ALTERNATE PATHWAYS OF PURINE METABOLISM IN SOME AFRICAN TRYPANOSOMES

BY

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DECLARATION

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declare that the content of this thesis is my work.

Any collaboration with colleagues is gratefully

acknowledged.



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ABSTRACT

The alternate pathways of purine metabolism in Trypanosoma conqolense; T. vivax and T. brucei were investigated. Enzymes of purine salvage pathway were detected in the trypanosomes although there were differences in activity. Purine nucleoside hydrolase was found in the trypanosomes but purine nucleoside phosphorylase was found only in T. conqol^nse. Adenine aminohydrolase, an enzyme usually associated with bacteria was detected in T. vivax. The trypanosomes studied lack adenosine deaminase but adenosine monophosphate deaminase was detected in T. brucei and T. congoiense. Deaminases provide points at which interconversion of purines occur; thus differences exist at the level of metabolic interconversion points. A proposal for the salvage of purines in these trypanosomes is presented.

Transport of purines in T. qambilense and T. congoiense, measured as uptake of triated adenosine was shown to take place by mediated mechanism. Two transport loci, one transporting adenosine and guanosine, and the other transporting thymidine was demonstrated. The inhibitors of nucleoside transport in animal cells, nitrobenzylthioinosine and dipyridamole, did not Inhibit the uptake of adenosine by T. gambiense. Nitrobenyylthioinosine which also binds tightly to cells did not bind to either T. gambiense or T. congoiense.

efflux of ^{3}H--thymidine from erythrocytes was used to measure damage to erythrocyte membrane in control mice and mice infected with trypanosomes. The efflux in infected mice was higher than in the controls. Since erythrocytes have a high rate of purine turnover, this may be a major source of exogenous purines for the trypanosomes.

The toxicity to mice and rats and trypanocidal activity of the tubercidin was confirmed by the present study. The toxicity of tubercidin was alleviated by intraperitoneal injection of nitrobenzylthioinosinate (5mkj/kg) 5 minutes earlier. Nitrobenzylthioinosinate neither had acute toxic nor trypanocidal effect at the doses tested and did not interfere with the trypanocidal effect of tubercidin. It is suggested that this nucleoside inhibitor. nitrobenzylthioinosinate, should be evaluated in combination with necleoside analogues in the therapy of African trypanosomiasis.

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LIST OF ABBREVIATIONS

TCA	Tricarboxylic acid
GAP	Glyceraldehyde-3-phosphate
DHAP	Dihydroxyacetone phosphate
GP	Glycerol-3-phosphate
diPGA	1, 3-diphosphoglycerate
PEP	Phosphoenolpyruvate
PGA	Phosphoglycerate
Adr	Adenosine
Ad	Adenine
Hx	Hypoxanthine
In	Inosine
INP	Inosine 5'-monophosphate
KDNA	Kinetoplast deoxynucleic acid
NBMPR	(nitrobenzylthioinosine) 6- (4-nitrobenzyl) thio -9-β-D-ribofuranosyl purine
NBMPR-P	(nitrobenzylthioinosinate) 6- (4-nitrobenzyl) thio -9-β-D-ribofuranosyl purine 5'-monophosphate
SHAM	Salicylhydroxamic acid
DFMO	Difluoromethyl ornithine
Tu	Tubercidine (7-deazaadenosine)
ip	Intraperitoneally
R-1-P	Ribose-1-phosphate
PRPP	5-phosphoribosyl 1-pyrophosphate
PRTase	Phosphoribosyltransferase
VSG	Variant surface glycoprotein
PSG	Phosphate saline glucose buffer
VAT	Variable antigen
PEI	Polyethyleneimine

BACKGROUND AND OBJECTIVES

Parasitic protozoa in general lack de novo synthesis of purine base (Bone and Steinert, 1956; Walsh and Sherman, 1968; Jaffe and Gutteridge, 1973; Gutteridge and Coombs, 1977) and must therefore rely on the salvage of purines from the host to meet their purine requirements. Salvage of purines consists basically of two events (a) transport of the purines across the cell membrane and (b) its conversion to nucleotides and subsequent incorporation into DNA and RNA. The reactions leading to the salvage of purine bases and nucleosides in animal cells have been well studied but its biological significance is not understood (Murray, 1971; Kornberg, 1957). Similar information on the salvage of purines in parasitic protozoa, especially African trypanosomes, is scanty; but a number of purine salvage enzymes have been reported for trypanosomatids. These include phosphoribosyltransferases in Leishmania (Tuttle and Krenitsky, 1979), T. cruzi (Gutteridge and Davies, 1981), malaria parasites (Lukow et al, 1973; Yamada and Sherman, 1981; Webster et al, 1982; Reyes et al, 1982); adenine deaminase in Leishmania (Konigk and Rasoul 1978; Marr et al, 1978a); C. fasciculata (Kidder et al, 1977); and hydrolase in T. gambiense (Schmidt et al, 1975). More detailed pathways have also been worked out for some of these trypanosomatids (Gutteridge and Davies, 1981; Reyes et al, 1982). Amongst the African trypanosomes, T. gambiense and T. rhodesiense

investigated (Schmidt *et al*, 1975; Fish *et al*, 1982a and b) and a pathway for the salvage of purines proposed.

It is generally accepted that African trypanosomes lack *de novo* purine synthesis and must depend on preformed exogenous purines. It is thus not unreasonable to assume that membrane transport mechanisms must be operative in these parasites.

In general, this aspect of purine metabolism has received little attention. A preliminary report on membrane transport of purines by *T. brucei* and *T. congolense* has been published (James and Born, 1980).

The normal plasma concentration of purine derivatives is of the order of 10^{-8} to 10^{-7} M in animals (Miyazaki *et al*, 1974). Could the erythrocytes which have a rapid turnover of purines (Adams and Harkness, 1973) be a major source of exogenous purines for the trypanosomes?

This study was thus undertaken to investigate the following:

1. salvage of purines and derivatives by *T. brucei*; *T. vivax* and *T. congolense*.
2. uptake of purines;
3. the role of erythrocytes as a source of exogenous purines;

and 4. possible application of the results from the study.

CHAPTER 1

INTRODUCTION

1.1 General

Trypanosomes are parasitic protozoa that cause serious diseases of humans and domestic animals in tropical Africa and South America. The genus, Trypanosoma is generally subdivided into two main sections: the stercoraria and the salivaria. The stercoraria parasite, T. cruzi, causes Chagas disease and infects an estimated ten million people in South America. The salivarian trypanosomes cause sleeping sickness in humans (T. rhodesiense and T. gambiense), nagana in cattle (T. brucei, T. vivax and T. congolense), surra in horses and camels (T. evansi) and dourine in horses (T. equiperdum).

1.2 Historical perspective

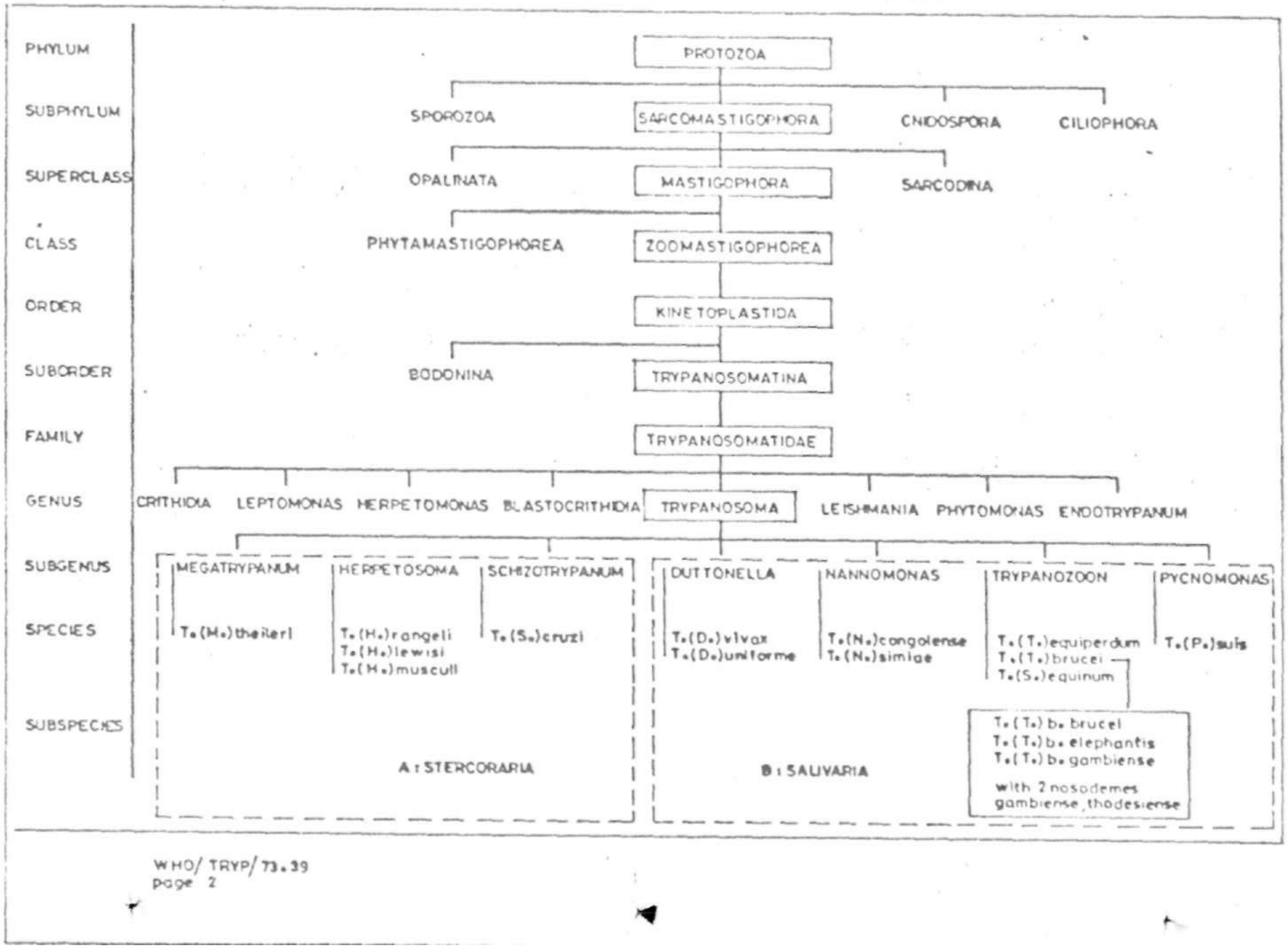
Trypanosomiasis has been known for centuries under various names before the causal organism was described. It has a unique distinction of being the only disease which has denied vast areas of land in Africa to all domestical animals except poultry (Hornby, 1952). The first recorded account of what appeared to be sleeping sickness disease was made by an Arab traveller Khaldoun (1373) who described the death of Sultan Mari Djata of the kingdom of Melle on the River Niger. Sultan died following a chronic disease in which excessive sleeping was a prominent symptom. It was not until 1843 that the first trypanosome was described by Gruby who found

the parasite in the blood of frog and named it Trypanosoma sanguinis. Later in 1878, trypanosome was found in the blood of rats in Bombay by Lewis and later it was named Trypanosoma lewisi. At this time, the relationship between trypanosome and sleeping sickness was not apparent or in fact recognized until Castellani (1903), a member of the first Royal Sleeping Sickness Commission sent to Lake Victoria to investigate the epidemic of sleeping sickness along the shore, found trypanosomes in the cerebrospinal fluid of a patient. This finding was later confirmed in a large number of patients. Other forms of trypanosomes were later discovered and identified: Trypanosoma congolense in 1904 by Broden; Trypanosoma vivax in 1905 by Ziemann. These discoveries generated tremendous interest in this disease and commissions were set up to investigate the devastating disease in many parts of Africa.

1.3 Classification of African trypanosomes

The systematics of mammalian trypanosomes has passed through several phases and finally in 1964, Hoare using a series of data collected over the years developed a classification based on phylogenetic, biological and morphological criteria. Trypanosomes transmitted by insect bites are distinguished as salivarian (salivary transmission) and stercorarian (faecal transmission) (Hoare, 1964). Further revisions were made by including differences in developmental characteristics to classify the two sections into seven subgenera (Table 1).

TAXONOMY OF SOME IMPORTANT MAMMALIAN TRYPANOSOMES.



The Trypanosoma brucei brucei or simply T. brucei is infectious to animals and is pathogenic to a variety of animal species. T. rhodesiense and T. gambiense which are pathogenic to man are subspecies of T. brucei and are regarded as separate nosodemes. The T. gambiense is differentiated from the T. rhodesiense by its low infectivity to laboratory animals and is well adapted to man in whom it causes infection of relatively low virulence, as opposed to the T. rhodesiense which usually causes acute infection.

The subgenus Nannomonas consists of T. congolense and T. simiae. The chief distinction between them being the high virulence of T. simiae to pigs. The Dutonella consists of T. vivax and T. uniforme. The development of these parasites in the vector takes place exclusively in the proboscis unlike T. congolense and T. brucei groups which take place in the midgut and salivary gland respectively.

1.4 Geography of trypanosomiasis

African trypanosomiasis is limited to the tropical zone of the continent by the distribution of the tsetse vector (Fig. 1). In the distribution map of human trypanosomiasis, it is believed that the typical T. gambiense form of infection occurs chiefly in West Africa and in parts of the Congo basin, but at times has appeared in some of the East African territories (Ford, 1971). T. gambiense is transmitted by the tsetse fly whose principal habitat is the dense vegetation along the rivers and forests; the common vectors being Glossina palpalis

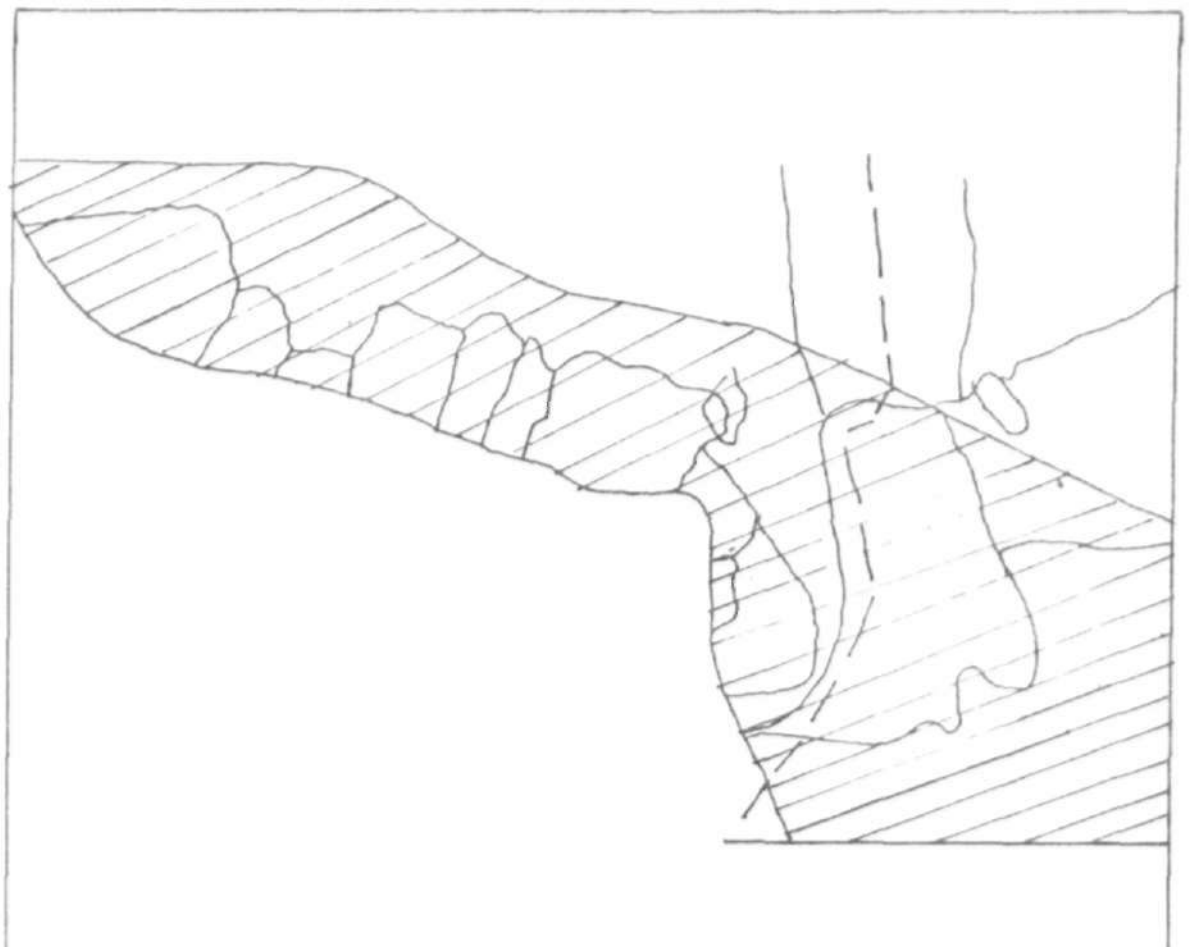


Fig. 1: Approximate indication of known foci of sleeping sickness in Africa (WHO 76142)

The shaded areas indicate the approximate foci of sleeping sickness in Africa. The broken line divides the area where T. gambiense is mostly found from the area where T. rhodesiense is found.

and G. fuscipes. The flies readily feed on man, bovids and aquatic reptiles but are quite flexible in their host preference.

The majority of T. rhodesiense infection occurs in East Africa but isolated cases have been reported from West Africa. In Nigeria, the main endemic areas for human trypanosomiasis lie in the northern part of the country although outbreaks have occurred in the south (Macfie and Gallagher, 1914), and in the Ahoada focus of the Delta region (Hutchinson and Watson, 1965).

The epizootiology of trypanosomiasis of livestock is more complex for it depends on three major biological elements: the host, the vector and the parasite. Although most species of Glossina can transmit T. congolense, it is evident from the distribution pattern that G. morsitans exhibits higher rates of infection than other species (Hoare, 1972). Equally the tsetse flies of the Morsitan group transmit T. vivax. Trypanosoma vivax is more important in West Africa than in East Africa since it is generally more virulent in West Africa. It has also established itself in Latin America where it is transmitted by blood sucking insects (Soltys and Woo, 1977). Studies carried out on cattle during their migration from the northern to the southern part of Nigeria show that T. vivax is the predominant parasite in the country (Unsworth, 1953; Mornet, 1954). Similar observations were described by Yesufu and Mshelbwala (1973) who examined not only the cattle but also the tsetse flies caught along the same route.

CHAPTER 2

BIOLOGY OF AFRICAN TRYPANOSOMES

2.1 Structural features of African trypanosomes

Trypanosomes are characterized by five main structural features (Fig. 2): a single flagellum, a single but often highly branched mitochondrion, a complex network of subpellicular microtubules and highly unusual microbody-like organelles (glycosomes) which contain the first nine enzymes of glycolysis (Opperdoes and Borst, 1977).

The nucleus of T. brucei is in a central or slightly posterior position with the golgi apparatus nearby. The latter is believed to play a part in the production of glycoproteins of the surface coat. The surface coat is a layer of glycoproteins covering the outer surface of the parasite including the flagellum. It occurs exclusively in blood forms and the preceding insect metacyclics and is the location of the variant antigens (Vickerman, 1969). It is also the cause of the almost neutral charge carried by the blood forms, by which they differ from the negatively charged uncoated forms (procyclics) and the blood cells. The kinetoplast and associated single mitochondrion constitute the extranuclear DNA. The degree of development of the mitochondrion is related to the metabolic potential, which in turn, depends on the more or less demanding conditions of the parasite's environment. For instance, the blood forms have a small mitochondrion, while culture forms and the insect midgut forms have a well developed mitochondrion.

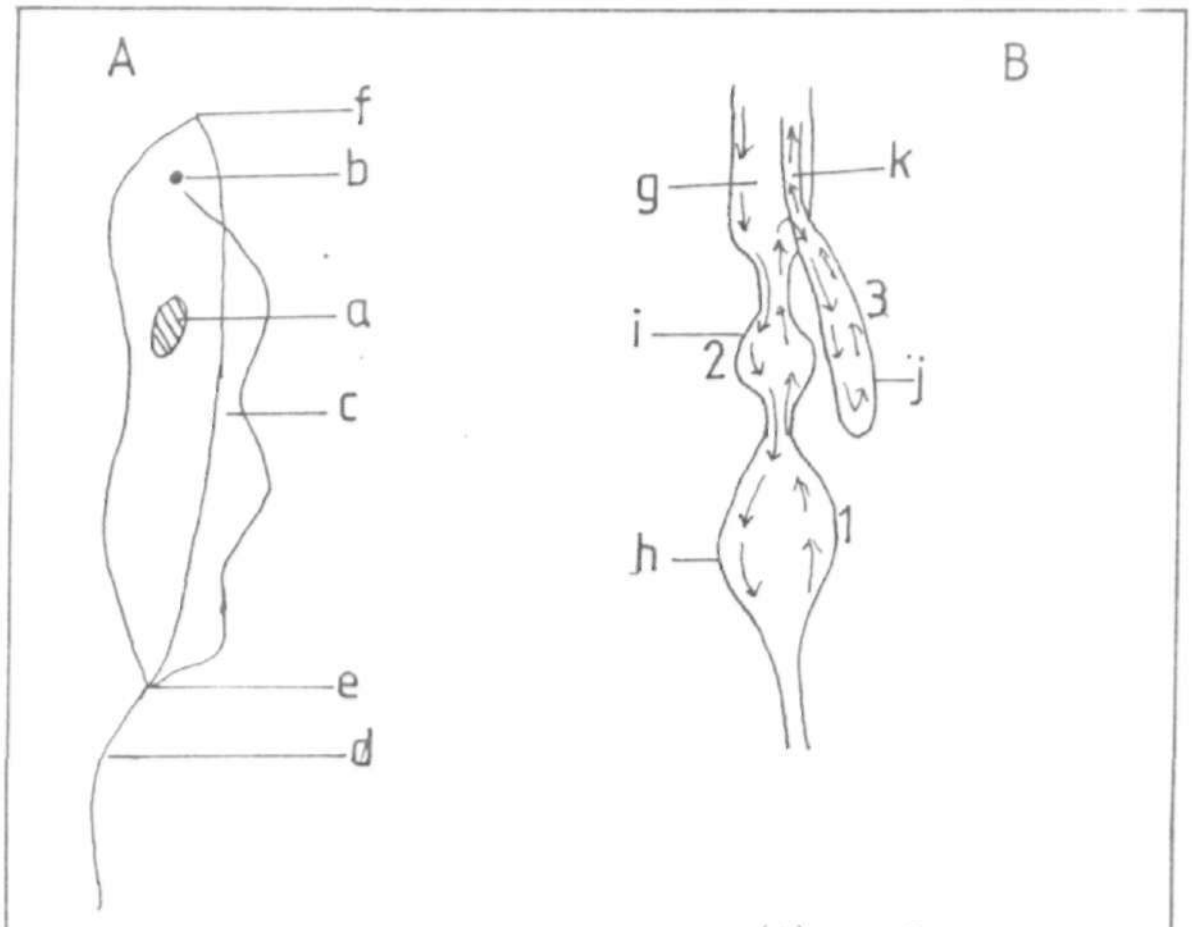


Fig. 2: A trypanosome trypomastigote (A) and T. gambiense development in tsetse fly (B) (Chandler and Read, 1961).

a. nucleus; b. kinetoplast; c. undulating membrane; d. flagellum; e. anterior end; f. posterior end; g. labial cavity; h. midgut; i. proventriculus; j. salivary gland; k. hypopharynx. 1. multiplication in midgut in trypanosome form. 2. long, slender trypanosomes in proventriculus. 3. passage to salivery glands and development there of crithidial and then infective trypanosome forms.

2.2 Life cycle of trypanosomes of man

The three trypanosomes belonging to the T. brucei species always occur in the form of trypomastigotes in the mammalian host. They are inoculated by the vector as metatrypanosomes, which are short, fairly slender forms (approximately 15 µm x 2.5 µm). After inoculation, while in the subcutaneous tissues, the organisms are converted to the so-called blood forms: long, slender forms (20 - 40 µm) and stumpy forms (15 - 25 µm x 3.5 µm) which occur in blood as well as tissue fluids. Some of the stumpy forms have the nucleus located near the posterior end, which is characteristic of the species, and are called the "posteronuclear" form. The slender forms have a long, free flagellum, up to 6 µm, and the position of the kinetoplast is subterminal, in contrast to the short, stumpy forms, in which the free part of the flagellum is usually less than 1 µm, or absent, while the kinetoplast is near the posterior end. Forms intermediate between slender and stumpy blood forms can occur. The slender forms predominate during rising parasitemias.

In the vector, the trypanosomes undergo morphological transformations, well described by Robertson (1913). The slender and stumpy blood forms, ingested by the tsetse fly with the blood meal, pass via the esophagus, the crop, and cardia (proventriculus) to reach the midgut, where presumably only the stumpy forms multiply and transform once again into slender trypamostigotes: the midgut forms (Fig. 2). The

latter have the kinetoplast located halfway between the posterior end and the nucleus, instead of in a terminal or subterminal position, as in the blood forms. In the salivary glands, the epimastigote form is taken, and these epimastigotes in turn change into metatrypanosomes, the only form by which mammals can be infected. Trypanosomes may also be present in the hemocoel (Mshelbwala 1972; Otieno 1973) but that does not necessarily exclude the above adopted cycle but may occur in parallel.

2.3 Life cycle of trypanosomes of livestock in Africa

Most trypanosomes pathogenic to domestic animals in Africa are transmitted cyclically by tsetse flies (Glossina sp.) exceptions being T. evansi and T. equiperdum. The disease in animals caused by tsetse-borne trypanosomes is known as nagana. The species of the genus Trypanosoma affecting animals in Africa have been assigned to the salivaria, which is divided into four subgenera, namely, Nannomonas, Duttonella, Trypanozoon and Pycnomonas (Hoare, 1972).

When meta-trypanosomes are inoculated by a tsetse fly into an animal, they change into normal blood forms and start to divide by binary fission. Soltys and Woo (1969) demonstrated the amastigote, promastigote, epimastigote and multinucleated stages in the spleen and the liver of mice inoculated with trypanosomes directly from rabbit that had been infected for longer than 3 weeks. After a tsetse fly has fed on an animal with trypanosomes in the blood, the

parasite undergoes a cyclical development which may take from 19 to 53 days. This period depends on several factors, among which temperature is particularly important. Only a small number or percentage of tsetse flies produce infective forms after feeding on an infected animal, however once the tsetse fly becomes infected, it remains so for the rest of its life.

The development of the trypanosomes ingested with the blood starts in the midgut, where they multiply as elongated trypomastigotes for some days and then migrate into the ectoperitrophic space and thence to the cardia. From the inner lumen of the cardia the flagellates migrate forward through the esophagus to the proboscis, where they are transformed into epimastigote forms. Finally, they reach the hypopharynx and change into metatrypanosomes, which are infective to the mammalian host. The trypanosome must undergo the complete cycle of development before it is infective except in the case of mechanical transmission. The entire developmental cycle of T. vivax in Glossina is confined to the proboscis. In the proboscis the trypanosome are transformed into typical epimastigote forms measuring 16 - 35 µm in length. They multiply intensively, giving rise to clusters of flagellates which eventually detach themselves from the wall and invade the hypopharynx where they are transformed into the "preinfective" trypomastigote forms. These forms are finally transformed into metatrypanosomes, which resemble the blood trypanosomes and are infective to animals. It takes

5 - 13 days for the whole cycle to be completed, this being partly dependent on the temperature. The flagellates may persist in the proboscis of the fly for up to 58 days. (Soltys and Woo, 1977).

2.4 Antigenic variation

It is characteristic of the salivarian trypanosomes that during the course of an infection, a sequence of populations of different antigenic types appears at irregular intervals, which is reflected by the relapsing parasitemias. These antigens enable the parasite to evade the host immune response. Variant antigens are present only while the parasites are in the vertebrate host, because, like the surface coat, their occurrence is restricted to the meta-cyclics and blood forms. Vickerman and Luckins (1969) demonstrated their location on the surface coat by electron microscopy, using ferritin-conjugated antibodies. The antigens seem to be soluble glycoproteins (Allasopp et al, 1971) with a single polypeptide chain of molecular weight 60,000 - 65,000 and containing 600 amino acid residues and 20 monosaccharide units (Cross, 1975; Barbet and McGuire, 1982). Antigenic variation is mediated through sequential expression of an extensive series of VSGS. Variant surface glycoproteins have strikingly different amino acid compositions. N-terminal amino acid sequences of variants studied were totally dissimilar, and peptide mapping and studies of C-terminal

peptides indicated immense variation in amino acid sequences between VSGs (Cross, 1978; Johnson and Cross, 1979). Carbohydrate content is also variable (Johnson and Cross, 1977).

There is predictable sequence of antigens arising from infection with one given strain of trypanosome (Gray, 1962; 1965). Any one variant developed from one strain reverts to one antigenic type after cyclical transmission through the vector (Gray, 1965; Vickerman, 1969). These constant features led to the suggestion that antigenic variation is an adaptive phenotypic phenomenon organized according to a pattern prefixed in the genetic information of the cell (Gray 1965; Vickerman, 1969). However, with recent studies using DNA recombinant technology, there is some understanding of the mechanism of antigenic variation. Using this method it has been shown that (a) each trypanosome in a stock contains the entire variant surface glycoprotein gene repertoire of that stock (Hoeijmakers et al, 1980). Diversity is therefore not generated by piecing gene segments together as occurs in antibody genes and (b) activation of some VSG genes involves a duplication of the gene and its transposition into an expression site (Borst et al, 1981). If there were a single active expression site per trypanosome, only one gene can be active at any time. This provides a simple though still speculative explanation for the tight intergenic exclusion seen between VSG genes.

CHAPTER 3

BIOCHEMISTRY OF AFRICAN TRYPANOSOMES

3.1 Intermediary Metabolism

In the mammal, bloodstream forms of *T. brucei* are entirely dependent on glucose for energy supply. These forms have no energy reserves and do not oxidize fatty acids or amino acids. The long, slender forms metabolize glucose aerobically to pyruvate, since they lack or have low levels of lactate dehydrogenase and tricarboxylic acid (TCA) cycle enzymes (Bowman and Flynn, 1976), but have a particulate terminal respiratory system, an L-α-glycerophosphate oxidase system (Grant and Sargent, 1960; Grant *et al*, 1961) (Fig. 3). Their rate of respiration is 50 times that of mammalian cells and consume an amount of glucose equal to their dry weight every hour (Fairlamb, 1982). Under anaerobic conditions or when the glycerophosphate oxidase is inhibited, glucose is metabolized at the same rate as during aerobic metabolism to form equimolar concentrations of pyruvate and glycerol. This is due to the fact that for each mole of triose phosphate converted to pyruvate, a mole of NAD+ is reduced to NADH, which, in turn, is reoxidized by a mole of DHAP to glycerophosphate. Glycerophosphate is then converted to glycerol, under the catalysis of glycerol kinase. This pathway is unique to bloodstream African trypanosomes and not found in other trypanosomatids.

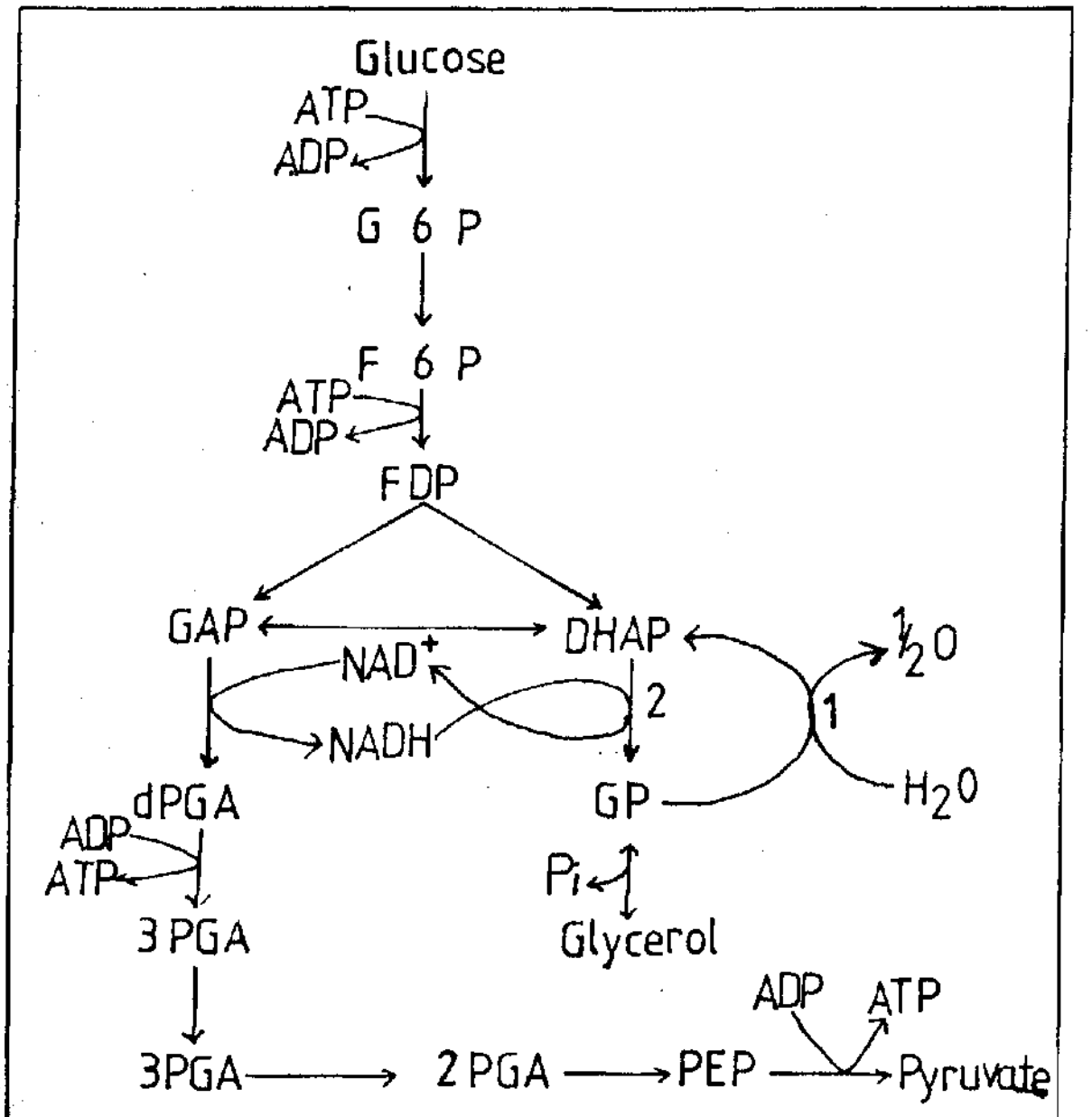


Fig. 3: Glycolytic pathway in long-slender bloodstream forms of T. brucei (Grant, 1966)

Enzymes 1. L-α-glycerophosphate oxidase;

2. L-α-glycerophosphate dehydrogenase.

In the short stumpy forms of T. brucei, some pyruvate can be further metabolized to acetate, succinate and a trace of CO_2. Although all the TCA cycle enzymes have been identified in these forms, the cycle is inoperative due to the extremely low activity of citrate synthetase and succinate dehydrogenase (Bowman and Flynn, 1976). The synthesis of TCA cycle enzyme is presumably an adaptation for survival in the insect midgut, since the long-slender forms do not generally infect tsetse flies.

The procyclics or the midgut forms of T. brucei contain a full complement of TCA cycle enzymes (Baernstein, 1963). Under anaerobic conditions, succinate is produced from pyruvate via malate and fumarate, involving fumarate reductase, similar to certain parasitic helminths. The main energy sources are amino acids, especially proline which is present in high concentrations in tsetse haemolymph. Proline is metabolized by proline oxidase and TCA cycle enzymes to aspartate and CO_2 (Bowman and Flynn, 1976).

3.2 Energy production in bloodstream form

Figure 3 shows that under aerobic conditions, bloodstream trypanosomes should obtain a net gain of 2ATP/mole of glucose used. Classically, one would expect that under anaerobic conditions, the net energy gain would be zero. However, studies have shown that ATP is produced from glycerophosphate and ADP under the catalysis of the highly active glycosomal

glycerol kinase (Hammond and Bowman, 1980). The glycosome contains the first nine enzymes of glycolysis (Opperdoes and Borst, 1977; Oduro *et al*, 1980), which may be associated within the organelle as a multi-enzyme complex (Oduro *et al*, 1980), that catalyse the following net reactions.

$$\text{Glucose} + [2]\,Pi + [2]\,DHAP \rightarrow [2]\,3PGA + [2]\,GP$$

$$\text{Glycerol} + Pi + [2]\,DHAP \longrightarrow 3PGA + [2]\,GP$$

In the overall reaction, the ADP/ATP and NAD^+/NADH ratios within the glycosome remain unchanged leading to the speculation that the glycosomal membrane acts as a permeability barrier for the entry of glycolytic intermediates, enabling *T. brucei* to maintain an extremely high glycolytic flux (Opperdoes and Borst, 1977). Thus, under anaerobic conditions, the glycosomal membrane permeability barrier to glycerophosphate would lead to accumulation of glycerophosphate, promoting the formation of ATP and glycerol from glycerophosphate and ADP (Hammond and Bowman, 1980).

3.3 Nucleic acid metabolism

Most metazoan cells possess two pathways leading to the synthesis of purine nucleotides. The *de novo* pathway (Buchanam, 1960) involves the synthesis of the purine molecule from simple precursors such as glycine and formate and the salvage pathway (Kornberg, 1957) which involves the utilization of preformed purine bases and nucleosides. The abnormalities of purine metabolism lead to inborn errors in humans.

Children with the most extreme degrees of purine nucleoside phosphorylase (PNP) deficiency have shown a profound hypouricemia and excretion of very little uric acid in urine as might be expected from the position of the enzyme defect (Seegmiller, 1979). Instead, they excrete inosine and guanosine in the urine. On molar basis, the total amount of purine excretion is comparable to the amount of urine acid excreted by children with Lesch-Nyhan disease who are deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase. In these two conditions, hypoxanthine is not reutilized for inosinic acid production. Adenosine deaminase, another enzyme of purine metabolism, is widely distributed in mammalian tissues, particularly in those of the reticuloendothelial system (Conway and Coke, 1939; Edwards et al, 1971). The activity of this enzyme is considerably elevated in the lymphoblasts of patients with acute lymphocytic and blast crisis chronic myeloid leukemias (Smyth and Harrap, 1975). Deficiency of adenosine deaminase is associated with combined immuno-deficiency disease, characterized by severely impaired humoral and cell mediated immunity and decreased numbers of peripheral blood lymphocytes (Giblett et al, 1972; Dissing and Knudsen, 1972). A gross deficiency of ecto-purine 5'-nucleotidase of the lymphocyte membrane to one-third of control values have been reported in congenital agammaglobulinemia in males. Affected males are unable to make gammaglobulins as a result of a B cell

dysfunction and therefore are particularly susceptible to recurrent bacterial infections.

Parasitic protozoa lack the ability to synthesize purines de novo but instead rely on salvage of preformed purine bases and nucleosides from host. The African trypanosomes have this biochemical peculiarity. Partial pathways for salvage and interconversion of adenine, adenosine and hypoxanthine have been demonstrated in T. gambiense (Schmidt et al, 1975; Fish et al, 1982a) and T. rhodesiense (Fish et al, 1982b). The enzymes, nucleoside hydrolase and phosphoribosyl transferases were shown to be active in these trypanosomes. Also, incorporation of purine bases and ribonucleosides were demonstrated in the bloodstream forms of T. brucei and T. congolense (James and Born, 1980). The efficiency of incorporation for purine bases was adenine > hypoxanthine > guanine > xanthine for T. gambiense and hypoxanthine > adenine > guanine > xanthine for T. rhodesiense (Fish et al, 1982a). Adenosine was taken up more efficiently in T. brucei and T. congolense than guanosine and inosine (James and Born, 1980).

All purine ribonucleosides are hydrolysed by the hydrolase of trypanosomes. In T. gambiense (Fish et al, 1982b; Schmidt et al, 1975) the efficiency of hydrolysis of the nucleosides was inosine > guanosine > adenosine > xanthosine while in T. rhodesiense (Fish et al 1982b) the efficiency

of cleavage was guanosine> adenosine > inosine > xanthosine. There is no adenase activity in T. gambiense (Schmidt et al, 1975; Fish et al, 1982b), but guanase activity is high in these trypanosomes (Fish et al, 1982b). Adenosine deaminase activity in T. vivax was reported by Akinrimisi (1981). In Trypanosoma cruzi (Gutteridge and Davies, 1981; Berens et al, 1981); Plasmodium (Lukon et al, 1973; Reyes et al, 1982); and C. fasciculata (Dewey and Kidder, 1973) several of purine salvage enzymes were domonstrated. Differences exist in the salvage pathway of adenosine utilization by these parasites (Fig. 4). Adenosine deaminase, purine nucleoside phosphorylase (with inosine or guanosine as substrates), hypoxanthine-guanine phosphoribosyltransferase and xanthine phosphoribosyltransferase were among the most active enzymes found in supernatant fractions of P. falciparum (Reyes et al, 1982). Xanthine oxidase was not detected in P. lophurae (Yamada and Sherman, 1981). Enzymes of nucleic acid metabolism involved in protein synthesis have been shown in bloodstream forms of T. brucei (Hans et al, 1975; Gbenle and Akinrimisi, 1981). A calcium-dependent endoribonuclease which is inhibited almost completely by 20 mM EDTA and cleaves poly(A) was isolated. The enzyme degrades adenine - containing polyribonucleotides to small oligonucleotides with 5'-phosphate and 3'-hydroxyl termi as final products (Gbenle and Akinrimisi, 1982).

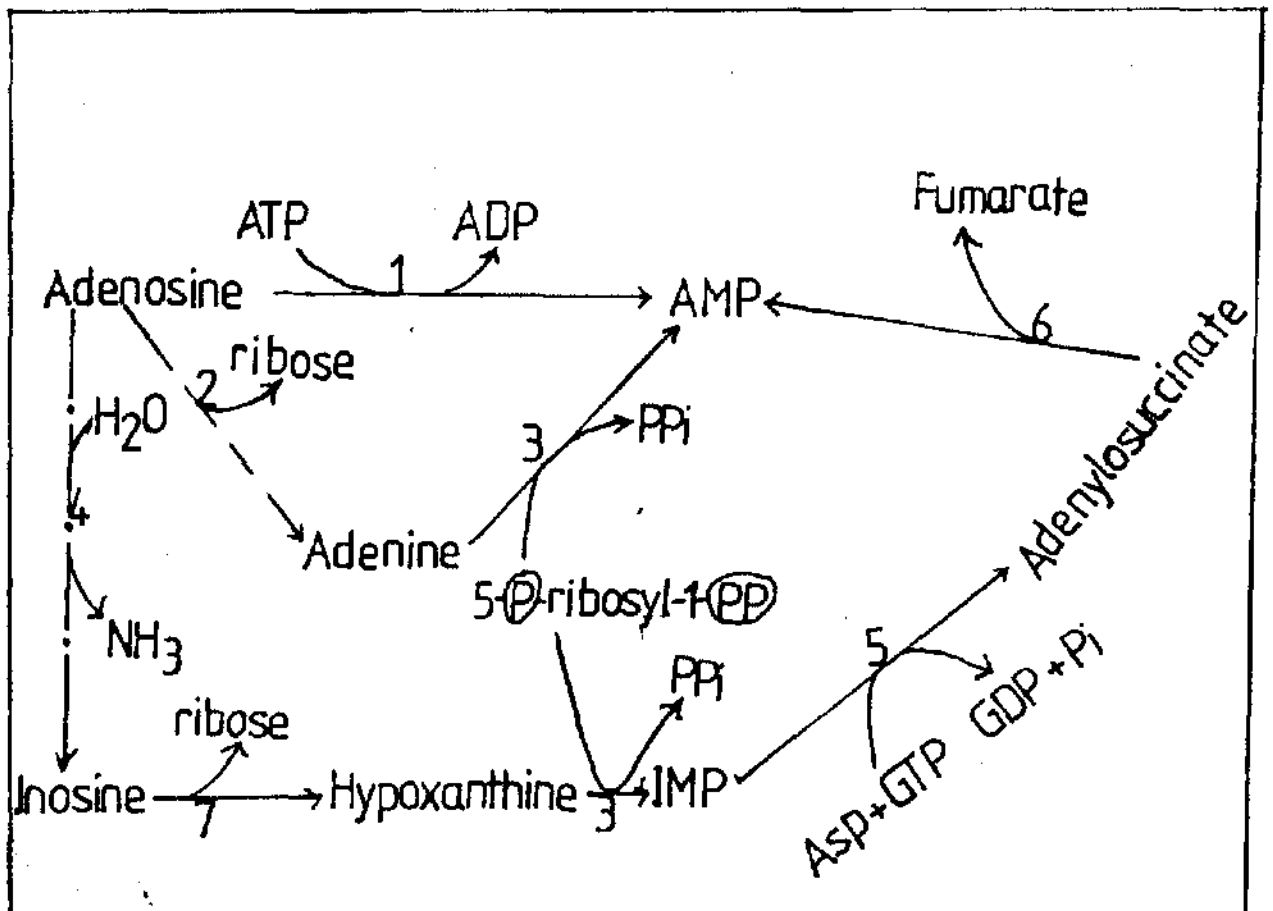


Fig. 4: Purine metabolism in *T. gambiense*; *P. chabaudi* and *T. cruzi*.

Solid lines indicate reactions common to all the parasites.

Broken line (--→): reaction present in *T. gambiense* (Schmidt *et al.*, 1975). *T. cruzi* (Gutteridge and Davies, 1981) but absent in *P. chabaudi* (Lukow *et al.*, 1973).

Broken line (-.-.→): reaction present in *T. cruzi* and *P. chabaudi* but absent in *T. gambiense*.

Enzyme activities: 1. adenosine kinase; 2. adenosine hydrolase/phosphorylase; 3. phosphoribosyltransferase; 4. adenosine deaminase; 5. adenylosuccinate synthetase; 6. adenylosuccinate lyase; 7. inosine hydrolase/phosphorylase.

All metazoan cells so far studied have both de novo and salvage pathways for pyrimidine synthesis. The de novo pathway involves the synthesis of the pyrimidine ring from aspartate and carbamyl phosphate, with orotic acid being a key intermediate in the pathway. The de novo pathway dominates in parasitic protozoa (Jaffe and Gutteridge, 1973). Plasmodium falciparum lacks the enzymes (uridine-cytidine kinase, deoxycytidine kinase, thymidine kinase) for the salvage of pyrimidine nucleosides (Reyes et al, 1982). These enzymes are key enzymes in the salvage pathway of pyrimidine metabolism.

3.4 Kinetoplast DNA

The kinetoplast DNA comprises a gigantic circular network of catenated circles of DNA, located in a special region of the mitochondrion at the base of the flagellum.

Each cell contains a single network and in T. brucei this comprises about 10% of the total cellular DNA with a mass of 4×10^9 daltons. Each network consists of about 6000 catenated small circular DNA molecules (Minicircles, 0.3 μm), interlaced with about 25 - 50 larger circles (maxicircles, 6 μm) (Fairlamb et al, 1978).

Minicircles in T. brucei are highly heterogeneous in sequence and may contain about 100 different minicircle sequences in a single network (Borst and Hoejimaker, 1979). Minicircles are not transcribed and their function is not known. The maxicircle is the genetic equivalent of mito-

chondrial DNA in higher eukaryotes (Fairlamb et al, 1978), and, in contrast to the minicircle, its sequence is homogenous and is evolving slowly.

How so complex a structure as kDNA is replicated and segregated into two identical daughter networks is an intriguing problem. Englund and Marini (1980) have proposed an attractive hypothesis where minicircles are released from the center of the network, replicated and then reattached at two sites opposite each other on the rim (Fig. 5). As network replication proceeds, an increasing proportion of the minicircles becomes nicked. The model will explain the rapid evolution of minicircle sequences due to random reattachment and hence distribution into the daughter networks. The hypothesis however does not explain how the maxicircles are replicated and segregated equally into the daughter networks, although maxicircle loops are known to cluster at the middle of the network, near the cleavage point, during replication (Fairlamb et al, 1978). The medical importance of research into kDNA is two-fold. First kDNA sequences may be used to distinguish strains that are morphologically and biochemically similar for epidemiological purposes. Second, an increased understanding of the biochemistry of kDNA may lead to the development of new antiparasitic agents. Many antitrypanosomal drugs bind selectively to kDNA. Specific inhibition of kDNA replication or expression result in the

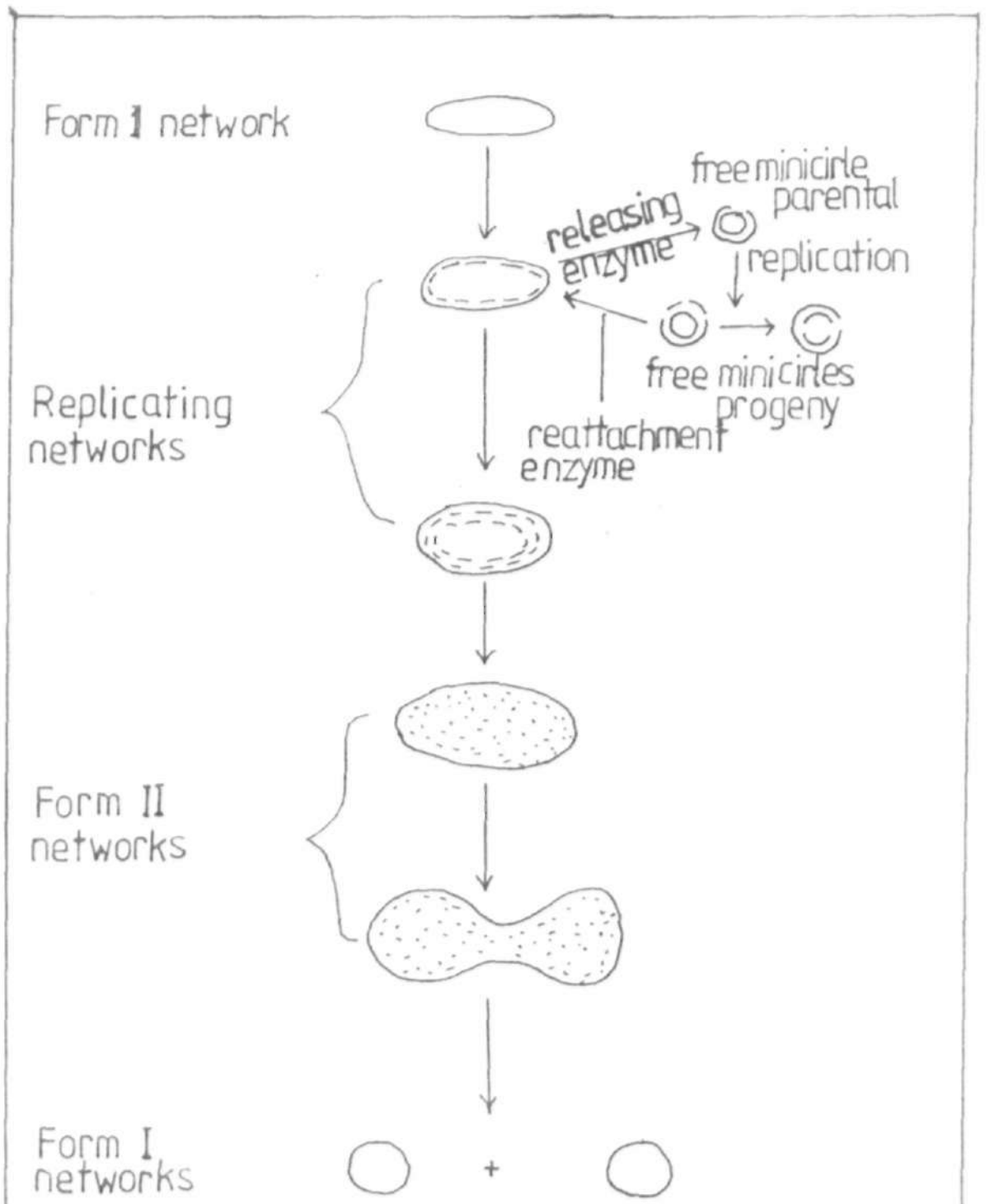


Fig. 5: Model for replication of a kinetoplast DNA network (Englund and Marini, 1980).

selective killing of T. cruzi, which appears to require functional kDNA throughout its life cycle (Fairlamb, 1982). This may not be so for bloodstream T. brucei which does not have active mitochondria. Indeed, some species such as T. evansi and T. equiperdum lack part or all of the kDNA network.

3.5 Some soluble enzymes of African trypanosomes

Ukoha (1980) demonstrated that seven soluble enzymes: alanine transferase (EC 2.6.1.2); aspartate amino transferase (EC 2.6.1.1); malate dehydrogenase (EC 1.1.137); glucose-phosphate isomerase (EC 5.3.1.9); glucose phosphotransferase (EC 2.7.5); malate oxidoreductase (EC 1.1.1.40) and glucose phosphate dehydrogenase (EC 1.1.1.49), from T. vivax; T. brucei; T. congolense; T. evansi and T. gambiense differ significantly in their isoenzyme patterns and electro-phoretic mobility. This has a consequence for epidemiological studies since isoenzyme patterns will be useful in identification of these trypanosomes.

3.6 Lipids

Phospholipids are the major portion of the total lipids in African trypanosomes. In T. rhodesiense, the lipid content of the bloodstream form is about 11.2% (Hutner and Provasoli, 1964). The phospholipids contain largely phosphatidylcholine; phosphatidylethanolamine and a smaller amount of phosphatidylinositol. Both the blood and culture forms contain sphingomyelin and phosphatidylserine, however both are

present in much smaller amounts in the culture form (Dixon and Williamson, 1970). Cholesterol is the principal sterol of T. rhodesiense (Dixon et al, 1971).

Pathogenic trypanosomes generate phospholipases (Tizard et al, 1978a). Within infected animals, phospholipases are released following trypanosome destruction and act on phospholipids to yield lyso-phospholipids and free fatty acids. Lysophospholipids are potent cytotoxins but are probably rapidly broken down to glycerophospholipids and more free fatty acids by the trypanosome lysophospholipids and in this way provoke endothelial damage (Carne and Onon, 1978). The action of trypanosome phospholipase on endogenous phospholipid results in vivo in the generation of relatively large quantities of free fatty acids. Those free fatty acids are surface-active and hence are both haemolytic and cytotoxic. Of the many fatty acids released, the major ones in both T. congolense and T. brucei are linoleic, palmitic and stearic acids which together account for about 75% of the total fatty acid content. Lesser amounts of oleic, gammalinoleic and arachidic acids are also present. It is not to be understood, however, that the release of free fatty acid is responsible for the anaemia in trypanosomiasis since there is no evidence for that.

3.7 Amino acids

Chappell et al (1972) analysed the free amino acid pools of the blood form of T. gambiense under a variety of conditions. The major amino acid (47%) present was found to

be alanine. The trypanosome is capable of taking up exogenous alanine, aspartate, glutamate and argenine. The T. gambiense also has amino acid biosynthetic capabilities, but the full understanding of the relationships between absorption, interconversion and biosynthesis is not known nor are the amino acid requirements known. There is evidence for the transamination of the aromatic amino acids, tryptophan, tyrosine and phenylalanine (Stibbs and Seed, 1975). The tryptophan is carboxylated and then converted to indole-3-ethanol and indole-3-acetate. The involvement of the tryptophan metabolites-indole-3-ethanol or tryptophol and indole-3-acetate in the pathogenesis of trypanosomiasis have been suggested (Tizard et al, 1978b). These compounds are capable of inducing nervous system depression and are immunosuppressive (Ackerman and Seed, 1976).

CHAPTER 4

MEMBRANE TRANSPORT OF PURINE NUCLEOSIDES IN AFRICAN TRYPANOSOMES

4.1 General principle

The study of transport of purine and pyrimidine bases and nucleosides across biological membranes has acquired some importance because of the discovery that specific membrane "carrier" systems exist for these compounds and that transport capacity may be closely coupled with physiological events such as cell growth and malignant transformations (Berlin and Oliver, 1975). In general, the low molecular weight compounds cross the biological membranes by one of several mechanisms. The hydrophobic molecules may permeate by passive diffusion because of their high solubility in the lipid of the plasma membrane. The rate of permeation depends on the oil: water partition coefficient of the compound. Hydrophilic compounds on the other hand that are unable to diffuse freely through a lipid bilayer and are large enough ($> 5\mathring{A}$) to be excluded from aqueous pores or channels, are often transported by specific carriers, presumably proteins that exist in the membrane. The mediated transport is characterized by Michaelis-Menten kinetics (saturability, and competition between related compounds for uptake) and temperature dependence. Active transport refers to those transport systems that involve energy expenditure either in form of ATP or in form of proton/ion gradient. It leads to

accumulation of metabolically unaltered substrate against an electro-chemical gradient. Facilitated diffusion is a carrier system mediated transport driven by electro-chemical concentration gradient. The characteristics of facilitated diffusion is rate saturability, non accumulative, temperature dependent and competitive inhibition. The kinetic analysis of transport processes in cells that metabolize nucleosides is complex. However the initial rates of the multistep uptake processes are taken to be those of the first step, that is transport. The nucleoside uptake processes involve transport of substrate across the cell membrane, phosphorylation by nucleoside monophosphate and diphosphate kinases and other metabolic fates including incorporation into nucleic acids.

4.2 Membrane transport of nucleosides in African trypanosomes

There is little information on the uptake and metabolism of purines and purine derivatives by the African trypanosomes. It is generally accepted that African trypanosomes lack the *de novo* purine synthesis and must therefore rely on exogenous purine sources (Bone and Steinert, 1956; Walsh and Sherman, 1968). It is not unreasonable therefore to assume that membrane transport mechanisms must be operating in them and are of great importance in meeting the nutritional needs of the parasites.

James and Born (1980) showed evidence of carrier mediated transport systems in T. congolense and T. brucei. Adenosine was taken up by these parasites more rapidly than inosine and the purine bases: adenine and hypoxanthine. Two transport loci were discernable in T. brucei, the first locus transports inosine, guanosine and also has a low affinity for adenosine. The second transport locus is specific for adenosine.

4.3 Nucleoside transport inhibitors

A number of compounds[1] chemically unrelated to nucleosides are recognized as inhibitors of nucleoside transport in animal cells (Lemkin and Hare, 1973; Cass and Paterson, 1973; Stefanovic et al, 1976; Lynch et al., 1978). Nitrobenzylthioinosine (NBMPR) is a specific nucleoside inhibitor, that is, it will not inhibit the uptake of purine bases, sugars or amino acids. The other compounds will inhibit nucleoside transport as well as uptake of purine bases and in some cases sugars. NBMPR inhibits the uptake of nucleosides by binding tightly to the membrane sites (Lauzon and Paterson, 1977). The adenosine analogue, 5'-O-sulfamoyl-adenosine was shown to inhibit adenine and adenosine uptake in T. rhodesiense and exerted its trypanocidal activity by denying the parasites the much needed purines (Jaffe et al, 1970). Also dipyridamole was shown to inhibit purine nucleoside uptake in T. brucei and T. congolense (James and Born, 1980) although it has no trypanocidal activity.

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1 Aflatoxin (Lemkin and Hare, 1973; Persantine (dipyridamole) (Philip et al., 1973). Cytochalasin B (Lynch et al. 1978) and NBMPR (Cass and Paterson, 1973).

CHAPTER 5

RATIONAL CHEMOTHERAPY OF AFRICAN TRYPANOSOMIASIS

5.1 General

Most of the drug action studies are often justified on the grounds that the knowledge of the action of the existing drugs will permit a more rational approach to chemotherapy. While it must be admitted that this is little more than a pious hope for the future, it cannot be denied that such studies have been highly rewarding: they have yielded much information about cell structure and function and have led to the identification of important differences between parasites and hosts.

For a rational approach to chemotherapy, detailed biochemical comparison between the parasite and host must be made: comparison of homologous enzymes; unique cell components or metabolic pathways in the parasite must be investigated to elucidate basis of selective toxicity of known drugs. Rational drug design is still in its infancy, partly because fundamental knowledge of many aspects of the parasite's metabolism is still lacking.

5.2 Glucose metabolism

Two drugs (suramin and melarsen oxide) currently used in the treatment of sleeping sickness inhibit glycolysis and energy production in T. brucei. Suramin is a potent in vitro inhibitor of the glycerophosphate oxidase and glycerophosphate

dehydrogenase (Fairlamb, 1981). It also inhibits respiration and glycolysis in intact cells only after several hours exposure to the drug *in vivo* (Fairlamb and Bowman, 1980). The delayed action of the drug is related to its slow uptake by *T. brucei*. The organic arsenical, melarsen, selectively inhibits pyruvate kinase in whole cells (Bowman and Flyn, 1976). Exposure of *T. brucei* to a combination of salicylhydroxamic acid and glycerol *in vitro* or *in vivo* was found to be trypanocidal (Clarkson and Brohn, 1976; Fairlamb *et al*, 1977). When glycerol and SHAM are administered in infected rodents, trypanosomes are rapidly cleared from the circulation but the animals relapse about one week later (Clarkson and Brohn, 1976; Evans and Brightman, 1980). This may be due to inadequate tissue concentrations of the drugs, a variable sensitivity to a "serum synergistic factor" implicated in lysis of trypanosomes *in vivo* or the distribution of the parasites in the host (Evans and Brightman, 1980; Amole and Clarkson, 1981).

5.3 Polyamines

Polyamines are low molecular weight aliphatics found universally in cells. They are made up of putrescine, spermidine and spermine; all three usually occur in eukaryotes (Cohen, 1978; Russel and Durie, 1978). The bloodstream *T. brucei* contain significant quantities of putrescine and spermidine, but not spermine (Bacchi *et al*, 1977; 1978). The synthesis of polyamines in higher eukaryotes is hormonally

regulated and closely controlled during cell cycle (Kano and Oka, 1976; Janne et al, 1978). Trypanosomes have a complex biochemical and antigenic changes during alterations from one form to another; such changes could be indicative of polyamine involvement in the life cycle. The absence of spermine in T. brucei is an indication of an intriguing difference between the parasite and metazoa cells. In flagellates, the NAD-α-glycerophosphate dehydrogenase, a key respiratory enzyme is stimulated by polyamine (Bacchi et al, 1978). It is conceivable thus that the polyamine metabolic pathways in trypanosomatids are attractive targets for chemotherapy in view of the rapid synthesis of polyamine in them (Bacchi et al, 1979). The synthesis of polyamines in T. brucei can be inhibited by an enzyme-activated inhibitor of ornithine decarboxylase - α - difluoromethy-lornithine (DFMO). Administration of DFMO in the drinking water of mice is curative for early T. brucei infections and co-administration of polyamines abolishes this effect, pointing to a specific vital function of polyamines in T. brucei.

5.4 Superoxide dismutase

Recent studies suggest that trypanosomes are more susceptible to cellular damage by activated oxygen species ($^{0}2-$) than mammalian cells (Fairlamb, 1982). NAD(P)H oxidase generates superoxide anion ($^{0}2-$) via one electron reduction of molecular oxygen. This $^{0}2-$ can undergo a

series of reactions to produce hydrogen peroxide (H_2O_2), hydroxyl radical (-OH) and perhaps, a singlet oxygen ($^1O_2$). Superoxide dismutase and catalase inhibit the production of activated oxygen in mammalian cells. These enzymes which destroy hydrogen peroxide are either very low or absent in trypanosomatids. Leishmania tropica has no detectable levels of catalase and glutathione peroxidase but has relatively high levels of superoxide dismutase which is cyanide-insensitive but azide and peroxide sensitive and furthermore is insensitive to diethyldithiocarbamate which inhibits some vertebrate enzymes (Meshnick and Eaton, 1981). Leishmania thus contain a superoxide dismutase different from its host's enzyme and a specific inhibitor of this enzyme might serve as an antileishmanial agent. Inhibition of glutathione synthesis by buthionine sulphoximine in T. brucei was found to be curative in mice (Arrick et al, 1981).

5.5 Nucleoside drugs

Trypanosomes are unable to synthesize purines de novo and therefore depend on preformed purines from the host. Purines can be interconverted by a multiplicity of routes, making chemotherapeutic attack a seemingly hopeless task. However, a number of nucleoside drugs, puromyain (6-dimethyl-amino-3'-p-methoxy-L-phenyl-alanylaminoadenosine); cordycepin (3'-deoxyadenosine); nucleocidin (5'-O-sulfamoyl 4'-fluoroadenosine) and tubercidin (7-deazaadenosine) have been shown to have trypanocidal activities (Hewitt et al,

1953; Tobie and Highman, 1956; Williamson, 1969). These nucleoside drugs have high toxicity. Allopurinol, a purine analogue, is metabolized differently by the host and parasite (Berens et al, 1980). In humans, allopurinol is converted to oxipurinol by xanthine oxidase, but not in T. cruzi and T. brucei, where xanthine oxidase is absent. In trypanosomes, the major metabolic pathway is to allopurinol ribonucleotide, which is converted to aminopyazolopyrimidine triphosphates and incorporated into RNA (Berens et al, 1980). Administration of allopurinol to mice with acute infections of T. cruzi significantly decreases parasitaemias and mortality rates and increases survival times (Avila and Avila, 1981).

5.6 Vaccination for trypanosomiasis

Control measures presently available for trypanosomiasis are either expensive or impracticable to apply on a large scale. The only possible alternative is the use of vaccines. Several approaches have been used in the development of vaccine against African trypanosomiasis; these include: (a) induction of resistance by use of non-specific immunostimulants; (b) use of surface coat antigens from the bloodstream form of the parasite; (c) use of plasma membrane antigens; metacyclic antigens and sub-cellular fractions from the parasites.

The administration of nonspecific immunostimulants like synthetic polyribomucleotides; Baccillus Calmette-Guerin; B. pertussis; Corynebacterium parvum prior to or at the same time as challenge with Trypanosoma congolense; T. rhodesiense or T. brucei significantly increased the survival time of infected mice (Singer et al, 1964; Murray and Morrison, 1979). African trypanosomes possess glycoprotein surface which covers the entire organism. This surface glycoprotein coat contains a variety of antigenic determinants; some are unique to the individual organism and yet others are found in the surface coats of all trypanosomes (Barbet and McGuire, 1978). The immunity induced by the unique determinants is protective but this protection is confired only to the organisms which bear these determinants, that is, organisms belonging to the same variable antigen type (VAT). Thus if VAT-specific antigens were used as field vaccines each VAT would require a separate vaccine and considering the enormous numbers of different VATS in each trypanosome species, one would be dealing with a cocktail vaccine consisting of thousands of different mixtures of VAT-specific antigens.

However, recent studies (Hadjuk and Vickerman, 1981; Esser et al, 1981) have shown that whereas serodeme of trypanosomes may consist of several bloodstream VATS, there are only few VATS in corresponding metacyclics. The overall VAT composition of the metacyclics of each serodeme is also constant, thus the metacyclics from tsetse infected with

one clone are similar, in type and composition, to those extruded by tsetse infected with any other clone belonging to the same serodeme. Studies in mice (Jenni and Brun, 1980), goats and cattle (Emergy et al, 1980) have indeed shown that immnunization with metacyclic antigens leads to the development of immunity against infected tsetse challenge. Such immunity, was however confined to the same serodeme. The immunized animals showed no protection against challenge with tsetse infected with different serodemes. Although vaccination using metacyclics appears to offer the best hope for vaccine development against African trypanosomiasis, some questions are yet to be answered. Some of these include determination of: (a) number of metacyclic VATS for each serodome; (b) number of different serodemes of each species of trypanosomes circulating in different geographical areas and (c) how stable they are. Also, the finding that s subset of VSG genes is not only unstable but evolves at a high rate (Frasch et al, 1982) makes the prospect of vaccination difficult.

CHAPTER 6

MATERIALS AND METHODS

6.1 Materials

The radioactive compounds: 8-^{14}C-adenine; 8-^{14}C-adenosine; 8-^{14}C-inosine; 8-^{14}C-hypoxanthine; ^{3}H-adenosine; ^{3}H-thymidine and G-^{3}H-NBMPR were obtained from either Moravek Biochemicals, Brea CA92621 or from Radiochemical Centre, Amersham, Bucks. The enzymes xanthine oxidase; pyruvate kinase and phosphoenol pyruvate dehydrogenase were obtained from Sigma Chemical Co. St. Louis, U.S.A. Percoll (density 1.13g/ml) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Earle's balanced salt solution 10 x without magnesium, bicarbonate and calcium was obtained from Grand Island Biological Co., Scotland. Cellulose thin layer sheets (13254) were obtained from Eastman Kodak Co., U.S.A. PEI cellulose sheets were from Applied Science, Milton Roy Laboratories, PA, 16801. The unlabelled NBMPR and NBMPR-P were gifts from Professor A.R.P. Paterson, Cancer Research Unit, McEarchern Laboratory, The University of Alberta, Edmonton, Canada. Tubercidin was a gift from Dr. J.E. Grady, UpJohn Company, Kalamazoo, Michigan, U.S.A. All the other chemicals used were of reagent grade.

Parasites: The parasites used in the study were *T. gambiense* (35A/2); *T. congolense* (58/98); *T. brucei* (ILRAD 2052). All the ILRAD stocks were cloned populations.

They were all maintained in rats or kept frozen in liquid nitrogen. The procyclics of T. congolense; T. vivax and T. brucei were maintained in culture medium in vitro.

Host animals: Normal BALB/C albino rats and lethally irradiated (900 rads in 4 mins or 600 rads in 5 mins) Wister rats weighing between 200-250g served as the blood donors for most of the studies. Each rat received approximately 10^5 organisms ip in a total volume of 0.5 ml. Mice, weighing between 18-22g, were used for the chemotherapy studies. Groups of mice were inoculated ip with trypomastigotes of either T. congolense or T. gambiense.

Blood collection: Blood samples were collected at the peak of parasitaemia by cardiac puncture using 3.8% sodium citrate made in 0.154M sodium chloride and 2% glucose as the anticoagulant. The blood samples from the rats were pooled and kept in ice-cold condition ($4^{\circ}C$).

6.2 Isolation of the trypanosomes: Two methods were employed in the large scale separation of the trypanosomes from the red blood cells.

In the first method, whole blood from parasitized animals was diluted with phosphate saline glucose (PSG) buffer, pH 8.0, and centrifuged at 450-500g for 5 min. The buffy coat fraction which contained most of the organisms was recovered and layered on DE-52 column previously equilibrated with PSG buffer. The trypanosomes were eluted from the column with the buffer as described by Lanham and

Godfrey (1970). The isolated parasites were concentrated by centrifugation and resuspended in either Tris buffer, pH 7.2 or phosphate buffer pH 7.0 at a concentration of 10^7-10^9 organisms per ml depending on the type of assay to be done.

In the second method for the isolation of the trypanosomes, whole blood containing the organisms were mixed with percoll: Earle Balanced Salt Solution (EBSS) in the ratio of 2:1 as described by Grab and Bwayo (1982). The percoll: EBSS-blood mixture was centrifuged at 20,000g for 30 min using either fixed angle rotor in a Beckman JA-20 centrifuge or swinging rotor in a Sorvall RC-5B (Du Pont Instruments). The resulting trypanosome-rich band which was well separated from the erythrocytes, was collected and the trypanosomes washed once with the PSG buffer and passed through DE 52 column (Lanham and Godfrey, 1970). Higher yields of trypanosomes were achieved by this method especially with T. vivax and T. congolense isolation.

6.3 Preparation of the isolated parasites. The method for the preparation of the parasites depends on the type of assay to be done with the trypanosomes.

Preparation of parasites for enzyme assay. The isolated parasites were resuspended in either 50mM phosphate buffer pH 7.0 or in Tris-HCl buffer pH 7.2 at a concentration of 5×10^8 to 10^9 organisms per ml. The buffers contained 0.1mM

dithiothreitol. The organisms were disrupted by either ultrasound sonicator (100 WATTS for 3 mins) (Braun-Sonic 1510; B. Braun Melsungen AG) in the cold (4°C) or by three cycles of freezing in liquid nitrogen and thawing at 37°C in a water bath. The total cell homogenate was subjected to highspeed centrifugation (20,000-24,000g) in refrigerated centrifuge for 45 mins. The supernatant fractions were used as the source of enzyme (Gutteridge and Davies, 1981). In some of the enzyme assays the crude protein extracts were dialysed against 1000 ml of Tris-HCl buffer overnight at 4°C with constant stirring.

Preparation of parasites for transport studies: The isolated trypanosomes were separated by the methods of Lanham and Godfrey (1970). The parasites were concentrated to a density of 10^7 parasites per ml. These were always kept in ice-cold condition until used.

6.4 Assay methods

Chromatography: The radiometric assay of the enzymes involved the analysis of the reaction mixture on cellulose thin layer sheets previously coated with 5 nmole carrier nucleoside, nucleotide or purine base. The thin layer plates were developed in 1.25M solution of ammonium sulphate, dried in the air and the carriers identified and marked under UV light. The zones containing the carrier purines were cut out in 1 cm strips and were counted as described below.

Determination of radioactivity. The thin layer after development as described in the previous section was further analysed by cutting out the carrier spots and determining the radioactivity in them. The radioactivity was determined by counting in either Packard scintillation spectrometer Model 3330 or in Beckman liquid scintillation spectrometer Model LS 100C using any of the following liquid phosphors (i) Toluene: 3.5g PPO; 0.05g POPOP in 1 litre of toluene (ii) Tritosol; 70ml ethlene glycol; 280ml absolute ethanol; 500ml triton-x-100; 1,150ml of xylene; 6g PPO; 0.1g POPOP in a total volume of 2 litres or (iii) Aquasol: a commercially available scintillant from New England Nuclear Boston, Mass.

The radioactivity on the parasites used for the transport assays was determined by digesting the parasites with 0.5ml of 0.5N KOH overnight in 1.5ml microcentrifuge. 0.2ml of the digests were transferred into scintillation vials and 10ml of tritosol phosphor added to them. These were then counted in the liquid scintillation spectrometer as above. The counting efficiency including the quenching corrections were variously determined and corrected. The quenching caused by the thin layer sheets was corrected by including the same sizes of the chromatogram sheet used in the tests but without any radioactivity and treating them in exactly the same manner as the tests before counting. Blank trypanosomes were counted to correct for quenching caused by the parasites. The efficiency of the counting was determined

always by using the standard supplied by the manufacturers of the scintillation spectrometer.

Enzymes: Two methods were employed for each enzyme assay.

Purine hydrolase (EC 2.4.2.1): This enzyme was assayed by spectrophotometric method of Tucker and Young (1976) and the radiometric method described by Tax and Veerkamp (1978). In the spectrophotometric method the assay medium contained 50 mM Tris-HCl buffer, pH 7.2; 1 MM purine nucleoside (adenosine or inosine) 0.6 units xanthine oxidase per ml and approximately 80-100 µg protein in a total volume of 1 ml. Incubations were done either at room temperature or at 37°C in a temperature controlled water bath for 5 min. The reaction was initiated by adding substrate and the initial rate of uric acid formation estimated by measuring the extinction at 293 nm using Beckman Model 25 recording spectrophotometer. Blank values were obtained by omitting the nucleoside from the reaction mixture. The molar extinction coefficient of uric acid was taken to be 12.5×10^3 (Kim et al, 1968).

In the radiotracer assay, the method described by Tax and Veerkamp (1978) was used. The final volume of 90 µl contained Tris-HCl buffer (pH 7.2); dithiothreitol 0.1 mM; EDTA 1 mM; about 20nmole radiolabelled nucleoside (0.05-1.0 µCi ^{14}C-nucleoside) diluted with 320 nmole unlabelled nucleoside, and approximately 80-120 µg protein. Reaction was terminated by boiling for 5 mins at 100°C. Aliquot from

the incubation medium was streaked on cellulose thin layer plates and developed as described previously. The crude protein extracts used for both assays were dialysed against 1000 ml Tris-buffer pH 7.2 overnight at 4°C.

Purine nucleoside phosphorylase (EC 2.4.2.1): The methods of Tucker and Young (1976) and Tax and Veerkamp (1978) were employed. The assay was however done in Tris buffer with phosphate ion added since the enzyme is more active in the presence of phosphate ion.

Adenosine deaminase (EC 3.5.4.4.): Spectrophotometry was used to quantitate adenosine deaminase using the coupled xanthine oxidase enzyme determination described by Mans and Koch (1960). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0; crude protein extract prepared from the trypanosomes as previously described (80-100 µg) and xanthine oxidase (0.16 units per ml) in a total volume of 1.0 ml. Reaction was initiated by addition of 10-100 ul of 0.04M adenosine and terminated by adding 2 ml of 0.3M NaOH. The optical density was read at 293 nm. The blank values were measured by omitting the purine nucleoside. The molar extinction of uric acid was taken to be 12.5×10^3 (Kim et al, 1968).

The radiotracer method used was that of Kidder et al, (1977). The assay mixture contained in a total volume of 90 µl consisted of Tris-HCl buffer (ph 7.2) 20 mM; dithiothreitol 0.1 mM; EDTA 1 mM; ^{14}C-adenosine (0.05-1.0 µCi) and crude protein

extract (80-100 µg). Reaction was terminated by boiling for 5 mins at 100°C. Aliquots of the incubation mixture were processed as described previously.

Adenine aminohydrolase (adenine deaminase; adenase EC 3.5.4.2):

The enzyme was assayed by the methods of Mans and Koch (1960) and Kidder et al. (1977) just as the adenosine deaminase. The substrate for the enzyme in this case was adenine.

Adenosine kinase (EC 2.7.1.20). The spectrophotometric method of Entner and Gonzalez (1961) and the radiotracer method of Lindberg et al. (1967) were used for the assays of the enzyme.

In the raiotracer assay method, the reaction mixture contained in a total volume of 200 µl, Tris-HCl buffer (pH 7.4) 25 mM; dithiothreitol, 0.1 mM; KCl, 50 mM; $MgCl_2$, 0.5 mM; ATP 0.7 mM; PEP, 0.25 mM; pyruvate kinase 0.005 mg protein; ^{14}C-adenosine (0.1 - 1 µCi) and approximately 100 µg crude protein extracts. In the time course assay, aliquots of the reaction mixture were removed at intervals and streaked on cellulose thin layer plates. For the kinetic determination, various concentrations of the purine nucleoside were used. The reaction was terminated by boiling at 100°C for 5 mins. The reaction mixtures were processed as previously described.

The spectrophotometric assay of this enzyme is based on the decrease in absorbance at 340 mµ in a couple multienzyme system; the molar extinction coefficient of NADH being 6.22 x

10^3 (Linberg et al. 1967). The reactions involved are as follows:-

ATP + Adr ⟶ ADP + AMP

ATP + AMP ⟶ 2ADP

3ADP + 3Phospho-enolpyruvate ⟶ 3ATP + 3 pyruvate

3 Pyruvate + 3 NADH + $3H^+$ ⟶ 3 Lactate + 3 NAD.

The ADP formed in the nucleoside reaction is reconverted to ATP in the presence of excess phospho-enolpyruvate and pyruvate kinase. The simultaneously formed pyruvate was converted to lactate in the presence of an excess of NADH and lactate dehydrogenase.

The assay mixture in a total volume of 1 ml consisted of 50 mM Tris-HCl buffer (pH 7.4); ATP 7 mM; PEP 0.25 mM; NADH 0.2 mM; $MgCl_2$ 0.5 mM; KCl 50 mM; lactate dehydrogenase 0.005 mg protein; pyruvate kinase 0.019 mg protein. Blank values were obtained by excluding the adenosine substrate in the incubation medium.

Purine phosphoribosyltransferase: AdePRTase (EC 2.4.2.7): Hypoxanthine-guanine PRTase (EC 2.4.2.8).

The reaction mixture based on method described by Gutteridge and Davies (1981) consists in a total volume of 130 µl of 40 mM Tris-HCl; 2 mM $MgCl_2$; 2 mM PRPP and ^{14}C-Adenine (0.5 µCi) or ^{14}C-Hypoxanthine (0.5 µci) and approximately 48 µg protein. Aliquots from the incubation medium were analysed as described previously. Enzyme activities were expressed as nmole/mg protein/min.

Transport assay: Isolated organisms were resuspended in Tris buffer pH 7.2 at a concentration of 1 - 2 x 10^7 parasites per ml. Each 10-ml tappered centrifuge tube in either a constant temperature water bath (at 37°C) for experiments performed at that temperature or at room temperature contained 1 ml of the ^{3}H-labeled nucleoside (and inhibitor in the case of inhibitor studies) at twice the final desired substrate and inhibitor concentrations. At appropriate intervals of time, 1 ml of the parasite suspension (prewarmed at 37°C or kept at room temperature) was pipetted into each centrifuge tube and incubated for the appropriate intervals of time. The incubations were terminated by diluting the mixture with four volumes of ice cold (4°C) Tris buffer and immediately centrifuging the tubes at 450g for 10 min in the cold (4°C). The pellet was washed three times by mixing and centrifuging in Tris-buffer (4°C) and finally drained of excess buffer. The pellet was then digested with 0.5 ml of 0.5N KOH overnight at room temperature. Aliquots (0.2 ml) of the KOH digest were taken into scintillation vials and counted with tritosol phosphor in a Beckman Model LS 100 C, Liquid scintillation spectrometer.

Efflux of ^{3}Hthymidine: The procedure for measuring the efflux of radioactivity from erythrocytes "loaded" with labeled thymidine was that of Cass and Paterson (1972). Erythrocytes collected from mice infected with trypanosomes and also from uninfected mice were washed three times in buffered saline (140 mM NaCl, 1.4 mM $mgSO_4$ and 18 mM N-tris (hydroxymethyl)

methyl-2-amino-ethane sulfonic acid at pH 7.4). The erythrocytes were "loaded" by incubating with 2.0 mM ^{3}H-thymidine for 40 min at 37^{o}C. This condition achieves an equilibrium distribution of labeled nucleoside. Loading was terminated by centrifugation at 1500 x g for 10 min. Since thymidine is not metabolized in erythrocytes (Oliver and Paterson, 1971), internal and external nucleoside concentrations were considered aqual at the end of the incubation and the intracellular nucleoside concentration was obtained by measuring the extracellular radioactivity.

Efflux of radioactive nucleosides from loaded cells was initiated by rapidly adding to 0.25 ml portions of loaded cells 2.5 ml volumes of either buffered saline alone or buffered saline containing the equilibrium concentration of 6 mM of non-radioactive thymidine. Radioactive thymidine originally present in the extracellular space of loaded cell sediments was diluted approximately 100-fold in this procedure (Cass and Paterson, 1973). Thymidine efflux was terminated after intervals of 2 min by adding 2.5 ml buffered saline containing 10 μM NBMPR, a potent nucleoside transport inhibitor (Cass and Paterson, 1973; Lauzon and Paterson, 1977). Zero time points were obtained by adding NBMPR 10 sec before addition of test medium. Separate reaction mixtures were prepared for each time point, and immediately after termination of efflux, portions of each mixture were centrifuged to obtain cell-free medium. Radioactivity present in cell-free

medium was determined using tritosol phosphor and liquid scintillation counting. The cell concentrations of individual reaction mixtures were obtained from the hematocrit measurements.

Binding assay: The organisms separated from the whole blood were resuspended in Tris buffer pH 7.2 to approximately 10^7 parasites per ml. Binding assays were initiated by the addition of ^{3}H-labelled NBMPR to the parasite suspension in 10 ml tappered centrifuge tubes (1 x 10^7 organisms in 2.0 ml final concentration). Intervals of binding were ended by centrifugation (450 x g for 10 min) and the supernatant fractions reserved for assay of ^{3}H-content. The pellets were dissolved in 0.5 ml of 0.5N KOH and assayed for ^{3}H-content by liquid scintillation counting using the xylene detergent-scintillant.

The ^{3}H-content of the supernatant fluid was determined by counting an aliquot (500 µl) before and after the assays to see whether there were depletions of radioactivity in the medium by the trypanosomes. Medium depletion of radioactivity had previously been used to study the binding of isotopic NBMPR to red cells (Ogbunude, 1979).

6.5 Chemotherapy study

The trypanosome, T. gambiense was obtained from white albino rats with parasitaemia above 5 x 10^7 organisms per ml. These were diluted to approximately 10^6 organisms per ml with normal saline and 0.2 ml of the suspension inoculated ip into

groups of male mice.

Acute toxicity tests: Acute toxic effects of ethidium bromide and tubercidin in mice were determined by giving single ip injections of the drugs to groups of ten mice. The drugs were made in 0.154M NaCl solution and the injection volumes were proportional to 0.2 ml per 20 g body weight whether the drugs were given singly or in combinations. The animals were observed for seven days for any deaths.

In vivo tests: Groups of mice (5 mice per group) were given single ip doses of ethidium or tubercidin and also tubercidin and NBMPR-P combination. The untreated groups received only saline solution in which the compounds were prepared. In another experiment, groups of mice inoculated with *T. gambiense* and showing approximately 10^7 parasites per ml of blood were given single doses of either tubercidin, ethidium or their combinations with or without NBMPR-P. Deaths were recorded daily for 30 days and tail blood checked for parasitaemia during this period.

In vitro tests: Trypanosoma gambiense isolated from blood of infected rats were incubated with tubercidin alone or in combination with NBMPR-P. The incubation medium, Ringer's phosphate buffered salt solution is supplemented with 10 mM glucose, 2% calf serum, streptomycin (50 µg/ml) and penincillin (100 iu/m) (York *et al*, 1929). Control parasites were incubated in the buffer solution containing no tubercidin or NBMPR. Samples from the incubation media were removed at interval to check the number of motile trypanosomes.

CHAPTER 7

RESULTS

7.1 Chromatography

The relative mobilities of the purine bases, nucleosides and nucleotides on cellulose thin-layers and polyethyleneimine-cellulose F (PEI-cellulose F) sheets are presented in Table 2. The cellulose-separated the purine bases and derivatives much better in this solvent system than the PEI-cellulose F which was unable to separate hypoxanthine from adenosine mono-phosphate. Trials with paper chromatography (data not presented) showed that it did not separate the nucleotides from the bases with the solvent systems tried.

7.2 Purine nucleoside hydrolase and phosphorylase

The purine nucleoside hydrolase catalyses the irreversible hydrolytic cleavage of purine nucleoside to purine base.

Purine nucleoside $\longrightarrow$ purine base + ribose sugar.

The purine nucleoside phosphorylase on the other hand catalyses reversible cleavage of purine nucleoside.

Purine nucleoside + Pi $\rightleftharpoons$ purine base + ribose-1-phosphate.

The assay of these enzymes in the African trypanosomes shows that in T. brucei (Fig. 6), the inclusion of phosphate in the assay medium did not change the rate of cleavage of adenosine or inosine. Also, purine nucleoside could not be synthesized from the purine base and ribose-1-phosphate (R-1-P) when the enzyme source was from T. brucei. Fig. 7 shows that phosphate stimulated adenosine cleavage but not

TABLE 2

R_f of purine bases and purine derivatives on thin-layer chromatography

Purine bases, nucleosides and nucleotides in approximately 5 nmole portions were chromatographed on either cellulose-thin layer sheets (Eastman 13254) or PEI-cellulose thin layer chromatography sheets using 1.25M ammonium sulphate solution as the solvent system.

Compound	Relative mobility (Rf)	
	Cellulose	PEI-cellulose
Adenosine	0.34	0.31
Adenine	0.25	0.20
Hypoxanthine	0.48	0.42
Inosine	0.70	0.68
Adenosine monophosphate	0.59	0.42
Inosine monophosphate	0.81	0.75

Fig. 6 Hydrolytic cleavage of adenosine and inosine by the hydrolase from T. brucei.

^{14}C-Adenosine (A) and ^{14}C-inosine (B) were separately incubated at 37°C with dialysed protein extracts of T. brucei trypomastigotes in Tris buffer (pH 7.2) without (O—O) and with phosphate (□—□). Samples (10 µl) of the incubation media were streaked on cellulose thin layers and developed in 1.25M ammonium sulphate solution. The zones on the chromatogram corresponding to adenine and hypoxanthine were cut out and analysed for radioactivity as described in Materials and Methods. The kinetic constants were determined from the double reciprocal plots (A2 and B2).

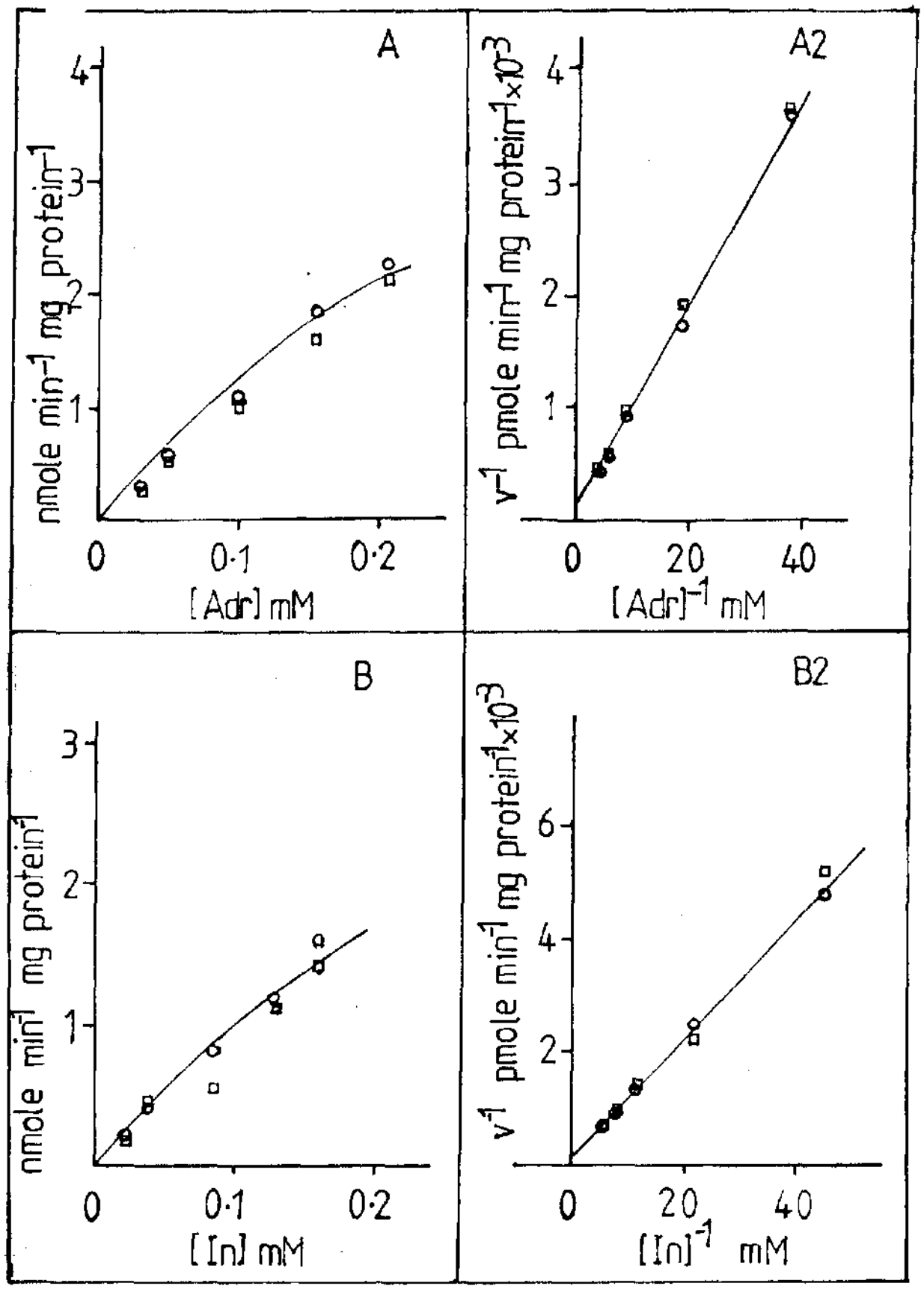
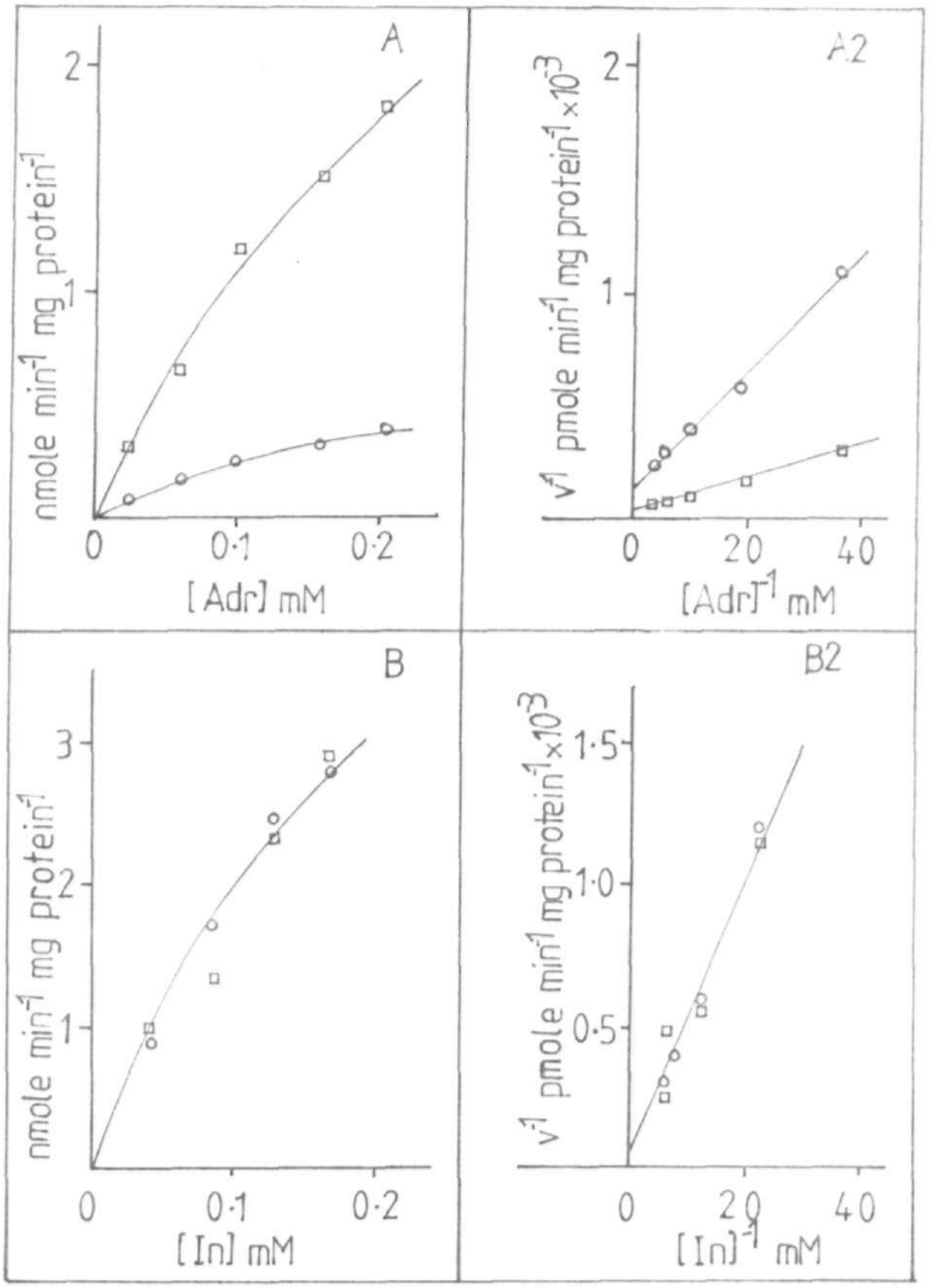

A
nmole min⁻¹ mg protein⁻¹
4
3
2
1
0
0·1
0·2
[Adr] mM
A2
v⁻¹ pmole min⁻¹ mg protein⁻¹×10⁻³
4
3
2
1
0
20
40
[Adr]⁻¹ mM
B
nmole min⁻¹ mg protein⁻¹
3
2
1
0
0·1
0·2
[In] mM
B2
v⁻¹ pmole min⁻¹ mg protein⁻¹×10⁻³
6
4
2
0
20
40
[In]⁻¹ mM

Fig. 7 A. Cleavage of adenosine by the phosphorylase from T. congolense.

B. Inosine cleavage by the hydrolase from T. congolense.

^{14}C-Adenosine (A) was incubated at 37°C with protein extract of T. congolense dialysed against Tris buffer (pH 7.2). The incubation was performed in increasing concentrations of labelled adenosine without (○—○) and with phosphate (□—□).

In another experiment, ^{14}C-inosine (B) was incubated with protein extract of T. congolense dialysed against Tris buffer (pH 7.2). The incubation was carried out as described above. Samples of media were streaked on cellulose thin layers and developed in 1.25M ammonium sulphate solution. The zones corresponding to adenine and hypoxanthine were cut out and the radioactivity associated with them determined as described in Materials and Methods.


A
nmole min-1 mg protein-1
2
1
0
0·1
0·2
[Adr] mM
A2
v-1 pmole min-1 mg protein-1 x10-3
2
1
0
20
40
[Adr]-1 mM
B
nmole min-1 mg protein-1
3
2
1
0
0·1
0·2
[In] mM
B2
v-1 pmole min-1 mg protein-1 x10-3
1·5
1·0
0·5
0
20
40
[In]-1 mM

inosine cleavage when the enzyme source was from T. congolense. With the same enzyme, it was possible to synthesize adenosine from adenine and R-1-P (Fig. 8). Adenosine cleavage by the enzyme from T. vivax (Fig. 9) was also unaffected by the presence of phosphate in the incubation medium.

7.3 Adenosine kinase

This enzyme catalyses the phosphorylation of adenosine. Adenosine + ATP ⟶ AMP + ADP. In the assay for the enzyme in T. brucei it was observed that under the assay condition, the adenosine was rapidly converted to adenine (Fig. 10) and AMP was not detected in the incubation medium over an hour of incubation. With T. congolense and T. vivax. AMP was detected in the assay medium (Fig. 11). Under the kinase assay condition, the rate of cleavage of adenosine in T. congolense and T. vivax was much lower than the rate of cleavage of adenosine in T. brucei. Almost 50% of the adenosine substrate was converted to adenine within an hour using T. congolense or T. vivax (Fig. 11). With T. brucei, conversion was almost complete within 5 min (Fig. 10).

7.4 Purine phosphoribosyltransferase

The purine phosphoribosyltransferase catalyses the transfer of ribosylphosphate from phosphoribosyl pyrophosphate to a purine base.

Purine base + PRPP ⟶ Purine monophosphate + PPi.

The enzyme is present in all the African trypanosomes studied (Figs. 12, 13 and 14). The activity was highest in T. brucei.

Fig. 8 Time course of adenosine synthesis from ^{14}C-adenine and ribose-1-phosphate with phosphorylase from T. congolense.

The protein extracts of T. congolense was incubated at 37°C with ^{14}C-adenine and ribose-1-phosphate in Tris buffer pH 7.2. The incubation medium was at intervals of time assayed for adenosine (□—□) and adenine (○—○) by taking samples (10 µl), streaking on cellulose thin layers and developing in 1.25M ammonium sulphate solution. The zones on the chromatogram corresponding to adenosine and adenine were cut out and the radioactivity associated with them determined as described in Materials and Methods.

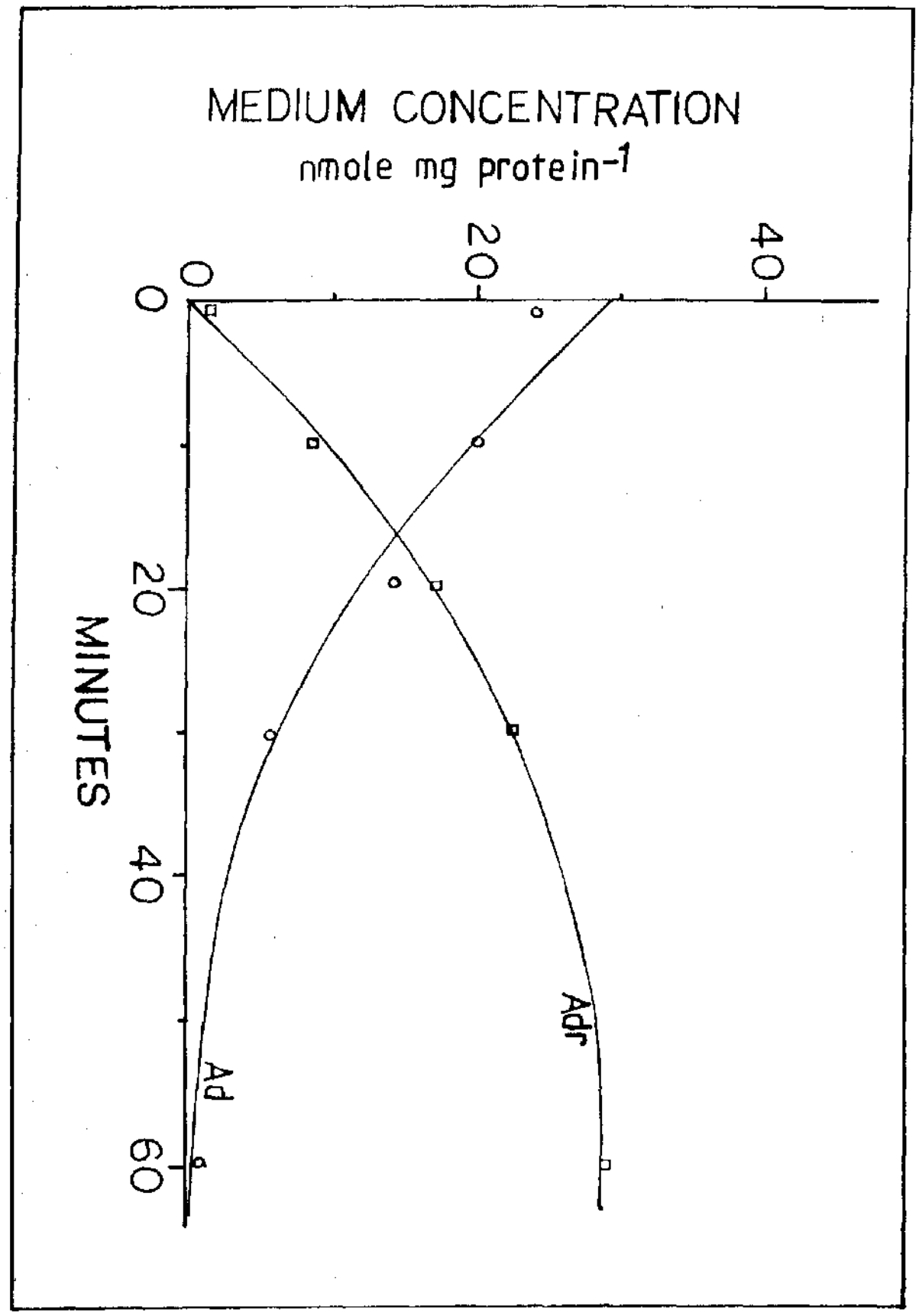

MEDIUM CONCENTRATION
nmole mg protein-1
0
20
40
MINUTES
0
20
40
60
Ad
Adr

Fig. 9 Hydrolytic cleavage of adenosine and inosine by the hydrolase from T. vivax.

^{14}C-Adenosine (A) and ^{14}C-inosine (B) were each incubated at 37°C with dialysed protein extracts of T. vivax trypomastigotes with and without inorganic phosphate as described in Materials and Methods. Assays were terminated by boiling at 100°C for 5 min and aliquots from the incubation media spotted on cellulose thin layers and developed in 1.25M ammonium sulphate solution. The zones on the thin layers corresponding to adenosine without (○——○) or with phosphate (□——□) and inosine without (○——○) or with phosphate (□——□) were assayed for radioactivity as described in Materials and Methods. From the double reciprol plots the kinetic constants were determined (A2 and B2).

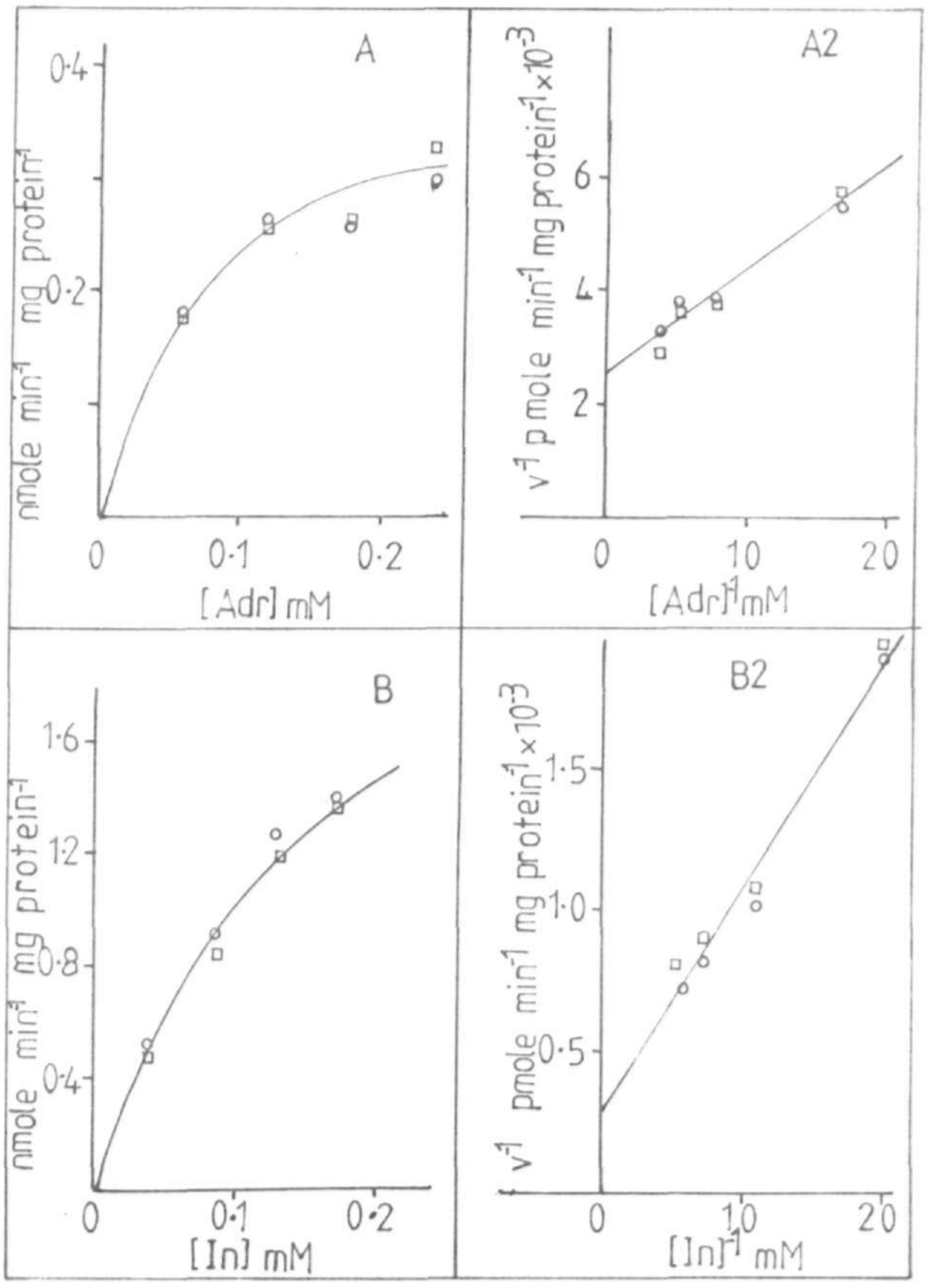

A
nmole min⁻¹ mg protein⁻¹
0·4
0·2
0
0·1
0·2
[Adr] mM
A2
v⁻¹ p mole min⁻¹ mg protein⁻¹ x10⁻³
6
4
2
0
10
20
[Adr]⁻¹ mM
B
nmole min⁻¹ mg protein⁻¹
1·6
1·2
0·8
0·4
0
0·1
0·2
[In] mM
B2
v⁻¹ pmole min⁻¹ mg protein⁻¹ x10⁻³
1·5
1·0
0·5
0
1·0
2·0
[In]⁻¹ mM

Fig. 10 Assay of adenosine kinase in extract of T. brucei

^{14}C-Adenosine was incubated at 37°C with protein extracts of T. brucei trypomastigotes as described in Materials and Methods. Aliquots (10 µl) of the incubation medium was removed at time intervals and spotted on cellulose thin layers and developed in 1.25M ammonium sulphate solution. The zones corresponding to adenosine (□——□) and adenine (○——○) were cut out and analysed for radioactivity as described in Materials and Methods. No AMP was detected over an hour incubation.

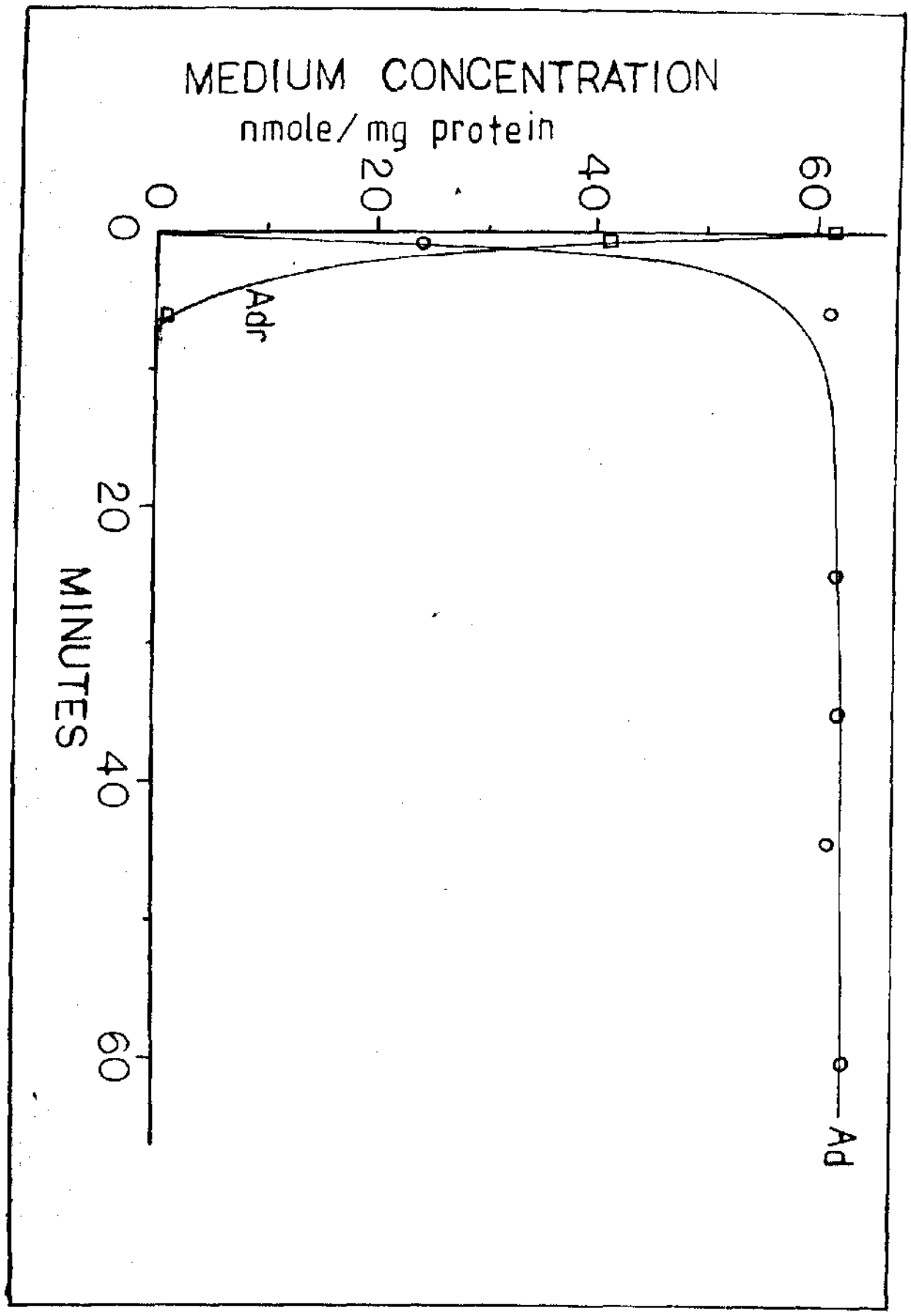

MEDIUM CONCENTRATION
nmole/mg protein
0
20
40
60
Adr
Ad
MINUTES
0
20
40
60

Fig. 11 Time course of adenosine kinase assay in extracts of T. congolense and T. vivax.

^{14}C-Adenosine was either incubated with protein extracts of T. congolense or that of T. vivax at 37°C as described in the Materials and Methods. Aliquots (10 µl) of the incubation media were removed at intervals of time spotted on cellulose thin layers and developed in 1.25M ammonium sulphate solution. The zones corresponding to adenosine (●——●); adenine (□——□); and AMP (○——○) were cut out and the radioactivity associated with them determined as described in Materials and Methods.

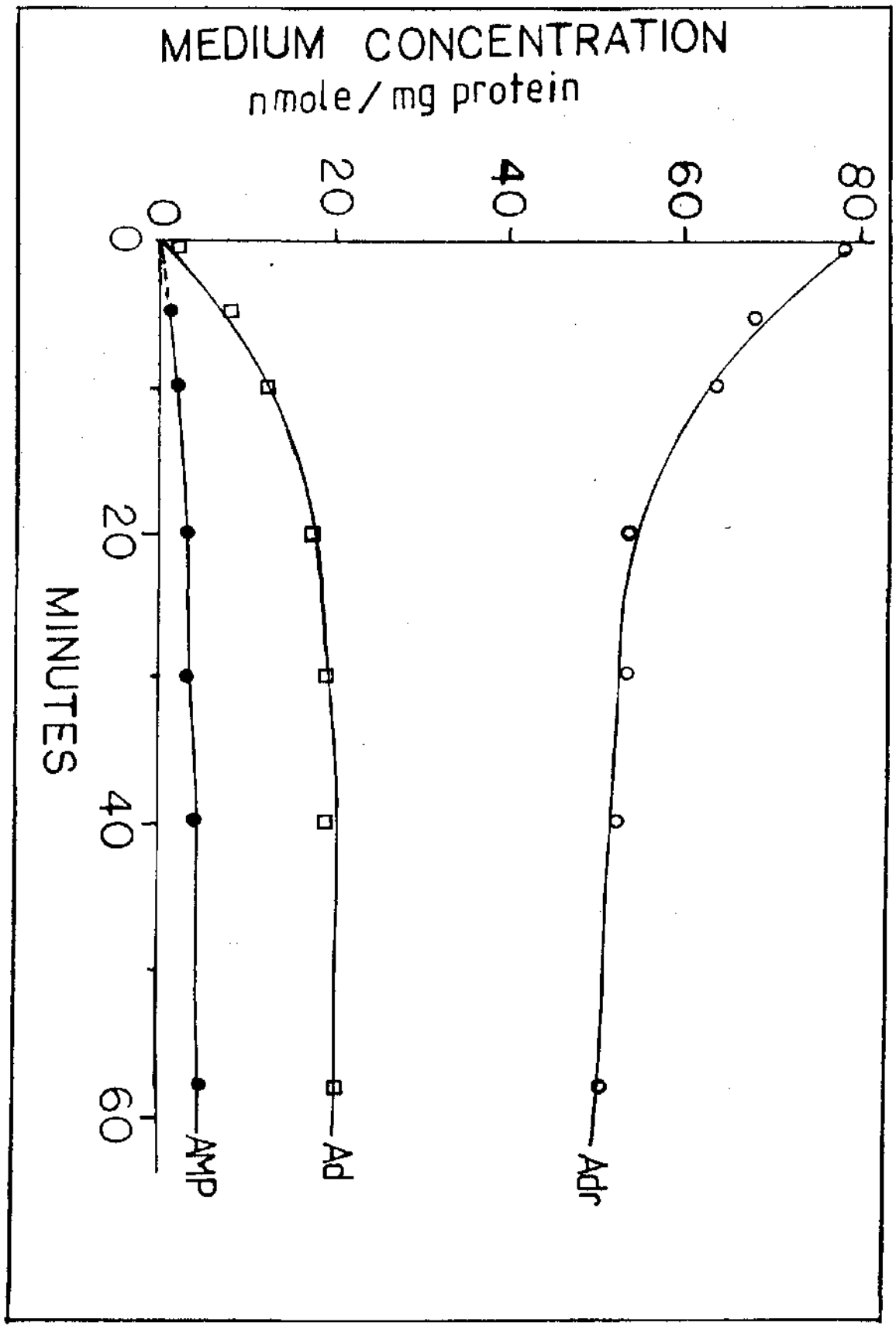
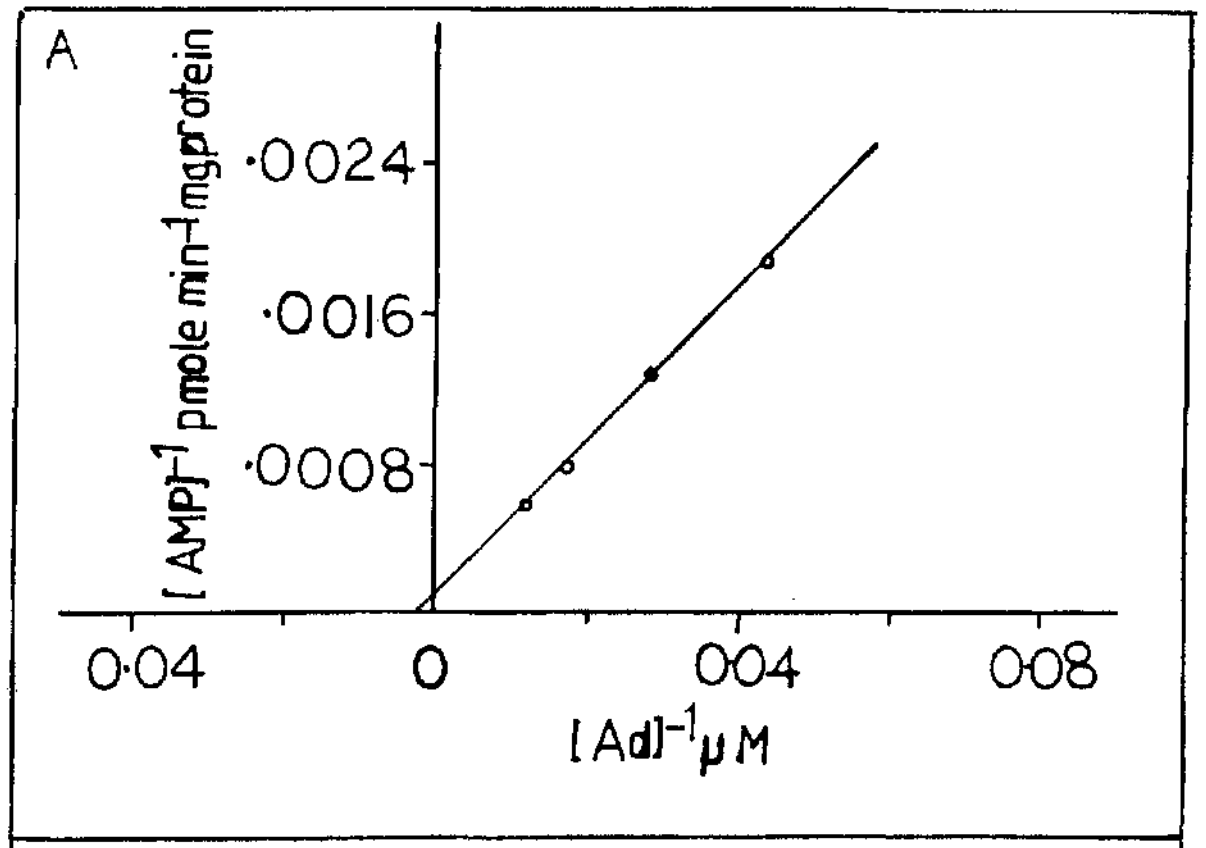

MEDIUM CONCENTRATION
nmole / mg protein
0
20
40
60
80
MINUTES
0
20
40
60
Adr
Ad
AMP

Fig. 12 Assay of purine phosphoribosyltransferase in extract from T. brucei.

^{14}C-Adenine and ^{14}C-hypoxanthine were each incubated with protein extract of T. brucei trypomastigotes at 37°C. The reactions were terminated by boiling at 100°C for 5 min and aliquots (10 µl) chromatographed on cellulose thin layers using a solution of 1.25M ammonium sulphate as the solvent system. The radioactivity associated with adenine and AMP zones on the chromatography sheets when ^{14}C-Adenine (A) was the substrate was determined. Also hypoxanthine and IMP zones were determined when ^{14}C-hypoxanthine (B) was the substrate. The kinetic constants were determined from the double reciprocal plots shown in the graph.


A
·0024
·0016
·0008
[AMP]$^{-1}$ pmole min^{-1}mg protein
0·04
0
004
008
[Ad]$^{-1}$ μ M

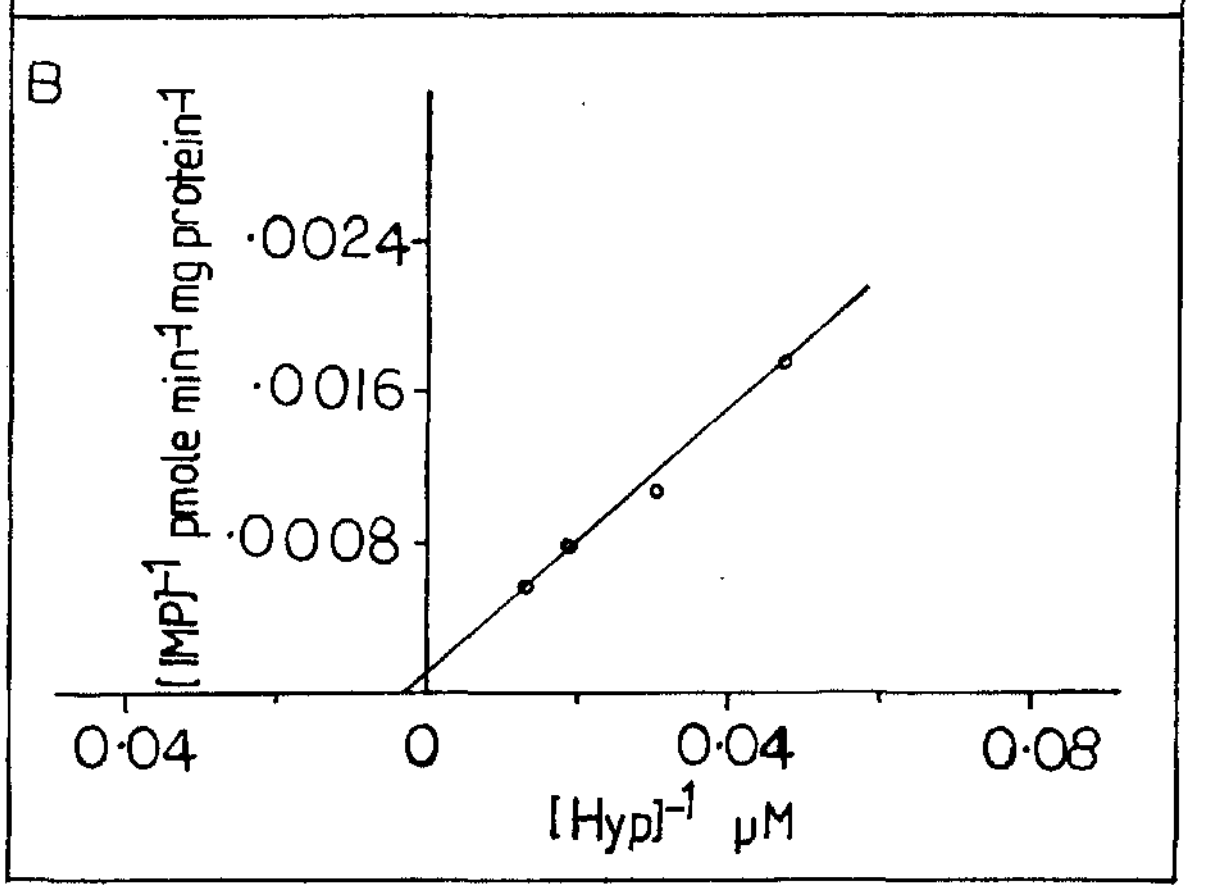

B
·0024
·0016
·0008
[IMP]$^{-1}$ pmole min^{-1} mg protein^{-1}
0·04
0
0·04
0·08
[Hyp]$^{-1}$ μM

Fig. 13 Assay of purine phosphoribosyltransferase in extract from T. congolense.

^{14}C-Adenine and ^{14}C-hypoxanthine were each incubated with protein extract of T. congolense trypomastigotes at 37°C. The reactions were terminated by boiling at 100°C for 5 min and aliquots (10 µl) chromatographed on cellulose thin layers using 1.25M ammonium sulphate solution as the solvent system. The radioactivity associated with adenine and AMP zones when ^{14}C-adenine (A) was substrate and hypoxanthine and IMP zones when ^{14}C-hypoxanthine (B) was the substrate were determined as described in Materials and Methods. The kinetic constants were determined from the double reciprocal plots.

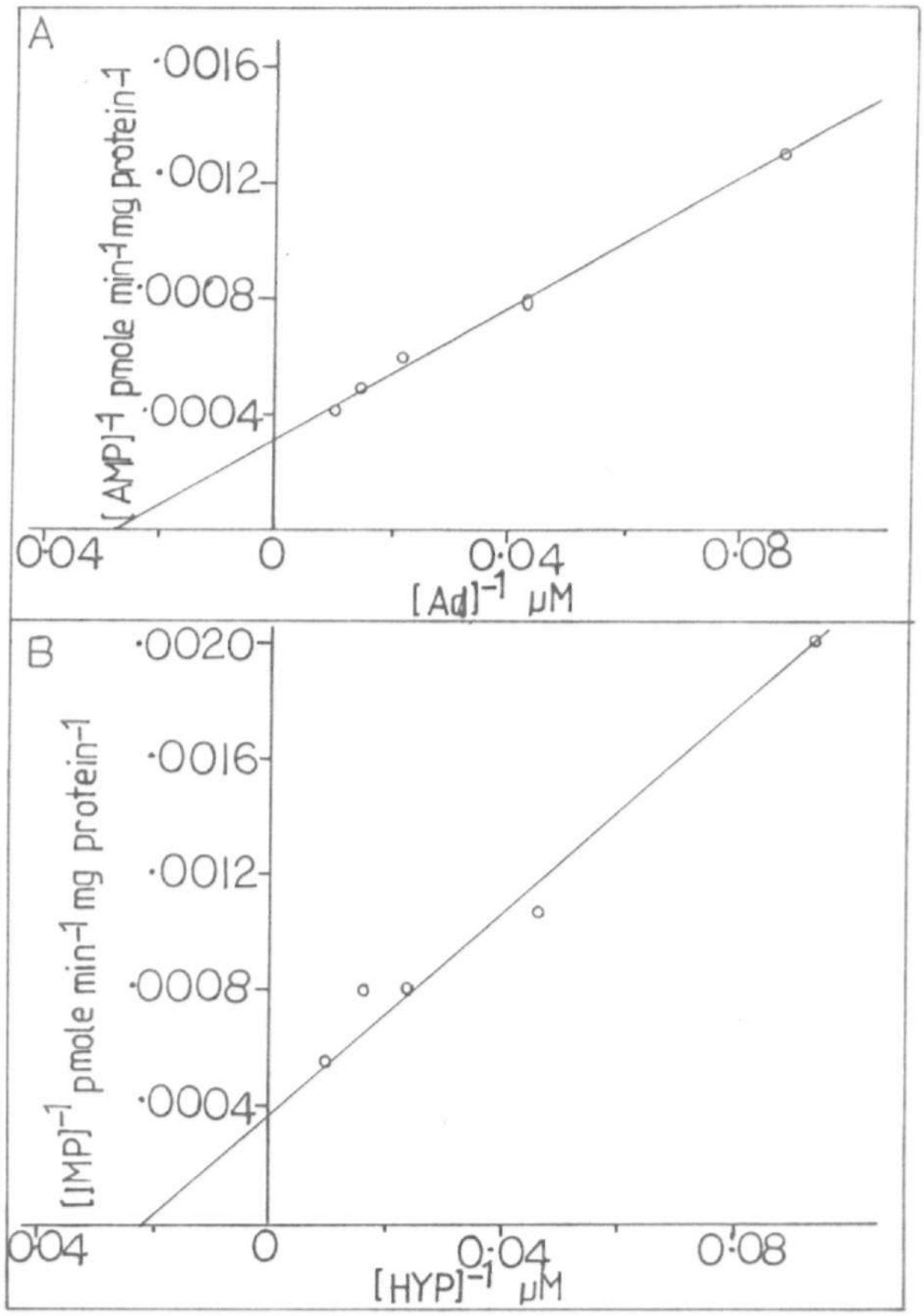
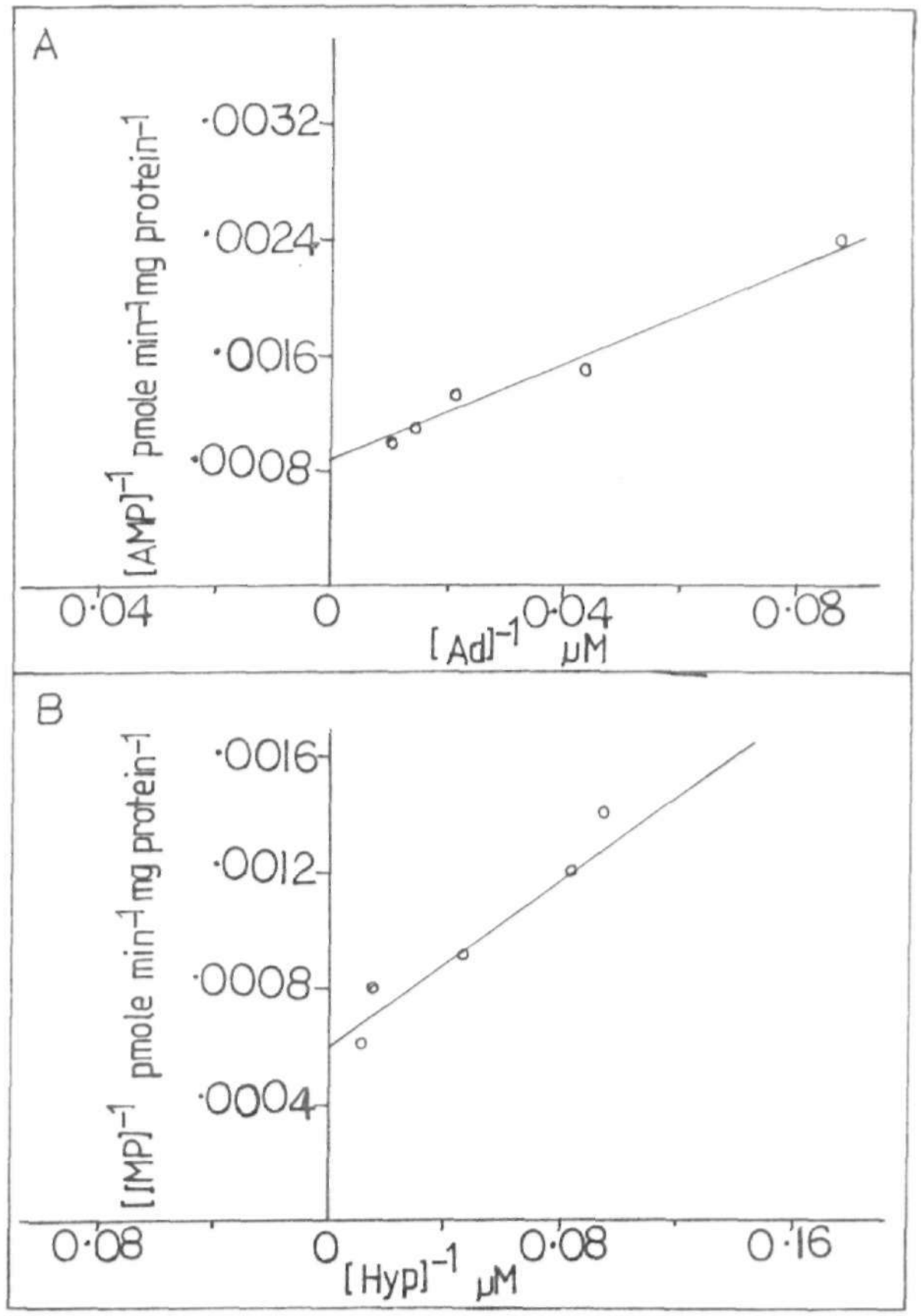

A
·0016
·0012
·0008
·0004
[AMP]⁻¹ pmole min⁻¹ mg protein⁻¹
0·04
0
0·04
0·08
[Ad]⁻¹ μM
B
·0020
·0016
·0012
·0008
·0004
[IMP]⁻¹ pmole min⁻¹ mg protein⁻¹
0·04
0
0·04
0·08
[HYP]⁻¹ μM

Fig. 14 Assay of purine phosphoribosyltransferase in extract from T. vivax.

^{14}C-Adenine and ^{14}C-hypoxanthine were each incubated separately with protein extract of T. vivax at 37°C. The incubation media were analysed by streaking aliquots (10 µl) on cellulose thin layers and devcloping on solution of 1.25M ammonium sulphate. The zones corresponding to adenine and hypoxanthine were cut out when ^{14}C-adenine (A) was the substrate and the IMP and hypoxanthine zones were cut out when ^{14}C-hypoxanthine (B) was the substrate. The radio-activity associated with them were determined as described in Materials and Methods. The kinetic constants were determined from the double reciprocal plots.


A
·0032
·0024
·0016
·0008
[AMP]⁻¹ pmole min⁻¹ mg protein⁻¹
0·04
0
0·04
0·08
[Ad]⁻¹ µM
B
·0016
·0012
·0008
·0004
[IMP]⁻¹ pmole min⁻¹ mg protein⁻¹
0·08
0
0·08
0·16
[Hyp]⁻¹ µM

Adenine phosphoribosyl transferase of these flagellates was not stable when kept frozen for two weeks; enzyme activity decreased to about 5% of the fresh sample after fourteen days of storage. The hypoxanthine phosphoribosyltransferase was more stable retaining 50% of its activity after fourteen days of storage in the cold (below $0^{\circ}C$).

7.5 Adenosine deaminase and adenine aminohydrolase

Adenosine deaminase catalyses the deamination of adenosine while adenine aminohydrolase, adenase or adenine deaminase catalyses adenine deamination.

Adenosine ——————→ Inosine

Adenine ——————→ hypoxanthine

All the trypanosomes examined so far (T. congolense; T. brucei; T. gambiense and T. vivax) whether the trypomastigotes or the procyclics lacked adenosine deaminase (Table 3) and only T. vivax has adenine aminohydrolase in both trypomastigotes or procyclic form (Fig. 15)

7.6 Adenosine 5'-monophosphate deaminase and inosine monophosphate nucleotidase

AMP deaminase catalyses deamination of adenosine monophosphosphate to inosine monophosphate while the IMP nucleotidase cleaves IMP to inosine.

TABLE 3

Activities of purine salvage enzymes in homogenates of T. congolense, T. vivax and T. brucei.

Enzyme activity	Purine substrate	Specific activity (nmole/min/mg protein)		
		T. congolense	T. vivax	T. brucei
Purine nucleoside hydrolase and phosphorylase	Adr.	1.4 ± 0.4 (3)	2.9 ± 0.4 (3)	153.2 ± 73.2 (3)
	Adr. + Phosphate	7.2 ± 0.6 (3)	3.2 ± 0.5 (3)	153.2 ± 73.2 (3)
	In	13.1 ± 1.4 (3)	23.4 ± 0.1 (3)	152.2 ± 88.6 (3)
	In + Phosphate	16.2 ± 1.6 (3)	18.9 ± 1.6 (3)	152.5 ± 88.6 (3)
Phosphoribosyltransferase	Ad.	3.55 ± 0.35 (2)	1.28 ± 0.08 (2)	19.9 ± 0.70 (2)
	Hyp.	2.14 ± 0.08 (2)	1.78 ± 0.04 (2)	9.48 ± 0.49 (2)
Adenosine deaminase	Adr.	n.d.	n.d.	n.d.
Adenine aminohydrolase	Ad	n.d.	121.1 ± 27.1 (2)	n.d.

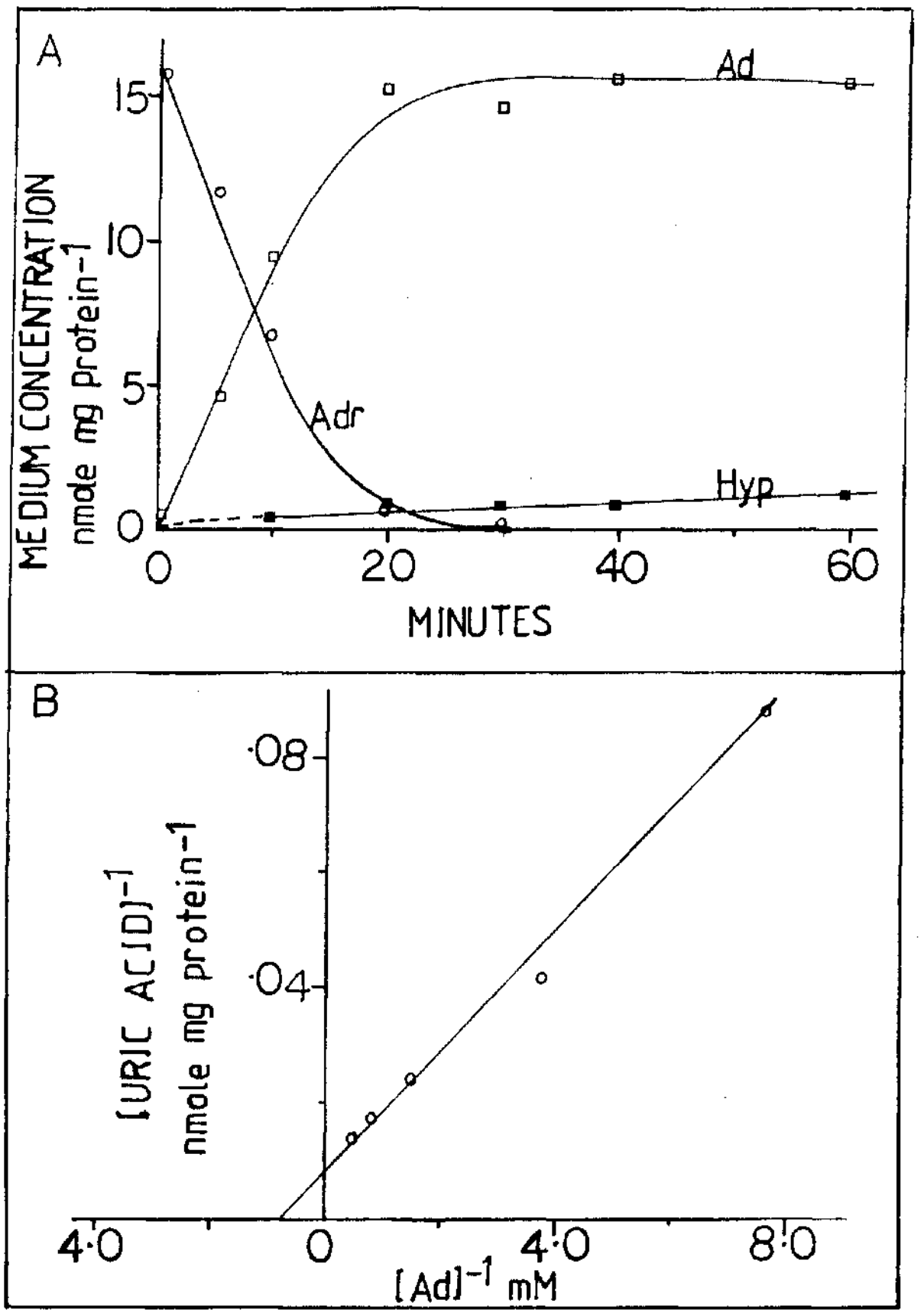
n.d. not detected; figures in parenthesis (no. of determinations)

Fig. 15. A. Time course of adenosine cleavage by extract of T. vivax.

B. Rate of adenine deamination by adenine aminohydrolase from T. vivax.

^{14}C-Adenosine (A) was incubated at 37°C with protein extract of T. vivax in buffer pH 7.2. Samples (10 µl) were removed at intervals and streaked on cellulose thin layers which were developed in 1.25M ammonium sulphate solution. The zones corresponding to adenosine (○——○); adenine (□——□) and hypoxanthine (■——■) were cut out and the radioactivity associated with them determined as described in Materials and Methods. No inosine intermediate was detected in the incubation medium.

In another experiment (B) various concentration of unlabelled adenine were incubated with protein extract of T. vivax and xanthine oxidase enzyme was added to the incubation medium. The uric acid production was measured spectrophotometrically as described by Mans and Koch (1960).


A
MEDIUM CONCENTRATION
nmole mg protein-1
15
10
5
0
Ad
Adr
Hyp
0
20
40
60
MINUTES
B
[URIC ACID]-1
nmole mg protein-1
·08
·04
4·0
0
4·0
8·0
[Ad]-1 mM

$$AMP \longrightarrow IMP + NH_3$$

$$IMP \longrightarrow Inosine + Pi$$

The enzymes were not assayed *per se* in this study but their presence in the trypanosomes (*T. brucei* and *T. congolense*) were shown by indirect evidence. Also the absence of AMP deaminase in *T. vivax* was also shown by indirect evidence. Figure 16 shows that when AMP was synthesized from adenine and PRPP in *T. congolense* and *T. brucei*, one of the end products was inosine; *T. vivax* gave only AMP; but inosine was generated when IMP was synthesized from hypoxanthine and PRPP (Fig. 17).

7.7 *Nucleoside uptake*

The uptake of ^{3}H-adenosine by trypomastigotes of *T. gambiense* and *T. congolense* when measured as a function of increasing substrate concentration showed a linear relationship (Figs. 18A and B). Guanosine competed for uptake with adenosine while thymidine did not affect the uptake of adenosine (Figs. 19A and 19B).

Efflux of ^{3}H-thymidine from the red cells of mice infected with *T. gambiense* was higher than from those of non-infected (Figs. 20A and B), but the number of NBMPR binding sites was similar in infected and non-infected mice (Fig. 21).

Fig. 16 Synthesis of AMP from adenine and phosphoribosyl pyrophosphate with adenine phosphoribosyltransferase from T. brucei (A) and T. congolense (B).

The protein extracts from T. brucei (A) and T. congolense (B) trypomastigotes were incubated at 37°C with ^{14}C-adenine; phosphoribosyl pyrophosphate and magnesium chloride in Tris buffer pH 7.2. The assays were terminated by boiling for 5 min at 100°C and aliquots (10 µl) of the media streaked on cellulose thin layers previously coated with carriers (AMP, inosine and IMP). The cellulose thin layers were developed in 1.25M ammonium sulphate solution and the zones corresponding to AMP (O——O); and inosine plus IMP (□—□) were cut out and the radioactivity associated with them determined as described in Materials and Methods.

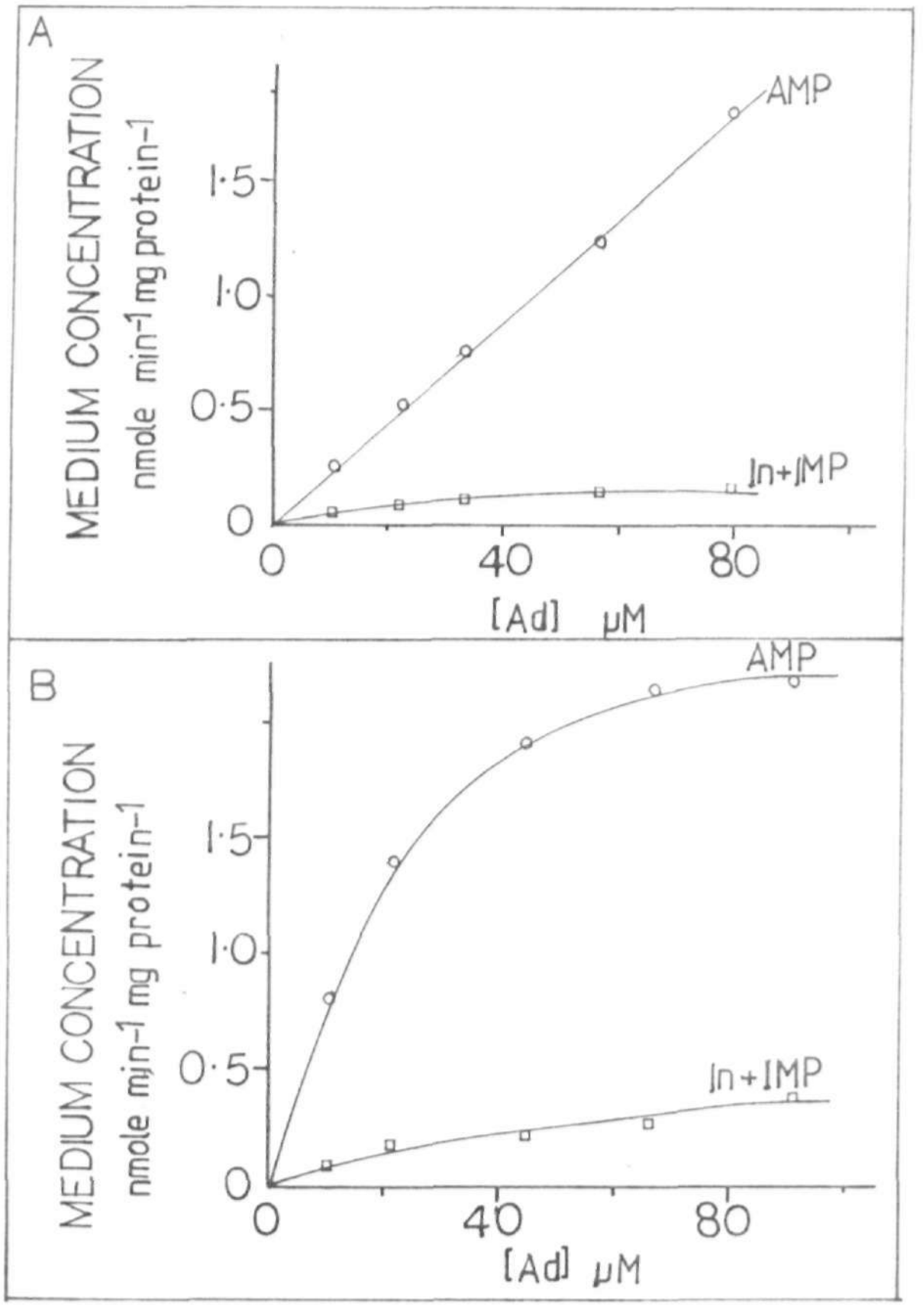

A
MEDIUM CONCENTRATION
nmole min-1 mg protein-1
1·5
1·0
0·5
0
0
40
80
[Ad] μM
AMP
In+IMP
B
MEDIUM CONCENTRATION
nmole min-1 mg protein-1
1·5
1·0
0·5
0
0
40
80
[Ad] μM
AMP
In+IMP

Fig. 17 Synthesis of IMP from hypoxanthine and phosphoribosyl pyrophosphate with extract from T. vivax.

The protein extract of T. vivax trypomastigotes was incubated at 37°C with ^{14}C-hypoxanthine and phosphoribosyl pyrophosphate in Tris buffer containing magnesium chloride. Incubation were terminated by boiling for 5 min at 100°C and measured volumes (10 µl) of the assay medium were applied on cellulose thin layers which were developed in 1.25M ammonium sulphate solution. The zones corresponding to IMP (○——○); inosine (□——□) were cut out and the radioactivity associated with them determined as described in Materials and Methods.

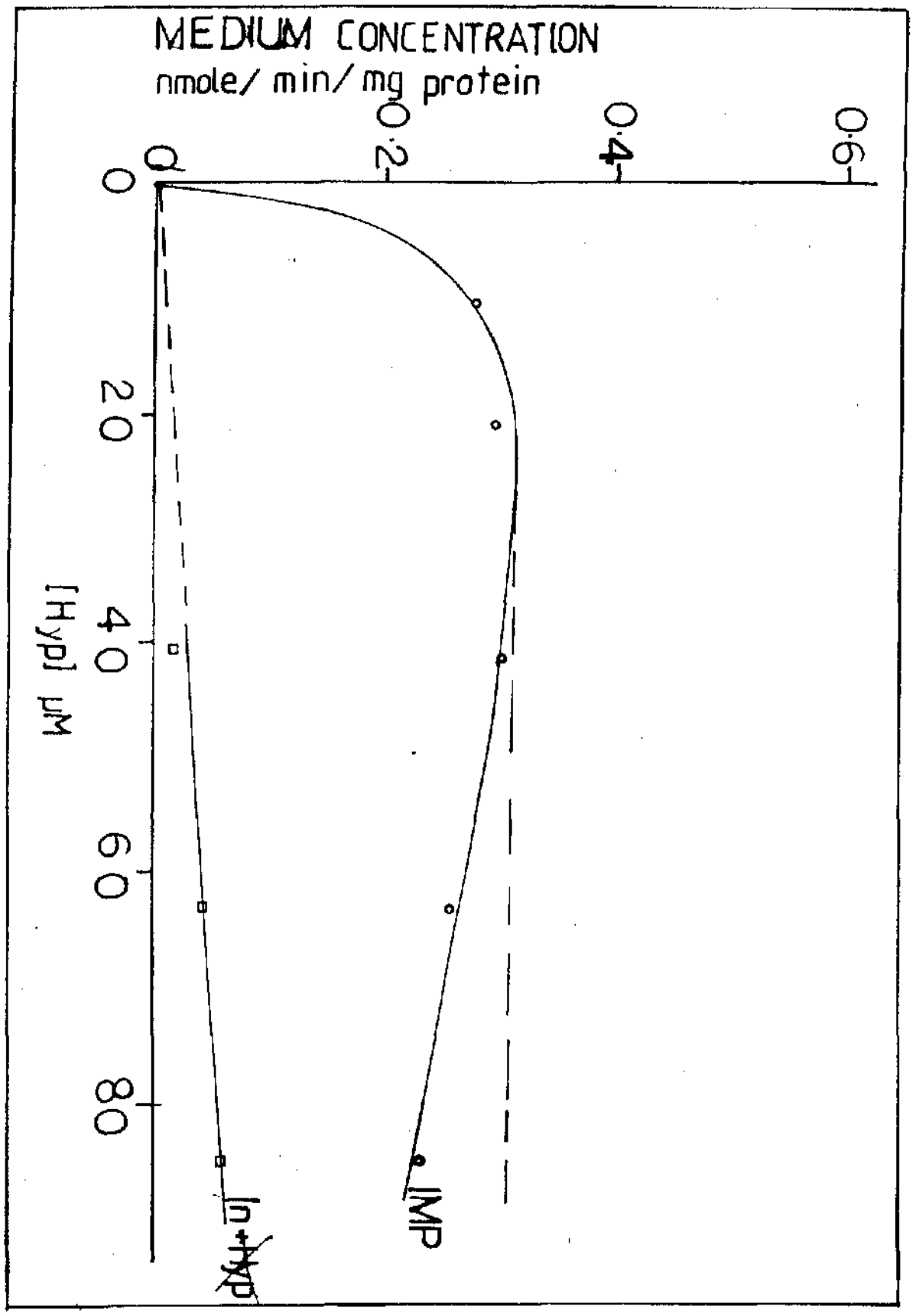

MEDIUM CONCENTRATION
nmole/ min/ mg protein
0
0·2
0·4
0·6
0
20
40
60
80
[Hyp] μM
In + IMP
IMP

Fig. 18 Uptake of ^{3}H-adenosine by T. gambiense (A) and T. congolense (B).

T. gambiense (A) and T. congolense (B) trypomastigotes were separately incubated with various concentrations (0.08 - 0.5mM) of ^{3}H-adenosine at 25°C. Assay intervals were terminated by centrifugation and the trypanosomes washed three times with buffer. The cellular content of ^{3}H was determined as described in Materials and Methods. The plots of concentrations of the substrate versus the rate of ^{3}H uptake is shown in the graph.

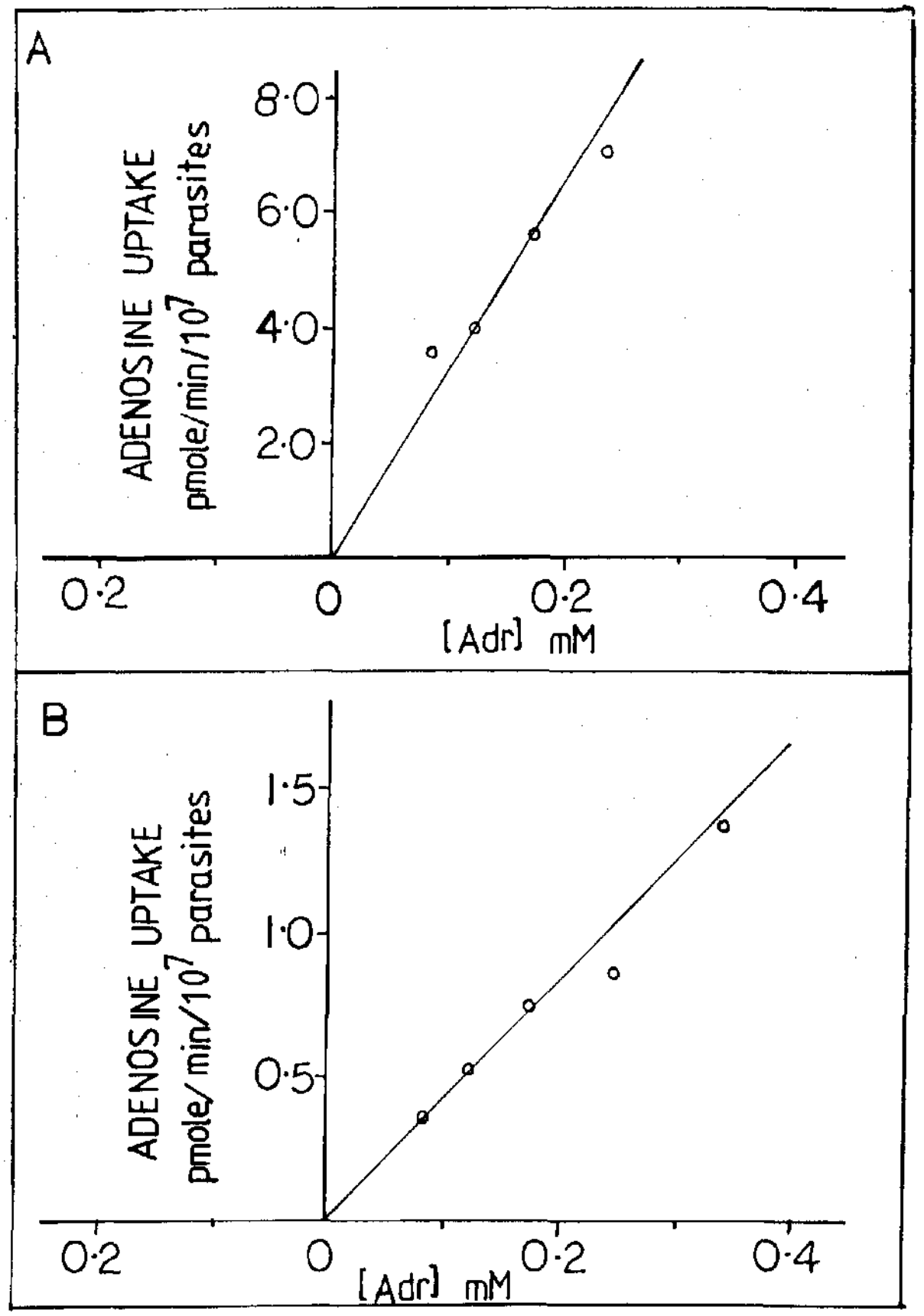

A
ADENOSINE UPTAKE
pmole/min/10^7 parasites
8·0
6·0
4·0
2·0
0·2
0
0·2
0·4
[Adr] mM
B
ADENOSINE UPTAKE
pmole/min/10^7 parasites
1·5
1·0
0·5
0·2
0
0·2
0·4
[Adr] mM

Fig. 19 Inhibition of ^{3}H-adenosine uptake by guanosine and thymidine in T. gambiense (A) and T. congolense (B).

The T. gambiense (A) and T. congolense (B) trypomastigotes were each incubated separately with labelled adenosine (0.08 - 0.5mM) at 25°C in buffered normal saline (○——○) and in the buffer containing guanosine (■——■) and thymidine (□——□). The cellular content of the trypanosomes after washing three times by centrifugation were measured as described in Materials and Methods. The Vmax of adenosine uptake calculated from the double reciprocal plots were 18.2 and 6.7 pmole/min/10^{7} trypanosomes for the T. gambiense and T. congolense respectively.

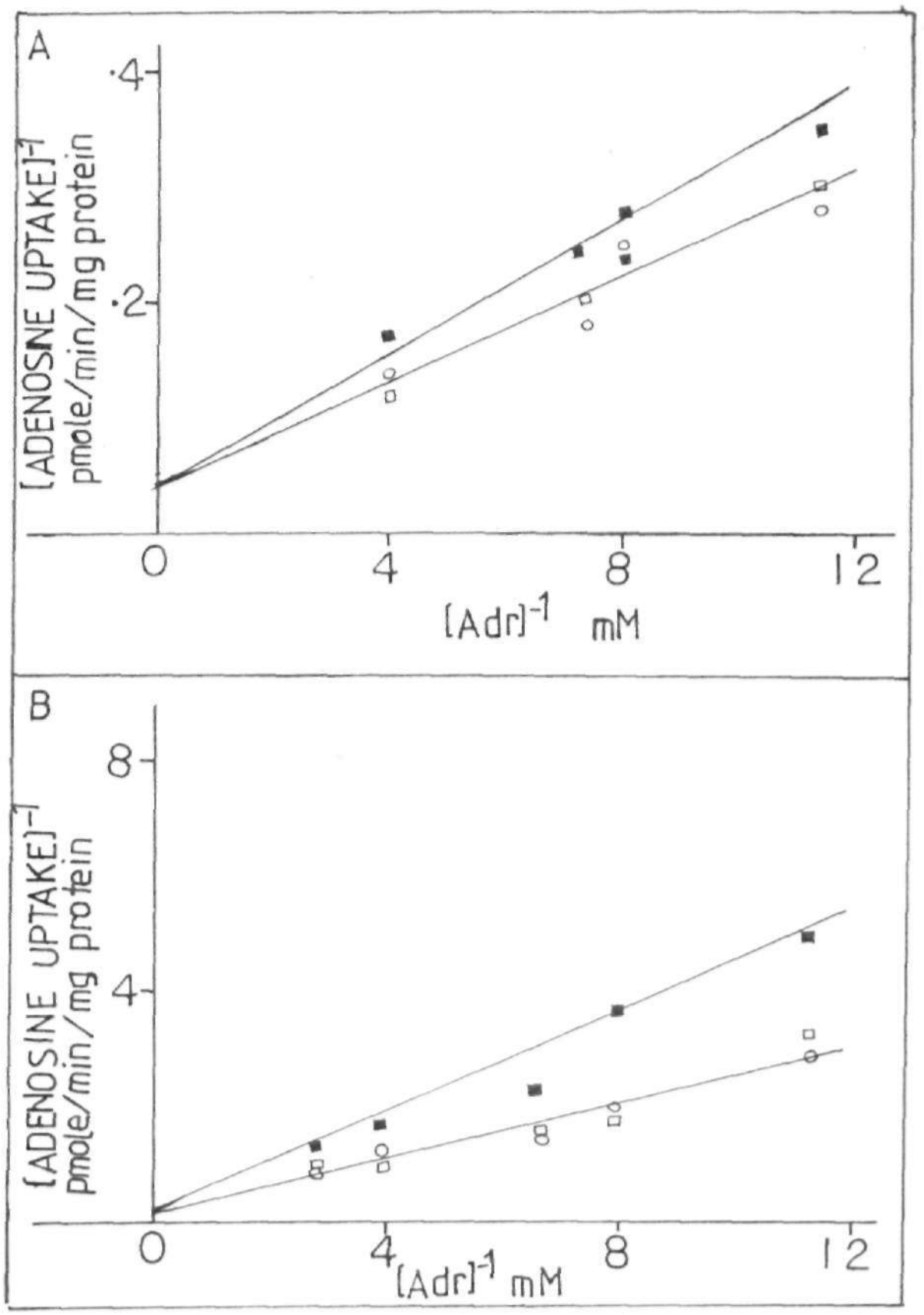

A
·4
·2
[ADENOSINE UPTAKE]⁻¹
pmole/min/mg protein
0
4
8
12
[Adr]⁻¹ mM
B
8
4
[ADENOSINE UPTAKE]⁻¹
pmole/min/mg protein
0
4
8
12
[Adr]⁻¹ mM

Fig. 20 Efflux of ^{3}H-thymidine from erythrocytes of mice uninfected and infected with T. gambiense

Erythrocytes from mice uninfected (A) and infected (B) with T. gambiense were loaded with 2.0 mM ^{3}H-thymidine as described in Materials and Methods. The erythrocytes were incubated in buffered saline only (○—○) and the buffered saline containing 6 mM non-labelled thymidine (□—□). Efflux of radioactivity in the incubation media were followed as described in Materials and Methods. Thymidine efflux expressed as µmoles/min/ml packed cells is indicated for each time course of apperance of radioactivity in the medium.

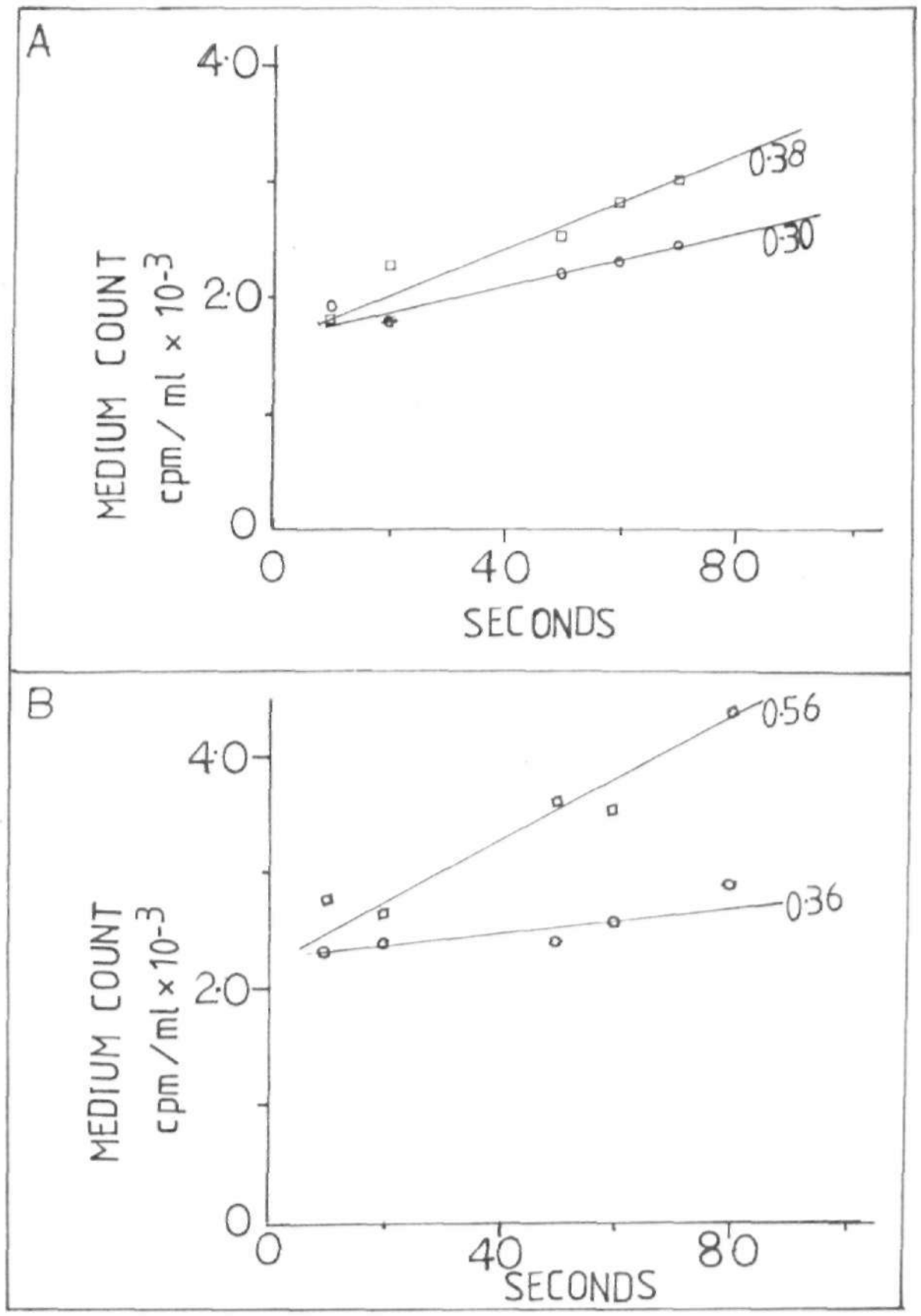
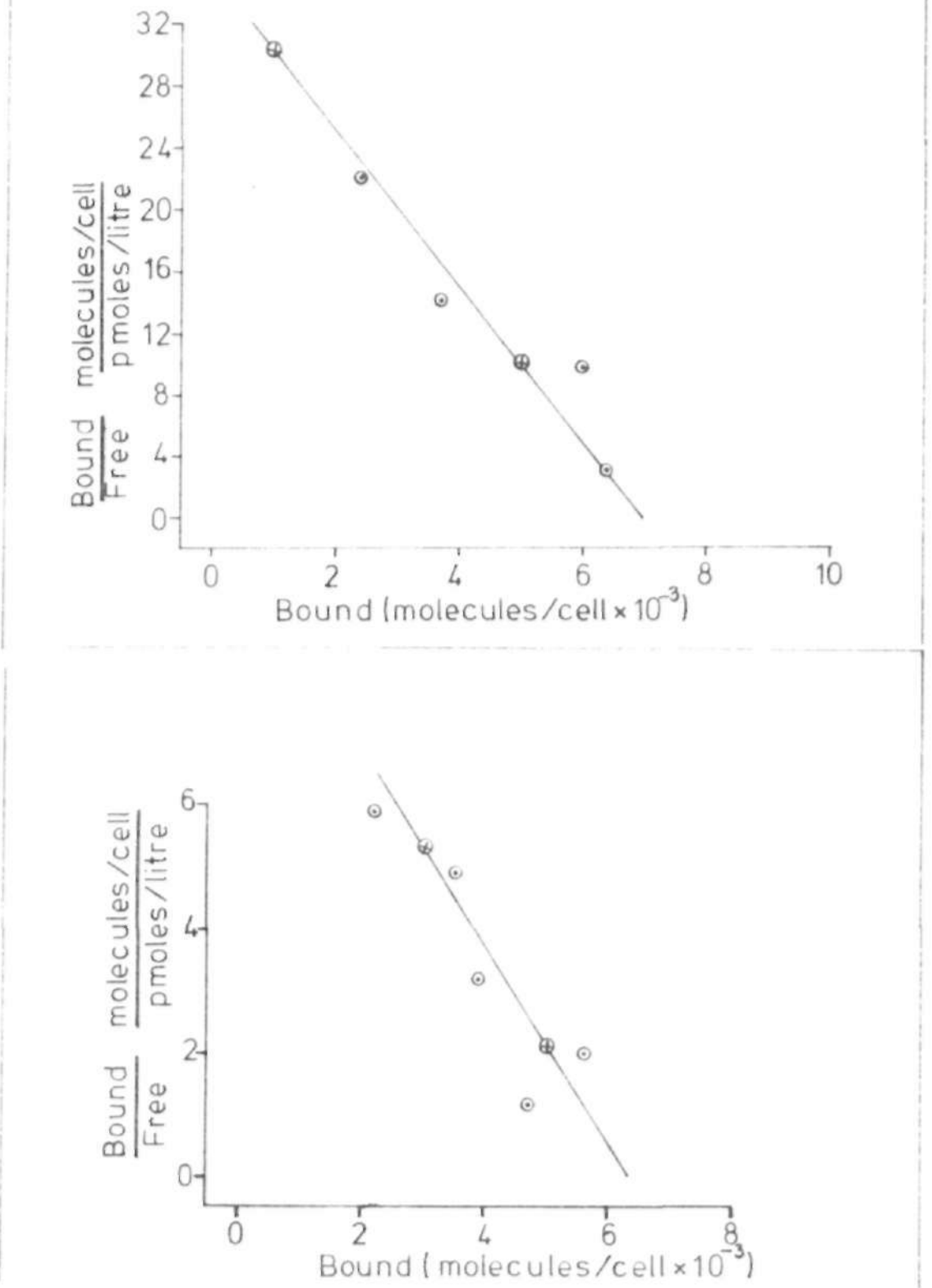

A
4·0
2·0
0
MEDIUM COUNT
cpm / ml x 10-3
0·38
0·30
0
40
80
SECONDS
B
4·0
2·0
0
MEDIUM COUNT
cpm / ml x 10-3
0·56
0·36
0
40
80
SECONDS

Fig. 21 Binding of NBMPR to erythrocytes from mice uninfected and infected with T. gambiense.

Washed erythrocytes from mice uninfected (A) and infected (B) were incubated at 25°C with graded concentrations of G-^{3}H-NBMPR without and with 5 µM NBMPR for 30 min. Binding was terminated by centrifugation and the medium content of ^{3}H determined. The amount of ligand taken up by erythrocytes was determined from the difference between the ^{3}H-content of the medium before and after incubation with erythrocytes (free NBMPR). The data obtained are presented in the form of a mass law (Scatchard) plot from which were obtained the dissociation constant of the bound NBMPR (10^{-9} M) and the maximum number of cellular binding sites (6.2 - 6.6 x 10^{3} sites/trypanosome).


Bound / Free molecules/cell / pmoles/litre
32
28
24
20
16
12
8
4
0
0
2
4
6
8
10
Bound (molecules/cell × 10⁻³)
Bound / Free molecules/cell / pmoles/litre
6
4
2
0
0
2
4
6
8
Bound (molecules/cell × 10⁻³)

7.8 Nucleoside inhibitors

The nucleoside transport inhibitor, nitrobenzylthioinosine did not bind to T. brucei or T. gambiense trypomastigotes (Fig. 22). Also nitrobenzylthioinosine and dipyridamole did not inhibit the uptake of adenosine in T. brucei (Fig. 23A) but dipyridamole interfered with adenosine uptake in T. congolense (Fig. 23B). The inhibition of adenosine uptake in T. congolense was competitive.

7.9 Chemotherapy studies

The LD50 for the drugs studied in mice is summarized in Table 4. The inclusion of nitrobenzylthioinosine at a concentration of 5 mg/kg abolished the toxicity of tubercidin and also of the tubercidin-ethidium combination (Table 4 and 5). Concurrent administration of tubercidin and NBMPR-P suppressed trypanosome infection in mice (Fig. 24). NBMPR -P did not block the trypanocidal action of tubercidin as illustrated in Fig. 25.

Fig. 22 Time course of G-^{3}H—NBMPR binding to *T. brucei* (A) and *T. gambiense* (B) trypomastigotes.

5nM G-^{3}H—NBMPR was incubated with either *T. brucei* (A) or *T. gambiense* (B) trypomastigotes at 26°C for the time intervals indicated with (○——○) and without nonisotopic 5 µM NBMPR (□——□). Incubation were terminated by centrifugation and the trypanosomes washed three times with normal saline. The radioactivity associated with the trypanosomes was determined as described in Materials and Methods.

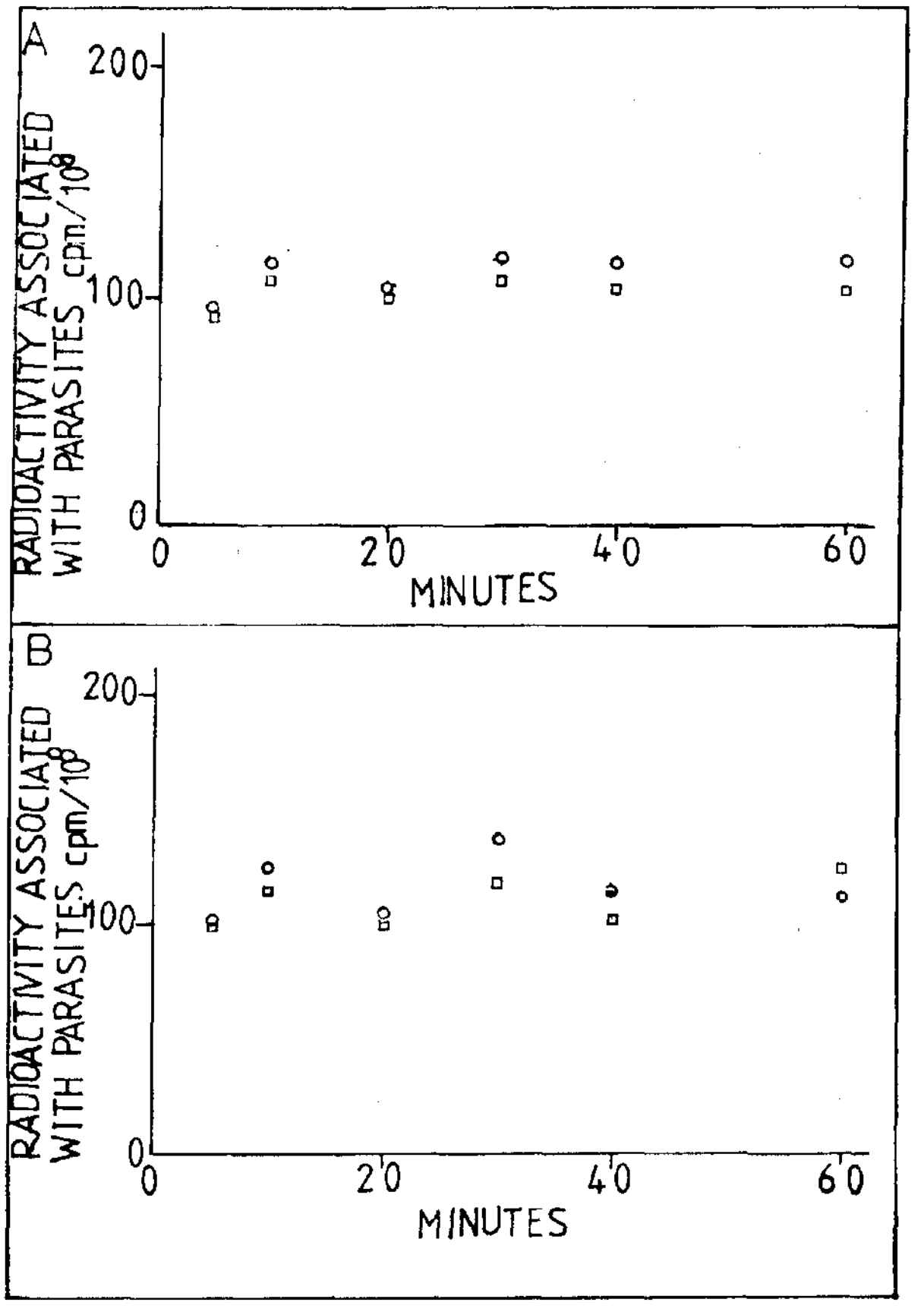

A
RADIOACTIVITY ASSOCIATED WITH PARASITES cpm/10⁸
200
100
0
0
20
40
60
MINUTES
B
RADIOACTIVITY ASSOCIATED WITH PARASITES cpm/10⁸
200
100
0
0
20
40
60
MINUTES

Fig. 23 Uptake of ^{3}H-adenosine with and without NBMPR and dipyridamole in T. gambiense (A) and T. congolense (B).

T. brucei (A) or T. congolense (B) trypomastigotes were incubated with concentrations of ^{3}H-adenosine at 26°C without (○——○); with NBMPR (□——□) and with dipyridamole (■——■). The assay was terminated by centrifugation and cellular content of ^{3}H determined as described in Materials and Methods.

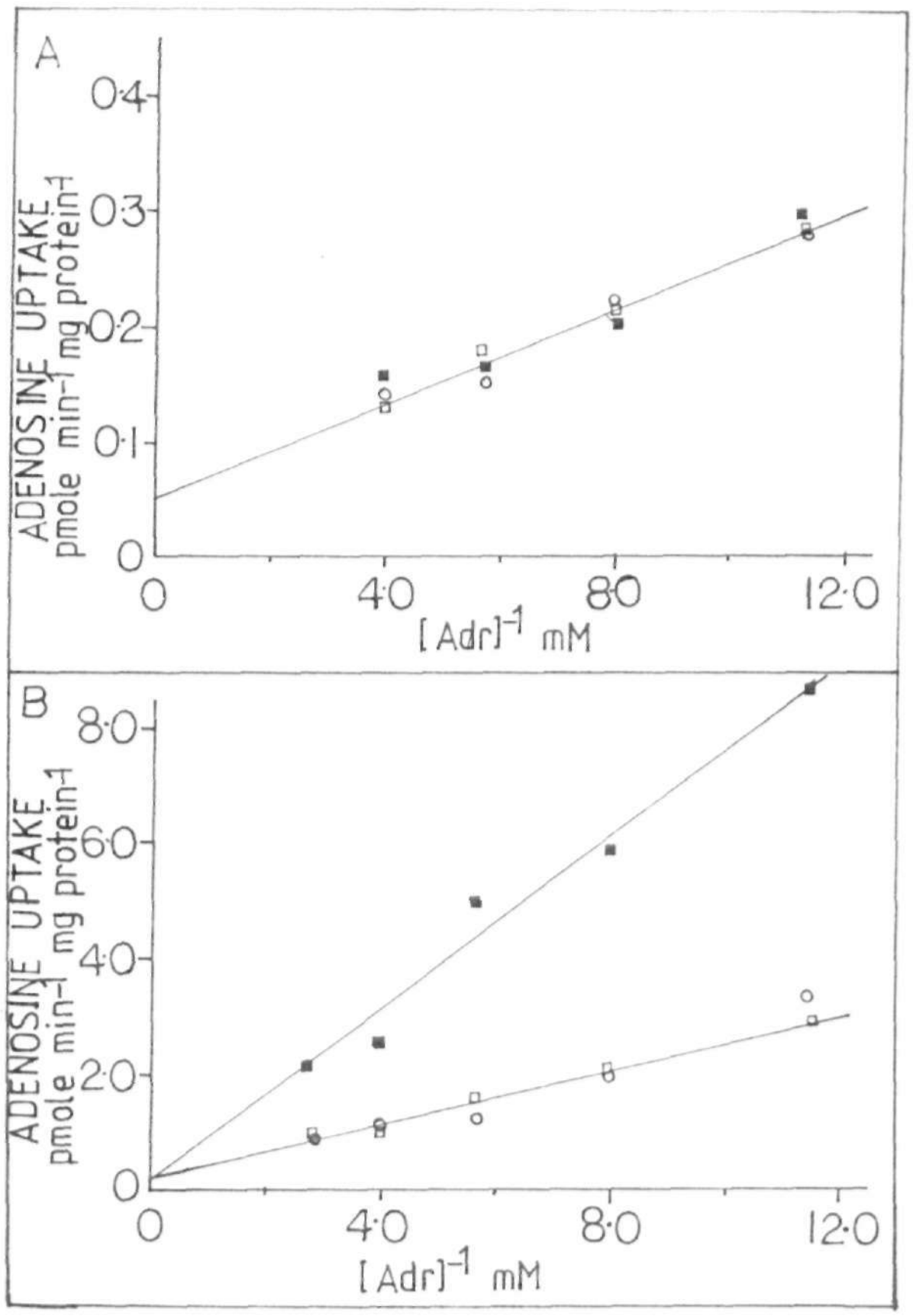

A
ADENOSINE UPTAKE
pmole min-1 mg protein-1
0·4
0·3
0·2
0·1
0
0
4·0
8·0
12·0
[Adr]-1 mM
B
ADENOSINE UPTAKE
pmole min-1 mg protein-1
8·0
6·0
4·0
2·0
0
0
4·0
8·0
12·0
[Adr]-1 mM

TABLE 4

Determination of LD50 for tubercidin and ethidium bromide in mice

Agent dosage (mg/kg)	Number surving on day 7 Number injected	LD50
Tubercidin		
12.5	4/10	
10.0	5/10	
8.0	7/10	10.7
6.3	8/10	(7.5-13.9)
4.0	9/10	
3.2	10/10	
Ethidium		
120	0/10	
100	5/10	74.1
75	7/10	(48.3-99.8)
50	8/10	
25	10/10	
Tubercidin-ethidium		
10.0/12.5	1/10	
5.0/12.5	3/10	
Tubercidin-ethidium-NBMPR-P		
10.0/12.5/5	10/10	
5.0/12.5/5	10/10	

Male BALB/C mice were given single ip injection of tubercidin, ethidium bromide and nitrobenzylthioinosinate aither singly or in comtination in dosages shown in the Table. Each mouse received an injection volume of 0.2ml per 20g body weight whether one or more drugs were given. Deaths were recorded daily for 7 days. The LD50 was determined as described by Tallarida and Jacob (1979).

TABLE 5

Inhibition of tubercidin toxicity in mice with nitrobenzylthioinosinate

Agent (dosage mg/kg)		Number surving up to 30 days / Number injected
Tubercidin		
25.0		0/10
18.7		0/10
12.5		4/10
6.3		8/10
3.1		10/10
Tubercidin/NBMPR-P		
25.0	5	10/10
18.7	5	10/10
12.5	5	10/10
6.3	5	10/10
3.1	5	10/10

Groups of mice (10 per group) were given single doses of either tubercidin or ethidium and NBMPR-P (5 mg/kg). The tubercidin were given at different doses as stated in the Table. The mice were observed for 30 days during which deaths were recorded.

Fig. 24 Trypanocidal activity of tubercidin with or without nitrobenzylthioinosinate

Groups of mice inoculated with T. gambiense were treated with either normal saline (O——O) which served as the control; 5 mg/kg tubercidin (Δ——Δ) or tubercidin and NBMPR-P (□——□) at 5 mg/kg each. The drugs were given on the days indicated (↓). Blood samples from the tails of the mice were examined daily. All the mice in the control group and the group that received tubercidin died in 3-4 days.

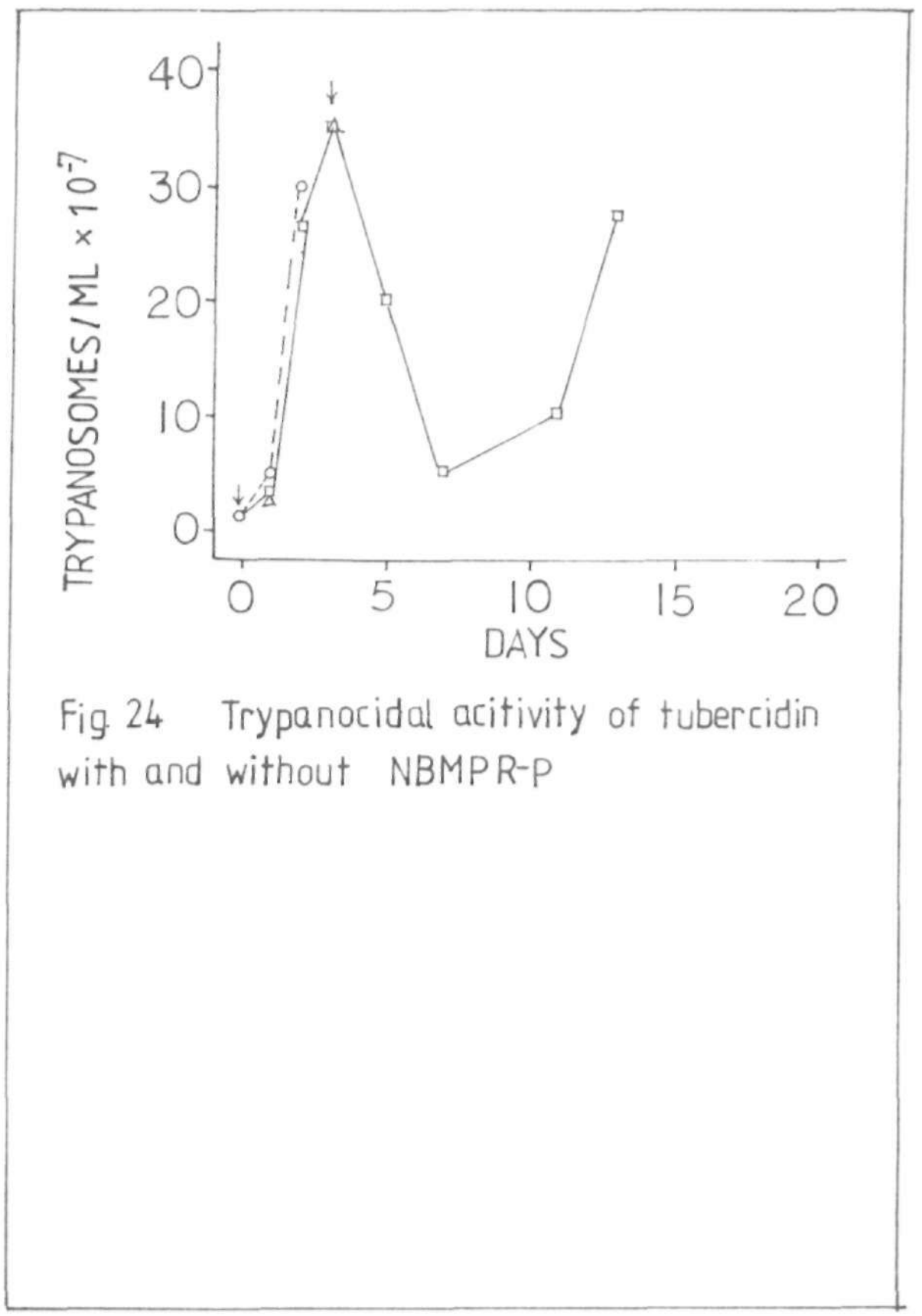
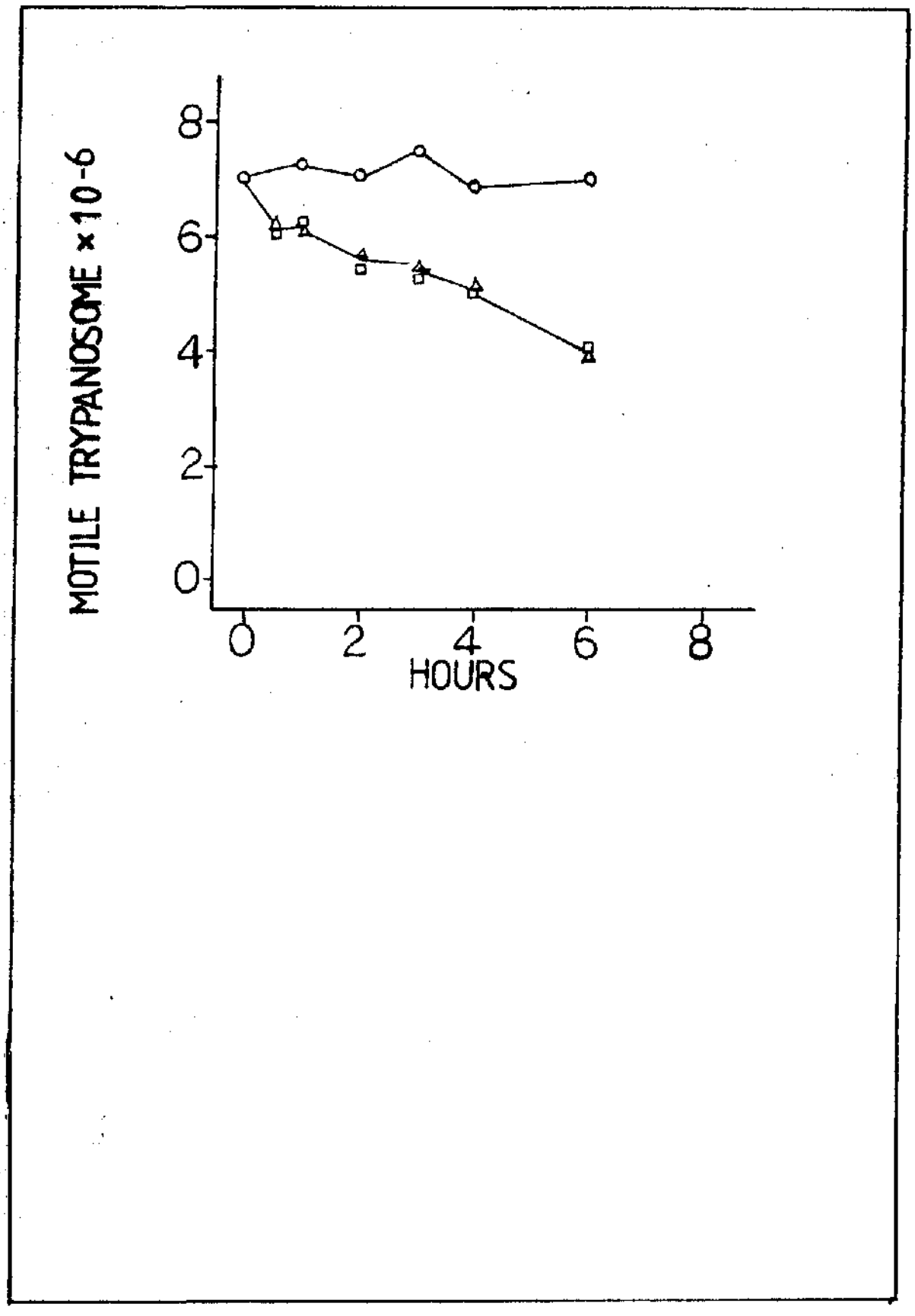


Fig 24 Trypanocidal acitivity of tubercidin with and without NBMPR-P

Fig. 25 Effect of tubercidin on the motility of *T. gambiense* trypomastigotes in vitro at 26°C.

T. gambiense trypomastigotes were incubated in the medium containing (a) buffer only to serve as the control (○——○); (b) 0.5 mM tubercidin (△——△); (c) 0.5 mM tubercidin and 5 µm NBMPR (□——□). Samples were removed at intervals shown in the graph to determine the number of motile parasites.


MOTILE TRYPANOSOME ×10-6
8
6
4
2
0
0
2
4
6
8
HOURS

CHAPTER 8

DISCUSSION

8.1 Chromatography

Literature search reveals a number of systems which have been employed for the separation of purine bases, nucleosides and nucleotides. Amongst these methods, paper (Litvak et al, 1969; Henderson et al, 1974) and thin-layer (Reyes et al, 1982; Berens et al, 1981; Marr et al, 1978) chromatography were commonly used. Trial experiments with paper chromatography did not give satisfactory results. Not only that the separations were unsatisfactory, the time required to develop the chromatograms were usually to long. For an example, using butanol : acetic acid : water (5: 3: 2 v/v/v) or 5% disodium orthophosphate-isoamyl alcohol mixture (v/v), it required 16 and 8 hours, respectively to develop the chromatogram in these solvent systems. With thin-layer chromatography, cellulose and PEI-cellulose were commonly used. The results shown in Table 2 evidently demonstrated that cellulose gave a better separation than the PEI-cellulose thin layer. The thin-layer chromatography when compared to the paper chromatography is superior not only in being able to resolve the purine bases from the nucleotides but also took less time (90 min) to develop in the solvent systems used for the separation.

8.2 Enzymes

Earlier studies based mainly on purine requirements for these parasites in semidefined media (Bone and Steinert, 1956;

Jaffe and Gutteridge, 1973) had shown that parasitic protozoa including the African trypanosomes, in general, lack the ability to synthesize purine bases de novo and instead they rely on preformed purine bases and nucleosides. A full complement of purine interconversion enzymes which would permit utilization of purine bases and nucleosides has been demonstrated in a number of flagellates; Plasmodium chabaudi (Lukow, et al, 1973); Trypanosoma cruzi (Gutteridge and Davies, 1981) and Leishmania (Marr et al, 1978) indicating that these parasites utilize preformed purine bases and nucleosides. Differences exist in the salvage pathway of purines in these parasitic protozoa (Fig. 4). However, it is not known whether differences exist in the salvage pathway among the African trypanosomes. Except for T. gambiense (Schmidt et al, 1975; Fish et al, 1982a and b), there has not been any systematic attempt to demonstrate and evaluate the salvage pathways for purines in other African trypanosomes.

A purine nucleoside upon entry into the cell could undergo scission to a purine base and ribose sugar by the action of purine hydrolase or a purine phosphorylase. The former enzyme, the hydrolase, is essentially found in bacteria (Soldo and Wagtendonk, 1961) but of recent has been described for a number of flagellates; C. fasciculata (Dewey and Kidder, 1973); T. cruzi (Gutteridge and Davies, 1981) and T. gambiense (Schmidt et al, 1975). Purine phosphorylase on the other hand is found both in animal cells (Tucker and Young, 1976; Murray,

1971) and in flagellates: T. cruzi (Gutteridge and Davies, 1981; Berens et al, 1981) and leishmania (Marr et al, 1978). In this study, the decision as to whether an enzyme is a hydrolase or a phosphorylase was made based on three criteria used by other workers (Gutteridge and Davies, 1981; Schmidt et al, 1975): (a) measurement of the enzyme activity of the protein extract dialysed in Tris buffer, (b) stimulation of these activities with inorganic phosphate, and (c) nucleoside production when radiolabelled bases were incubated with ribose-1-phosphate under the conditions used for the hydrolase assay.

The failure to stimulate adenosine and inosine cleavage by T. brucei enzyme (Fig. 6) Suggests that purine nucleosides are cleaved by hydrolase as in T. gambiense (Schmidt et al, 1975). This is confirmed by the inability of the enzyme to catalyse the synthesis of purine nucleoside from the purine base and ribose-1-phosphate. In contrast, the cleavage of adenosine by the enzyme from T. congolense was stimulated by phosphate (Fig. 7) while inosine cleavage was not affected suggesting that the former was cleaved by phosphorylase while the latter was cleaved by hydrolase. The cleavage of adenosine by phosphorylase was confirmed by synthesis of adenosine from adenine and ribose-1-phosphate (Fig. 8). Assays of crude protein extracts from T. vivax showed that both adenosine and inosine were cleaved by purine nucleoside hydrolase (Fig. 9).

Since assays were done with crude extracts, the rate values reported in Table 3 gave only indications of the activities and the contributions of these enzymes in the salvage of purines in trypanosomes. Apparently, the T. brucei metabolized the nucleosides at a rate much faster than the T. vivax and T. congolense. The specific activities for the cleavage of adenosine and inosine are similar in T. brucei while in T. vivax and T. congolense the specific activity for inosine cleavage is greater than that for adenosine cleavage. Could this represent a preference for inosine and hypoxanthine in T. vivax and T. congolense? In T. cruzi, it had been suggested that adenine and hypoxanthine are preferred substrates for the parasite (Berens et al, 1981). The relative importance of the purine substrates, inosine and hypoxanthine and indeed the other purine bases and nucleosides will be dealt with when other purine salvage enzymes and the uptake processes have been discussed.

It has often been assumed without proof that purine necleoside kinases are present in African trypanosomes. Nucleosides upon entry into cells are rapidly metabolized by phosphorylation either by the direct action of purine nucleoside kinase or by first being hydrolysed to a purine base before being phosphorylated to a nucleotide by the enzyme phosphoribosyltransferase. In animal cells, the former pathway, that is the direct phosphorylation of nucleosides by the kinases dominates; purine nucleoside phosphorylase

activities are usually very low or virtually absent (Murray, 1971). In the flagellates, the purine nucleosides usually undergo scission before phosphorylation (Gutteridge and Davies, 1981; Schmidt et al, 1975; Dewey and Kidder, 1973) although in some others like malaria parasites, both the hydrolase and phosphorylase are not detectable, thus they most likely use kinases like animal cells and in fact adenosine kinase has been partially purified from P. chabaudi (Schmidt et al, 1974; Lukow et al, 1973; Konigk, 1976; Yamada and Sherman, 1981). The rapid conversion of adenosine to adenine by purine hydrolase from T. brucei and the absence of AMP in the assay medium after an hour of incubation period (Fig. 10) suggested that adenosine kinase was either absent in the trypanosome or the activity was too low in the organism to be detected. The result was reproducible and even when the assay was repeated using spectrophotometric method, no adenosine kinase activity was detected. This is consistent with the undetectable level of this enzyme in T. brucei. When the protein extracts from T. congolense or T. vivax were assayed for this enzyme, some AMP (Fig. 11) was detected suggesting that adenosine kinase may be present in the two trypanosomes. Although, some adenosine kinase activity was detected in T. congolense and T. vivax it is doubtful whether this pathway contributes significantly to the salvage of purine nucleosides in the parasites. This point will be discussed further when the other pathways for purine nucleotide formation in the parasites are considered.

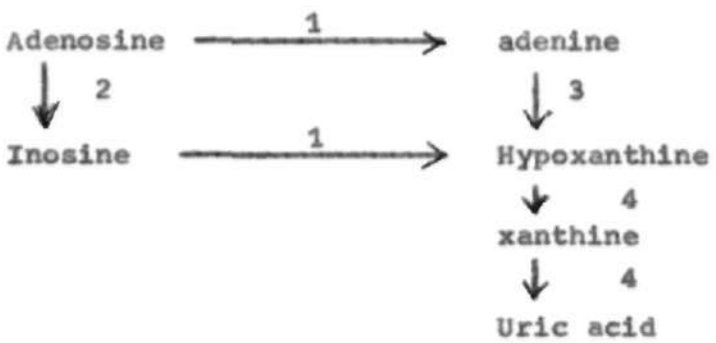
It was first shown in 1955 that purine bases are converted to ribonucleotides by enzymes present in yeast autolyzates in the presence of phosphoribosyl pyrophosphate (Kornberg *et al*, 1955). Since then these enzymes first called pyrophosphorylases and now phosphoribosyltransferases, have been found in a variety of organisms from procaryotes to mammals, including the humans (Olsen and Milman, 1974; 1977). While mammalian cells (Henderson, 1968; Olsen and Milman, 1974) and malarial parasite, *P. chabaudi* (Walter and Konigk, 1974) have H x PRTase, *E. coli* (Miller *et al*, 1972) and the flagellate kinetoplastid, *C. fasciculata* (Kidder *et al*, 1979) have phosphoribosyltransferase specific for guanine or hypoxanthine, that is, GuaPRTase and H x PRTase are separate enzymes in *E. coli* and *C. fasciculata*. In this study, no attempt was made to determine whether there are separate phosphoribosyltransferases for guanine and hypoxanthine rather the study was meant to establish the presence of phosphoribosyltransferase and evaluate the role of the enzyme in the salvage of purines in African trypanosomes. As seen in the results of the assays (Table 3), all the trypanosomes studied have appreciable activity of this enzyme.

Purine bases were taken up more efficiently into the nucleotide pools than the ribonucleosides, for example, percent incorporation of the purine bases and their respective ribonucleosides into mononucleotides are respectively adenine and adenosine 23.0% and 2.1% in *T. congolense*; 17.5% and 3.5% in *T. vivax*; hypoxanthine and inoine

18.0% and 4.0% in T. congolense and 26.0% and 4.1% in T. vivax. This indicated that the major pathway from a purine base to its ribonucleotide in these parasites is direct via purine PRTase instead of a sequential conversion from base to ribonucleotide. This enzyme would play a significant role in the uptake of purine bases by the trypanosomes. T. brucei has a higher activity of the purine PRTase and indeed of the other enzymes than the T. congolense and T. vivax (Table 3). This would correlate with the higher metabolic activity observed in T. brucei (Von Brand, 1951). Trypanosoma vivax in general appeared to exhibit a rate of metabolism intermediate between that of the T. brucei and T. congolense (Desowitz, 1956).

Besides phosphorylation, there are other possible fates like deamination which an incoming adenosine or adenine undergoes before finally being phosphorylated. Adenosine is toxic to cells (Dewey et al, 1978; Hovi et al, 1976) and it has been suggested that ADP formed from it was largely responsible through inhibition of phosphoribosyl pyrophosphate synthetase. It is therefore essential that the cell develops a system to prevent accumulation of adenosine or adenine nucleotides. Malaria parasites (Reyes et al, 1982; Lukow et al, 1973); T. cruzi (Gutteridge and Davies, 1981) and animal cells (Wax and Veerkamp, 1978; Murray, 1971) have adenosine deaminase which converts excess adenosine to inosine. The enzyme, adenosine deaminase is used by cells to protect themselves against

excess adenine nucleotides. All the African trypanosomes examined to-date (T. vivax; T. congolense; T. gambiense; T. brucei) lack the enzyme. Also adenine aminohydrolase which could deaminate adenine to hypoxanthine is present only in T. vivax (Table 3). How then do these parasites deal with the excess adenine nucleotides? Before discussing this point further, it is important to note that adenine aminohydrolase is essentially a bacterial enzyme which has not been reported in animal cells (Hartenstein and Fridovich, 1967; Whitely and Douglas, 1951; Whitely, 1952) but of recent found in a number of flagellates: C. fasciculata (Kidder et al, 1977); L. tarentolae (Kidder and Nolan, 1979) and L. donovani (Marr et al, 1978). The presence of deaminase in T. vivax was obtained when it was observed that uric acid was formed when adenosine and xanthine oxidase were incubated with the protein extract of this parasite. Evidently two routes could lead to uric acid production: either via adenosine.



Enzymes: (1) Purine nucleoside hydrolase (2) adenosine deaminase (3) adenine aminohydrolase (4) xanthine oxidase.

conversion to inosine and then to hypoxanthine or through conversion to adenine and then to hypoxanthine as shown overleaf. Hypoxanthine is then converted to uric acid by xanthine oxidase. The pathway operating was delineated by using radiotracer technique and searching for the intermediates. Adenine and hypoxanthine were the only intermediates (Fig. 15) recovered leading to the conclusion that adenine aminohydrolase was present in T. vivax. Also ^{14}C-adenine was deaminated to ^{14}C-hypoxanthine when incubated with the protein extract of T. vivax. This again confirms the presence of adenine aminohydrolase in the trypanosome. In T. vivax, this enzyme may have similar regulatory role as adenosine deaminase to control the level of adenine nucleotides. In addition, the enzyme could provide the interconversion point for purines in T. vivax. This latter point is important because it has been shown that one purine base or nucleoside, for example, adenine, guanine, adenosine or inosine could support growth of flagellates (Jaffe and Gutteridge, 1973). Also it was demonstrated that ^{14}C-labelled hypoxanthine and inosine would incorporate equally into adenine and guanine nucleotides. These studies imply the existence of a branch point where interconversion of purines would take place in flagellates. In T. vivax the interconversion point is obvious but not for the others (T. brucei, T. congolense and T. gambiense), however since

these trypanosomes lacked adenosine deaminase and adenine aminohydrolase, the only interconversion point must be at IMP where AMP deaminase will convert AMP to IMP. The presence of AMP deaminase in T. congolense and T. brucei was shown by indirect evidence. When AMP was synthesized from the purine base adenine, and PRPP, one of the end products was inosine. This indicates the presence of active deaminase and IMP-nucleotidase since these trypanosomes lacked adenosine deaminase.

AMP ⟶ IMP ⟶ Inosine

A similar study showed that T. vivax lacked AMP deaminase but has active IMP nucleotidase. The presence of the latter enzyme was shown by synthesizing IMP starting with hypoxanthine. The analysis of the incubation medium showed inosine. When adenine was used for the synthesis of AMP, no inosine was recovered in the assay medium showing the absence of AMP deaminase in T. vivax. This means that the metabolic interconversion point is at the IMP in T. brucei and T. congolense and at hypoxanthine in T. vivax. This may have some significance, in chemotherapy especially of T. vivax which has a unique interconversion point. In addition to the above, T. vivax can easily be distinguished from the other African trypanosomes which affect cattle (T. brucei and T. congolense) by the presence of adenine aminohydrolase.

In summary, a pathway is proposed for the salvage of purines by African trypanosomes (Fig. 26). Unlike the

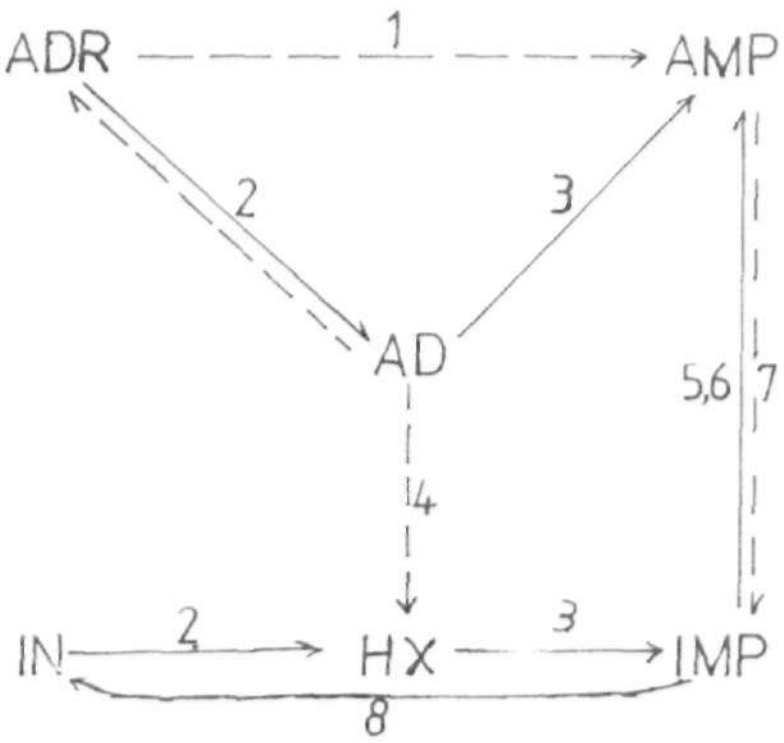


Fig. 26

NOMENCLATURE OF ENZYMES OF PURINE METABOLISM

Enzyme Number	Common Name	Systematic Name	EC Number
1	Adenosine kinase	ATP: adenosine 5'-phosphotransferase	2.7.1.20
2	Purine nucleoside Phosphorylase	Purine nucleoside: orthophosphate ribosyltransferase	2.4.2.1
2	Purine nucleoside hydrolase	N-riboyl purine hydrolase	3.2.2.7
3	Adenine phosphoribosyltrans-ferase	AMP: pyrophosphorylase transphosphoribosidase	2.4.2.7
4	Adenine deaminase	Adenine aminohydrolase	3.5.4.2
6	Adenylosuccinate synthetase	IMP: L-aspartate ligase (GDP)	6.3.4.4
7	AMP deaminase	AMP aminohydrolase	3.5.4.6
8	5-Nucleotidase	5-Ribonucleotide phosphohydrolase	3.1.3.5
9	Hypoxanthine (guanine) Phosphoribosyltransferase	IMP: pyrophosphorylase transphosphoribosidase	2.4.2.8
-	IMP dehydrogenase	IMP. NAD Oxidoreductase	1.2.1.14
-	Nucleoside permease	-	-

proposal by Schmidt et al, (1975), this one takes care of differences between strains of African trypanosomes.

8.3 Nucleoside transport

There is little information on transport and even on uptake of radiolabelled purines and purine derivatives by the African trypanosomes. Nucleoside transport is the process leading to the translocation of nucleoside across the biological membranes while the nucleoside uptake processes involve transport of substrate across the cell membrane, phosphorylation and other metabolic fates including incorporation into nucleic acids. Purine salvage enzymes have been demonstrated in earlier section to be present in African trypanosomes and these enzymes are bound to affect the substrate pool levels and subsequent rates of membrane transport in the trypanosomes. Consequently the Vmax reported are those of uptake and not transport.

In other trypanosomatids, it has been shown, for example, in leishmania (Hansen et al, 1982) that three different transport loci are present for the transport of purine bases and nucleosides. One locus transports inosine, while the second is specific for adenosine and the third transport locus takes up the purine bases, adenine and hypoxanthine. In malaria parasite, P. berghei (Hansen et al, 1980), a common transport locus was found for adenosine, hypoxanthine and inosine while another locus was specific for adenine. In African trypanosomes, it was shown that in T. congolense and T. brucei (James and Born, 1980), inosine

and guanosine are taken up by the same system for which their affinity is high, but which also has a low affinity for adenosine. In addition, another transport locus specific for adenosine was also described. These workers suggested further that adenosine is the most important purine for these trypanosomes.

In this study with T. brucei and T. congolense, it was shown that a linear relationship existed when adenosine uptake was measured as a function of increasing concentration of adenosine (Fig. 18). This suggests that mediated uptake predominated at the concentrations studied (less than 0.5 mM). A hyperbolic relationship would have indicated mixed uptake kinetics with diffusion predominating at the higher substrate concentration. The inhibition of labelled adenosine uptake by higher concentration of unlabelled adenosine support mediated uptake. The term "inhibition" may however result in some confusion when used to describe the action of non-labelled substrate on a labelled substrate of the same molecular species. Ruff and Read (1974) have explained that the addition of an unlabelled compound could not affect the rate of uptake of the labelled substrate into the cell. However, when labelled and non-labelled molecules of the same substrate were binding to a limited number of sites on the surface of the membrane, competition for those available sites would ensue. Consequently, the observed inhibitions are assumed to be the result of competition for available transport loci and is useful in delineating mediated

and diffusion systems. The competitive inhibition of adenosine uptake by guanosine (Fig. 19) also offers further evidence for a carrier mediated transport system. The non-interference of adenosine uptake by thymidine suggested that in these trypanosomes, at least two functional transport loci are present; one transports the purine nucleosides, adenosine and guanosine and the other transports the pyrimidine nucleoside, thymidine. It is difficult to conclude on the relative importance of the purine bases and their derivatives on the basis of uptake experiments as expounded by James and Born (1981). This is because in uptake assays where permeants are metabolized, it is difficult to measure transport as the rate of transport is influenced by the enzymes involved in the metabolism of the permeants. In the case of adenosine uptake, the enzymes, phosphorylase in T. congolense and hydrolase in T. brucei would rapidly break down adenosine to adenine. Phosphoribosyltransferase would then convert the purine base to ribonucleotide. In the earlier section, it was shown that 50% of adenosine was converted to adenine in 30 seconds in T. brucei. In the light of this, it is difficult to see how a 2-min incubation period used by these authors would represent transport of adenosine. The enzyme studies would lead one to conclude that purine bases are more important to the parasites than the nucleosides. This, however needs to be correlated with transport studies. It is more likely that the parasites would encounter purine

bases in their environment than the purine nucleosides (Marr et al, 1978). There is some avidence that purines, mainly as nucleosides, are continously released from various tissues (Rubio and Berne, 1969). There is also a rapid turnover of adenosine in the nucleotides of erythrocytes in normal blood (Adams and Harkness, 1973; Henderson and Lepage, 1959). This point begs a question as to whether erythrocytes contributed in any way to meeting the purine requirements of the trypanosomes. For malarial parasites which are intraerythrocytic, it has been demonstrated that numerous changes take place in the membrane function and morphology of the erythrocytes by permitting the parasites to freely obtain nutrients from the surrounding (Cenedella et al, 1969; Overman, 1948; Dunn, 1969). African trypanosomiasis cause anemia in mammals but the exact mechanism by which this happens is not known (Fiennes, 1954; Mackenzie and Cruickshank, 1973; Kobayashi et al, 1976). Amongst the possible mechanisms postulated including immunological (Woo and Kobayashi, 1975; Herbert and Inglis, 1973) and fatty acid mediation (Tizard et al, 1978), one point is obvious, it is not certain why trypanosomiasis should cause anemia. Trypanosome infection causes an increased rate of erythrocyte destruction with a consequent shortening of the erythrocyte life-span (Mamo and Holmes, 1975). It is with this view that erythrocytes may undergo some changes, that the investigation of its permeability to nucleosides was undertaken. Erythrocytes from mice infected with

trypanosomes (T. congolense, T. brucei, or T. gambiense) were used for the studies. In measuring the transport rates, accelerative exchange diffusion was used. Accelerative exchange diffusion occurs when the rate of outward transfer of permeant is accelerated by inward transfer of the same or a related permeant present at the opposite membrane face. In the study, the accelerative exchange diffusion of nucleosides was examined using procedures based on measurements of outward transport of thymidine which is not phosphorylated or cleaved by erythrocytes (Oliver and Paterson, 1971). This procedure had previously been used to investigate permeant specificity of nucleoside transport in erythrocytes (Cass and Paterson, 1972). The higher efflux rate of ^{3}H-thymidine observed with the erythrocytes from mice infected with trypanosomes than the erythrocytes from uninfected mice (Fig. 20), suggested that there was some membrane abnormality leading to increased permeability to the nucleoside. Since a higher concentration of non-labelled thymidine increased the rate of outflow of the ^{3}H-thymidine, it is concluded that the increased efflux is mediated by a specific transport carrier system. The efflux was inhibited by the specific nucleoside transport inhibitor, nitrobenzylthioinosine. This inhibitor has been shown to inhibit mediated uptake in a variety of animal cells including erythrocytes (Lauzon and Paterson, 1977; Pickard and Paterson, 1972). This is a clear evidence supporting the involvement of carrier mediated system in the efflux of the

^{3}H-thymidine and not increase in simple diffusion. One of the ways in which trypanosomes would affect the membrane carrier proteins would be by the action of the phospholipases believed to be generated by pathogenic trypanosomes (Tizard et al, 1978). The phospholipases acting on the membrane surface lipids of the erythrocytes would "free" the carrier proteins on the erythrocyte membrane. The re-orientation of the carrier protein could affect the activity in a number of ways: (i) reduce the activity or cause it to lose the activity completely; (ii) the activity may be unaffected; and (iii) the activity could be enhanced as seen in this case. Whatever may be the mechanism, it is obvious from the study that the rate of efflux of nucleoside was enhanced in erythrocytes from mice infected with the trypanosomes. Could the erythrocyte which has a high nucleoside turnover be a major source of purine base and nucleosides for the parasites even though the concentration of purine derivatives is very low in normal plasma, measuring 10^{-8} to 10^{-7}M (Miyazaki et al, 1974)?

8.4 Nucleoside inhibitor

The nucleoside transport inhibitor had been shown to bind tightly but reversibly to sites on the membrane of cells (Lauzon and Paterson, 1977; Pickard et al, 1973). The binding of the inhibitor was also shown to correlate with the inhibition of thymidine and uridine in human erythrocytes (Cass et al, 1974) so that the determination of the number of the NBMPR binding sites on cells would give idea of the

number of nucleoside transporter sites on the cells. The observation that the number of NBMPR binding sites on the erythrocytes from mice infected with trypanosomes and those from uninfected mice were same (Fig. 21), suggested that the nucleoside transporter sites on these erythrocytes were unaffected by infection with trypanosomes. The carrier proteins were only activated by the parasites as earlier suggested.

Dipyridamole, also a nucleoside transport inhibitor in animal cells (Olsson, 1972; Paterson et al, 1980; Kolassa et al, 1978) and in African trypanosomes (James and Born, 1980) was found to be ineffective in T. **brucei** although James and Born (1980) could demonstrate inhibition of adenosine by this compound in their studies with their T. brucei strain. This study showed competitive inhibition of adenosine uptake in T. congolense (Fig. 23), just as the former workers (James and Born, 1980) had demonstrated. NBMPR was unable to bind to either T. brucei or T. congolense trypomastigotes (Fig. 22). The inability of this compound to bind to the trypanosomes correlated with its non inhibition of nucleoside uptake in the parasites.

8.5 Chemotherapy studies

It has been shown earlier in the study that the nucleoside transport inhibitor, NBMPR would bind to mouse erythrocytes. It also inhibits nucleoside uptake in erythrocytes (Pickard et al, 1973). In addition, NBMPR was shown to be unable to bind to either T. brucei, T. gambiense or T. congolense and did not interfere with their nucleoside uptake. The chemotherapy study was thus

designed as a practical application of these findings.

The NBMPR and tubercidin together in the incubation medium had a similar effect on the motility of the trypanosomes as the tubercidin alone (Fig. 25), indicating that NBMPR did not interfere with the trypanocidal action of NBMPR. Although NBMPR-P, which is a phosphorylated NBMPR, was used for all the in vivo studies because it is more soluble in water than NBMPR. Previous study had shown that NBMPR-P per se did not bind to cells but is dephosphorylated at the cell surface, and the resulting NBMPR bound to be cell and inhibits nucleoside transport (Ogbunude, 1979). It is apparent from the result summarized in Table 5 that NBMPR-P significantly alleviated the acute toxicity of tubercidin. NBMPR-P did not affect the acute toxicity of ethidium consistent with previously published finding that NBMPR is a specific inhibitor of nucleoside transport (Lynch et al, 1978; Cass and Paterson, 1979). The biochemical basis for the NBMPR-P protection of mice against the acute toxicity of nucleoside analogues appears to be due to the reduction of access of the drugs to tissues or organs sensitive to the toxic drugs. The trypanosomes which did not have the binding sites for the inhibitor was not protected and thus take up the nucleoside drugs which are known to enter cells by way of the nucleoside-specific transport as their physiological counterparts (Paterson et al, 1981). The study suggests that therapy of African trypanosomiasis with nucleoside drugs and the nucleoside inhibitor, NBMPR, may be beneficial.

CHAPTER 9

POSSIBLE APPLICATIONS OF THIS STUDY

If trypanosomes rely on exogenous purines, analysis of the purine salvage pathways and the uptake mechanisms of these parasites might show up some differences from the corresponding mechanisms in the animal hosts. Such differences could be exploited in the design of drugs selective for the trypanosomes. The knowledge could also aid in rational use of the drugs currently available.

It was shown in the study that T. brucei, T. vivax and T. congolense cleave inosine with hydrolase enzyme. This information will be useful in predicting some reactions of nucleoside analogue compounds in the trypanosomes, for an example, formycin B, an analogue of inosine, will be expected to be effective in the treatment of trypanosomiasis. This is predictable from this study and from properties of the compound. Formycin B is neither phosphorylated nor hydrolysed by animal cells but it is hydrolysed and phosphorylated by bacterial and viral cells (Umezawa et al, 1967). It is an antiviral as well as bacteriacidal agent but has very low toxicity for animal cells, LD_{50}: 1000mg/kg (Umezawa et al, 1965; Takeuchi et al, 1966). The compound is activated by phosphorylation and is toxic by incorporation into the RNA of parasites (Marr et al, 1978).

The difference between the bacterial and animal cells with regard to the enzymes that will phosphorylate

formycin B is that animal cells will cleave the compound with the phosphorylase enzyme before conversion to the nucleotide form using hx-guaPRTase while bacteria will cleave the compound to the purine base before phosphorylation with phosphoribosyl-transferase. Formycin B is not a substrate for phosphorylase (Sheen et al, 1968). The trypanosomes (T. vivax, T. brucei; T. congolense and T. gambiense) use hydrolase for inosine cleavage and would therefore hydrolyse the inosine analogue to its base and then convert purine base to ribonucleotide (Fig. 27). On the other hand if formycin which is an adenosine analogue is to be used; in vitro, formycin will be effective in only T. vivax. It is not likely to be effective in T. brucei, T. gambiense and T. congolense under similar condition. The T. brucei will cleave formycin to its purine base just as the T. vivax but the T. vivax will further deaminate the formycin base to formycin b base which is then converted to formycin B monophosphate by phosphoribosyl-transferase (Fig. 28). T. brucei lacks adenine aminohydrolase as had been shown in the study. T. congolense uses phospho-rylase for the cleavage of adenosine and will be unable to cleave the compound.

In vivo, a lot of reactions could take place including the deamination of the formycin by the animal cell. The activity thus under in vivo condition, will depend among other things, on the rate of deamination of the compound by the animal cell but it will still be effective in T. vivax whether deaminated or not.

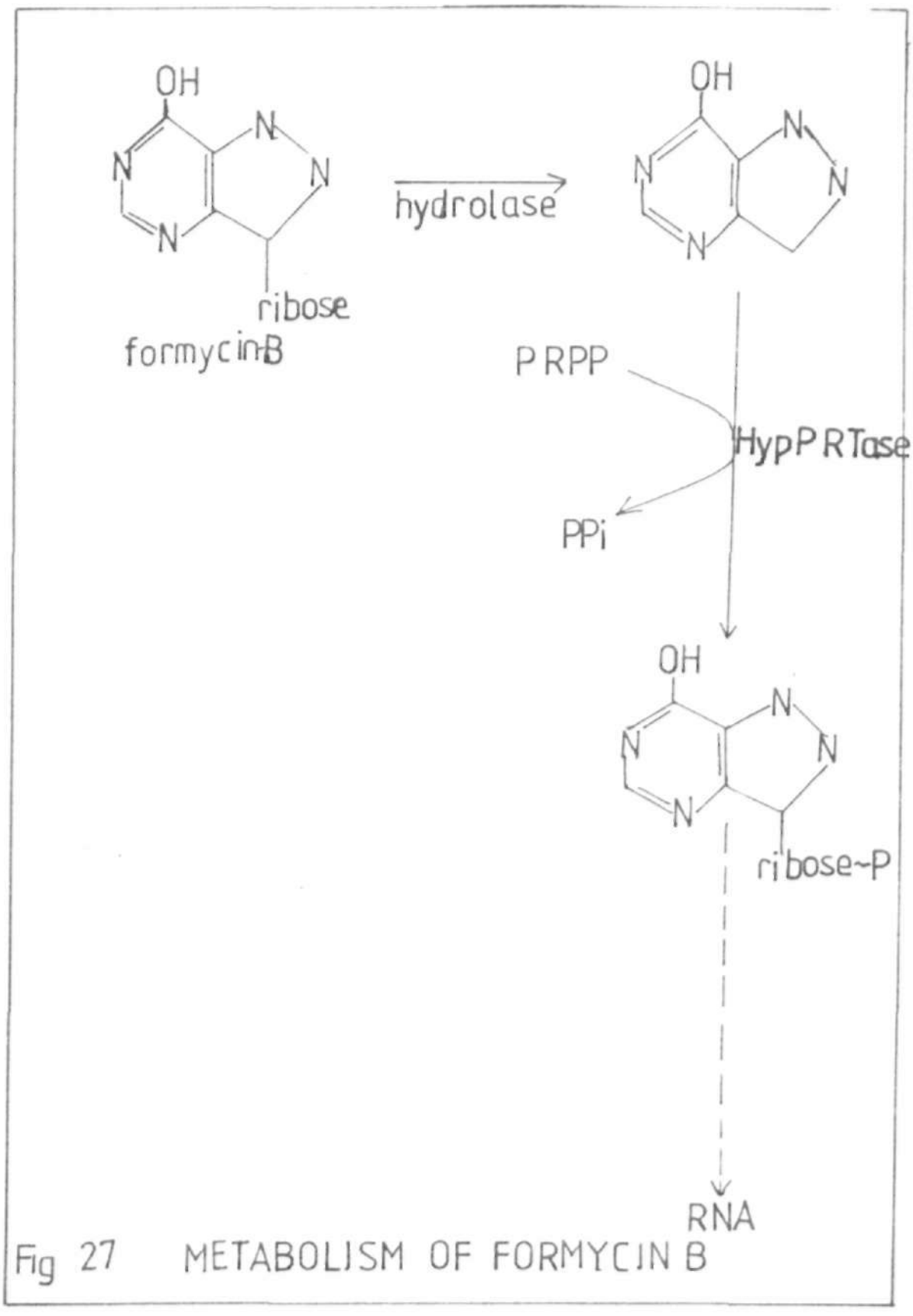


Fig 27 METABOLISM OF FORMYCIN B

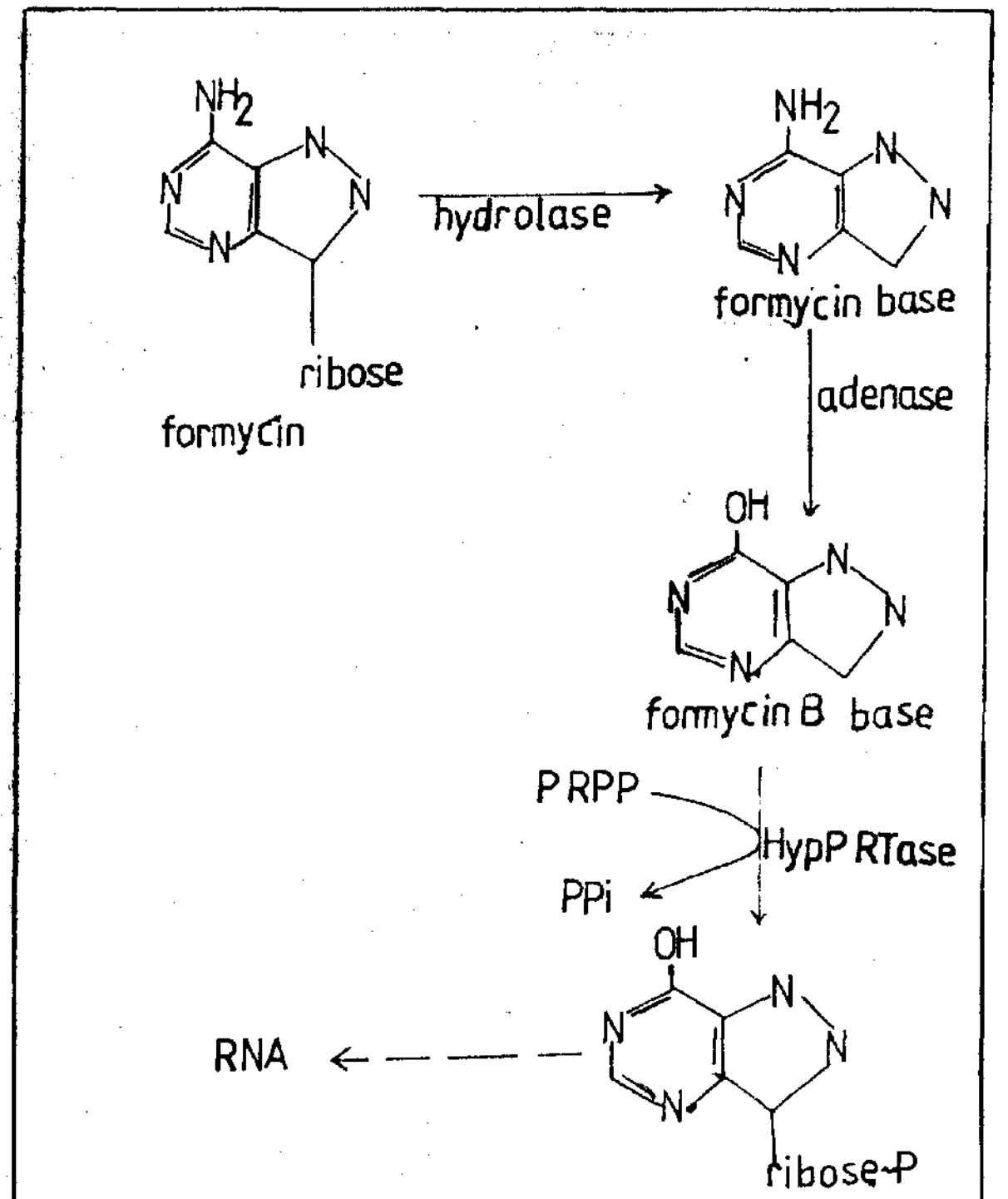


Fig 28 METABOLISM OF FORMYCIN IN I.VIVAX

The absence of phosphorylase and adenosine kinase in T. brucei means that there is no alternate pathway for the salvage of the purine base, adenine and the only pathway by which this can be salvaged is via phosphoribosyltransferase. Inhibitors of this enzyme activity should therefore block the growth of T. brucei. The presence of adenine amino-hydrolase or adenase in T. vivax could also be used to advantage in the chemotherapy of the trypanosome. If a specific inhibitor of this enzyme can be found, there is bound to be some inhibition of growth of the parasite. Already coformycin and deoxycoformycin have been found to inhibit the enzyme in C. fasciculata resulting in inhibition of growth of the parasite in vitro (Kidder and Nolan, 1979).

As regards the uptake studies, if the transport aspects of nucleoside uptake is well characterized, specific nucleoside drugs could be designed. Nucleoside inhibitors which would effect the animal cells just like nitrobenzylthioinosine without effecting the trypanosomes could be designed and used to advantage in the chemotherapy of trypanosomiasis.

CHAPTER 10

SUMMARY AND SUGGESTION FOR FURTHER WORK

10.1 Summary

In summary, the following conclusions were reached from the study.

1. Enzymes of purine salvage are present in T. brucei; T. congolense and T. vivax. Differences in levels of activity were observed. While T. congolense cleaves adenosine phosphorolytically, all the trypanosomes examined hydrolytically cleaved inosine and adenosine.

All the trypanosomes lack adenosine deaminase and with the exception of T. vivax, all the African trypanosomes examined including T. gambiense lack adenine aminohydrolase. T. brucei had no detectable adenosine kinase activity. AMP deaminase is present in T. brucei T. congolense but not detected in T. vivax.

2. Two nucleoside transporter loci were detected on T. gambiense and T. congolense. One locus takes up adenosine and guanosine while the second transports thymidine.

3. Erythrocytes from mice infected with either T. congolense; T. brucei or T. gambiense showed higher rate of efflux of ^{3}H-thymidine than control erythrocytes from uninfected mice. This finding suggests that erythrocytes may be a source for purine base and purine

derivatives for the trypanosomes.

4. The nucleoside transport inhibitor, nitrobenzylthioinosine known to bind to several animal cells did not bind to either T. brucei; T. congolense or T. gambiense trypomastigotes. The inhibitor also failed to inhibit the uptake of adenosine in these trypanosomes. The inability of NBMPR to inhibit the nucleoside uptake in these trypanosomes correlated with the lack of NBMPR binding sites on the organisms. Dipyridamole, another nucleoside transport inhibitor, did not inhibit adenosine uptake in T. gambiense but inhibited the uptake of adenosine in T. congolense.

5. The toxicity of nucleoside analogue, tubercidin was alleviated when given concurrently with nitrobenzylthioinosinate. The nitrobenzylthioinosinate was not per se trypanocidal and did not affect the trypanocidal activity of tubercidin. The biochemical basis for the in vivo protection of the experimental mice by NBMPR was as a result of the blockage of uptake of the toxic nucleoside by the animal cell.

10.2 Suggestions for further works

It is recommended that further work be undertaken on two enzymes involved in purine salvage. These enzymes, adenine aminohydrolase found only in T. vivax and purine phosphoribosyltransferase found in all the trypanosomes

should be purified and characterized. Specific inhibitors to them should be sought. In the area of chemotherapy, it is recommended that the nucleoside transport inhibitor nitrobenzylthioinosinate should be tried with the other nucleoside analogues known to have trypanocidal activity but are toxic to animals for example nucleocidin. It is further recommended that the study be extended to larger animals.

REFERENCES

Ackerman, S.B. and Seed, J.R. (1976). The effects of tryphophol on immune responses and its implications towards trypanosome - induce immunosuppression; Experientia, 32; 645-647.

Adams, A and Harkness, R.A. (1973). 'Purine uptake from arythrocytes by rabbit tissue in vivo', Biochemical Society Transactions, 1, 138-139.

Akinrimisi, E.O. (1981). The biochemist in the control of Trypanosomiasis - A review. Proceedings of the First National Conference on Tsetse and Trypanosomiasis Research in Nigeria, Kaduna 10 - 12 August. Edit. Ilemobade pp. 170-178, Shereef Sarem Press, Zaria.

Allsopp, B.A., Njogu, A.R. and Humphrey, K.C. (1971). Nature and localization of Trypanosoma brucei subgroup exoantigen and its relationship to 4S antigen; Experimental Parasitology, 29, 271-284.

Amole, B.O. and Clarkson, A.B. (1981). 'Trypanosoma brucei; Host parasite interaction in parasite destruction by salicylhydroxamic acid and glycerol in mice, Experimental Parasitology, 51, 133-140.

Arrick, B.A. Griffth, O.W. and Ceremi, A. (1981). Inhibition of glututhione synthesis as a chemotherapeutic strategy for trypanosomiasis, Journal of Experimental Medicine, 153, 720-725.

Avila, J.L. and Avila, A.A. (1981). 'Trypanosoma cruzi; Allopurinol in the treatment of mice with experimental acute Changas disease', Experimental Parasitology, 51, 204-208.

Bacchi, C.J., Lipschik, G.Y. and Nathan, H.C. (1977). 'Polyamines in trypanosomatids', Journal of Bacteriology, 131, 657-661.

Bacchi, C.J., Hutner, S.H., Lambros, C. and Lipschik, G.Y. (1978). 'Polyamine function in trypanosomatids: activation of cytoplasmic cx-glycerophosphate dehydrogenase by cationic trypanocides', In Advances in Polyamine Research, 2, 129-138.

Bacchi, C.J., Vergara, C., Garofalo, J., Lipschik, G.Y. and Hutner, S.H. (1979). 'Synthesis and content of polyamines in bloodstream Trypanosoma brucei', Journal of Protozoology, 26, 484-488.

Barbet, A.F. and McGuire, T.C. (1978). Cross-reacting determinants in variant-specific surface antigens of African trypanosomes. Procedings of National Academy of Science, 75, 1979-1982.

Barbet, A.F., and McGuire, T.C. (1982). Purification of Trypanosoma brucei variable surface glycoproteins: analysis of degradation accuring during isolation. Parasitology, 85, 511-522.

Baernstein, H.D. (1963). 'A review of electron transport mechanisms in parasitic protozoa', Journal of Parasitology, 49, 12-21.

Berens, R.L., Marr, J.J., Nelson, D.J. and Lafon, S.W. (1980). 'Antileishmanial effect of allopurinol and allopurinol ribonucleoside on intracellular forms of Leishmania donovani', Biochemical Pharmacology, 29, 2397-2398.

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.

Bernards, A; Van der Ploeg, L.H.T; Frasch, A.C.C.; Borst, P; Boothroyd, J.C., Coleman, S. and Cross, G.A.M. (1981). Activation of trypanosome surface glycoprotein genes involves a gene duplication-transposition leading to an altered 3'-end. Cell, 27, 497-505.

Berlin, R.D. and Oliver, J.M. (1975). 'Membrane transport of purine and pyrimidine bases and nucleosides in animal cells', International Review of Cytology, 42, 287-336.

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

Borst, P. and Hoeijmakers, J.H.J. (1979). 'A reviews kinetoplast DNA', Plasmid, 2, 20-40.

Borst, P., Frasch, A.C.C., Bernards, A., Vander Ploeg, L.H.T., Hoeijmakers, J.H.J., Arnberg, A.C. and Cross, G.A.M. (1981). 'DNA rearrangements involving the genes for variant antigens in Trypanosoma brucei. Cold Spring Harbor Symposium Quarterly Journal in Biology, 45, 935-943.

Bowman, I.B.R. and Flynn, I.W. (1976). 'Oxidative metabolism of trypanosomes', In Biology of the kinetoplastida, Lumsden, W.H.R. and Evans, D.A. eds. Vol.I, pp. 435-476. Academic Press, New York.

Broden, A. (1904). 'Les infections, 'a trypansomes au Congo du l'homme et les animaux', Bulletin de Societe' Etudes Colon (Brussels) February.

Buchanam, J.M. (1960). 'Biosynthesis of purine nucleosides', The Nucleic Acids Chargaff, E. and Davidson, J.N. eds. Vol.23, pp. 303-322.

Cantrell, W. (1958). 'Mutation rate and antigenic variation in Trypanosoma equiperdum, Journal of Infectious Diseases, 103, 263-271.

Carne, H.R. and Onon, E.O. (1978). 'Action of Corynebacterium ovis exotoxin on endothelial cells of blood vessels', Nature (London), 271, 246-248.

Cass, C.E. and Paterson, A.R.P. (1972). 'Mediated transport of nucleosides in human erythrocytes. Accelerative exchange diffusion of uridine and thymidine and specificity toward pyrimidine nucleosides as permeants', Journal of Biological Chemistry, 247, 3314-3320.

Cass, C.E. and Paterson, A.R.P. (1973). 'Mediated transport of nucleosides by human erythrocytes specificity toward purine nucleosides as permeants', Biochemica et Biophysica Acta, 291, 734-746.

Cass, C.E., Gaudette, L.A. and Paterson, A.R.P. (1974). 'Mediated transport of nucleosides in human erythrocytes. Specific binding of the inhibitor nitrobenzylthioinosinosine to nucleoside transport sites in the erythrocyte membrane', Experimental cell Research, 105, 427-435.

Castellani, A. (1903). 'Trypanosoma in sleeping sickness', British Medical Journal, pt.1 1218.

Cenedella, R.J., Jarrell, J.J. and Saxa, L.H. (1969). 'Lipid synthesis in vitro from ^{14}C-Oleic acid and 6 - ^{3}H-glucose by intraerythrocytic plasmodium berghei', Military Medicine, 139, 1045-1055.

Chandler, A.C. and Read, C.P. (1961). 'Hemoflagellates II. Trypanosomes. Introduction to Parasitology. 10th ed. pp. 131-160. John Wiley and Sons Inc. New York.

Chappell, L.H. Southworth, G.C. and Read, C.P. (1972). 'Short-interval absorption and metabolism of some amino-acids in Trypanosoma gambiense', Parasitology, 64, 379-387.

Clarkson, A.B. and Brohn, F.H. (1976). 'Trypanosomiasis: An approach to chemotherapy by the inhibition of carbohydrate catabolism', Science, 194, 204-205.

Cohen, S.S. (1978). 'What do the polyamines do? ' Nature (London), 274, 209-210.

Cross, G.A.M. (1975). 'Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei', Parasitology, 71, 393-417.

Cross, G.A.M. (1978). Antigenic variation in trypanosomes. Proceedings of Royal Society (London), 202, 55-72.

Conway, E.J. and Cooke, R. (1939). Deaminases of adenosine and adenylic acid in blood and tissues. Biochemical Journal, 33, 479-492.

Dissing, J. and Knudsen, B. (1972). Adenosine deaminase deficiency and combined immuno-deficiency syndrome. Lancet, 2, 1316.

Desowitz, R.S. (1970). 'Immunity in mammalian hosts: African trypanosomes', In Immunity to Parasitic Animals, Vol. 2, pp. 551-596, Appleton, New York.

Dewey, V.C. and Kidder, G.W. (1973). 'Partial purification and properties of a nucleoside hydrolase from Crithidia', Archives of Biochemistry and Biophysics, 157, 380-387.

Dewey, V.C., Kidder, G.W. and Nolan, L.L. (1978). 'Mechanism of inhibition of Crithidia fasciculata by adenosine and adenosine analogos', Biochemical Pharmacology, 17, 1479-1485.

Dixon, H. and Williamson, J. (1970). 'The lipid composition of blood and culture forms of Trypanosoma lewisi and Trypanosoma rhodesiense compared with that of their environment', Comparative Biochemistry and Physiology, 33B, 111-128.

Dixon, H., Ginger, C.D. and Williamson, J. (1971). 'The lipid metabolism of blood and culture forms of Trypanosoma lewisis and Trypanosoma rhodesiense', Comparative Biochemistry and Physiology, 39B, 247-266.

Dunn, M.J. (1966). 'Alterations of red blood-cell sodium transport during malarial infection, Journal of Clinical Investigation, 48, 674-684.

Edwards, Y.H., Hopkinson, D.A. and Harris, H. (1971). Adenosine deaminase isoenzymes in human tissues. Annals of Human Genetics, 35, 207-219.

Englund, P.T. and Marini, J.C. (1980). 'The Replication of kinetoplast DNA', American Journal of Tropical Medicine and Hygiene, 29, 1064-1069.

Entner, N. and Gonzalez, G. (1961). 'Nucleoside mono- and diphosphate kinase of Ascaris lumbricoidis, ' Biochemica Biophysica Acta, 47, 52-60.

Esser, K.M., Scheenbechler, M.J., Guigrich, J.B. and Diggs, C.L. (19 (1981). Monoclonal antibody analysis of Trypanosoma rhodesiense metacyclic antigen types. Federation Proceedings, 40, 1011-1018.

Evans, D.A. and Brightman, C.A. (1980). 'Pleomorphism and the problem of recrudescent parasitaemia following treatment with salicyl-hydroxamic acid (SHAM) in African trypanosomiasis', Transactions of the Royal Society of Tropical Medicine and Hygiene, 74, 601-604.

Fairlamb, A.H. Opperdoes, F.R. and Borst, P. (1977). 'New approaches to screening drugs for activity against African trypanosomes', Nature (London), 265, 270-271.

Fairlamb, A.H., Weislogel, P.O., Hoeijmakers, J.H.J. and Borst, P. (1978). 'Isolation and characterization of kinetoplast DNA from bloodstream form of Trypanosoma brucei', Journal of cell Biology, 76, 293-309.

Fairlamb, A.H. and Bowman, I.B.R. (1980). 'Uptake of the trypanocidal drug suramin by bloodstream forms of Trypanosoma brucei and its effect on respiration and growth in vivo', Molecular and Biochemical Parasitology, 1, 315-333.

Fairlamb, A.H. (1981). 'Alternate metabolic pathways in protozoan energy metabolism', Parasitology, 82, 1 - 3 and 22-33.

Fairlamb, A. (1982). 'Biochemistry of trypanosomiasis and rational approaches to chemotherapy', Trends in Biochemical Sciences, 7, 249-253.

Fiennes, R.N.T.W. (1954). 'Hematological studies in trypanosomiasis of cattle', Veterinary Record, 66, 423-434.

Fish, W.R., Marr, J.J. and Berens, R.L. (1982a). Purine metabolism in Trypanosoma brucei gambiense. Biochemica et Biophysica Acta, 714, 422-428.

Fish, W.R., Looker, D.L., Marr, J.J. and Berens, R.L. (1982b). Purine metabolism in the bloodstream forms of Trypanosoma gambiense and Trypanosoma rhodesiense. Biochemica et Biophysica Acta, 719, 223-231.

Ford, J. (1971). 'Geographical patterns of trypanosome distribution', In the Role of Trypanosomiasis in African Ecology, pp. 91-109. Clarendon Press, Oxford.

Frasch, A.C.C., Borst, P. and Van den Burg, J. (1982). 'Rapid evolution of genes coding for variant surface glycoproteins in trypanosomes', Gene, 17, 197-211.

Gbenle, G.O. and Akinrimisi, E.O. (1981). Isolation and partial characterization of a calcium-dependent endoribonuclease of Trypanosoma brucei cytoplasm. Biochemical International, 2, 219-228.

Gbenle, G.O. and Akinrimisi, E.O. (1982). Studies on the catalytic properties of the calcium-dependent endoribonuclease of Trypanosoma brucei cytoplasm. Molecular and Biochemical Parasitology, 5, 213-220.

Giblett, E.R., Anderson, J.E., Cohen, F., Pollara, B. and Meuwissen, H.J. (1972). Adenosine deaminase deficiency in two patients with severe impaired cellular immunity. Lancet, 2, 1067-1069.

Grant, P.T. and Sargent, J.R. (1960). 'Properties of L-α-glycerophosphate oxidase and its role in the respiration of Trypanosoma rhodesiense', Biochemical Journal, 76, 229-237.

Grant, P.T., Sargent, J.R, and Ryley, J.F. (1961). 'Respiratory systems in the trypanosomatidae', Biochemical Journal, 81, 200-206.

Grant, P.T. (1966). 'The selective inhibition of energy-yielding reactions', Symposium of the Society for General Microbiology, 16, 281 - 293.

Grey, A.R. (1962). 'The influence of antibody on serological variation of Trypanosoma brucei', Annals of Tropical Medicine and Parasitology, 56, 4-13.

Grey, A.R. (1965). 'Antigenic variation in a strain of Trypanosoma brucei transmitted by Glossina Morsitans and Glossina Palpalis', Journal of General Microbiology, 41, 195-214.

Grab, D.J. and Bwayo, J.J. (1982). Isopycnic isolation of African trypanosomes on percoll gradients formed in situ. Acta Tropica, 39, 363-366.

Gruby, D. (1843). 'Recherches et observations sur une nouvelle espece d'hematozoaire', Trypanosoma sanguinis, Comptes Rendus de l'Academie, des Sciences, 17, 1134.

Gutteridge, W.E., and Coombs, G.H. (1977). 'Nucleic acid metabolism', In Biochemistry of Parasitic Protozoa, pp. 69-88, University Park Press, London.

Gutteridge, W.E. and Davies, H.J. (1981). 'Enzymes of purine salvage in Trypanosoma cruzi', Federation of European Biochemical Society Letters, 127, 211 - 214.

Hadjuk, S.K. and Vickerman, K. (1981). 'Antigenic variation in cyclically transmitted Trypanosoma brucei. Variable antigen type composition of the first parasitemia in mice bitten by trypanosome-infected Glossina morsitans, Parasitology, 83, 609-621.

Hammond, D.J. and Bowman, I.B.R. (1980). 'Studies on glycerol kinase and its role in ATP synthesis in Trypanosoma brucei', Molecular and Biochemical Pharmacology, 2, 63-91.

Hans, J., Linden, G. and Stuart, K. (1975). Mitochondrial and cytoplasmic ribosomes and their activity in blood and culture from Trypanosoma brucei. Journal of cell Biology, 65, 103-111.

Hansen, B.D., Sleeman, H.K. and Pappas, P.W. (1980). 'Purine base and nucleoside uptake in Plasmodium berghei and host erythrocytes', Journal of Parasitology, 66, 205-212.

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

Henderson, J.F. and Lepage, G.A. (1959). 'Transport of adenine-8-^{14}C among mouse tissues by blood cells', Journal of Biological chemistry, 234, 3219-3223.

Henderson, J.F. (1968). 'Kinetic properties of hypoxanthine-guanine and adenine phosphoribosyltransferase', Federation of American Society of Experimental Biology (Proceedings), 27, 1053-1054.

Henderson, J.F., Fraser, J.H. and McCoy, E.E. (1974). 'Methods for the study of purine metabolism in human cells in vitro, Clinical Biochemistry, 7, 339-358.

Herbert, W.J. and Inglis, M.D. (1973). 'Immunization of mice against Trypanosoma brucei infection by the administration of released antigen adsorbed to erythrocytes. Transactions of Royal Society of Tropical Medicine and Hygiene, 67, 268.

Hewitt, R.I., Wallace, W.S., Gumble, A.R., Gill, E.R. and Williams, J.H. (1953). 'Experimental chemotherapy of Trypanosomiasis III. Effect of achromycin against Trypanosoma equiperdum and Trypanosoma cruzi', American Journal of Tropical Medicine and Hygiene, 2, 254-256.

Hoars, C.A. (1964). Morphological and taxonomic studies on mammalian trypanosomes, x. Revision of systematics', Journal of Protozoology, II. 200-207.

Hoare, C.A. (1972). The trypanosomes of mammals', A zoological monograph, Blackwell, Oxford, 1972.

Hoeijmakers, J.H., Frasch, A.C.C., Bernards, A., Borst, P. and Cross, G.A.M. (1980). Novel expression-linked copies of the genes for variant surface antigens in trypanosomes', Nature (London), 284, 78 - 80.

Hornby, H.E. (1952). 'Animal trypanosomiasis in Eastern Africa', London, His Master's Stationery Office, pp. 1949.

Hovi, I., Smyth, J.F., Allison, A.C. and Williams, S.C. (1976). 'Role of adenosine deaminase in lymphocyte proliferation', Clinical and Experimental Immunology, 23, 395-403.

Hutchinson, M.P. anf Watson, H.J.C. (1965). 'Human trypanosomiasis', World Health Organization Course in Trypanosomiasis, N.I.T.R., Kaduna, pp. 3-85.

Hutner, S.H. and Provasoli, L. (1964). 'Comparative biochemistry of flagellates', In Protozoa Vol. 3, pp. Academic Press, New York.

Jaffe, J.J., McCormack, J.J. and Meymerian, E. (1970). Trypanocidal properties of 5'-O-Sulfamoyladenosine, a close structural analog of nucleocidin. Experimental Parasitology, 28, 535-543.

Jaffe, J.J. and Gutteridge, W.E. (1973). 'Purine and pyrimidine metabolism in Protozoa', In P. de puytorac and Crain Actualities Protozoologiques. Universite de Clermont, Clermot - Ferrand, 1, 23-35.

James, D.M. and Born, G.V.R. (1980). Uptake of purine bases and nucleosides in African trypanosomes', Parasitology, 81, 383-393.

Janne, J., Poso, H. and Raina, A. (1978). Polyamines in rapid growth and cancer', Biochemica Biophysica Acta Review of Cancer), 473, 241-293.

Jenni, L. and Brun, R. (1980). In vitro cultivation of pleomorphic Trypanosoma brucei stocks: A possible source of variable antigens for immunization studies. Transactions of Royal Society of Tropical Medicine and Hygiene, 75, 150-153.

Johnson, J.G. and Cross, G.A.M. (1977). Carbohydrate composition of variant-specific surface antigen glycoproteins from Trypanosoma brucei, Journal of protozoology, 24, 587-591.

Johnson, J.G. and Cross, G.A.M. (1979). Selective cleavage of variant surface glycoproteins from Trypanosoma brucei. Biochemical Journal, 178, 689-697.

Kano, K. and Oka, T. (1976). 'Polyamine transport and metabolism in mouse mammary gland', Journal of Biological Chemistry, 251, 2795-2800.

Khaldoun, I. (1373). 'Histoire des berberes et des dynasties musculmanes de l'Afrique septentrionale. Traduite de L'Arebe par le Baran de Slave', Nouv Edition, Vol. 2, pp. 114, Paris.

Kluder, G.W., Davey, W.C. and Nolan, L.L. (1977). 'Adenine deaminase from a eukaryotic animal cell, Crithidia fasciculata Archives of Biochemistry and Biophysics, 183, 7-12.

Kidder, G.W., Nolan, L.L. and Dewey, V.C. (1979a), The purine phosphoribosyltransferases of Crithidia fasciculata, Journal of Parasitology, 65, 520-525.

Kidder, G.W. and Nolan, L.L. (1979b). Adenine amino-hydrolases: occurrence and possible significance in trypanosomid flagellates', Proceedings of National Academy of Sciences (USA) 76, 3670-3672.

Kim, B.K., Sungman, C. and Parks, R.E. (1968). Purine nucleoside phosphorylase from human erythrocytes. 1. Purification and properties. Journal of Biological chemistry, 243, 1763 - 1770.

Kobayashi, A., Tizard, I.R. and Woo, P.T.K. (1976). Studies on the anemia in experimental African trypanosomiasis. II. The pathogenesis of the anemia in calves infected with Trypanosoma congolense, American Journal of Tropical Medicine and Hygiene, 25, 401-406.

Kolassa, N., Plank, B. and Turnheim, K. (1978). 'pH and temperature dependence of adenosine uptake in human erythrocytes', European Journal of Pharmacology, 2, 345-351.

Konigk, E. (1976). Comparative aspects of nucleotide biosynthesis in pathogenic protozoan', In Biochemistry of Parasites and Host - parasite relationships (ed. H. Van de Bossche) pp. 51-58. Elsevier/North Horland Biomedical Press - Amsterdam.

Konigk, E. and Rasoul, S.A. (1978). Catabolism of adenosine 5'-monophosphate in promastigotes of Leishmania tropica Tropemendizin und Parasitologie, 29, 319-322.

Kornberg, A., Leiberman, I. and Simms, E.S. (1955). 'Enzymatic synthesis of purine nucleotides', Journal of Biological chemistry, 215, 417-427.

Kornberg, A. (1957). 'Pathways of enzymatic synthesis of nucleotides and polynucleotides', The Chemical Basis of Heredity, McElroy, W.D. and Glass, B. eds. pp. 579-608. John Hopkins Press, Baltimore, Maryland.

Lanham, S.M. and Godfrey, D.G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose', Experimental Parasitology, 28, 521-534.

Lauzon, G.J. and Paterson, A.R.P. (1977). 'Binding of the nucleoside transport inhibitor, nitrobenzylthioinosine to HeLa cells', Molecular Pharmacology, 13, 883-891.

Lemkin, J.A. and Hare, J.D. (1973). 'Nucleoside transport in normal and polyma-transformed cells: Kinetic differences following adenosine and serum or insulin stimulation', Biochimica Biophysica Acta, 318, 113-122.

Lewis, T.R., (1878). 'Flagellated organisms in the blood of Healthy rats', Quarterly Journal of Microscopical Sciences, 19, 109.

Linberg, B., Klenow, H. and Hansen, K. (1967). 'Some properties of partially purified mammalian adenosine kinase', Journal of Biological Chemistry, 242, 350-356.

Litvak, S., Boeckx, R.L.O. and Dakshinomurti, K. (1969). 'A new paper chromatography solvant system resolving pyrimidine-riboside pyrimidine deoxyriboside mixtures', Analytical Biochemistry, 30, 474-477.

Lowry, O.H., Rosenbrough, N.J., Farr, A.C. and Randall, P.J. (1951). Protein measurement with Folin-Phenol reagent. Journal of Biological Chemistry, 139, 265-275.

Lukow, I., Schmidt, G., Walter, R.D. and Konigk, E. (1973). 'Adenosinmonophasphat - salvage synthese bei Plasmodium chabaudi', Zeitschrift-Tropenmedun Parasitologie, 24, 500-504.

Lynch, T.P., Lauzon, G.J., Naik, S.R., Cass, C.E. and Paterson, A.R.P. (1978). 'Inhibition of nucleoside uptake in HeLa cells by nitrobenzylthioinosinate', Biochemical Pharmacology, 27, 1303-1304.

Macfie, J.W.S. and Gallagher, G.H. (1914). Sleeping sickness in the Eket District of Nigeria. Annals of Tropical Medicine and Parasitology, 8, 379-410.

Mackenzie, P.K.I. and Cruickshank, J.G. (1973). 'Phagocytosis of erythrocytes and leucocytes in sheep infected with Trypanosoma congolense. Research in Veterinary Science, 15, 256-262.

Mamo, E. and Holmes, P.K. (1975). 'The erythrokinetics of Zebu cattle chronically infected with Trypanosoma congolense, Research in Veterinary Science, 18, 105-106.

Mans, R.J. and Koch, A.L. (1960). Metabolism of adenosine and deoxyadenosine by growing cultures of Escherichia coli', Journal of Biological Chemistry, 235, 450-456.

Marr, J.J., Berens, R.L. and Nelson, D.J. (1978a). 'Purine metabolism in Leishmania donovani and Leishmania braziliensis', Biochimica et Biophysica Acta, 544, 360-371.

Marr, J.J., Berens, R.L. and Nelson, D.J. (1978b). 'Anti-trypanosomal effect of allopurinol: Conversion in vivo to aminopyrazolopyrimidine nucleotides by Trypanosoma cruzi', Science, 20, 1018-1020.

Meshnick, S.R. and Eaton, J.W. (1981). 'Leishmanial superoxide dismutase: a possible target for chemotherapy', Biochemical Biophysical Research Communication, 102, 970-976.

Miller, R.L., Ramsey, G.A., Krenitsky, T.A. and Elion, G.B. (1972). 'Guanine phosphoribosyltransferase form Escherichia coli, specificity and properties. Biochemistry, II, 4723-4731.

Miyazaki, H., Nambu, K., Minaki, Y., Hashimoto, M. and Nakamura, K. (1974). 'The role of direct phosphorylation in the mechanism of adenosine utilization for nucleotide synthesis in mouse erythrocytes', Journal of Biochemistry, 76, 409-417.

Mornet, P. (1954). 'Les trypanosomes pathogenes de l'A.O.F. Considerations sur leur repartition, leur frequence, les taux d'infestation des animaux domestigues', Bulletin de Societe' Pathologie, 47, 709.

Mshelbwala, A.S. (1972). Trypanosoma brucei infections in the haemocoel of tsetse flie', Transactions of Royal Society of Tropical Medicine and Hygiene, 66, 637-643.

Murray, A.W. (1971). 'The biological significance of purine salvage', Annual Review of Biochemistry, 40, 811-826.

Murray, M. and Morrison, W.I. (1979). Nonspecific induction of increased resistance in mice to Trypanosoma congolense and T. brucei by immunostimulants. Parasitology, 79, 349-356.

Oduro, K.K., Bowman, I.B.R. and Flynn, I.W. (1980). 'Trypanosoma brucei: activities and subcellular distribution of glycolytic enzymes from differently disrupted cells', Experimental Parasitology, 50, 240-250.

Ogbunude, P.O.J. (1979). 'Interaction of the nucleoside transport inhibitor, nitrobenzylthioinosine 5'-monophosphate, with HeLa cells and erythrocytes', unpublished M.Sc. thesis, University of Alberta, Edmonton, Canada.

Oliver, J.M. and Paterson, A.R.P. (1971). 'Nucleoside transport. I.A. mediated process in human erythrocytes. Canadian Journal of Biochemistry, 49, 262-270.

Olsson, R.A., Snow, J.A., Gentry, M.K. and Frick, G.P. (1972). 'Adenosine uptake by canine heart', Circulation Research, 31, 767-778.

Olsen, A.S. and Milman, G. (1974). Chinese hampster hypoxanthine-guanine phosphoribosyltransferase structural and catalytic properties', Journal of Biological Chemistry, 249, 4030-4037.

Olsen, A.S. and Milman, G. (1977). Human hypoxanthine phosphoribosyltransferase. Purification and properties', Biochemistry, 16, 2501-2505.

Opperdoes, F.R. and Borst, P. (1977). 'Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome', Federation of European Biochemical Society Letters, 80, 360-364.

Otieno, L.H. (1973). 'Trypanosoma (Trypanozoon) brucei in the haemolymph of experimentally infected young Glossina morsitans', Transactions of the Royal Society of Tropical Medicine and Hygiene, 67, 886-887.

Overman, R. (1948). 'Reversible cellular permeability alteractions in disease. In vivo studies on sodium, potassium and chloride concentrations in erythrocytes of the malarious monkey', American Journal of Physiology, 152, 113-121.

Paterson, A.R.P., Lau, E.Y., Dahlig, E. and Cass, C.E. (1980). 'A common basis for inhibition of nucleoside transport by dipyridamole and nitrobenzylthioinosine? Molecular Pharmacology, 18, 40-44.

Paterson, A.R.P., Kolassa, N. and Cass, C.E. (1981). 'Transport of nucleoside drugs in animal cells', Pharmaceutical Therapeutics 12, 515-536.

Pays, E., Van Meirvenne, N., Le Ray, D. and Steinert, M. (1981). Gene duplication and transposition linked to antigenic variation in Trypanosoma brucei. Proceedings of National Academy of Science (US) 78, 2673-2677.

Philip, R.B., Francey. I. and McElroy, F. (1973). 'Effect of aggregation and adenosine uptake', Thrombosis Research 3, 35-50

Pickard, M. A. and Paterson, A.R.P. (1972). 'Use of 4-nitrobenzylthioinosine in the measurement of rates of nucleoside transport in human erythrocytes', Canadian Journal of Biochemistry, 50, 839-840.

Pickard, M.A., Brown, R.R., Paul, B. and Paterson, A.R.P. (1973). 'Binding of the nucleoside transport inhibitor 4-nitrobenzylthioinosine to erythrocyte membranes', Canadian Journal Biochemistry, 51, 666-672.

Reyes, P., Rathod, P.K., Sanchez, D.J., Mrema, J.E.R., Riekmann, K.H and Heildrich, H.G. (1982). Enzymes of purine and pyrimidine metabolism from the human malaria parasite, Plasmodium falciparum', Molecular and Biochemical Parasitology, 5, 275-290.

Robertson, M. (1913). 'Notes on the life history of Trypanosoma gambiense', Report on Sleeping Sickness Commission of Royal Society, 3, 119-142.

Rubio, R. and Berne, R.M. (1969). Release of adenosine by the normal myocardium in dogs and its relationship to the regulation of coronary resistance', Circulation Research, 25, 407-415.

Ruff, M.D. and Read, C.P. (1974). 'Specificity of carbohydrate transport in Trypanosoma equiperdum, Parasitology, 68, 103-115.

Russell, D.H. and Durie, B.G.M. (1978). 'Polyamines as biochemical markers of normal and malignant growth', Progress in Cancer Research Therapeutics, 8, Raven Press, New York.

Schmidt, G., Walter, R.D. and Konigk, E. (1974). 'Adenosine kinase from normal mouse erythrocytes and from Plasmodium chabaudi: partial purification and characterization', Tropenmedizin und Parasitologie 25, 301-308.

Schmidt, G., Walter, R.D. and Konigk, E. (1975). 'A purine nucleoside hydrolase from Trypanosoma gambiense, purification and properties , Tropenmedium und Parasitology, 26, 19-26.

Sheen, M.R., Kim, B.K. and Parks, R.E. (Jr) (1968). 'Purine nucleoside phosphorylase from human erythrocytes, III. Inhibition by the inosine analog formycin B. of the isolated enzyme and of nucleoside metabolism in intact erythrocytes and sarcoma 180 cells', Molecular Pharmacology, 4, 293-299.

Singer, I., Kimble, E.T, and Ritts, R.E. (1964). Alteractions of the host- parasite relationship by administration of endotoxin to mice with infection with trypanosomes. Journal of Infectious Diseases, 114, 243-249.

Smyth, J.F. and Harrap, K.R. (1975). Adenosine deaminase activity in leukemia. British Journal of Cancer, 31, 544-549.

Soldo, A.T. and Van Wagtendonk, W.J. (1961). 'Nitrogen metabolism in Paramecium aurelia', Journal of Protozoology, 8, 41-55.

Soltys, M.A. and Woo, P.T.K. (1969). 'multiplication of Trypanosoma brucei and Trypanosoma congolense in vartebrate hosts', Transactions of the Royal Society of Tropical Medicine and Hygiene, 63 490-494.

Soltys, M.A. and Woo, P.T.K. (1977). 'Trypanosomes producing disease in livestock in Africa', In Parasitic Protozoa, 1, 239-268, Academic Press, New York.

Stefanovic, V., Mandel. P. and Rosenberg, A. (1976). Ecto 5'-nucleotidase of intact culture C_6 Rat Glioma cells. Journal of Biological Chemistry, 251, 3900-3905.

Stibbs, H.H. and Seed, J.R. (1975). 'Metabolism of tyrosine and phenylalanine in Trypansoma brucei gambiense', International Journal of Biochemistry, 6, 197-203.

Tallarida, R.J. and Jacob, L.S. (1979). Quantal dose-response relation. The Dose-Response relation in Pharmacology, pp. 50-121. Springer-Verlas, New York.

Takeuchi, T., Iwanaga, J., Aoyazi, T. and Umezawa, H. (1966) 'Antiviral effect of formycin and formycin B', Journal of Antibiotics, 19, 286-287.

Tax, W.J.M. and Veerkamp, J.H. (1978). 'Activity of adenosine deaminase and purine nucleoside phosphorylase in erythrocytes and lymphocytes of man, horse and cattle', Camparative Biochemistry and Physiology, 61B, 439-441.

Tizard, I., Nielsen K.H., Seed, J.R. and Hall, J.E. (1978a). 'Biologicallly active products from African trypanosomes', Microbiology Review, 42, 661.

Tizard, I.R., Holmes, W.L. and Nielsen, K. (1978b). Mechanisms of the anamia in trypanosomiasis: Studies on the role of the haemolytic fatty acids derived from Trypanosoma congolense, Tropenmedum Parasitologie, 29, 108-114.

Tucker, E.M. and Young, J.D. (1976). Genetic variation in the purine nucleoside phosphorylase activity of sheep red cells', Animal blood Groups and Biochemical Genetics, 7, 109-117.

Tuttle, J.W. and Krenitsky, T.A. (1979). Purine phosphoribosyl-transferases from Leishmania donovani. Journal of Biological Chemistry, 255, 909-916.

Ukoha, A.I. (1980). 'Electrophoretic characterization of some soluble enzymes of Trypanosoma vivax, Unpublished Ph.D. dissertation. Ahmadu Bello University.

Umezawa, H., Sawa, T., Fukagawa, Y., Koyama, G., Murase, M., Hamada, M. and Takeuchi, T. (1965). Transformation of formycin to formycin B and their biological activities', Journal of Antibiotics, 18A, 178-181.

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', Journal of Antibiotics, 204, 308-316.

Unsworth, K. (1953). Studies on Trypanosoma vivax. VIII. Observations on the incidence and pathogenicity of Trypanosoma vivax in Zebu cattle in Nigeria', Annals of Tropical Medicine and Parasitology, 47, 361.

Vickerman, K. (1969). 'On the surface coat and flagella adhensions in trypanosomes', Journal of Cell Science, 5, 163-193.

Vickerman, K. and Luckins, A.G. (1969). 'Localization of variable antigens in the surface coat on Trypanosoma brucei using ferratin conjugated antibody', Nature (London) 224, 1125-1126.

Von Brand, T. (1951). 'Physiology of blood flagellates', In Parasitic Infections in Man, Most, H. (ed.), pp. 90, Columbia University Press, New York.

Walsh, C.J. and Sherman, I.W. (1968). 'Purine and Pyrimidine synthesis by the avian malaria parasite, Plasmodium lophurae', Journal of Protozoology, 15, 763-770.

Walter, R.D. and Konigk, E. (1974). 'Hypoxanthine-guanine phosphroibosyltransferase and adenine phosphoribosyltransferase from Plasmodium chabaudi, purification and properties', Zeitschrift Tropenmedun Parasitologie, 25, 227-235.

Watkins, J.F. (1964). 'Observations on antigenic variation in a strain of Trypanosoma brucei growing in mice. Journal of Hygiene, 62, 69-80.

Webster, H.K., Haut, M.J., Martin, L.K. and Hildebrandt, P.K. (1982). Purine and pyrimidine nucleotide profles during synchronous malaria infection (Plasmodium knowlesi) in the rhesus monkey. Internationa Journal of Parasitology, 12, 75-79.

Williamson, J. (1969). 'Nucleoside trypanocides', Parasitology, 59, 9 p.

Woo, P.T.K. and Kobayashi, A. (1975). 'Studies on the anemia in experimental African trypanosomiasis. I. Preliminary communication as the mechanism of the anemia', Annals of Belgium Society of Tropical Medicine, 55, 37-45.

Whitely, H.R. and Douglas, H.C. (1951). 'The fermentation of purines by Micrococus lactilyticus' Journal of Bacteriology, 61, 605-616.

Whitely, H.R. (1952). 'The fermentation of purines by Hicrococus aerogenes', Journal of Bacteriology, 63> 163-175.

Yamada, K.A. and Sherman, I.W. (1981). •Purine metabolism by the avian malarial parasite Plasmodium lophurae'$_t$ Molecular and Biochemical Parasitology, ^3, 253-264.

Yesufu, H.H. and Mshelbwala, A.Li. (1973). Trypanosomiasis survey in cattle and tsetse flies along a trade cattle route in Southern Nigeria[9], Annals of Tropical Medicine and P arasltology, 67, 307-312.

Yorke, W., Adams, A.R.D. and Murgatroyd, F. (1929). 'Studies in chemotherapy X. A. Method for maintaining pathogenic trypanosomes alive in vitro at 37 C for 24 hours. Annals of Tropical Medicine and Parasitology, 23, 501—518.

Zlemann, h. (1905). •Beitrag zur trypanosomenfrage*, CbL B^Jctp 38$_f$ 307-429.