which is subsequently decarboxylated by OMP decarboxylase (ODCase) to yield UMP.

Three main approaches have been used to establish the existence of de novo synthesis of pyrimidine nucleotides in parasitic protozoa.

1. Growth requirements

The development of minimal defined media that contain no pyrimidines but which support the growth of parasitic protozoa has shown that de novo synthesis of UMP occurs in these organisms (references given in section 2.2.2.). Indeed most parasitic protozoa

Fig. 3. De novo synthesis of UMP. Enzymes: 1, carbamoylphosphate synthetase (EC 2.7.2.5); 2, aspartate transcarbamylase (EC 2.1.3.2); 3, dihydroorotase (EC 3.5.2.3); 4, dihydroorotate dehydrogenase (EC 1.3.3.1); 5, orotate phosphoribosyltransferase (EC 2.4.2.10); 6, orotidine-5'-monophosphate decarboxylase (EC 4.1.1.23). Abbreviation: PRPP, phosphoribosylpyrophosphate.

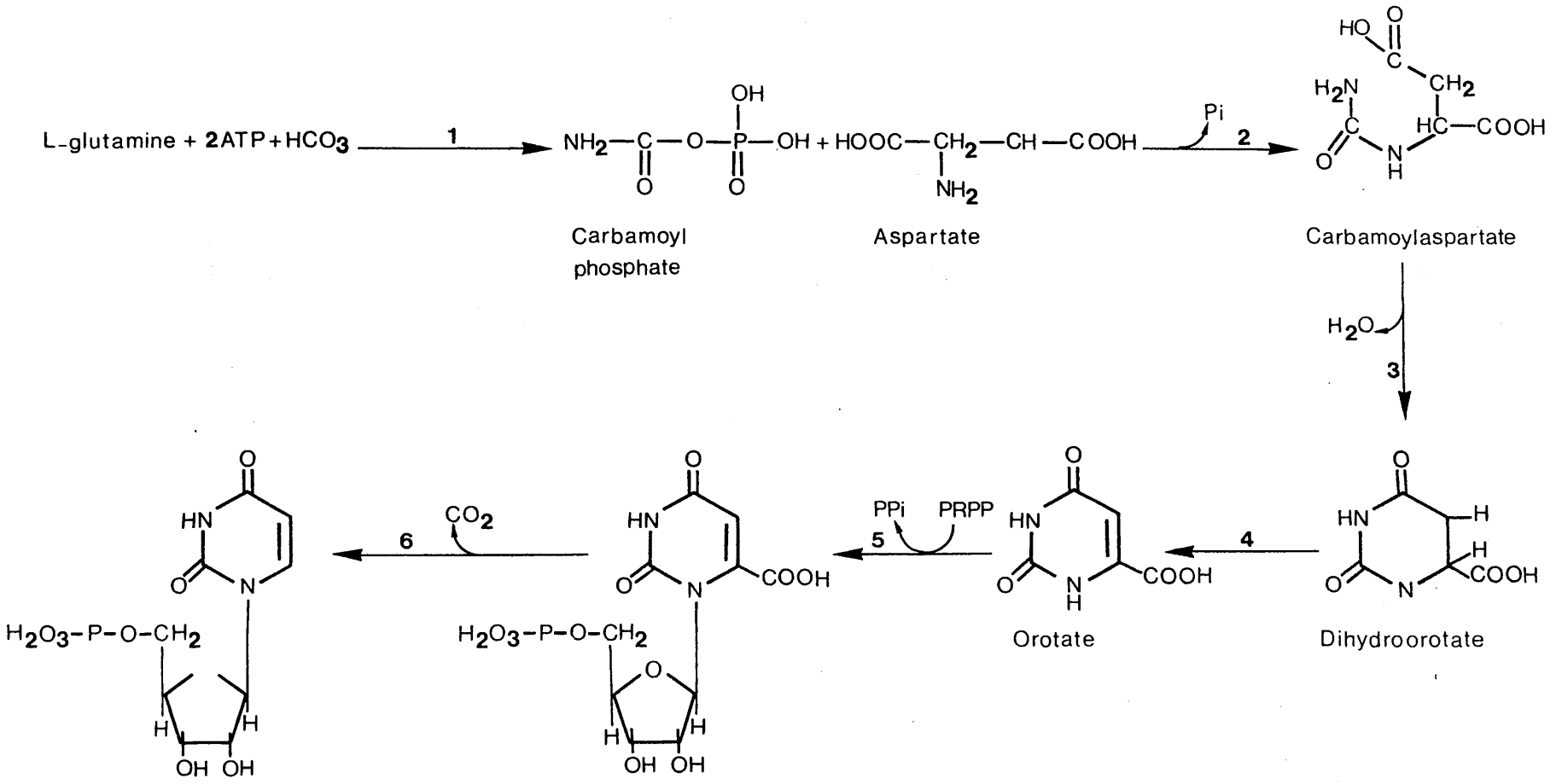

L-glutamine + 2ATP + HCO3
1
Carbamoyl phosphate
Aspartate
Pi
2
Carbamoylaspartate
H2O
3
Dihydroorotate
4
Orotate
PPi
PRPP
5
Orotidine 5'-phosphate
CO2
6
Uridine 5'-phosphate

Fig.3

studied appear to be able to synthesise UMP de novo (Gutteridge and Coombs 1977). Exceptions are trichomonads and Tetrahymena. Nutritional studies have shown that Trichomonas vaginalis (Linstead 1981) and Tritrichomonas foetus (Wang et al. 1984a) and the ciliate Tetrahymena pyriformis (Kidder et al. 1984) are unusual in that they require preformed pyrimidines for their growth, suggesting that these organisms are incapable of de novo synthesis of pyrimidine.

2. Labelling of nucleic acid pyrimidines by bicarbonate, orotate and aspartate.

Initial evidence for the presence of de novo biosynthesis came from the observation that radioactive bicarbonate, orotate and aspartate were incorporated into nucleic acid pyrimidines. Gutteridge and Gaborak (1979) demonstrated the incorporation of bicarbonate into nucleic acids of all forms of T. cruzi; low incorporation of orotate was also observed. In earlier studies, the incorporation of orotic acid into T. cruzi had not been detected (Yoneda et al. 1974a). Similarly L. donovani promastigotes (Lafon et al. 1982), T. gondii (Perrotto et al. 1971) and B. rodhaini (Irvine and Young 1979) incorporated orotic acid only at very low levels. It is now accepted, however, that the ability to incorporate orotic acid can be a poor index of pyrimidine biosynthetic capacity, mainly because some cells are relatively impermeable to this intermediate. Nevertheless, it has been shown that orotic acid is utilised for pyrimidine biosynthesis in T. equiperdum (Rubin et al. 1962), E. histolytica (Booden et al. 1973), P. knowlesi (Gutteridge and Trigg 1970), B. divergens and B. major (Irvin and Young 1979). Interestingly, C. fasciculata has been shown to incorporate the ureide carbon of citrulline into pyrimidines, this possibly occurs via carbamoylphosphate synthesis (Kidder et al. 1976). In addition

P. lophurae (Walsh and Sherman 1968) and T. gondii (Schwartzman and Pfefferkorn 1981) can incorporate bicarbonate and aspartate, respectively, into the pyrimidines of nucleic acids. These results confirm that the parasites are capable of synthesizing pyrimidine de novo. On the other hand, the anaerobic flagellates, Trichomonas vaginalis (Heyworth et al. 1984; Wang and Cheng 1984b), Tritrichomonas foetus (Jarrol et al. 1983; Wang et al. 1983b) and G. lamblia (Lindmark and Jarrol 1982; Aldritt et al. 1985) are known to lack de novo pyrimidine synthesis as confirmed by their inability to incorporate bicarbonate, aspartate and orotate into the pyrimidines of nucleic acids.

3. Enzyme analyses

The pyrimidine biosynthetic enzymes of members of the Kinetoplastida have been studied in some detail. All six enzymes have been detected in trypomastigote, epimastigote and amastigote forms of T. cruzi (Hammond and Gutteridge 1980), trypomastigotes of T. brucei and promastigotes of L. m. amazonensis (Hammond and Gutteridge 1982), promastigotes of L. tropica and culture forms of C. fasciculata (Hill et al. 1981a). The first three enzymes of this pathway, CPS, ATCase and DHOase are cytosolic in trypanosomatids similarly to other cells and evidence has recently emerged that these enzymes exist as separate unassociated proteins in C. luciliae (Tampitag and O'Sullivan 1986). This is in contrast to mammalian systems where these activities copurify as a multienzyme complex (Jones 1980). The last three enzymes of the pathway of members of the Kinetoplastida are of particular interest. In mammalian systems, the enzyme involved in the conversion of dihydroorotate to orotate is a dehydrogenase, which is particulate, mitochondrial and intimately connected to the respiratory chain to which it passes electrons

directly, probably at the ubiquinone level (Jones 1980). In contrast, the isofunctional enzyme of the Kinetoplastida is soluble and consumes oxygen but is not linked to the respiratory chain (Hammond and Gutteridge 1984). The results of earlier studies with C. fasciculata (Kidder and Nolan 1973; Kidder et al. 1976), several species of trypanosomes and L. enriettii (Gutteridge et al. 1979) lead to the postulation that the enzyme responsible for this conversion is a hydroxylase which required tetrahydrobiopterin for maximum activity. Such a hydroxylase mechanism, however, was not consistent with the findings of Gero and Coombs (1982a) working on amastigote or promastigote forms of L. m. mexicana. Recently, Pascal et al. (1983) presented evidence that the enzyme purified from C. fasciculata and T. brucei exhibits a characteristic flavin electronic spectrum suggesting that dihydroorotate oxidation is mediated by flavoprotein oxidases rather than pterin-linked hydroxylases. Consequently they have suggested that the name dihydroorotate oxidase is appropriate for the enzyme. The last two enzymes of UMP synthesis, OPRTase and ODCase, which are cytosolic and exist as an enzyme complex in higher eukaryotes (Jones 1980) appear to be particulate in trypanosomatids (Rubin et al. 1962); Gero and Coombs 1980a; Hammond and Gutteridge 1982) and associated with the glycosomes at lease in T. brucei trypomastigotes, L. m. amazonensis promastigotes (Hammond et al. 1981), T. cruzi epimastigotes (Hammond and Gutteridge 1983) and C. luciliae (Pragobpol et al. 1984a). It has been suggested that the glycosomal location of the OPRTase-ODCase enzymes could be favourable for the direct channeling of OMP, the product of OPRTase, to ODCase, the next enzyme in the pathway. Hammond and Gutteridge (1983), however, were unable to detect latency of OPRTase and they found that various inhibitors were as effective against the enzyme activity of intact glycosomes as that in broken

glycosomal preparations. They suggested, therefore, that both enzymes are located on the outside of the glycosome and are freely accessible to the cytoplasm. A particularly intriguing finding was that the promastigotes of L. m. mexicana contained 40 times more OPRTase/ODCase activity than amastigotes (Gero and Coombs 1980a). In addition OPRTase has also found in extracts of L. tarentolae and Herpetomonas species (Hassan and Coombs 1986b) These differences found between the trypanosomatid and mammalian enzymes provide promising targets for chemotherapeutic attack.

Despite the apparent lack of de novo pyrimidine synthesis from radiolabeled precursors in Trichomonas vaginalis, there is uncertainty as to whether the enzymes are present. Hewyorth et al. (1979) reported the presence of all six enzymes of UMP synthesis in T. vaginalis, whereas Hill et al. (1981a) confirmed the presence of only CPS. Subsequent attempts to detect OPRTase and ODCase in T. vaginalis extracts proved negative (Gero and Coombs 1980b; Heyworth et al. 1984), which could explain why the parasite is devoid of pyrimidine de novo synthesis. The lack of detectable levels of CPS, ATCase, DHOase, DHODHase and OPRTase in Tritrichomonas foetus (Jarrol et al. 1983) and G. lamblia (Lindmark and Jarrol 1982) correlates well with the observed lack of de novo pyrimidine synthesis in these parasites.

Toxoplasma gondii has been reported to have all six enzymes of the de novo UMP synthesis (Hill et al. 1981a; O'Sullivan et al. 1981; Asai et al. 1983). CPS, ATCase and DHOase exist as separate entities. DHODHase is particulate, sensitive to respiratory chain inhibitors and is probably connected to oxygen utilisation through the respiratory chain; though the nature of linkage remains to be established. OPRTase and ODCase copurified in a single protein

complex, with the former being completely inhibited with oxipurinol but not allopurinol. The lack of detectable levels of CPS and DHOase, together with the low activities of ATCase, OPRTase and ODCase, in the oocyst of E. tennella suggests that the de novo synthesis of pyrimidine is of little importance to this resting stage of the parasite's life cycle (Hill et al. 1981a). As yet there is no information available on the enzymes of de novo pyrimidine biosynthesis in the trophozoit forms of Eimeria species.

Plasmodium species have been shown to contain all the enzymes required for the synthesis of UMP by the de novo pathway (Hill et al. 1981b; Reyes et al. 1982; Gero et al. 1984). As in fungi (Makoff and Radford 1978) and other higher eukaryotes (Jones 1980), CPS and ATCase in P. berghei are associated in a single enzyme complex (Hill et al. 1981b). The DHODHases from various plasmodia are particulate and linked to the respiratory chain; their activity is inhibited by respiratory chain inhibitors (Krooth et al. 1969; Gutteridge et al. 1979; Gero et al. 1981a, 1981b, 1984). Hammond et al. (1985b) suggested that the inhibition of DHODHase by 2-(4-t-butylcyclohexyl)-3-hydroxy-1,4-naphthoquinone is due to inhibition of the respiratory chain at the ubiquinone step, where it differs significantly from mammals, The compound causes a gradual decrease in UTP levels and cessation of P. falciparum growth in vitro. OPRTase and ODCase are mainly soluble in Plasmodium species (Walsh and Sherman 1968; O'Sullivan and Ketley 1980; Gero et al. 1981b) and reside on separate and distinct proteins in P. falciparum (Rathod and Reyes 1983). Recently Scott et al. (1986), from studies the effect of pyrazofurin on P. falciparum in vitro, suggested that the inhibition of ODCase might be its mechanism of action. Plasmodium species rely entirely on UMP synthesis for their pyrimidine requirements as they are unable to salvage pyrimidines or their nucleosides and therefore

this pathway offers several opportunities for the design of new antimalarial drugs (Sherman 1979).

A functional pathway of de novo pyrimidine synthesis has also been demonstrated in various Babesia species (Gero and Coombs 1982b; Gero et al. 1983; Holland et al. 1983). CPS, ATCase and DHOase all are soluble, with the former being unstable. DHODHase is particulate and is inhibited by respiratory chain inhibitors (Gero and Coombs 1982b); it seems likely that it is associated with the respiratory chain to which it passes electrons via ubiquinone. OPRTase and ODCase are soluble and neither activity was inhibited by allopurinol or oxipurinol. It remains to be determined whether the first three enzymes or the last two enzymes exist as complexes or as separate proteins.

As yet, no information is available on the enzymes of de novo pyrimidine biosynthesis in Theileria, Entamoeba and Acanthamoeba species.

The regulation of pyrimidine nucleotide biosynthesis in microorganisms has been extensively studied. It appears to be mediated by inhibition of the first two enzymes, CPS and ATCase, by CTP or UTP and also by repression or depression of the synthesis of individual enzymes. There are some data on the regulation of pyrimidine biosynthesis in a few parasitic protozoa. For instance, in T. gondii ATCase does not appear to be the controlling enzyme of the pathway as it is relatively insensitive to feedback inhibition by UTP and CTP (Asai et al. 1983). Similarly, the lack of inhibition or stimulation by pyrimidine nucleotides of the ATCase of Plasmodium berghei suggests that CPS could be the point of metabolic control in this parasite (Hill et al. 1981b). In P. falciparum, CPS, which

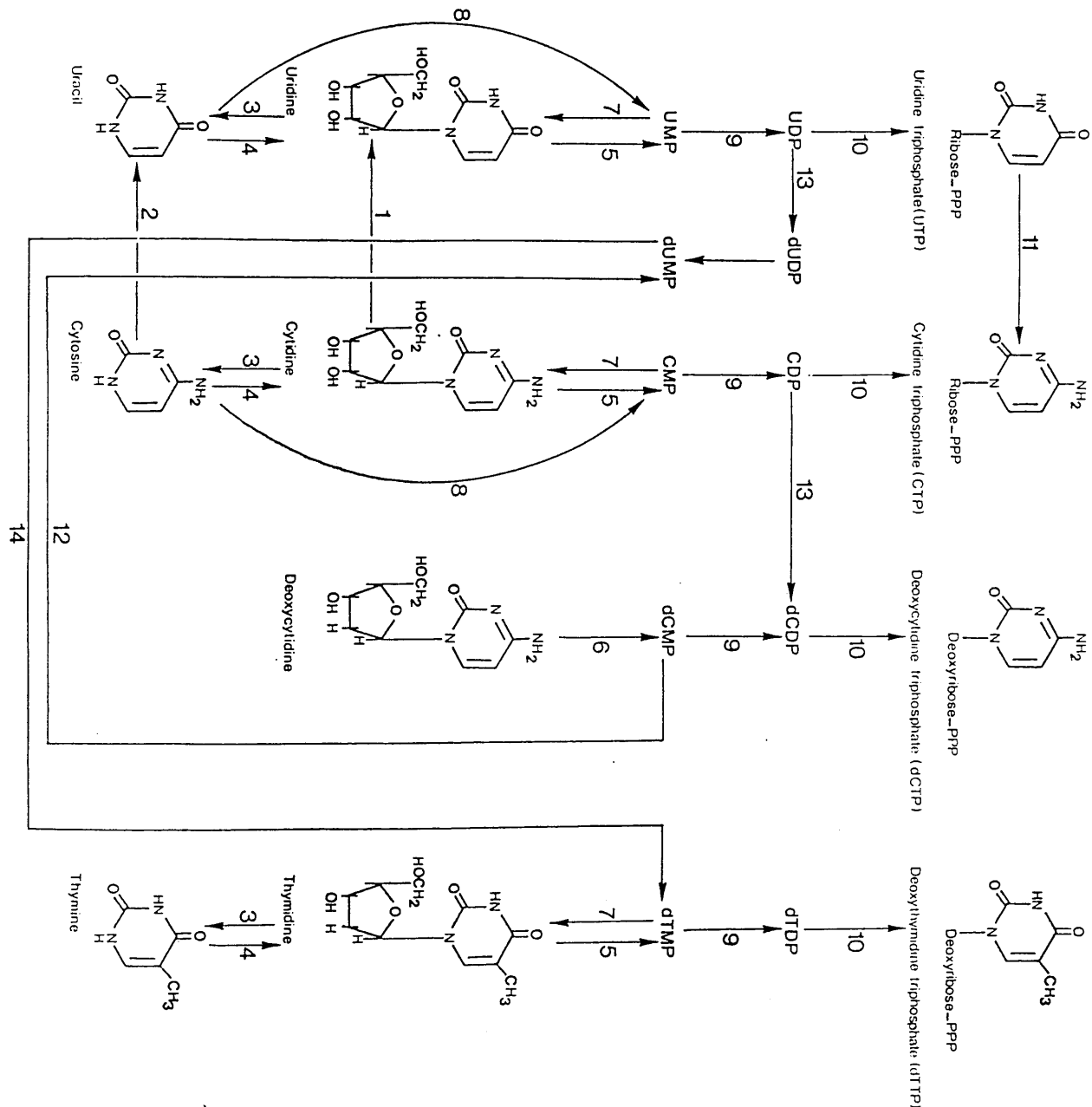
utilises glutamine more effectively than ammonia, appears to be a locus of control as it is inhibited by UTP (Gero et al. 1984).

2.3.3. Pyrimidine salvage

As described above, most groups of parasitic protozoa can synthesise pyrimidines de novo. Nevertheless, most parasitic protozoa so far studied also have some salvage pathways. The importance of these pathways as a source of pyrimidine nucleotides, or their role in the regulation of the cellular concentrations of these nucleotides, varies greatly. The evidence for the existence of the salvage pathways comes mainly from studies demonstrating the incorporation of radioactive uracil, uridine, cytosine, cytidine, thymine and thymidine into nucleic acid pyrimidines and also from the isolation and characterization of the enzymes involved in pyrimidine salvage. These are shown schematically in Fig. 4. The pyrimidine nucleosides can be cleaved by pyrimidine nucleoside phosphorylase or pyrimidine nucleosidase (hydrolase) to their correspondent bases and ribose-1-phosphate. Thymidine phosphorylase is present in most cell types (Peters and Veerkamp 1983). Pyrimidine nucleoside phosphorylase also occurs in some bacteria and can catalyse the synthesis of pyrimidine nucleosides from bases and ribose-1-phosphate (Neuhard 1983). The pyrimidine nucleosides can also be converted by pyrimidine nucleoside kinase to their nucleotides. The other route for the synthesis of UMP is by uracil phosphoribosyltransferase (UrPRTase) from uracil and PRPP. In most organisms, the only route for cytosine utilisation is by deamination to uracil catalysed by cytosine deaminase. The search for cytosine deaminase in a variety of parasitic protozoa has so far proved negative, whereas the enzyme of other microorganisms has been well studied (Neuhard 1983). Cytidine deaminase is another enzyme that occurs in some cells, this

Fig. 4. Salvage and interconversion of pyrimidines. Enzymes: 1, cytidine deaminase (EC 3.5.4.5); 2, cytosine deaminase (EC 3.5.4.1); 3, nucleosidase (EC 3.2.2.8) or phosphorylase (catabolic) EC 2.4.2.3, EC 2.4.2.4); 4, phosphorylase (anabolic) (EC 2.4.2.3); 5, nucleoside kinase (EC 2.7.1.48, EC 2.7.1.21); 6, deoxycytidine kinase (EC 2.7.1.74); 7, nucleotidase (EC 3.1.3.5); 8, phosphoribosyltransferase (EC 2.4.2.9.); 9, nucleoside-5-monophosphate kinase (EC 2.7.4.14); 10, nucleoside-5'-diphosphate kinase (EC 2.7.4.6); 11, CTP synthetase (EC 6.3.4.2); 12, deoxycytidylate deaminase (EC 3.5.4.12); 13, ribonucleotide reductase (EC 1.17.4.1); 14, thymidylate synthetase (EC 2.1.1.45).

Fig.4



Modified from Gutteridge and Coombs 1977

deaminates cytidine/deoxyctidine to uridine/deoxyuridine which can then be utilised for UMP/dUMP synthesis.

2.3.3.a. Trypanosomatids

Rubin et al. (1962) and Momparler and Jaffe (1965) studied the utilisation of pyrimidines by T. equiperdum and found that uracil is readily incorporated into nucleic acids possibly via UrPRTase. Gutteridge and Gaborak (1979) reported that uracil is incorporated by amastigotes at much lower rate than by epimastigotes and trypomastigotes of T. cruzi. In confirmation of this finding, UrPRTase was present in epimastigotes and trypomastigotes but could not be detected in amastigote forms (Hammond and Gutteridge 1982). These workers also reported that UrPRTase is soluble enzyme and quite distinct from glycosomal OPRTase. UrPRTase has also been demonstrated in cell free extracts of L. m. amazonensis, L. tarentolae, C. fasciculata (Hammond and Gutteridge 1982) and C. luciliae (Tampitag and O'Sullivan 1986). The utilisation of uridine for nucleic acid synthesis has been reported for various trypanosomes (Chang et al. 1978; Gutteridge and Gaborak 1979), leishmanial species (Bachrach et al. 1979; Mukkada et al. 1985) and C. fasciculata (Cosgrove et al. 1979). Uridine phosphorylase and uridine nucleosidase have been detected in T. cruzi, T. brucei, various leishmanial species, C. fasciculata, C. luciliae and Herpetomonas species (Dewey and Kidder 1973; Koszalka and Krenitsky 1979; Hammond and Gutteridge 1982; Miller et al. 1984; Tampitag and O'Sullivan 1986; Hassan and Coombs 1986b). Uridine kinase was not found in L. m. amazonensis, T. cruzi, T. brucei (Hammond and Gutteridge 1982) and C. luciliae (Tampitag and O'Sullivan 1986). Similarly T. equiperdum seems deficient in uridine kinase (Jaffe 1961) but it has been postulated, although with no experimental

evidence presented, that C. fasciculata have this activity (Dewey et al. 1978). This enzymic deficiency in members of the Kinetoplastida stands in contrast to the observation that uridine kinase is present in most mammalian cells thus far studied (Kepler and Holstege 1982). Thus, it appears that uridine utilisation by trypanosomatids is likely to involve conversion to uracil via uridine phosphorylase or nucleosidase with the uracil subsequently being converted to UMP by UrPRTase. All forms of T. cruzi have been found able to utilize cytidine for nucleic acid synthesis (Gutteridge and Gaborak 1979) whereas T. equiperdum has extremely limited ability to incorporate cytosine or cytidine (Momparler and Jaffe 1965). Cytidine deaminase is present in C. fasciculata and T. cruzi (Kidder 1984), leishmanias, Herpetomonas and T. brucei (Hassan and Coombs 1986b). In addition, cytidine nucleosidase is found in C. fasciculata (Dewey and Kidder 1973) and L. donovani (Koszalka and Krenitisky 1979). It has been inferred that the inability of L. tropica and L. donovani to utilise deoxycytidine is due to lack of deoxycytidine kinase (Konigk 1976). This contrasts markedly with the situation in T. gambiense, where the uptake of deoxycytidine is significant (Konigk 1976). Thymidine but not thymine is utilised effectively by most trypanosomes for nucleic acid synthesis (Pizzi and Taliaferro 1960; Al Chalabi and Gutteridge 1977a; Cosgrove et al. 1979; Gutteridge and Gaborak 1979; Lafon et al. 1982; Mukkada et al. 1985). Thymidine phosphorylase is reported to be present in various trypanosomes but not C. oncopelti (Al Chalabi and Gutteridge 1977a), leishmanias, Herpetomonas and C. fasciculata (Hassan and Coombs 1986b). Of the range of trypanosomatids studied, only L. m. mexicana promastigotes and C. fasciculata have been found to contain thymidine nucleosidase (Hassan and Coombs 1986b). Thymidine kinase has been observed in T. b. rhodesiense, and to be controlled by feedback inhibition from TTP

(Chello and Jaffe 1972), whereas L. donovani has been found to contain thymidine phosphotransferase instead (Krenitsky et al. 1980).

2.3.3.b. Trichomonads

Pyrimidine salvage becomes indispensable for trichomonads as well as Giardia as they apparently lack de novo pyrimidine biosynthesis (Wang 1983). Trichomonas vaginalis (Wang and Cheng 1984b; Heyworth et al. 1984) and Tritrichomonas foetus (Jarrol et al. 1983; Wang et al. 1983b) can incorporate uracil, uridine, cytidine and thymidine into pyrimidine nucleotides. Cytosine and thymine are not incorporated. Whereas uracil is most effectively utilised by T. foetus, uridine and cytidine are preferentially utilised by Trichomonas vaginalis. Both parasites are able to incorporate thymidine into DNA, possibly via particulate thymidine phosphotransferase (Wang 1983) although this enzyme was not found in a different strain of T. vaginalis (in another study) (Miller and Linstead 1983). The apparent absence of thymidylate synthetase in trichomonads (see section 2.3.5.) makes thymidine phosphotransferase essential for DNA synthesis and so an attractive target for chemotherapeutic attack. Tritrichomonas foetus has very high UrPRTase activity which enables incorporation of uracil into UMP as one major pyrimidine salvage pathway; Trichomonas vaginalis, however, has very little of this activity. UrPRTase seems to be of crucial importance to the Tritrichomonas foetus for it has been observed that 5-flourouracil, which specifically inhibits this enzyme, is an inhibitor of growth of T. foetus in vitro (Wang et al. 1983b). It seems most likely that the incorporation of uridine and cytidine into T. foetus takes place either through a phosphotransferase or the prior conversion to uracil by the actions of uridine phosphorylase and cytidine deaminase (Wang 1983). It has

been reported that uridine kinase was also found in extracts of T. foetus (Jarrol et al. 1983), suggesting the existence of an another route for converting uridine to UMP, although this was not confirmed by Wang et al (1983b). Indeed, published results indicate that there are several distinct differences in pyrimidine salvage between strains of trichomonads. For instance, Trichomonas vaginalis strains ATCC 30001 (Wang and Cheng 1984b) and Bushby (Miller and Linstead 1983; Heyworth et al. 1984). Pyrimidine nucleoside phosphotransferase are reported to predominate in the former whereas pyrimidine nucleoside kinases are present in the latter. Uridine kinase has been recently isolated from both strains. The results support the previous work on the Bushby strain as it has been shown that the enzyme does not phosphorylate thymidine (Miller and Miller 1985b). It is yet to be determined whether these discrepancies can be explained by differences in growth conditions or analytical methodology or are true strain differences.

2.3.3.c. Giardia lamblia

The means of pyrimidine salvage in G. lamblia are very similar to those of trichomonads in that uracil and thymidine are salvaged predominantly by UrPRTase and thymidine phosphotransferase, respectively, to supply necessary nucleotides for the parasites (Lindmark and Jarrol 1982; Aldritt et al. 1985b). Uridine and cytidine may also be degraded to uracil through the actions of uridine nucleosidase and cytidine deaminase before utilisation for nucleotide synthesis. Uracil and uridine may also be converted to cytosine nucleotides probably via CTP synthetase. The major differences in pyrimidine salvage between G. lamblia and trichomonads seems to be that Giardia contains cytidine nucleosidase and cytosine PRTase activities.

2.3.3.d. Amoebae

Autoradiographic studies have shown that E. histolytica can incorporate thymidine and uridine into DNA and RNA, respectively (Albach et al. 1966; Sharma et al 1969). Booden et al. (1976) have also shown that E. histolytica can incorporate cytidine, uracil, cytosine and thymine into nucleic acids. It was also reported that uridine and cytidine are taken up by the amoeba by a carrier-mediated transport system whereas uracil, cytosine, thymine and thymidine enter via passive diffusion (Booden et al. 1978). Acanthamoeba species have also been shown to incorporate uridine and thymidine into their nucleic acids (McIntosh and Chang 1971; Jantzen 1974). As yet, however, there have been no reports on the pyrimidine salvage enzymes that occur in these amoebae.

2.3.3.e. Toxoplasma gondii

Studies on T. gondii growing in mutant host cells deficient in pyrimidine salvage activities showed that the parasite incorporated uracil, uridine and deoxyuridine (Pfefferkorn 1981). Similarly it was shown that labelled thymidine and cytidine were incorporated into parasite nucleic acid (Perrotto et al. 1971; Yoneda et al. 1974b). It has been suggested that uracil and uridine enter the nucleotides of T. gondii through the action of UrPRTase (Pfefferkorn 1978) and uridine kinase (O'Sullivan et al. 1981), respectively. Uridine phosphorylase has also been detected in extracts of T. gondii (Pfefferkorn and Pfefferkorn 1977).

2.3.3.f. Eimeria species

Little is known about pyrimidine salvage in Eimeria species. Unfortunately, no information is yet available about pyrimidine salvage enzymes in Eimeria species. Autoradiographic experiments

with Eimeria-infected host cells revealed incorporation of uridine and cytidine but not thymidine into the nucleic acids of the parasite (Roberts et al. 1970; Ouellette et al. 1973). It is possible that uridine becomes phosphorylated when it enters eimerian parasites, and then may be incorporated directly into RNA or may be reduced and then dephosphorylated to yield deoxythymidylic acid, thus methylation of the latter would result in thymidylic acid avialable for DNA synthesis (Ouellette et al. 1974). However, these findings could suggest a possible key role of uridine for pyrimidine nucleotide synthesis by Eimeria. Interestingly, many uridine analogues exhibit activity against Eimeria species (Ryley et al. 1974; Matsuno et al. 1984; Kobayashi et al. 1986).

2.3.3.g. Plasmodium species

There is now ample evidence to suggest that Plasmodium species are unable to utilise preformed pyrimidine bases and nucleosides and must, therefore, obtain them by de novo synthesis (Sherman 1979). It has been reported that of a variety of radioactive pyrimidines tested (uracil, uridine, cytosine, cytidine, thymine and thymidine) none was utilised by the intraerythrocytic form of the parasite (Bungener and Nielson 1967; Walsh and Sherman 1968; Gutteridge and Trigg 1970; Van Dyke et al. 1970; Lantz et al. 1971; Neame et al. 1974). Konigk (1976) found that the uptake of deoxycytidine by P. chabaudi-infected erythrocytes could possibly occur via deoxycytidine kinase; this pathway could provide deoxycytidylate which becomes deaminated to deoxyuridylate, the substrate of the thymidylate synthetase. Most recently, Reyes et al. (1982) presented evidence that P. falciparum apparently lacks uridine-cytidine kinase, deoxycytidine kinase, thymidine kinase, and cytidine deaminase for salvage of pyrimidine

nucleotides, thereby confirming that the salvage of pyrimidine bases and nucleosides pathway is not occurring. UrPRTase was detected, but at a very low activity level.

2.3.3.h. Piroplasms

Irvin and colleague (1978, 1979) reported that murine species of Babesia (B. microti and B. rodhaini) incorporated labelled uridine and thymidine into nucleic acids to a greater extent than bovine species (B. divergens and B. major). This implies that the murine strains rely more on salvage pathways for their pyrimidine requirements, whereas de novo synthesis is more important in the bovine strains. Irvin and Stagg (1977) also reported that the incorporation of thymidine into T. parva was high enough to predict the presence of thymidine kinase; uridine was also incorporated but without evidence of specific localisation. As yet,however, there have not been any reports on the occurrence of pyrimidine salvage enzymes in Babesia and Theileria species.

2.3.3.i. Tetrahymena species

The inability of T. pyriformis, to synthesize the pyrimidine ring makes them dependent upon an exogenous supply of pyrimidine for growth (Hill 1972). Extensive examination of the enzymes in T. pyriformis extracts revealed the presence of highly active cytidine/deoxycytidine deaminase, thymidine phosphorylase, uridine/deoxyuridine phosphorylase, UrPRTase and thymidine kinase activities. In contrast cytidine-uridine kinase and cytosine PRTase could not be detected (Hill and Chambers 1967; Hill 1972; Wykes and Prescot 1968; Yuyama et al. 1979).

2.3.4. Interconversion of pyrimidine nucleotides

The pathways for UMP biosynthesis (whether synthesized de novo or via salvage of preformed pyrimidine bases and nucleosides) represent only the first of three equally important phases of nucleotide biosynthesis. A second phase consists of the conversion of UMP to UTP and CTP, whereas the third phase involves the formation of deoxyribonucleotides. Once UMP is available, it can be phosphorylated by kinases through UDP to UTP in a two step reaction involving ATP as the phosphoryl donor. UTP is then aminated to CTP by the enzyme CTP synthetase which utilises glutamine (mammals) or ammonia (bacteria) as the amine donor.

As yet, no direct information is available on CTP synthetase in parsitic protozoa. Although there are no reports on the presence of this enzyme in trypanosomes or Leishmania, it has been shown that both UTP and CTP are produced (Hammond and Gutteridge 1984; Marr and Berens 1985). Similarly for Trichomonas vaginalis (Heyworth et al. 1984) and Tritrichomonas foetus (Wang et al. 1983b), incubation of cells with either (^{3}H) cytidine or (^{3}H) uridine resulted in labelling of both uracil and cytosine nucleotide pools which, together with the finding of cytidine deaminase, suggests that CTP synthetase is present in these parasites.

A continuous and balanced supply of deoxyribonucleoside triphosphates is required during DNA synthesis. In mammalian cells, these are produced by the reduction of ribonucleotides. This reduction occurs at the ribonucleoside diphosphate level and is catalysed out by ribonucleoside diphosphate reductase (Thelander and Reichard 1979). This reaction requires thioredoxin, thioredoxin reductase and NADPH as a cofactor (Fig. 5). In some bacteria; such as Lactobacillus leichmannii, the reduction of ribonucleotides is catalysed by a single complex protein which reduces all four

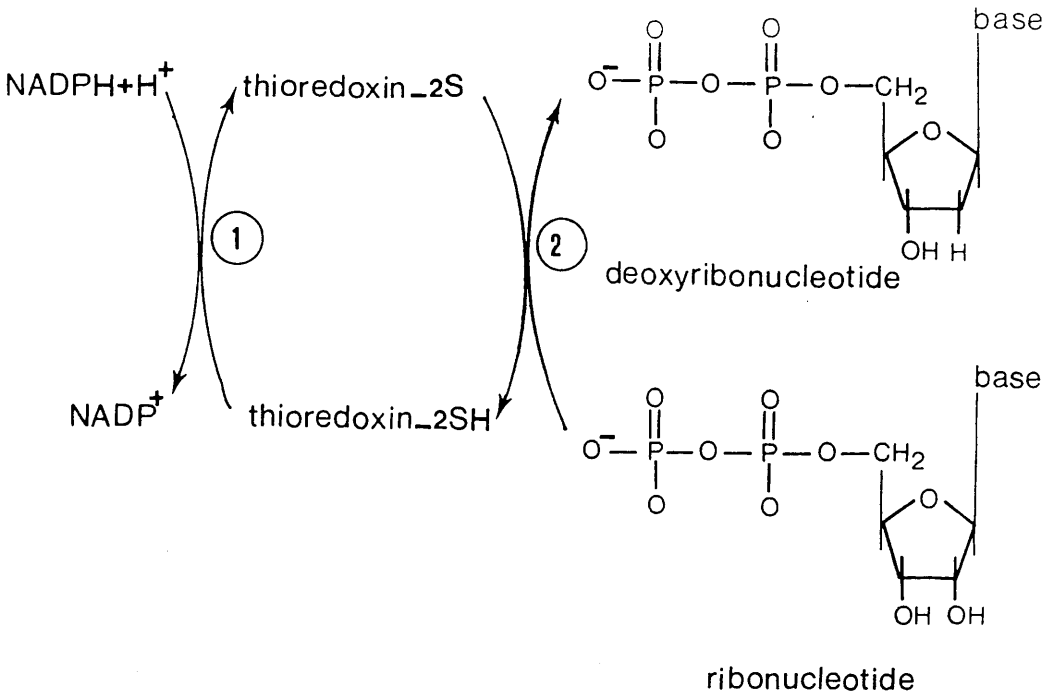


Fig. 5. Transformation of ribonucleotide to deoxyribonucleotide form. Enzymes: 1, thioredoxin reductase (EC 1.6.4.5); 2, ribonucleotide reductase (EC 1.17.4.1).

naturally occurring ribonucleoside triphosphates (ATP, CTP, UTP, and GTP) to their corresponding 2'-deoxyribonucleoside triphosphates (dATP, dCTP, dUTP and dGTP) (Mollgaard and Neuhard 1983). This enzyme has an absolute requirement for 5'-deoxy-5-adenosylcobalamine (coenzyme B12) and can use lipoic acid or dithiothreitol or thioredoxin as reducing agents.

There is little known about the formation of deoxyribonucleotides in parasitic protozoa. Some evidence has been obtained for some parasites indicating that the reduction occurs at the diphosphate level. Ribonucleoside diphosphate reductase has been shown to be present in T. foetus (Wang 1983) and constitutes the first report of this enzyme in any parasitic protozoa. The enzyme is not dependent upon coenzyme B12 and also is insensitive towards hydroxyurea (Wang 1983) with the latter not effecting the parasite's growth. Hydroxyurea, however, is a specific inhibitor of most ribonucleoside diphosphate reductases, and its addition to growing cultures of C. fasciculata (Cosgrove et al. 1979) and T. gondii (Kasper and Pfefferkorn 1982) resulted in a rapid inhibition of the cell division in these organisms. Hydroxyurea also inhibits the growth of L. tarentolae (Simpson and Braly 1970) and T. b. brucei (Brun 1980) and growth and transformation of L. m. mexicana (Hart et al. 1981). These tentative conclusions about the presence of the enzyme, however, must be confirmed by direct enzyme assays. In contrast to T. foetus, Trichomonas vaginalis lacks ribonucleoside diphosphate reductase but contains instead deoxyribonucleoside phosphotransferase (Wang and Cheng 1984a). This enzyme can transfer the phosphate group from p-nitrophenylphosphate to thymidine, deoxyadenosine, deoxyguanosine and deoxycytidine with the formation of TMP, dAMP, dGMP and dCMP, respectively (Wang and Cheng 1984a).

The enzyme has an estimated molecular weight of 200,000 daltons, apparent K_m values of 2-3 mM for the four deoxyribonucleosides and optimal activity in the range pH 5.0-6.0. It seems likely that this enzyme may provide the main mechanism for supplying T. vaginalis deoxyribonucleotides and so provides a good target for antitrichomonial chemotherapy (Wang and Cheng 1984a).

2.3.5. Biosynthesis of thymine nucleotides

The formation of thymine nucleotides is a strategically important part of the complex of metabolic events which provides nucleotide building blocks for DNA replication. The strategic importance and the complex reactions leading to thymidylate have led to suggestions that DNA synthesis may be influenced or even regulated through such reactions. Deoxythymidylic acid (TMP) is formed from deoxyuridylic acid (dUMP) by the enzyme thymidylate synthetase (TS) (Fig. 6). In this reaction, a methylene group and two reducing equivalents are transferred from 5,10-methylenetetrahydrofolate to dUMP. The products are TMP and dihydrofolate. Regeneration of tetrahydrofolate occurs in a reaction catalysed by dihydrofolate reductase (DHFR) with NADPH as the electron donor. Thymidylate synthetase and DHFR exist as a bifunctional protein in the Trypanosomatidae (Al Chalabi and Gutteridge 1977b; Ferone and Roland 1980; Yaseen and Al Chalabi, 1980; Garrett et al. 1984), Plasmodium species (Ferone et al. 1970, Pattanakitsakul et al. 1985) and E. tenella (Wang et al. 1975; Coles et al. 1980; Garrett et al. 1984), whereas the enzymes from bacteria, yeast and mammalian cells are distinct and readily separable (Blakley 1969). It has been reported that L. major promastigotes, selected for resistance to the DHFR inhibitor methotrexate or the TS inhibitor 10-propargyl-5,8 dideazofolate, overproduce the bifunctional TS-DHFR and show two

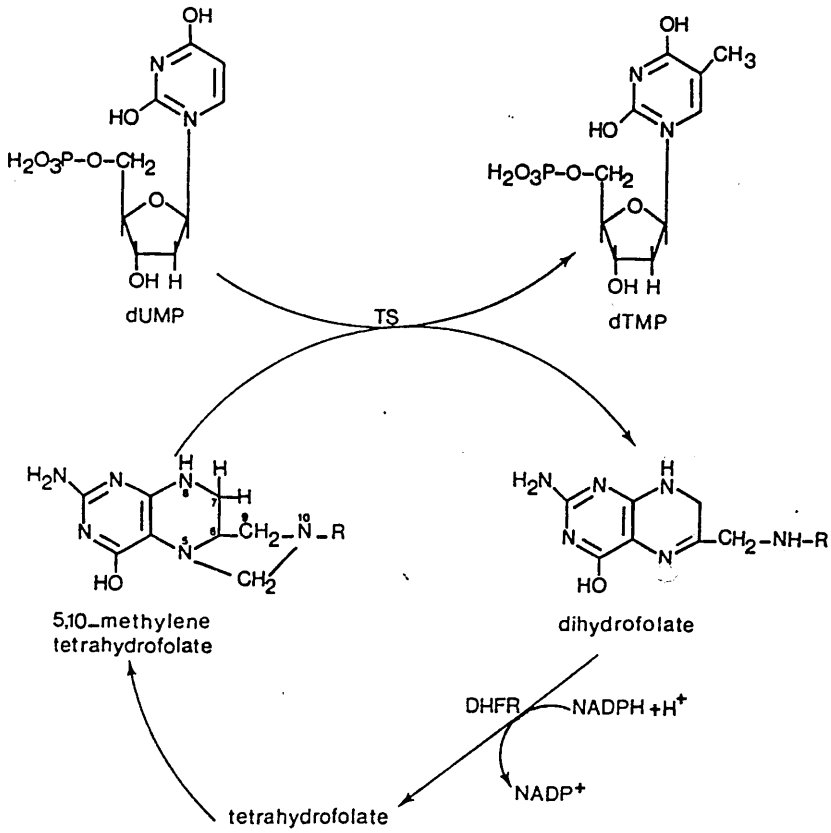


Fig. 6. Methylation of dUMP to dTMP and its relation to folic acid metabolism. TS, thymidylate synthetase (EC 2.1.1.45); DHFR, dihydrofolate reductase (EC 1.5.1.3).

distinct and uniform amplified regions of DNA (Coderre et al. 1983; Beverly et al. 1984; Garvey et al. 1985; Washtein et al. 1985). The bifunctional TS-DHFR has been purified to homogeneity from the promastigotes of L. major and found to have two identical subunits of molecular weight 55,000 daltons (Meek et al. 1985). The protein has been found to be extremely susceptible to proteolysis, which results in selective destruction of TS activity and the generation of fragments of 36,000 daltons and 20,000 daltons (Garvey and Santi 1985). The limited proteolysis does not separate the protein into its two components (TS and DHFR), however, but rather it is cleaved at a highly vulnerable site of the TS sequence. Data from proteolysis experiments, amino acid sequencing and nucleic acid sequencing indicate that DHFR occurs on the N-terminal region of the protein whereas TS is towards the carboxy terminal region (Garvey and Santi 1985). In C. fasciculata, the resistance to methotrexate has found to be associated with a decrease in the rate of transport of the drug into the cell and not with amplification of the DHFR gene as in the case of L. major (Dewes et al. 1986). On the other hand, investigation of pyrimethamine resistance in Plasmodium species revealed both an increased amount of DHFR as well as changes in its kinetic properties (Ferone et al. 1970; Diggens et al. 1970). Recent evidence, however, indicates that the resistance to pyrimethamine in P. chabaudi (Sirawaraporn and Yuthavong 1984) and in P. falciparum (Walter 1986) is not correlated with an increase in DHFR content but is most likely due to genetic changes leading to a structurally different enzyme. Trichomonas vaginalis, Tritrichomonas foetus and G. lamblia were found to lack TS and DHFR, which enables them to grow normally in the presence of potent antifolates or TS inhibitors such as methotrexate, pyrimethamine, trimethoprim and 6-

azauridine (Wang 1983). Thymidylate synthetase and DHFR also could not be detected in E. histolytica and E. invadens (Garrett et al. 1984).

References

Adam, K.M.G. 1959. The growth of Acanthamoeba sp. in a chemically defined medium. J. Gen. Microbiol. 21, 519-529.

Albach, R.A., Shaffer, J.G. and Watson, R.H. 1966. Autoradiographic studies of H^3-thymidine uptake in Entamoeba histolytica in CLG medium. J. Protozool. 13, 349-355.

Al Chalabi, K. and Gutteridge, W.E. 1977a. Catabolism of deoxythymidylate in some trypanosomatids. Parasitol. 74, 299-312.

Al Chalabi, K. and Gutteridge, W.E. 1977b Presence and properties of thymidylate synthase in trypanosomatids. Biochim. Biophys. Acta 481, 71-79.

Aldritt S.M. and Wang, C.C. 1986. Purification and characterization of guanine phosphoribosyltransferase from Giardia lamblia. J. Biol. Chem. 261, 8528-8533.

Aldritt, S.M., Tien, P. and Wang, C.C. 1985. Pyrimidine salvage in Giardia lamblia. J. Exp. Med. 161, 437-445.

Asai, T., O'Sullivan, W.J., Kobayashi, M., Gero, A.M., Yokogawa, M. and Tatibana M. 1983. Enzymes of the de novo pyrimidine biosynthetic pathway in Toxoplasma gondii. Mol. Biochem. Parasitol. 7, 89-100.

Avila, J.L., Avila, A. and Casanova, M. 1981. Differential metabolism of allopurinol and derivatives in Trypanosoma rangeli and T. cruzi culture forms. Mol. Biochem. Parasitol. 4, 265-272.

Avila, J.L., Avila, A., Munoz, E and Munoz, H. 1983. Trypanosoma cruzi: 4-aminopyrazolopyrimidine in the treatment of experimental chagas' disease. Exp. Parasitol. 56 236-240.

Avila, J.L., Avila, A. and Munoz, H. 1984. Differences in allopurinol and 4-aminopyrazolopyrimidine metabolism in drug-sensitive and insensitive strains of Trypanosoma cruzi. Mol. Biochem. Parasitol. 11 51-60.

Avila, J.L., Poleger M.A. and Robin R.K. 1986. Action of pyrazolopyrimidine derivative on Trypanosoma rangeli culture forms. Comp. Biochem. Physiol. 83C 291-294.

Azevedo, H.P. and Roitman, I. 1984. Cultivation of Trypanosoma cruzi in defined media. In Genes and Antigens of Parasites, Morel C.M. ed., Proceedings of a Course Sponsored by UNDP/World Bank/WHO. Special Programme for Research and Training in Tropical Diseases. Rio de Janeiro, Brazil, pp. 29-36.

Bachrach, U., Brem, S., Wertman, S.B., Schnur, S.L. and Greenblatt, C.L. 1979. Leishmania spp: effect of inhibitors on growth and on polyamine and macromolecular synthesis. Exp. Parasitol. 48, 464-470.

Berens, R.L., Marr, J.J., Burn, R., 1980a. Pyrazolopyrimidine metabolism in african trypanosomes: metabolic similarities to Trypanosoma cruzi and Leishmania species. Mol. Biochem. Parasitol. 1, 69-73.

Berens, R.L., Marr, J.J., Nelson, D.J. and Lafon, S.W. 1980b. Antileishmanial effect of allopurinol and allopurinol ribonucleoside on intracellular forms of Leishmania donovani. Biochem. Pharmacol. 29, 2397-2398.

Berens, R.L., Marr, J.J., Lafon, S.W. and Nelson, D.J. 1981. Purine metabolism in Trypanosoma cruzi. Mol. Biochem. Parasitol. 3, 187-196.

Berens, R.L., Marr, J.J., Cruz, F. and Nelson, D.J. 1982. Effect of allopurinol on Trypanosoma cruzi: metabolism and biological activity in intracellular and bloodstream froms. Antimicrob. agents Chemother. 22, 657-661.

Berens, R.L., Marr, J.J., Looker, D.L., Nelson, D.J. and Lafon, S.W. 1984. Efficacy of pyrazolopyrimidine ribonucleosides against Trypanosoma cruzi: studies in vitro and in vivo with sensitive and resistant strains. J. Infect. Dis. 150, 602-608.

Berens, R.L., Looker, D.L. and Marr, J.J. 1985. Purine ribonucleoside metabolism in Giardia lamblia: implification for chemotherapy. Fed. Proc. 44, 1333.

Berman, J.D. and Webster, H.K. 1982. In vitro effects of mycophenolic acid and allopurinol against Leishmania tropica in human macrophages. Antimicrob. Agents Chemother. 21, 887-891.

Beverly, S.M., Coderre, J.A., Santi, D.V. and Schimke, R.T. 1984. Unstable DNA amplifications in methotrexate-resistant Leishmania consist of extrachromosomal circles which relocalise during stabilization. Cell 38, 431-439.

Blakley, R.L. 1969. The Biochemistry of Folic Acid and Related Pteridines, North Holland, Amsterdam.

Bone, G.J. and Steinert, M. 1956. Isotopes incorporated in the nucleic acids of Trypanosoma mega. Nature (London) 178, 308-309.

Booden, T., Shaffer, J.G. and Albach, R.A. 1973. Purine and pyrimidine nucleotide anabolism by Entamoeba histolytica. P. 52. In P. de Puytorac and J. Grain (eds.), Progress in Protozoology. Fourth International Congress, University de Clermont, Clermont Ferrand, France.

Booden, T., Albach, R.A. and Boonlayangoor, P. 1978. Uptake of selected pyrimidines by axenically grown Entamoeba histolytica. Arch. Invest. Med. (Mex.) 9, 133-140.

Booden, T., Boonlayangoor, P. and Albach, R.A. 1976. Incorporation of purines and pyrimidines into axenically grown Entamoeba histolytica. J. Parasitol. 62, 641-643.

Boonlayangoor, P., Albach, R.A., Stern, M.L. and Booden, T. 1978. Entamoeba histolytica: uptake of purine bases and nucleosides during axenic growth. Exp. Parasitol. 45, 225-233.

Boonlayangoor, P., Albach, R.A. and Booden, T. 1980. Purine nucleotide synthesis in Entamoeba histolytica. Arch. Invest. Med. (Mex.) 11, 83-88.

Brun, R. 1980. Hydroxyurea: effect on growth, structure, and (^{3}H) thymidine uptake of Trypanosoma brucei procyclic culture forms. J. Protozool. 27, 122-128.

Brun, R. and Schonenberger, 1979. Cultivation and in vitro cloning of procyclic culture forms of Trypanosoma brucei in a semi-defined medium. Acta Trop. 36, 289-292.

Bungener, W. and Nielsen, G. 1967. Nukleinsaurenstoffwechsel bei experimenteller Malaria. I. Untersuchungen uber den Einbau von thymidin uridin und adenosin in malariaparasiten (Plasmodium berghei und Plasmodium vinckei). Z. Tropenmed. Parasitol. 18, 456-462.

Carson, D.A. and Chang, K-P. 1981. Phosphorylation and antileishmanial activity of formycin B. Biochem. Biophys. Res. Comm. 100, 1377-1383.

Ceron, C.R., Caldas, R., Felix, C., Mundim, M. and Roitman I. 1979. Purine metabolism in trypanosomatids. J. Protozool. 26, 479-483.

Chang, K-P., Steiger, R.F., Dave, C. and Cheng, Y. 1978. Effects of methylglyoxal bis (guanylhydrazone) on trypanosomatid flagellates: Inhibition of growth and nucleoside incorporation in Trypanosoma brucei. J. Protozool. 25, 145-149.

Chello, P.L. and Jaffe, J.J. 1972. Comparative properties of trypanosomal and mammalian thymidine kinase. Comp. Biochem. Physiol. 43B, 543-562.

Coderre, J.A., Beverley, S.M., Schimke, R.T. and Santi, D.V. 1983. Overproduction of a bifunctional thymidylate synthetase-dihydrofolate reductase and DNA amplification in methotrexate-resistant Leishmania tropica. Proc. Natl. Acad. Sci. USA. 80, 2132-2136.

Cohen, A., Ullman, B. and Martin, D.W. 1979. Characterisation of a mutant mouse lymphoma cell with deficient transport of purine and pyrimidine nucleosides. J. Biol. Chem. 254, 122-116.

Cohen, A., Barankiewicz, J., Lederman, H. and Gelfand, E. 1983. Purine and pyrimidine metabolism in human T lymphocytes. J. Biol. Chem. 258, 12334-12340.

Coles, A.M., Swoboda, B. and Ryley, J. 1980. Thymidylate synthetase as a chemotherapeutic target in the treatment of avian coccidiosis. J. Protozool. 27, 502-506.

Conrad, P.A. 1986. Uptake of tritiated nucleic acid precursors by Babesia bovis in vitro. Int. J. Parasit. 16, 263-268.

Cosgrove, W.B., Skeen, M. and Hajduk, S.L. 1979. Effects of hydroxyurea on Crithidia fasciculata. J. Protozool. 26, 643-648.

Daddona, P.E., Wiesmann, W.P., Lambros, C., Kelley, W.N. and Webster, H.K. 1984. Human malaria parasite adenosine deaminase. J. Biol. Chem. 259, 1472-1475.

Davies, M.J., Ross, A.M. and Gutteridge, W.E. 1983. The enzymes of purine salvage in Trypanosoma cruzi, Trypanosoma brucei and Leishmania mexicana. Parasitol. 87, 211-217.

De Pierre, J.W. and Karnovsky, M.L. 1973. Plasma membranes of mammalian cells: a review of methods for their characterization and isolation. J. Cell Biol. 56, 275-303.

Dewes, H., Ostergaard, H.L. and Simpson, L. 1986. Imparied drug uptake in methotrexate resistant Crithidia fasciculata without changes in dihydrofolate reductase activity or gene amplification. Mol. Biochem. Parasitol. 19, 149-161.

Dewey, V.C. and Kidder, G.W. 1973. Partial purification and properties of a nucleoside hydrolase from Crithidia. Arch. Biochem. Biophys. 157, 380-387.

Dewey, V.C. and Kidder, G.W. 1977. Inhibitory action of the adenine anolog, 4-aminopyrazolo(3,4-d)pyrimidine in Crithidia fasciculata. Can. J. Biochem. 55, 110-112.

Dewey, V.G., Kidder, G.W. and Nolan, L.L. 1978. Mechanism of inhibition of Crithidia fasciculata by adenosine and adenosine analogs. Biochem. Pharmacol. 27, 1479-1485.

Diggens, S.M., Gutteridge, W.E. and Trigg, P.I. 1970 Altered dihydrofolate reductase associated with a pyrimethamine-resistant Plasmodium berghei produced in a single step. Nature (London) 228, 579-580.

Dewyer, D.M. and Gottlieb, M. 1984. Surface membrane localisation of 3'- and 5'-nucleotidase activities in Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 10, 139-150.

Emeh, J.K., Omotainse, S.O. and Olotu, C.O. 1986. Presence of adenine aminohydrolase in Onchocerca gutturosa. IRCS Med. Sci. 14, 262-263.

Eubank, W.B. and Reeves, R.E. 1981. Growth inhibition of axenic Entamoeba histolytica by tubercidin and Ara-A. Am. J. Trop. Med. Hyg. 30, 900-902.

Ferone, R. and Roland, S. 1980. Dihydrofolate reductase: thymidylate synthase, a bifunctional polypeptide from Crithidia fasciculata. Proc. Natl. Acad. Sci. USA. 77, 5802-5806.

Ferone, R., O'Shea, M. and Yoeli, M. 1970. Altered dihydrofolate reductase associated with drug-resistance transfer between rodent plasmodia. Science 167, 1263-1264.

Fernandes, J. and Kimura, E. 1973. Biosynthesis of purines and pyrimidines by different forms of Trypanosoma cruzi. p. 137. In P. de Puytorac and J. Grain (eds.), Progress in Protozoology. Fourth International Congress, University de Clermont, Clermont Ferrand, France.

Fish, W.R., Looker, D.L., Marr, J.J. and Berens, R.L. 1982a. Purine metabolism in the bloodstream forms of Trypanosoma gambiense and Trypanosoma rhodesiense. Biochim. Biophys. Acta. 719, 223-231.

Fish, W.R., Marr, J.J. and Berens, R.L. 1982b. Purine metabolism in Trypanosoma brucei gambiense. Biochim. Biophys. Acta. 714, 422-428.

Fontenelle, L.J. and Henderson, J.F. 1969. An enzymatic basis for the inability of erythrocytes to synthesise purine ribonucleotides de novo. Biochim. Biophys. Acta. 177, 175-176.

Garrett, C.E., Coderre, J.A., Meek, T., Garvey, E., Claman, D.M., Beverley, S.M. and Santi, D.V. 1984. A bifunctional thymidylate synthetase dihydrofolate reductase in protozoa. Mol. Biochem. Parasitol. 11, 257-265.

Garvey, E. and Santi, D.V. 1985. Limited proteolysis of the bifunctional thymidylate synthase-dihydrofolate reductase from Leishmania tropica Proc. Natl. Acad. Sci. USA 82, 7188-7192.

Garvey, E., Coderre, J.A. and Santi, D.V. 1985. Selection and properties of Leishmania tropica resistant to 10-propargyl-5, 8-dideazafolate, an inhibitor of thymidylate synthetase. Mol. Biochem. Parasitol. 17, 79-91.

Gero, A.M. and Coombs, G.H. 1980a. Orotate phosphoribosyltransferase and orotidine 5-phosphate decarboxylase in two parasitic kinetoplastid flagellates FEBS letters, 118, 130-132.

Gero, A.M. and Coombs, G.H. 1980b. Pyrimidine biosynthetic enzymes in Leishmania mexicana mexicana and other parasitic protozoa. Parasitol. 81 V.

Gero, A.M. and Coombs, G.H. 1982a. Leishmania mexicana: conversion of dihydroorotate to orotate in amastigotes and promastigotes. Exp. Parasitol. 54, 185-195.

Gero, A.M. and Coombs, G.H. 1982b. Pyrimidine biosynthetic enzymes in Babesia hylomysci. Int. J. Parasitol. 12, 377-382.

Gero, A.M., Finney, K.G., Bennett, J.C. and O'Sullivan, W.J. 1981a. Comparative studies on dihydroorotate dihydrogenase from P. berghei and the mouse reticulocyte. Aust. J. Exp. Biol. Med. Sci. 59, 477-490.

Gero, A.M., Tetley, K., Coombs, G.H. and Phillips, R.S. 1981b. Dihydroorotate dehydrogenase, orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase in Plasmodium falciparum. Trans. Roy. Soc. Trop. Med. Hyg. 75, 719-720.

Gero, A.M., O'Sullivan, W.J., Wright, I.G. and Mahoney, D.F. 1983. The enzymes of pyrimidine biosynthesis in Babesia bovis and Babesia bigemina. Aust. J. Exp. Biol. Med. Sci. 61, 239-243.

Gero, A.M., Brown, G.V. and O'Sullivan, W.J. 1984. Pyrimidine de novo synthesis during the life cycle of the intraerythrocytic stage of Plasmodium falciparum. J. Parasitol. 70, 536-541.

Gottlieb, M. 1985. Enzyme regulation in a trypanosomatid: effect of purine starvation on levels of 3'-nucleotidase activity. Science, 227, 72-74.

Gottlieb, M. and Dwyer, D.M. 1983. Evidence for distinct 5'-and 3'-nucleotidase activities in the surface membrane fraction of Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 7, 303-317.

Gottlieb, M., Gardiner, P.R. and Dwyer, D.M. 1986. 3'-Nucelotidase activity in procyclic and bloodstream stages of Trypanosoma rhodesiense. Comp. Biochem. Physiol. 83B, 63-69.

Gutteridge, W.E. and Coombs, G.H. 1977. Biochemistry of Parasitic Protozoa, MacMillan Press, London.

Gutteridge, W.E. and Davies, M.J. 1981. Enzymes of purine salvage in Trypanosoma cruzi. FEBS Letters, 127, 211-214.

Gutteridge, W.E. and Davies, M.J. 1982. Properties of the purine phosphoribosyltransferases of Trypanosoma cruzi. FEBS Microbiol. Letters 13, 207-212.

Gutteridge, W.E. and Gaborak, M. 1979. A re-examination of purine and pyrimidine synthesis in the three main forms of Trypanosoma cruzi. Int. J. Biochem. 10, 415-422.

Gutteridge, W.E. and Trigg, P.I. 1970. Incorporation of radioactive precursors into DNA and RNA of Plasmodium knowlesi in vitor. J. Protozool. 17, 89-96.

Gutteridge, W.E., Dave, D. and Richards, W. 1979. Conversion of dihydroorotate to orotate in parasitic protozoa. Biochim. Biophys. Acta 582, 390-401.

Guttman, H.N. 1966. The first defined medium for Trypanosoma spp. J. Protozool. 13, Supplement No. 59.

Hamet, M., Bonissol, C. and Cartier, P. 1980. Enzymatic activities of purine and pyrimidine metabolism in nine Mycoplasma species contaminating cell cultures. Clin. Chim. Acta, 103, 15-22.

Hammond, D.J. and Gutteridge, W.E. 1980. Enzymes of pyrimidine biosynthesis in Trypanosoma cruzi. FEBS Letters, 118, 259-262.

Hammond, D.J. and Gutteridge, W.E. 1982. UMP synthesis in the kinetoplastida. Biochem. Biophys. Acta 718, 1-10.

Hammond, D.J. and Gutteridge, W.E. 1983. Studies on the glycosomal orotate phosphoribosyltransferase of Trypanosoma cruzi. Mol. Biochem. Parasitol. 7, 319-330.

Hammond, D.J. and Gutteridge, W.E. 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Mol. Biochem. Parasitol. 13, 243-261.

Hammond, D.J., Gutteridge, W.E. and Opperdoes, F.R. 1981. A novel location for two enzymes of de novo pyrimidine biosynthesis in trypanosomes and Leishmania. FEBS Letters 128, 27-29.

Hammond D.J., Aman, R.A. and Wang, C.C. 1985a. The role of compartmentation and glycerol kinase in the synthesis of ATP within the glycosome of Trypanosoma brucei. J. Biol. Chem. 260, 15646-15654.

Hammond, D.J., Burchell, J.R. and Pudney, M. 1985b. Inhibition of pyrimidine biosynthesis de novo in Plasmodium falciparum by 2-(4-t-butylcyclohexyl)-3-hydroxy-1, 4-napthoquinone in vitro. Mol. Biochem. Parasitol. 14, 97-109.

Hansen, B.D., Sleeman, H.K. and Pappas, P.W. 1980. Purine base and nucleoside uptake in Plasmodium berghei and host erythrocytes. J. Parasitol 66, 205-212.

Hansen, B.D., Arbelo, J., Walkony, J.F. and Hendricks, L.D. 1982. The specificity of purine base and nucleoside uptake in promastigotes of Leishmania braziliensis panamensis. Parasitol. 85, 271-282.

Hansen, B.D., Webster, H.K., Hendricks, L.D. and Pappas, H.C. 1984. Leishmania mexicana: Purine metabolism in promastigotes, axenic amastigotes and amastigotes derived from vero cells. Exp. Parasitol. 58, 101-109.

Hart, D.T. and Opperdoes, F.R. 1984. The occurrence of glycosomes (microbodies) in the promastigote stage of four major Leishmania species. Mol. Biochem. Parasitol. 13, 159-172.

Hart, D.T., Vickerman, K. and Coombs, G.H. 1981. Transformation in vitro of Leishmania mexicana amastigotes: nutritional requirements and the effect of drugs. Parasitol. 83, 529-541.

Hassan, H.F. and Coombs, G.H. 1985a. Leishmania mexicana: Purine metabolising enzymes of amastigotes and promastigotes. Exp. Parasit. 59, 139-150.

Hassan, H.F. and Coombs, G.H. 1985b. Purine phosphoribosyltransferases of Leishmania mexicana mexicana and other flagellate protozoa. Comp. Biochem. Physiol. 82B, 773-779.

Hassan, H.F. and Coombs, G.H. 1986a. De novo synthesis of purines by Acanthamoeba castellanii and A. astronyxis. IRCS Med Sci. 14, 559-560.

Hassan, H.F. and Coombs, G.H. 1986b. A comparative study of the purine- and pyrimidine-metabolising enzymes of a range of trypanosomatids. Comp. Biochem. Physiol. 84B, 217-223.

Hassan, H.F. and Coombs, G.H. 1986c. Purine-metabolising enzymes in Entamoeba histolytica. Mol. Biochem. Parasitol 19, 19-25.

Hassan, H.F. and Coombs, G.H. 1986d. Purine salvage by Acanthamoeba castellanii. Int. J. Parasitol, in press.

Hassan, H.F. and Coombs, G.H. 1986e. Phosphomonoesterases of Leishmania mexicana mexicana and other flagellates. Submitted to Mol. Biochem. Parasitol.

Hassan, H.F., Mottram, J.C. and Coombs, G.H. 1985. Subcellular localisation of purine-metabolising enzymes in Leishmania mexicana mexicana. Comp. Biochem. Physiol. 81B, 1037-1040.

Hassan, H.F., Phillips, R.S. and Coombs, G.H. 1986. Purine-metabolising enzymes in Babesia divergens. Zeit. Parasitenkunds, in press.

Heyworth, P.G., Gutteridge, W.E. and Ginger, C.D. 1979. Pyrimidine biosynthesis in Trichomonas vaginalis. Parasitol. 79 xli.

Heyworth, P.G., Gutteridge, W.E. and Ginger, C.D. 1982. Purine metabolism in Trichomonas vaginalis. FEBS Lett 141, 106-110.

Heyworth, P.G. Gutteridge, W.E. and Ginger, C.D. 1984. Pyrimidine metabolism in Trichomonas vaginalis. FEBS Letters 176, 55-60.

Hill, D.L. 1972. The Biochemistry and Physiology of Tetrahymena. Academic Press, New York and London.

Hill, D.L. and Chambers, P. 1967. The purine and pyrimidine metabolism of Tetrahymena pyriformis. J. Cell. Physiol. 69, 321-330.

Hill, B., Kilsby, J., Rogerson, G.W., McIntosh, R.T. and Ginger, C.D. 1981a. The enzymes of pyrimidine biosynthesis in a range of parasitic protozoa and helminths. Mol. Biochem. Parasitol. 2, 123-134.

Hill, B., Kilsby, J., McIntosh, R.T., Wrigglesworth, R. and Ginger, C.D. 1981b. Pyrimidine biosynthesis in Plasmodium berghei. Int. J. Biochem. 13, 303-310.

Holland, J.W., Gero, A.M. and O'Sullivan, W.J. 1983. Enzymes of de novo pyrimidine biosynthesis in Babesia rodhaini. J. Protozool. 30, 36-40.

Hupe, D.J., Azzolina, B.A. and Behrens, N.D. 1986. IMP dehydrogenase from the intracellular parasitic protozoan Eimeria tenella and its inhibition by mycophenolic acid. J. Biol. Chem. 261, 8363-8369.

Iovannisci, D.M. and Ullman, B. 1984. Characterisation of a mutant Leishmania donovani deficient adenosine kinase activity. Mol. Biochem. Parasitol. 12, 139-151.

Iovannisci, D.M., Goebel, D., Allen, K., Kaur, K. and Ullman, B. 1984a. Genetic analysis of adenine metabolism in Leishmania donovani promastigotes. J. Biol. Chem. 259, 14617-14623.

Iovannisci, D.M., Kaur, K., Young, L. and Ullman, B. 1984b. Genetic analysis of nucleoside transport in Leishmania donovani. Mol. Cell. Biol. 4, 1013-1019.

Irvin, A.D. and Stagg, D.A. 1977. Theileria parva: purine and pyrimidine metabolism and the action of folate antagonists in parasitized bovine lymphoid cells. Exp. Parasitol. 41, 172-185.

Irvin, A.D. and Young, E.R. 1977. Possible in vitro test for screening drugs for activity against Babesia and other blood protozoa. Nature 269, 407-409.

Irvin, A.D. and Young, E.R. 1979. Further studies on the uptake of tritiated nucleic acid precursors by Babesia spp. of cattle and mice. Int. J. Parasitol. 9, 109-114.

Irvin, A.D., Young, E.R. and Purnell, R.E. 1978. The in vitro uptake of tritiated nucleic acid precursors by Babesia spp. of cattle and mice. Int. J. Parasitol. 8, 19-24.

Ishizuka, M., Sawa, T., Hori, S., Takayama, H., Takeuchi, T. and Umezawa H. 1968. Biological studies on formycin and formycin B. J. Antibiot. (Tokyo) 21, 5-12.

Jackson, R.C., Lui, M., Boritzki, T., Morris, H. and Weber, G. 1980. Purine and pyrimidine nucleotide patterns of normal, differentiating and regenerating liver and of hepatomas in rats. Cancer Res. 40, 1286-1291.

Jaffe, J.J. 1961. The effect of 6-azauracil upon Trypanosoma equiperdum. Biochem. Pharmacol. 8, 216-223.

James, D.H. and Born, G.V. 1980. Uptake of purine bases and nucleosides in African trypanosomes. Parasitol. 81, 383-393.

Jantzen, H. 1974. Die Entwicklung von Acanthamoeba castellanii zur cyste mit und ohne veranderung des genaktivitatsmusters. J. Protozool. 21, 791-795.

Jarrol, E.L., Lindmark, D.G. and Paolella, P. 1983. Pyrimidine metabolism in Tritrichomonas foetus. J. Parasit. 69, 846-849.

Jones, M.E. 1980. Pyrimidine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis. Annu. Rev. Biochem. 49, 253-279.

Kasper, L.H. and Pfefferkorn, E.R. 1982. Hydroxyurea inhibition of growth and DNA synthesis in Toxoplasma gondii: characterization of a resistant mutant. Mol. Biochem. Parasitol. 6, 141-150.

Keppler, D. and Holstege, A. 1982. Pyrimidine nucleotide metabolism. In Metabolic Compartmentation (Sies, H., ed.), Academic Press, London. pp. 147-203.

Kidder, G.W. 1982. Adenosine kinase from Trypanosoma cruzi. Biochem. Biophys. Res. Comm. 107, 381-388.

Kidder, G.W. 1984. Characteristics of cytidine aminohydrolase activity in Trypanosoma cruzi and Crithidia fasciculata. J. Protozool. 31, 298-300.

Kidder, G.W. and Dewey, V.C. 1948. Studies on the biochemistry of Tetrahymena XIV. The activity of a natural purines and pyrimidines. Proc. Natl. Acad. Sci. USA. 34, 566-574.

Kidder, G.W. and Dutta, B.N. 1958. The growth and nutrition of Crithidia fasciculata. J. Gen. Microbiol. 18, 621-638.

Kidder, G.W. and Nolan, L.L. 1973. Pteridine requiring dihydroorotate hydroxylase from Crithidia fasciculata. Biochem. Biophys. Res. Comm. 53, 929-936.

Kidder, G.W. and Nolan, L.L. 1979. Adenine aminohydrolase: Occurrence and possible significance in trypanosomid flagellates. Proc. Nat. Acad. Sci. USA 76, 3670-3672.

Kidder, G.W. and Nolan, L.L. 1981. The in vivo and in vitro action of 4-amino-5-imidazolecarboxamide in trypanosomid flagellates. Mol. Biochem. Parasitol. 3, 265-269.

Kidder, G.W. and Nolan, L.L. 1982. Xanthine phosphoribosyltransferase in Leishmania: Divalent cation activation. J. Protozool. 29, 405-409.

Kidder, G.W., Dewey, V.C. and Nolan, L.L. 1976. Enzymes of the orotate biosynthetic pathway in Crithidia fasciculata. Can. J. Biochem. 54, 32-41.

Kidder, G.W., Dewey, V.C. and Nolan, L.L. 1977. Adenine deaminase of a eukaryotic animal cell, Crithidia fasciculata. Arch. Biochem. Biophys 183, 7-12.

Kidder, G.W., Dewey, V.C. and Nolan, L.L. 1978. Transport and accumulation of purine bases by Crithidia fasciculata. J. Cell. Physiol. 96, 165-170.

Kidder, G.W., Nolan, L.L. and Dewey, V.C. 1979. The purine phosphoribosyltransferases of Crithidia fasciculata. J. Parasit. 65, 520-525.

Kobayashi, N., Matsuno, T., Hariguchi, F., Yamazaki, T. and Imai, K. 1986. Eimeria tenella, E. necatrix, E. acervulina, E. maxima and E. brunetti: potent anticoccidial activity of an uridine analog, 1-(B-D-ribofuranosyl)-2(1H)-pyrazinone 4-oxide. Exp. Parasitol. 61, 42-47.

Konigk, E. 1976. Comparative aspects of nucleotide biosynthesis in parasitic protoxoa. In Van den Bossche H., ed. Biochemistry of parasites and host parasite relationships. Amsterdam, North Holland, pp 51-58.

Konigk, E. 1977. Salvage syntheses and their relationship to nucleic acid metabolism. Bull. W.H.O. 55, 249-252.

Konigk, E. 1978. Purine nucleotide metabolism in promastigotes of Leishmania tropica: Inhibitory effect of allopurinol and analogues of purine nucleosides. Tropenmed. Parasit. 29, 435-438.

Konigk, E. and Putfarken, B. 1980. Stage specific differences of a perhaps signal-transferring system in Leishmania donovani. Tropenmed. Parasit. 31, 421-424.

Konigk, E. and Rasoul, S.A. 1978. Catabolism of adenosine 5'-monophosphate in promastigotes of Leishmania tropica. Tropenmed. Parasit. 28, 319-322.

Koszalka, G.W. and Krenitsky, T.A. 1979. Nucleosidases from Leishmania donovani. J. Biol. Chem. 254, 8185-8193.

Koszalka, G.W. and Krenitsky, T.A. 1985. 5'-Methylthioadenosine phosphorylase from Leishmania donovani. Pediat. Res. 19, 761.

Krenitsky, T.A., Neil, S., Elion, G.B. and Hitchings, G. 1969a. Adenine phosphoribosyltransferase from monkey liver. J. Biol. Chem. 244, 4779-4784.

Krenitsky, T.A., Papionnou, R. and Elion, C.B. 1969b. Human hypoxanthine phosphoribosyltransferase. J. Biol. Chem. 244, 1263-1270.

Krenitsky, T.A., Neil, S., and Miller, R.L. 1970. Guanine and xanthine phosphoribosyltransferase activities of Lactobacillus casci and Escherichia coli. J. Biol. Chem. 245, 2605-2611.

Krenitsky, T.A., Koszalka, G.W., Tuttle, J.V., Adamczyk, D.L., Elion, G.E. and Marr, J.J. 1980. Purine salvage enzymes in man and Leishmania donovani. In Purine Metabolism in Man (Rapado A. and Watts R.W.E., eds.), Part III, pp. 51-56, Plenum Publishing Crop., New York.

Krenitsky, T.A., Rideout, J.L., Koszalka, G.W., Inmon, R., Chao, E., Elion, G.B., Latter, V. and Williams, R. 1982. Pyrazolo(3,4-d) pyrimidine ribonucleosides as anticoccidials. J. Med. Chem. 25, 32-35.

Krooth, R.S., Wuu, K. and Ma, R. 1969. Dihydroorotic acid dehydrogenase: introduction into erythrocyte by the malaria parasite. Science 164, 1073-1075.

Lafon S.W. and Nelson D.J. 1985. Purine metabolism in the intact sporozoites and merozoites of Eimeria tenella. Mol. Biochem. Parasitol. 14, 11-22.

Lafon, S.W., Nelson, D.J., Berens, R.L. and Marr, J.J. 1982. Purine and pyrimidine salvage pathways in Leishmania donovani. Biochem. Pharmacol. 31, 231-238.

Lantz, C.H., Van Dyke, K. and Carter, G. 1971. Plasmodium berghei: in vitro incorporation of purine derivatives into nucleic acids. Exp. Parasitol. 29, 402-416.

Lepage, G. and Junga, I. 1963. Use of nucleoside in resistance to 6-thioguanine. Cancer Res. 23, 739-743.

Levine, R., Hoogenraad, N. and Kretchner, N. 1974. A review: biological and clinical aspects of pyrimidine metablism. Pediat. Res. 8, 724-734.

Lindmark, D.G. and Jarrol, E.L. 1982. Pyrimidine metabolism in Giardia lamblia trophozoites. Mol. Biochem. Parasitol. 5, 291-296.

Linstead, D. 1981. New defined and semi-defined media for cultivation of the flagellate Trichomonas vaginalis. Parasitol. 3, 125-137.

Lo, H. and Wang, C.C. 1985. Purine salvage in Entamoeba histolytica. J. Parasitol. 71, 662-669.

Lobelle-Rich, P.A. and Reeves, R.E. 1983. The partial purification and characterization of adenosine kinase from Entamoeba histolytica. Am. J. Trop. Med. Hyg. 32, 976-979.

Looker, D.L., Berens, R.L. and Marr, J.J. 1983. Purine metabolism in Leishmania donovani amastigotes and promastigotes. Mol. Biochem. Parasitol. 9, 15-28.

Looker, D.L., Marr, J.J. and Berens R.L. 1986. Mechanisms of action of pyrazolopyrimidines in Leishmania donovani. J. Biol. Chem. 261, 9412-9415.

Lowy, B., Williams, M. and London, I.M. 1962. Enzymatic deficiencies of purine nucleotide synthesis in the human erythrocyte. J. Biol. Chem. 237, 1622-1625.

Lukow, I., Schmidt, G., Walter, R.D. and Konigk, E. 1973. Adenosinmonophosphat-salvage-synthese bei Plasmodium chabaudi. Z Tropenmed. Parasit. 24, 500-504.

Makoff, A.J. and Radford, A. 1978. Genetic and biochemistry of carbamoylphosphate biosynthesis and its utilisation in the pyrimidine biosynthetic pathway. Microbiol. Rev. 42, 307-328.

Manandhar, M.S. and Van Dyke, K. 1975. Detailed purine salvage metabolism in and outside the free malarial parasite. Exp. Parasitol. 37, 138-146.

Marr, J.J. 1983. Pyrazolopyrimidine metabolism in the patogenic hemoflagellates. In: Singer, T.P., Mansour, T., Ondarza, R. (eds). Biochemical basis of drug action. Academic Press, New York. pp. 147-157.

Marr, J.J. 1984. The chemotherapy of leishmaniasis. In Parasitic diseases: The Chemotherapy, Vol. 2, Mansfield J.M. (ed.). Marcel Dekker, New York, pp. 201-227.

Marr, J.J. and Berens R.L. 1983. Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Mol. Biochem. Parasitol. 7, 339-356.

Marr, J.J. and Berens, R.L. 1985. Purine and pyrimidine metabolism in Leishmania. In Leishmaniasis, Chang K-P and Bray eds. pp. 65-78. Elsevier Science Publishers B.V. (Biomedical Division).

Marr, J.J., Berens, R.L. and Nelson, D.J. 1978a. Purine meabolism in Leishmania donovani and Leishmania braziliensis. Biochem. biophys. Acta 544, 360-371.

Marr, J.J. Berens, R.L. and Nelson, D.J. 1978b. Antitrypanosomal effect of allopurinol: conversion in vivo to aminopyrazolopyrimidine nucleotides by Trypanosoma cruzi. Science 201, 1018-1020.

Marr, J.J., Berens, R.L., Nelson, D.J., Krenitsky, T.A., Spector, T., Lafon, S. and Elion, G.B. 1982. Antileishmanial action of 4-thiopyrazolo(3,4-d)pyrimidine and its ribonucleoside. Biochem. Pharmacol. 31, 143-148.

Martin, D.W. 1981. Metabolism of purine and pyrimidine nucleotides. In Harper's review of Biochemistry, Martin D.W., Mayers P.A. and Rodwell V.W. eds., Lange Medical Publications, California, pp. 331-348.

Matsuno T., Kobayashi H., Hariguchi, F., Vamazaki, T., Imai, K. and Onaga, H., 1984. Eimeria tenella, E. necatrix, E. acervulina and E. maxima: Anticoccidal activity of 1,6-dihydro-6-oxo-2-pyrazinecarboxylic acid 4-oxide. Exp. Parasitol. 57, 55-61.

McIntosh, A.H. and Chang, R.S. 1971. A comparative study of 4 strains of Hartmanellid amoebae. J. Protozool. 18, 632-636.

McLaughlin, J. 1981. Association of adenylate kinase with the glycosome of Trypanosoma rhodesiense. Biochem. Int. 2, 345-353.

Meek, T.D., Garvey, E.P. and Santi, D.V. 1985. Purification and characterization of the bifunctional thymidylate synthetase-dihydrofolate reductase from methotrexate-resistant Leishmania tropica. Biochem. 24, 678-686.

Melo, N.M., De Azevedo, H., Roitman, I. and Mayrink, W. 1985. A new defined medium for cultivating Leishmania promastigotes. Acta Trop. 42, 137-141.

Miller, R.L. and Linstead, D. 1983. Purine and pyrimidine metabolizing activities in Trichomonas vaginalis extracts. Mol. Biochem. Parasitol. 7, 41-51.

Miller, R.L. and Miller, W.H. 1985a. Purine salvage enzymes in Trichomonas vaginalis. Pediat. Res. 19, 766.

Miller, R.L. and Miller, W.H. 1985b. Trichomonas vaginalis: purification and characterization of the nucleoside kinases. International symposium on trichomonads and trichomoniases. pp. 39. Prague, Czechoslovakia.

Miller, R.L., Ramsey, G.A., Krenitsky, T.A. and Elion, G.B. 1972. Guanine phosphoribosyltransferase from Escherichia coli. Biochem. 11, 4723-4731.

Miller, R.L., Adamczyk, D.L. and Miller, W.H. 1979. Adenosine kinase from rabbit liver. J. Biol. Chem. 254, 2339-2345.

Miller, R.L., Adamczyk, D.L., Rideout, J.L. and Krenitsky, T.A. 1982. Purification, characterization, substrate and inhibitor specificity of adenosine kinase from several Eimeria species. Mol. Biochem. Parasitol. 6, 209-223.

Miller, R.L., Sabourin, C.L.K. and Krenitsky, T.A. 1984. Nucleoside hydrolase from Trypanosoma cruzi. J. Biol. Chem. 259, 5073-5077.

Miller, R.L., Nelson, D.J., Krenitsky, T.A., Berens, R.L. and Marr, J.J. 1986. Antigiardial activity of arabinosyladenine and arabinosylguanine. J. Cell. Biochem. Supplement 10A, 166.

Mitchell, A. and Finch, L. 1977. Pathways of nucleotide biosynthesis in Mycoplasma mycoides subsp. mycoides. J. Bacteriol. 130, 1047-1054.

Mollgaard, H. and Neuhard, J. 1983. Biosynthesis of deoxythymidine triphosphate. In Munch-Peterson A. ed. Metabolism of nucleotides, and nucleobases in microorganisms. pp. 149-201. Academic Press. London.

Momparler, R.L. and Jaffe, J.J. 1965. Effect of azaserine on the incorporation of ^{14}C-labeled purines and pyrimidines into the acid soluble and nucleic acid fractions of Trypanosoma equiperdum. Biochem. Pharmacol. 14, 255-262.

Muirhead, K.M. and Bishop, S.H. 1974. Purification of adenylosuccinate synthetase from rabbit skeletal muscle. J. Biol. Chem. 249, 459-464.

Mukkada, A.J., Meade, J.C., Glaser, T.A. and Bonventre, P.F. 1985. Enhanced metabolism of Leishmania donovani amastigotes at acid pH: an adaptation for intracellular growth. Science 229, 1099-1101.

Mundim, M. and Roitman, I. 1977. Extra nutritional requirements of artificially aposymbiotic Crithidia deanei. J. Protozool. 24, 329-331.

Mundim, M., Roitman, I., Hermans, M. and Kitajima, E.W. 1974. Simple nutrition of Crithidia deanei, a reduviid trypanosomatid with an endo symbiont. J. Protozool. 21, 518-521.

Murray, A.W. 1971. The biological significance of purine salvage. Annu. Rev. Biochem. 40, 811-826.

Nagy, M., Taty, M. and Heslot, H. 1973. Regulation of the biosynthesis of purine nucleotides in Schizosccharomyces pombe. III. Kinetic studies of adenylosuccinate synthetase. Biochim. Bihys Acta, 309, 1-10.

Neal, R.A., Croft, S.L. and Nelson, D.J. 1985. Antileishmanial effect of allopurinol ribonucleoside and the related compounds, allopurinol, thiopurinol, thiopurinol ribonucleoside, and of formycin B, sinefungin and the lepidine WR6026. Trans. Roy. Soc. Trop. Med. Hyg. 79, 122-128.

Neame, K.D., Brownbill, P.A. and Homewood, C.A. 1974. The uptake and incorporation of nucleosides into normal erythrocytes and erythrocytes containing Plasmodium berghei. Parasitol. 69, 329-335.

Nelson, D.J., Bugge, C.J., Krasny, H.C., and Elion, G.B. 1973. Formation of nucleotides of (6-^{14}C) allopurinol and (6-^{14}C) oxipurinol in rat tissues and effects on uridine nucleotide pools. Biochem. Pharmacol. 22, 2003-2022.

Nelson, D.J., Bugge, C.J., Elion, G.B., Berens, R.L. and Marr, J.J. 1979a. Metabolism of pyrazolo (3,4-d) pyrimidine in Leishmania braziliensis and Leishmania donovani. J. Biol. Chem. 254-3959-3964.

Nelson, D.J., Lafon, S.W., Tuttle, J.V., Miller, W.H., Miller, R.L., Krenitisky, T.A., Elion, G.B., Berens, R.L. and Marr, J.J. 1979b. Allopurinol ribonucleoside as an antileishmanial agent, biological effects, metablism and enzymatic phosphyorylation. J. Biol. Chem. 254, 11544-11549.

Nelson, D.J., Lafon, S.W., Jones, T.E., Spector, T., Berens, R.L. and Marr, J.J. 1982 The metabolism of formycin B in Leishmania donovani. Biochem. Biophys. Res. Comm. 108, 349-354.

Neuhard, J. 1983. Utilization of preformed pyrimidine bases and nucleosides. In Munch-Petersen A. ed. Metabolism of nucleotides, nucleosides and nucleobases in microorganisms. pp 96-148. Academic Press, London.

Newton, B.A. 1957. Nutritional requirements and biosynthetic capabilities of the parasitic flagellate Strigomonas oncopelts. J. Gen. Microbiol. 17, 708-717.

Nolan, L.L. 1984. Partial purification and characterization of guanine aminohydrolase from Trypanosoma cruzi. Curr. Microbiol. 11, 217-220.

Nolan, L.L. and Kidder, G.W. 1980. Inhibition of growth and purine metabolising enzymes of trypanosomid flagellates by N-methyladenine. Antimicrob. Agents Chemoth. 17, 567-571.

Nolan, L.L., Berman, J.D. and Giri, L. 1984. The effect of formycin B on mRNA translation and uptake of purine precursors in Leishmania mexicana. Biochem. Int. 9, 207-218.

Nygaard, P. 1983. Utilization of preformed purine bases and nucleosid. In Munch-Petersen A. ed. Metabolism of nucleotides, nucleosides and nucleobases in microorganisms. pp. 28-93. Academic Press, London.

O'Donovan, G.A. and Neuhard, J. 1970. Pyrimidine metabolism in microorganisms. Bacteriol. Rev. 34, 278-343.

Ogbunude, P.O. and Ikediobi, C.O. 1983. Comparative aspects of purine metabolism in some African trypanosomes. Mol. Biochem. Parasit. 9, 279-287.

Ogbunude, P.O., Ikediobi, C.O. and Ukoha, A.I. 1985. Adenosine cycle in African trypanosomes. Ann. Trop. Med. Parasitol. 79, 7-11.

Okochi, V.I., Abaelu, A.M. and Akinrimisi, E.O. 1983. Studies on the mechanism of adenosine transport in Trypanosoma vivax. Biochem. Int. 6, 129-139.

Opperdoes F.R., Markos A. and Steiger, R.F. 1981. Localization of malate dehydrogenase, adenylate kinase and glycolytic enzymes in glycosomes and the threonine pathway in the mitochondrion of cultured procyclic trypomastigotes of Trypanosoma brucei. Mol. Biochem. Parasitol. 4, 291-309.

O'Sullivan, W.J. and Ketley, K. 1980. Biosynthesis of uridine monophosphate of Plasmodium berghei. Ann. Trop. Med. Parasitol. 74, 109-114.

O'Sullivan, W.J., Johnson, A.M., Finney, K.G., Gero, A.M., Hogan, E., Holland, H.N. and Smithers, G.W. 1981. Pyrimidine and purine enzymes in Toxoplasma gondii. Aust. J. Exp. Biol. Med. Sci. 59, 763-767.

Ouellette, C.A., Strout, R.G. and McDougald, L.R. 1973. Incorporation of radioactive pyrimidine nucleosides into DNA and RNA of Eimeria tenella cultured in vitro. J. Protozool. 20, 150-153.

Ouellette, C.A., Strout, R.G. and McDougald, L.R. 1974. Thymidylic acid synthesis in Eimeria tenella cultured in vitro. J. Protozool. 21, 398-400.

Pascal, R.A., Trang, N.L., Cerami, A. and Walsh, C. 1983. Purification and properties of dihydroorotate oxidase from Crithidia fasciculata and Trypanosoma brucei. Biochem. 22, 171-178.

Pattanakitsakul, S., Ratanaphan, A. and Ruenwongsa, P. 1985. Comparative studies on thymidylate synthetase from Plasmodium berghei and mouse reticulocytes. Comp. Biochem. Physiol. 80B, 583-587.

Pereira, N.M. and Konigk, E. 1981. A nucleotidase from Leishmania tropica promastigotes: Partial purification and properties. Tropenmed. Parasit. 32, 209-214.

Perrotto, J., Keister, D.B. and Gelderman, A.H. 1971. Incorporation of precursors into Toxoplasma DNA. J. Protozool. 18, 470-473.

Peters, G.J. and Veerkamp, J.H. 1983. Purine and pyrimidine metabolism in peripheral blood lymphocytes. Int. J. Biochem. 15, 115-123.

Pfefferkorn, E.R. 1978. Toxoplasma gondii: The enzymic defect of a mutant resistant to 5-fluorodeoxyuridine. Exp. Parasitol. 44, 26-35.

Pfefferkorn, E.R. 1981. Toxoplasma gondii and the biochemistry of intracellular parasitism. TIBS 6, 311-313.

Pferfferkorn, E.R. and Pfefferkorn, L.C. 1977. Specific labeling of intracellular Toxoplasma gondii with uracil. J. Protozool. 24, 449-453.

Pfefferkorn, E.R. and Pffefferkorn, L.C. 1977a. Toxoplasma gondii: specific labeling of nucleic acids of intracellular parasites in Lesch-Nyhan cells. Exp. Parasitol. 41, 95-104.

Pierre, K. and LePage, G. 1968. Formation of inosine-5'-monophosphate by a kinase in cell free extracts of Ehrlich ascites cells in vitro. Proc. Soc. Exp. Biol. Med. 127, 432-440.

Pizzi, T. and Taliaferro, W.H. 1960. A comparative study of protein and nucleic acid synthesis in different species of trypanosomes. J. Infect. Dis. 107, 100-107.

Pragobpol, S., Gero, A.M., Lee, C.S. and O'Sullivan, W.J. 1984. Orotate phosphoribosyltransferase and orotidylate decarboxylase from Crithidia luciliae: subcellular location of the enzymes and a study of substrate channeling. Arch. Biochem. Biophys. 230, 285-293.

Rainey, P. and Santi, D.V. 1983. Metabolism and mechanism of action of formycin B in Leishmania. Proc. Natl. Acad. Sci USA. 80, 288-292.

Rainey, P., Garret, C.E., and Santi, D.V. 1983. The metabolism and cytotoxic effects of formycin B in Trypanosoma cruzi. Biochem. Pharmacol. 33, 749-752.

Rasenack, J., Pausch, J. and Gerok, W. 1985. De novo pyrimidine biosynthesis in isolated rat hepatocytes. J. Biol. Chem. 260, 4145-4150.

Rathod, P.K. and Reyes, P. 1983. Orotidylate-metabolising enzymes of the human malarial parasite, Plasmodium falciparum, differ from host cell enzymes. J. Biol. Chem. 258, 2852-2855.

Reeves, R.E. and West, B. 1980. Entamoeba histolytica: Nucleic acid precursors affecting axenic growth. Exp. Parasitol 49, 78-82.

Reyes, P., Rathod, P.K., Sanchez, D.J., Mrema, J., Rieckman, K.H. and Heidrich, H. 1982. Enzymes of purine and pyrimidine metabolism from the human malaria parasite, Plasmodium falciparum. Mol. Biochem. Parasitol. 5, 275-290.

Roberts, W., Eisner, V., Shigematsu, A. and Hammond, D.M. 1970. Lack of incorporation of ^{3}H-thymidine into Eimeria callospermophili in cell cultures. J. Parasitol. 56, 833-834.

Roitman, C., Roitman, I. and De Azevedo, H.P. 1972. Growth of an insect trypanosomatid at 37C in a defined medium. J. Protozool. 19, 346-349.

Roitman, I., Angluster, J. and Fiorini, J.E. 1985. Nutrition of the trypanosomatids Crithidia acanthocephali and C. harmosa. Ribose and adenosine: substrates for C. acanthocephal: J. Protozool 32, 490-492.

Rubin, R.J., Jaffe, J.J. and Handschumacher, R.E. 1962. Qualitative differences in the pyrimidine metabolism of Trypanosoma equiperdum and mammals as characterized by 6-azauracil and 6-azauridine. Biochem. Pharmacol. 11, 563-572.

Ryley, J.F. and Wilson, R.G. 1972. Growth factor antagonism studies with Coccidia in tissue cultures. Zeit. Parasitenkund. 40, 31-34.

Ryley, J.F., Wilson, R.G. and Betts, M.G. 1974. Anticoccidial activity of an azauracil derivative. Parasitol. 68, 69-79.

Schimandle, C.M. and Sherman, I.W. 1983. Characterization of adenosine deaminase from the malarial parasite, Plasmodium lophurae and its host cell, the duckling erythrocyte. Biochem. Pharmacol. 32, 115-122.

Schimandle, C.M., Tanigoshi, L., Mole, L.A. and Sherman, I.W. 1985. Purine nucleoside phosphorylase of the malarial parasite, Plasmodium lophurae. J. Biol. Chem. 260, 4455-4460.

Schmidt, G., Walter, R.D. and Konigk, E. 1974. Adenosine kinase from normal mouse erythrocytes and from Plasmodium chabaudi: partial purification and characterization. Tropenmed. Parasit. 25, 301-308.

Schmidt, G., Walter, R.D. and Konigk, E. 1975. A purine nucleoside hydrolase from Trypanosoma gambiense, purification and properties. Tropenmed. Parasit. 26, 19-26.

Scholar, E.M. and Calabresi, E.M. 1973. Identification of the enzymatic pathways of nucleotide metabolism in human lymphocytes and leukemia cells. Cancer Res. 33, 94-103.

Schwartzman, J.D. and Pfefferkorn, E.R. 1981. Pyrimidine synthesis by intracellular Toxoplasma gondii. J. Parasitol. 67, 150-158.

Schwartzman, J.D. and Pfefferkorn, E.R. 1982. Toxoplasma gondii: purine synthesis and salvage in mutant host cells and parasites. Exp. Parasitol. 53, 77-86.

Scott, H.V., Gero, A.M. and O'Sullivan, W.J. 1986. In vitro inhibition of Plasmodium falciparum by pyrazofurin, an inhibitor of pyrimidine biosynthesis de novo. Mol. Biochem. Parasitol. 18, 3-15.

Sharma, N.N. Albach, R.A. and Shaffer, J.G. 1969. Autoradiographic studies of uridine-5-H^3 uptake in Entamoeba histolytica in the GLC medium. J. Protozool. 16, 405-411.

Sherman, I.W. 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol. Rev. 43, 453-495.

Simpson, L. and Braly, P. 1970. Synchronization of Leishmania tarentolae by hydroxyurea. J. Protozool. 17, 511-517.

Sirawaraporn, W. and Yuthavong, Y. 1984. Kinetic and molecular properties of dihydrofolate reductase from pyrimethamine-sensitive and pyrimethamine-resistant Plasmodium chabaudi. Mol. Biochem. Parasitol. 10, 355-367.

Spector, T. and Miller, R.L. 1976. Mammalian adenylosuccinate synthetase. Biochim. Biophys. Acta. 445, 509-517.

Spector, T. and Jones, T.E. 1982. Guanosine 5-monophosphate reductase from Leishmania donovani. A possible chemotherapeutic target. Biochem. Pharmacol. 31, 3891-2897.

Spector, T., Jones T.E. and Elion, G.B. 1979. Specificity of adenylosuccinate synthetase and adenylosuccinate lyase from Leishmania donovani. Selective amination of antiprotozoal agent. J. Biol. Chem. 254, 8422-8426.

Spector, T., Berens, R.L. and Marr, J.J. 1982. Adenylosuccinate synthetase and adenylosuccinate lyase from Trypanosoma cruzi. Biochem. Pharmacol. 31, 225-229.

Spector, T., Jones, T.E., Lafon, S.W., Nelson, D.J. Berens, R.L. and Marr, J.J. 1984. Monophosphates of formycin B and allopurinol riboside: interaction with leishmanial and mammalian succino-AMP synthetase and GMP reductase. Biochem. Pharmacol. 33, 1611-1617.

Steiger, R.F. and Steiger, E. 1977. Cultivation of Leishmania donovani and Leishmania braziliensis in defined medium: nutritional requirements. J. Protozool. 24, 437-441.

Strauss, P.R., Henderson, J.F. and Goodman, M.G. 1985. Nucleosides and lymphocytes - an overview. Proc. Soc. Exp. Biol. Med. 179, 413-418.

Tampitag, S. and O'Sullivan, W.J. 1986. Enzymes of pyrimidine biosynthesis in Crithidia luciliae. Mol. Biochem. Parasitol. 19, 125-134.

Thelander, L. and Reichard, P. 1979. Reduction of ribonucleotides. Ann. Rev. Biochem. 48, 133-158.

Tracy, S.M. and Sherman, I.W. 1972. Purine uptake and utilization by the avian malaria parasite Plasmodium lophurae. J. Protozool. 19, 541-549.

Trager, W. 1974. Nutrition and biosynthetic capabilities of flagellates: problems of in vitro cultivation and differentiation. In Ciba Foundation Symposium 20, pp. 225-246, Elsevier, Amsterdam.

Tremblay, G.C., Crandall, D.E., Knott, C.E. and Alfant, M. 1977. Orotic acid biosynthesis in rat liver: studies on the source of carbamoylphosphate. Arch. Biochem. Biophys. 178, 264-277.

Trigg, P.I. and Gutteridge, W.E. 1971. A minimal medium for the growth of Plasmodium knowlesi in dilution cultures. Parasitol. 62, 113-123.

Trigg, P.I., Gutteridge, W.E. and Williamson, J. 1971. The effects of cordycepin on malaria parasites. Trans. Roy. Soc. Trop. Med. Hyg. 65, 514-520.

Tuttle, J.V. and Krenitsky, T.A. 1980. Purine phosphoribosyltransferases from Leishmania donovani. J. Biol. Chem. 255, 909-916.

Ullman, B., Kaur, K. and Watts, T. 1983. Genetic studies on the role of the nucleoside transport function in nucleoside efflux, the inosine efflux and purine biosynthesis. Mol. Cell. Biol. 3, 1187-1196.

Umezawa, H., Sawa, T., Fukagawa, Y., Homma, I., Ishizuka, M. and Takeuchi, T. 1967. Studies on formycin and formycin B in cells of Ehrlich carcinoma and E. coli. J. Antibiot (Tokyo). 20, 308-316.

Van Dyke, L., Tremblay, G.C. Lantz, C.H. and Szustkiewicz, C. 1970. The source of purines and pyrimidines in Plasmodium berghi. Am. J. Trop. Med. Hyg. 19, 203-208.

Walsh, C.J. and Sherman, I.W. 1968. Purine and pyrimidine synthesis by the avian malaria parasite Plasmodium lophurae. J. Protozool 15, 763-770.

Walter, R.D. 1986. Altered dihydrofolate reductase in pyrimethamine-resistant Plasmodium falciparum. Mol. Biochem. Parasitol. 19, 61-66.

Walter, R.D. and Konigk, 1974. Hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase from Plasmodium chabaudi, purification and properties. Z. Tropend. Parasitol. 25, 227-235.

Wang, C.C. 1983. Purine and pyrimidine metabolism in Trichomonadidae and Giardia: Potential targets for chemotherapy. In: Singer T.P., Mansour, T., Ondarza, R. (eds) Biochemical basis of drug action, Academic Press, New York, pp. 133-145.

Wang, C.C. 1984. Parasite enzymes as potential targets for antiparasitic chemotherapy. J. Med. Chem. 27, 1-9.

Wang, C.C. and Simashkevich, P.M. 1981. Purine metabolism in the protozoan parasite Eimeria tenella. Proc. Natl. Acad. Sci. U.S.A. 78, 6618-6622.

Wang, C.C. and Aldritt, S. 1983. Purine salvage networks in Giardia lamblia. J. Exp. Med. 158, 1703-1712.

Wang, C.C. and Cheng, H.W. 1984a. The deoxyribonucleoside phosphotransferase of Trichomonas vaginalis. J. Exp. Med. 160, 987-1000.

Wang, C.C. and Cheng, H.W. 1984b. Salvage of pyrimidine nucleosides by Trichomonas vaginalis. Mol. Biochem. Parasitol. 10, 171-184.

Wang, C.C., Stotish, R.L. and Poe, M. 1975. Dihydrofolate reductase from Eimeria tenella: rationalization of chemotherapeutic efficacy of pyrimethamine. J. Protozool. 22, 564-568.

Wang, C.C., Simashkevich, P.M. and Stotish, R.L. 1979. Mode of anticoccidial action of aprinocid. Biochem. Pharmacol. 28, 2241-2248.

Wang, C.C., Verham, R., Rice, A. and Tzeng, S. 1983a. Purine salvage by Tritrichomonas foetus. Mol. Biochem. Parasitol. 8, 325-337.

Wang, C.C., Verham, R., Tzeng, S., Aldritt, S. and Cheng, H.W. 1983b. Pyrimidine metabolism in Tritrichomonas foetus. Proc. Natl. Acad. Sci. U.S.A. 80, 2564-2568.

Wang, C.C., Wang, A.L. and Rice, A. 1984a. Tritrichomonas foetus: partly defined cultivation medium for study of the purine and pyrimidine metabolism. Exp. Parasitol. 57, 68-75.

Wang, C.C., Verham, R., Cheng, H.W., Rice, A. and Wang, A.L. 1984b. Differential effects of inhibitors of purine metabolism on two trichomonad species. Biochem. Pharmacol. 33, 1323-1329.

Washtien, W.L., Grumont, R. and Santi, D.V. 1985. DNA amplification in antifolate-resistant Leishmania. J. Biol. Chem. 260, 7809-7812.

Webster, H.K. and Whaun, J.M. 1982. Antimalarial properties of bredinin. J. Clin. Invest. 70, 461-469.

Webster, H.K., Whaun, J.M., Walker, M.D. and Bean, T. 1984. Synthesis of adenosine nucleotides from hypoxanthine by human malaria parasites (Plasmodium falciparum) in continuous erythrocyte culture: inhibition by hadacidin but not alanosine. Biochem. Pharmacol. 33, 1555-1557.

Wykes, J.R. and Prescott, D.M. 1968. The metabolism of pyrimidine compounds by Tetrahymena pyriformis. J. Cell. Physiol. 72, 173-184.

Yamada, K.A. and Sherman, I.W. 1981a. Purine metabolism by the avian malarial parasite Plasmodium lophurae. Mol. Biochem. Parasitol. 3, 253-264.

Yamada, K.A. and Sherman, I.W. 1981b. Purine metabolizing enzymes of Plasmodium lophurae and its host cell, the duckling (Anas domesticus) erythrocyte. Mol. Biochem. Parasitol. 2, 349-358.

Yaseen, M.S. and Al-Chalabi, K.A. 1980. Studies on properties and inhibition of thymidylate synthetase in promastigote forms of Leishmania tropica and Leishmania donovani. Iraqi J. Sci. 21, 534-550.

Yoneda, S. 1971. Some aspects of the purine metabolism of Trypanosoma cruzi in tissue culture. Rev. Bras. Pesquisas Med. Biol. 4, 205-218.

Yoneda, S., Carvalho, R. and Quiroga, M. 1974a. Aspects of pyrimidine biosynthesis of Trypanosoma cruzi. Rev. Inst. Med. Trop. Sao Paulo, 16, 324-327.

Yoneda, S., Carvalho, R. and Castellani, B.R. 1974b. Synthesis of pyrimidic base by intracellular forms of Toxoplasma gondii in cell culture. Rev. Inst. Med. Trop. Sao Paulo, 16, 328-331.

Yuyama, S., Corff, S.C. and Young, P.G. 1979. Levels of true thymidine kinase and nucleoside phosphotransferase in two strains of Tetrahymena pyriformis under different growth conditions. Comp. Biochem. Physiol. 62B, 325-328.

Chapter 3

LEISHMANIA MEXICANA MEXICANA: PURINE METABOLISING ENZYMES OF AMASTIGOTES AND PROMASTIGOTES

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SUMMARY

Culture promastigote and isolated amastigote forms of Leishmania m. mexicana have been surveyed for the presence of enzymes involved in purine metabolism. Quantitative but not qualitative differences between the enzymes of two forms were discovered. There were found to be significant differences between the enzyme content of L.m.mexicana and that reported for L.donovani. Extracts of both parasite forms of L.m. mexicana were found to have higher adenine deaminase and guanine deaminase than adenosine deaminase. There appeared to be two distinct nucleosidases; one active on nucleosides, the other on deoxynucleosides. Phosphorylase could be detected only in the catabolic direction. Nucleotidases were present, but were more active on 3' - than 5' - nucleotides. Phosphoribosyltransferase and nucleoside kinase activities were detected in both forms. Nucleotide-interconverting enzymes were found to be present, with IMP dehydrogenase being the most active. Cell fractionation experiments revealed that, in the promastigote, enzyme separation within the parasite may play an important part in regulating cellular purine metabolism.

Index Descriptors: Leishmania mexicana mexicana, promastigote, amastigote; Protozoa, parasitic; Metabolism; Enzyme; Purine.

INTRODUCTION

All parasitic protozoa examined to date appear to be incapable of synthesizing purine *de novo* and so are dependent on salvage pathways (Gutteridge and Coombs, 1977). This conclusion comes from detailed investigations of various forms of species of *Trypanosoma* (Ceron *et al.* 1979; Gutteridge and Gaborak, 1979; Berens *et al.* 1981; Gutteridge and Davies, 1981; Fish *et al.* 1982a, 1982b), *Leishmania* (Marr *et al.*1978; Davies *et al.* 1983), *Trichomonas* (Heyworth *et al.* 1982; Miller and Lindstead,1983; Wang *et al.*1983), *Plasmodium* (Walsh and Sharman, 1968; Lukow *et al.*, 1973; Yamada and Sherman, 1981; Reyes *et al.* 1982) and *Eimeria* (Wang and Simashkevich, 1981).

There have been several reports on the occurrence and properties of various purine metabolizing enzymes of the promastigotes of the human pathogens *Leishmania donovani* (Marr *et al.* 1978; Koszalka and Krenitsky, 1979; Spector *et al.* 1979a; Konigk and Putfarken, 1980; Nolan and Kidder, 1980; Tuttle and Krenitsky, 1980; Kidder and Nolan, 1981; Lafon *et al.* 1982, Spector and Jones, 1982; Gottlieb and Dywer, 1983), *L.major* (*L.tropica*) (Konigk, 1978; Konigk and Abdel Rasoul, 1978), *L.braziliensis* and *L.mexicana* (Davies *et al.* 1983; Kidder and Nolan, 1979, 1982; Nolan and Kidder, 1980). Very little has been reported, however, on the enzymes of purine metabolism in the leishmanial amastigote. Konigk and Putfarken (1980) inferred that there are stage-specific differences in the AMP-catabolizing reactions of amastigotes and promastigotes of *L.donovani* and this was confirmed by Looker

et al. (1983), who also detected phosphoribosyltransferase and nucleosidase activities in amastigotes of L.donovani. There have been no reports, however, on purine metabolizing enzymes of the amastigote of L.m.mexicana or other cutaneous leishmanias.

In this investigation, comparative studies were performed on extracts of amastigotes and promastigotes of L.m.mexicana in order to characterise the enzymes involved in purine salvage pathways. Special emphasis has been placed on identifying differences between the two forms, in an attempt to elucidate the special adaptations of the amastigote to its environment in the mammalian host. Such important enzymes or metabolic pathways could be targets for chemotherapeutic attack of this parasite.

MATERIALS AND METHODS

Isolation, Cultivation and Fractionation of Parasites.

Leishmania m.mexicana amastigotes were isolated from cutaneous lesions in female NIH mice (Hacking and Churchill, Huntingdon, Cambridge) or CBA mice (Department of Zoology, University of Glasgow, Glasgow G12 8QQ) by a method involving saponin lysis of contaminating erythrocytes and ion exchange chromatography to remove leukocytes (Hart et al. 1981). Promastigotes of L.m.mexicana were grown in vitro in HOMEM medium (Berens et al. 1976) with 10% (v/v) heat inactivated foetal calf serum at 26 C as described previously (North and Coombs, 1981). Crude homogenates of parasites in 50 mM Tris, pH 7.0, containing 0.1mM DTT and (except for studies on phosphoribosyltransferase and kinase activities)

1mM EDTA were obtained by two periods of 15s sonication, separated by a 30s cooling period, using a MSE Soniprep 150. Homogenates were fractionated by centrifugation at 105,000 Xg at 4 C for 1 h and the resultant supernatant used as source of the enzyme in all experiments except where specified. With amastigotes, leupeptin was added at 100 µg/ml to the parasite suspension before sonication for some experiments.To investigate the subcellular localisation of enzymes, L.m.mexicana promastigotes were gently lysed by mixing with acid-washed alumina (sigma type 305) and fractionated by differential centrifugation at 2100 Xg for 10 min, 15,800 Xg for 10 min and 240,000 Xg for 1 h as described previously (Coombs et al. 1982). The four fractions produced were frozen and thawed three times to disrupt organelles and assayed for purine salvage enzymes.

Enzyme analyses.

The composition of the assay mixtures and the extinction coefficients used to calculate the enzyme activities are summarised in Table I. All enzyme activities were assayed spectrophotometrically at 26 C. The rate of change in absorbance resulting from the conversion of substrates to products was monitored at the appropriate wavelength using a Pye Unicam SP800 ultraviolet spectrophotometer at a full scale setting of 0-0.2 absorbance units. Final assay mixtures had a total volume of 1.0ml and 1cm pathlength quartz cuvettes were used. Reaction mixtures were preincubated to 26 C and the reaction usually was started by the addition of enzyme. All assays were carried out in triplicate in each experiment. One unit of enzyme activity was defined as the amount of enzyme that will catalyze the conversion of 1 nmol of substrate to product per minute at 26 C. All specific activities were calculated with reference to sample

TABLE I

Spectrophotometric Assay Conditions

Enzyme	EC	Substrate	Final concentration (m*M*)	Other constituent of reaction mixture[a]	Product	nm (mM^{-1} cm^{-1})[b]	References
Adenine deaminase	3.5.4.2	Adenine	0.1	0.02 U XOD	Urate	293 (10.6)	
Guanine deaminase	3.5.4.3	Guanine	0.05	0.02 U XOD	Urate	293 (8.4)	Kalckar 1947*a*
Adenosine deaminase	3.5.4.4	Adenosine	0.1	0.005 U PNP, 0.02 U XOD	Urate	293 (10.6)	Kalckar 1947*a*
AMP deaminase	3.5.4.6	AMP	0.1		IMP	265 (−7.6)	Kalckar 1947*b*
Nucleosidase or phosphorylase (catabolic)[c]	3.2.2.1 or 2.4.2.1	Inosine	0.5	0.02 U XOD	Urate	293 (10.6)	Koszalka and Krenitsky 1979
		(r/d)[d] Guanosine	0.05		Guanine	252 (−4.4)	Koszalka and Krenitsky 1979
		(r/d) Adenosine	0.1		Adenine	255 (−2.0)	Koszalka and Krenitsky 1979
		Xanthosine	0.5	0.02 U XOD	Urate	302 (5.6)	Koszalka and Krenitsky 1979
Phosphorylase (anabolic)	2.4.2.1	Adenine	0.1	1 m*M* R-1-P or dR-1-P, 0.4 U ADA	Inosine	261 (−6.4)	Miller and Lindstead 1983
		Hypoxanthine	0.1	1 m*M* R-1-P or dR-1-P	Inosine	249 (1.4)	Miller and Lindstead 1983
		Guanine	0.05	1 m*M* R-1-P or dR-1-P	Guanosine	255 (4.2)	Miller and Lindstead 1983
		Xanthine	0.1	1 m*M* R-1-P or dR-1-P	Xanthosine	248 (4.4)	Miller and Lindstead 1983
Nucleotidase	3.1.3.6	3′-Nucleotide	2.5	100 m*M* KCl, 1 m*M* $CoCl_2$			Gottlieb and Dwyer 1983
	3.1.3.5	5′-Nucleotide	5	100 m*M* KCl, 10 m*M* $MgCl_2$			Gottlieb and Dwyer 1983
		IMP	0.25	5 m*M* $MgSO_4$, 0.1 m*M* EDTA, 32 m*M* $(NH_4)_2SO_4$, 0.005 U PNP, 0.02 U XOD	Urate	292 (11.5)	Tuttle and Krenitsky 1980
Phosphoribosyltransferase (PRTase)	2.4.2.7	Adenine	0.1	5 m*M* $MgSO_4$, 1 m*M* PRPP, buffer pH 9.2	AMP	255 (3.3)	Tuttle and Krenitsky 1980
	2.4.2.8	Guanine	0.05	5 m*M* $MgSO_4$, 1 m*M* PRPP	GMP	255 (4.2)	Tuttle and Krenitsky 1980
	2.4.2.8	Hypoxanthine	0.1	5 m*M* $MgSO_4$, 1 m*M* PRPP	IMP	243 (2.2)	Tuttle and Krenitsky 1980
	2.4.2.22	Xanthine	0.1	5 m*M* $MgSO_4$, 1 m*M* PRPP	XMP	250 (3.9)	Tuttle and Krenitsky 1980
Kinase	2.7.1.20	Nucleoside	0.1	25 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* PEP, 0.2 m*M* NADH, 1 m*M* ATP, 0.125 U PK, 0.1 U LDH	Nucleotide	340 (−18.66)	Lindberg *et al.* 1967
	2.7.4.3	AMP	0.1	As per nucleoside	ADP	340 (−12.44)	Lindberg *et al.* 1967
	2.7.4.8	GMP	0.1	As per nucleoside	GDP	340 (−12.44)	Lindberg *et al.* 1967
Adenylosuccinate lyase	4.3.2.2	Succino AMP	0.05	5 m*M* EDTA	AMP	280 (−10.7)	Spector *et al.* 1979*a*
GMP reductase	1.6.6.8	GMP	0.1	5 m*M* DTT, 1 m*M* EDTA, 0.1 m*M* NADPH	IMP	340 (−6.22)	Spector *et al.* 1979*b*
IMP dehydrogenase	1.2.1.14	IMP	2.3	0.1 *M* KCl, 0.28 m*M* NAD	XMP	340 (6.22)	Miller and Adamczyk 1976
Adenylosuccinate synthetase	6.3.4.4	IMP	0.25	0.125 m*M* GTP, 5 m*M* $MgCl_2$, 8 m*M* KCl, 0.2 m*M* NADH, 0.125 U PK, 0.1 U LDH, 4 m*M* aspartate[e]	S-AMP	280 (11.7)	Spector *et al.* 1979*a*
GMP synthetase	6.3.5.2	XMP	0.125	1.25 m*M* ATP, 10 m*M* $MgSO_4$, 60 m*M* $(NH_4)_2SO_4$, 0.5 m*M* PEP, 0.2 m*M* NADH, 0.125 U PK, 0.1 U LDH	GMP	340 (12.44)	Spector *et al.* 1974

Note. Abbreviations used as indicated under Materials and Methods.

[a] When not included otherwise, the buffer used was 50 mM Tris–HCl.

[b] Wavelength monitored (extinction coefficient).

[c] In phosphorylase assays, 20 mM sodium phosphate was added.

[d] (r/d) = ribo/deoxyribo.

[e] 50 mM Hepes buffer was used.

protein and measured at the enzyme's pH optimum, except where indicated otherwise. Adenine phosphoribosyltransferase (APRTase) was assayed at pH 9.2 in order to eliminate interference from adenine deaminase. The apparent K_m's for some substrates were calculated by the Lineweaver-Burk method. Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Enzyme nomenclature.

A variety of names have been used by previous workers for what were apparently the same enzymes activities. In this communication, the following enzyme names are used throughout (alternatives used in the literature are given in parentheses): adenine deaminase (adenase, adenine aminohydrolase); guanine deaminase (guanase, guanine aminohydrolase); adenosine deaminase (adenosine aminohydrolase); AMP deaminase (AMP aminohydrolase); nucleosidase (purine ribonucleosidase, nucleoside hydrolase, nucleoside cleavage); phosphorylase (purine nucleoside phosphorylase); AMP kinase (adenylate kinase); GMP kinase (guanylate kinase); adenylosuccinate lyase (succino-AMP lyase, adenosuccinate AMP-lyase); GMP reductase (guanylate reductase); IMP dehydrogenase (IMP: NAD+ oxidoreductase); adenylosuccinate synthetase (succino-AMP synthetase, IMP: L-aspartate ligase (GDP)) and GMP synthetase (guanylate synthetase, XMP: L-glutamine amidoligase (AMP)).

Materials.

Purine bases, nucleosides, 2' - deoxynucleosides, nucleoside monophosphates, D-ribose-1-phosphate (R-1-P), 2-deoxy-D-ribose-1-phosphate (dR-1-P), NADH, phosphoenolpyruvate (PEP), 5-phosphoribosyl-1-pyrophosphate

(PRPP), bovine milk xanthine oxidase (XOD), calf intestine adenosine deaminase (ADA), rabbit muscle pyruvate kinase (PK) and lactate dehydrogenase (LDH) were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K., NAD^+, NADPH and calf spleen purine nucleoside phosphorylase (PNP) were purchased from Boehringer Mannheim Corp. Leupeptin was supplied by the Protein Research Foundation, Osaka, Japan. Other reagents were of analytical grade and obtained from BDH Laboratory Chemical Division, Poole, Dorset or Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

RESULTS

The specific activities at the pH optimum of the various purine salvage enzymes which were detected in high speed supernatant fractions of promastigote and amastigote forms of L.m.mexicana are given in Tables II and III respectively. Higher activities of several enzymes were detected in amastigotes lysed in the presence of 100 µg/ml leupeptin than in lysates produced in its absence. The lower activities in the latter case presumably were due to inactivation of the enzymes by the amastigote proteinases that are present at very high activity (Coombs, 1982). It has been suggested that inhibition of the amastigote proteinase by leupeptin is a sensible precaution to take in many studies of amastigote proteins (Pupkis and Coombs, 1984).

Deaminases.

Adenine deaminase, guanine deaminase and adenosine deaminase were present in both promastigotes and amastigotes, whereas AMP deaminase was found only with small organelle fractions of the cell (see Table V). The activity of adenine deaminase was 5 times greater in the promastigote

TABLE II

Activities of Purine Salvage Enzymes in Promastigote Forms of *Leishmania mexicana mexicana*

	Deaminase	Nucleosidase	Phosphorylase (catabolic)	Phosphorylase (anabolic)	Kinase	PRTase	Nucleotidase
pH optimum	[a]	[b]	7.0	[c]	6.0	7.4	[e]
Adenine	156 ± 8 (4)			n.d. < 3 (2)		15 ± 4 (3)[d]	
Adenosine	15 ± 1 (3)	n.d. < 2 (3)	n.d. < 5 (3)		47 ± 6 (4)		
Deoxyadenosine		n.d. < 2 (3)	n.d. < 5 (3)		40 ± 5 (6)		
5′-AMP	n.d. < 2 (3)				48 ± 17 (3)		23 ± 2 (3)
3′-AMP							70 ± 6 (3)
dAMP					34 ± 17 (3)		
Hypoxanthine				n.d. < 13 (2)		55 ± 6 (3)	
Inosine		172 ± 9 (6)	97 ± 45 (4)		26 ± 4 (3)		
Deoxyinosine		75 ± 5 (6)	15 ± 6 (3)		28 ± 7 (3)		
IMP					31 ± 1 (2)		76 ± 7 (3)
dIMP					34 ± 4 (2)		
Guanine	60 ± 5 (5)			n.d. < 4 (2)		120 ± 9 (3)	
Guanosine		101 ± 21 (5)	17 ± 11 (3)		27 ± 6 (3)		
Deoxyguanosine		60 ± 6 (5)	n.d. < 2 (3)		27 ± 7 (3)		
GMP					36 ± 12 (2)		52 ± 6 (3)
dGMP					40 ± 17 (2)		
Xanthine				n.d. < 4 (2)		19 ± 2 (3)	
Xanthosine		52 ± 4 (3)	34 ± 14 (3)		24 ± 5 (3)		
XMP					62 ± 6 (3)		24 ± 1 (3)

Note. The activities given are in nmol min^{-1} mg $protein^{-1}$, and are the means (±SD or range) from the number of experiments given in parentheses. n.d., not detectable.

[a] pH optimum for adenine deaminase was 7.4 and for guanine deaminase, 8.0; adenosine deaminase and AMP deaminase were assayed at pH 7.4.

[b] pH optimum for nucleosidase was 5.5 and for deoxyribonucleosidase was 6.5.

[c] Assayed at pH 6.8.

[d] Assayed at pH 9.2.

[e] 5′-Nucleotidase assayed at pH 7.0; 3′-nucleotidase assayed at pH 8.5. Activity in homogenate, assayed by the method of Gottlieb and Dwyer 1983.

TABLE III

Activities of Purine Salvage Enzymes in Amastigote Forms of *Leishmania mexicana mexicana*

	Deaminase	Nucleosidase	Phosphorylase (catabolic)	Phosphorylase (anabolic)	Kinase	PRTase	Nucleotidase
pH optimum	[a]	[b]	7.0	[c]	6.0	7.4	[e]
Adenine	45 ± 11 (5)/ 48 ± 7 (3)			n.d. < 1 (2)		n.d. < 2 (3)/6 ± 5 (3)[d]	
Adenosine	9 ± 2 (4)/ 8 ± 2 (4)	n.d. < 3 (3)	ND/n.d. < 4 (2)		4 ± 3 (3)/ 34 ± 10 (3)		
Deoxyadenosine		n.d. < 3 (3)	ND/n.d. < 4 (2)		3 ± 0.7 (2)/ 22 ± 9 (3)		
5'-AMP	n.d. < 0.7 (3)				2 ± 0.6 (2)/ 26 ± 13 (3)		12 ± 1 (3)/ 13 ± 2 (3)
3'-AMP							53 ± 4 (3)/ 54 ± 7 (3)
Hypoxanthine				n.d. < 6 (2)		n.d. < 2 (3)/16 ± 5 (4)	
Inosine		85 ± 4 (2)/ 87 ± 7 (4)	ND/11 ± 2 (3)		2 ± 0.1 (2)/ 13 ± 6 (2)		
Deoxyinosine		86 ± 14 (3)/ 89 ± 13 (3)	ND/6 ± 1 (3)		2 ± 1 (2)/ 16 ± 3 (3)		
IMP							16 ± 1 (2)/ 15 ± 1 (2)
Guanine	69 ± 17 (3)/ 67 ± 10 (3)			n.d. < 2 (2)		n.d. < 1 (3)/11 ± 9 (3)	
Guanosine		10 ± 1 (3)/ 10 ± 3 (3)	ND/n.d. < 2 (2)		0.9 ± 0.2 (2)/ 17 ± 6 (3)		
Deoxyguanosine		4 ± 3 (3)	ND/n.d. < 2 (2)		2 ± 0.1 (2)/ 19 ± 6 (5)		
GMP					0.7 ± 0.4 (2)/ 2 ± 1 (3)		17 ± 1 (2)/ 14 ± 1 (2)
Xanthine				n.d. < 2 (2)		n.d. < 1 (3)/4 ± 3 (3)	
Xanthosine		103 ± 16 (3)/ 148 ± 23 (7)	ND/6 ± 2 (3)		3 ± 2 (2)/ 12 ± 3 (3)		
XMP							17 ± 2 (2)/ 12 ± 1 (2)

Note: For each enzyme, the first and second activities given are those detected in homogenate supernatants prepared in the absence and presence of leupeptin (100 μg/ml), respectively. The activities were assayed and presented as described in Table II. n.d., not detectable; ND, not determined.

[a] pH optimum for adenine deaminase was 7.4 and for guanine deaminase, 7.0; adenosine deaminase and AMP deaminase were assayed at pH 7.4.

[b] pH optimum for nucleosidase was 5.5 and for deoxynucleosidase, 6.0.

[c] Assayed at pH 6.8.

[d] Assayed at pH 9.2.

[e] 5'-Nucleotidase assayed at pH 7.0; 3'-nucleotidase assayed at pH 8.5. Activity in homogenate, assayed by the method of Gottlieb and Dwyer 1983.

than the amastigote, but the pH optimum remained the same and the apparent K_m for adenine differed little: amastigote, 0.45 x 10^{-5} M; promastigote, 0.50 x 10^{-5} M. Guanine deaminase was similarly active in both forms, however there were significant differences in the pH optimum and apparent Km's for guanine : amastigote, pH 7.0, 10^{-5} M; promastigote, pH 8.0, 3 x 10^{-5} M. The guanine deaminase activity declined sharply after approximately 5 min incubation with both promastigote and amastigote samples.

Nucleosidases.

All of the ribonucleosides and 2' -deoxyribonucleosides tested, with the exception of ribo- and deoxyriboadenosine, were cleaved to their respective bases by extracts of L.m.mexicana promastigotes. The ribonucleosides were cleaved at higher rates than their corresponding 2' -deoxyribonucleoside derivatives. Optimum activity was observed at pH 5.5 with inosine, guanosine and xanthosine as substrates and at pH 6.5 with deoxyinosine and deoxyguanosine as substrates. The apparent Michaelis constants for ribo- and deoxyriboinosine were 0.6 x 10^{-5} M and 1 x 10^{-5} M respectively, while that for guanosine was 0.2 x 10^{-5} M.

Nucleosidase and deoxynucleosidase of amastigotes were found to be different from the promastigote enzymes in several respects. Firstly, the activity with deoxyinosine as substrate was found to be optimal at pH 6.0 not pH 6.5. Secondly, purine ribonucleosides and their corresponding 2' -deoxyribonucleosides were found to be cleaved at very similar rates. Thirdly, the hydrolysis of deoxyinosine, although initially rapid, declined sharply after a few minutes, unlike the promastigote activity. Fourthly, the relative activities against different substrates was found

to be xanthosine>inosine = deoxyinosine>guanosine>deoxyguanosine, whereas with promastigote enzymes the activities were in the order of inosine> guanosine>deoxyinosine>deoxyguanosine>xanthosine. In absolute terms, the rates of cleavage of inosine, guanosine, and deoxyguanosine were higher in promastigotes than amastigotes, whereas those of xanthosine and deoxyinosine were higher in amastigotes. As with the promastigote, no hydrolysis of ribo- and deoxyriboadenosine could be detected with the amastigotes.

Phosphorylases.

None of the purine bases tested were converted to their respective ribonucleosides by the extracts of either parasite form in the presence of either ribose-1-phosphate or deoxyribose-1-phosphate. These data indicate that L.m.mexicana does not possess a phosphorylase activity in the anabolic direction. To test the possibility that phosphorylase could play a part in the cleavage of nucleosides, the effect of phosphate (20mM) on nucleoside hydrolysis was studied. Phosphorylase acting on inosine, deoxyinosine and xanthosine was detected in both forms of the parasite, although the rate was higher with the promastigote. The phosphate-dependent conversion of guanosine to guanine was detected only with the promastigote. No activity was detected for adenosine with either amastigotes or promastigotes. The pH optimum for phosphorylase was significantly higher than for the nucleosidases.

Kinases.

Purine nucleoside kinase activity in extracts of promastigotes was optimal at pH 6.0. Magnesium ions were essential for enzyme activity,

the rate of which was related to the concentrations of both Mg^{++} and ATP (Fig.1). The highest enzyme rate was obtained with 2×10^{-3} M Mg^{++} and 10^{-3} M ATP. At lower concentrations of each, the activity was less, whereas increases in the ATP and Mg^{++} concentrations above these levels, either singly or together, resulted in inhibition of the enzyme. The Michaelis constants were determined to be 1.5×10^{-6} M for adenosine and 1.0×10^{-4} M for ATP. Promastigote adenosine kinase exhibited broad specificity with respect to nucleoside triphosphate, though the activity was highest with ATP as phosphate donor.

Extracts of amastigotes were assayed for adenosine kinase activity using the conditions optimal for the promastigote enzyme. The activity detected was some eight times greater in lysates produced in the presence of leupeptin, suggesting that it protected the enzyme. Kinase activities with inosine, guanosine, xanthosine and their respective deoxyribonucleosides were detected in the amastigote. AMP and GMP kinase activities also were present in both parasite forms (Tables II and III).

Phosphoribosyltransferases.

Purine phosphoribosyltransferase (PRTase) activities were detected in promastigotes of L.m.mexicana. Parasite adenine deaminase activity significantly interfered with the assay of adenine PRTase. To minimize this, adenine PRTase was assayed at pH 9.2, where the adenine deaminase activity was insignificant. The rates of activity detected with different substrates was in the order guanine>hypoxanthine>xanthine> adenine. The activities were stimulated by divalent cations (5mM) and it was found that Mg^{++} was more effective than Mn^{++}, Zn^{++} or Co^{++}. It is interesting to note, however, that $MnSO_4$ (5mM), unlike the other divalent

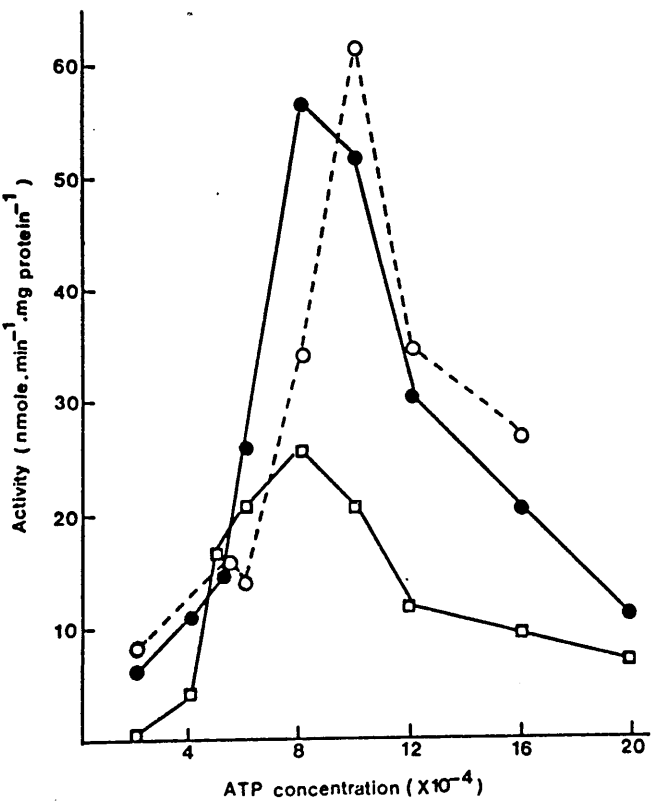

FIG. 1. The effect of ATP concentration on the adenosine kinase activity of *Leishmania mexicana mexicana* promastigotes at various Mg^{2+} concentrations. Key: (●)10^{-3}; (○)2×10^{-3}; (□)5×10^{-4} *M* Mg^{2+}.

cations tested, markedly increased the rate of change of absorbance of an incubation mixture containing buffer, PRPP and extract but no purine base. This endogenous activity made it impossible to measure, spectrophotometrically, the precise purine PRTase activities in the presence of Mn^{++}.

PRTase activities were detectable in amastigote extracts only when leupeptin had been used. Even then, the amastigote activities were significantly lower than in the promastigote (Tables II and III). The relative rates of PRTase activities detected in amastigotes were hypoxanthine>guanine>adenine>xanthine.

Nucleotidases.

5^{1}- and 3^{1} -Nucleotidase activities towards several substrates were present in homogenates of both promastigotes and amastigotes (Tables II and III).

Enzymes catalyzing purine nucleotide interconversions.

Adenylosuccinate lyase, adenylosuccinate synthetase, IMP dehydrogenase, GMP reductase and GMP synthetase were all detected in both amastigotes and promastigotes, although only when leupeptin was used were the latter two enzymes detectable in amastigote extracts (Table IV). IMP dehydrogenase was the most active enzyme of the five in both forms of the parasite. Promastigote adenylosuccinate lyase was stimulated three fold by 5mM EDTA, was most active at pH 7.8 and had a K_m for adenylosuccinate of 2.3×10^{-6} M.

Subcellular localisation of enzymes.

The activities recovered in the various cell fractions of L.m.

TABLE IV

Specific Activities of Enzymes of *Leishmania mexicana mexicana* Involved in Interconversion of Purine Nucleotides

Enzyme	Promastigote	Amastigote	
		− Leupeptin[a]	+ Leupeptin
Adenylosuccinate lyase	19 ± 1 (6)	4 ± 2 (3)	12 ± 6 (9)
Adenylosuccinate synthetase	2 ± 0.4 (6)	1 ± 0.3 (4)	2 ± 0.5 (7)
IMP dehydrogenase	151 ± 12 (4)	17 ± 9 (3)	56 ± 10 (7)
GMP reductase	5 ± 0.7 (5)	n.d. < 0.5 (2)	2 ± 0.3 (2)
GMP synthetase	6 ± 1 (2)	n.d. < 0.3 (2)	7 ± 5 (3)

Note. The activities given are in nmol min^{-1} mg $protein^{-1}$, and are the means (±SD or range) from the number of experiments given in parentheses. n.d., not detectable.

[a] Amastigotes were lysed in the presence or absence of leupeptin at 100 μg/ml.

mexicana promastigotes are shown in Table V . Adenine deaminase and guanine deaminase activities were present mainly in the cytosolic fraction. In contrast, AMP deaminase activity was detected only in the small organelle fraction, whereas adenosine deaminase was recovered in all fractions. The nucleosidases were mainly cytosolic. Nucleoside kinase activities were recovered in all fractions, but the highest amounts were in fractions P_1 and P_2. In contrast, most of GMP kinase was recovered in the cytosolic fraction. Adenine PRTase was recovered in S but was not detectable in any other fraction. Guanine PRTase also appeared to be largely cytosolic, although a significant proportion was recovered in fraction P_2. IMP nucleotidase was found to be largely particulate with more than 80% of the activity being recovered in the particulate fractions. IMP dehydrogenase was detected in all fractions, with a large proportion being in P_3, the microsomal fraction, whereas adenylosuccinate lyase was mainly cytosolic.

DISCUSSION

The pathways implicated with purine salvage in L.m.mexicana by this study are shown in Fig.2. The results suggest that the differences between amastigotes and promastigotes probably are quantitative rather than qualitative. The presence of highly active adenine deaminase, guanine deaminase and nucleosidases points to the parasite xanthine and hypoxanthine phosphoribosyltransferase activities being of central importance to both developmental forms. This suggestion is supported by the previous observation that L.m.mexicana amastigotes and promastigotes incorporate exogenous hypoxanthine in large amounts into their nucleic

TABLE V

Distribution of Enzymes in Subcellular Fractions of *Leishmania mexicana mexicana* Promastigotes[a]

Enzyme	P_1	P_2	P_3	S	Percentage recovery
Adenine deaminase	10 ± 5	5 ± 3	4 ± 0.8	81 ± 8	134 ± 15
Guanine deaminase	n.d.[b]	n.d.	n.d.	100	n.a.[c,d]
Adenosine deaminase	22 ± 8	27 ± 4	14 ± 4	44 ± 4	121 ± 12
AMP deaminase	n.d.	100	n.d.	n.d.	n.a.[d]
Inosine nucleosidase	1 ± 0.9	<0.5 ± 0.2	7 ± 6	91 ± 7	110 ± 9
Guanosine nucleosidase	13 ± 3	5 ± 4	<2 ± 0.8	81 ± 5	128 ± 35
Adenosine nucleosidase	n.d.	n.d.	n.d.	n.d.	n.a.[d]
Xanthosine nucleosidase	n.d.	n.d.	n.d.	100	n.a.[d]
2-Deoxyinosine nucleosidase	n.d.	n.d.	n.d.	100	n.a.[d]
2-Deoxyguanosine nucleosidase	25 ± 6	n.d.	n.d.	53 ± 6	n.a.[d]
2-Deoxyadenosine nucleosidase	n.d.	n.d.	n.d.	n.d.	n.a.[d]
Adenosine kinase	31 ± 4	29 ± 6	15 ± 6	25 ± 6	104 ± 11
Inosine kinase	27 ± 6	39 ± 8	12 ± 4	22 ± 6	107 ± 5
Guanosine kinase	30 ± 4	30 ± 4	15 ± 7	24 ± 5	127 ± 12
Xanthosine kinase	33 ± 6	33 ± 6	13 ± 5	20 ± 7	72 ± 36
AMP kinase	38 ± 5	25 ± 5	14 ± 8	23 ± 4	71 ± 16
GMP kinase	29 ± 11	9 ± 3	5 ± 1	58 ± 12	91 ± 21
Adenine PRTase	n.d.	n.d.	n.d.	100	n.a.[d]
Guanine PRTase	8 ± 2	14 ± 2	4 ± 0.5	74 ± 6	98 ± 28
IMP nucleotidase[e]	23 ± 6	36 ± 4	26 ± 11	16 ± 4	99 ± 7
IMP dehydrogenase	19 ± 7	13 ± 4	35 ± 3	34 ± 13	49 ± 17
Adenylosuccinate lyase	22 ± 3	<8 ± 3	<5 ± 0.5	66 ± 0.9	126 ± 44
Hexokinase[f]	16 ± 6	54 ± 7	14 ± 5	12 ± 6	97 ± 10
Succinic dehydrogenase[f]	43 ± 24	35 ± 26	23 ± 14	<1	101 ± 31
RNA/Protein[f]	0.07 ± 0.06	0.07 ± 0.05	0.37 ± 0.16	0.06 ± 0.04	n.a.
Glucose-6-phosphate dehydrogenase[f]	6 ± 6	6 ± 2	5 ± 2	81 ± 6	98 ± 6

[a] The figures (means ± SD from three experiments) represent the activity present in each fraction as a percentage of the total activity recovered.

[b] n.d., not detectable.

[c] n.a., not applicable.

[d] Activity not detectable in homogenates.

[e] Assayed by the method of Tuttle and Krenitsky 1980.

[f] Data from Coombs *et al.* 1982.

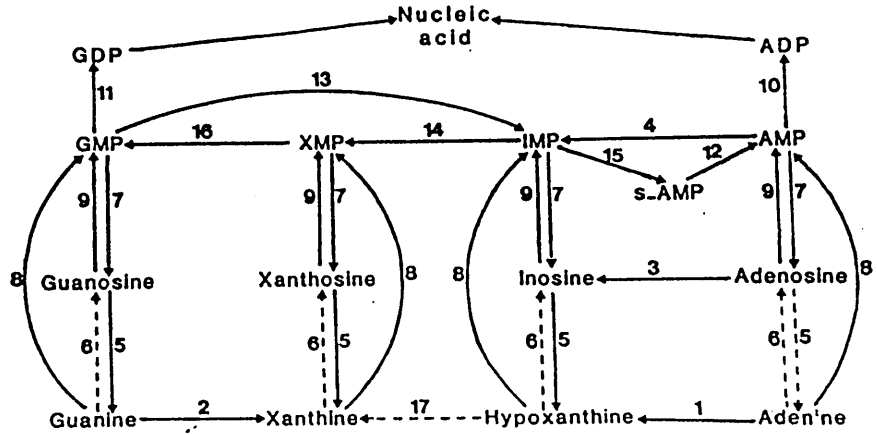

FIG. 2. Purine salvage pathways of *Leishmania mexicana mexicana*. (→) pathways present; (- - - →) pathways not detectable. Key to enzymes: 1, adenine deaminase; 2, guanine deaminase; 3, adenosine deaminase; 4, AMP deaminase; 5, nucleosidase or phosphorylase (catabolic); 6, phosphorylase (anabolic); 7, nucleotidase; 8, phosphoribosyltransferase; 9, nucleoside kinase; 10, AMP kinase; 11, GMP kinase; 12, adenylosuccinate lyase; 13, GMP reductase; 14, IMP dehydrogenase; 15, adenylosuccinate synthetase; 16, GMP synthetase; 17, xanthine oxidase.

acids (Capaldo and Coombs, 1983). The nucleotides produced by the activity of the phosphoribosyltransferases could be converted to AMP and GMP by other enzymes present (adenylosuccinate synthetase, adenylosuccinate lyase, IMP dehydrogenase and GMP synthetase) and so incorporated into RNA and DNA. In this respect, L.m.mexicana appears to be similar to L.donovani (Looker et al. 1983) and several other trypanosomatids studied (Berens et al. 1981; Ceron et al. 1979; Fish et al. 1982a, 1982b; LaFon et al. 1982; Marr et al. 1978). The present study shows, however, that in various respects purine metabolism in L.m.mexicana differs significantly from that of L.donovani.

Leishmania m.mexicana apparently differs quite considerably from L.donovani in adenine and adenosine metabolism. L.donovani amastigotes possess an active adenosine deaminase which catalyzes the conversion of the nucleoside to inosine which can be metabolised subsequently to hypoxanthine (Konigk and Putfarken, 1980; Looker et al. 1983). In contrast, L.donovani promastigotes lack adenosine deaminase, and, although adenine deaminase is present, the major route for adenosine metabolism is thought to be phosphorylation to AMP. It has been postulated that the differences in metabolism of the two developmental forms is related to the interpretation of environmental signals (Konigk and Putfarken, 1980). Such stage-specific adenosine metabolism apparently does not exist in L.m.mexicana. The presence in both parasite forms of adenosine deaminase and adenosine kinase and the apparent absence of adenosine nucleosidase and adenosine phosphorylase suggests that exogenous adenosine, which is incorporated in large amounts by both stages (Capaldo and Coombs, 1983), is converted either to AMP by adenosine kinase or to hypoxanthine via adenosine deaminase and inosine

nucleosidase. It is not known how the differences between L.m.mexicana and L.donovani are related to their respective environments; further study is necessary to unravel this fascinating problem.

Two nucleosidases have been reported for L.donovani, one specific for ribonucleosides, the other for deoxyribonucleosides (Koszalka and Krenitsky, 1979). The evidence presented in this paper concerning the relative rates of hydrolysis of ribonucleosides and deoxyribonucleosides in the two forms of L.m.mexicana and the pH optima with the different substrates, together with the different sensitivities of the two activities to a range of inhibitors (unpublished data), suggests that L.m.mexicana also contains two distinct enzymes.

Leishmania m.mexicana appears also to differ from L.donovani with respect to nucleoside kinase activities. Unlike this latter organism (Looker et al. 1983), activities were detected in L.m.mexicana with all four purine nucleosides and, in addition, the specific activities towards the different nucleosides were similar in both forms of the parasite.

The absence of phosphorylase activity in the anabolic direction suggests that purine bases can be salvaged only via phosphoribosyltransferases. Nucleosides other than adenosine could be cleaved by nucleosidase or phosphorylase and the resulting bases then converted to their respective nucleotides by phosphoribosyltransferases. The specific activities of the phosphoribosyltransferases reported in this paper are quite similar to the levels reported by Looker et al. (1983) for L.donovani and the previous work with promastigotes of L.donovani (Tuttle and Krenitsky, 1980). The enzymes' requirement for divalent cations is similar to previous reports for xanthine

phosphoribosyltransferase in L.mexicana (Kidder and Nolan, 1982). The distribution profiles for adenine and guanine PRTases suggests that they are distinct enzymes, as has also been found with L.donovani (Tuttle and Krenitsky, 1980). The recovery of a significant proportion of guanine PRTase in the P_2 fraction suggests that this enzyme may be associated with glycosomes as has been reported in Trypanosoma cruzi (Gutteridge and Davies, 1982). Studies on the subcellular organisation of PRTases in L.m.mexicana involving separation of parasite fractions by isopycnic centrifugation on linear sucrose gradients have demonstrated that xanthine, guanine and hypoxanthine PRTase activities are present in glycosomes but adenine PRTase is not (see chapter 4).

In contrast to observations with the promastigotes of L.donovani, adenylosuccinate lyase was found to be more active than adenylosuccinate synthetase in both forms of L.m.mexicana (Spector et al. 1979b). The level of GMP reductase was quite similar to that reported to be present in L.donovani (Spector and Jones, 1982). There have been no previous reports on the activity of IMP dehydrogenase in trypanosomatids. The relatively high activity of the enzyme and its potentially key role in synthesis of GMP make this an interesting enzyme for further analysis. The apparent location of a significant part of this activity in the microsomal fraction of L.m.mexicana, whereas the kinase activities and IMP nucleotidase were present in the small organelle fraction and the nucleosidases were found in the cytosol, suggests that there is separation within the cell of at least some of the anabolic and catabolic enzymes which would compete for the same substrates. It seems likely that such separation plays an important role in regulating cellular purine metabolism.

Previous studies have demonstrated how differences in the properties of leishmanial and host cell purine metabolizing enzymes may be exploited in chemotherapy (Pfaller and Marr, 1974; Marr and Berens, 1977, 1983; Nelson, et al. 1979a, 1979b, 1982; Carson and Chang, 1981; Berman et al. 1983, Rainy and Santi, 1983). It is the amastigote that is the main target for antileishmanial drugs. The study reported in this paper is the first stage in an investigation of L.m.mexicana amastigote enzymes and their potential as targets in chemotherapy.

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REFERENCES

BERENS, R.L., BRUN, R., AND KRASSNER, S.M. 1976. A simple monophasic medium for axenic culture of Hemoflagellates. Journal of Parasitology 62, 360-365.

BERENS, R.L., MARR, J.J., LaFON, S.W., AND NELSON, D.J. 1981. Purine metabolism in Trypanosoma cruzi. Molecular and Biochemical Parasitology 3, 187-196.

BERMAN, J.D., RAINEY, P., AND SANTI, D.V. 1983. Metabolism of formycin B by Leishmania amastigotes in vitro. Comparative metabolism in infected and uninfected human macrophages. Journal of Experimental Medicine 158, 252-257.

CAPALDO, J., AND COOMBS, G.H. 1983. New approaches to the chemotherapy of Leishmaniasis. Parasitology 87, xli.

CARSON, D.A., AND CHANG, K.P. 1981. Phosphorylation and anti-leishmanial activity of formycin B. Biochemical and Biophysical Research Communications 100, 1377-1383.

CERON, C.R., CALDAS, R.A., FELIX, C.R., MUNDIM, M.H., AND ROITMAN, I. 1979. Purine metabolism in Trypanosomatids. Journal of Protozoology 26, 479-483.

COOMBS, G.H. 1982. Proteinases of Leishmania mexicana and other flagellate protozoa. Parasitology 84, 149-155.

COOMBS, G.H., CRAFT, J.A., AND HART, D.T. 1982. A comparative study of Leishmania mexicana amastigotes and promastigotes. Enzyme activities and subcellular locations. Molecular and Biochemical Parasitology 5, 199-211.

DAVIES, M.J., ROSS, A.M., AND GUTTERIDGE, W.E. 1983. The enzymes of purine salvage in Trypanosoma cruzi, Trypanosoma brucei and

Leishmania mexicana. Parasitology 87, 211-217.

FISH, W.R., MARR, J.J., AND BERENS, R.L. 1982a. Purine metabolism in Trypanosoma brucei gambiense. Biochimica et Biophysica Acta 714, 422-428.

FISH, W.R., LOCKER, D.L., MARR, J.J., AND BERENS, P.L. 1982b. Purine metabolism in the bloodstream forms of Trypanosoma gambiense and Trypanosoma rhodesiense. Biochemica et Biophysica Acta 719, 223-231.

GOTTLIEB, M., AND DWYER, D.M. 1983. Evidence for distinct 5'- and 3'-nucleotidase activities in the surface membrane fraction of Leishmania donovani promastigotes. Molecular and Biochemical Parasitology 7, 303-317.

GUTTERIDGE, W.E., AND COOMBS, G.H. 1977. Biochemistry of parasitic protozoa. MacMillan Press Ltd., London.

GUTTERIDGE, W.E., AND GABORAK, M. 1979. A re-examination of purine and pyrimidine synthesis in the three main forms of Trypanosoma cruzi. International Journal of Biochemistry 10, 415-422.

GUTTERIDGE, W.E., AND DAVIES, M.J. 1981. Enzymes of purine salvage in Trypanosoma cruzi. FEBS Letters 127, 211-214.

GUTTERIDGE, W.E. AND DAVIES, M.J. 1982. Properties of the purine phosphoribosyltransferases of Trypanosoma cruzi. FEMS Microbiology Letters 13, 207-212.

HART, D.T., VICKERMAN, K., AND COOMBS, G.H. 1981. A quick, simple method for purifying Leishmania mexicana amastigotes in large numbers. Parasitology 82, 345-355.

HEYWORTH, P.G., GUTTERIDGE, W.E., AND GINGER, C.D. 1982. Purine metabolism in Trichomonas vaginalis. FEBS Letters 141, 106-110.

KALCKAR, H.M. 1947a. Differential spectrophotometry of purine compounds by means of specific enzymes. I. Determination of hydroxypurine compounds. Journal of Biological Chemistry 167, 429-443.

KALCKAR, H.M. 1947b. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. Journal of Biological Chemistry 167, 461-475.

KIDDER, G.W., AND NOLAN, L.L. 1979. Adenine aminohydrolase: Occurrence and possible significance in trypanosomid flagellates. Proceedings of the National Academy of Science of the U.S.A. 76, 3670-3672.

KIDDER, G.W., AND NOLAN, L.L. 1981. The in vivo and in vitro action of 4-amino-5-imidazolecarboxamide in trypanosomatid flagellates. Molecular and Biochemical Parasitology 3, 265-269.

KIDDER, G.W., AND NOLAN, L.L. 1982. Xanthine phosphoribosyltransferase in Leishmania: Divalent cation activation. Journal of Protozoology 29, 405-409.

KONIGK, E. 1978. Purine nucleotide metabolism in promastigotes of Leishmania tropica: Inhibitory effect of allopurinol and analogues of purine nucleosides. Tropenmedizin Parasitologie 29, 435-438.

KONIGK, E., AND RASOUL, S.A. 1978. Catabolism of adenosine 5 -monophosphate in promastigotes of Leishmania tropica. Tropenmedizin Parasitologie 28, 319-322.

KONIGK, E., AND PUTFARKEN, B. 1980. Stage-specific differences of a perhaps signal-transferring system in Leishmania donovani. Tropenmedizin Parasitologie 31, 421-424.

KOSZALKA, G.W. AND KRENITSKY, T.A. 1979. Nucleosidases from Leishmania donovani. Pyrimidine ribonucleosidase, purine ribonucleosidase, and a novel purine 2' -deoxyribonucleosidase. Journal of Biological

Chemistry 254, 8185-8193.

LaFON, S.W., NELSON, D.J., BERENS, R.L., AND MARR, J.J. 1982. Purine and pyrimidine salvage pathways in Leishmania donovani. Biochemical Pharmacology 31, 231-238.

LINDBERG, B., KLENOW, H., AND HANSEN, K. 1967. Some properties of partially purified mammalian adenosine kinase. Journal of Biological Chemistry 242, 350-356.

LOCKER, D.L., BERENS, R.L., AND MARR, J.J. 1983. Purine metabolism in Leishmania donovani amastigotes and promastigotes. Molecular and Biochemical Parasitology 9, 15-28.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., AND RANDALL. R.J. 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

LUKOW, I., SCHMIDT, G., WALTER, R.D., AND KONIGK, E. 1973. Adenosinmono-phosphate-salvage-synthese bei Plasmodium chabaudi. Tropenmedizin Parasitologie 24, 500-504.

MARR, J.J., AND BERENS, R.L. 1977. Antileishmanial effect of alopurinol. II. Relationship of adenine metabolism in Leishmania species to the action of allopurinol. Journal of Infectious Diseases 136, 724-732.

MARR,J., NELSON,D.J.,AND BERENS, R.1978. Purine metabolism in Leishmania donovani and Leishmania braziliensis. Biochimica et Biophysica Acta 544, 360-371.

MARR, J.J., AND BERENS, R.L. 1983. Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Molecular and Biochemical Parasitology 7, 339-356.

MILLER, R.L., AND ADAMCZYK, D.L. 1976. Inosine 5' -monophosphate dehydrogenase from Sarcoma 180 cells. Substrate and inhibitor

specificity. Biochemical Pharmacology 25, 883-888.

MILLER, R.L., AND LINDSTEAD, D. 1983. Purine and pyrimidine metabolizing activities in Trichomonas vaginalis extracts. Molecular and Biochemical Parasitology 7, 41-51.

NELSON, D.J., BUGGE, C.J.L., ELION, G.B., BERENS, R.L., AND MARR, J.J. 1979a. Metabolism of pyrazolo (3,4-d) pyrimidines in Leishmania braziliensis and Leishmania donovani. Allopurinol, oxipurinol, 4-aminopyrazolo (3,4,-d) pyrimidines. Journal of Biological Chemistry 254, 3959-3964.

NELSON, D.J., LaFON, S.W., TUTTLE, J.V., MILLER, W.H., MILLER, R.L., KRENITSKY, T.A., ELION, G.B., BERENS, R.L., AND MARR, J.J. 1979b. Allopurinol ribonucleoside as antileishmanial agent. Biological effects, metabolism, and enzymatic phosphorylation. Journal of Biological Chemistry 254, 11544-11549.

NELSON, D.J., LaFON, S.W., JONES, T.E., SPECTOR, A., BERENS, R.L., AND MARR, J.J. 1982. The metabolism of formycin B in Leishmania donovani. Biochemical and Biophysical Research Communications 108, 349-354.

NOLAN, L.L., AND KIDDER, G.W. 1980. Inhibition of growth and purine-metabolizing enzymes of Trypanosomid flagellates by N^6-methyladenine. Antimicrobial Agents Chemotherapy 17, 567-571.

NORTH, M.J., AND COOMBS, G.H. 1981. Proteinases of Leishmania mexicana amastigotes and promastigotes: Analysis by gel electrophoresis. Molecular and Biochemical Parasitology 3, 293-300.

PFALLER, M.A., AND MARR, J.J. 1974. Antileishmanial effect of allopurinol. Antimicrobial Agents and Chemotherapy 5, 469-472.

PUPKIS, M.F., AND COOMBS, G.H. 1984. Purification and characterisation of proteolytic enzymes of Leishmania mexicana mexicana amastigotes and

promastigotes. Journal of General Microbiology, 130,2375-2383.

RAINEY, P., AND SANTI, D.V. 1983. Metabolism and mechanism of action of formycin B in Leishmania. Proceedings of the National Academy of Science of the U.S.A. 80, 288-292.

REYES, P., RATHOD, P.K., SANCHEZ, D.J., MREMA, J.E.K., RIECKMANN, K.H. AND HEIDRICH, H.G. 1982. Enzyme of purine and pyrimidine metabolism from the human malaria parasite, Plasmodium falciparum. Molecular and Biochemical Parasitology 5, 275-290.

SPECTOR, T., MILLER, R.L., FYFE, J.A., AND KRENITSKY, T.A. 1974. GMP synthetase from Escherichia coli B-96. Biochimica et Biophysica Acta 370, 585-591.

SPECTOR, T., JONES, T.E., AND MILLER, R.L. 1979a. Reaction mechanism and specificity of human GMP reductase. Substrates, inhibitors, activators, and inactivators. Journal of Biological Chemistry 254, 2308-2315.

SPECTOR, T., JONES, T.E., AND ELION, G.B. 1979b. Specificity of adenylosuccinate synthetase and adenylosuccinate lyase from Leishmania donovani. Selective amination of antiprotozoal agent. Journal of Biological Chemistry 254, 8422-8426.

SPECTOR, T., AND JONES, T.E. 1982. Guanosine 5'-monophosphate reductase from Leishmania donovani. A possible chemotherapeutic target. Biochemical Pharmacology 31, 3891-3897.

TUTTLE, J.V., AND KRENITSKY, T.A. 1980. Purine phosphoribosyltransferase from Leishmania donovani. Journal of Biological Chemistry 255, 909-916.

WALSH, C.J., AND SHERMAN, I.W. 1968. Purine and pyrimidine synthesis by the avian malarial parasite, Plasmodium lophurae. Journal of

Protozoology 15, 763-770.

WANG, C.C., VERHAM, R., RICE, A., AND TZENG, S. 1983. Purine salvage by Tritrichomonas foetus. Molecular and Biochemical Parasitology 8, 325-337.

WANG, C.C., AND SIMASHKEVICH, P.M. 1981. Purine metabolism in the protozoan parasite Eimeria tenella. Proceeding of the National Academy of Science of the U.S.A. 78, 6618-6622.

YAMADA, K.A., AND SHERMAN, I.W. 1981. Purine metabolizing enzymes of Plasmodium lophurae and its host cell, the duckling (Anas domesticus) erythrocyte. Molecular and Biochemical Parasitology 2, 349-358.

Chapter 4

SUBCELLULAR LOCALISATION OF PURINE-METABOLISING ENZYMES IN *LEISHMANIA MEXICANA MEXICANA*

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Abstract - 1. Leishmania mexicana mexicana cultured promastigotes were fractionated by isopycnic centrifugation on linear sucrose gradients.

2. Guanine, hypoxanthine and xanthine phosphoribosyltransferase activities were found to be associated with glycosomes, whereas adenine phosphoribosyltransferase was cytosolic.

3. 3'- and 5'- nucleotidases and IMP dehydrogenase were shown to be particulate, the former two possibly being associated with the plasma membrane, IMP dehydrogenase with the endoplasmic reticulum.

4. Nucleosidases and deaminases were found to be cytosolic.

5. The results demonstrate that intracellular separation of enzymes could play a part in the regulation of the parasite's purine metabolism.

INTRODUCTION

Leishmanias have been found to lack de novo purine synthesis and require an exogenous purine for growth (Marr et al. 1978). It has been shown that Leishmania differ from mammalian cells in their ability to metabolise some purine analogues through the salvage pathways, the enzymes concerned therefore provide opportunities for the development of new antileishmanial agents (Nelson et al. 1979; Rainey and Santi, 1983). There have been several reports on purine metabolism in various Leishmania species (Marr et al. 1978; Koszalka and Krenitsky, 1979; Spector et al. 1979; Tuttle and Krenitsky, 1980; Lafon et al. 1982; Spector and Jones, 1982; Looker et al. 1983; Hassan and Coombs, 1984, 1985), but as yet little attention has been paid to the subcellular localisation of the purine-metabolising enzymes within the cell. Investigations of Leishmania mexicana mexicana cultured promastigotes

by fractionation involving differential centrifugation showed that some of the purine salvage enzymes were located in the particulate fractions of the parasite (Hassan and Coombs, 1985). We have now extended these studies by using isopycnic centrifugation and have elucidated the organellar localisation of many of the purine-metabolising enzymes of L. m. mexicana promastigotes

MATERIALS AND METHODS

Promastigote growth and fractionation

Leishmania mexicana mexicana promastigotes were grown at 26°C and harvested as described previously (Coombs et al. 1982). The promastigotes (approximately $2x10^{10}$ cells per experiment) were washed, lysed with alumina and subjected to isopycnic centrifugation using a linear sucrose gradient (1.15-1.24 g/ml) as described by Mottram and Coombs (1985). The final gradient was recovered in 2 ml fractions which were assayed for enzyme activities, the data being analysed and represented as described previously (Mottram and Coombs, 1985).

Enzyme assays

All assays were carried out at 26°C. Adenine deaminase (EC 3.5.4.2.), inosine and 2-deoxyinosine nucleosidase (EC 3.2.2.1), adenylosuccinate lyase (EC 4.3.2.2) and IMP dehydrogenase (EC 1.2.1.14) were assayed spectrophotometrically as described previously (Hassan and Coombs, 1985). 3'- and 5'-nucleotidase (EC 3.1.3.6 and 3.1.3.5, respectively) were assayed colorimetrically using the procedure described by Gottlieb and Dwyer (1983). Purine phosphoribosyltransferases (PRTases) (adenine PRTase, EC 2.4.2.7; guanine and hypoxanthine PRTase, EC 2.4.2.8; xanthine PRTase, EC 2.4.2.22) were assayed radiochemically in an reaction mixture (total volume 150 µl) containing 50 mM Tris-HCl, pH 7.4, 0.1 mM (2-^{3}H) adenine (50mCi mmol^{-1}) or

(8-^{14}C) guanine (5 mCi mmol $^{-1}$) or (G -^{3}H) hypoxanthine (850 mCi mmol $^{-1}$) or (6-^{14}C) xanthine (18 mCi mmol $^{-1}$), 1 mM sodium salt of 5- phosphoribosyl-1-pyrophosphate (PRPP), divalent cations as their sulphates 5 mM $MgSO_4$ for guanine PRTase, 5 mM $CoSO_4$ for xanthine PRTase and 0.75 mM $MnSO_4$ for adenine PRTase and hypoxanthine PRTase and an appropriate amount of enzyme to catalyze nucleotide synthesis at a linear rate for the period of the incubation. Reactions were initiated by the addition of substrate, incubation was for 5 min (guanine PRTase), 10 min (adenine PRTase and hypoxanthine PRTase) or 30 min (xanthine PRTase) and the reactions were stopped by heating the reaction mixture in a boiling water bath for 3 min. The mixtures subsequently were centrifuged at 2100 g for 5 min and the substrate and product in 10 µl aliquots of the supernatants were separated by ascending thin layer chromatography on PEI cellulose F precoated sheets using water for the adenine PRTase assays (R_f values: adenine, 0.287; AMP, 0.0) and potassium phosphate buffer, pH 6.8/saturated ammonium sulfate/n-propanol (100/60/2, v/v) for the hypoxanthine PRTase, guanine PRTase and xanthine PRTase assays (R_f values: hypoxanthine, 0.37; IMP, 0.67; guanine, 0.22; GMP, 0.53; xanthine, 0.22; XMP, 0.47). In each case, 10 nmol of nonradioactive substrate and product were added as carriers during TLC separation. Spots corresponding to the purine base and 5'- ribonucleotide were visualised under ultraviolet light, cut out and assessed for radioactivity using a scintillation cocktail containing 0.35% (w/v) 2,5-diphenyloxazole, 0.005% (w/v) 1,4-di-2-(5- phenyloxazolyl) benzene and 3% (v/v) Triton X100 in toluene

MATERIALS

(2-^{3}H) Adenine, 24 Ci mmol $^{-1}$; (G-^{3}H) hypoxanthine, 2.3 Ci mmol $^{-1}$ and (6-^{14}C) xanthine, 54 mCi mmol $^{-1}$, were all obtained from Radiochemical Center,

Amersham, England. (8-^{14}G) Guanine hydrochloride, 55.5 mCi mmol^{-1}, was purchased from New England Nuclear, West Germany. PEI cellulose F precoated sheets were obtained from BDH, Glasgow, Scotland. All other chemicals were obtained from Sigma Chemical Co.Ltd., Poole, Dorset, England.

RESULTS

The distribution profiles of purine-metabolizing enzymes on the linear sucrose gradients are presented in Figures 1 and 2. Appropriate marker enzymes for different cell compartments in Leishmania mexicana mexicana promastigotes (namely, glycosomal hexokinase, mitochondrial oligomycin-sensitive ATPase and cytosolic pyruvate kinase (Mottram and Coombs, 1985)) are also included in these figures for comparison. Phosphoribosyltransferase activities with guanine, hypoxanthine and xanthine banded at a density of 1.225-1.230 g/ml, similar to the glycosomal marker enzyme hexokinase. A portion of each of these activities (29%, 33% and 4%, respectively) was recovered in the cytosolic fractions. In contrast, adenine PRTase was almost entirely recovered in the cytosolic fraction. The distribution profiles of 3'- and 5' nucleotidases were similar, with a peak of activity at 1.155-1.160 g/ml. Nucleotidase activity was detected in all the fractions, however, which was probably a reflection of some of the plasma membrane being spread throughout the gradient, as has been shown by transmission electron microscopy (unpublished). IMP dehydrogenase showed a prominant peak of activity at 1.160-1.165 g/ml. Inosine nucleosidase activity was mostly recovered in the cytosolic fraction, as were the activities of adenine deaminase and 2'-deoxyinosine nucleosidase (data not shown), whereas adenylosuccinate lyase was mainly present in the cytosolic fraction but also showed a small but distinct peak at 1.19-1.20 g/ml, the same density as the mitochondrial marker oligomycin-sensitive ATPase

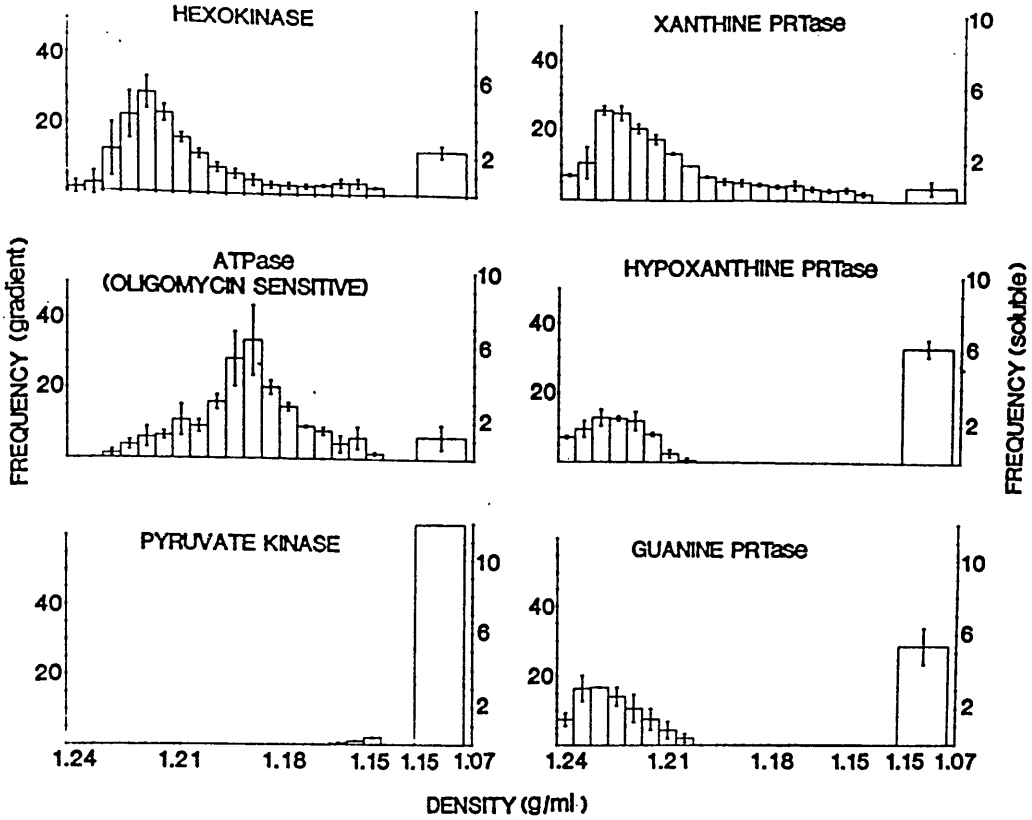

Fig. 1.

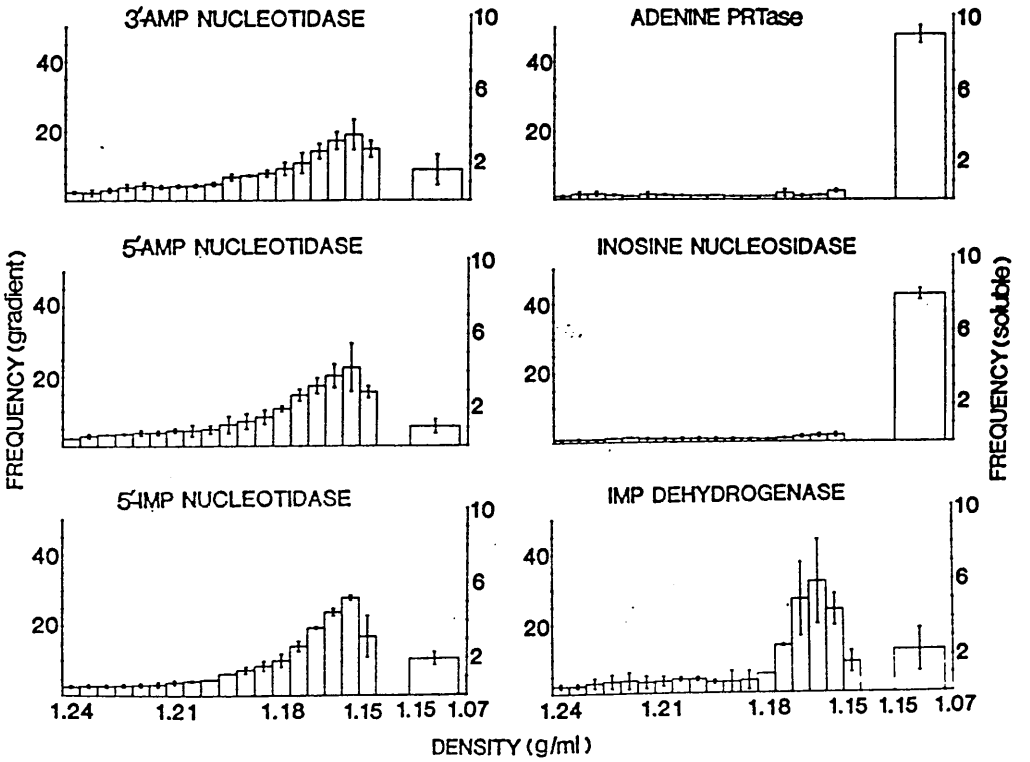

Fig. 2.

Figs 1 and 2. Distribution profiles of enzymes in *L. m. mexicana* promastigote lysates after isopycnic centrifugation on 1.24–1.15 g/ml linear sucrose gradients. The left hand axis represents the frequency of the gradient (ρ = 1.24–1.15 g/ml) and the right hand axis represents the frequency of the soluble fractions combined as one histogram bar (ρ = 1.15–1.07 g/ml). Histograms show the means (±SE) from three experiments. Mean percentage recoveries ranged from 81% (IMP dehydrogenase) to 105% (xanthine PRTase).

DISCUSSION

The finding that hypoxanthine and guanine PRTase activities are associated with glycosomes in Leishmania mexicana mexicana promastigotes is in agreement with the results of Gutteridge and Davies (1982) for Trypanosoma cruzi epimastigotes and so suggests that, in this respect, the organelles from the two species are similar. This study, however, has established for the first time that glycosomes contain xanthine PRTase and so adds another enzyme to the lengthening list of those known to be present in these trypanosomatid-specific organelles (Opperdoes, 1982). The pyrimidine biosynthetic enzyme orotate PRTase has also been found to be associated with the glycosomes of Leishmania promastigotes (Hammond and Gutteridge, 1982). The relevance of the glycosomal location of purine and pyrimidine PRTases is unclear, but the common requirement of the enzymes for PRPP suggests that glycosomes may have a transport system for this compound or that the purine PRTases may be located on the surface of the organelle as has been suggested for orotate PRTase (Hammond and Gutteridge, 1983). The presence of these enzymes in glycosomes, which have no counterpart in mammalian cells, make them attractive targets in the development of new antileishmanial drugs.

Gottlieb and Dwyer (1983) reported the presence of 5'- and 3'- nucleotidase activities on the plasma membrane of L. donovani, the latter enzyme being thought of as a marker for this structure. Both 5'- and 3'- nucleotidases are largely sedimentable in both amastigotes and cultured promastigotes of L. m. mexicana (see chapter six). The recovery of the two nucleotidase activities throughout the gradient suggests that upon cell lysis the plasma membrane was disrupted into segments of different densities, although the peak activity at 1.155-1.160 g/ml suggests that the membrane segments were concentrated at this density. The peaking of IMP dehydrogenase activity at the higher density of 1.160-1.165 g/ml suggests that it is not associated with the plasma membrane and it appears likely that the enzyme is located in the endoplasmic reticulum,

as has been described for higher eukaryotic cells (Wu and Scringeour, 1973).

The results suggest that in L. m. mexicana promastigotes there is intracellular separation of some of the purine-metabolising enzymes and that whereas the catabolism of purines and nucleosides occurs mainly in the cytosol, the anabolic processes leading to nucleotides for RNA and DNA synthesis are associated with cell organelles. Such a situation would mean that channelling of purines, nucleosides and nucleotides between subcellular compartments is an integral part of leishmanial purine metabolism.

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REFERENCES

Coombs G.H., Craft J.A. and Hart D.T. (1982) A comparative study of Leishmania mexicana amastigotes and promastigotes. Enzyme activities and subcellular locations. Mol. Biochem. Parasit. 5, 199-211.

Gottlieb M. and Dwyer D.M. (1983) Evidence for distinct 5'- and 3'-nucleotidase activities in the surface membrane fraction of Leishmania donovani promastigotes. Mol. Biochem. Parasit. 7, 303-317

Gutteridge W.E. & Davies M.J. (1982) Properties of the purine phosphoribosyltransferases of Trypanosoma cruzi. FEMS Microbiol. Letters 13, 207-212.

Hammond D.J. and Gutteridge W.E. (1982) UMP synthesis in the kinetoplastida. Biochem. biophys. Acta 718, 1-10.

Hammond D.J. and Gutteridge W.E. (1983) Studies on the glycosomal orotate phosphoribosyltransferase of Trypanosoma cruzi. Mol. Biochem Parasit. 7 319-330.

Hassan H.F. and Coombs G.H. (1984) Purine and pyrimidine metabolism in Leishmania mexicana mexicana. Parasit 89, ii-iii.

Hassan H.F. and Coombs G.H. (1985) Leishmania mexicana: Purine-metabolising enzymes of amastigotes and promastigotes. Exp. Parasit. 59,139-150.

Koszalka G.W. and Krenitsky T.A. (1979) Nucleosidases from Leishmania donovani: Pyrimidine ribonucleosidase, purine ribonucleodidase and a novel purine 2-deoxyribonucleosidase. J. biol. Chem. 254, 8185-8193.

Lafon S.W., Nelson D.S., Berens R.L. and Marr J.J. (1982) Purine and pyrimidine salvage pathways in Leishmania donovani. Biochem. Pharm. 31, 231-238.

Looker D.L., Berens R.L. and Marr J.J. (1983) Purine metabolism in Leishmania donovani amastigotes and promastigotes. Mol. Biochem. Parasit. 9, 15-28.

Marr J.J., Berens R.L. and Nelson D.J. (1978) Purine metabolism in Leishmania donovani and Leishmania braziliensis. Biochem. biophys Acta 544, 360-371.

Mottram J.C. and Coombs G.H. (1985) Leishmania mexicana: Subcellular distribution of enzymes in amastigotes and promastigotes. Exp. Parasit. 59, 265-274.

Nelson D.J., Lafon S.W., Tuttle J.V., Miller W.H., Miller R.L., Krenitsky T.A., Elion G.B., Berens R.L. and Marr J.J. (1979) Allopurinol ribonucleoside as an antileishmanial agent, biological effects, metabolism and enzymic phosphorylation. J. biol. Chem. 254, 11544-11549.

Opperdoes F.R. (1982) The glycosome. Ann. N.Y. Acad. Sci. 386, 543-545.

Rainey P. and Santi D.V. (1983) Metabolism and mechanism of action of formycin B in Leishmania. Proc. Nat. Acad. Sci. U.S.A. 80, 288-292.

Spector T., Jones T.E. and Elion G.B. (1979). Specificity of adenylosuccinate synthetase and adenylosuccinate lyase from Leishmania donovani. J. biol Chem. 254, 8422-8426

Spector T. and Jones T.E. (1982). Guanosine 5-monophosphate reductase from Leishmania donovani. Biochem. Pharm., 31, 3891-3897

Tuttle J.V. and Krenitsky T.A. (1980). Purine phosphoribosyltransferases from Leishmania donovani. J. biol. Chem. 255, 909-916.

Wu T. and Scrimgeour K.G. (1973) Properties of inosinic acid dehydrogenase from Bacillus subtilis. I. Purification and physical properties. Can. J. Biochem. 51 1380-1390

Chapter 5

PURINE PHOSPHORIBOSYLTRANSFERASES OF *LEISHMANIA MEXICANA MEXICANA* AND OTHER FLAGELLATE PROTOZOA

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Abstract - 1. Amastigotes and cultured promastigotes of Leishmania mexicana mexicana and L. m. amazonensis, cultured promastigotes of L. donovani and L. tarentolae, and the culture forms of Crithidia fasciculata, Herpetomonas muscarum muscarum and H. m. ingenoplastis all possessed four phosphoribosyltransferase (PRTase) activities: adenine PRTase, hypoxanthine PRTase, guanine PRTase and xanthine PRTase.

2. The enzymes of L. m. mexicana required divalent cations for activity; Mn^{2+} or Co^{2+} produced maximal activity in most cases.

3. Hypoxanthine PRTase, guanine PRTase and xanthine PRTase from all organisms were sedimentable in part, suggesting that they may occur within glycosomes.

4. The enzymes of L. m. mexicana cultured promastigotes were inhibited by a range of purine analogues.

INTRODUCTION

Previous investigations have revealed that phosphoribosyltransferases (PRTases) are the enzymes principally involved in the utilization of purines by the two main forms of Leishmania mexicana mexicana (Hassan and Coombs, 1984, 1985) as has also been suggested for other Leishmania species and a range of other parasitic protozoa (Wang, 1984). The finding of the presence of highly active adenine deaminase, guanine deaminase and nucleosidases in both amastigotes and promastigotes of L. m. mexicana (Hassan and Coombs, 1985) and promastigotes of other Leishmania species (Kidder and Nolan, 1979; Konigk and Rasoul, 1978; Koszalka and Krenitsky, 1979; Marr et al., 1978; Nolan and Kidder, 1980; Looker et al., 1983) further emphasised the importance of hypoxanthine PRTase (HPRTase) and xanthine PRTase (XPRTase) to these parasites. Leishmanial HPRTase is an especially interesting enzyme because it metabolises allopurinol to its ribotide, which accumulates in large quantities in the parasite and is responsible for the antileishmanial activity of this drug (Tuttle and Krenitsky, 1980; Nelson et al., 1979). In contrast, allopurinol is a poor substrate for mammalian HPRTase and its ribotide is produced only in minute quantities within the cells (Nelson et al., 1973). The existence in leishmanial parasites of XPRTase, an enzyme not found in mammalian cells, makes it another potential target for antileishmanial agents. In our previous studies on PRTases, it was shown that guanine PRTase (GPRTase), HPRTase and XPRTase were associated with the glycosomes of promastigotes of L. m. mexicana (Hassan et al., 1985), as has been reported also for epimastigotes of a related trypanosomatid Trypanosoma cruzi (Gutteridge and Davies, 1982). We have extended these studies by investigating the enzymes of L. m. mexicana amastigotes and promastigotes with respect to divalent cation requirements and sensitivity to purine analogues and compared these enzymes with those in a variety of other trypanosomatids.

MATERIALS AND METHODS

Isolation, cultivation and fractionation of parasites

Amastigotes of L. m. mexicana (M379) and L. m. amazonensis (LV78) were isolated from cutaneous lesions in female CBA mice (Department of Zoology, University of Glasgow, Glasgow G12 8QQ) and balb/c mice (Liverpool School of Tropical Medicine), respectively, as described by Hart et al. (1981). Promastigotes of L. m. mexicana (M379), L. m. amazonensis (LV78) and L. donovani (LV9) were grown in vitro at 26°C in HOMEM medium (Berens et al., 1976) with 10% (v/v) heat inactivated foetal calf serum as described previously (North and Coombs, 1981). Leishmania tarentolae, Crithidia fasciculata (Anopheles strain ATCC 11745), Herpetomonas muscarum muscarum (ATCC 30260) and H. m. ingenoplastis (ATCC 30269) were grown at 26°C in LIT medium with 10% (v/v) heat inactivated foetal calf serum (Camargo, 1964). Gentamicin sulphate was added at 25 µg/ml to all culture media to inhibit bacterial growth. Parasites were harvested by centrifugation at 3,000 X g at 4°C for 10 min and washed twice in 50 mM Tris-HCI buffer, pH 7.4, containing 0.1 mM dithiothreitol. For most studies, the pelleted cells were suspended in 2 ml of the above buffer and lysed by sonication for two periods of 15 s, separated by a 30 s cooling period, using a MSE Soniprep 150 fitted with an exponential probe at 4 µ amplitude. The crude homogenates produced were fractionated by centrifugation at 105,000 X g at 4°C for 1 h and the resultant pellets were resuspended in the above buffer to the volume of the supernatant fraction. With amastigotes, the proteinase inhibitor leupeptin was added at 100 µg/ml to the parasite suspension before sonication for some experiments. To study the subcellular localisation of enzymes, L. m. mexicana promastigotes were gently lysed with alumina and fractionated by differential centrifugation exactly as described previously (Coombs et al., 1982).

Analytical methods

The standard assay conditions for the PRTases, which were used except were stated otherwise, were as follows: Adenine PRTase (APRTase EC 2.4.2.7), 50 mM Tris-HCI, pH 7.5, 0.75 mM $MnSO_4$, 1 mM PRPP, 0.1 mM (2-^{3}H)adenine (50 mCi $mmol^{-1}$); GPRTase (EC 2.4.2.8), 50 mM Tris-HCI, pH 8.0, 5 mM $MgSO_4$, 1 mM PRPP, 0.1 mM (8-^{14}C)guanine (5 mCi $mmol^{-1}$); HPRTase (EC 2.4.2.8), 50 mM Tris-HCI, pH 9.0, 0.75 mM $MnSO_4$, 1 mM PRPP, 0.1 mM (G-^{3}H)hypoxanthine (850 mCi $mmol^{-1}$); XPRTase (EC 2.4.2.22), 50 mM Tris-HCl, pH 8.0, 5mM $CoSO_4$, 1 mM PRPP, 0.1mM (6-^{14}C) xanthine(18 mCi $mmol^{-1}$). Assay mixtures had, unless otherwise specified, a final volume of 100 µl and contained an amount of extract sufficient to catalyse nucleotide synthesis at a linear rate for the period of incubation. The reaction was routinely preincubated with PRPP at 26°C for 5 min before the reaction was initiated by the addition of purine base. Control assays were run without PRPP. Incubation was at 26°C for 5 min (GPRTase) or 10 min (APRTase, HPRTase and XPRTase) and the reaction was stopped by heating the reaction mixture in a boiling water bath for 3 min. The resultant precipitated protein was removed by centrifugation at 2100 X g for 5 min and aliquots (10 µl) of the supernatant were spotted on to PEI cellulose F sheets in the presence of 10 nmol of purine base and corresponding 5'-ribonucleotide as carriers. Plates were developed by ascending chromatography in water (for AMP formation) or in 0.1 M potassium phosphate buffer, pH 6.8/saturated $(NH_4)_2SO_4$/n-propanol (100/60/2 (v/v)) (for GMP, IMP and XMP formation). The carrier spots were visualised with ultraviolet light, they had the following R_f values: adenine, 0.29; AMP, 0.0; guanine, 0.22; GMP, 0.53; hypoxanthine, 0.37; IMP, 0.67; xanthine, 0.22; XMP, 0.47. The spots of the chromatograms containing the purine and 5'-ribonucleotide were cut out and counted for radioactivity using a scintillation cocktail containing 0.35% (w/v) 2,5 diphenyloxazole, 0.005% (w/v) 1,4-di-2-(5-phenyloxazolyl)-benzene and 3% (v/v) Triton X100 in toluene. One unit of enzyme activity was defined as

the amount of enzyme that catalyses the conversion of 1 nmol of substrate to product per min.

PRTase activities were determined at a range of pH obtained using the following buffers: potassium phosphate, 50 mM (pH 6.0-7.0); Tris-HCl, 50 mM (pH 7.0-9.0) and CHES, 50 mM (pH 9.0-9.8). The apparent K_ms for substrate and PRPP were calculated by the Lineweaver-Burk method. For inhibitor studies, the extract was preincubated with the inhibitor for 10 min at 26°C and the assay were carried out as described above. Inhibition is expressed as the % reduction in activity compared with the activity under standard conditions. PRTase activities of flagellate protozoa other than L. m. mexicana were measured under the standard assay conditions.

Adenine deaminase (EC 3.5.4.2) was assayed spectrophotometrically at 26°C as described previously (Hassan and Coombs, 1985). Protein concentrations were estimated by the method of Lowry et al., (1951) with bovine serum albumin as standard.

Materials

(2-^{3}H) Adenine, 24 Ci mmol^{-1}, (G-^{3}H) hypoxanthine, 2.3 Ci mmol^{-1} and (6-^{14}C) xanthine, 54 mCi mmol^{-1}, were obtained from Amersham International, England. (8-^{14}C) Guanine hydrochloride, 55.5 mCi mmol^{-1} was purchased from New England Nuclear, West Germany. 5-Phosphoribosyl-1-pyrophosphate (PRPP), purine bases and 5'-ribonucleotides were all obtained from Sigma Chemical Co Ltd., Dorset, Poole, England. PEI cellulose F precoated sheets (Merck) were obtained from BDH, Glasgow, Scotland.

RESULTS

Leishmania m. mexicana was found to contain high activity of adenine deaminase (Hassan and Coombs, 1985) which could interfere with the assay of APRTase. To eliminate this problem, APRTase was assayed in the presence of 0.75 mM MnSO4. This inhibited the activity of adenine deaminase by 85%,

whereas APRTase was fully activated (Fig. 1).

The effect of pH on the activities of L. m. mexicana PRTases is shown in Fig. 2. With amastigote extracts, the pH optimum for GPRTase, HPRTase and XPRTase was 8.5, the activity declining sharply on both sides. With promastigote extracts, the activity with guanine was optimal in the range pH 7.5-9.0, while with hypoxanthine and xanthine optimal activity was at pH 9.0 and 8.0, respectively. In contrast, APRTase from both forms was most active at pH 7.5.

The divalent cation activation of PRTases from promastigote and amastigote forms of L. m. mexicana is shown in Figs 3 and 4, respectively. It was found for the promastigote enzymes that the relative efficiencies of the cations at 1 mM in stimulating the activities were $Mn^{2+} > Mg^{2+} > Co^{2+} > Zn^{2+}$ for GPRTase, $Mn^{2+} > Zn^{2+} > Mg^{2+} > Co^{2+}$ for HPRTase, $Co^{2+} > Mn^{2+} > Zn^{2+} > Mg^{2+}$ for XPRTase and $Co^{2+} > Mn^{2+} > Mg^{2+} > Zn^{2+}$ for APRTase. Amastigote enzymes were affected somewhat differently from those of promastigotes, notably with respect to the marked stimulation of HPRTase by Co^{2+} (1 mM) and APRTase by Zn^{2+} (0.25 mM). In many instances, it was found that the divalent cations were inhibitory at higher concentrations.

Using the standard assay conditions, GMP production catalysed by L. m. mexicana promastigote extract increased linearly with time up to 5 min, while the formation of IMP, XMP and AMP was linear with time for 10 min. The purine PRTase activities detected in extracts of promastigotes and amastigotes forms of L. m. mexicana are given in Table 1. With all four substrates, the activities of promastigotes were appreciably higher than those of amastigotes. The activities detected in amastigotes lysed in the absence of leupeptin were significantly lower than those in extracts produced in its presence. The relative rates of PRTase activities detected in promastigotes were guanine > hypoxanthine > adenine > xanthine, whereas those detected in amastigotes were guanine > hypoxanthine > xanthine > adenine.

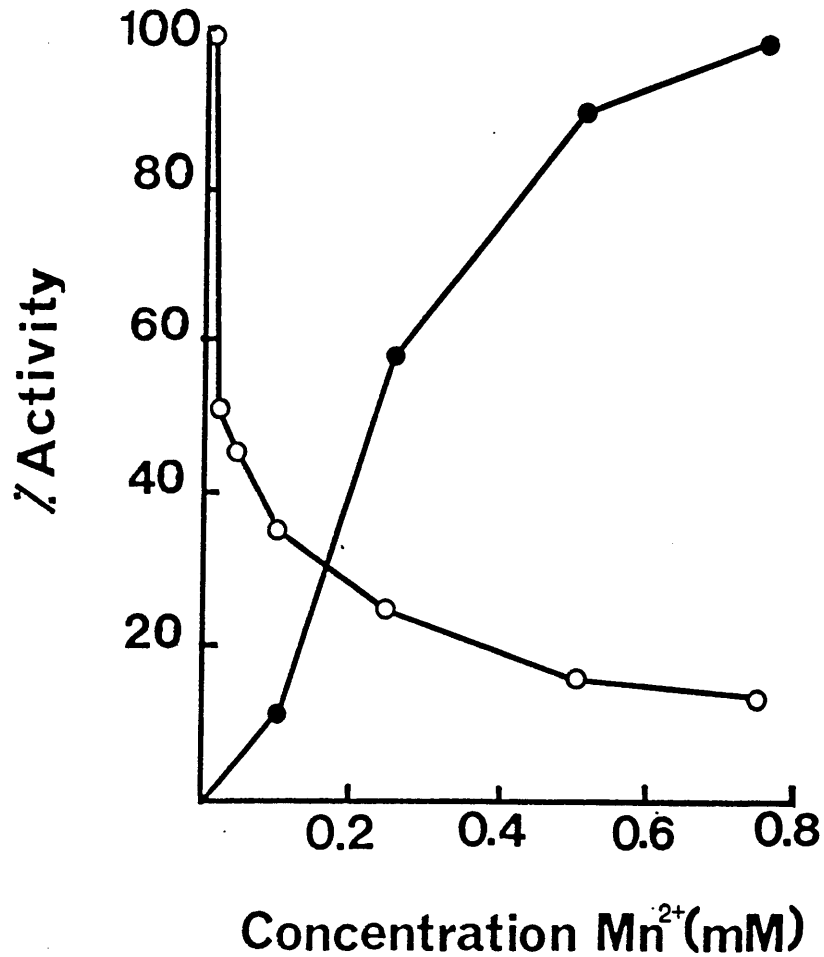

Fig. 1. The effect of Mn^{2+} concentration on the APRTase (●) and adenine deaminase (○) activities of *Leishmania m. mexicana* promastigotes.

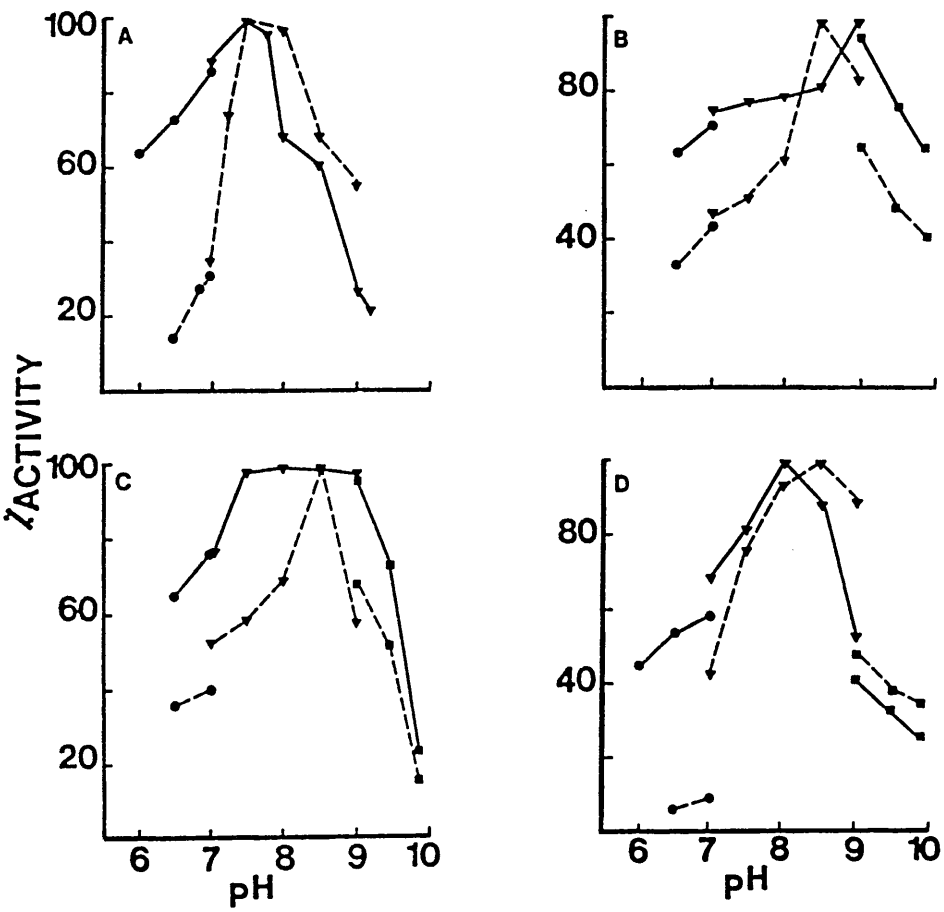

Fig. 2. *Leishmania m. mexicana* promastigote (—) and amastigote (---) purine phosphoribosyltransferase activities at a range of pH. A, APRTase; B, HPRTase; C, GPRTase; D, XPRTase. The buffers used (all 50 mM) were: potassium phosphate (●); Tris–HCl (▼) and CHES (■). The ordinates show the enzyme activity as percentage of the maximum.

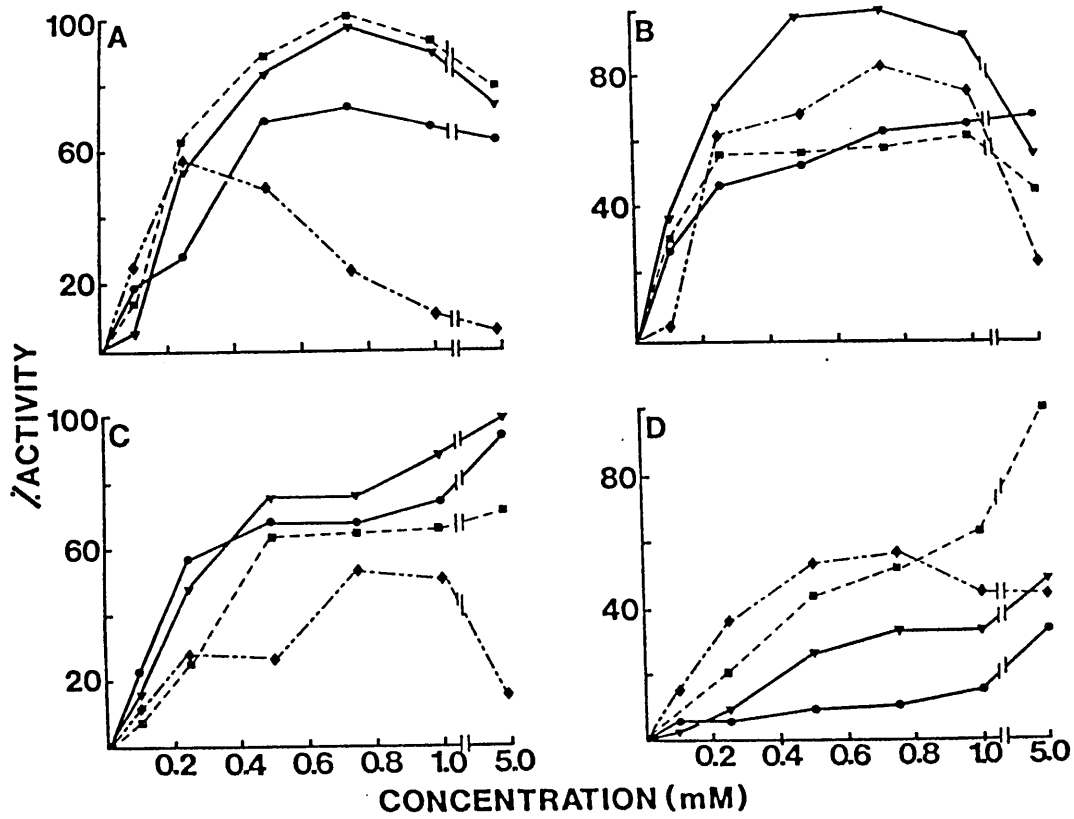

Fig. 3. The effect of Mg^{2+} (●), Mn^{2+} (▼), Co^{2+} (■) and Zn^{2+} (◆) on the purine phosphoribosyltransferase activities of *Leishmania m. mexicana* promastigotes. A, APRTase; B, HPRTase; C, GPRTase; D, XPRTase.

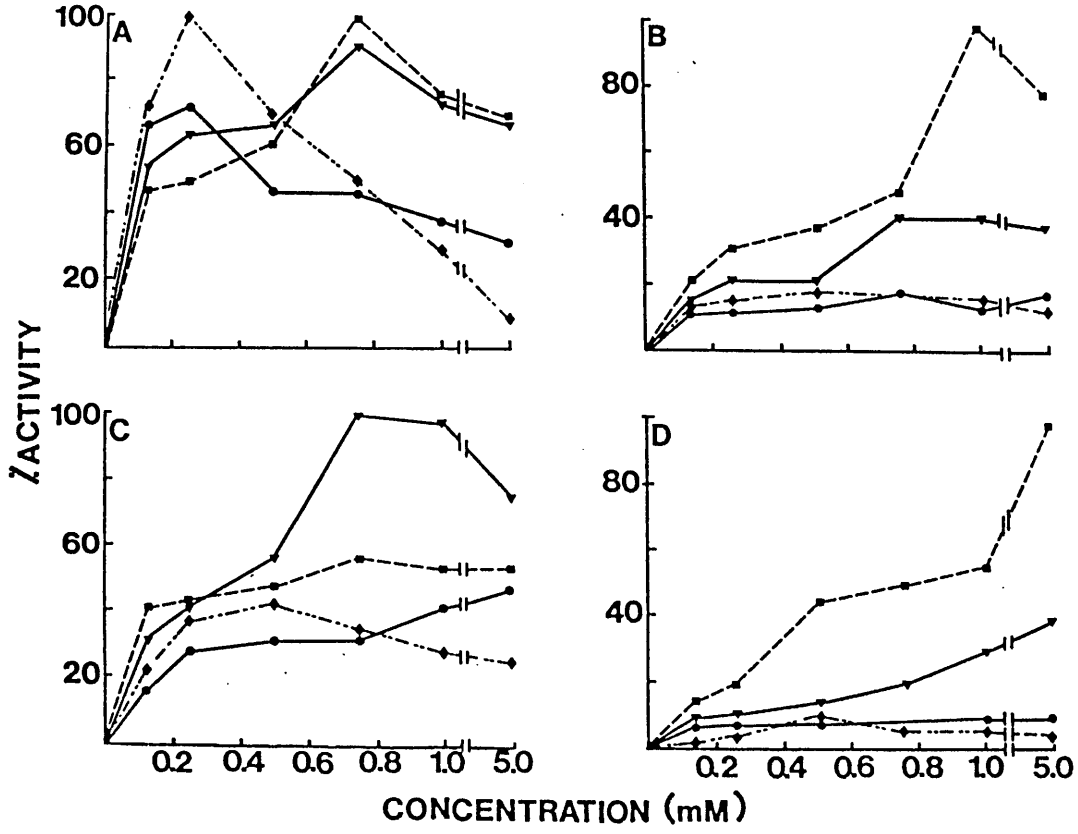

Fig. 4. The effect of Mg^{2+} (●), Mn^{2+} (▼), Co^{2+} (■) and Zn^{2+} (◆) on the purine phosphoribosyltransferase activities of *Leishmania m. mexicana* amastigotes. A, APRTase; B, HPRTase; C, GPRTase; D, XPRTase.

Table 1. Specific activities and some properties of purine phosphoribosyltransferases in promastigotes and amastigotes of *Leishmania m. mexicana*

		APRTase	HPRTase	GPRTase	XPRTase
Promastigote	Specific activity[a]	17 ± 1	21 ± 1	30 ± 3	11 ± 1
	K_m: Substrate (μM)	250	220	200	100
	PRPP (μM)	500	270	800	65
	% Sedimentability[b]	6 ± 2	20 ± 6	36 ± 4	31 ± 4
Amastigote	Specific acivity I[a]	3.0 ± 0.8	5 ± 2	10 ± 1	4.0 ± 0.9
	II[a]	0.5 ± 0.1	1.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
	K_m: Substrate (μM)	71	200	18	500
	PRPP (μM)	48	48	62	142
	% Sedimentability[b]	45 ± 7	40 ± 4	32 ± 5	51 ± 1

[a]The activities are given in nmol/min/mg protein and are the means ± S.D. from three experiments. The activities detected in amastigote lysates produced in the presence (I) and absence (II) of leupeptin (100 μg/ml) are given.

[b]The activity sedimented in the 105,000 *g* pellet as a % of the total activity originally detected. The figures given are the means ± SD from three experiments.

The apparent K_m values for each substrate also are given in Table 1, together with data on sedimentability of the PRTase activities in crude homogenates (sonicates) of the two parasite forms. GPRTase, HPRTase and XPRTase were sedimentable in part, although the greater proportion of the enzyme activities were recovered in the supernatant fraction. The activities recovered in the subcellular fractions of L. m. mexicana promastigotes are shown in Table 2. A significant proportion of guanine, hypoxanthine and xanthine PRTases were recovered in fraction P_2 which also contained many of the glycolytic activities and was rich in glycosomes (Coombs et al., 1982), whereas APRTase was mainly cytosolic.

The inhibitory effect of various purine analogues on the PRTases from promastigotes of L. m. mexicana are given in Table 3. Replacement of the 6-amino group of adenine or the 6-hydroxy group of guanine and hypoxanthine by a thiol group produced potent inhibitors such as 6-mercaptopurine, 2-amino-6-mercaptopurine and their ribotides. Substitution of other groups (methylthio, methylamino, and dimethyl) in the 6 position led to poorer inhibitors. Of the pyrazolopyrimidines tested, thiopyrazolopyrimidine inhibited APRTase and HPRTase more effectively than GPRTase. Allopurinol, however, was a poorer inhibitor of APRTase than GPRTase and HPRTase. 4-Aminopyrazolopyrimidine inhibited APRTase appreciably but was relatively ineffective against GPRTase and HPRTase. None of the purine analogues tested was very effective as an inhibitor of XPRTase, 6-methylmercapto-purine and thiopyrazolopyrimidine were the most active. Tubercidin also had a large effect against the PRTases, APRTase and HPRTase being especially sensitive. Formycin B and sinefungin, however, had only minor inhibitory activity.

Data on the purine PRTase activities in other trypanosomatid species are presented in Table 4. Each of the four activities was detected in each protozoan, although the levels of activity differed significantly as did the

Table 2. The distribution of purine phosphoribosyltransferase activities in subcellular fractions of *Leishmania m. mexicana* promastigotes[a]

Enzyme	P_1	P_2	P_3	S	% Recovery
APRTase	3	2	1	94	148
HPRTase	14	13	6	67	91
GPRTase	9	14	6	71	95
XPRTase	12	28	9	51	85
Hexokinase[b]	16	54	14	12	97
Pyruvate kinase[b]	1	3	1	62	67
Succinic dehydrogenase[b]	43	35	23	<1	101
RNA[b]	6	5	55	18	84
Protein[b]	17	16	19	42	94

[a]The figures given are the activity present in each fraction as a percentage of the total activity recovered.
[b]Data from Coombs *et al.*, 1982.

Table 3. The effect of inhibitors on *Leishmania m. mexicana* promastigote purine phosphoribosyltransferase activities

Compound	APRTase	HPRTase	GPRTase	XPRTase
4-Aminoimidazole-5-carboxamide	25	0	32	20
4-Aminoimidazole-5-carboxamide riboside	1	5	0	23
6-Mercaptopurine	96	62	88	50
6-Mercaptopurine riboside	92	89	71	44
2-Amino-6-mercaptopurine	97	87	90	26
2-Amino-6-mercapto-purine riboside	95	33	39	39
6-Methylaminopurine	57	49	23	29
6-Methylmercaptopurine	49	27	20	54
6-Methylmercaptopurine riboside	40	67	0	37
Dimethylallylaminopurine	38	59	3	20
Dimethylallylaminopurine riboside	36	53	13	13
Allopurinol	38	81	73	47
4-Aminopyrazolo (3,4-d) pyrimidine	59	14	0	31
4-Amino-6-hydroxypyrazolo(3,4-d) pyrimidine	45	19	35	30
4-Thiopyrazolo (3,4-d)pyrimidine	90	89	18	66
Tubercidin	80	98	54	50
Formycin B	0	4	0	17
Sinefungin	0	2	2	24

The figures given are the % inhibition produced by the compounds at 1 mM (formycin B, 0.1 mM).

Table 4. Specific activities and percentage sedimentability of purine phosphoribosyltransferase activities in homogenates of trypanosomatids

	APRTase		HPRTase		GPRTase		XPRTase	
	Specific activity[a]	% Sediment[b]	Specific activity[a]	% Sediment	Specific activity[a]	% Sediment	Specific activity[a]	% Sediment
Leishmania m. amazonensis (Amastigote)	2.0 ± 0.5	53 ± 3	12 ± 1	38 ± 2	6 ± 1	51 ± 10	18 ± 1	45 ± 1
Leishmania m. amazonensis (Promastigote)	6 ± 1	10 ± 2	10 ± 2	17 ± 2	19 ± 3	26 ± 4	7 ± 1	68 ± 6
Leishmania donovani	19 ± 4	13 ± 1	9 ± 3	30 ± 1	21 ± 2	40 ± 6	5 ± 1	49 ± 7
Leishmania tarentolae	13 ± 6	17 ± 6	8 ± 1	42 ± 11	27 ± 7	53 ± 6	7 ± 2	55 ± 8
Crithidia fasciculata	13 ± 3	11 ± 6	3 ± 1	30 ± 5	18 ± 5	18 ± 3	4 ± 1	49 ± 3
Herpetomonas m. muscarum	13 ± 2	13 ± 7	0.5 ± 0.1	80 ± 10	10 ± 4	44 ± 10	4 ± 1	49 ± 1
Herpetomonas m. ingenoplastis	11 ± 3	31 ± 7	0.3 ± 0.1	46 ± 3	9 ± 3	43 ± 8	6 ± 2	50 ± 4

[a] The activities given are in nmol/min/mg protein and are the means ± SD from three experiments

[b] The activity sedimented in the 105,000 *g* pellet as a % of the total activity originally present. The figures are the means ± SD from three experiments.

extent to which the enzyme could be sedimented by centrifugation.

DISCUSSION

The results presented confirm that L. m. mexicana contains enzymes to convert adenine, guanine, hypoxanthine and xanthine to their monophosphates and it is evident that these enzymes are present at similar activities to those found in other Leishmania species (Kidder and Nolan, 1982; Konigk, 1978; Davies et al., 1983; Looker et al., 1983; this communication). The enzymes from L. m. mexicana, however, appear to differ significantly, both with respect to K_m and pH optima, from those of other Leishmania species. The apparent K_ms of purine substrates for the enzymes of L. m. mexicana promastigotes (Table 1) were much higher than those reported for L. donovani promastigotes (Tuttle and Krenitsky, 1980), whereas distinct differences in the pH optimum for APRTase and HPRTase were observed (8.4-9.1 and 6.9, respectively, for L. donovani promastigotes as compared with 7.5 and 9.0 for the same enzymes in L. m. mexicana promastigotes). The assay conditions used to determine the K_ms in the present study differed from those previously used for L. donovani, not only with respect to pH but also ions. This possibly accounts in part for the differences observed; it has been postulated that cations change the affinity of the enzymes for their substrates (Kidder et al., 1979). The divalent cation activation of L. m. mexicana APRTase, GPRTase and HPRTase observed in this study are quite similar to those reported for the isofunctional enzymes in the related trypanosomatid Crithidia fasciculata (Kidder et al., 1979) and the sensitivity of L. m. mexicana XPRTase activity to ions is similar to those previously reported for four species of Leishmania (Kidder and Nolan, 1982).

The lower activities of PRTases in amastigote lysates produced in the absence rather than the presence of leupeptin presumably were due to inactivation of the enzymes by the amastigote cysteine proteinase that is present at very high activity (Coombs, 1982; Pupkis and Coombs, 1984). It

has been suggested that inhibition of the amastigote proteinase by leupeptin is a sensible precaution to take in many studies of amastigote proteins (Pupkis and Coombs, 1984). Even when leupeptin was used, however, the PRTase activities were much lower in amastigotes than in promastigotes of L. m. mexicana, which could be a reflection of the lower growth rate of the amastigotes.

The findings that HPRTase, GPRTase and XPRTase in L. m. mexicana are apparently membrane bound in part, and were recovered with the particulate fractions of promastigotes, relate well with our previous report that they are associated with glycosomes (Hassan et al., 1985).

Purine PRTases have been investigated previously not only in Leishmania species, the activities in cultured promastigote forms of L. donovani, L. m. amazonensis and L. tarentolae being similar to those detected in this study, but also in several other parasitic protozoa including Plasmodium chabaudi (Walter and Konigk, 1974), Crithidia fasciculata (Kidder et al., 1979), Eimeria tenella (Wang and Simashkevich, 1981), Trypanosoma cruzi (Gutteridge and Davies, 1982), Trichomonas vaginalis (Miller and Lindstead, 1983), Tritrichomonas foetus (Wang et al., 1983) and Giardia lamblia (Wang and Aldritt, 1983). There have been no previous reports, however, on the PRTases in L. m. amazonensis amastigotes and Herpetomonas spp. or on XPRTase in C. fasciculata. The present study has shown that these hemoflagellates also possess all four PRTase activities. The finding that HPRTase, GPRTase and XPRTase were sedimentable in part in all of these trypanosomatids gives some support to the suggestion that these PRTases are glycosomal in all of the kinetoplastida.

The potent inhibitions by 6-mercaptopurine and 2-amino-6-mercaptopurine of HPRTase and GPRTase were probably due to competition with the purine base for the same substrate (PRPP). The activity observed with 6-mercaptopurine riboside and 2-amino-6-mercaptopurine riboside may have been due to the cleavage of these ribosides by parasite nucleosidase or phosphorylase

(Koszalka and Krenitsky, 1979; Hassan and Coombs, 1985) during the preincubation period and the subsequent inhibition of the PRTases by the released 6-mercaptopurine and 2-amino-6-mercaptopurine, respectively. The antileishmanial affects of allopurinol and thiopyrazolopyrimidine are probably due to their conversion to their respective ribonucleotides *in vivo* and subsequent inhibition of nucleotide metabolism (Nelson *et al.*, 1979). Allopurinol and thiopyrazolopyrimidine have been shown to be efficient substrates for HGPRTase (the enzyme possessing both HPRTase and GPRTase activity) from *L. donovani* (Tuttle and Krenitsky, 1980), as has 6-mercaptopurine. The inhibitions observed with these compounds in this study could similarly be due to their competing as substrates for the enzymes. The high sensitivity of the leishmanial PRTases to tubercidin make this analogue interesting for further investigation, especially since it is a potent inhibitor of both *in vitro* growth of promastigotes and amastigote transformation (see chpter 12).

The data on the distribution of the enzymes in cell fractions indicates that, in *L. m. mexicana*, APRTase is distinct from the other PRTases (Table 2; Hassan *et al.*, 1985). The relative insensitivity of XPRTase to inhibition by purine analogues (Table 3) suggests that it may be a different protein from HPRTase and GPRTase. These latter two activities are catalysed by the same enzyme in many cells including many protozoa (Tuttle and Krenitsky, 1980). It has not been established whether this is also the case with *L. m. mexicana*, the data presented, however, clearly demonstrate that although the two activities are similar in various properties they are affected differently both by divalent cations and some purine analogues. We are at present undertaking the purification of each of the PRTase enzymes present in *L. m. mexicana* promastigotes with the aim of elucidating the number present as well as gaining further insight into the properties of these important leishmanial enzymes.

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REFERENCES

Berens R.L., Brun R. & Krassner S.A. (1976) A simple monophasic medium for axenic culture of hemoflagellates. J. Parasit. 62, 360-365.

Camargo E.P. (1964) Growth and differentiation in Trypanosoma cruzi. Origin of metacyclic trypanosomes in liquid media. Rev. do Inst. de Med. Trop. Sao Paulo 6, 93-100.

Coombs G.H. (1982) Proteinases of Leishmania mexicana and other flagellate protozoa. Parasit. 84, 149-155.

Coombs G.H., Craft J.A. & Hart D.T. (1982) A comparative study of Leishmania mexicana amastigotes and promastigotes. Enzyme activities and subcellular locations. Mol. Biochem. Parasit. 5, 199-211

Davies M.J., Ross A.M. & Gutteridge W.E. (1983) The enzymes of purine salvage in Trypanosoma cruzi, Trypanosoma brucei and Leishmania mexicana. Parasit. 87, 211-217

Gutteridge W.E. & Davies M.J. (1982) Properties of the purine phosphoribosyltransferases of Trypanosoma cruzi. FEMS Microbiol. Letters 13, 207-212.

Hart D.T., Vickerman K. & Coombs G.H. (1981) A quick, simple method for purifying Leishmania mexicana amastigotes in large numbers. Parasit. 82, 345-355.

Hassan H.F. & Coombs G.H. (1984) Purine and pyrimidine metabolism in Leishmania mexicana mexicana. Parasit. 89, ii-iii.

Hassan H.F. & Coombs G.H. (1985) Leishmania mexicana: Purine metabolising enzymes of amastigotes and promastigotes. Exp. Parasit. 59, 139-150.

Hassan H.F., Mottram J.C. & Coombs G.H. (1985) Subcellular localisation of purine-metabolising enzymes in Leishmania mexicana mexicana. Comp. Biochem. Physiol., 81B, 1037-1041.

Kidder G.W. & Nolan L.L. (1979) Adenine aminohydrolase: Occurrence and possible significance in trypanosomid flagellates. Proc. Nat. Acad. Sci. U.S.A. 76, 3670-3672.

Kidder G.W. & Nolan L.L. (1982) Xanthine phosphoribosyltransferase in Leishmania: Divalent cation activation. J. Protozool. 29, 405-409.

Kidder G.W., Nolan L.L. & Dewey V.C. (1979) The purine phosphoribosyltransferases of Crithidia fasciculata. J. Parasit. 65, 520-525.

Konigk E. (1978) Purine nucleotide metabolism in promastigotes of Leishmania tropica: Inhibitory effect of allopurinol and analogues of purine nucleosides. Tropenmed. Parasit. 29, 435-438.

Konigk E. & Rasoul S.A. (1978) Catabolism of adenosine 5-monophosphate in promastigotes of Leishmania tropica. Tropenmed. Parasit. 28, 319-322.

Koszalka G.W. & Krenitsky T.A. (1979) Nucleosidases from Leishmania donovani. J. biol. Chem. 254, 8185-8193.

Looker D.L., Berens R.L. & Marr J.J. (1983) Purine metabolism in Leishmania donovani amastigotes and promastigotes. Mol. Biochem. Parasit. 9, 15-28.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with the folin phenol reagent. J. biol. Chem. 193, 265-275.

Marr J.J., Berens R.L. & Nelson D.J. (1978) Purine metabolism in Leishmania donovani and Leishmania braziliensis. Biochem. biophys. Acta 544, 360-371.

Miller R.L. & Lindstead D. (1983) Purine and pyrimidine metabolising activities in Trichomonas vaginalis extracts. Mol. Biochem. Parasit. 7, 41-51.

Nelson D.J., Bugge C.J., Krasny H.C., & Elion G.B. (1973) Formation of nucleotides of (6-^{14}C) allopurinol and (6-^{14}C) oxipurinol in rat tissues and effects on uridine nucleotide pools. Biochem. Pharm. 22,

2003-2022.

Nelson D.J., Lafon S.W., Tuttle J.V., Miller W.H., Miller R.L., Krenitisky T.A., Elion G.B., Berens R.L. & Marr J.J. (1979) Allopurinol ribonucleoside as an antileishmanial agent, biological effects, metabolism and enzymatic phosphyorylation. J. biol. Chem. 254, 11544-11549.

Nolan L.L. & Kidder G.W. (1980) Inhibition of growth and purine metabolising enzymes of trypanosomid flagellates by N-methyladenine. Antimicrob. Agents Chemoth. 17, 567-571.

North M.J. & Coombs G.H. (1981) Proteinases of Leishmania mexicana amastigotes and promastigotes: analysis by gel electrophoresis. Mol. Biochem. Parasit. 3, 293-300.

Pupkis M.F. & Coombs G.H. (1984) Purification and characterisation of proteolytic enzymes of Leishmania mexicana mexicana amastigotes and promastigotes. J. Gen. Microbiol. 130, 2375-2383.

Tuttle J.V. & Krenitsky T.A. (1980) Purine phosphoribosyltransferases from Leishmania donovani. J. biol. Chem. 255, 909-916.

Walter R.D. & Konigk E. (1974) Hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase from Plasmodium chabaudi, purification and properties. Tropenmed. Parasit. 25, 227-235.

Wang C.C. (1984) Parasite enzymes as potential targets for antiparasitic chemotherapy. J. Med. Chem. 27, 1-9.

Wang C.C. & Aldritt S. (1983) Purine salvage networks in Giardia lamblia. J. Exp. Med. 158, 1703-1712.

Wang C.C. & Simashkevich P.M. (1981) Purine metabolism in the protozoan parasite Eimeria tenella. Proc. Natl. Acad. Sci. U.S.A. 78, 6618-6622

Wang C.C., Verham R., Rice A. & Tzeng S. (1983) Purine salvage by Tritrichomonas foetus. Mol. Biochem. Parasit. 8, 325-337.

Chapter 6

PHOSPHOMONOESTERASES OF *LEISHMANIA MEXICANA MEXICANA* AND OTHER FLAGELLATES

Submitted as: Hassan, H.F. and Coombs, G.H. 1986. Phosphomonoesterases of *Leishmania mexicana mexicana* and other flagellates.

Abstract

Amastigotes and log-phase promastigotes of Leishmania mexicana mexicana contained distinct acid phosphatase, 3'-nucleotidase and 5'-nucleotidase activities, distinguishable by their responses to pH and inhibitors. Both tartrate-sensitive and tartrate-resistant acid phosphatase was present in the two forms, amastigotes possessed less tartrate-resistant acid phosphatase than promastigotes. A tartrate-sensitive acid phosphatase was secreted into the medium in large amounts during the growth in vitro of L.m. mexicana promastigotes. The 5'-nucleotidase activity of both parasite forms was inhibited by ammonium molybdate, sodium tartrate and, to less extent, by sodium flouride whereas 3'-nucleotidase was inhibited by EDTA. All three activities were shown to be present on the external surface of both amastigotes and promastigotes. The three phosphomonoesterase activities were also detected in extracts of L. m. amazonensis, L. donovani, L. tarentolae, Crithidia fasciculata, Herpetomonas muscarum muscarum, H. m. ingenoplastis and Trichomitus batrachorum whereas 5'-nucleotidase was not detected in Trypanosoma brucei brucei extract and 3'-nucleotidase was absent from extracts of Trichomonas vaginalis and Tritrichomonas foetus.

Key words: Leishmania mexicana mexicana; Amastigotes; Promastigotes; Trypanosomatids; Trichomonads; Acid phosphatase; 3'-Nucleotidase; 5'-Nucleotidase.

Introduction

Parasite surface membranes are of special interest as they are the prime site of interaction between the parasite and its host and so they are intimately involved in many cellular events including the transport of essential nutrients and the protection of the parasite from the host's immune response (1). The plasma membranes of a variety of parasites have been purified in recent years and this has allowed a detailed examination of their many components. Some of the most exciting findings have come from investigations of trypanosomatids, most notably the leishmanias (2-7).

Leishmania donovani promastigotes have been shown to possess three distinct phosphomonoesterase activities (acid phosphatase, 3'-nucleotidase and 5'-nucleotidase) all of which are localised, at least in part, on the external surface of the plasma membrane (3-5). The surface acid phosphatase of L. donovani promastigotes appears to comprise three activities. The main one is tartrate-resistant and the enzyme responsible has been purified (8) and shown to inhibit markedly the oxidative metabolism of neutrophils (9); the implication is that this enzyme may play a crucial role in enabling of the parasite to avoid the microbicidal activity of macrophages. Acid phosphatase also occurs intracellularly in L. donovani promastigotes as well as being secreted in large amounts (6). Other species of Leishmania and other trypanosomatids also possess acid phosphatases (10-12) but they have been studied in less detail.

3'-Nucleotidase, an enzyme that does not have a mammalian counterpart, has also been purified from L. donovani promastigotes. Although highly active as a 3'-nucleotidase, there are suggestions that the physiological function is as a nuclease (13). Despite its potential interest, most of the findings so far have come from studies of culture promastigotes of leishmanias. There is as yet

relatively little information on the 3'-nucleotidases of other parasitic protozoa, although similar enzymes have been reported to be present in Crithidia fasciculata (14), Trypanosoma rhodesiense (15) and Entamoeba histolytica (16).

5'-Nucleotidase is thought to be a usual constituent of the plasma membrane of mammalian cells (17) and a similar enzyme has also been detected in promastigotes of several leishmanial species (4, 18-19); it seems likely that they play a key part in processing exogenously available nucleotides to a form suitable for transport into the cell.

These three activities may well play key parts in the interaction of flagellate parasites with their hosts and the information on the leishmanial enzymes that has been reported so far provides a most fascinating indication of their possible roles. As yet, however, studies on the leishmanial enzymes have been largely limited to cultured promastigotes and mainly those of L. donovani. The purpose of this study was to determine to what extent the enzyme activities differ between species, information which should give a greater insight into their role, and, crucially, the situation in leishmanial amastigotes. Log-phase promastigotes of leishmanias are thought to correspond to the stage that occurs in the sandfly midgut. Not only do these forms differ from amastigotes, which reside in mammalian macrophages, but also they appear to be significantly different from the promastigotes mainly responsible for initiating infections in mammals (20).

Materials and Methods

Isolation, cultivation and fractionation of parasites. Amastigotes of L. m. mexicana (M 379) were isolated from cutaneous lesions in female CBA mice (Zoology Department, Glasgow University,

Glasgow G12 8QQ) as described previously (21). Promastigotes of L. m. mexicana (M 379), L.m. amazonensis (LV 78), L. donovani (LV 79) and L. tarentolae, culture forms of C. fasciculata (Anopheles strain ATCC 11745), Herpetomonas muscarum muscarum (ATCC 30260) and H. m. ingenoplastis (ATCC 30269) and procyclic trypomastigotes of Trypanosoma brucei brucei (Stock EATRO 1125) were cultured as described previously (21). Trichomonas vaginalis (G3), Tritrichomonas foetus (F2) and Trichomitus batrachorum (B2) were cultured in modified MDM medium as described previously (22). Parasites at mid-log phase of growth were harvested by centrifugation at 3000 x g at 4°C for 10 min and all except trichomonads washed twice in 50 mM Tris-HCl buffer, pH 7.2, containing 0.85% NaCl. Trichomonads were washed in 0.25 M sucrose. The cells were stored as pellets at -70°C until use, whereupon they were suspended in the buffered saline supplemented with 0.1 mM dithiothreitol and lysed by sonication for two periods of 15 s, separated by a 10 s cooling period, using a MSE Soniprep 150 fitted with an exponential probe at 4 μm amplitude. The crude homogenates produced were either used directly or fractionated at 105,000 x g at 4°C for 1 h with the resultant supernatants and pellets being separated and the latter resuspended in the above buffer to the volume of the supernatant fraction.

Enzyme analyses 3'-Nucleotidase (EC 3.1.3.6) and 5'-nucleotidase (EC 3.1.3.5) activities were assayed by determining the amount of inorganic phosphate released from the substrate. The standard assay conditions for the nucleotidases, which were used unless otherwise specified, were as follows. 3'-Nucleotidase: 50 mM Tris-maleate (pH 7.5 for leishmanial amastigotes or pH 8.0 for

leishmanial promastigotes and other flagellates); 1 mM $CoCl_2$; 5 mM 3'-AMP and sample protein. 5'-Nucleotidase: 50 mM Tris-maleate, pH 6.5 (leishmanial promastigotes and other flagellates) or 50 mM sodium acetate, pH 5.5 (leishmanial amastigotes); 1 mM $MgCl_2$; 5 mM 5'-AMP and sample protein. Incubations were for 30 min at 42°C in a final volume of 1 ml and involved an amount of extract sufficient to catalyse the reaction at a linear rate for period of the incubation. The assay mixtures were routinely preincubated for 3 min at 42°C before the reaction was initiated by the addition of enzyme sample. The reaction was terminated by addition of 1 ml 8% (w/v) trichloroacetic acid and chilling in an ice bath. The precipitated protein was removed by centrifugation at 2100 x g for 5 min and the phosphate concentration in the supernatant estimated by the method of Fiske and Subbarow (23). Acid phosphatase (EC 3.1.3.2) was assayed in a reaction mixture (0.4 ml) containing 50 mM sodium acetate (pH 4.5 for leishmanial amastigotes or pH 5.5 for leishmanial promastigotes and other flagellates), 5 mM p-nitrophenylphosphate (PNPP) and an amount of extract sufficient to catalyse the reaction at a linear rate for the 30 min incubation at 42°C. The reaction was initiated by the addition of enzyme sample and terminated by the addition of 1 ml of 0.1 N NaOH. The released p-nitrophenol was estimated spectrophotometrically at 410 nm using an extinction coefficient of 14.3×10^{-3} M^{-1} cm^{-1}. Acid phosphatase activity towards other substrates was assayed by determining the amount of inorganic phosphate released during incubation for 30 min at 42°C. The inorganic phosphate was estimated by the method of Fiske and Subbarow (23) following termination of the reaction by addition of 1 ml 8% (w/v) trichloroacetic acid. Inosine nucleosidase was assayed as described previously (19).

The effect of pH on the enzyme activities was determined by

using the following buffers: sodium acetate, 50 mM (pH 3.5-6.0) and Tris-maleate, 50 mM (pH 6.0-9.0). The apparent K_m values for substrate were determined by the Lineweaver-Burk method (24). The effects of inhibitors on enzyme activities were assessed by preincubating the extract with the inhibitor for 10 min before initiating the assay. Protein concentrations were estimated by the method of Lowry et al. (25), with bovine serum albumin as standard.

Phosphomonoesterase activities of living L. m. mexicana Studies on the nucleotidase and acid phosphatase activities of living promastigotes and amastigotes of L. m. mexicana were carried out using the method of Gottlieb and Dwyer (3). Purified amastigotes (1 x 10^8/ml) and mid-log phase promastigotes (5 x 10^7/ml) were incubated for 30 min at 30°C (amastigotes) or 25°C (promastigotes) in a reaction mixture containing 50 mM Tris-maleate, pH 7.0, 0.85% (w/v) NaCl and 5 mM PNPP or 2.5 mM 3'-AMP or 2.5 mM 5'-AMP. At appropriate time points, aliquots of the incubation mixture were centrifuged at 2100 x g for 5 min and the supernatants were analysed for the released inorganic phosphate (nucleotidases) or p-nitrophenol (acid phosphatase) as described above. The activities of living parasites were compared with those of parasite lysates containing the same amount of protein and assayed under identical conditions.

Released phosphomonoesterase activities. The acid phosphatase released by L. m. mexicana promastigotes into growth medium was studied using the following procedure. Promastigotes were removed from late-log phase cultures by centrifugation at 3,000 x g for 10 min at 4°C and the resultant supernatants were filtered (0.22 μm millipore filter) and concentrated by freeze-drying for 24 h . The concentrated medium was resuspended in 50 mM Tris-HCl, pH 7.2, with

0.1 mM dithiothreitol and aliquots were assayed for acid phosphatase as described above. Aliquots of whole cultures (i.e. containing promastigotes) also were assayed for acid phosphatase activity, using 0.1% (v/v) Triton to ensure cell lysis.

Electron microscopic studies on the localisation of leishmanial phosphomonoesterases. For detection of acid phosphatase, amastigotes (1×10^8/ml) or promastigotes (5×10^7/ml) were fixed in 0.5% (v/v) glutaraldehyde (amastigotes) or 1% (v/v) glutaraldehyde (promastigotes) in 0.1M sodium cacodylate, pH 7.2, containing 5% (w/v) sucrose (SCS) for 30 min at 4°C. Following fixation, cells were washed twice in SCS, twice in 50 mM sodium acetate, pH 5.0, containing 5% (w/v) sucrose (SAS) and resuspended in SAS with 3.3 mM $Pb(NO_3)_2$ and 12.5 mM disodium glycerol-2-phosphate as substrate. After 30 min incubation at 37°C, the cells were washed twice in SAS and post-fixed with 1% (v/v) OsO_4 for 1 h at 4°C. The cells were then washed twice with distilled water, stained for 20 min with 0.5% aqueous (w/v) uranyl acetate, washed again with distilled water, dehydrated in a graded series of ethanols, embeded in Epon, sectioned and examined unstained or after staining with uranyl acetate and lead citrate as described previously (26). For detection of 3'- and 5'-nucleotidases, living cells were incubated at a density of 1×10^8/ml (amastigotes) or 5×10^7/ml (promastigotes) in the medium described by Dwyer and Gottlieb (5) which comprised 40 mM Tris-maleate, pH 7.2, 4 mM $Mg(NO_3)_2$, 100 mM NaCl, 20 mM KCl, 40 mM sucrose, 1 mM $Pb(NO_3)_2$ and 1 mM 3'-AMP or 5'-AMP. After incubation for 30 min at 37°C (amastigotes) or 25°C (promastigotes) the reaction was terminated by addition of an equal volume of 2% (v/v) glutaraldehyde in 0.2M sodium cacodylate, pH 7.2, and kept on ice for 30 min. The cells were then washed twice in 0.1 M sodium cacodylate, pH 7.2, post fixed with 1%

(v/v) OsO_4 for 1 h at 4°C and processed as described above.

Materials. 3'-AMP (sodium salt), 5'-AMP (sodium salt), glycerol-2-phosphate (disodium) and p-nitrophenyl phosphate (magnesium salt) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England. All other chemicals were obtained from the sources listed previously (16, 19).

Results

The phosphomonoesterase activities detected in homogenates of amastigotes and promastigotes of L. m. mexicana are given in Table 1. The level of each of the three activities was slightly higher in promastigotes than in amastigotes and in both forms the activities towards the three substrates were in the order 3'-AMP > PNPP > 5'-AMP. The recoveries of the phosphomonoesterase activities in the particulate and soluble fractions are also given in Table 1. The major proportion of both nucleotidases was recovered in the particulate fraction of both parasite forms, which is consistent with these activities being largely membrane-bound. In contrast, only just over half of the acid phosphatase activity was sedimented suggesting that this activity occurs in multiple forms.

The three monoesterase activities of L. m. mexicana were examined in more detail in order to distinguish them. The effects of pH are shown in Fig. 1. Acid phosphatase activity was found to be maximal at pH 4.5 and 5.5 for amastigotes and promastigotes, respectively, there being little activity towards PNPP at alkaline pH. 5'-AMP nucleotidase activity was maximal at pH 5.5 for amastigotes and at pH 6.5 for promastigotes, whereas 3'-AMP nucleotidase showed high activity over a broad range of pH (7.0-8.5), the apparent optima being 7.5 for amastigotes and 8.0 for

Table 1. Activities and some properties of phosphomonoesterases in L. m. mexicana

	Acid phosphatase	3'-AMP nucleotidase	5'-AMP nucleotidase
Amastigotes			
pH optimum	4.5	7.5	5.5
Specific activity[a]	66 ± 4	69 ± 7	24 ± 4
% Sedimentability[b]	56 ± 6 (101 ± 5)	89 ± 2 (96 ± 2)	70 ± 7 (93 ± 6)
K_m (mM)	1.1	0.64	0.86
Promastigotes			
pH optimum	5.5	8.0	6.5
Specific activity[a]	88 ± 9	76 ± 4	37 ± 5
% Sedimentability[b]	58 ± 4 (98 ± 5)	90 ± 1 (97 ± 4)	81 ± 3 (99 ± 7)
K_m (mM)	0.66	0.58	0.72

[a] The mean activities ± SD in homogenates expressed in nmol/min/mg protein.

[b] The activity sedimented in the 105,000 x g pellet as a % of the total activity recovered. The figures are the mean ± SD from three experiments with the mean percentage activity recovered ± SD given in parentheses.

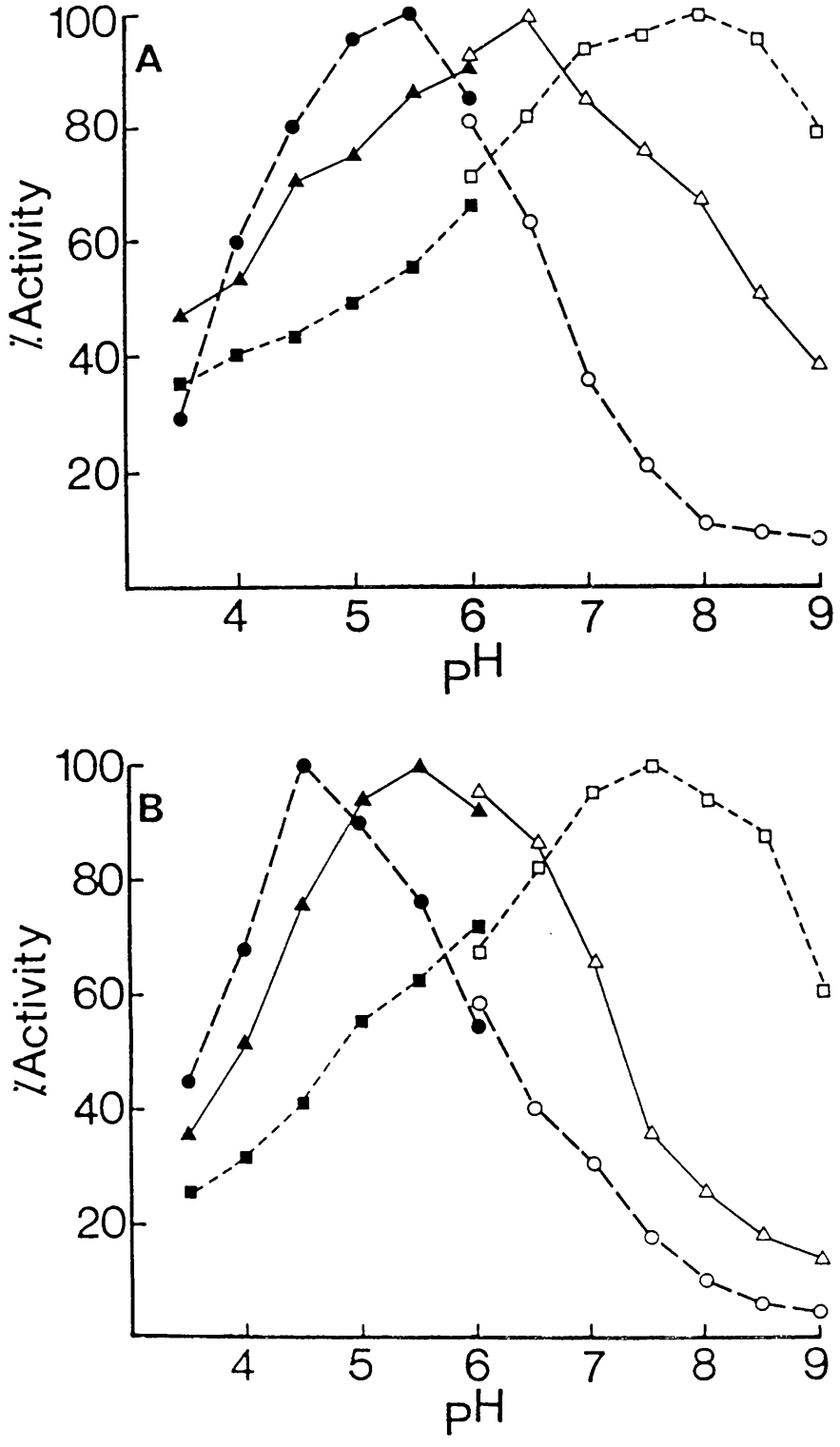

Fig.1 The pH dependence of Leishmania m. mexicana promastigotes (A) and amastigotes (B) phosphomonoesterases. The buffers used (50 mM) were sodium acetate (● , ■ , ▲) and Tris-maleate (○ , □ , △). The ordinates show the enzyme activity as percentage of the maximum. Key: ● , ○ , acid phosphatase; ■ , □ , 3'-nucleotidase; ▲ , △ , 5'-nucleotidase.

promastigotes.

The effects of several cations on the phosphomonoesterase activities of particulate fractions of amastigotes and promastigotes of L. m. mexicana were investigated using chlorides of Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}. The only significant effect observed was the small stimulation (approximately 15%) of both amastigote and promastigote 5'-AMP nucleotidase activity by 1 mM Mg^{2+} and the greater stimulation (around 50%) of 3'-AMP nucleotidase of both parasite forms by 1 mM Co^{2+}.

The three phosphomonoesterase activities of L. m. mexicana were also differently sensitive to various inhibitors (Table 2). Molybdate proved to be an effective inhibitor of acid phosphatase and 5'-nucleotidase but had no effect on 3'-nucleotidase. Fluoride, tartrate and dithionite inhibited amastigote acid phosphatase to a greater extent than the promastigote activity. 5'-Nucleotidase of both forms was inhibited to a considerable degree by fluoride and tartrate but affected little by dithionite. EDTA was the only compound found to inhibit 3'-nucleotidase to a great extent, dithionite caused only a small inhibition.

A range of other potential substrates were used to give more information on the specificity of the L. m. mexicana phosphomonoesterases, the results are presented in Table 3. Acid phosphatase was most active towards PNPP, although fructose-6-phosphate, glycerol-2-phosphate and glucose-6-phosphate were all hydrolysed by both amastigotes and promastigotes extracts. Fructose-6-phosphate was hydrolysed more rapidly than glucose-6-phosphate by both forms. Of the 3'-nucleotides tested, 3'-AMP and 3'-IMP were the best substrates, although 3'-UMP, 3'-GMP and 3'-CMP were also hydrolysed. Phosphate was also released from each of the 5'-

Table 2. Effects of inhibitors on the phosphomonoesterase activities of the 105,000 x g pellets of L. m. mexicana amastigotes and promastigotes[a]

Inhibitor	Concentration (mM)	Acid phosphatase		3'-AMP nucleotidase		5'-AMP nucleotidase	
		Amastigotes	Promastigotes	Amastigotes	Promastigotes	Amastigotes	Promastigotes
Ammonium molybdate	10	89 ± 3	91 ± 1	NI	NI	85 ± 1	87 ± 1
	1	89 ± 3	88 ± 2	NI	NI	78 ± 3	85 ± 1
	0.1	87 ± 1	79 ± 6	NI	NI	74 ± 4	84 ± 2
Sodium fluoride	10	91 ± 1	68 ± 1	NI	NI	35 ± 6	33 ± 4
	1	89 ± 2	46 ± 8	NI	NI	19 ± 8	25 ± 5
	0.1	34 ± 8	NI	NI	NI	7 ± 4	14 ± 4
Sodium tartrate	10	86 ± 1	54 ± 4	NI	NI	50 ± 1	57 ± 3
	1	61 ± 9	38 ± 5	NI	NI	38 ± 2	37 ± 11
	0.1	30 ± 9	5 ± 1	NI	NI	29 ± 1	12 ± 4
Sodium dithionite	10	73 ± 1	33 ± 1	15 ± 6	9 ± 4	10 ± 4	6 ± 3
	1	30 ± 13	5 ± 1	6 ± 3	7 ± 2	4 ± 2	1 ± 1
	0.1	3 ± 1	NI	NI	NI	NI	NI
EDTA	10	NI	NI	68 ± 6	76 ± 6	4 ± 1	4 ± 1
	1	NI	NI	49 ± 7	62 ± 6	4 ± 2	2 ± 1
	0.1	NI	NI	7 ± 3	11 ± 4	NI	NI

[a] The figures given are the % inhibition relative to the activity in the absence of inhibitors (means ± SD).
NI, no inhibition detected.

Table 3. Activities of L. m. mexicana phosphomonoesterases on different substrates[a]

Acid phosphatase			3'-Nucleotidase			5'-Nucleotidase		
Substrate	Amastigote	Promastigote	Substrate	Amastigote	Promastogote	Substrate	Amastigote	Promastigote
p-Nitrophenylphosphate	78 ± 4	97 ± 9	3'-AMP	125 ± 14	133 ± 15	5'-AMP	34 ± 4	58 ± 7
Glycerol-2-phosphate	21 ± 2	30 ± 5	3'-IMP	102 ± 10	139 ± 14	5'-IMP	34 ± 3	82 ± 8
Fructose-6-phosphate	15 ± 2	43 ± 4	3'-UMP	95 ± 10	84 ± 9	5'-UMP	63 ± 6	55 ± 5
Glucose-6-phosphate	9 ± 2	15 ± 3	3'-GMP	46 ± 4	42 ± 3	5'-GMP	35 ± 8	34 ± 10
			3'-CMP	49 ± 5	49 ± 8	5'-CMP	19 ± 4	11 ± 3
						TMP	49 ± 5	104 ± 6

[a] The figures given are specific activities (in nmol/min/mg 105,000 x g pellet protein) and are the means ± SD from replicate experiments.

nucleotides tested, although in this case there were significant differences between the affinity of the amastigote and promastigote enzymes towards some of the substrates. The nucleotidases of L. m. mexicana displayed no activities toward 2'-AMP.

Living amastigotes and promastigotes of L. m. mexicana were found to hydrolyse PNPP, 3'-AMP and 5'-AMP (Fig. 2). Cell lysates hydrolysed PNPP at significantly greater rates than intact promastigotes and amastigotes. In contrast, homogenates and living parasites hydrolysed 3'-AMP at the same rate whereas 5'-AMP was broken down slightly more rapidly by homogenates than by intact parasites.

In order to examine whether acid phosphatase was released from growing promastigotes of L. m. mexicana, the activity of this and inosine nucleosidase in log-phase culture medium was assessed. It was found that a very high proportion (83 ± 10%) of the total acid phosphatase in log-phase cultures was recovered in culture medium after removal of the cells. In contrast, inosine nucleosidase, which is at high activity in the parasite and mainly cytosolic (27), could not be detected in culture medium after removal of the promastigotes. From the limit of detection of the enzyme assay, it was calculated that at most 2% of total inosine nucleosidase activity could have been present in the culture medium; this confirms that there had been insignificant cell lysis. The extracellular acid phosphatase activity was found to be maximally active in the range of pH 4 - pH 6 and markedly inhibited by both sodium fluoride (92% at 1 mM) and sodium tartrate (89% at 0.1 mM). In contrast, 3'-AMP nucleotidase and 5'-AMP nucleotidase activities were not detectable in the culture medium following the removal of cells. Using the lower limit of detection for each enzyme, it was calculated that at most 5% and 8%, respectively, of total activities in the cultures could have been

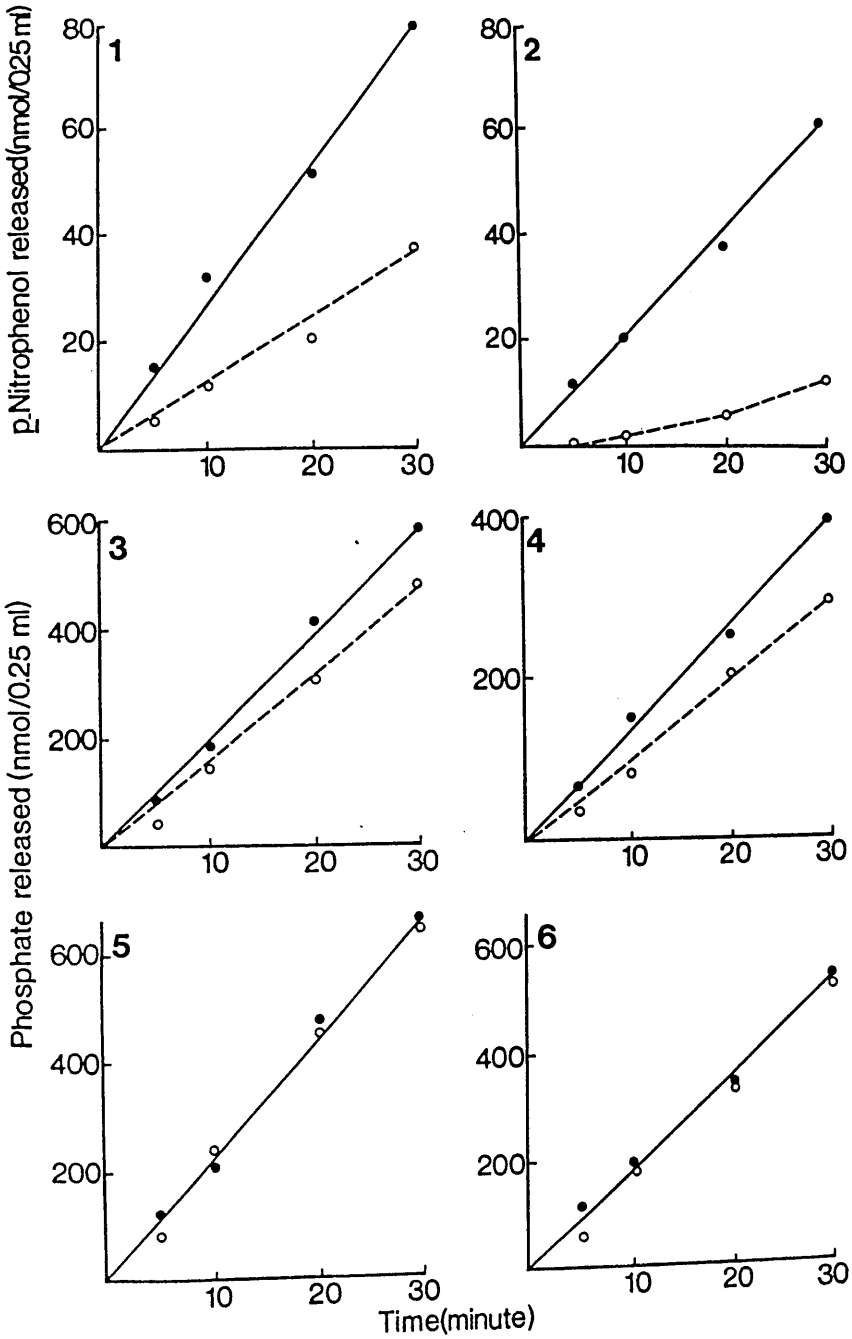

Fig. 2. Phosphomonoesterase activities of living Leishmania m. mexicana and parasite lysates. Key: 1,3,5, promastigotes; 2,4,6, amastigotes; ● , parasite lysates; ○ , living parasites; 1, 2, acid phosphatase; 3,4, 5'-AMP nucleotidase; 5, 6, 3'-AMP nucleotidase.

present in the medium.

Phosphomonoesterases were studied cytochemically by using lead as the capture agent of the phosphate liberated in the hydrolysis of glycerol-2-phosphate, 3'-AMP and 5'-AMP. For L. m. mexicana promastigotes and with each substrate, the reaction product appeared as electron dense particles distributed over the cell surface (Fig. 3a-c). No reaction product was observed on the plasma membrane when the substrate was omitted from the incubation medium. With amastigotes of L. m. mexicana, acid phosphatase was primarily detected in megasomes (26). Surface staining of acid phosphatase was consistently observed (Fig. 3d), but it was much less intense than for promastigotes (Fig. 3a). Reaction product deposition was also observed using the acid phosphatase procedure described but with 1 mM CMP as substrate, whereas there was no deposit apparent when 1 mM PNPP was used. It was much more difficult to detect surface nucleotidases with amastigotes than with promastigotes. Using the same conditions that were successful with promastigote enzymes, reaction product was found but only over parts of the surface of some amastigotes, as shown for example in Fig. 3e. Modification of the incubation conditions including varying the pH (6.8-7.8), incubation time (5-30 min), incubation temperature (0-37^{o}C) and also using cerium chloride as the capture agent instead of lead proved no more successful.

The activities of acid phosphatase, 5'-nucleotidase and 3'-nucleotidase detected in other trypanosomatids and three trichomonads are given in Table 4. Acid phosphatase was present in all species with high levels of activity and 5'-AMP nucleotidase was detected in all except Trypanosoma b. brucei, although the activity in trichomonads was relatively low. 3'-AMP nucleotidase was present in

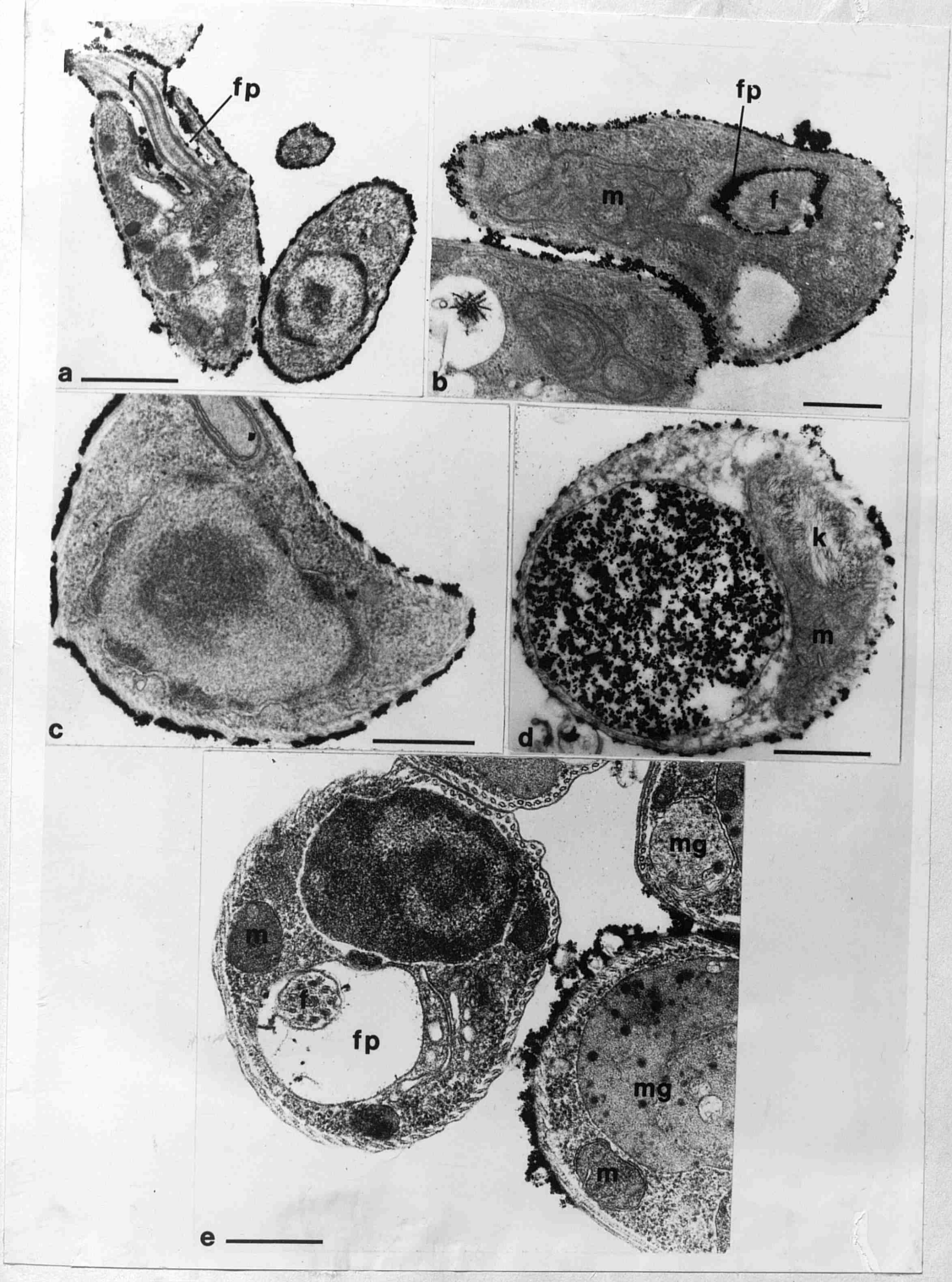

Fig. 3. Ultrastructural localisation of acid phosphatase (a, bar = 1 µm), 3'-AMP nucleotidase (b, bar = 0.5 µm), 5'-AMP nucleotidase (c, bar = 0.25 µm), acid phosphatase (d, bar = 0.25 µm) and 3'-AMP nucleotidase (e, bar = 0.5 µm) in promastigotes (a-c) and amastigotes (d-e) of Leishmania m. mexicana using lead as the capture agent. Abbreviations: f, flagellum; fp, flagellar pocket; k, kinetoplast; m, mitochondrial profile; mg, megasome.

Table 4. Phosphomonoesterase activities of a range of flagellates.

	Acid phosphatase		3'-AMP nucleotidase		5'-AMP nucleotidase	
	Specific activity[a]	% Sediment[b]	Specific activity[a]	% Sediment[b]	Specific activity[a]	% Sediment[b]
L. m. amazonensis	62 ± 3	65 ± 7	105 ± 11	88 ± 2	55 ± 2	75 ± 6*
L. donovani	84 ± 2	68 ± 4	67 ± 1	90 ± 6	8 ± 2	77 ± 2*
L. tarentolae	39 ± 4	61 ± 3**	24 ± 4	71 ± 6*	17 ± 2	60 ± 4*
C. fasciculata	174 ± 6	67 ± 3**	202 ± 15	93 ± 3*	94 ± 11	75 ± 6
H. m. muscarum	63 ± 13	51 ± 8**	14 ± 1	71 ± 6	20 ± 2	47 ± 2
H. m. ingenoplastis	129 ± 10	36 ± 6	23 ± 3	75 ± 1	20 ± 4	50 ± 4
T. b. brucei	34 ± 4	54 ± 1	48 ± 4	80 ± 3	< 0.2	na
T. vaginalis	53 ± 3	60 ± 6	< 0.1	na	0.6 ± 0.2	44 ± 1
T. foetus	56 ± 3	60 ± 5	< 0.1	na	2 ± 0.2	48 ± 5*
T. batrachorum	85 ± 7	47 ± 1	0.6 ± 0.1	64 ±1*	5 ± 1	67 ± 1

[a] The activities given are those in parasite homogenates, are expressed in nmol/min/mg protein and are the means ± SD from replicate experiments.

[b] The activity sedimented in the 105,000 x g pellet as a % of the total activity recovered. The figures are the means ± SD from replicate experiments. The mean total activity recovered was between 90-100% except were indicated * or ** for which mean total recoveries were 85 - 90% and 110-120%, respectively.

na, not applicable.

all trypanosomatids, although at different activities, whilst it was not found in Trichomonas vaginalis or Tritrichomonas foetus but was detected, albeit at a very low level, in Trichomitus batrachorum. Most of the activities detected were to a large extent sedimentable by centrifugation (Table 4).

Discussion

The present investigation has shown that in several respects the phosphomonoesterases of L. m. mexicana appear to be similar to those of L. donovani (3-5). There appear to be three groups of enzyme that can be distinguished by their responses to pH (Fig. 1) and inhibitors (Table 2). It seems, however, that each can use a variety of substrates (Table 3) and it is probable that at least some of the activities measured are due to multiple enzymes. This is supported by the finding that certainly acid phosphatase and probably 5'-nucleotidase occur both membrane-bound and in the matrix of the cells and that acid phosphatase is excreted in large amounts. Leishmania donovani 3'-nucleotidase was found upon purification to be a heterogenous complex of proteins, although there were suggestions that these may have been due to proteolysis upon cell lysis (13). The significance of the multiplicity of acid phosphatase activities, the relationship, if any, of the different forms, and their natural substrates are intriguing questions that remain to be answered.

Another unresolved question concerns the functional significance of the enzymes. We have shown in this study that all three phosphomonoesterase activities occur on the surface membrane of amastigotes as well as promastigotes of L. m. mexicana; this apparently is also the situation with L. donovani (3,5), although the evidence for amastigotes is yet to be published. The finding that acid phosphatase staining was less abundant on the surface of

amastigotes of L. m. mexicana than occurred on promastigotes does not provide support for the suggestion that the surface enzyme is mainly involved in the interaction of the parasite with macrophages. A similar conclusion can be drawn from the inhibition results (Table 2) that show amastigotes possess less tartrate-resistant acid phosphatase, the enzyme of L. donovani that inhibits the oxidative metabolism of neutrophils (9). The cytochemical staining method, however, is not easily quantified and the results with 3'-nucleotidase and 5'-nucleotidase suggest that the amastigote surface may be able to rearrange and remove the surface labelling quite rapidly. Thus these results should be interpreted with caution.

The information gained on the distribution of the enzyme activities amongst the protozoa provides some insight into their possible functions. The results of this study (Table 4) show that whereas acid phosphatase activity is present in all flagellates investigated, 5'-nucleotidase is apparently absent from T. b. brucei, confirming the previous findings of McLaughlin (28) and Gottlieb et al. (15) with T. rhodesiense, and 3'-nucleotidase is a characteristic of the trypanosomatids studied but not all trichomonads. 3'-Nucleotidase has been found previously in C. fasciculata (14) but it apparently does not occur in all trypanosomatids for it was not detected in T. cruzi (15). Interestingly it also occurs in E. histolytica (16) but has been reported to be absent from Babesia divergens (29), Acanthamoeba species (30) and T. vaginalis (31). 5'-Nucleotidase is more widely distributed being found in all of these four parasites as well L. tropica (18). There is, however, little information yet on whether the enzymes are present on the surface of these parasites. The occurrence of all three activities on the outer surface of L. donovani is well established and we have now shown this to hold for L. m. mexicana. The situation appears to be the same for

L. m. amazonensis (32) and C. fasciculata (14) but interestingly promastigotes of L. major, both mid-log and metacyclic, appear to lack an acid phosphatase on the surface (32). In contrast, the insect flagellate H. m. muscarum possesses an highly active surface acid phosphatase, although H. m. ingenoplastis does not (32). 3'-Nucleotidase occurs on the surface of procyclic T. rhodesiense (15) but the location of acid phosphatase in this parasite has not been reported.

There is some evidence that the 3'-nucleotidase activity of leishmanias may be a different expression of an enzyme that functions as a nuclease in vivo (13), presumably hydrolysing nucleic acids available in the parasites' environment. The resultant nucleotides could be further catabolised by the surface localised 5'-nucleotidase to yield nucleosides which could be taken into the cells more easily. Such a sequential action of the two activities could be beneficial to leishmanias and other trypanosomatids in both their insect and mammalian hosts and studies on the consumption and incorporation of nucleic acids and nucleotides by these protozoa should help to elucidate the enzymes roles. The lack of 5'-nucleotidase in T. b. brucei, however, casts some doubt on the proposed role of 3'-nucleotidase present as nuclease unless the parasite can use 5'-nucleotidase directly.

The situation concerning the possible functions of acid phosphatase is even more intriguing. The excretion of large amounts of the enzyme from both L. donovani and L. m. mexicana promastigotes provided evidence that this may be a general property of leishmanias promastigotes; recently, however, it was reported that a similar excretion does not occur with L. major (32,33). It is yet to be confirmed if excretion also occurs with amastigotes, although there

is evidence that it does with L. donovani at least (33), and so could be involved with survival in macrophages. At present some nutritional role seems equally likely at least for the parasite in the sandfly; information from further studies on insect flagellates may provide the answer. The extracellular enzyme appears to differ from the main surface localised activity, certainly with respect to inhibition by tartrate and fluoride. The finding that a tartrate-resistant acid phosphatase purified from L. donovani promastigotes (and thought to be the same as the surface bound enzyme) inhibited the oxidative metabolism of neutrophils raised the possibility that this enzyme may be crucial for survival of Leishmania in macrophages (9). If this is so, it would be expected to be a leishmania-specific feature and a key enzyme in the stages of Leishmania that infect macrophages, that is metacyclics and amastigotes, being unimportant to non-infective promastigotes such as those in log-phase culture and insect trypanosomatids. As yet there is no report that such a correlation exists, although the finding that a more virulent line of L. donovani possesses higher acid phosphatase activity is relevant (34). The presence of surface acid phosphatase on C. fasciculata, if confirmed, would argue against the enzyme's only role being related to survival in macrophages, as does our observations for H. m. muscarum, whereas the lack of activity on the surface of L. major promastigotes shows that the enzyme is not essential for infection of mammals (33). It may well be, however, that leishmanias differ, at least in part, in their survival mechanisms and that there are several surface acid phosphatases with individual roles. Understanding the function of these activities would be aided by elucidation of their natural substrates. As shown in this and other studies, several sugar phosphates can be hydrolysed. Their hydrolysis would supply a usable energy source to the parasite,

although not all of them are likely to be available in both or either of the sandfly gut or macrophages. The recent report that phosphoprotein and inositol phosphatase are also hydrolysed adds support to the suggestion that at least one acid phosphatase is involved in modulating the parasite's environment (35).

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References

1. Chang, K-P. and Fong, D. (1983) Cell biology of host-parasite membrane interactions in leishmaniasis. In: Cytopathology of Parasitic Disease, Ciba Foundation Symposium 99, pp. 113-137, Pitman Press, Bath, Great Britain.

2. Dwyer, D.M. and Gottlieb, M. (1985) The biochemistry of Leishmania surface membranes. In: Leishmaniasis (Chang, K-P. and Bray, R.S. eds.) pp. 32-47, Elsevier Science Publications, Amsterdam, New York, Oxford.

3. Gottlieb, M. and Dwyer, D.M. (1981) Leishmania donovani: surface membrane acid phosphatase activity of promastigotes. Exp. Parasitol. 52, 117-128.

4. Gottlieb, M. and Dwyer, D.M. (1983) Evidence for distinct 5'- and 3'-nucleotidase activities in the surface membrane fraction of Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 7, 303-317.

5. Dwyer, D.M. and Gottlieb, M. (1984) Surface membrane localization of 3'- and 5'-nucleotidase activities in Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 10, 139-150.

6. Gottlieb, M. and Dwyer, D.M. (1982) Identification and partial characterization of an extracellular acid phosphatase activity of Leishmania donovani promastigotes. Mol. Cell. Biol. 2, 76-81.

7. Glew, R.H., Czuczman, M.S., Diven, W.F., Berens, R.L., Tope, M.T. and Katsoulis, D.E. (1982) Partial purification and characterization of particulate acid phosphatase of Leishmania donovani promastigotes. Comp. Biochem. Physiol. 72B, 581-590.

8. Remaley, A.T., Das, S., Campbell, P.I., La Rocca, G.M., Tope, M.T. and Glew, R.H. (1985) Characterization of Leishmania donovani acid phosphatases. J. Biol. Chem. 260, 880-886.

9. Remaley, A.T., Kuhns, D.B., Basford, R.E., Glew, R.H. and Kaplan, S.S. (1984) Leishmanial phosphatase blocks neutrophil O_2 production. J. Biol. Chem. 259, 11173-11175.

10. Eeckhout, Y. (1972) Studies on acid hydrolases and on catalase of the trypanosomatid Crithidia luciliae. In: Comparative Biochemistry of Parasites (Van den Bossche, H., ed.), pp. 297-315, Academic Press, London.

11. Nagakura, K., Tachibana, H. and Kaneda, Y. (1985) Alteration of the cell surface acid phosphatase concomitant with the morphological transformation in Trypanosoma cruzi. Comp. Biochem. Physiol. 81B, 815-817.

12. Letelier, M., Repetto, Y., Aldunate, J. and Morello, A. (1985) Acid and alkaline phosphatase activity in Trypanosoma cruzi epimastigotes. Comp. Biochem. Physiol. 81B, 47-51.

13. Zlotnick, G.W., Wyman, C. and Gottlieb, M. (1985) Identification of the active subunits of the 3'-nucleotidase of Leishmania donovani. Fed. Proc. 44, 1332.

14. Gottlieb, M. (1985) Enzyme regulation in a trypanosomatid: effect of purine starvation on levels of 3'-nucleotidase activity. Science, 227, 72-74.

15. Gottlieb, M., Gardiner, P.R. and Dwyer, D.M. (1986) 3'-Nucleotidase activity in procyclic and bloodstream stages of Trypanosoma rhodesiense. Comp. Biochem. Physiol. 83B, 63-69.

16. Hassan, H.F. and coombs, G.H. (1986) Purine-metabolising enzymes in Entamoeba histolytica. Mol. Biochem. Parasitol. 19, 19-25.

17. DePierre, J-W. and Karnovsky, M.L. (1973) Plasma membranes of mammalian cells: a review of methods for their characterization and isolation. J. Cell Biol. 56, 275-303.

18. Pereira, N.M. and Konigk, E. (1981) A nucleotidase from Leishmania tropica promastigotes: partial purification and properties. Tropenmed. Parasitol. 32, 209-214.

19. Hassan, H.F. and Coombs G.H. (1985) Leishmania mexicana: purine metabolising enzymes of amastigotes and promastigotes. Exp. Parasitol. 59, 139-150.

20. Mallinson, D.J. and Coombs, G.H. (1986) Molecular characterization of the metacyclic forms of Leishmania. IRCS Med. Sci. 14, 557-558.

21. Hassan, H.F. and Coombs, G.H. (1986) A comparative study of the purine- and pyrimidine-metabolising enzymes of a range of trypanosomatids. Comp. Biochem. Physiol. 84B, 217-223.

22. Lockwood, B.C., North, M.J. and Coombs, G.H. (1984) Trichomonas vaginalis, Tritrichomonas foetus, and Trichomitus batrachorum: Comparative proteolytic activity. Exp. Parasitol. 58, 245-253.

23. Leloir, L.F. and Cardini, C.E. (1957) Characterization of phosphorus compounds by acid lability. Methods Enzymol. 3, 840-850.

24. Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 658-666.

25. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

26. Coombs, G.H., Tetley, L., Moss, V.A. and Vickerman, K. (1986) Three dimensional structure of the Leishmania amastigote as revealed by computer-aided reconstruction from serial sections. Parasitol. 22, 13-23.

27. Hassan, H.F., Mottram, J.C. and Coombs, G.H. (1985) Subcellular localisation of purine-metabolising enzymes in Leishmania mexicana mexicana. Comp. Biochem. Physiol. 81B, 1037-1040.

28. McLaughlin, J. (1982) Subcellular distribution of particle-associated antigens in Trypanosoma rhodesiense. J. Immunol. 128, 2656-2663.

29. Hassan, H.F., Phillips, R.S. and Coombs G.H. (1986) Purine-metabolising enzymes in Babesia divergens. Z. Parasitenkd. in press.

30. Hassan, H.F. and Coombs G.H. (1986) Purine salvage by Acanthamoeba castellanii. Int. J. Parasitol. in press.

31. Miller, R.L. and Lindstead, D. (1983) Purine and pyrimidine metabolising activities in Trichomonas vaginalis. Mol. Biochem. Parasitol. 7, 41-51.

32. Coombs, G.H., Hassan, H.F., Lockwood, B.C., Mallinson, D.J., North, M.J., Alunda, J.M. and Tetley, L. (1986) Phosphomono-esterases and proteinases of leishmanias. In: Host-Parasite Molecular Recognition and Interaction in Protozoal Infections (Chang, K-P. ed.) in press.

33. Dwyer, D.M. (1986) The roles of surface membrane enzymes and transporters in the survival of Leishmania. In: Host-Parasite Molecular Recognition and Interaction in Protozoal Infections (Chang, K-P. ed.) in press.

in press.

34. Katakura, K. (1986) Studies of infectivity of Leishmania donovani promastigotes: isolation, intracellular behaviour and biological characteristics of virulent lines. Jikeikai Med. J. 33, 157-173.

35. Das, S., Saha, A.K., Remaley, A.T., Glew, R.H., Dowling, J.N., Kajiyoshi, M. and Gottlieb, M. (1986) Hydrolysis of phosphoproteins and inositol phosphates cell surface phosphatase of Leishmania donovani. Mol. Biochem. Parasitol. 20, 143-153.

Chapter 7

A COMPARATIVE STUDY OF THE PURINE- AND PYRIMIDINE-METABOLISING ENZYMES OF A RANGE OF TRYPANOSOMATIDS

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Abstract 1. A range of trypanosomatids (amastigotes and cultured promastigotes of Leishmania mexicana mexicana, cultured promastigotes of L. m. amazonensis, L. donovani and L. tarentolae, culture forms of Crithidia fasciculta, Herpetomonas muscarum muscarum and H. m. ingenoplastis and procyclic trypomastigotes of Trypanosoma brucei brucei) have been surveyed for the presence of purine- and pyrimidine- metabolising enzymes.

2. Several common features were observed, including the presence of nucleosidases, catabolic phosphorylases, phosphoribosyltransferases, kinases and cytidine deaminase and the apparent absence of AMP deaminase, anabolic purine phosphorylase and cytosine deaminase.

3. Significant differences between species were discovered, notably in adenine and adenosine metabolism.

4. Nucleoside phosphotransferase active on inosine was detected in insect trypanosomatids but not in L. m. mexicana.

INTRODUCTION

There have been numerous studies on purine and pyrimidine metabolism in the Trypanosomatidae (see Hammond and Gutteridge, 1984). These have revealed many interesting and unusual features of this area of trypanosomatid metabolism, including a variety of enzymes (notably purine nucleoside phosphotransferase, EC 3.7.1.77; purine nucleosidase, EC 3.2.2.1; adenine deaminase, EC 3.5.4.2; xanthine phosphoribosyltransferase, EC 2.4.2.22; adenylosuccinate synthetase, EC 6.3.4.4; dihydroorotate oxidase, EC 1.3.3.1; orotate phosphoribosyltransferase, EC 2.4.2.10; and orotidylate decarboxylase, EC 4.1.1.23) that either do not have a mammalian equivalent or are very different from it and so provide the basis for the rational design of chemotherapeutic agents (Krenitsky et al. 1980; Marr and Berens, 1983; Wang, 1984). Although a great deal of information has been gathered already, there are still many deficiencies in our knowledge. Some aspects, for instance the enzymes involved in the interconversions of purine nucleotides, have been little investigated and even much of the data on the more studied areas are not comparable as they were obtained in a variety of laboratories using very different procedures (see Hammond and Gutteridge, 1984). The present study was undertaken with the aim of filling some of these gaps in our knowledge. Eight species, from four genera, of trypanosomatid have been surveyed for the presence of purine- and pyrimidine-metabolising enzymes so as to give an overview of the areas of similarity and differences. An additional aim was to determine whether any of the insect flagellates provided a good source of material for studying key enzymes (e.g. purine nucleoside phosphotransferase) that are present at only very low levels in the important mammalian parasites.

MATERIALS AND METHODS

Isolation, cultivation and fractionation of parasites

Amastigotes of L. m. mexicana (M379) were isolated from cutaneous lesions in female CBA mice (Zoology Department, Glasgow University, Glasgow G12 8QQ) as described by Hart et al. (1981). Promastigotes of L. m. mexicana (M379), L. m. amazonensis (LV 78) and L. donovani (LV9) were grown at 26°C in HOMEM medium (Berens et al. 1976) with 10% heat inactivated foetal calf serum as described previously (North and Coombs, 1981). The procyclic trypomastigote form of Trypanosoma brucei brucei (Stock EATRO 1125) was grown in the same medium as leishmanial promastigotes. Leishmania tarentolae, Crithidia fasciculata (Anopheles strain ATCC 11745), Herpetomonas muscarum muscarum (ATCC 30260) and H. m. ingenoplastis (ATCC 30269) were cultured as described previously (Hassan and Coombs, 1985a). Parasites were harvested by centrifugation at 3,000 X g at 4°C for 10 min and washed twice in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM dithiothreitol. Parasites were lysed in the above buffer by sonication involving two 15 sec periods, separated by a 30 sec cooling period, using a MSE Soniprep 150 fitted with an exponential microprobe at 4 amplitude microns. Homogenates were fractionated by centrifugation at 105,000 X g at 4°C for 1 h and the resultant supernatant was used as source of the enzyme in all cases except orotate phosphoribosyltransferase, for which crude homogenate was used. With amastigotes, the proteinase inhibitor leupeptin was added at 100 μg/ml to the parasite suspension before sonication.

Enzyme Assays

All enzyme activities except purine phosphoribosyltransferases and purine nucleoside phosphotransferase were assayed spectrophotometrically using 1cm light path cuvettes and a Perkin-

Elmer Lambda 5 uv/vis Spectrophotometer. The sample chamber was maintained at 26°C. Unless otherwise specified, assay mixtures were of a total volume of 1 ml and were preincubated at 26°C with the reaction being started by the addition of enzyme. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 nmol of substrate to product per minute at 26°C. All activities given are means (± SD) from at least 3 experiments. Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

a. Purine-metabolising enzymes. Adenine deaminase (EC 3.5.4.2), adenosine deaminase (EC 3.5.4.4), guanine deaminase (EC 3.5.4.3), AMP deaminase (EC 3.5.4.4), catabolic nucleosidase and phosphorylase (EC 3.2.2.1 and EC 2.4.2.1), anabolic phosphorylase (EC 2.4.2.1), nucleoside kinase (EC 2.7.1.20), adenylosuccinate lyase (EC 3.4.2.2), GMP reductase (EC 1.6.6.8), adenylosuccinate synthetase (EC 6.3.4.4) and GMP synthetase (EC 6.3.5.2) were all assayed as described previously (Hassan and Coombs, 1985b). Adenine phosphoribosyltransferase (PRTase) (EC 2.4.2.7); guanine-hypoxanthine PRTase (EC 2.4.2.8) and xanthine PRTase (EC 2.4.2.22) were assayed radiochemically as described by Hassan and Coombs (1985a). Purine nucleoside phosphotransferase (EC 2.7.1.77) was also assayed radiochemically as described by Hassan and Coombs (1986).

b. Pyrimidine-metabolising enzymes. The composition of the assay mixtures and the extinction coefficients used to calculate the enzyme activities are summarised in Table 1.

Materials

Chemicals were obtained from the sources listed previously (Hassan and Coombs, 1985a, 1985b).

Table 1. Spectrophotometric assay conditions of pyrimidine-metabolising enzymes

Enzyme	EC	Substrate	Final concentration (mM)	Other constituents of reaction mixture[a]	Product	nM($mM^{-1}cm^{-1}$)[b]
Cytidine deaminase	3.5.4.5	Cytidine	0.5		Uridine	286 (-2.5)
		2'-deoxycytidine	0.5		2'-deoxyuridine	286 (-2.8)
Cytosine deaminase	3.5.4.1	Cytosine	0.5		Uracil	286 (-1.1)
Nucleosidase or Phosphorylase (catabolic)[d]	3.2.2.8 or 2.4.2.3	(r/d)[c] Uridine	0.4		Uracil	282 (-1.6)
		Thymidine	0.4		Thymine	290 (-1.0)
		Ortidine	0.1		Orotate	265 (-5.4)
Phosphorylase (anabolic)	2.4.2.3	Uracil	0.4	1 mM R-1-P	Uridine	282 (1.6)
		Thymine	0.4	1 mM R-1-P	Thymidine	290 (1.0)
		Orotate	0.1	1 mM R-1-P	Orotidine	265 (5.4)
Phosphoribosyltransferase	2.4.2.10	Orotate	0.1	5 mM $MgSO_4$, 1 mM PRPP, 50 mM Tris-HCl, pH 7.8	OMP	295 (-3.95)

[a]The buffer used (except were otherwise indicated) was 50 mM Tris–HCl, pH 7.2.
[b]Wavelength monitored (extinction coefficient).
[c]r d = ribo/deoxyribo.
[d]In phosphorylase assays, 20 mM KH_2PO_4 was added.

RESULTS

The activities of purine-metabolising enzymes detected in the soluble fractions of the trypanosomatids investigated are given in Tables 2, 3 and 4. Adenine deaminase was present with high levels of activity in all species except for T. b. brucei, whilst adenosine deaminase was detected only in the two subspecies of L. mexicana and in T. b. brucei. Guanine deaminase was present in all species except H. m. ingenoplastis. The guanine deaminase activity declined sharply after approximately 5 min incubation in all cases. AMP deaminase was not detected in any of the organisms.

The purine ribonucleosides inosine, guanosine and xanthosine were cleaved to their respective bases (hypoxanthine, guanine and xanthine) by extracts of all species, whereas adenosine was cleaved only by T. b. brucei. To test the possibility that phosphorylase could play a part in the cleavage of nucleosides, the effect of the addition of phosphate (20 mM) on nucleoside hydrolysis was studied. It was found that all species exhibited phosphorylase activity towards inosine and guanosine whereas the activity towards xanthosine was not detected in T. b. brucei and H. m. ingenoplastis. The phosphate-dependent conversion of adenosine to adenine (adenosine phosphorylase) was detected only in extracts of L. donovani and T. b. brucei whereas only T. b. brucei converted purine bases to their corresponding ribonucleosides (anabolic phosphorylase) (Table 2).

Purine phosphoribosyltransferase (PRTase) activities toward adenine, guanine, hypoxanthine and xanthine were detected in all the species, albeit at a rather low level in some cases (Table 3). Trypanosoma b. brucei had the lowest adenine PRTase, guanine PRTase and xanthine PRTase activities of all the organisms. Guanine PRTase was generally the most active PRTase, and the ratio of guanine

Table 2. Activities of purine catabolic enzymes in trypanosomatids

Enzyme	Substrate	Leishmania m. mexicana[a]	Leishmania m. amazonensis	Leishmania donovani	Leishmania tarentolae	Crithidia fasciculata	Herpetomonas m. muscarum	Herpetomonas m. ingenoplastis	Trypanosoma b. brucei
Deaminase	Adenine	156 ± 8	63 ± 17	105 ± 9	55 ± 8	68 ± 8	69 ± 7	44 ± 8	nd < 0.7
	Adenosine	15 ± 1	3 ± 1	nd < 1	nd < 1	nd < 0.8	nd < 1	nd < 0.8	7 ± 2
	Guanine	60 ± 5	41 ± 6	10 ± 1	98 ± 13	59 ± 11	130 ± 9	nd < 1	4 ± 1
	AMP	nd < 2	nd < 0.8	nd < 1	nd < 0.8	nd < 0.6	nd < 0.8	nd < 0.6	nd < 1
Nucleosidase	Inosine	172 ± 9	42 ± 8	205 ± 10	55 ± 8	49 ± 6	108 ± 10	104 ± 6	74 ± 7
	Adenosine	nd < 2	nd < 2	nd < 3	nd < 2	nd < 1	nd < 2	nd < 2	20 ± 6
	Guanosine	101 ± 21	47 ± 17	59 ± 12	38 ± 8	33 ± 6	32 ± 9	12 ± 6	32 ± 2
	Xanthosine	52 ± 4	11 ± 6	156 ± 9	34 ± 8	91 ± 21	28 ± 8	79 ± 24	6 ± 2
Phosphorylase	Inosine	97 ± 45	53 ± 8	11 ± 3	30 ± 10	14 ± 2	78 ± 19	99 ± 12	8 ± 3
	Adenosine	nd < 5	nd < 2	10 ± 2	nd < 2	nd < 1	nd < 2	nd < 2	8 ± 2
	guanosine	17 ± 11	19 ± 4	21 ± 2	8 ± 2	26 ± 10	26 ± 7	8 ± 5	10 ± 4
	Xanthosine	34 ± 14	12 ± 5	23 ± 6	26 ± 14	40 ± 4	15 ± 2	nd < 2	nd < 1

The activities given are in nmol/min/mg protein and are the means ± SD from at least of three experiments. nd, not detectable with limit of detection given.

[a]Data from Hassan and Coombs, 1985b.

Table 3. Activities of purine anabolic enzymes in trypanosomatids

Enzyme	Substrate	Leishmania m. mexicana[a]	Leishmania m. amazonensis	Leishmania donovani	Leishmania tarentolae	Crithidia fasciculata	Herpetomonas m. muscarum	Herpetomonas m. ingenoplastis	Trypanosoma b. brucei
Phosphorylase	Adenine	nd < 3	nd < 2	nd < 2	nd < 2	nd < 1	nd < 2	nd < 1	3 ± 1
	Guanine	nd < 4	nd < 1	nd < 1	nd < 1	nd < 0.8	nd < 1	nd < 0.8	3 ± 1
	Hypoxanthine	nd < 13	nd < 1	nd < 2	nd < 1	nd < 0.9	nd < 1	nd < 0.9	nd < 1
	Xanthine	nd < 4	nd < 1	nd < 2	nd < 1	nd < 0.9	nd < 1	nd < 0.8	nd < 2
PRTase	Adenine	32 ± 2	25 ± 3	38 ± 4	19 ± 7	21 ± 5	22 ± 2	10 ± 1	2.0 ± 0.3
	Guanine	76 ± 6	40 ± 5	44 ± 7	29 ± 9	27 ± 6	9 ± 3	12 ± 2	0.8 ± 0.2
	Hypoxanthine	41 ± 6	20 ± 1	10 ± 4	9 ± 1	2 ± 1	0.8 ± 0.2	0.4 ± 0.1	5 ± 1
	Xanthine	25 ± 5	8 ± 3	6 ± 1	10 ± 5	5 ± 2	6 ± 1	11 ± 6	0.03 ± 0.01
Kinase	Adenosine	47 ± 6	10 ± 3	4 ± 1	18 ± 3	57 ± 15	17 ± 5	23 ± 6	1.0 ± 0.2
	Inosine	26 ± 4	10 ± 1	4 ± 1	17 ± 2	46 ± 18	16 ± 4	19 ± 7	1.0 ± 0.2
	Guanosine	27 ± 6	8 ± 2	3 ± 1	16 ± 7	48 ± 20	14 ± 6	13 ± 5	1.0 ± 0.2
	Xanthosine	24 ± 5	7 ± 3	2 ± 1	13 ± 4	38 ± 9	17 ± 3	11 ± 4	nd < 0.7
Phospho-transferase	Inosine	nd < 0.06	ND	ND	0.3 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	ND

The activities given are in nmol/min/mg protein and are the means ± SD from three experiments. nd, not detectable with limit of detection given.

ND, not determined.

[a]Data from Hassan and Coombs, 1985b.

PRTase to hypoxanthine PRTase ranged from 1.8:1 with L. m. mexicana to 30:1 with H. m. ingenoplastis. Hypoxanthine PRTase was 100 fold lower in activity in H. m. ingenoplastis than in L. m. mexicana.

The purine nucleoside kinase activities were rather similar for all substrates with each species. The level of activity, however, ranged from >50 nmol min^{-1} $(mg\ protein)^{-1}$ with C. fasciculata to approximately 1 nmol min^{-1} $(mg\ protein)^{-1}$ for T. b. brucei; with the latter organism, xanthosine kinase was not detectable. Purine nucleoside phosphotransferase towards inosine using p-nitrophenylphosphate as phosphate donor was present, but only at a low level, in extracts of C. fasciculata, L. tarentolae and Herpetomonas spp., whereas no activity was detected with L. m. mexicana.

Enzymes (adenylosuccinate lyase, adenylosuccinate synthetase, GMP synthetase and GMP reductase) which catalyse purine nucleotide interconversions were all detected in all species studied, although at relatively low activity in most cases (Table 4).

The activities detected of enzymes potentially involved in pyrimidine salvage are given in Table 5. Cytidine deaminase was detected in all species, whereas deoxycytidine deaminase was present only in L. m. mexicana cultured promastigotes and C. fasciculata. Cytosine deaminase was not detected in any organism. Uridine nucleosidase was present at relatively high level of activity in all trypanosomatids, whilst deoxyuridine nucleosidase was not found in L. mexicana subspecies and L. tarentolae. In contrast, nucleosidase acting on thymidine was detected only in L. m. mexicana cultured promastigotes and C. fasciculata, whereas orotidine nucleosidase was absent from all species. Catabolic phosphorylase activities were widespread, with activities towards deoxyuridine (L. donovani and T. b. brucei) and orotidine (T. b. brucei) being the only ones that were

Table 4. Activities of purine nucleotide interconversion enzymes in trypanosomatids

	Adenylosuccinate lyase	Adenylosuccinate synthetase	GMP synthetase	GMP reductase
Leishmania m. mexicana[a]	19 ± 1	2.0 ± 0.4	6 ± 1	2.0 ± 0.7
Leishmania m. amazonensis	12 ± 2	4 ± 1	3.0 ± 0.5	2.0 ± 0.5
Leishmania donovani	11 ± 4	5.0 ± 0.5	3 ± 1	8 ± 1
Leishmania tarentolae	9 ± 1	10 ± 4	3 ± 1	3 ± 1
Crithidia fasciculata	6 ± 2	5 ± 1	23 ± 3	0.9 ± 0.4
Herpetomonas m. muscarum	4 ± 1	12 ± 4	28 ± 12	1.0 ± 0.7
Herpetomonas m. ingenoplastis	6 ± 2	13 ± 6	6 ± 2	1.0 ± 0.5

The activities given are in nmol/min/mg protein and are the means ± SD from three experiments.
[a]Data from Hassan and Coombs, 1985b.

Table 5. Activities of pyrimidine metabolising enzymes in trypanosomatids

Enzyme	Substrate	Leishmania m. mexicana (amastigote)	Leishmania m. mexicana (promastigote)	Leishmania m. amazonensis	Leishmania donovani	Leishmania tarentolae	Crithidia fasciculata	Herpetomonas m.muscarum	Herpetomonas m. ingenoplastis	Trypanosoma b. brucei
Deaminase	Cytidine	5 ± 1	26 ± 7	6 ± 2	11 ± 6	8 ± 4	15 ± 4	4 ± 1	20 ± 7	4 ± 2
	2'-deoxycytidine	nd < 1	14 ± 6	nd < 0.5	nd < 1	nd < 2	13 ± 6	nd < 1	nd < 0.7	nd < 2
	Cytosine	nd < 4	nd < 1	nd < 1	nd < 1	nd < 2	nd < 1	nd < 1	nd < 0.7	nd < 2
Nucleosidase	Uridine	9 ± 2	52 ± 7	21 ± 5	42 ± 2	44 ± 14	143 ± 47	25 ± 10	108 ± 10	7 ± 2
	2'-deoxyuridine	nd < 3	nd < 6	nd < 1	4 ± 2	nd < 1	65 ± 28	19 ± 8	14 ± 6	4 ± 1
	Thymidine	nd < 5	13 ± 1	nd < 1	nd < 2	nd < 2	39 ± 16	nd < 2	nd < 1	nd < 1
	Orotidine	nd < 1	nd < 3	nd < 0.2	nd < 0.3	nd < 0.3	nd < 0.3	nd < 0.2	nd < 0.3	nd < 0.5
Phosphorylase (catabolic)	Uridine	2 ± 1	13 ± 2	15 ± 5	14 ± 4	26 ± 9	120 ± 34	67 ± 18	61 ± 4	11 ± 5
	2'-deoxyuridine	2 ± 1	9 ± 1	8 ± 5	nd < 1	21 ± 5	176 ± 27	96 ± 30	21 ± 9	nd < 2
	Thymidine	1 ± 1	8 ± 1	3 ± 1	8 ± 4	7 ± 4	92 ± 12	26 ± 8	38 ± 16	2 ± 1
	Orotidine	1 ± 0.5	4 ± 2	6 ± 2	5 ± 2	9 ± 2	7 ± 1	7 ± 4	7 ± 4	nd < 0.5
Phosphorylase (anabolic)	Uracil	4 ± 2	4 ± 2	6 ± 3	nd < 1	nd < 1	12 ± 3	18 ± 2	13 ± 6	6 ± 2
	Thymine	nd < 2	nd < 2	nd < 3	nd < 2	nd < 2	18 ± 2	14 ± 5	16 ± 8	6 ± 1
	Orotate	nd < 0.2	nd < 0.2	nd < 0.2	nd < 0.2	nd < 0.2	nd < 0.2	nd < 0.4	nd < 0.1	nd < 0.4
PRTase	Orotate	3 ± 2	67 ± 14	74 ± 15	90 ± 23	45 ± 11	80 ± 15	33 ± 19	44 ± 16	2 ± 1

The activities given are in nmol/min/mg protein and are the means ± SD from three experiments. nd, not detectable with the limit of detection given.

not detectable. Notably high nucleosidase and phosphorylase activities towards uridine, deoxyuridine and thymidine were found in C. fasciculata with the herpetomonads also possessing relatively high levels of most of these activities. All trypanosomatids investigated except L. donovani and L. tarentolae were able to convert uracil to uridine in the presence of ribose-1-phosphate, suggesting that their uridine phosphorylase activities are reversible. Thymine was also converted to thymidine by T. b. brucei, C. fasciculata and Herpetomonas spp. Comparatively high orotate PRTase activity was detected in homogenates of most of the organisms, although only low activity was detected in L. m. mexicana amastigotes and T. b. brucei.

DISCUSSION

The results obtained in this study can only be used as a guideline to the levels of enzyme activity present in the trypanosomatids as it was not confirmed that all assays were carried out under optimal conditions of, for instance, pH. Nevertheless, this is the first general survey of the purine- and pyrimidine-metabolising enzymes in this range of trypanosomatids and various conclusions can be made. The study has revealed that there are significant differences between species with respect to adenine and adenosine metabolism. These are summarised schematically in Fig. 1. The distinct differences between species in the content of adenine deaminase, adenosine deaminase and adenosine phosphorylase could form the basis of a means of species identification. The finding of adenosine deaminase in T. b. brucei is in agreement with the results of Davies et al. (1983) but not with Ogbunude and Ikediobi (1983). On the other hand, although the apparent lack of adenosine deaminase in L. donovani is in agreement with previous observations (Looker et al., 1983), Davies et al. (1983) reported that L. m. amazonensis also

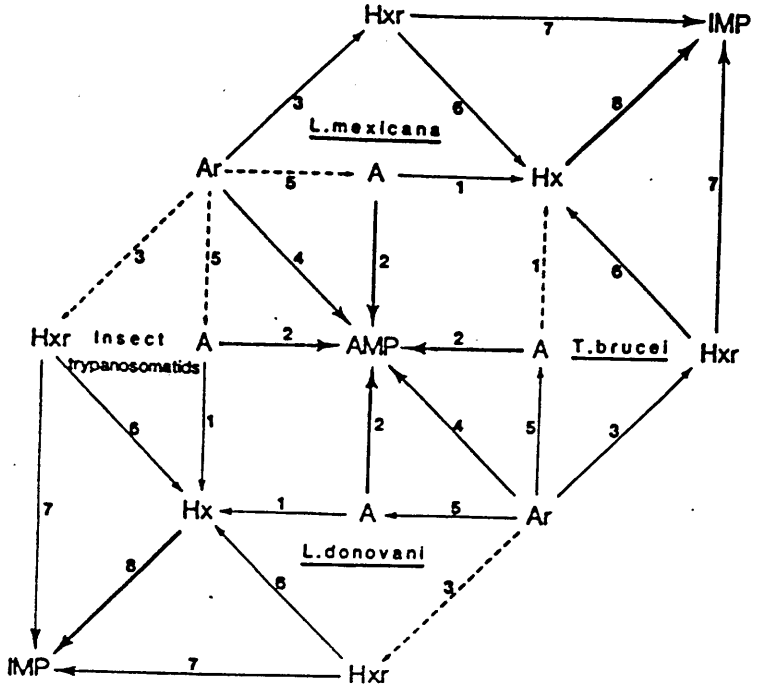

Fig. 1. Purine metabolism in trypanosomatids. (→) pathways present; (---→) pathways not detectable. Key to enzymes: 1, adenine deaminase; 2, adenine phosphoribosyltransferase; 3, adenosine deaminase; 4, adenosine kinase; 5, adenosine phosphorylase; 6, inosine nucleosidase; 7. inosine kinase; 8, hypoxanthine phosphoribosyltransferase. Abbreviations: A, adenine; Ar, adenosine; AMP, adenosine 5′-monophosphate; Hx, hypoxanthine; Hxr, inosine; IMP, inosine 5′-monophosphate.

lacked the enzyme but contained adenine deaminase. In our study, however, we found that L. m. amazonensis possessed both activities, similarly to L. m. mexicana. It is possible that these differences in enzyme activity in the same subspecies could be due to the use of different strains, growth conditions or analytical methodology. We have reported previously that the stage-specific adenosine metabolism found in L. donovani, with cultured promastigotes containing adenine deaminase and amastigotes adenosine deaminase (Konigk and Putfarken, 1980; Looker et al., 1983), apparently does not exist in L. m. mexicana (Hassan and Coombs, 1985b). The present results suggest that in this respect L. m. amazonensis may be similar to L. m. mexicana, although we must await the results of a study of amastigote enzymes for confirmation of this. The presence of highly active adenine deaminase in C. fasciculata is consistent with previous observations (Kidder et al., 1977; Kidder and Nolan, 1979) and it is interesting that the enzyme also occurs in the two herpetomonads.

The data obtained in the present study suggest that all trypanosomatids possess nucleosidases active towards inosine, guanosine and xanthosine. Such activities have been observed previously in extracts of L. donovani (Koszalka and Krenitsky, 1979), L. tropica (Konigk, 1978), C. fasciculata (Dewey and Kidder, 1973), T. gambiense (Schmidt et al., 1975) and T. cruzi (Miller et al., 1984). Catabolic phosphorylase towards inosine, guanosine and xanthosine also are a common feature, although rather surprisingly they have been reported previously for only one flagellate, Trichomonas vaginalis (Miller and Linstead, 1983). The existence of adenosine phosphorylase in extracts of T. b. brucei and L. donovani relate well with the results of Davies et al. (1983) for T. brucei, Krenitsky et al. (1980) for L. donovani and Konigk (1978) for L.

tropica. In the absence of anabolic phosphorylase (except T. b. brucei), the only pathways for the conversion of purine bases to nucleotides were expected to be phosphoribosyltransferases. The confirmation of the presence of PRTase activities towards all four purine bases in all trypanosomatids tested shows that it is these enzymes that enable the protozoa to salvage purine bases and to channel them as nucleotide components into metabolic processes. The specific activities of the PRTases reported in this study are quite similar to the levels reported by Looker et al. (1983) for L. donovani and the previous work with C. fasciculata (Kidder et al., 1979) and T. brucei (Davies et al., 1983).

The levels of nucleoside kinase activities detected in the trypanosomatids suggests that the nucleosides produced during nucleotide degradation can be used to regenerate nucleotides which can then be further phosphorylated. It is also possible that the nucleoside kinases could enable nucleoside analogues to enter the pathway of purine nucleotide synthesis as has been demonstrated for tubercidin with the purified adenosine kinase of Entamoeba histolytica (Lobelle-Rich and Reeves, 1983) and with ethylthio- and cinnamylthio-pyrazolo(3,4-d) pyrimidine ribonucleosides in Eimeria tenella and E. brunetti (Miller et al., 1982). Adenosine kinase activity for L. donovani found in this study was eight fold higher than that reported previously for the same species (Looker et al., 1983). With p-nitrophenylphosphate as the phosphate donor, phosphorylation of inosine could be detected only in the insect trypanosomatids. Purine nucleoside phosphotransferase is known to be present in leishmanias, but whereas allopurinol riboside and formycin B are good substrates, nucleosides are not (Krenitsky et al., 1980). We are at present investigating the substrate specificities of crithidial and herpetomonad phosphotransferase; it is possible that

they could be good model enzymes to study.

Enzyme measurements using supernatant fractions from trypanosomatids have shown that all the species studied have enzymes for the conversion of GMP and IMP to AMP. Of these enzymes, adenylosuccinate lyase, adenylosuccinate synthetase (Spector et al, 1979) and GMP reductase (Spector and Jones, 1982) have been purified from L. donovani promastigotes and suggested as targets for chemotherapeutic attack of this human pathogen. If the enzymes are similar in all trypanosomatids, a drug acting on one of them should have a broad spectrum of activity.

The data presented in this study show clearly that all the species contain an enzyme which can deaminate cytidine to uridine. The levels of activity reported here are similar to those reported for C. fasciculata and T. cruzi (Kidder, 1984) but is lower than those reported for T. vaginalis (Wang and Cheng, 1984) and Giardia lamblia (Aldritt et al., 1985). Nevertheless, cytidine deaminase seems likely to be a primary means of supplying uridine in these organisms. Our results demonstrate that the uridine utilisation is likely to involve conversion to uracil via uridine nucleosidase with the uracil being converted to UMP by uracil PRTase. The latter enzyme has been detected in members of the kinetoplastida (Hammond and Gutteridge, 1982). No attempts were made in the present work to study pyrimidine nucleoside kinases, but uridine kinase has been undetectable in all kinetoplastida studied previously (Hammond and Gutteridge, 1982). Thymidine kinase was isolated from T. b. rhodesiense (Chello and Jaffe, 1972) whereas L. donovani was found to contain thymidine phosphotransferase instead (Krenitsky et al., 1980). With the exception of L. m. mexicana promastigotes and C. fasciculata, the conversion of thymidine to thymine required

inorganic phosphate indicating that the enzyme involved was a phosphorylase rather than nucleosidase, as was demonstrated previously for a number of trypanosomatids (Al Chalabi and Gutteridge, 1977) and Tritrichomonas foetus (Jarrel et al., 1983). The level of activity found for orotate PRTase are in agreement with the previous reports for L. m. mexicana (Gero and Coombs, 1980), T. brucei (Hammond and Gutteridge, 1982) and C. fasciculata (Hill et al., 1981). It is particularly intriguing that the promastigotes of L. m. mexicana contained 20 times more activity than amastigotes.

This study has revealed the common features of purine and pyrimidine metabolism in trypanosomatids, as well as where there is species variation. It therefore opens the way for more detailed investigations of key trypanosomatid enzymes to help in the elucidation of the special adaptations of individual species and stages and to establish which enzymes provide good targets for chemotherapeutic attack.

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REFERENCES

Al Chalabi K. and Gutteridge W.E. (1977) Catabolism of deoxythymidylate in some trypanosomatids. Parasit. 74, 299-312.

Aldritt S.M., Tien P. and Wang C.C. (1985) Pyrimidine salvage in Giardia lamblia. J. Exp. Med. 161, 437-445.

Berens R.L., Brun R. and Krassner S.A. (1976) A simple monophasic medium for axenic culture of hemoflagellates. J. Parasit. 62, 360-365.

Chello P.L. and Jaffe J.J. (1972) Comparative properties of trypanosomal and mammalian thymidine kinase. Comp. Biochem. Physiol. 43B, 543-562.

Davies M.J., Ross A.M. and Gutteridge W.E. (1983) The enzymes of purine salvage in Trypanosoma cruzi, Trypanosoma brucei and Leishmania mexicana. Parasit. 87, 211-217.

Dewey V.C. and Kidder G.W. (1973) Partial purification and properties of a nucleoside hydrolase from Crithidia. Arch. Biochem. Biophys. 157, 380-387.

Gero A.M. and Coombs G.H. (1980) Orotate phosphoribosyltransferase and orotidine 5-phosphate decarboxylase in two parasitic kinetoplastid flagellates FEBS letters, 118, 130-132.

Hammond D.J. and Gutteridge W.E. (1982) UMP synthesis in the kinetoplastida. Biochem. Biophys. Acta 718, 1-10.

Hammond D.J. and Gutteridge W.E. (1984) Purine and pyrimidine metabolism in the Trypanosomatidae. Mol. Biochem. Parasit. 13, 243-261.

Hart D.T., Vickerman K. and Coombs G.H. (1981) A quick, simple method for purifying Leishmania mexicana amastigotes in large numbers. Parasit. 82, 345-355.

Hassan H.F. and Coombs G.H. (1985a) Purine phosphoribosyltransferases of Leishmania mexicana mexicana and other flagellate protozoa. Comp. Biochem. Physiol., 82B,773-779.

Hassan H.F. and Coombs G.H. (1985b) Leishmania mexicana: Purine metabolizing enzymes of amastigotes and promastigotes. Exp. Parasitol. 59, 139-150.

Hassan H.F. and Coombs G.H. (1986) Purine metabolizing enzymes in Entamoeba histolytica. Molec. Biochem. Parasitol, 19, 19-25.

Hill B., Kilsby J., Rogerson G.W., McIntosh R.T. and Ginger C.D. (1981). The enzymes of pyrimidine biosynthesis in a range of parasitic protozoa and helminths. Mol. Biochem. Parasit. 2, 123-134.

Jarrol E.L., Lindmark D.G. and Paolella P. (1983) Pyrimidine metabolism in Tritrichomonas foetus. J. Parasit. 69, 846-849.

Kidder G.W. (1984) Characteristics of cytidine aminohydrolase activity in Trypanosoma cruzi and Crithidia fasciculata. J. Protozool. 31, 298-300.

Kidder G.W., Dewey V.C. and Nolan L.L. (1977) Adenine deaminase of a eukaryotic animal cell, Crithidia fasciculata. Arch. Biochem. Biophys. 183, 7-12.

Kidder G.W. and Nolan L.L. (1979) Adenine aminohydrolase: Occurrence and possible significance in trypanosomid flagellates. Proc. Nat. Acad. Sci. USA 76, 3670-3672.

Kidder G.W., Nolan L.L. and Dewey V.C. (1979) The purine phosphoribosyltransferases of Crithidia fasciculata. J. Parasit. 65, 520-525.

Konigk E. (1978) Purine nucleotide metabolism in promastigotes of Leishmania tropica: Inhibitory effect of allopurinol and analogues of purine nucleosides. Tropenmed. Parasit. 29, 435-438.

Konigk E. and Putfarken B. (1980) Stage specific differences of a perhaps signal-transferring system in Leishmania donovani. Tropenmed. Parasit. 31, 421-424.

Koszalka G.W. and Krenitsky T.A. (1979) Nucleosidases from Leishmania donovani. J. biol. Chem. 254, 8185-8193.

Krenitsky T.A., Koszalka G.W., Tuttle J.V., Adamczyk D.L., Elion G.E. and Marr J.J. (1980) Purine salvage enzymes in man and Leishmania donovani. In Purine Metabolism in Man (Rapado A. and Watts R.W.E., eds.), Part III, pp. 51-56, Plenum Publishing Corp., New York.

Lobelle-Rich P.A. and Reeves R.E. (1983) The partial purification and characterization of adenosine kinase from Entamoeba histolytica. Am. J. Trop. Med. Hyg. 32, 976-979.

Looker D.L., Berens R.L. and Marr J.J. (1983) Purine metabolism in Leishmania donovani amastigotes and promastigotes. Mol. Biochem. Parasit. 9, 15-28.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) Protein measurement with the folin phenol reagent. J. biol. Chem. 193, 265-275.

Marr J.J. and Berens R.L. (1983) Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Mol. Biochem. Parasit. 7 339-356.

Miller R.L., Adamczyk D.L., Rideout J.L. and Krenitsky T.A. (1982) Purification, characterization, substrate and inhibitor specificity of adenosine kinase from several Eimeria species. Mol. Biochem. Parasit. 6, 209-223.

Miller R.L. and Linstead D. (1983) Purine and pyrimidine metabolizing activities in Trichomonas vaginalis extracts. Mol. Biochem. Parasit. 7, 41-51.

Miller R.L., Sabourin C.L.K. and Krenitsky T.A. (1984) Nucleoside hydrolase from Trypanosoma cruzi. J. biol. Chem. 259, 5073-5077.

North M.J. and Coombs G.H. (1981) Proteinases of Leishmania mexicana amastigotes and promastigotes: Analysis by gel electrophoresis. Mol. Biochem. Parasit. 3, 293-300.

Ogbunude P.O. and Ikediobi C.O. (1983) Comparative aspects of purine metabolism in some African trypanosomes. Mol. Biochem. Parasit. 9, 279-287.

Schmidt G., Walter R.D. and Konigk E. (1975) A purine nucleoside hydrolase from Trypanosoma gambiense, purification and properties. Tropenmed. Parasit. 26, 19-26.

Spector T. and Jones T.E. (1982) Guanosine 5-monophosphate reductase from Leishmania donovani. A possible chemotherapeutic target. Biochem. Pharm. 31, 3891-2897.

Spector T., Jones T.E. and Elion G.B. (1979) Specificity of adenylosuccinate synthetase and adenylosuccinate lyase from Leishmania donovani. Selective amination of antiprotozoal agent. J. biol. Chem. 254, 8422-8426.

Wang C.C. (1984) Parasite enzymes as potential targets for antiparasitic chemotherapy. J. Med. Chem. 27, 1-9.

Wang C.C. and Cheng H.W. (1984) Salvage of pyrimidine nucleosides by Trichomonas vaginalis. Mol. Biochem. Parasit. 10, 171-184.

Chapter 8

PURINE METABOLISING ENZYMES IN *ENTAMOEBA HISTOLYTICA*

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SUMMARY

The enzymes that catalyse the salvage of purines in Entamoeba histolytica trophozoites have been surveyed. Adenine deaminase (EC 3.5.4.2), adenosine deaminase (EC 3.5.4.4), guanine deaminase (EC 3.5.4.3), adenine phosphoribosyltransferase (PRTase) (EC 2.4.2.7), xanthine PRTase (EC 2.4.2.22) and hypoxanthine PRTase (EC 2.4.2.8) were all detected in cell homogenates but only at low activities, whereas AMP deaminase (EC 3.5.4.6) and guanine PRTase (EC 2.4.2.8) were not found. Phosphorylases (EC 2.4.2.1) active in both anabolic and catabolic directions were present and all nucleosides tested were phosphorylated by kinases (EC 2.7.1.15, EC 2.7.1.20, EC 2.7.1.73). 3'-Nuclectidase (EC 3.1.3.6) and 5'-nucleotidase (EC 3.1.3.5) were found, the former being mainly particulate. Nucleotide interconversion enzymes (adenylosuccinate lyase, EC 4.3.2.2; adenylosuccinate synthetase, EC 6.3.4.4; IMP dehydrogenase, EC 1.2.1.14; GMP synthetase, EC 6.3.5.2 and GMP reductase, EC 1.6.6.8) were not detected. The results suggest that in E. histolytica the main route of nucleotide synthesis is from the individual bases through the actions of phosphorylases and kinases.

Key Words: Entamoeba histolytica; Metabolism; Enzymes; Purines.

INTRODUCTION

Studies reported by Boonlayangoor et al (1) have shown that Entamoeba histolytica lacks the ability to synthesize purine de novo and therefore must rely on purines from its host. These purines enter the amoeba via some form of carrier-mediated transport system (2) and then are converted into nucleotides and eventually nucleic acid (3). As yet, however, there has been no survey of the purine salvage enzymes that occur in this parasite. The only report

concerns adenosine kinase; the enzyme was partially purified and its substrate specificity characterized (4).

The differences in purine metabolism between Entamoeba and man, where both de novo synthesis and salvage pathways are significant, mean that it should be possible to design purine analogues that are selectively toxic to this important human pathogen (5-7). The present study was undertaken to establish which are the main routes of purine salvage in E. histolytica and which enzymes offer the most promise as targets for chemotherapeutic attack.

MATERIALS AND METHODS

Materials (2-^{3}H) Adenine, (2-^{3}H) adenosine, (G-^{3}H) hypoxanthine, (6-^{14}C) xanthine, (8-^{14}C) inosine and (8-^{14}C) IMP were obtained from Amersham International, England. (8-^{14}C) Guanine was purchased from New England Nuclear, West Germany. In all cases, purity was $\geq$ 97%. All other chemicals were obtained from Sigma Chemical Co. Ltd., Dorset, Poole, England. PEI cellulose F precoated sheets (Merck) were obtained from BDH, Glasgow, Scotland.

Parasite growth and breakage. Entamoeba histolytica (strain 200:NIH) was grown at 37°C in TYI-S-33 medium (8) with 10% (v/v) heat inactivated horse serum, 100 U sodium benzylpenicillin ml^{-1} and 100 µg streptomycin sulphate ml^{-1}. Cultures at late log phase of growth were chilled in an ice bath for 5 min and the detached parasites subsequently harvested by centrifugation at 500 x g for 5 min. Parasites were suspended to a density of 1-5 x 10^{6} cells ml^{-1} in 50 mM Tris, pH 7.0, containing 0.9% (w/v) NaCl and lysed by sonication for two periods of 10 s, separated by a 10 s cooling period, using a MSE Soniprep 150 fitted with an exponential probe at 4 µ amplitude.

The crude homogenates produced were used as source of the enzyme in all experiments except where specified. For some experiments, crude homogenates were fractionated by centrifugation at 105,000 x g for 1 h at 4°C and the resultant pellets were resuspended in the above buffer to the volume of the supernatant fraction.

Analytical methods.

Radiochemical assays. All assays were carried out at 37°C. All assay mixtures, unless otherwise specified, had a final volume of 100 µl and contained an amount of extract sufficient to catalyze the reaction at a linear rate for the period of the incubation. Linearity was confirmed by taking samples at different time points. Reactions were initiated by the addition of substrate to the preincubated (3 min at 37°C) reaction mixture and were terminated by immersion in boiling water for 3 min. The precipitated protein was removed by centrifugation at 2100 x g for 5 min, and aliquots (10 µl) of the supernatant were spotted on to PEI cellulose F sheets together with 10 nmol of both substrate and product as carriers and the reactants separated by ascending chromatography. The carrier spots were visualised with ultraviolet light, cut out and counted for the radioactivity as previously described (9). The radioactivity recovered in substrate and product spots usually accounted for >96% of that originally applied. One unit of enzyme activity was defined as the amount of enzyme that catalyses the conversion of one nmol of substrate to product per min. The following standard assay conditions were used, unless otherwise specified.

Adenine deaminase (EC 3.5.4.2), adenosine deaminase (EC 3.5.4.4) and guanine deaminase (EC 3.5.4.3):50 mM PIPES, pH 6.8; 0.2 mM (2-^{3}H) adenine (50 mCi mmol^{-1})or (2-^{3}H) adenosine (50 mCi mmol^{-1}) or (8-^{14}C) guanine (5 mCi mmol^{-1}). The incubation was for 1 h. The solvents

used for chromatography were 0.1M potassium phosphate, pH 6.8/saturated ammonium sulphate/n-propanol (100/60/2, v/v) for adenine deaminase and adenosine deaminase (R_F values: adenine, 0.16; hypoxanthine, 0.37; adenosine, 0.26; inosine, 0.55) and butyl alcohol/methyl ethyl ketone/ water/formic acid (44/44/10/0.26, v/v) for guanine deaminase (R_F values: guanine, 0.13; xanthine, 0.33).

Phosphorylase, anabolic direction (EC 2.4.2.1): 50 mM PIPES, pH 6.4; 1 mM ribose-1-phosphate; 0.2 mM (2-^{3}H) adenine (50 mCi mmol^{-1}) or (G-^{3}H) hypoxanthine (850 mCi mmol^{-1}) or (8-^{14}C) guanine (5 mCi mmol^{-1}) or (6-^{14}C) xanthine (18 mCi mmol^{-1}). The incubation was for 10 min and 0.1 M LiCl was used as chromatography solvent (R_F values: adenine, 0.26; adenosine, 0.40; hypoxanthine, 0.42; inosine, 0.63; guanine, 0.24; guanosine, 0.46; xanthine, 0.26; xanthosine, 0.51).

Nucleosidase (EC 3.2.2.1) and phosphorylase, catabolic direction (EC 2.4.2.1): 50 mM PIPES, pH 6.8; 0.2 mM (2-^{3}H) adenosine (50 mCi mmol^{-1}) or (8-^{14}C) inosine (2.5 mCi mmol^{-1}); 20 mM KH_2PO_4 (phosphorylase assay only). The incubation period was 10 min and 0.1 M LiCl was used as the chromatography solvent.

Adenine phosphoribosyltransferase (APRTase) (EC 2.4.2.7), hypoxanthine-guanine PRTase (EC.2.4.2.8) and xanthine PRTase (EC 2.4.2.22):50 mM PIPES, pH 6.8; 5 mM $MgCl_2$; 1 mM phosphoribosyl-1-pyrophosphate and 0.2 mM of radiolabelled purine bases (specific activities as above). The incubation was for 1 h and distilled, deionised water was the chromatography solvent (R_F values: adenine, 0.28; AMP, 0.0; guanine, 0.24; GMP, 0.0; hypoxanthine, 0.45; IMP, 0.0; xanthine, 0.26; XMP, 0.0).

Phosphotransferase (EC 2.7.1.77): 0.1M sodium acetate, pH 5.4; 100 mM p-nitrophenylphosphate; 1 mM (8-^{14}C) inosine (2.5 mCi mmol^{-1}). The incubation was for 1 h and distilled, deionised water was used as

chromatography solvent (R_F values: inosine, 0.7; IMP, 0.0).

IMP dehydrogenase (EC 1.2.1.14): 50 mM PIPES, pH 6.8; 100 mM KCl; 0.3 mM NAD; 0.2 mM (8 -^{14}C) IMP (2.5 mCi mmol^{-1}). Incubation was for 1 h. Chromatography was carried out in two stages, using firstly 0.5 M sodium formate, pH 3.4, after which the chromatogram was dried, and secondly 50% (v/v) methanol in water. The R_F values (relative to formate) were: IMP, 0.5; XMP, 0.37.

Spectrophotometric assays AMP deaminase (EC 3.5.4.6), nucleosidase (EC 3.2.2.1) and phosphorylase (EC 2.4.2.1) with guanosine and xanthosine as substrates, adenylosuccinate lyase (EC 4.3.2.2), GMP reductase (EC 1.6.6.8), adenylosuccinate synthetase (EC 6.3.4.4) and GMP synthetase (EC 6.3.5.2) were assayed at 37°C as described previously (10). Nucleoside kinase (EC 2.7.1.20) and adenylate kinase (EC 2.7.4.3) were assayed at 37°C as described by Lobelle-Rich and Reeves (4).

3'-AMP nucleotidase (EC 3.1.3.6) and 5'-AMP nucleotidase (EC 3.1.3.5) were assayed in reaction mixtures (0.5 ml) containing, respectively: 50 mM Tris-maleate, pH 7.5, 5 mM $MgCl_2$ and 5 mM 3'-AMP; 50 mM sodium acetate, pH 6.0, 5 mM $MgCl_2$ and 5 mM 5'-AMP. After incubation at 37°C for 30 min, the reactions were terminated by addition of one ml of 8% (w/v) trichloroacetic acid. The precipitated protein was centrifuged at 2100 x g for 5 min and aliquots of the supernatant were used to determine the released phosphate concentration according to the Fiske-Subbarow method (11).

Acid phosphatase (EC 3.1.3.2) was assayed in a reaction mixture (0.25 ml) containing 50 mM sodium acetate, pH 5.0, 5 mM $MgCl_2$ and 5 mM p-nitrophenylphosphate. After incubation for 30 min at 37°C, 1 ml of 0.1 N NaOH was added and the absorbance at 405 nm was measured.

The apparent K_m values for some substrates were determined by the Lineweaver Burk method (12). In the inhibitor studies, the extract was preincubated at 37°C with the inhibitor for 10 min and the assay were carried out as described above. Inhibition is expressed as the percent reduction in activity compared with the activity under standard conditions. Protein concentrations were estimated by the method of Lowry et al. (13) with bovine serum albumin as standard.

RESULTS

The activities of the various purine anabolic and catabolic enzymes detected in crude homogenates of E. histolytica are given in Table 1. Adenine deaminase, adenosine deaminase and guanine deaminase were present at only low levels, whereas AMP deaminase was below the level of detection. The amoebal extracts catalysed the conversion of nucleosides to their respective bases. These conversions (nucleosidase activity in Table 1) could be due to either nucleosidases or phosphorylases; the latter enzyme activities would be dependent upon endogenous phosphate. The presence of phosphorylases was confirmed by the finding that the conversions were much more rapid after the addition of 20 mM inorganic phosphate to the reaction mixture. The phosphorylase activities given in Table 1 represent the increase in activity upon addition of inorganic phosphate. There were significant differences in the pH profile, K_m values and inhibitor sensitivity of the phosphorylase activities towards inosine and adenosine. Maximum activity was at pH 6.8 and 7.4, respectively, (Fig. 1) and the apparent K_m values for adenosine and inosine were 9.5×10^{-5}M and 3.3×10^{-4}M, respectively (Fig. 1). The effects of various purine analogues on the phosphorylase activities are given in Table 2. The adenosine analogues formycin A,

TABLE I

The activities of purine metabolising enzymes in *E. histolytica*

Substrate	Deaminase	Nucleosidase	Phospho-rylase (catabolic)	Phospho-rylase (anabolic)	PRTase	Kinase	Nucleotidase
Adenine	0.15 ± 0.03			16 ± 2	0.09 ± 0.04		
Adenosine	0.20 ± 0.07	0.3 ± 0.1	4 ± 1			4 ± 2	
5′-AMP	nd < 0.08					18 ± 3	20 ± 3
3′-AMP							9 ± 2
Hypoxanthine				10 ± 3	0.11 ± 0.02		
Inosine		2 ± 1	16 ± 3			4 ± 1	
5′-IMP						5 ± 1	2 ± 1
Guanine	0.4 ± 0.1			25 ± 6	nd < 0.01		
Guanosine		3 ± 1	9 ± 2			3 ± 1	
5′-GMP						7 ± 2	7 ± 3
Xanthine				4 ± 1	0.09 ± 0.01		
Xanthosine		4 ± 1	12 ± 4			5 ± 1	
5′-XMP						6 ± 3	nd < 1

The activities are given in nmol min^{-1} (mg protein)$^{-1}$ and are the means ± S.D. from at least three experiments. nd: not detectable.

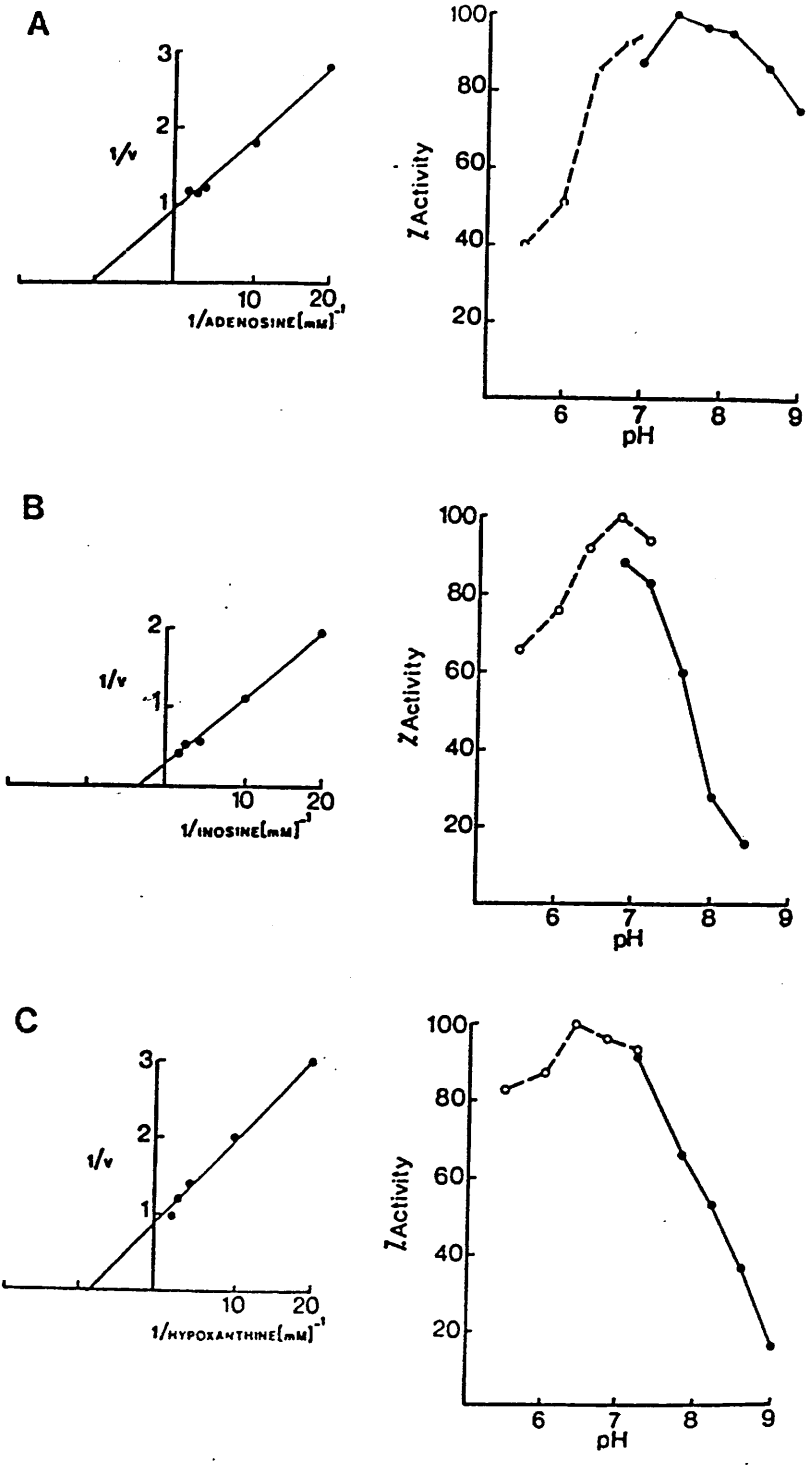

Fig. 1. Lineweaver–Burk plots and pH profiles for *E. histolytica* phosphorylases. (A) Adenosine phosphorylase; (B) inosine phosphorylase; (C) hypoxanthine phosphorylase. The buffers used (both 50 mM) were PIPES (o) and Tris-HCl (•).

TABLE II

The effect of inhibitors on *E. histolytica* adenosine phosphorylase and inosine phosphorylase activities

Inhibitor	% Inhibition[a]	
	Adenosine phosphorylase	Inosine phosphorylase
4-Amino-5-imidazolecarboxamide riboside	20 ± 5	34 ± 12
6-Mercaptopurine riboside	67 ± 12	64 ± 15
6-Mercaptopurine arabinoside	82 ± 6	71 ± 8
2-Amino-6-mercaptopurine riboside	62 ± 24	75 ± 10
Tubercidin	25 ± 2	90 ± 1
Formycin B	21 ± 4	89 ± 6
Formycin A	92 ± 4	18 ± 8
Cordycepin (3′-deoxyadenosine)	86 ± 4	30 ± 14
Adenine-9-β-D-arabinofuranoside (Ara-A)	90 ± 5	14 ± 6
4-Aminopyrazolo (3,4-d)-pyrimidine 2′-deoxyriboside	52 ± 6	47 ± 15

[a] The figures given are the % inhibition produced by the compounds at 1 mM and are the means ± S.D. from three experiments.

cordycepin and Ara A all markedly inhibited the phosphorolysis of adenosine, while the nucleoside analogues formycin B and tubercidin had similarly great effects on the phosphorolysis of inosine. The data indicate that E. histolytica probably possesses two distinct phosphorylases, one specific for adenosine and other active on inosine.

All purine bases tested were converted at a high rate to their respective nucleosides in the presence of ribose-1-phosphate. It was confirmed by using a second separation system (that used for the adenine deaminase assay) that adenine was indeed being phosphorylated to adenosine and that under these assay conditions it was deaminated to hypoxanthine at only a very low rate. The pH optimum for anabolic phosphorylase with hypoxanthine as substrate was found to be at 6.4 and the apparent K_m for hypoxanthine was 1.1×10^{-4} M (Fig. 1). Anabolic phosphorylase was very largely (94 ± 8%) recovered in the soluble fraction of the cell, the residual activity probably being trapped in the pellet rather than membrane-bound.

Purine PRTase activities toward adenine, hypoxanthine and xanthine were detected in amoebal extracts. The PRTase activities found, however, were very low compared with the activities present in various other parasitic protozoa (14) and under the conditions of assay only approximately 1% of the total radioactivity was present as 5'-ribotides after the 60 min incubation. Guanine PRTase was not detectable. This lack of activity was not due to the conversion of guanine to xanthine. It was confirmed by using the separation system for the guanine deaminase assay that very little xanthine was produced in the guanine PRTase assay. The amoebal extracts contained relatively high nucleoside kinase activities capable of converting nucleosides to 5'-ribotides (Table 1). Kinase activities were found

with all nucleosides tested. Nucleotide kinases also were present (Table 1). On the other hand, nucleoside phosphotransferase using p-nitrophenylphosphate as phosphate donor was detected only at very low activity (0.3 nmol/min/mg protein, with inosine as substrate).

5'-Nucleotidase activities toward several substrates were also detected (Table 1). The highest activity was found with AMP as substrate, GMP and IMP were also hydrolysed whereas no activity was found with XMP. With AMP substrate, 5'-nucleotidase was approximately two fold higher in activity than 3'-nucleotidase. Attempts were made to characterise these enzymes in more detail and acid phosphatase was included in this study for comparative purposes. The distinguishing features of these enzymes are given in Table 3 and Fig. 2. The data suggest that the two nucleotidases differ from each other and acid phosphatase.

Enzymes (adenylosuccinate lyase, adenylosuccinate synthetase, IMP dehydrogenase, GMP synthetase and GMP reductase) which catalyse nucleotide interconversions in many parasitic protozoa were not detectable (limits of detection: 0.4, 0.4, 0.1, 0.3 and 0.5 nmol min^{-1} mg $protein^{-1}$, respectively) in extracts of E. histolytica.

DISCUSSION

The main pathways of purine salvage in E. histolytica as suggested by the results of this study are shown schematically in Fig. 3. The presence of high activities of phosphorylases which function in the anabolic direction and nucleoside kinases and the very low activities of PRTases suggests that nucleotides are synthesized from bases mainly via nucleosides.

The differences observed between the pH profiles and the effects of nucleoside analogues upon the phosphorylase activities towards adenosine and inosine suggests that they are separate proteins. It

TABLE III

Distinguishing properties of phosphomonoesterases from *E. histolytica*

Property	Acid phosphatase	5′-Nucleotidase	3′-Nucleotidase
pH optimum	5.0	6.0	7.5
Specific activity[a]	36 ± 6	20 ± 3	9 ± 2
% Sedimentability[b]	89 ± 5 (124)	45 ± 2 (90)	82 ± 6 (88)
Inhibitors[c]			
tartrate	83 ± 6	76 ± 5	0
fluoride	67 ± 9	85 ± 5	12 ± 4

[a] Specific activities given are in nmol min^{-1} (mg protein)$^{-1}$ and are the means ± S.D. from three experiments.

[b] The activity sedimented in the 105 000 × *g* pellet as a percentage of the total activity recovered. The figures are the means ± S.D. from three experiments with the mean percent activity recovered given in parentheses.

[c] The figures given are the percentage inhibition produced by the compounds at 10 mM and are the means ± S.D. from three experiments.

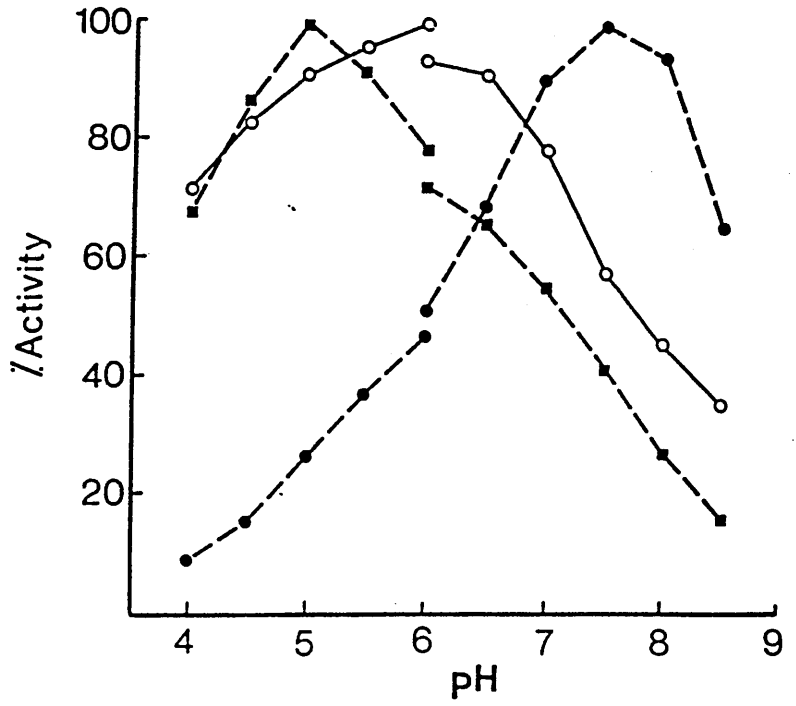

Fig. 2. The pH profiles for *E. histolytica* 5′-nucleotidase (○), 3′-nucleotidase (●) and acid phosphatase (■). The buffers used (both 50 mM) were sodium acetate (pH 4.0–6.0) and Tris maleate (pH 6.0–8.5). The ordinate gives the enzyme activity as percentage of the maximum.

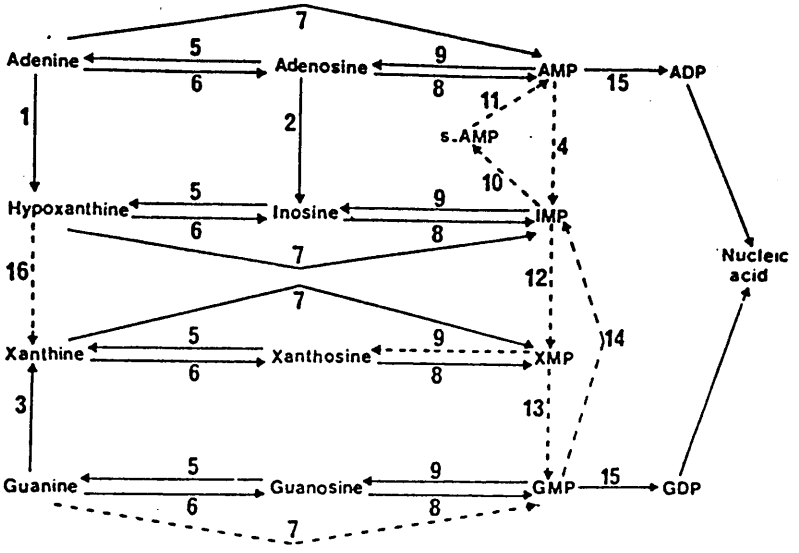

Fig. 3. Purine salvage pathways of *E. histolytica*. (→) pathways present; (--›) pathways not detectable. Key to enzymes: 1, adenine deaminase; 2, adenosine deaminase; 3, guanine deaminase; 4, AMP deaminase; 5, nucleosidase or phosphorylase (catabolic); 6, phosphorylase (anabolic); 7, phosphoribosyltransferase; 8, nucleoside kinase; 9, nucleotidase; 10, adenylosuccinate synthetase; 11, adenylosuccinate lyase; 12, IMP dehydrogenase; 13, GMP synthetase; 14, GMP reductase; 15, nucleotide kinase; 16, xanthine oxidase.

would appear likely that they are adenosine phosphorylase (EC 2.4.2.4) and purine nucleoside phosphorylase (EC 2.4.2.1) as occur in many eukaryotes. Adenosine phosphorylase has been found in several parasitic protozoa including Leishmania donovani (15), L. tropica (16), Trypanosoma cruzi (17), T. congolense (18) and Tritrichomonas foetus (19), whereas purine nucleoside phosphorylase has been detected in L. m. mexicana (10), Trichomonas vaginalis (20) T. foetus (19) and Plasmodium lophurae (21).

The detection of relatively high nucleoside kinase activities in E. histolytica explains the findings of Boonlayangoor et al (1) that most of the radiolabelled nucleosides incubated with the parasite were rapidly incorporated into nucleotide and is in agreement with the previous report of the presence of adenosine kinase (4). The apparent absence of enzymes involved in nucleotide interconversions suggests that E. histolytica relies on adenosine kinase and guanosine kinase for its requirement of adenine and guanine nucleotides.

McLaughlin and Meerovitch (22) reported that the surface membrane of E. invadens possessed acid phosphatase but no specific 5'-AMP nucleotidase activity. In contrast, Takeuchi et al (23) reported that nucleotidases with ADP and ATP as substrates were associated with the plasma membrane of E. histolytica. The findings in this study that 45% of the 5'-AMP nucleotidase activity was sedimentable could be explained by a surface membrane location of part of the activity; this will have to be confirmed, however, by fractionation studies. Nevertheless, the demonstration of high amoebal activity of nucleotidase towards 5'-AMP helps to explain the reports of Reeves and West (24) that the addition of AMP to a depleted medium produced sustained growth of E. histolytica. There have been no reports previously on the presence of 3'-nucleotidase in

Entamoeba species. The high sedimentability (82%) of this activity indicates that this enzyme is associated with membrane, possibly the plasma membrane. This enzyme appears to be quite similar to that reported to be present in Leishmania donovani (25) and Trypanosoma rhodesiense (26) with respect to pH optimum and insensitivity towards tartrate and flouride. The elucidation of the part played by these amoebal nucleotidases must await further studies, although clearly, if it is confirmed that they are located on the plasma membrane, a likely role is in the provision of nucleosides which are then transported across the cell membrane and used in nucleotide synthesis.

The results of this study demonstrate that in many respects amoebal purine metabolism is considerably different from that of mammalian cells (27). Interestingly, overall it is quite similar to that of Trichomonas vaginalis, another anaerobic, parasitic pathogen (20). The marked differences found with Tritrichomonas foetus (19), however, show that such mechanisms of purine salvage are not a common feature of anaerobic sarcomastigophora.

ACKNOWLEDGEMENTS

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REFERENCES

1. Boonlayangoor, P., Albach, R.A. and Booden, T. (1980) Purine nucleotide synthesis in Entamoeba histolytica: a preliminary study. Arch. Invest. Med. (Mex.) 11, 83-88.
2. Boonlayangoor, P., Albach, R.A., Stern, M.L. and Booden, T. (1978) Entamoeba histolytica: uptake of purine bases and nucleosides during axenic growth. Exp. Parasitol. 45, 225-233.
3. Booden, T., Boonlayangoor, P. and Albach, R.A. (1976) Incorporation of purines and pyrimidines into axenically grown Entamoeba histolytica. J. Parasitol. 62, 641-643.
4. Lobelle-Rich, P.A. and Reeves, R.E. (1983) The partial purification and characterization of adenosine kinase from Entamoeba histolytica Am. J. Trop. Med. Hyg. 32, 976-979.
5. Nakamura, M. and Jonsson, S. (1957) The effect of antimetabolites on the growth of Entamoeba histolytica. Purine and pyrimidine analogs. Arch. Biochem. Biophys. 66, 183-189.
6. Eubank, W.B. and Reeves, R.E. (1981) Growth inhibition of axenic Entamoeba histolytica by tubercidin and ARA-A. Am. J. Trop. Med. Hyg. 30, 900-902.
7. Wang, C.C. (1984) Parasite enzymes as potential targets for antiparasitic chemotherapy. J. Med. Chem. 27, 1-9.
8. Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978) A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans. Roy. Soc. Trop. Med. Hyg. 72, 431-432.
9. Hassan, H.F., Mottram, J.C. and Coombs, G.H. (1985) Subcellular localisation of purine-metabolising enzymes in Leishmania mexicana mexicana. Comp. Biochem. Physiol. 81B, 1037-1040.
10. Hassan, H.F. and Coombs, G.H. (1985) Leishmania mexicana: Purine metabolizing enzymes of amastigotes and promastigotes. Exp. Parasitol. 59, 139-150.

11. Leloir, L.F. and Cardini, C.E. (1957) Characterization of phosphorus compounds by acid lability. Meth. Enzymol. 3, 840-850.

12. Lineweaver, H. and Burk, D. (1934) The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 658-666.

13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

14. Hassan, H.F. and Coombs, G.H. (1985) Purine phosphoribosyltransferases of Leishmania mexicana mexicana and other parasitic protozoa. Comp. Biochem. Physiol. 82B,773-779.

15. Krenitsky, T.A., Koszalka, G.W., Tuttle, J.V., Adamczyk, D.L. Elion, G.B. and Marr, J.J. (1980) Purine salvage enzymes in man and Leishmania donovani In: Purine Metabolism in Man-III (Rapado, A. and Watts, R.W.E., eds.), pp. 51-56, Plenum Publishing Corp., New York.

16. Konigk, E. (1978) Purine nucleotide metabolism in promastigotes of Leishmania tropica: Inhibitory effect of allopurinol and analogues of purine nucleosides. Tropenmed. Parasit. 29, 435-438.

17. Gutteridge, W.E. and Davies, M.J. (1981) Enzymes of purine salvage in Trypanosoma cruzi. FEBS Lett. 127, 211-214.

18. Ogbunude, P.O. and Ikediobi, C.O. (1983) Comparative aspects of purine metabolism in some african trypanosomes. Mol. Biochem. Parasitol. 9, 279-287.

19. Wang, C.C., Verham R., Rice, A. and Tzeng, S. (1983). Purine salvage by Tritrichomonas foetus. Mol. Biochem. Parasitol. 8, 325-337.

20. Miller, R.L. and Lindstead, D. (1983) Purine and pyrimidine metabolizing activities in Trichomonas vaginalis. Mol. Biochem. Parasitol. 7, 41-51.

21. Schimandle, C.M., Tanigoshi, L., Mole, L.A. and Sherman, I.W. (1985) Purine nucleoside phosphorylase of the malarial parasite, Plasmodium lophurae. J. Biol. Chem. 260, 4455-4460.

22. McLaughlin, J. and Meerovitch, E. (1975) The surface membrane and cytoplasmic membranes of Entamoeba invadens (Rodhain 1934) - I. Gross chemical and enzymatic properties. Comp. Biochem. Physiol. 52B, 477-486.

23. Takeuchi, T., Kobayashi, S., Masuda, M., Tanabe, M., Miura, S. and Fujiwara, T. (1981) Entamoeba histolytica: Localization and characterization of Ca^{2+}- dependent nucleotidases. Int. J. Parasitol. 11, 209-215.

24. Reeves, R.E. and West, B. (1980) Entamoeba histolytica: Nucleic acid precursors affecting axenic growth. Exp. Parasitol. 49, 78-82.

25. Gottlieb, M. and Dwyer, D.M. (1983) Evidence for distinct 5'- and 3'- nucleotidase activities in the surface membrane fraction of Leishmania donovani promastigotes. Mol. Biochem. Parasitol. 7, 303-317.

26. Gardiner, P.R., Gottlieb, M. and Dwyer, D.M. (1982) A 3'-nucleotidase activity associated with the surface membrane of an african trypanosome. J. Protozool. 29, 471.

27. Murray, A.W. (1971) The biological significance of purine salvage. Ann. Rev. Biochem. 40, 811-826.

Chapter 9

DE NOVO SYNTHESIS OF PURINES BY ACANTHAMOEBA CASTELLANII AND A. ASTRONYXIS

Published as: Hassan, H.F. and Coombs, G.H. 1986. De novo synthesis of purines by Acanthamoeba castellanii and A. astronyxis. IRCS Medical Science, 14, 559-560.

Introduction

A supply of purine nucleotides is essential for the growth and functioning of cells, not only because of their roles as precursors of nucleic acids but also because they participate as activated intermediates in energy metabolism and other group-transfer reactions and as coenzymes. Most metazoans so far investigated have been found capable of synthesizing purines de novo. In contrast, all parasitic protozoa so far studied, with the exception of the endosymbiont-containing Crithidia oncopelti and C. deanei, rely entirely on salvaging preformed purine bases and nucleosides (1).

Acanthamoeba species are small, free-living amoebae that occur widespread within soils. They have received considerable attention recently, however, because of the discovery that certain strains cause primary amoebic meningoencephalitis in humans and animals (2). It has long been known that the growth of Acanthamoeba species can not be impaired by deprivation of exogenous purines, which led to the postulation that they can synthesize these substances (3). Until now, however, there have been no reported attempts to establish that this occurs. We describe here that Acanthamoeba species are capable of de novo synthesis of purines.

Materials and methods

Acanthamoeba castellanii (CCAP 1501/1a) and A. astronyxis (CCAP 1534/1) were grown at 25°C in a liquid monophasic medium containing amoebal saline (AS) (4), mycological peptone (Oxoid) (2%, w/v) and maltose (1.8%, w/v). Cultures at late-log phase of growth (approximately 10^6 organisms (ml culture medium $^{-1}$) were detached by agitation and harvested by centrifugation at 500 x g for 5 min, washed twice with AS and resuspended to a cell density of 2-3 x 10^6

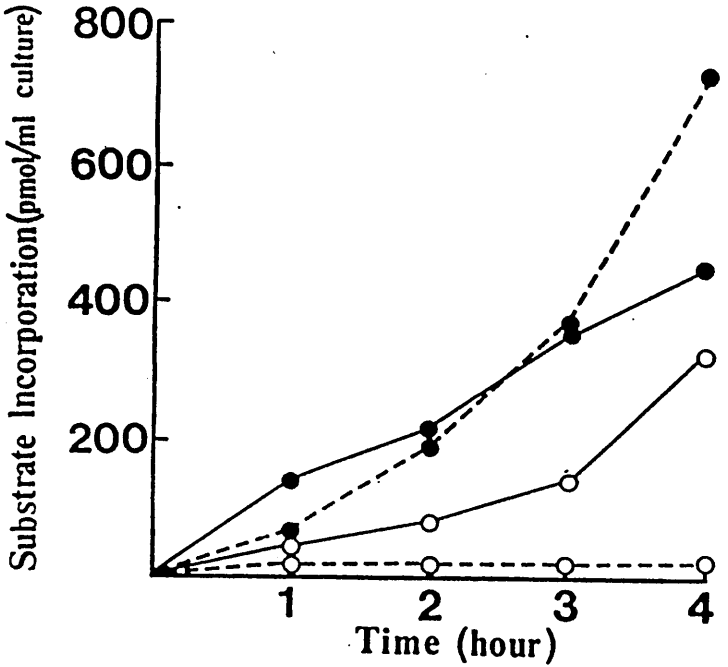
trophozoites ml^{-1} in 8 ml prewarmed (25°C) AS containing maltose (1.8%, w/v). Substrate was then added to a concentration of 5 μCi ml^{-1} (0.1mM) and the cells were incubated at 25°C for 4 h . The radiolabelled substrates used were: (2-^{3}H) adenine, 24 Ci/mmol; (2-^{3}H) glycine, 10.9 Ci/mmol; (U-^{14}C) leucine, 330 mCi/mmol. Following the incubation, cells were centrifuged and washed twice as above and subsequently resuspended in 1 ml of cold 0.2 N perchloric acid (PCA) at 4°C for 30 min. The cold acid-insoluble precipitate was then collected on a millipore filter (0.45 μm), washed with 50 ml of cold 0.2 N PCA, air dried and estimated for radioactivity as described previously (5). In other experiments, using a modification of the method of Gutteridge and Gaborak (6), the distribution of radiolabel into various cell fractions was investigated. The labelled cells were first treated twice with cold 0.2 N PCA at 4°C for 30 min to yield the cold acid-soluble (pool) fraction. The precipitated material was extracted once with ethanol/ether (1:1, v/v) at 45°C for 30 min to yield the lipid fraction and then twice with 0.2 N PCA at 95°C for 30 min to yield the hot acid-soluble (nucleic acid) fraction. The residual material was designated the protein fraction. Aliquots (0.5 ml) of each fraction were mixed with 5 ml of Hydro Luma (May & Baker Ltd) and assessed for radioactivity. Acid hydrolysis of the nucleic acid fraction in 12 N PCA at 100°C for 1 h was performed according to Gutteridge and Gaborak (6). This procedure yielded purine bases, which were separated by ascending chromatography on PEI cellulose F sheets (BDH, Glasgow) using isopropanol:HCl:H_2O (65:16.6:18.4, v/v) as solvent (R_F values: adenine, 0.33; guanine, 0.19) (7). Leishmania mexicana mexicana (M379) amastigotes and promastigotes were cultured and harvested as described previously

(8). Purified amastigotes (1-2 x 10^7 ml^{-1}) and mid-log phase promastigotes (10^6 ml^{-1}) were incubated for 4 h at 37°C and 25°C, respectively, in HOMEM medium containing 5 μCi ml^{-1} (0.1 mM) of radiolabelled precursors and processed as described above.

Results and discussion

There was a progressive incorporation of (2-^{3}H) glycine into both the cold acid-soluble and cold acid-insoluble fractions of *Acanthamoeba* species during the 4 h incubations. A typical result is shown in Fig. 1. In contrast, glycine was not incorporated to a detectable level into the pool fractions of either form of *L. m. mexicana*, although incorporation into macromolecules occurred at a high rate. The results from the fractionation experiments (Table 1) show convincingly that a large amount of the glycine incorporated into the macromolecules of the two *Acanthamoeba* species was into amoebal nucleic acids, suggesting that *de novo* synthesis of purines had occurred. The success of the fractionation procedure was confirmed by the recovery of the majority of the adenine and leucine incorporated in the nucleic acid and protein fractions, respectively. In contrast, only very small amounts of glycine were recovered in the nucleic acid fraction of the leishmanial cells, confirming that *L. m. mexicana* is similar to other *Leishmania* species in being unable to synthesize purine *de novo* (9). After hydrolysis of the nucleic acid fractions from *Acanthamoeba* and separation of the resultant bases by thin layer chromatography, most of the radiolabel was recovered in areas corresponding to the adenine and guanine spots (Fig. 2) confirming that most of the glycine had been utilized for nucleotide synthesis.

It is evident from the results obtained that *Acanthamoeba* species are capable of *de novo* synthesis of purine nucleotides. In



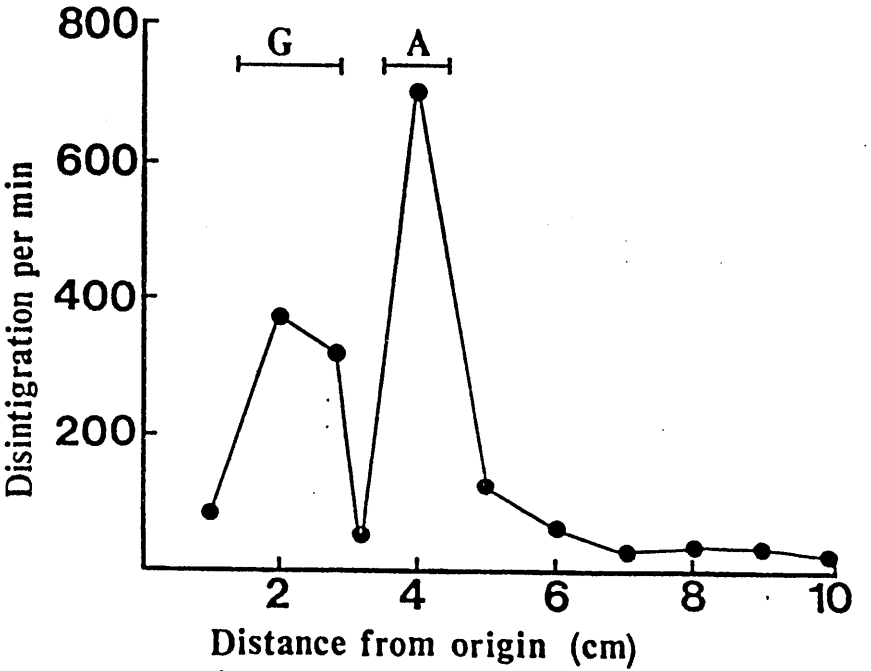


Figure 1 (left): Time courses of [2-^{3}H]-glycine incorporation into cold acid-soluble (– – –) and cold acid-insoluble (———) fractions by Acanthamoeba castellanii (●) *and* Leishmania m. mexicana *promastigotes (○). Figure 2 (right): Chromatography of nucleic acid hydrolysate of* Acanthamoeba castellanii *on PEI cellulose. The R_f values for guanine (G) and adenine (A) are indicated by bars.*

Table 1. Incorporation of substrates into macromolecules of *Acanthamoeba* species and *Leishmania m. mexicana*

Substrate	Relative incorporation[a]											
	A. castellanii			*A. astronyxis*			*Leishmania m. mexicana* Promastigotes			*Leishmania m. mexicana* Amastigotes		
	Lipid	Nucleic acid	Protein	Lipid	Nucleic acid	Protein	Lipid	Nucleic acid	Protein	Lipid	Nucleic acid	Protein
(2-^{3}H)Adenine	2 ± 1	98 ± 1	0.1 ± 0.1	4 ± 1	96 ± 5	< 0.1	0.2	99	0.1	ND	ND	ND
(2-^{3}H)Glycine	8 ± 4	70 ± 1	22 + 3	3 ± 1	81 ± 7	16 ± 7	43	<0.1	57	31	1	67
(U-^{14}C)Leucine	7 ± 1	1 ± 1	92 ± 3	7 ± 2	< 1	93 ± 1	4	0.1	96	1	4	95

[a]Results are expressed as the percentage of the total radioactivity recovered in the cold acid-insoluble material (means ± range from replicate experiments with *Acanthamoeba* spp). ND, not determined.

this respect, they appear to be unique amongst the parasitic protozoa studied to date. Certainly they differ from the related parasitic amoeba Entamoeba histolytica, a widespread human pathogen, which lacks this ability and depends instead on salvage reactions (10). Acanthamoeba species can also salvage purines (see Chapter 10). The situation in another soil amoeba, Naegleria fowleri, which can parasitise man with fatal consequences (2) is not known. These opportunistic parasitic amoebae cause infections for which at present there is no reliable chemotherapy. The finding that Acanthamoeba species can synthesize purines de novo highlights an area of possible chemotherapeutic attack as well as giving an indication that drugs inhibiting purine salvage may be ineffective against these parasites.

References

1. Gutteridge, W.E. and Coombs, G.H. (1977) "Biochemistry of Parasitic Protozoa", MacMillan Press, London.
2. Warhurst, D.C. (1985) Parasitol. Today, 1, 24-28.
3. Adam, K.M. (1959) J. gen. Microbiol., 21, 519-529.
4. Page, F.C. (1967) J. Protozool., 14, 709-724.
5. Hassan, H.F. and Coombs, G.H. (1985) Comp. Biochem. Physiol., 81B, 1037-1040.
6. Gutteridge, W.E. and Gaborak, M. (1979) Int. J. Biochem., 10, 415-422.
7. Fink, K., Gline, R. and Fink, R. (1963) Anal. Chem., 35, 389-398.
8. Coombs, G.H. (1982) Parasitol., 84, 149-155.
9. Marr, J.J., Berens, R.L. and Nelson, D.J. (1978) Biochim. Biophys. Acta, 544, 360-371.

10. Hassan, H.F. and Coombs, G.H. (1986) Mol. Biochem. Parasitol., 19, 19-25

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Chapter 10

PURINE SALVAGE BY *ACANTHAMOEBA CASTELLANII*

To be published as: Hassan, H.F. and Coombs, G.H. 1987. Purine salvage by *Acanthamoeba castellanii*. International Journal for Parasitology.

Abstract - Hassan H.F. and Coombs G.H. 1986. Purine salvage by Acanthamoeba castallanii. International Journal for Parasitology. Trophozoites of Acanthamoeba castellanii were found to incorporate a range of purine bases and nucleosides into parasite nucleic acids. Results from competition studies suggest that A. castellanii is capable of interconverting purine nucleotides. The amoebae contain deaminase, phosphorylase, kinase, phosphoribosyltransferase and 5'-nucleotidase activities towards a number of purine compounds. The results of both the incorporation studies and the enzyme analyses suggest that hypoxanthine is of central importance in the parasite's purine metabolism.

INDEX KEY WORDS: Acanthamoeba castellanii; purine salvage enzymes; phosphoribosyltransferase; nucleoside kinase; 5'-nucleotidase.

INTRODUCTION

Acanthamoebae have attracted many investigators because of their cosmopolitan distribution, their simple but interesting life cycle involving extensive cellular differentiation, and the recent discovery that certain strains are pathogenic to humans and animals (Warhurst, 1985). The ease with which they can be handled and the development of a defined medium that supports their axenic growth have greatly aided studies of their biochemistry (Byers, Akins, Maynard, Lefken & Martin, 1980). Despite these advantages, however, many aspects of the metabolism of acanthamoebae, including that of purines, remain largely unexplored.

We reported recently (Hassan & Coombs, 1986a) that two *Acanthamoeba* species are able to incorporate glycine into nucleic acids, suggesting the occurrence of purine *de novo* synthesis. In this respect acanthamoebae differ from all other parasitic protozoa investigated so far - these have been found to rely entirely upon salvage pathways to satisfy their purine requirements (Gutteridge & Coombs, 1977). To date, however, there have been no reports concerning purine salvage reactions in acanthamoebae. In contrast, there have been several detailed studies on purine uptake and the enzymes involved in their metabolism in the human parasite *Entamoeba histolytica* and other amoebae (Lo & Wang, 1985; Hassan & Coombs, 1986b). Such studies are important as they provide a firm foundation for biochemical studies of encystment and cell differentiation of these parasites and also help us to understand how the parasites are adapted to their environment and interact with their host. Purine salvage reactions of parasitic protozoa have also attracted particular attention as they appear to offer good opportunities for chemotherapeutic attack (Marr & Berens, 1983). There is at present no reliable form of drug therapy for infections of *Acanthamoeba*. For

these reasons, we have measured purine incorporation by two species of Acanthamoeba and examined lysates of these parasites for the enzymes likely to be responsible for purine salvage.

MATERIALS AND METHODS

Growth and fractionation of the parasites. Acanthamoeba castellanii (CCAP 1501/1a) and A. astronyxis (CCAP 1534/1) were cultured and harvested as described previously (Hassan & Coombs, 1986a). Parasites (5 x 10^6 trophozoites/ml) in 50 mM Tris-HCl, pH 7.0, containing 0.1 mM dithiothreitol were lysed by sonication for two periods of 10 s, separated by a 10 s cooling period, using a MSE Soniprep 150 fitted with an exponential probe at 4 μm amplitude. The homogenates produced were fractionated by centrifugation at 105,000 x g at 4°C for 1 h and the resultant supernatants were used as source of the enzymes in all experiments except where specified.

Incorporation studies. Acanthamoeba species were resuspended in amoebal saline (Page, 1967) containing maltose (1.8%, w/v) to a cell density of 2-3 x 10^6 trophozoites/ml. The radiolabelled substrates ((2-^{3}H) adenine, 888 GBq/mmol; (2-^{3}H) adenosine, 925 GBq/mmol; (8-^{14}C) guanine, 2.1 GBq/mmol; (5-^{3}H) guanosine, 814 GBq/mmol; (G-^{3}H) hypoxanthine, 85 GBq/mmol; (8-^{14}C) inosine, 1.9 GBq/mmol; (6-^{14}C) xanthine, 2.0 GBq/mmol) were each added to 185 kBq ml^{-1} with the final substrate concentration being 0.1 mM and the cells were incubated at 25°C for 4 h. Following the incubation, cells were harvested by centrifugation, washed with amoebal saline, and the incorporation of radiolabel into either the perchloric acid (PCA)-insoluble fraction or the nucleic acid fraction determined using the methods described previously (Hassan & Coombs, 1986a).

Enzyme analyses. Unless otherwise stated, the reaction mixtures were 100 μl in volume and incubation was at 25°C for 10 min. The

reactions were initiated by the addition of substrate and terminated by heating in a boiling water bath for 3 min. The mixtures were subsequently centrifuged at 2100 x g for 5 min and 10 µl aliquots of the resultant supernatants were applied, together with 10 nmol of substrate and product as carriers, on to polyethyleneimine (PEI) cellulose F precoated sheets and the reactants separated by ascending chromatography (see below). Ultraviolet absorbing areas were cut out and counted for radioactivity as described previously (Hassan & Coombs, 1986b). Linearity of reaction rates was confirmed by taking samples at different time points. Control assays were run without extract. The radioactivity recovered in substrate and product spots usually counted for > 96% of that originally applied. One unit of enzyme activity was defined as the amount of enzyme that catalyses the conversion of one nmol of substrate to product per minute.

The standard assay conditions, which were used unless otherwise specified, were as follows: Adenine deaminase (EC 3.5.4.2), adenosine deaminase (EC 3.5.4.4) and guanine deaminase (EC 3.5.4.3): 50 mM Tris-HCl, pH 7.2; 0.2 mM (2-^{3}H) adenine (1.85 GBq mmol^{-1}) or (2-^{3}H) adenosine (1.85 GBq mmol^{-1}) or (8-^{14}C) guanine (185 MBq mmol^{-1}). Phosphorylase, anabolic direction (EC 2.4.2.1): 50mM Tris-HCl, pH 7.2; 1mM ribose-1-phosphate; 0.2 mM (2-^{3}H) adenine (1.85 GBq mmol^{-1}) or (8-^{4}C) guanine (185 MBq mmol^{-1}) or (G-^{3}H) hypoxanthine (31.4 MBq mmol^{-1}) or (6-^{14}C) xanthine (666 MBq mmol^{-1}). Nucleosidase (EC 3.2.2.1) and phosphorylase, catabolic direction (EC 2.4.2.1): 50 mM Tris-HCl, pH 7.4; 0.2 mM (2-^{3}H) adenosine (1.85 GBq mmol^{-1}) or (5-^{3}H) guanosine (1.85 GBq mmol^{-1}) or (8-^{14}C) inosine (92.5 MBq mmol^{-1}); 20 mM KH_2PO_4 (phosphorylase assay only). Adenine phosphoribosyltransferase (PRTase) (EC 2.4.2.7), hypoxanthine-guanine PRTase (EC 2.4.2.8) and xanthine PRTase (EC 2.4.2.22): 50 mM Tris-HCl (at the pH optimum for each enzyme as indicated below); 5 mM

$MgSO_4$; 1 mM phosphoribosyl-1-pyrophosphate (PRPP); 0.2 mM of radiolabelled purine bases (specific activities as above). Nucleoside kinases (EC 2.7.1.15, EC 2.7.1.73, EC 2.7.1.23): 50 mM Tris-HCl, pH 7.4; 1 mM ATP; 2mM $MgCl_2$; 25 mM KCl; 0.2 mM phosphoenolpyruvate; 4 IU rabbit muscle pyruvate kinase ml^{-1}; 0.2 mM radiolabelled purine nucleosides (specific activities as above).

The components of the enzyme reactions were separated using the following systems. I. Distilled deionised water was used as chromatography for adenine PRTase, hypoxanthine PRTase, xanthine PRTase and nucleoside kinases (R_F values: adenine, 0.28; adenosine, 0.50; AMP, 0.0; hypoxanthine, 0.45; inosine, 0.70; IMP, 0.0; guanosine, 0.54; GMP, 0.0; xanthine, 0.26; XMP, 0.0). II. 0.1 M Potassium phosphate, pH 6.8/saturated ammonium sulphate/n-propanol (100/60/20, v/v) was used for adenine deaminase, adenosine deaminase, anabolic phosphorylase with adenine, hypoxanthine and xanthine as substrates, nucleosidase and catabolic phosphorylase with adenosine and inosine as substrates (R_F values: adenine, 0.16; hypoxanthine, 0.37; adenosine, 0.26; inosine, 0.55; xanthine, 0.22; xanthosine, 0.40). III. Butyl alcohol/methyl ethyl ketone/ water/ formic acid (44/44/10/0.26, v/v) was used for guanine deaminase, guanine phosphorylase, guanine PRTase, guanosine nucleosidase and guanosine phosphorylase (R_F values: guanine, 0.13; guanosine, 0.50; xanthine, 0.33; GMP, 0.0).

IMP dehydrogenase (EC 1.2.1.14) and nucleoside phosphotransferase (EC 2.7.1.77) were assayed radiochemically as described previously (Hassan & Coombs, 1986b) whereas AMP deaminase (EC 3.5.4.6), adenylosuccinate lyase (EC 4.3.2.2), GMP reductase (EC 1.6.6.8), adenylosuccinate synthetase (EC 6.3.4.4) and GMP synthetase (EC 6.3.5.2) were assayed spectrophotometrically as described

previously (Hassan & Coombs, 1985). 3'-AMP nucleotidase (EC 3.1.3.6), 5'-AMP nucleotidase (EC 3.1.3.5) and acid phosphatase (EC 3.1.3.2) were assayed colorimetrically as described previously (Hassan & Coombs, 1986b).

Phosphoribosyltransferase and nucleotidase activities at a range of pH were determined using sodium acetate, 50 mM (pH 4.0 - 6.0), and Tris-maleate, 50 mM (pH 6.0 - 8.5). The apparent K_m values for some substrates were determined by the Lineweaver-Burk method (1934). For inhibitors and activator studies, the extract was preincubated with the compound for 10 min at 25°C and then assayed as described above. Inhibition is expressed as the % reduction in activity compared with that under standard conditions. Protein concentrations were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Materials. Chemicals were of analytical grade and obtained from the sources listed previously (Hassan & Coombs, 1986b).

RESULTS

Each of the purine bases and nucleosides tested was incorporated into the nucleic acid fraction of A. castellanii (Table 1). Hypoxanthine was incorporated at the highest rate, although its rate of incorporation and that of guanine were not significantly different. Adenine, adenosine and guanosine were incorporated at quite similar rates, lower than that of hypoxanthine, whereas the rates of inosine and xanthine incorporation were relatively low. The effects of adding other purines, at 1 mM, on the incorporation of each purine base are shown in Fig. 1 . Adenine inhibited the incorporation of hypoxanthine by about 60%, whereas it had relatively little inhibitory effect on guanine and xanthine incorporation. Guanine had a moderate inhibitory effect on the

TABLE 1. Incorporation of purines into the nucleic acids of *Acanthamoeba castellanii*

Purine	Incorporation (nmol/2 x 10^6 cell/4h)*
Adenine	387 ± 117 (3)
Guanine	562 ± 76 (3)
Hypoxanthine	712 ± 225 (4)
Xanthine	176 ± 36 (2)
Adenosine	445 ± 101 (2)
Guanosine	320 ± 145 (2)
Inosine	213 ± 61 (2)

* Mean ± S.D. (number of experiments)

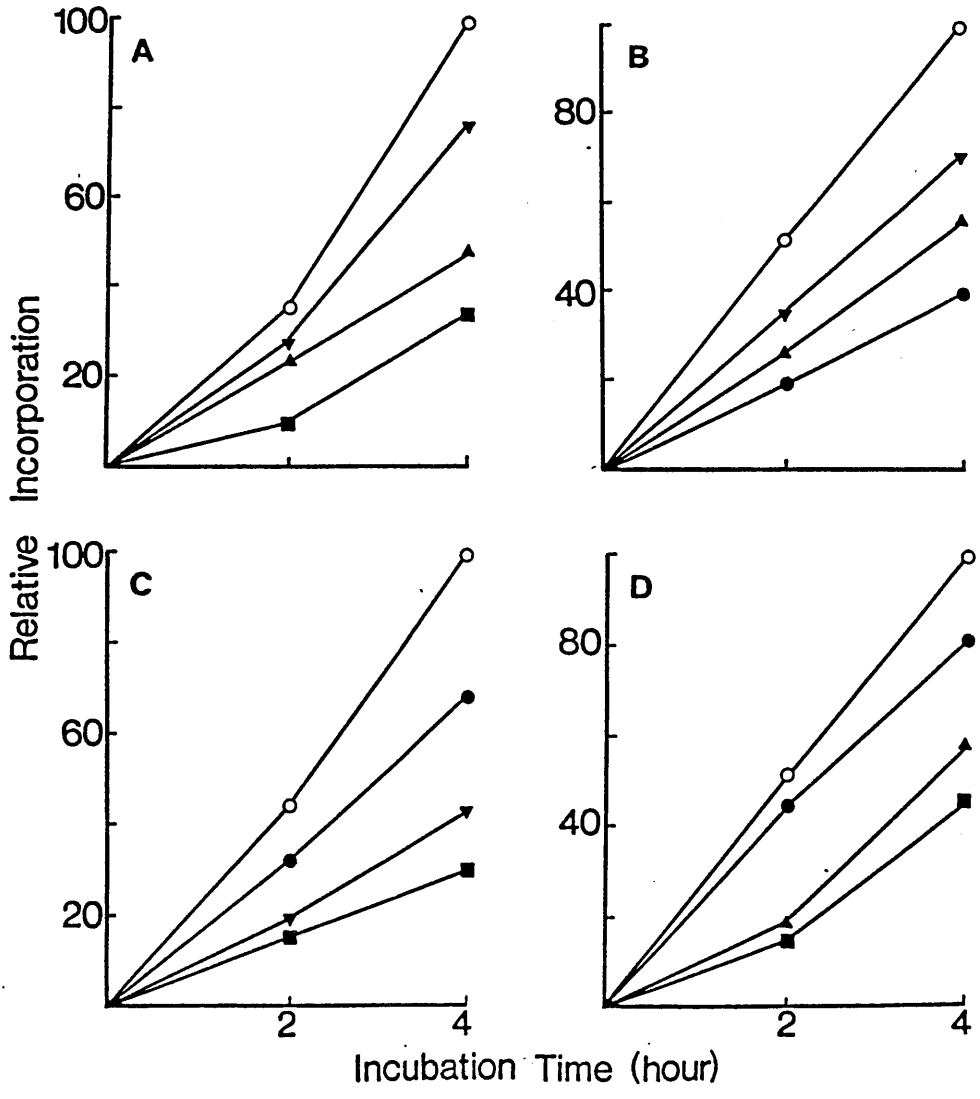

FIG. 1. Purine incorporation by Acanthamoeba castellanii; the effect of other purines. The graphs show the incorporation of adenine(A), hypoxanthine(B), guanine(C) and xanthine(D) into the cold PCA-insoluble fraction of A. castellanii. The radiolabelled purine was present either in the absence (O) or presence of other purines (● , adenine; ▲ , hypoxanthine; ■ , guanine; ▼ , xanthine). The data are expressed as the incorporation relative to that after 4 h in the absence of competing purine.

incorporation of each of the labelled purines, whereas hypoxanthine was found to reduce quite markedly the incorporation all of the other purine bases. Xanthine, on the other hand, was found to have rather little effect on adenine and hypoxanthine incorporation, although it inhibited guanine incorporation by > 50%. These competitive interactions may have been due, in part, to the purines competing for PRPP but they also suggest that A.castellanii is capable of interconverting most of the purine nucleotides to some extent, and in particular adenine and guanine nucleotides. This finding was substantiated by the detection of the enzymes involved in these interconversions.

The activities of the various purine-metabolising enzymes detected in the soluble fractions of A.castellanii are given in Table 2. Adenosine deaminase activity was relatively high in Acanthamoeba extracts. This activity was inhibited 83% by coformycin at 10^{-4} M. Assaying adenosine nucleosidase, adenosine phosphorylase and adenosine kinase in the presence of 10^{-4} M coformycin, however, did not result in higher specific activities being obtained; this suggests that the adenosine concentration was not limiting under the conditions used. Adenine deaminase and guanine deaminase activities were detected at similar levels; xanthine was found to be the only product formed from guanine in the absence of PRPP giving an indication that xanthine oxidase was absent. AMP deaminase activity was not detected.

The soluble fraction of A. castellanii showed only low activities of the enzymes converting nucleosides to their respective bases (nucleosidases). The rate of conversion of adenosine to adenine was not increased upon addition of 20 mM inorganic phosphate to the reaction mixtures. By contrast, this addition stimulated the rates of hydrolysis of inosine and guanosine, to hypoxanthine and

TABLE 2. The activities of purine-metabolising enzymes in Acanthamoeba castellanii.

Substrate	Deaminase	Phosphorylase*	Phosphorylase	PRTase	Kinase	Nucleotidase+
Adenine	4 ± 1		2 ± 0.1	13 ± 2		
Adenosine	9 ± 2	0.6 ± 0.1 (0.4 ± 0.2)			13 ± 1	
5'-AMP	< 1				67 ± 13	75 ± 15
3'-AMP						< 1
Hypoxanthine			2 ± 0.5	22 ± 5		
Inosine		1 ± 0.1 (0.5 ± 0.3)			0.2 ± 0.1	
5'-IMP						43 ± 9
Guanine	3 ± 1		10 ± 1	19 ± 4		
Guanosine		5 ± 2 (1 ± 0.2)			3 ± 1	
5'-GMP					6 ± 2	37 ± 11
Xanthine			4 ± 1	2 ± 0.5		
Xanthosine		1 ± 0.2 (3 ± 1)				

The activities are given in nmol/min/mg protein and are the means ± SD from replicate experiments.

*The figures in parentheses represent the apparent nucleosidase activities.

+Activity in 105,000 x g sedimentable pellet.

guanine 2-fold and 5-fold respectively; suggesting the presence of phosphorylase activities. The Acanthamoeba extracts also converted all purine bases to their corresponding nucleosides in the presence of ribose-1-phosphate (anabolic phosphorylase). The high activity towards guanine correlates well with the relatively high catabolic phosphorylase activity.

Purine phosphoribosyltransferase activities towards adenine, guanine and hypoxanthine were detected at levels sufficient to account for the rates of incorporation of purine bases observed with whole organisms. The activity towards adenine was optimal at pH 7.4, pH 7.8 was the optimum for hypoxanthine and guanine, whereas with xanthine optimal activity was in the range pH 7.0 - 8.2. The activities were stimulated by addition of divalent cations at 5 mM and in each case it was found that Mg^{2+} was more effective than Mn^{2+} or Co^{2+}. All four activities were recovered mainly in the soluble fractions of the cells. The apparent K_m values were 2.5×10^{-4} M for guanine and 3.3×10^{-4} M for adenine and hypoxanthine.

Nucleoside kinase activities were detected in parasite extracts. The rates of activity with different substrates was in the order adenosine > guanosine > inosine. Nucleotide kinases also were present, the activity with AMP being 11-fold higher than that towards GMP. Purine nucleoside phosphotransferase towards inosine and using p-nitrophenylphosphate as phosphate donor was not detectable (< 0.08 nmol/min/mg protein).

5'-AMP nucleotidase was present at high activity whereas 3'-AMP nucleotidase was not detected. About 95% of 5'-AMP nucleotidase activity was present in the particulate fraction after centrifugation at 105,000 x g for 1 h. Phosphatase towards p-nitrophenylphosphate was also present at high activity (98 ± 13 nmol/min/mg sedimentable

protein) and also found to be largely particulate, with more than 75% of the activity being recovered in the 105,000 x g sediment. These two activities were different, however, with respect to pH profile, divalent cation activation and sensitivity to inhibitors. 5'-Nucleotidase was active over a broad pH range, with 7.8 being optimal, whereas phosphatase was optimal at pH 5.5. Of the several divalent cations (Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+}) tested, only Mg^{2+} was found to stimulate 5'-AMP nucleotidase activity. In contrast, it produced no increase in phosphatase activity at either pH 5.5 or 7.8. 5'-Nucleotidase was unaffected by sodium tartrate (10 mM) and inhibited only a little (18%) by sodium fluoride (10 mM). Both of these compounds proved to be potent inhibitors of phosphatase (about 85% at 10 mM). IMP and GMP were also hydrolysed by 5'-nucleotidases. The apparent K_m value of nucleotidase for 5'-AMP as substrate was found to be 0.2 mM.

Enzymes (adenylosuccinate lyase, adenylosuccinate synthetase, IMP dehydrogenase, GMP synthetase and GMP reductase) which catalyse nucleotide interconversions in many parasitic protozoa were detected in soluble extracts of A. castellanii. The specific activities were 4 ± 1, 0.4 ± 0.1, 3 ± 1, 2 ± 1 and 11 ± 1 nmol/min/mg protein, respectively.

All the results above are from experiments with A. castellanii. Very similar data were obtained with A. astronyxis, these are not given in order to save space.

DISCUSSION

Studies on purine metabolism of parasitic protozoa have revealed many interesting and unusual features that distinguish the parasites' metabolism from that of mammals and so are potentially vulnerable to chemotherapeutic attack (Hammond & Gutteridge, 1984; Marr & Berens,

1983; Wang, 1984). Consequently, purine metabolism of a range of parasitic protozoans has been extensively investigated. The present study, however, is the first on the purine-metabolising enzymes of acanthamoebae and it has revealed that A. castellanii possesses enzymes capable of salvaging purine bases and nucleosides. We have recently reported that Acanthamoeba species are capable of de novo synthesis of purines, glycine being utilized for the synthesis of both adenine and guanine nucleotides (Hassan & Coombs, 1986a). The results of the present investigation, notably the high rate of incorporation of hypoxanthine into Acanthamoeba nucleic acids and the relatively high activity of hypoxanthine PRTase, suggest, however, that purine salvage pathways are a major source of purines for the organism. The finding that all purine bases and nucleosides tested were incorporated into the organism, and that each purine base antagonised to some extent the incorporation of each of the others, provide strong indications that there are several ways in which purines can be salvaged and interconverted in vivo. The results from the enzyme analyses (summarised in Fig. 2) support such an hypothesis.

The relative rates of incorporation of exogenous purines found, together with the levels of enzyme activities detected, suggest that hypoxanthine is an especially important source of purine for nucleotide synthesis in this parasite.. The IMP produced from hypoxanthine by hypoxanthine PRTase could be converted both to adenine nucleotides by the combined action of adenylosuccinate synthetase and adenylosuccinate lyase and to guanine nucleotide by IMP dehydrogenase and GMP synthetase. In this respect, A. castellanii appears to be rather similar to many other parasitic protozoa studied, including Leishmania species, Crithidia fasciculata and Herpetomonas species (Hassan & Coombs, 1986c). The presence of

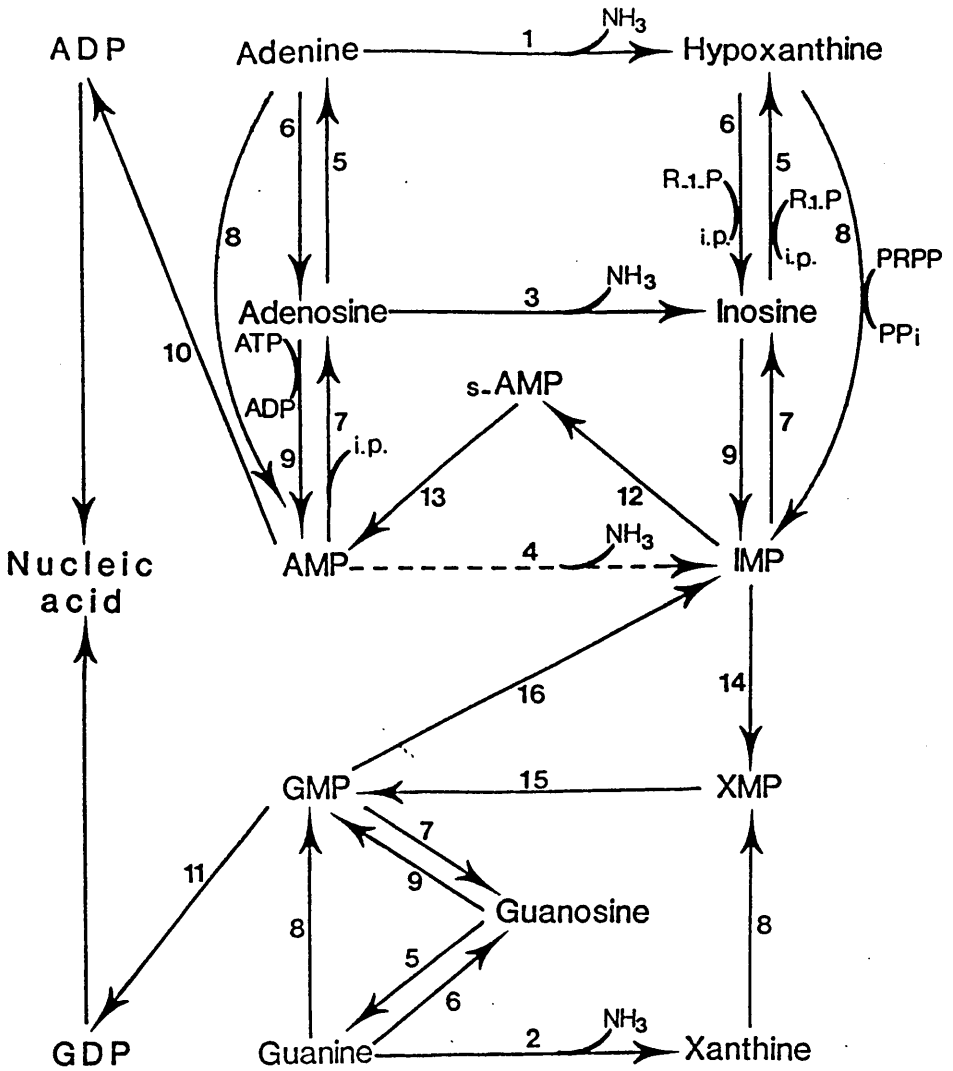

Fig. 2. Purine salvage pathways of Acanthamoeba castellanii (⟶) pathways present; (⇢) pathway not detectable. Key to enzymes: 1, adenine deaminase; 2, guanine deaminase; 3, adenosine deaminase; 4, AMP deaminase; 5, phosphorylase (catabolic); 6, phosphorylase (anabolic); 7, nucleotidase; 8, phosphoribosyltransferase; 9, nucleoside kinase; 10, AMP kinase; 11, GMP kinase; 12, adenylosuccinate synthetase; 13, adenylosuccinate lyase; 14, IMP dehydrogenase; 15, GMP synthetase; 16, GMP reductase. Abbreviations: s-AMP, adenylosuccinic acid; i.p., inorganic phosphate; PPi, inorganic pyrophosphate; R.1.P, ribose-1-phosphate; PRPP, phosphoribosylpyrophosphate. Note: the conversion of nucleosides to their bases can also be catalysed by nucleosidases, which were detected at low levels of activity in A. castellanii

adenine PRTase and adenosine kinase, however, suggests that Acanthamoeba is also capable of using adenine and adenosine directly for adenine nucleotide synthesis. Similarly guanine nucleotide can also be synthesised directly from guanine and guanosine through the actions of guanine PRTase, guanine phosphorylase and guanosine kinase. The AMP kinase and GMP kinase activities which were demonstratible in parasite extracts correlates well with the expectation that ATP and GTP are the most required purine nucleotides. The presence of guanine deaminase and adenine deaminase indicates that these two bases can be converted to xanthine and hypoxanthine, respectively. The observation that the addition of adenine greatly reduced the incorporation of hypoxanthine into the parasite provides some evidence that the deamination of adenine occurs in vivo. The relatively high activity in Acanthamoeba of adenosine deaminase suggests that the deamination of adenosine also occurs in the organism; the inosine produced could be converted to hypoxanthine and then IMP.

5'-Nucleotidase of Acanthamoeba species has been studied previously and considered to be a constituent of the plasma membrane (Schultz & Thompson, 1969). The present study has confirmed that 5'-nucleotidase of A. castellanii appears to be quite similar, both with respect to the apparent K_m towards 5'-AMP (0.2 mM) and pH optimum (7.8), to that reported to be present in the related amoeba Naegleria fowleri (K_m, 0.18 mM; pH optimum, 9.0) (Lowery & McLaughlin, 1985). It differs more, however, from the activity of Leishmania tropica (K_m, 0.62 mM; pH optimum, 5.6) (Pereira & Konigk, 1981) and other trypanosomatids (unpublished). The insensitivity of acanthamoeba 5'-nucleotidase towards tartrate and flouride is in contrast to the enzyme of Entamoeba histolytica which is inhibited by both compounds

(Hassan & Coombs, 1986b). The likely role of 5'-nucleotidase in Acanthamoeba is dephosphorylation of exogenous nucleotides to nucleosides which can then be transported across the cell membrane into the cell whereupon they can be converted to nucleotides by one of the routes described above. This differences with respect to nucleotidase is not the only way in which purine metabolism of Acanthamoeba species and E. histolytica differs. In contrast to Acanthamoeba, E. histolytica apparently lacks the ability to interconvert adenine and guanine nucleotides through formation of IMP and it also largely relies on phosphorylase and kinase activities for salvage synthesis of purine nucleotides (Lo & Wang, 1985; Hassan & Coombs, 1986b). As yet, the situation in another pathogenic soil amoeba, N. fowleri, is not known. It has been shown (Nakamura & Johnsson, 1957), that some purine analogues inhibit the growth of E. histolytica, the sensitivity of Acanthamoeba to these and related compounds is yet to be established. The enzyme data obtained so far for the two parasitic amoebae, however, suggests that they are likely to be differently sensitive to such inhibitors.

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REFERENCES

BYERS T.J., AKINS R.A., MAYNARD B.J., LEFKEN R.A. & MARTIN S.M. 1980. Rapid growth of Acanthamoeba in defined media; Induction of encystment by glucose-acetate starvation. Journal of Protozoology 27: 216-219.

GUTTERIDGE W.E. & COOMBS G.H. 1977. Biochemistry of Parasitic Protozoa. Macmillan Press, London.

HAMMOND D.J. & GUTTERIDGE W.E. 1984. Purine and pyrimidine metabolism in the Trypanosomatidae. Molecular and Biochemical Parasitology. 13: 243-261.

HASSAN H.F. & COOMBS G.H. 1985. Leishmania mexicana: Purine-metabolising enzymes of amastigotes and promastigotes. Experimental Parasitology 59: 139-150.

HASSAN H.F. & COOMBS G.H. 1986a. De novo synthesis of purines by Acanthamoeba castellanii and A. astronyxis. IRCS Medical Science 14: 559-560.

HASSAN H.F. & COOMBS G.H. 1986b. Purine-metabolising enzymes in Entamoeba histolytica. Molecular and Biochemical Parasitology 19: 19-25.

HASSAN H.F. & COOMBS G.H. 1986c. A comparative study of purine- and pyrimidine-metabolising enzymes of a range of trypanosomatids. Comparative Biochemistry and Physiology 84B: 217-223.

LINEWEAVER H. & BURK D. 1934. The determination of enzyme dissociation constants. Journal of American Chemical Society 56: 658-666.

LO H.S. & WANG C.C. 1985. Purine salvage in Entamoeba histolytica. Journal of Parasitology 71: 662-669.

LOWERY D.M. & MCLAUGHLIN J. 1985. Subcellular distribution of hydrolases in Naegleria fowleri. Journal of Protozoology 32: 616-621.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275.

MARR J.J. & BERENS R.L. 1983. Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Molecular and Biochemical Parasitology 7: 339-356.

NAKAMURA M. & JONSSON S. 1957. The effect of antimetabolites on the growth of Entamoeba histolytica. Purine and pyrimidine analogues. Archives of Biochemistry and Biophysics 66: 183-189.

PAGE F.C. 1967. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of Hartmanella and 3 of Vahlkampfia. Journal of Protozoology 14: 499-521.

PEREIRA N.M. & KONIGK E. 1981. A nucleotidase from Leishmania tropica promastigotes: Partial purification and properties. Tropenmedizin und Parasitologie 32: 209-214.

SCHULTZ T.M. & THOMPSON J.E. 1969. Enrichment of 5'-nucleotidase in membrane fragments isolated from Acanthamoeba species. Biochimica et Biophysica Acta 193: 203-211.

WANG C.C. 1984. Purine and pyrimidine metabolism in Trichomonadidae and Giardia: Potential targets for chemotherapy. In: Biochemical Basis of Drug Action, (Edited by SINGER T.P., MANSOUR T. & ONDARZA R.) pp. 133-145 Academic Press, New York.

WARHURST D.C. 1985. Pathogenic free-living amoebae. Parasitology Today 1: 24-28.

Chapter 11

PURINE-METABOLISING ENZYMES IN BABESIA DIVERGENS

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ABSTRACT. Extracts of Babesia divergens were examined for the enzymes which catalyse purine salvage. Adenosine deaminase (EC 3.5.4.4), guanine deaminase (EC 3.5.4.3), inosine phosphorylase (EC 2.4.2.1), purine phosphoribosyltransferases (EC 2.4.2.7, EC 2.4.2.8, EC 2.4.2.22) and nucleoside kinases (EC 2.7.1.15, EC 2.7.1.20, EC 2.7.1.73) were all detected at relatively high activities, whereas nucleotide interconverting enzymes were not detected. Coformycin and 4-amino-5-imidazolecarboxamide were found to be potent inhibitors of adenosine deaminase and guanine deaminase, respectively. The results suggest that B. divergens is capable of synthesizing purine nucleotides via two routes, one involving purine phosphoribosyltransferases and the other employing nucleoside kinases.

INTRODUCTION

Purine metabolism in many parasitic protozoa differs significantly from that of their host (Gutteridge and Coombs 1977). Not only are the parasites unable to synthesize de novo the purine ring, but also several of the enzymes responsible for the salvage of purine bases and nucleosides have substrate specificities different from their mammalian counterparts (Wang 1984a). The differences between isofunctional enzymes have been elegantly exploited by the development of a variety of purine analogues that have been found to possess interesting antiprotozoal activity. Notably, inosine analogues have high potential as antitrypanosomatid agents (Marr and Berens 1983). These findings, together with those for other parasitic protozoa including trichomonads (Wang 1984b), amoebae (Hassan and Coombs 1986a) and Plasmodium species (Walsh and Sherman 1968; Yamada and Sherman 1981; Reyes et al. 1982), have confirmed that purine metabolism is a prime area for investigation in the search for host-parasite differences that can be used in the design of new antiprotozoal drugs.

There is, however, little known about this aspect of metabolism in piroplasms, although there is an indication that some antibabesial drugs may affect purine incorporation (Irvin and Young 1977). The uptake of exogenous adenine, adenosine and hypoxanthine into Babesia-infected-erythrocytes has been studied, and it was suggested that hypoxanthine was likely to be the major purine source in vivo (Irvin et al. 1978; Irvin and Young 1979). There have not, however, been any reports on the occurrence of purine-metabolising enzymes in Babesia species nor indeed of the absence of a de novo synthetic pathway. In this investigation, we have surveyed extracts of Babesia divergens for a range of enzymes potentially involved in the salvage of purines by this important cattle parasite.

MATERIALS AND METHODS

MATERIALS

The (2-^{3}H) adenine, 24 Ci mmol^{-1}, (2-^{3}H) adenosine, 25 Ci mmol^{-1}, (5-^{3}H) guanosine, 22 Ci mmol^{-1}, (G-^{3}H) hypoxanthine, 2.3 Ci mmol^{-1}, (8-^{14}C) inosine, 52 mCi mmol^{-1}, (8-^{14}C) IMP, 54 mCi mmol^{-1} and (8-^{14}C) xanthine, 54 mCi mmol^{-1} were all obtained from Amersham International, England. The (8-^{14}C) guanine, 55.5 mCi mmol^{-1}, was purchased from New England Nuclear, F.R.G. In all cases, purity was > 97%. PEI cellulose F precoated sheets (Merck) were obtained from BDH, Glasgow, Scotland. Coformycin was obtained from Calbiochem Behring, Cambridge, England and was dissolved in 0.1 M sodium phosphate buffer, pH 8.0, before use. All other chemicals were obtained from Sigma Chemical Co. Ltd., Dorset, Poole, England.

Growth, purification and lysis of the parasites.

The bovine isolate of B. divergens that had been adapted for growth in splenectomised rats as previously described (Phillips 1984) was used. The parasite was maintained by serial passage through 3-6 month old Wistar rats that had been splenectomised whilst under ether anaesthesia at least one week prior to infection. The donor rats for this study were inoculated intravenously with 2 x 10^{6} parasitised erythrocytes and their blood was collected into heparin (10 IU ml blood^{-1}) three days later when the parasitaemia was between 30 and 65%. The blood collected (20 ml) was diluted five fold with PBS (0.02 M sodium phosphate, pH 7.4, 0.14 M NaCl), the blood cells washed three times with PBS by centrifugation at 1600 x g for 10 min and finally suspended in 100 ml of PBS. The erythrocytes were freed of leukocytes by passing the blood cell suspension through a cellulose-powder column (Whatman CF 11) (Richards and Williams 1973). The parasites were released from host erythrocytes by suspension of

the cell preparation in 10-20 ml 0.2% (w/v) NaCl, the suspension being gently stirred for 10-20 min at room temperature. The isolated parasites were washed three times in PBS, using centrifugation at 10,000 x g for 2 min, suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M dithiothreitol, and lysed by sonication for two periods of 15 s, separated by a 20 s cooling period, using a MSE Soniprep 150 fitted with an exponential probe at 4 μm amplitude. The crude homogenate produced was fractionated by centrifugation at 105,000 x g at 4°C for 1 h and the resultant supernatant was used as source of the enzyme in all experiments except where specified. Material derived from uninfected blood subjected to the same procedure as above was used as a control. Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Enzyme analyses.

Purine-metabolising enzymes were assayed by the following procedures. Unless otherwise stated, reactions were in a total volume of 100 μl and involved incubation at 37°C for 10 min. Linearity of rates under standard conditions was achieved by adjusting the enzyme concentration so that no more than 20% of the substrate was converted to product by the end of incubation and confirmed by taking samples at three time points during the course of the reaction. The mixtures were pre-equilibrated to the incubation temperature for 3 min before the reactions were initiated by the addition of substrate. The reactions were terminated by heating in a boiling water bath for 3 min after which the precipitated protein was removed by centrifugation at 2100 x g for 5 min. Samples (10 μl) of the resultant supernatants were spotted on to PEI cellulose F sheets in the presence of 10 nmol of both substrate and product as carriers. The plates were developed by ascending chromatography in one of

three solvents systems: Solvent I, 0.1 M potassium phosphate, pH 6.8/saturated ammonium sulphate/n-propanol (100/60/2, v/v) (R_F values: adenine, 0.16; hypoxanthine, 0.37; adenosine, 0.26; inosine, 0.55; xanthine, 0.22; xanthosine, 0.40); solvent II, butyl alcohol/methyl ethyl ketone/water/formic acid (44/44/10/0.26, v/v) (R_F values: guanine, 0.13; guanosine, 0.50; xanthine, 0.33; GMP, 0.0); solvent III, distilled deionised water (R_F values: adenine, 0.28; adenosine, 0.50; AMP, 0.0; hypoxanthine, 0.45; inosine, 0.70; IMP, 0.0; guanosine, 0.54; GMP, 0.0; xanthine, 0.26; XMP, 0.0). The carrier spots were visualised under ultraviolet light, cut out and counted for radioactivity as previously described (Hassan and Coombs 1985a). The radioactivity recovered in substrate and product spots usually accounted for > 96% of that originally applied. Controls comprised either the complete assay mixture stopped at zero time or incubations without substrate. The standard assay mixtures, which were used unless otherwise specified, consisted of 50 mM Tris-HCl, pH 7.4, 0.2 mM of the appropriate radiolabelled purine bases or nucleosides (adenine, 50 mCi $mmol^{-1}$; adenosine, 50 mCi $mmol^{-1}$; guanine 5 mCi $mmol^{-1}$; guanosine, 50 mCi $mmol^{-1}$; hypoxanthine, 850 mCi $mmol^{-1}$; inosine, 2.5 mCi $mmol^{-1}$; xanthine, 18 mCi $mmol^{-1}$) and sample protein. The following were also used, as indicated: 1mM ribose-1-phosphate for anabolic phosphorylase assays; 20 mM KH_2PO_4 for catabolic phosphorylase assays; 1mM phosphoribosyl-1-pyrophosphate/5 mM $MgSO_4$ for phosphoribosyltransferase (PRTase) assays; 1mM ATP/2 mM $MgCl_2$/25 mM KCl/0.2 mM phosphoenolpyruvate/4 IU rabbit muscle pyruvate kinase ml^{-1} for nucleoside kinase assays; 100 mM p-nitrophenylphosphate, 0.1 M sodium acetate, pH 5.4, instead of Tris-HCl for phosphotransferase assays. Coformycin (10^{-4} M) was included in the assays of adenosine nucleosidase, adenosine phosphorylase and

adenosine kinase. Solvent I was used in the chromatographic separation for the adenine deaminase (EC 3.5.4.2), adenosine deaminase (EC 3.5.4.4), anabolic phosphorylase (EC 2.4.2.1) with adenine, hypoxanthine or xanthine as substrate, nucleosidase (EC 3.2.2.1) and catabolic phosphorylase towards adenosine and inosine (EC 2.4.2.1) assays. To take into account the effects of the inosine nucleosidase activity present, adenosine deaminase activity was calculated on the basis of the sum of label recovered in both inosine and hypoxanthine spots. Solvent II was used for the guanine deaminase (EC 3.5.4.3), guanine phosphorylase (EC 2.4.2.1), guanine PRTase (EC 2.4.2.8), guanosine nucleosidase (EC 3.2.2.1) and guanosine phosphorylase (EC 2.4.2.1) assays and Solvent III for the adenine PRTase (EC 2.4.2.7), hypoxanthine PRTase (EC 2.4.2.8), xanthine PRTase (EC 2.4.2.22), nucleoside kinases (EC 2.7.1.15, EC 2.7.1.20, EC 2.7.1.73) and phosphotransferase (EC 2.7.1.77) assays.

IMP dehydrogenase (EC 1.2.1.14) was assayed radiochemically as described previously (Hassan and Coombs 1986a). AMP deaminase (EC 3.5.4.6), nucleosidase and phosphorylase with xanthosine as substrate, adenylosuccinate lyase (EC 4.3.2.2), GMP reductase (EC 1.6.6.8), adenylosuccinate synthetase (EC 6.3.4.4) and GMP synthetase (EC 6.3.5.2) were assayed spectrophotometrically as described previously (Hassan and Coombs 1985b). 3'-AMP and 5'-AMP nucleotidases (EC 3.1.3.6 and EC 3.1.3.5, respectively) were assayed colorimetrically as described previously (Hassan and Coombs 1986a). Adenosine deaminase, guanine deaminase and PRTase activities were determined at a range of pH obtained using the following buffers: Tris-maleate, 50 mM (pH 6.0-7.0) and Tris-HCl, 50 mM (pH 7.0-9.0). The apparent K_m values for some substrates were determined by the Lineweaver-Burk method (1934).

RESULTS

The specific activities of the purine-metabolising enzymes detected in the soluble fraction of B. divergens are given in Table 1. The corresponding activities in material derived from uninfected blood are also included in the Table for comparison. In each case, the enzyme activity was apparently absent or insignificant in the material derived from uninfected blood, whereas there were high levels of activity of many enzymes in parasite lysates.

Adenosine deaminase and guanine deaminase were both present at high activity, whereas adenine deaminase was present with low levels of activity and AMP deaminase was below the level of detection. Adenosine deaminase and guanine deaminase were both active over a broad pH range (7.4-8.2) and the apparent K_m values for adenosine and guanine were 4×10^{-4} M and 3×10^{-4} M, respectively. Examination of the radiolabel distribution in sequential segments of the TLC plate confirmed that adenosine deaminase competes with other enzymes for adenosine as substrate. Coformycin (10^{-4} M) inhibited adenosine deaminase by 92% and its addition to the assay mixtures resulted in apparent two-fold increases in the activities of adenosine nucleosidase, adenosine phosphorylase and adenosine kinase. Consequently, it was routinely included in the assay mixtures for these enzymes. Guanine deaminase was found to compete for the substrate guanine in the assays of guanine phosphorylase and guanine PRTase such that approximately 8% of it was converted to xanthine by the termination of the assay. The 4-amino-5-imidazolecarboxamide at 10^{-4} M inhibited guanine deaminase by 84%, but addition of this compound to the assay mixtures for guanine phosphorylase and guanine PRTase did not result in increases in the activities detected. This suggests that the substrate concentration was not rate limiting under the conditions used.

Table 1 The activities of purine-metabolizing enzymes in Babesia divergens

Substrate	Deaminase	Nucleosidase	Phosphorylase	Phosphorylase	PRTase	Kinase
Adenine	0.6 ± 0.1 (<0.06)			0.2 ± 0.1 (<0.04)	12 ± 2 (0.04)	
Adenosine	23 ± 3 (0.06)	2 ± 1 (<0.1)	3 ± 0.4 (0.06)			7 ± 1 (<0.1)
AMP	< 1 (< 1)					26 ± 3 (< 1)
Hypoxanthine				29 ± 0.7 (0.1)	19 ± 1 (0.02)	
Inosine		6 ± 1 (<0.06)	11 ± 3 (1)			6 ± 1 (<0.01)
Guanine	28 ± 4 (0.4)			8 ± 0.8 (0.9)	13 ± 4 (0.2)	
Guanosine		2 ± 0.8 (<0.02)	5 ± 1 (0.04)			15 ± 4 (0.06)
GMP						2 ± 1 (< 1)
Xanthine				4 ± 0.6 (0.1)	0.6 ± 0.1 (<0.01)	
Xanthosine		3 ± 1 (< 1)	5 ± 2 (< 1)			< 1

The activities are given in nmol min^{-1} (mg protein)$^{-1}$ and are the means ± SD from replicate experiments. The figures in parentheses represent the activities in material derived from uninfected blood.

Purine nucleoside phosphorylase activity towards adenosine, inosine, guanosine and xanthosine and active in both catabolic and anabolic directions was detected in B. divergens extracts. The values obtained for apparent nucleosidase activities were less than the catabolic phosphorylase rates such that these conversions could have been due to phosphorylase using endogenous phosphate. The presence of nucleosidase, however, cannot be excluded. Purine phosphoribosyltransferase activities towards adenine, guanine and hypoxanthine were relatively high, whereas that towards xanthine was present only at a low level. The activity was optimal at pH 7.4 towards adenine and at pH 7.8 with guanine and hypoxanthine as substrates. Nucleoside kinase activity was found with all nucleosides tested with the exception of xanthosine. The relative activities towards different substrates was found to be guanosine > adenosine > inosine. AMP kinase activity also was present in parasite extracts, being 13-fold higher in activity than GMP kinase.

Purine nucleoside phosphotransferase towards inosine using p-nitrophenylphosphate as phosphate donor was below the limit of detection (0.09 nmol/min/mg protein). 5'-AMP nucleotidase activity was present, albeit at a low level (5 nmol/min/mg protein in homogenates); the activity was largely particulate, with more than 85% being recovered in the particulate fraction. 3'-AMP nucleotidase activity was undetectable in homogenates (less than 0.7 nmol/min/mg protein). Similarly none of the enzymes (adenylosuccinate lyase, adenylosuccinate synthetase, IMP dehydrogenase, GMP synthetase and GMP reductase) involved in purine nucleotide interconversions could be detected in soluble extracts of B. divergens. The limits of detection of these enzymes were 0.7, 0.7, 1.0, 0.6, 1.0 nmol/min/mg protein, respectively.

DISCUSSION

This is the first reported survey of purine-metabolising enzymes in a piroplasm and it has revealed that Babesia divergens possesses enzymes capable of salvaging purine bases and nucleosides. These enzymes probably are of prime importance in the provision of purine nucleotides for the parasite, especially if, as seems likely, babesias are similar to most other parasitic protozoa in being unable to synthesise purines de novo. The only exceptions to this rule so far found are the occasional, opportunistic parasites, Acanthamoeba species (Hassan and Coombs 1986b).

This study represents only the first stage in a full analysis of the pathways operating in the salvage of purines by B. divergens and characterization of the key enzymes involved. Nevertheless, the results produced so far give strong indications of the most important metabolic routes in the parasite. The high activities of adenosine deaminase and inosine phosphorylase suggest that B. divergens may be similar to malaria parasites (Daddona et al. 1984; Schimandle and Sherman 1983; Schimandle et al. 1985) in converting exogenous adenosine mainly to hypoxanthine before incorporation of the latter into nucleotides through the action of hypoxanthine PRTase. Such a metabolic sequence could help to explain the findings from the uptake studies of Irvin and his colleagues (1978 and 1979). The presence of adenosine kinase, however, suggests that this nucleoside could also be converted directly to its nucleotide. Similarly, the detection of kinase activities towards other nucleosides indicates that nucleotide synthesis is mediated both directly from the base by the action of the appropriate PRTase and also via the nucleoside. Moreover, the lack of detectable purine nucleotide interconverting enzymes in B.

divergens suggests that these routes (purine PRTase and nucleoside kinase) may be mainly responsible for the independent production of both adenine and guanine nucleotides. In this respect B. divergens would be relatively similar to Trichomonas vaginalis (Heyworth et al. 1982) and Entamoeba histolytica (Hassan and Coombs 1986a) but differ from Plasmodium species which, although adenylosuccinate synthetase is the only enzyme detected so far (Lukow et al. 1973), can convert hypoxanthine into both nucleotide types via IMP (Yamada and Sherman 1981; Reyes et al. 1982). It would be premature, however, to rule out nucleotide interconversions as being significant to piroplasms, especially taking into account that hypoxanthine is incorporated into the parasite so readily (Irvin and Young 1979); it may simply be that the enzymes are at relatively low specific activities. Further studies following the fate of incorporated purines into parasite nucleotides are required to clarify the situation. The high activity of guanine deaminase is also rather surprising and is, as yet, unexplained, especially as only low activities of xanthine-metabolising enzymes were detected. The enzyme activities partially characterised (adenosine deaminase, guanine deaminase and the PRTases) appear to be quite similar to those of Plasmodium species (Walter and Konigk 1974; Schimandle and Sherman 1983) and trypanosomatids (Kidder and Nolan 1981; Nolan 1984; Hassan and Coombs 1985a and b) with respect to their pH optimum, substrate K_m and sensitivity to inhibitors; more detailed analysis of the individual enzymes are required to determine the full extent of the similarities and differences between isofunctional mammalian and piroplasm enzymes. Overall, therefore, the main contribution of the present study has been the confirmation that purine-metabolising

enzymes are present in B. divergens and the pointers it has given to the enzymes that hold the most promise for further study.

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REFERENCES

Daddona PE, Wiesmann WP, Lambros C, Kelley WN, Webster HK (1984) Human malaria parasite adenosine deaminase. J Biol Chem 259:1472-1475.

Gutteridge WE, Coombs GH (1977) Biochemistry of Parasitic Protozoa, MacMillan Press, London.

Hassan HF, Coombs GH (1985a) Purine phosphoribosyltransferases of Leishmania mexicana mexicana and other flagellate protozoa. Comp Biochem Physiol 82B: 773-779.

Hassan HF, Coombs GH (1985b) Leishmania mexicana: Purine-metabolizing enzymes of amastigotes and promastigotes. Exp Parasitol 59: 139-150.

Hassan HF, Coombs GH (1986a) Purine-metabolising enzymes in Entamoeba histolytica. Mol Biochem Parasitol 19: 19-25.

Hassan HF, Coombs GH (1986b) De novo synthesis of purines by Acanthamoeba castellanii and A. astronyxis. IRCS Med Sci 14: 559-560.

Heyworth PG, Gutteridge WE, Ginger CD (1982) Purine metabolism in Trichomonas vaginalis. FEBS Lett 141: 106-110.

Irvin AD, Young ER (1977) Possible in vitro test for screening drugs for activity against Babesia and other blood protozoa. Nature 269: 407-409.

Irvin AD, Young ER, Purnell RE (1978) The in vitro uptake of tritiated nucleic acid precursors by Babesia spp. of cattle and mice. Int J Parasitol 8:19-24.

Irvin AD, Young ER (1979) Further studies on the uptake of tritiated nucleic acid precursors by Babesia spp. of cattle and mice. Int J Parasitol 9:109-114.

Kidder GW, Nolan LL (1981) The in vivo and in vitro action of 4-amino-5-imidazolecarboxamide in trypanosomid flagellates. Mol Biochem Parasitol 3:265-269.

Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56: 658-666.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Lukow I, Schmidt G, Walter RD, Konigk E (1973) Adenosinmonophosphat-salvage-synthese bei Plasmodium chabaudi. Z Tropenmed Parasit 24: 500-504.

Marr JJ, Berens RL (1983) Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Mol Biochem Parasitol 7: 339-356.

Nolan LL (1984) Partial purification and characterization of guanine aminohydrolase from Trypanosoma cruzi. Curr Microbiol 11: 217-220.

Phillips RS (1984) Babesia divergens in splenectomised rats. Res Vet Sci 36: 251-255.

Reyes P, Rathod PK, Sanchez DJ, Mrema J, Rieckman KH, Heidrich H (1982) Enzymes of purine and pyrimidine metabolism from the human malaria parasite, Plasmodium falciparum. Mol Biochem Parasitol 5: 275-290.

Richards WH, Williams SG (1973) The removal of leucocytes from malaria infected blood. Ann Trop Med Parasitol 67: 249-250.

Schimandle CM, Sherman IW (1983) Characterization of adenosine deaminase from the malarial parasite, Plasmodium lophurae and its host cell, the duckling erythrocyte. Biochem Pharmacol 32: 115-122.

Schimandle CM, Tanigoshi L, Mole LA, Sherman IW (1985) Purine nucleoside phosphorylase of the malarial parasite, Plasmodium lophurae. J Biol Chem 260: 4455-4460.

Walsh CJ, Sherman IW (1968) Purine and pyrimidine synthesis by the avian malaria parasite Plasmodium lophurae. J. Protozool 15: 763-770.

Walter RD, Konigk E (1974) Hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase from Plasmodium chabaudi, purification and properties. Z Tropenmed Parasit 25: 227-235.

Wang CC (1984a) Parasite enzymes as potential targets for antiparasitic chemotherapy. J Med Chem 27: 1-9.

Wang CC (1984b) Purine and pyrimidine metabolism in Trichomonadidae and Giardia: Potential targets for chemotherapy. In: Singer TP, Mansour T, Ondarza R (eds) Biochemical basis of drug action, Academic Press, New York, pp 133-145.

Yamada KA, Sherman IW (1981) Purine metabolizing enzymes of Plasmodium lophurae and its host cell, the duckling (Anas domesticus) erythrocyte. Mol Biochem Parasitol 2: 349-358.

Chapter 12

THE EFFECTS OF PURINE ANALOGUES ON THE GROWTH *IN VITRO* OF *LEISHMANIA MEXICANA MEXICANA* PROMASTIGOTES

To be submitted as: Hassan, H.F. and Coombs, G.H. 1986. The effects of purine analogues on the growth *in vitro* of *Leishmania mexicana mexicana* promastigotes.

SUMMARY

Several purine analogues were found to be potent inhibitors of *Leishmania mexicana mexicana* growth *in vitro*. Especially effective were allopurinol, 4-aminopyrazolo(3,4-d)pyrimidine, 4-mercaptopyrazolo (3,4-d)pyrimidine, formycin A, formycin B, tubercidin, 6-thioguanosine, 6-methylpurine and sinefungin. Other trypanosomatids were similarly inhibited by allopurinol, tubercidin and sinefungin. The anti-leishmanial effects of allopurinol, 4-aminopyrazolo(3,4-d)pyrimidine, 4-mercaptopyrazolo(3,4-d)pyrimidine and tubercidin were variously antagonised by adenine, hypoxanthine, adenosine, inosine and *p*-nitro-benzylthioinosine.

INTRODUCTION

Leishmanias differ from mammalian cells in that they lack the ability to synthesize purine de novo and so rely entirely upon salvage pathways to provide the purines necessary for growth (Marr & Berens, 1985; Hassan & Coombs, 1986a). Some of the enzymes involved in these pathways have been shown to differ significantly from isofunctional mammalian enzymes (notably nucleoside hydrolase, hypoxanthine-guanine phosphoribosyltransferase, adenylosuccinate synthetase and GMP reductase), whereas others catalyse reactions that do not occur in the host (for instance adenine deaminase, xanthine phosphoribosyltransferase and nucleoside phosphotransferase) (Koszalka & Krenitsky, 1979; Kidder & Nolan, 1979; Spector, Jones & Elion, 1979; Tuttle & Krenitsky, 1980; Carson & Chang, 1981; Hassan & Coombs, 1985,1986b). Both categories of enzyme provide targets for chemotherapeutic exploitation and attempts to design drugs to do this have led to the discovery that some purine analogues, such as allopurinol, allopurinol riboside and formycin B, have significant antileishmanial activity (Nelson et al., 1979a; Rainey & Santi, 1983; Marr et al., 1984; Avila et al., 1986; Looker, Marr & Berens, 1986). The results so far suggest that this is a very promising approach to discovering new drugs for the treatment of leishmaniasis. Consequently, we have investigated the effects of a range of purine analogues on the growth of Leishmania m.mexicana promastigotes, and the factors which effect their toxicity, in order to extend our knowledge of their potential as antileishmanial agents.

MATERIALS AND METHODS

Parasite growth

Promastigotes of Leishmania m.mexicana (M 379), L.m.amazonensis (LV 78) and L.donovani (LV 9) were grown at 26°C in HOMEM medium with

10% (v/v) heat inactivated foetal calf serum as described previously (Hassan & Coombs, 1986b). Leishmania tarentolae and Crithidia fasciculata (Anopheles strain ATCC 11745) were cultured in LIT medium as described previously (Hassan & Coombs, 1986b). Gentamicin sulphate was added at 25 μg/ml to all culture media to inhibit bacterial growth.

Drug susceptibility experiments

The test protocol used in these experiments was as follows. Cultures were initiated at $5x10^5$ - $1x10^6$ parasites/ml and drugs were added, to appropriate concentrations, 24h later. For studies on the antagonism of the activity of purine analogues by purines or other analogues, the antileishmanial compounds were present at a molar ratio of 10:1 or 5:1 as indicated. Drug solutions were freshly prepared and sterilised using a millipore filter (0.22 μm). The numbers of motile parasites present in the cultures were counted daily, using an improved Neubauer hemocytometer, until parasite growth entered stationary phase. The efficacy of some compounds is given in terms of the LD_{50} and LD_{90} - the minimum concentration of drug used that reduced the number of promastigotes present after 3 days by 50% and 90%, respectively.

Materials

Allopurinol, 4-aminopyrazolo(3,4-d)pyrimidine (4APP) and 4-amino-6-hydroxypyrazolo(3,4-d)pyrimidine were purchased from Aldrich Chemical Company, Dorset, U.K. Other reagents were of analytical grade and obtained from Sigma Chemical Company, Poole, Dorset, U.K.

RESULTS

Many of the purine analogues tested were found to inhibit the growth of L.m.mexicana promastigotes in vitro (Figures 1 and 2 and

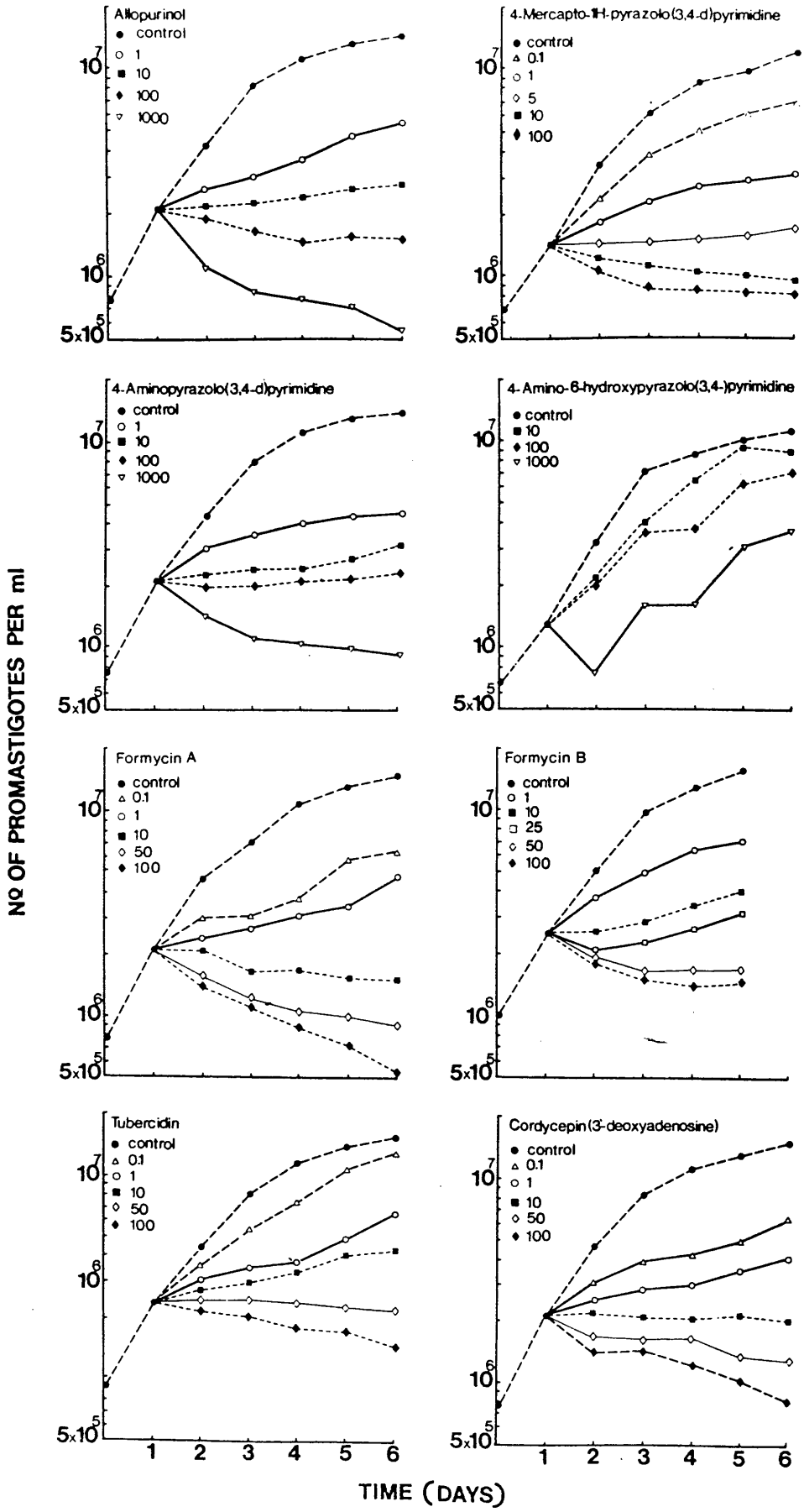

Fig.1

Figures 1 and 2. The effect of purine analogues on the growth of cultured promastigotes of L.m.mexicana. The figures given on the graph are the concentrations of compound tested and are in μM.

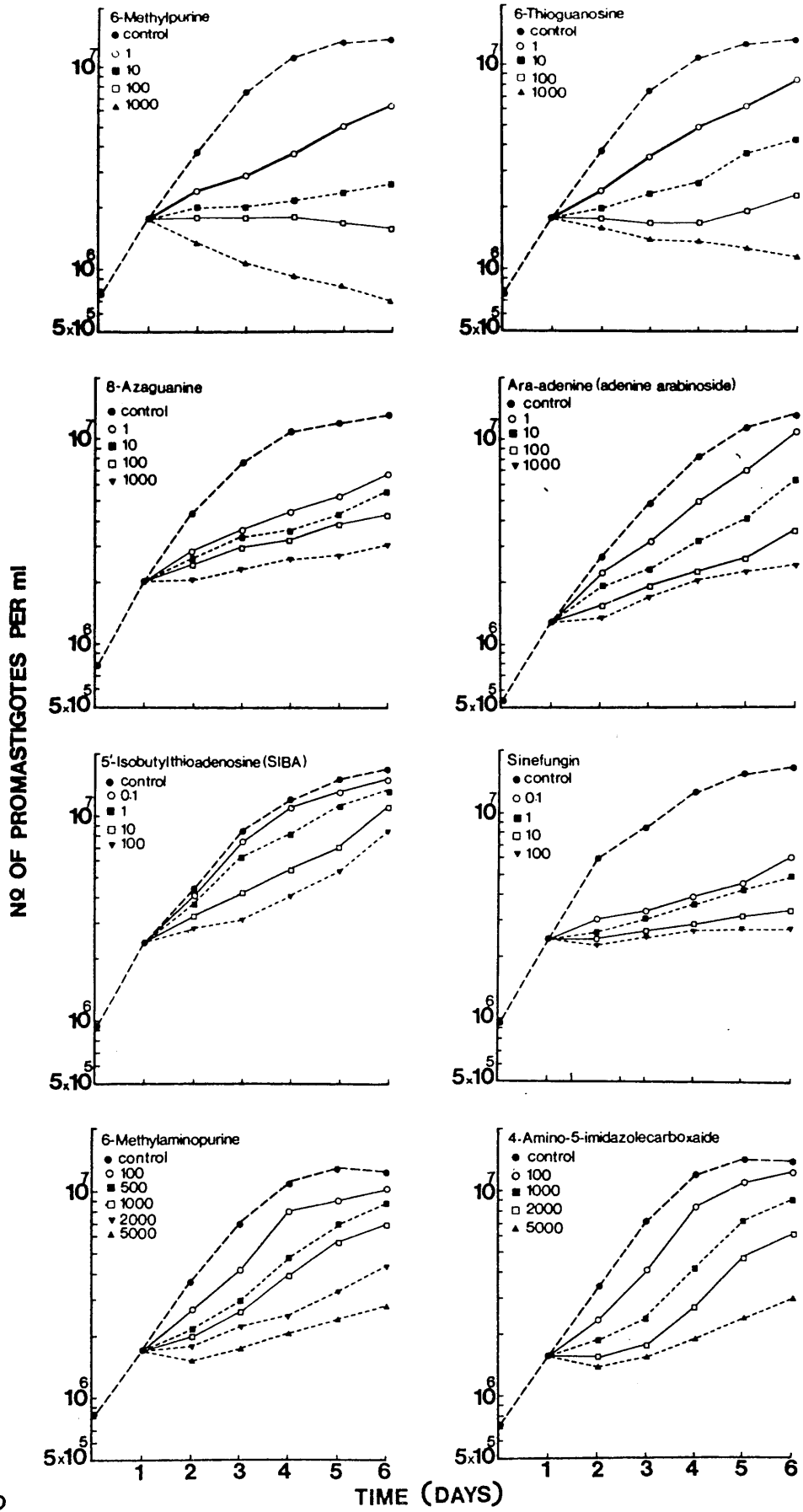
6-Methylpurine
control
1
10
100
1000
6-Thioguanosine
control
1
10
100
1000
8-Azaguanine
control
1
10
100
1000
Ara-adenine (adenine arabinoside)
control
1
10
100
1000
5'-Isobutylthioadenosine (SIBA)
control
0.1
1
10
100
Sinefungin
control
0.1
1
10
100
6-Methylaminopurine
control
100
500
1000
2000
5000
4-Amino-5-imidazolecarboxaide
control
100
1000
2000
5000
Nº OF PROMASTIGOTES PER ml
TIME (DAYS)

Fig.2

Table I). Pyrazolopyrimidine compounds proved to be potent growth inhibitors even at low concentrations. For instance, allopurinol and 4-aminopyrazolo(3,4-d)pyrimidine (4APP), at a concentration of 1 μM, inhibited the growth by 3 days by 61% and 67%, respectively. 4-Mercaptopyrazolo(3,4-d)pyrimidine (4MPP) was the most potent compound and caused 43% inhibition at 0.1 μM. Formycin A, formycin B, tubercidin and cordycepin all exhibited antileishmanial activity and at 1 μM reduced the parasite numbers by 3 days by 68%, 56%, 59% and 73%, respectively. Sinefungin, allopurinol, tubercidin and 5'-S-isobutylthioadenosine (SIBA) were also tested for activity against other Leishmania species and Crithidia fasciculata (Figure 3). Sinefungin and tubercidin were almost equally effective against all these parasites, whereas allopurinol affected them differently; L.m.amazonensis and C.fasciculata being quite sensitive, L.donovani less so, whereas L.tarentolae was relatively resistant. SIBA, on the other hand, was found to be active against L.m.amazonensis, L.tarentolae and L.donovani only when at high concentration and it had no significant effect on C.fasciculata.

It was found that promastigotes of L.m.mexicana cultured for 5 days in the presence of 10 μM or 100 μM allopurinol or 4APP resumed normal growth when subcultured into drug-free medium (data not shown), confirming that at these concentrations the drugs are leishmanistatic. The toxicities of allopurinol, 4APP and 4MPP to L.m.mexicana promastigotes were antagonised by adenine or hypoxanthine (Figure 4). Adenine was more effective than hypoxanthine in reversing the growth inhibition induced by 4APP, whereas the two compounds were similarly effective at reversing the inhibitory effect of allopurinol and 4MPP. Growth inhibition by formycin B or tubercidin was not reversed by adenine or hypoxanthine (data not shown). Similarly, the activity of tubercidin was unaffected by adenosine or inosine, whereas p-

Table I. The effect of purine analogues on the growth *in vitro* of *L.m.mexicana* promastigotes.

Compound*	LD_{50} (μM)	LD_{90} (μM)	Highest concentration tested (μM)
4-Aminopyrazolo(3,4-d) pyrimidine-2'-deoxyriboside	100	NA	1000
2-Amino-6-mercaptopurine	500	NA	5000
Hypoxanthine arabinoside	1-10	> 100	1000
Azahypoxanthine	1000	NA	1000
6-Mercaptopurine	< 50	1000	5000
6-Mercaptopurine riboside	50	5000	5000
6-Mercaptopurine arabinoside	100	NA	1000
6-Methylmercaptopurine riboside	250	1000	5000
Kinetin riboside	> 100	NA	1000
4-Aminoimidazolecarboxamide riboside	NI	NA	5000

* Serial five- or tenfold dilutions were used.

NI, not inhibitory; NA, 90% inhibition not achieved.

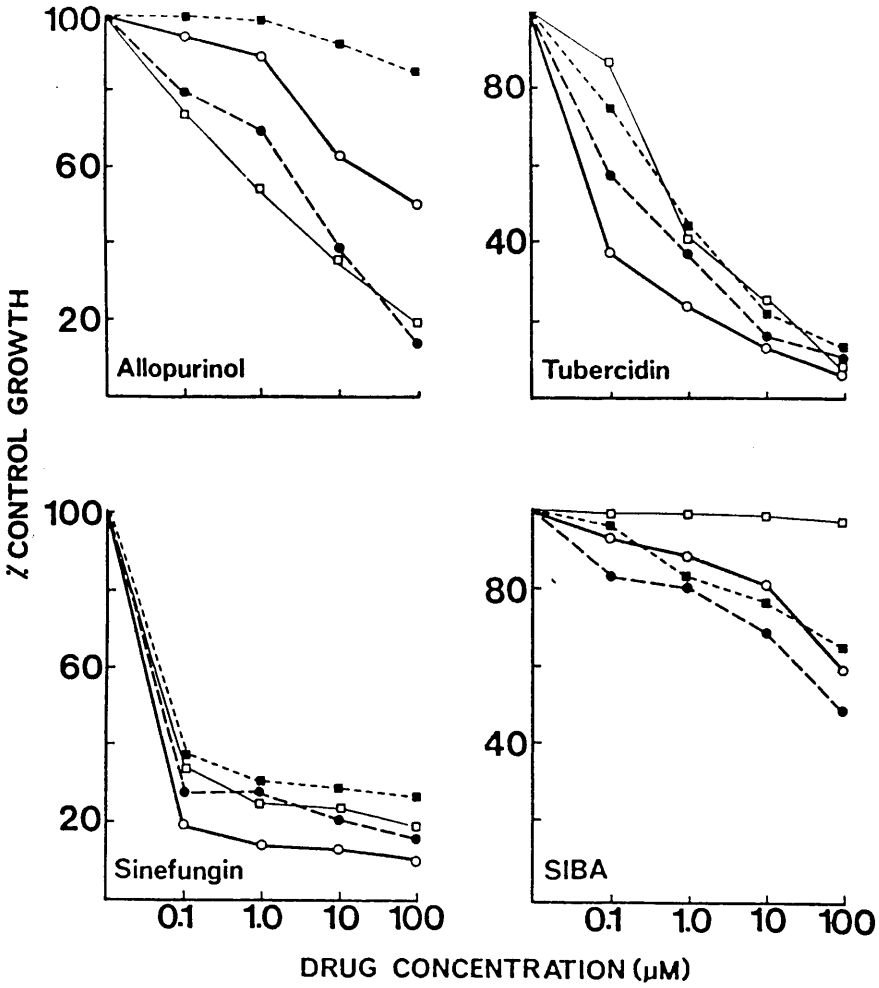

Figure 3. The effects of purine analogues on the growth of L.m.amazonensis (●), L.donovani (○), L.tarentolae (■) and C.fasciculata (□). The ordinate gives the number of viable organisms present after 3 days (L.tarentolae and C.fasciculata) or 5 days (L.m.amazonensis and L.donovani), expressed as a percentage of the number in the control (no drug).

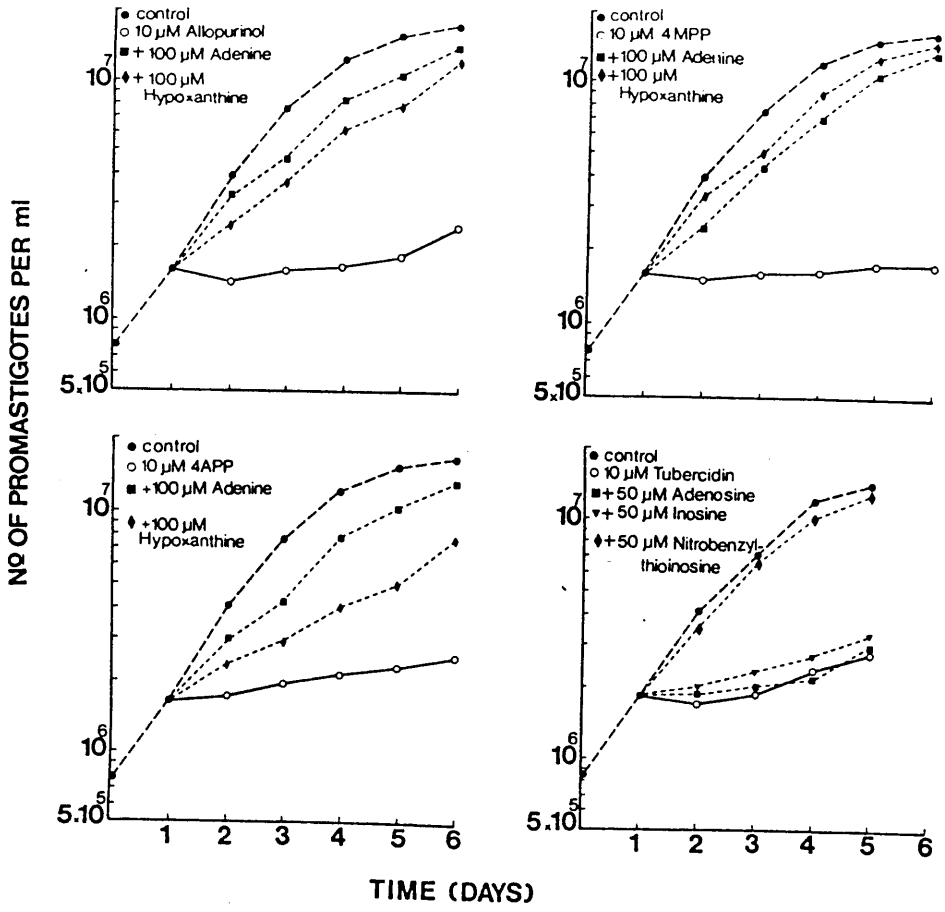

Figure 4. Effects of adenine, hypoxanthine, adenosine and p-nitrobenzylthioinosine on the toxicity of allopurinol, 4-aminopyrazolo(3,4-d)pyrimidine (4APP), 4-mercaptopyrazolo(3,4-d)pyrimidine (4MPP) and tubercidin to L.m.mexicana promastigotes.

nitrobenzylthioinosine, a known inhibitor of the adenosine transporter of various cells (Lynch et al., 1978), was found to be very effective at blocking the cytotoxicity of tubercidin (Figure 4).

DISCUSSION

The results presented are in agreement with those from previous studies, mainly with L.donovani, showing that allopurinol and 4APP have antileishmanial activity (Marr & Berens, 1977; Nelson et al., 1979a; Looker, Marr & Berens, 1986) and that the effect is leishmanistatic rather than leishmanicidal. It has been demonstrated that the selective toxicity of allopurinol and 4APP for L.donovani may in part be due to their conversion to ribotides by the parasite's hypoxanthine phosphoribosyltransferase and adenine phosphoribosyltransferase activities, respectively, and subsequent innibition of nucleotide metabolism (Nelson et al., 1979a; Marr & Berens, 1983; Looker, Marr & Berens, 1986). We have reported that allopurinol and 4APP also inhibit L.m.mexicana hypoxanthine phosphoribosyltransferase and adenine phosphoribosyltransferase, respectively, and this could be due to their competing as substrates for the parasite enzymes (Hassan & Coombs, 1985). These findings provide one possible explanation as to how hypoxanthine and adenine antagonise the antileishmanial activities of allopurinol and 4APP (Figure 4). Alternatively, the IMP formed from hypoxanthine may compete with allopurinol ribotide for adenylosuccinate synthetase. On the other hand, the antagonism of allopurinol activity by adenine could be due to its deamination to hypoxanthine or its direct utilisation by adenine phosphoribosyltransferase to produce AMP, which would compete with 4APP ribotide for conversion to the triphosphate and incorporation into RNA. The finding that hypoxanthine is less active than adenine at antagonising 4APP is consistent with our previous results that 4APP was relatively ineffective against

hypoxanthine phosphoribosyltransferase (Hassan & Coombs, 1985) and also suggests that the antagonistic effect of adenine is probably mediated, at least in part, at the level of adenine phosphoribosyltransferase. The finding that adenine and hypoxanthine can antagonise the activity of these purine analogues is relevant to their potential as antileishmanials. Their in vitro activity clearly is likely to be affected by the concentration of purines present. Methods of reducing this could have a profound effect on the efficacy of the compounds; this may provide a novel approach to increasing the effectiveness of these known antileishmanials.

It is of interest to note that 4-aminopyrazolo(3,4-d)pyrimidine-2'-deoxyriboside (4APP-2'-deoxyriboside) is several fold less active than 4-aminopyrazolo(3,4-d)pyrimidine against L.m.mexicana promastigotes (Table I and Figure 1). Similar findings have also been reported for other Leishmania species promastigotes (Avila et al., 1986). In this respect, it must be remembered that allopurinol and its riboside are converted to nucleotides by different enzymatic reactions (Nelson et al., 1979b); the former being catalysed by hypoxanthine phosphoribosyltransferase whereas the latter is mediated by nucleoside phosphotransferase. Similar enzymatic differences in the metabolism of 4APP and 4APP-2'-deoxyriboside by Leishmania species are suggested by our results and those of Avila et al. (1986). The activity of 4MPP against L.m.mexicana confirms the results of Marr et al. (1982) using various Leishmania species in vitro. The susceptibility of L.m.mexicana promastigotes towards formycin B observed in this study was quite similar to those reported for other leishmanial species (Berman et al., 1983; Neal, Croft & Nelson, 1985). The mode of action of formycin B has been shown to be associated with its conversion to formycin B 5'-monophosphate by the nucleoside phosphotransferase; this

nucleotide is subsequently aminated to formycin A ribotide, then converted to di- and triphosphates that become incorporated into RNA (Rainey & Santi, 1983). The activity of adenosine analogues (formycin A, cordycepin and adenine arabinoside) against L.m.mexicana promastigotes could be due to the ability of the parasite to metabolise these compounds via nucleoside kinase to their respective monoribotides, then to their triphosphates and so eventually incorporate them into nucleic acids; L.m.mexicana promastigotes contain high activities of nucleoside kinases (Hassan & Coombs, 1986b).

Tubercidin was found to be very active against L.m.mexicana promastigotes; this may in part due to the fact that it is actively transported across the plasma membrane (Iovannisci et al., 1984). The reversal of tubercidin activity by p-nitrobenzylthioinosine provides further evidence for the transport of this toxic nucleoside; p-nitrobenzylthioinosine is specific inhibitor of nucleoside transport in various cells (Lynch et al., 1978). This inosine analogue can be used to reduce the toxicity of tubercidin to mammals and such dual therapy has a possible application in malaria. The present finding, however, suggests that this approach may be less successful in leishmaniasis.

Sinefungin and SIBA have also been previously reported to have activity against various leishmanial species (Bachrach et al., 1980; Paolantonacci, Lawrence & Robert-Gero, 1985) and our present results are consistent with those findings. The mode of action of these drugs on Leishmania remains to be resolved. It has been postulated that the toxicity of sinefungin for mammalian cells is due to inhibition of polyamine synthesis or protein methylases (Robert-Gero et al., 1980). SIBA can also interfere with polyamine metabolism by inhibiting methylthioadenosine phosphorylase (Robert-Gero et al., 1980). We have detected this enzyme in L.m.mexicana, L.donovani and L.tarentolae (specific activity: 15±3, 17±2, and 6±2 nmol/min/mg protein,

respectively, unpublished data).

The antileishmanial effect of azaguanine could be due to its conversion to monoribotide by hypoxanthine-guanine phosphoribosyl-transferase, the analogue has been shown to be a substrate for the enzyme from L.donovani (Tuttle & Krenitsky, 1980), and its further phosphorylation to the triphosphate derivative which could be incorporated into nucleic acids. The 4-aminoimidazolecarboxamide and 6-methylaminopurine activities could have been due to inhibition of guanine deaminase, this activity of L.m.mexicana was inhibited by both compounds (I_{50}s:7.5×10^{-4}M and 2×10^{-4}M, respectively, unpublished data). Similar results have also been reported for other Leishmania species (Nolan & Kidder, 1980; Kidder & Nolan, 1981).

This study has confirmed that several purine analogues possess activity against promastigotes of L.m.mexicana and other species at such a level as to justify studies of their activity against amastigotes both in vitro and in vivo. A few have been studied in this way already (Berman, 1985), we await the results for the others with interest.

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REFERENCES

Avila, J.L., Polegre, M.A., Avila, A. & Robins, R.K. (1986). Action of pyrazolopyrimidine derivatives on american Leishmania spp. promastigotes. Comparative Biochemistry and Physiology 83C, 285-289.

Bachrach, U., Schnur, L.F., El-on, J., Greenblatt, C.L., Pearlman, E., Robert-Gero, M. & Lederer, E. (1980). Inhibitory activity of sinefungin and SIBA (5'-deoxy-5'-S-isobutylthioadenosine) on the

growth of promastigotes and amastigotes of different species of Leishmania. FEBS Letters 121, 287-291.

Berman, J.D. (1985). Experimental chemotherapy of leishmaniasis. In Leishmaniasis (Chang, K-P. & Bray, R.S., Eds.), pp. 111-138. Elsevier-Science Publications, Amsterdam, New York, Oxford.

Berman, J.D., Lee, L.S., Robins, R.K. & Revankar, G. (1983). Activity of purine analogs against Leishmania tropica within human macrophages in vitro. Antimicrobial Agents and Chemotherapy 24, 233-236.

Carson, D.A. & Chang, K-P. (1981). Phosphorylation and antileishmanial activity of formycin B. Biochemical and Biophysical Research Communications 100, 1377-1383.

Hassan, H.F. & Coombs, G.H. (1985). Purine phosphoribosyltransferases of Leishmania mexicana mexicana and other flagellate protozoa. Comparative Biochemistry and Physiology 82B, 773-779.

Hassan, H.F. & Coombs, G.H. (1986a). De novo synthesis by Acanthamoeba castellanii and A.astronyxis. IRCS Medical Science 14, 559-560.

Hassan, H.F. & Coombs, G.H. (1986b). A comparative study of the purine - and pyrimidine-metabolising enzymes of a range of trypanosomatids. Comparative Biochemistry and Physiology 84B, 217-223.

Iovannisci, D., Kaur, K., Young, L. & Ullman, B. (1984). Genetic analysis of nucleoside transport in Leishmania donovani. Molecular and Cellular Biology 4, 1013-1019.

Kidder, G.W. & Nolan, L.L. (1979). Adenine aminohydrolase: occurrence and possible significance in trypanosomatid flagellates. Proceedings of the National Academy of Science USA 76, 3670-3672.

Kidder, G.W. & Nolan, L.L. (1981). The in vivo and in vitro action of 4-amino-5-imidazolecarboxamide in trypanosomatid flagellates. Molecular and Biochemical Parasitology 3, 265-269.

Koszalka, G.W. & Krenitsky, T. (1979). Nucleosidases from Leishmania donovani. Journal of Biological Chemistry 254, 8185-8193.

Looker, D.L., Marr, J.J. & Berens, R.L. (1986). Mechanisms of action of pyrazolopyrimidines in Leishmania donovani. Journal of Biological Chemistry 261, 9412-9415.

Lynch, T., Lauzon, G., Naik, S., Cass, C. & Paterson, A. (1978). Inhibition of nucleoside uptake in HeLa cells by nitrobenzylthioinosine. Biochemical Pharmacology 27, 1303-1304.

Marr, J.J. & Berens, R.L. (1977). Antileishmanial effect of allopurinol. Journal of Infectious Diseases 136, 724-732.

Marr, J.J. & Berens, R.L. (1983). Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. Molecular and Biochemical Parasitology 7, 339-356.

Marr, J.J. & Berens, R.L. (1985). Purine and pyrimidine metabolism in Leishmania. In Leishmaniasis (Chang, K-P. & Bray, R.S., Eds.), pp. 65-78. Elsevier Science Publications, Amsterdam, New York, Oxford.

Marr, J.J., Berens, R.L., Nelson, D.J., Krenitsky, T.A., Spector, T., Lafon, S. & Elion, G. (1982). Antileishmanial action of 4-thiopyrazolo (3,4-d)pyrimidine and its ribonucleoside. Biochemical Pharmacology 31, 143-148.

Marr, J.J., Berens, R.L., Cohn, N., Nelson, D.J. & Klein, R. (1984). Biological action of inosine analogs in Leishmania and Trypanosoma spp. Antimicrobial Agents and Chemotherapy 25, 292-295.

Neal, R.A., Croft, S.L. & Nelson, D.J. (1985). Antileishmanial effect of allopurinol ribonucleoside and the related compounds, allopurinol, thiopurinol, thiopurinol ribonucleoside, and of formycin B, sinefungin and the lepidine WR6026. Transaction of the Royal Society of Tropical Medicine and Hygiene 79, 122-128.

Nelson, D.J., Bugge, C., Elion, G., Berens, R.L. & Marr, J.J. (1979a). Metabolism of pyrazolo(3,4-d)pyrimidines in Leishmania donovani and Leishmania braziliensis. Journal of Biological Chemistry 254, 3959-3964.

Nelson, D.J., Lafon, S., Elion, G., Berens, R.L. & Marr, J.J. (1979b). Allopurinol riboside as an antileishmanial agent. Journal of Biological Chemistry 254, 11544-11549.

Nolan, L.L. & Kidder, G.W. (1980). Inhibition of growth and purine metabolising enzymes of trypanosomatid flagellates by N-methyladenine. Antimicrobial Agents and Chemotherapy 17, 567-571.

Paolantonacci, P., Lawrence, F. & Robert-Gero, M. (1985). Differential effect of sinefungin and its analogs on the multiplication of three Leishmania species. Antimicrobial Agents and Chemotherapy 28, 528-531.

Rainey, P. & Santi, D. (1983). Metabolism and mechanism of action of formycin B in Leishmania. Proceedings of the National Academy of Science USA 80, 288-292.

Robert-Gero, M., Pierre, A., Vedel, M., Enouf, J., Lawrence, F., Raies, A. & Lederer, E. (1980). Analogues of S-adenosylhomocysteine as in vitro inhibitors of transmethylases and in vivo inhibitors of viral oncogenesis and other cellular events. In Enzyme Inhibitors (Brodbeck, U., Ed.), pp. 61-74. Verlag Chemie, Weinheim, Federal Republic of

Germany.

Spector,T., Jones, T. & Elion, G. (1979). Specificity of adenylosuccinate synthetase and adenylosuccinate lyase from Leishmania donovani. Journal of Biological Chemistry 254, 8422-8426.

Tuttle, J. & Krenitsky, T. (1980). Purine phosphoribosyltransferases from Leishmania donovani. Journal of Biological Chemistry 255, 909-916.