# UPTAKE AND METABOLISM OF PURINE NUCLEOTIDE AND PYRIDOXAL PHOSPHATE PRECURSORS IN TRYPANOSOMA BRUCEI BRUCEI.

BY

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This thesis is dedicated to my parents.

I hereby declare that this thesis has been composed by myself. The work of which this is a record has been carried out by myself and all sources of information have been acknowleged by means of references.

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**ABSTRACT OF THE THESIS**

The uptake and metabolism of the $B_6$ vitamers pyridoxine and pyridoxal by T.brucei (TREU 55) was investigated. The results of these investigations indicate that these vitamers are taken up by simple diffusion followed by metabolic trapping. Both pyridoxine and pyridoxal were found to be metabloised to pyridoxal phosphate in whole cells. The majority of pyridoxal taken up appears to remain as pyridoxal (perhaps protein bound) the remainder being slowly phosphorylated to pyridoxal phosphate by pyridoxal kinase.The majority of pyridoxine taken up was found to be rapidly metabolised to pyridoxal. Evidence was also found supporting the hypothesis that pyridoxine may be phosphorylated to pyridoxine phosphate and then rapidly converted to pyridoxal phosphate.

The uptake of adenosine, adenine and hypoxanthine by T.brucei (TREU 55) was also investigated and found to be mediated by high affinity active transport systems (Km < 1μM). The presence of two (possibly three) separate transporters at the level of the plasma membrane was demonstrated by the use of nucleoside analogue inhibitors of uptake which showed differing affinities for each of the transporters. The adenosine transport protein has been tentatively identified one or possibly two proteins with molecular weights of 51 and 57KDa by affinity labelling experiments using radiolabelled dialdehyde adenosine as a probe for the transport protein. These experiments were carried out using both whole cells and partially purified plasma membranes.

The metabolism of adenosine. adenine and hypoxanthine to nucleotides was also investigated. Adenosine was found to be very rapidly metabolised to adenine in a cell lysate by adenosine

hydrolase as opposed to a phosphorylase as is normally found in mammalian tissues. The adenine formed in this reaction was shown to be converted to AMP by the action of adenosine phosphoribosyltransferase. Hypoxanthine was also rapidly converted to nucleotide (IMP) by a hypoxanthine phosphoribosyl transferase which as in mammalian systems appeared to show some specificity towards guanine as substrate.

The transport systems for adenosine, adenine and hypoxanthine appeared to be under some form of control since uptake was shown to stop after two minutes although metabolism of the material already taken up continued. Preliminary results are presented which implicate phosphorylation by a protein kinase as the regulatory mechanism since high levels of AMP have been shown to stimulate the phosphorylation of a protein with a molecular weight of 51KDa.

## ABREVIATIONS

A.....Adenine

Ar.....Adenosine

APRT.....Adenine phosphoribosyltransferase

DIDS.....4,4'-Diisothiocyanatostilbene-2,2'
-disulphonic acid

DTT.....Dithiothreitol

EDTA.....Ethylenediamine tetra-acetic acid

G.....Guanine

Gr.....Guanosine

HEPES.....N-2-Hydroxyethylpiperazine-N'-
2 ethanesulphonic acid

Hx.....Hypoxanthine

HxPRT.....Hypoxanthine phosphoribosyltransferase

HxGPRT.....Hypoxanthine/guanine
phosphoribosyltransferase

IDA.....5'-Iodo-5'-deoxyadenosine

Ir.....Inosine

KDa.....Kilo Daltons

Km.....$K_m$

LDH.....Lactate dehydrogenase

NBTI.....p-Nitrobenzyl-6-thioinosine

PA.....Pyridoxal

PAC.....Pyridoxic acid

PAM.....Pyridoxamine

PAMP.....Pyridoxamine-5-phosphate

PAP.....Pyridoxal-5-phosphate

P.C.V.....Packed cell volume

3-PGA.....3-phosphoglyceric acid

Pi.....Inorganic phosphate

PK.....Pyruvate kinase

PMSF.....Phenylmethylsulphonylfluoride

PN.....Pyridoxine

PNP.....Pyridoxine-5-phosphate

SDS.....Sodium dodecylsulphate

SITS.....4-Acetamido-4'-isothiocyanatostilbene
-2,2'-disulphonic acid

TEMED.....NNN'-N'-Tetra-methyl-1,2-diaminoethane

TREU.....Trypanosomiasis Research
Edinburgh University

W.H.O.....World Health Organisation

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# 1. INTRODUCTION

## 1.1 NATURAL HISTORY OF SLEEPING SICKNESS AND CONTROL OF THE DISEASE.

African sleeping sickness (trypanosomiasis) is caused by trypanosomes of the brucei group which are not limited to Africa but are spread throughout the world (Ormerod,1979). T.rhodesiense and T.gambiense are responsible for the acute and chronic forms of the human disease respectively. T.brucei appears to be infective only to cattle, some game animals and rodents.

Both human and cattle forms (Nagana) of Trypanosomiasis are major health and economic problems in Africa. It is estimated that some 20,000 people die from this disease per year and since 1974 83,000 head of cattle have died, at a cost of £ 11 million, from the disease in the North-East of Africa alone. The disease is transmitted by an insect vector, the tse-tse fly, which in Mozambique and Zimbabwe is extending its range at a rate of $10km^2$ per year since independence wars and cutbacks in control measures. The affected area covers 322,000 $km^2$ which at present supports 650,000 head of cattle which could be increased to 6.7 million head if the tse-tse fly or trypanosomes were eliminated.

Eradication of the tse-tse fly is to be attempted by spraying vast areas of Zimbabwe, Zambia, Malawi and Mozambique with the insecticide, Endosulfan. Initial trials are to be conducted by spraying 20,600 $km^2$ although the effects of such large scale spraying on the ecosystem are unknown. Alternatives to spraying have been considered such as the release of sterile male flies (Dame,1970). The main problems with this approach are the reduction of the fly populations to manageable levels before release of the sterile flies

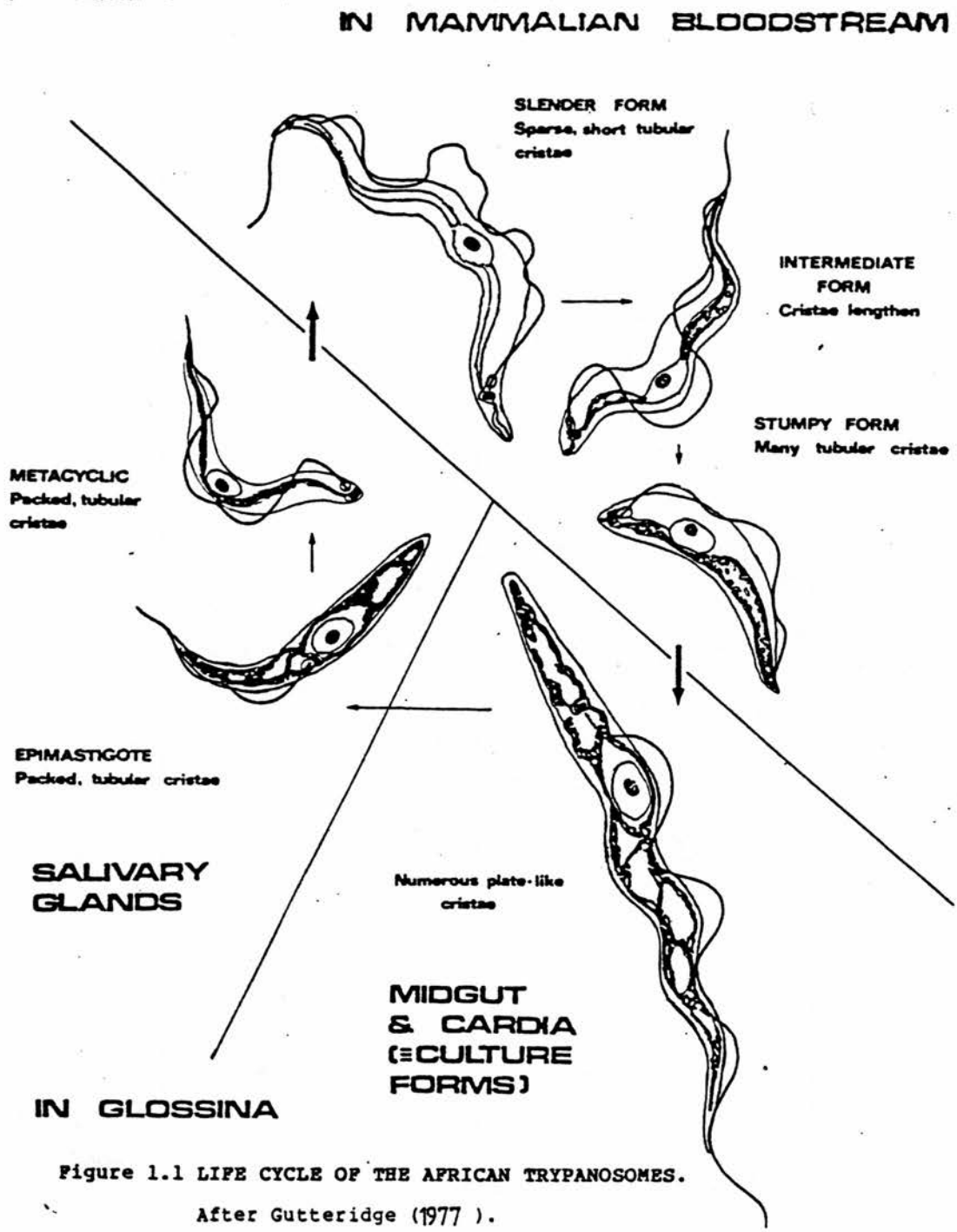



Figure 1.1 LIFE CYCLE OF THE AFRICAN TRYPANOSOMES.

After Gutteridge (1977 ).

and the production of sterile male flies in sufficient numbers (WHO, 1977).

Another method for control of the disease is to remove the reservoir of infection, which in largely agricultural areas resides in man and his domestic animals, by selective chemotherapy. This method would not work in the bush area since the reservoir of infection resides in the wild game animals. Unfortunately there are few drugs available for this strategy, those in use being suramin and pentamidine for prophylaxis and cure in early stages of the disease with melarsoprol and nitrofurazone used for curative purposes in the late stages of the disease. The melaminyl arsenical drugs are used in the late stages of the disease since of the drugs listed above only they are capable of crossing the blood brain barrier. These drugs also have serious side-effects and resistant strains of trypanosomes are beginning to appear in the wild. These problems can only be solved by the development of new drugs (see Van den Bossche(1978) and Marr et al.(1978b)).

### 1.1.1 Life Cycle of the African Trypanosomes.

The life cycle of the pleomorphic African trypanosomes can be considered in two parts. The insect stage and the mammalian host stage, as summarised in Figure 1.1. When a tse-tse fly takes a blood meal from an infected mammal short stumpy trypomastigotes are ingested and lodge in the fly's gut where they differentiate into procyclic forms which are biochemically equivalent to culture forms of trypanosomes. The metabolic potentials of each of the morphological forms encountered in the life cycle are summarised in Table 1.1. In the gut of the fly the procyclic forms divide by

Table 1.1: Comparison of the metabolic status of the different forms of *T. brucei*.

| Parameter | Blood trypomastigote forms | | Culture procyclic Trypomastigote forms |
|---|---|---|---|
| | Long slender | Short stumpy | |
| glycolysis | + | + | + |
| TCA cycle | - | Partial | + |
| L-α-glycerophosphate oxidase | + | + | Low |
| Proline oxidase | - | Low | + |
| Cytochrome oxidase | - | - | + |
| Cyanide sensitivity | - | - | Partial |
| Mitochondria | Very few tubular cristae | Tubular cristae | Plate like cristae |
| Growth temperature ($^{o}$C) | 37 | 37 | 25 |

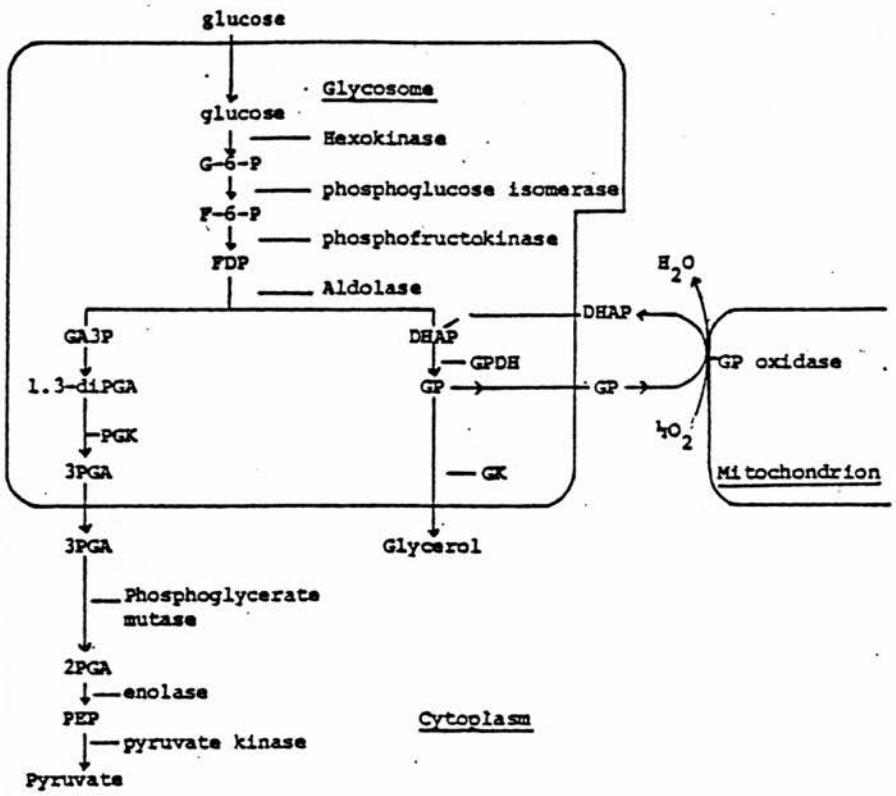
+, present -, absent. Based on Table 1 of Gutteridge and Rogerson (1979).

longitudinal binary fission and eventually migrate to the fly's salivary glands where they differentiate further into the epimastigote form and the metacyclic form which is infective to mammals. The mammalian host is infected when the fly takes a blood meal and metacyclic forms are released from the fly's salivary glands. In the host bloodstream the trypanosomes differentiate into the long slender trypomasigote form which lacks the TCA cycle enzymes (see Table 1.1) and divides by longitudinal binary fission. As the numbers of trypomastigotes in the host's bloodstream increases a fever results until the host's immune system recognises the infection and destroys the trypanosomes. However not all of the trypanosomes are destroyed since they have the ability to change their antigenic glycoprotein coat which covers the cell surface. This ability to evade the host's immune system results in repeated bouts of fever the frequency of which depends on the trypanosome species causing the infection. The process of antigenic variation has been recently reviewed by Turner(1982) as has the mechanism of release of the glycoprotein coat (Turner et al.,1985). This antigenic variation allows a relapsing parasitaemia to continue until the long slender trypomastigotes invade the host's central nervous system which eventually results in death if not treated. As the numbers of long slender trypomastigotes increase a certain proportion differentiate into short stumpy trypomastigotes which are infective to the tse-tse fly so completing the life cycle.

### 1.1.2 Glycolysis in Bloodstream Trypanosomes.

The changes in trypanosomal metabolism which occur during the life cycle are summarised in Table 1.1 which shows that in the

Figure 1.2: The general metabolism and compartmentation in T. brucei.




The scheme was constructed from results published by Oduro (1977); Opperdoes et al. (1977) and Opperdoes and Borst (1977)

mammalian bloodstream the trypanosomes rely totally on glycolysis for their ATP production. The general metabolism of glucose and the distribution of the glycolytic enzymes in bloodstream forms of T.brucei are shown in Figure 1.2. It has been suggested that the enzymes of glycolysis ( hexokinase to phosphoglycerate kinase) are contained within the glycosome to maintain optimal concentrations of enzymes, subtrates and co-factors for glycolysis. (Opperdoes & Borst,1977; Oduro,1977). Similar glycosome like organelles have been identified in C.fasciculata and T.cruzi leading to the suggestion that the glycosome is a feature of all the Kinetoplastida (Taylor et al.,1980a).

## 1.2 UPTAKE AND TRANSPORT : GENERAL CONSIDERATIONS.

Throughout this thesis the following distinctions are made between uptake and transport. "Uptake" is used to denote accumulation of exogenously added radiolabelled substrate regardless of any metabolic conversions of the substrate, in the same sense as used by Berlin and Oliver(1975). "Transport" is used when describing the transfer or translocation of a substrate across a membrane as mediated by a saturable selective carrier. The action of this carrier may or may not be dependent on an energy source. If transport is dependent on an energy source other than a concentration gradient of substrate across the membrane and free unmetabolised substrate accumulates against its concentration gradient, it will be described as "active transport".

### 1.2.1 Methods of Uptake and Transport.

Uptake of solutes from the external environment can occur by

three different mechanisms as listed below.

1) Uptake may be by simple non-carrier mediated diffusion with or without subsequent trapping by metabolism or intracellular binding. This method of uptake does not involve a transport step as defined above (Section 1.2).

2) Uptake may be mediated by facilitated diffusion involving the transport of the solute across the membrane by a carrier molecule. This mechanism of uptake may or may not involve metabolism of the transported solute in order to maintain a gradient of solute across the membrane.

3) Uptake may be mediated by an active transport system involving a solute carrier in the membrane, as for facilitated diffusion, but in this case the system is capable of accumulating solute against its concentration gradient by using an energy source to drive transport. Active transport is therefore not dependent on metabolism of the solute taken up to maintain a favourable concentration gradient for uptake. Each of the above transport and uptake systems will now be considered in more detail.

Uptake by simple diffusion is exemplified by the uptake of neutral lipophilic compounds which can diffuse through the hydrophobic lipid bilayer of a membrane. The uptake of salicylic acid from the stomach by the epithelial cells of the stomach lining is by diffusion. In the acid environment of the stomach the weak acid becomes protonated which abolishes the negative charge of the free carboxyl group allowing the salicylate to partition into the plasma membranes of the stomach epithelial cells. From the plasma membrane the salicylate diffuses into the cytoplasm of the cells where it dissociates at the higher intracellular pH once again becoming

negatively charged preventing exit back through the plasma membrane. The rate of uptake by diffusion is therefore limited by the solute's partition co-efficient between the aqueous phase and the lipid phase of the membrane and also its pK if a weak acid or base (Leib & Stein,1971; Graff et al.,1977). Once the solute has entered the cell it may be metabolised which will maintain a concentration gradient of solute across the membrane allowing diffusion to continue. In the case of a weak acid, such as salicylate, the solute may be accumulated against its concentration gradient without metabolism if the pH inside the cell is higher than outside. This occurs since it is the protonated form of the acid which diffuses across the membrane to equilibrium and at the higher intracellular pH dissociates according to its pKa therefore more of the solute will be required on the cytoplasmic side of the membrane to give equal concentrations of the protonated form across the membrane. This effect is described in more detail in Section 2.19 as a useful tool in the measurement of $\Delta$pH across a membrane. Uptake by simple diffusion is usually characterised by being non-saturable, non-inhibitable by solute analogues and showing little temperature dependence.

Facilitated diffusion differs from simple diffusion, described above, in that a carrier molecule is involved in the translocation of the solute across the membrane. Once the solute has entered the cell the diffusion gradient may be maintained by metabolism of the solute. It is also possible that the transported solute or another solute with affinity for the carrier may recombine with the carrier and be transported back across the membrane. This effect can be of use in determining the initial rates of transport in metabolically enfeebled cells as described in Section 1.2.2. Facilitated diffusion is

incapable of accumulation of solute against its concentration gradient but allows diffusion of the solute across the membrane to be independent of the lipid solubility of the solute. Facilitated diffusion is characterised by the ability to saturate transport and by transport rates showing marked temperature dependence. The specificity also allows inhibition of transport by analogues of the carriers natural substrate or substrates. Examples of facilitated diffusion are detailed in Section 1.2.2.

Active transport, like facilitated diffusion, is mediated by a specific carrier molecule in the membrane, the nature of which is determined by the energy source to which transport is coupled. Active transport can be subdivided into three different types.

a) The transport of a solute may require the direct hydrolysis of one or more ATP molecules.

b) The transport of a solute may be coupled to a pre-existing gradient of another solute across the membrane or which is established by another transport system.

c) The transport of a solute may be concomitant with the solute's metabolism.

The first type (a) of active transport is restricted to the translocation of ions across a membrane as typified by the $Na^+/K^+$-ATPase, $H^+$-ATPase and the $Ca^{2+}$-ATPase of plasma membranes, mitochondria, chloroplasts and sarcoplasmic reticulum of striated muscle cells. These transport systems are responsible for the maintenance of the internal ionic environment of the cell,synthesis of ATP,generation of ion gradients to be used by type b transports as defined above and control of muscle contraction depending on their location and the organism in which they are found. The $Na^+/K^+$-ATPase

is responsible for the maintenance of a membrane potential in most animal cells and regeneration of the membrane potential in nerve cells after conduction of an action potential, which involves influx of $Na^+$and efflux of $K^+$(Korenbrot,1977). The $Na^+$ and $K^+$ gradients generated by this ion pump can be used as energy sources for co-transport of other solutes in kidney proximal tubular epithelial cells and intestinal epithelium. (See Section 1.2.2).

Proton translocating ATPases can be differentiated into two types, the plasma membrane type involved in generating ion gradients for b type active transport systems (Goffeau & Slayman,1981; Villalobo,1982) or the ATP synthetase type typical of mitochondria, bacteria and chloroplasts (Fillingame,1981; Njus et al.,1981; Nelson,1981). The bacterial $H^+$-ATPase can work in either direction, generating a proton gradient for use in transport or using a proton gradient for the synthesis of ATP. In anaerobic bacteria the ATPase functions to generate a proton gradient for use in transport (Abrams,1976).

The $Ca^{2+}$-ATPase of the sarcoplasmic reticulum is responsible for control of striated muscle contraction by controlling cytoplasmic concentrations of calcium ion as reviewed by Korenbrot(1977). Many different cell types have been shown to possess a plasma membrane $Ca^{2+}$-ATPase which keeps cytoplasmic calcium ion concentrations below 1μM. The functions,properties and distributions of the $Ca^{2+}$-ATPases, and the other ATPases mentioned above, are extensively reviewed in "Transport ATPases" (Annals of the New York Academy of Science vol.402 (1982)).

The final type of active transport to be considered, involving concomitant transport and metabolism (type c), is referred to as

group translocation. The best characterised system of this type is the phosphotransferase system for sugar transport in E coli . The details of this active transport mechanism are reviewed by Oxender(1972) and Boos(1974). The net result of transport is that a sugar molecule is translocated across the bacterial plasma membrane and at the same time is phosphorylated via a series of reactions involving phosphate transfer from the glycolytic intermediate, phosphoenol pyruvate.

Having outlined the various types of transport which can occur in prokaryotic and eukaryotic cells, methods of investigating these systems will be considered.

### 1.2.2 Methods of Investigating Uptake and Transport.

Investigation of transport as distinct from uptake can be complicated by metabolism or catabolism of the solute once it has entered the cell. This problem has been well documented in the case of nucleoside transport in animal cells. It has been found that uptake of nucleosides by animal cells is apparently linear over a time period of 1-10 minutes (Plagemann,1971a&b; Plagemann & Richey,1974; Zylka & Plagemann,1975; Loike & Horwitz,1976; Lynch et al.,1978: Mizel & Wilson,1972; Paterson et al.,1975; Schuster & Hare,1971). It was therefore assumed that measurements of the rates of uptake in this time period were a reflection of the initial rates of transport from which the kinetic parameters of transport were estimated. The assumption that initial rates of transport were being measured was supported by the observation that simple Michaelis-Menten kinetics were obeyed which was suggestive of a

single, saturable, rate-limiting step for uptake and also the kinetic constants measured for uptake were lower than those measured for the metabolism of substrate in cell lysates. It was also noted that the concentration ratio of substrate inside:outside never exceeded unity. These observations supported the view that uptake occurred by facilitated diffusion followed by metabolic trapping. However it was found that the experiments, in which uptake rates were being measured over a time period greater than one minute, were measuring the rates of formation of nucleotide at steady state intracellular concentrations of nucleoside (Wohlhueter et al.,1976 & 1979; Plagemann,1971b; Lum et al.,1979; Marz et al.,1979). These authors showed that transport allowed equilibration of intra- and extracellular substrate within a few seconds at substrate concentrations below or near to the Km for transport.

Measurement of initial rates of uptake may therefore require methods which allow very short incubation times and rapid sampling. It is also advantageous if metabolism of the transported solute can be reduced or eliminated. Reduction of metabolism can be accomplished by using mutant cell lines deficient in the enzymes required for metabolism of the transported solute, a method first used in mammalian cells by Kessel & Shurin(1968) using a mouse leukemia cell line. This technique has since been applied by many authors in the study of nucleoside transport.(Lieu et al.,1971; Plagemann et al.,1978; Witney & Taylor,1978; Murphy et al.,1977)

Metabolism of substrates which require ATP or PRPP can be reduced if cellular ATP is depleted by preincubation of the cells in glucose free media containing KCN and iodoacetate (Plagemann & Erbe,1973; Plagemann et al.,1976; Wolhueter et al.,1978). This method

has the disadvantage that it may decrease cellular integrity (Plagemann et al.,1976; Gazitt et al.,1976). Substrate metabolism may also be reduced by the use of enzyme inhibitors specific for the metabolising enzymes. However this requires that the inhibitors used do not have any effect on transport (Lum et al.,1979).

A third method of reducing the interference of metabolism when measuring transport rates is to use non-metabolisable substrates for the transport assay (Kessel,1978; Ungenmach & Hegner, 1978). However this method requires that the analogue used in the transport assay uses the same carrier to enter the cell as the substrate being investigated and of course all kinetic parameters estimated apply only to the analogue used and may bear little relation to the parameters of transport for the natural substrate.

Reduction of metabolism makes the assay of transport alone less difficult but since the carriers may allow the equilibration of substrate across the membrane in a matter of seconds rapid sampling techniques are still required if initial rates of transport are to be measured. Many methods have been devised for the determination of initial rates of transport, the most frequently used being described below. In experiments in which the influx of substrate into the cell is measured the cells can be removed from the external medium by centrifugation through an inert oil layer such as silicon oil with a density greater than that of the incubation medium but less than that of the cells. The amount of substrate taken up or transported can be measured in the cell pellet or, depending on the amounts taken up, the depletion of substrate in the incubation medium can be measured. This procedure has been applied to measurements of the distribution of various compounds across mitochondrial membranes (Werkheiser &

Bartley,1957), the influx of labelled uridine into erythrocytes (Oliver & Paterson,1971) and nucleoside and base influx in cultured cells (Wohlhueter et al.,1976 & 1978; Ungenmach & Hegner, 1978; Strauss et al.,1976; Pofit & Strauss,1977).This technique was also used in a study of the uptake of the trypanocidal drug pentamidine by trypanosomes (Damper and Patton,1976a). This technique has the advantage that the cells can be centrifuged into various stopping solutions of the appropriate density which can stop metabolism of any substrates transported. The main disadvantage of this technique is that the cells carry some of the incubation medium containing substrate through the oil phase giving an over-estimate of transport or uptake. This carry-through can be corrected for by including a labelled compound in the incubation medium which can not penetrate the cells, such as inulin or L-glucose.

Cells cultured on glass cover slips can be used in rapid assays of transport since they can be dipped into incubation medium containing the compound to be transported for a few seconds and then rinsed in ice cold buffer. This technique obviously requires that the cells being studied can be cultured fixed to glass, but it also suffers from a more serious drawback. During the rinse in buffer, even at low temperatures, efflux of transported substrate is a major consideration. It has been calculated that Novikoff cells treated in this way could lose upto 50% of their thymidine pool during a 43 second wash period (Wohlhueter et al.,1979). Another disadvantage of this technique is the small amount of material which can be used in any one assay.

Rapid filtration of cells from the suspending medium by "Millipore" filters has also been used as a sampling technique for

estimating efflux of substrates from preloaded cells by measuring substrate released into the filtrate (Cabantchik & Ginsburg, 1977). Estimation of transport or uptake by this method suffers from problems of quantitative estimation of substrate taken up due to high and inconsistent background binding of radiolabelled substrates to the filters, (Section 3.1).

The use of accelerated exchange diffusion (carrier mediated) has been used to measure the kinetic parameters of nucleoside transport in many types of animal cell. This method involves loading cells with radiolabelled substrate and measuring the rates of efflux of this substrate in response to different concentrations of a second substrate which uses the same carrier. The labelled substrate can be present on either side of the membrane in this type of experiment. The various experimental protocols which can be used in this type of assay have been extensively reviewed by Plagemann & Wohlhueter(1980) and Young & Jarvis(1983). This type of experiment requires that many substrates use the same transporter and that there is no metabolism of any of the substrates used.

The methods of analysis of transport described above are applicable to estimation of the kinetic properties of transport systems. However other methods are required for more detailed investigations of transport and uptake systems. Identification and purification of carrier molecules requires assay systems for measuring the activity of the carrier as it is purified and methods of labelling the carrier so that it can be identified. The hexose transporter of erythrocytes was purified using reconstitution of transport activity into phospholipid vesicles as an assay of the

amount of carrier present (Kasahara & Hinkle, 1976 & 1977). This method was also applied in the purification of the anion channel from erythrocytes (Ross & McConnell,1977) and the ATP/ADP nucleotide carrier of mitochondria (Schertzer & Racker,1976). Identification of the transport protein can be accomplished by radiolabelling the transporter with reactive radioactive analogues of transported substrates or tightly binding inhibitors of transport. The anion channel of the erythrocyte was identified by radiolabelling the channel with the channel blockers SITS and DIDS (Cabantchick & Rothstein,1974). This methodology has also been applied to the identification of the protein responsible for ATP/ADP exchange across the mitochondrial membrane using the radiolabelled inhibitors atractyloside and bongkrekic acid (Brandolin et al.,1974; Block et al.,1981). The nucleotide transporter of the bovine chromaffin granule has been identified by labelling with the radioactive reactive substrate analogues p-fluorosulphonyl adenosine and 8-azido ATP (Grüniger,1982). A variation on this labelling technique was used to identify the protein associated with lactose permease activity in E.coli. by labelling it with N-[U-$^{14}$C] ethylmaleimide after protecting it with substrate during a pre-incubation with unlabelled N-ethylmaleimide (Fox & Kennedy,1965).

Effects of temperature on uptake and transport can provide information as to the type of transport system operating, if one is present, and its environment. The value of the activation energy calculated from an Arrhenius plot of transport or uptake rates can give an indication as to whether substrate entry into the cell is carrier-mediated or by simple diffusion (Dixon & Webb,1964; Goldman et al.,1968; Damper & Patton,1976b). Sharp breaks in Arrhenius plots

may be a reflection of temperature dependent phase transitions in the lipid phase of the membrane. A change from liquid crystalline state to fluid state would result in a lowering of the activation energy of transport if the transport mechanism requires movement of the carrier protein (Plagemann & Erbe,1975; Stein & Rozengurt,1975; Zylka & Plagemann,1975)

The methodologies described above are applicable to the study of active transport systems, but since active transport is coupled to an energy source it is susceptible to inhibition by methods other than those described above. It has been shown that nucleoside transport in E.coli. K12 (Munch-Petersen & Pihl,1980) E.coli. B (Roy-Burman et al.,1978) and S.typhimurium (Rader & Hochstadt,1976) is coupled to a proton gradient which is generated by proton pumping electron transport chains. This makes uptake susceptible to inhibition by metabolic inhibitors which interfere with electron transport such as sodium azide or protonophores which render the plasma membrane permeable to protons such as 2,4-dinitrophenol and FCCP. The lactose permease (West & Michell,1973) and dicarboxylic acid (Ramos & Kabach,1977) transport systems of E.coli are also coupled to proton gradients and show the same susceptibility to inhibition by electron transport inhibitors and protonophores. The bacterium Halobacterium halobium uses a light driven proton pump (bacterial rhodopsin) to generate a transmembrane proton gradient (Bogomolni et al.,1976), which in turn maintains a gradient of $Na^+$ across the membrane using a sodium proton antiport system to pump sodium ions out of the cell (Lanyi & MacDonald,1976). Vesicles can be prepared from the membrane of this organism which are capable of accumulating amino-acids against their concentration gradients when illuminated. The uptake of

the amino acid glutamate appears to be dependent on the presence of a sodium ion gradient alone, sodium ions being co-transported across the membrane (Lanyi et al.,1976). Other amino-acids transported appear to be dependent on the sodium ion gradient and a membrane potential generated by the ion pumps. The requirement for a membrane potential was shown by the use of ionophores such as valinomycin and gramicidin which are capable of allowing selected ions (depending on the ionophores specificity) to pass across the membrane and so collapse or generate membrane potentials depending on the experimental conditions. The properties and specificities of most of the commonly used ionophores are reviewed by Pressman(1976). Many other bacterial transport systems have been shown to be dependent on $Na^+$ or $H^+$ gradients. Melibiose and glutamate transport in E.coli are dependent on a sodium gradient (Tsuchiya et al.,1977a & 1977b) as are the proline transport system in Mycobacterium phlei (Hirata et al.,1974) and melibiose in Salmonella (Tokuda & Kaback,1977). The transport of lactate and alanine (Collins et al.,1976) and accumulation of galactosides (Flagg & Wilson,1976) in E.coli are driven by a pH gradient across the plasma membrane. The various methods, both hypothetical and proven, for coupling transport of solutes to metabolism via proton and other ion gradients in microorganisms has been comprehensively reviewed by Eddy(1978).

Some of the transport systems described above have been investigated in preparations of membrane vesicles. This technique is described in detail by Muner & Kine(1980) as applied to mammalian cells but the principles apply to all cell types if vesicles can be made from their plasma membranes.

Active transport in eukaryotic cells has also been shown to be coupled to ion gradients and membrane potentials. The best studied mammalian systems are those for the transport of amino acids and glucose by intestinal epithelial cells in which these solutes are taken up by co-transport with sodium ions (Kaunitz et al.,1982) and glucose $Na^+$ co-transport by the proximal tubule of the kidney (Hopfer & Groseclose,1980; Koepsell; et al.,1983). These systems are susceptible to inhibition and manipulation in the same manner as the bacterial systems already described.

In summary the active transport systems described above which are dependent on ion gradients and membrane potentials can be investigated by using ionophores to manipulate the membrane potential and magnitude of the ion gradients. The use of specific ionophores is detailed in Sections 3.6 to 3.6.8.

Having described the methods available for the study of transport and uptake of solutes their application to the study of the transport systems of trypanosomes will now be considered.

### 1.2.3 Trypanosomal Transport Systems.

Since trypanosomes are parasitic organisms they rely on their hosts to provide many of the nutrients they require. The African trypanosomes, for example, are incapable of de novo synthesis of the purine ring (Fish et al.,1982a) as are T.cruzi (Berens et al.,1981; Gutteridge & Gaborak,1979) and Leishmania spp. (Marr et al.,1978a). They therefore obtain all their purines from the host organism. For this purpose they have evolved transport systems for the uptake of these nutrients. The transport of purine nucleosides and bases and their subsequent metabolism will be considered in detail in Section

1.4.1. The most extensively studied uptake systems in trypanosomes are the carbohydrate and amino acid uptake systems. These systems will be considered in turn.

The uptake and transport of glucose and other carbohydrates by trypanosomes have received very little attention compared to the metabolism of these compounds. Carbohydrate transport has been shown to be carrier-mediated in T.gambiense (Southworth & Read,1969 & 1970) and T.rhodesiense (Seed et al.,1965) and the specificities of the carriers investigated. The results presented by Southworth & Read(1969 & 1970) indicate the presence of two loci for the uptake of carbohydrate, one specific for the uptake of glucose, mannose and glycerol, the other specific for fructose and glucosamine. These specificities differ from those reported by Ruff & Read(1974) for T.equiperdum which was found to have two loci for carbohydrate transport but one specific for hexoses and one for glycerol. The transport of glucose across the trypanosomal plasma membrane has been shown to be the rate limiting step in glucose metabolism by Gruenberg et al.(1978) who also showed that glucose transport was by facilitated diffusion. The glucose transported was rapidly phosphorylated by hexokinase so maintaining a diffusion gradient for glucose across the membrane. This facilitated diffusion uptake contrasts with the active transport of glucose by Leishmania donovani (Zilberstein & Dwyer.1984) indicated by this organism's ability to accumulate glucose and the partially metabolised glucose analogue, 2-deoxyglucose, against their concentration gradients. The transport can be inhibited by metabolic poisons and the uncoupler 2,4-dinitrophenol which suggests the coupling of transport to an energy source, the nature of which has yet to be elucidated.

The uptake of amino acids by T.brucei has been shown to be a carrier-mediated process for the amino-acids lysine, glycine, alanine and leucine although at high concentration (>1.0mM) leucine and lysine uptake appears to be by simple diffusion. The uptake of ornithine, aspartate, arginine and glutamate did not show saturation kinetics suggesting that they enter the cell by passive diffusion (Southworth & Read,1972). The apparent diffusion of leucine and lysine at high concentrations may be due to increasing the intracellular concentrations of these amino acids to a point where some metabolising step in the cell becomes saturated. An increase in external concentration will therefore cause an increase in intracellular concentration via a linear relationship whether or not a carrier exists so long as it is not saturated. T.brucei has been shown to have four distinct amino acid transporters (Voorheis.1971) designated, according to their specificites, as follows.

(a) Neutral amino acid carrier one ($N_1$), transporting glycine, alanine, serine and threonine.

(b) Neutral carrier two ($N_2$), transporting valine, leucine, proline andmethionine.

(c) Acid carrier one ($A_1$), transporting glutamic acid.

(d) Basic carrier one ($B_1$), transporting lysine.

Threonine uptake via carrier $N_1$ is mediated by active transport (Voorheis,1971; Fricker et al.,1984) but the energy source has not yet been defined. Voorheis(1977) has shown that transport is independent of $Na^+$ concentration, which is in agreement with Fricker et al.(1984), and is also independent of a proton gradient. Voorheis(1980) also provided evidence that transport was dependent on a glycolytic intermediate from before the aldolase reaction. Fricker

et al.(1984) proposed that the uptake of threonine may be dependent on an exchange system with alanine which is formed from pyruvate by transamination and accumulates in the cell to high concentrations (Chappell et al.,1972). However no direct evidence for this hypothesis is available. Uptake of amino acids by T.gambiense has been investigated by Hansen(1979) who found five transport loci specific for different groups of amino-acids on the basis of mutual inhibition or stimulation of uptake between amino acids. This author in contrast to the results presented by Fricker et al.(1984) and Voorheis(1977) found threonine uptake to be $Na^+$ dependent and sensitive to inhibition by ouabain. This disagreement in the mode of transport for threonine may be due to species differences. In addition to membrane transport systems trypanosomes can take up proteins by endocytosis which are digested in lysosomes to release amino acids. Endocytosis has been shown to mediate the uptake of the albumin bound trypanocide suramin (Fairlamb & Bowman,1980) and other macromolecules and serum proteins (Langreth & Balber,1975; Opperdoes & Vanroy,1982).

A recent study of trypanosomal transport in connection with the post-translational modification of the glycoprotein coat has shown that ethanolamine is taken up by facilitated diffusion in T.brucei (Rifkin & Fairlamb,1985). It has also been reported that the plasma membrane of T.rhodesiense contains a calcium pumping ATPase which is implicated in the regulation of intracellular calcium concentrations (McLaughlin,1985).

In summary, the little information available regarding trypanosomal transport systems indicates that there is wide diversity in the methods of transport used for the same solute. The dependence

| ENZYME | EC No. |
|---|---|
| AMINE OXIDASE | 1.4.3.6 |
| GLUTAMATE FORMYLTRANSFERASE | 2.1.2.6 |
| GLYCINE SYNTHETASE | 2.1.2.10 |
| δ-AMINO LAEVULINATE SYNTHETASE | 2.3.1.37 |
| KYNURENINASE | 3.7.1.3 |
| THREONINE ALDOLASE | 4.1.2.5 |
| PHENYLSERINE ALDOLASE | 4.1.2.6 |
| DIHYDROSPHINGOSINE-1-PHOSPHATE ALDOLASE | 4.1.2.27 |
| TRYPTOPHANASE | 4.1.99.1 |
| D-SERINE DEHDRATASE | 4.1.99.2 |
| THREONINE DEHYDRATASE | 4.2.1.16 |
| TRYPTOPHAN SYNTHETASE | 4.2.1.20 |
| CYSTATHIONINE SYNTHASE | 4.2.1.22 |
| GLUCOSAMINATE AMMONIA LYASE | 4.3.1.9 |

Table 1·2 ENZYMES REQUIRING PYRIDOXAL PHOSPHATE AS CO-FACTOR

of trypanosomes on exogenous supplies of nutrients and enzyme co-factors warrants further investigation into their modes of transport with a view to determining differences between the host and parasite.

## 1.3 FUNCTIONS AND BIOSYNTHESIS OF VITAMIN $B_6$.

Vitamin $B_6$ is synthesised by all higher plants and many microorganisms but is required preformed by some fungi,protozoa and bacteria. The vitamin occurs naturally in three major forms, which are pyridoxine(PN), pyridoxal(PA) and pyridoxamine(PAM). Very low levels of these three compounds are found in animal tissues and bacteria, most $B_6$ occurring as the corresponding phosphate esters. Of the three phosphorylated forms of $B_6$, pyridoxal-5-phosphate(PAP),pyridoxamine-5-phosphate(PAMP) and pyridoxine-5-phosphate(PNP) only PAP and PAMP have functions as co-enzymes.

Pyridoxal-5-phosphate and pyridoxamine-5-phosphate function as co-factors in many enzyme reactions. The most common reaction in which these co-factors are involved is transamination. In this reaction the enzyme bound PAP is converted to PAMP by donation of the amino group of the amino acid, the PAP being regenerated when the amino group is transferred to the keto acid. Vitamin $B_6$ is involved in many other reactions of amino acid metabolism including decarboxylation and desulfhydration. Vitamin $B_6$ is also required for the oxidation of amines, phosphorylase activity of muscle, conversion of tryptophan to nicotinic acid and conversion of amino acids to biogenic amines. Some of the enzymes which use PAP as a co-factor are listed in Table 1.2.

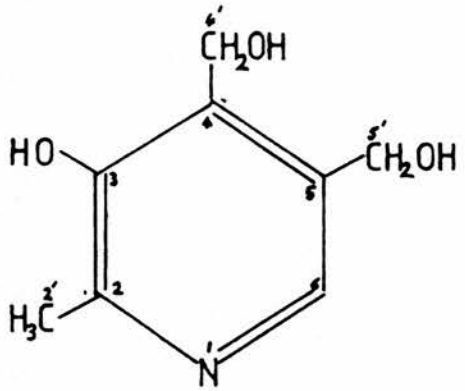



Figure 1·3 STRUCTURE OF PYRIDOXINE.

The biosynthesis of vitamin $B_6$ has been reviewed by Plaut et al.(1974) detailing the results of tracer experiments in which various bacteria and yeasts were grown in media containing radiolabelled glycerol, glucose, pyruvate and serine and the distribution of label in synthesised $B_6$ was determined. Hill and Spencer, (1973) and Hill et al.(1972) proposed that dihydroxyacetone phosphate condenses with acetaldehyde derived from pyruvate to yield a pentulose-1-phosphate intermediate which could then condense with glyceraldehyde-3-phosphate the intermediate so formed being subsequently converted to PAP. More recent work by Vella et al.(1981) has shown that the two carbon unit C-2-2'(See Figure 1.3 for structure) of PN is derived from pyruvate and provides some evidence that acetyl thiamine pyrophosphate is the final precursor metabolite of pyruvate before incorporation into the $B_6$ molecule. A detailed description of the labelling experiments described briefly above is outside the scope of this introduction. However it should be borne in mind that the results presented in the references quoted above indicate only which compounds are capable of being utilised in the synthesis of vitamin $B_6$; none of the hypothetical intermediates required in the proposed syntheses have been detected. Also the enzymes required for the synthesis of some of these intermediates have not been detected in any of the yeast or bacteria known to be capable of the synthesis of vitamin $B_6$. Whether or not the African trypanosomes can synthesise vitamin $B_6$ is unknown; however Tetrahymena geleii, a ciliated protozoan, has been shown to require vitamin $B_6$ preformed for normal growth in culture(Kidder and Dewey, 1949a & 1949b).

### 1.3.1 Uptake of Vitamin $B_6$.

Uptake of PN by the yeast Saccharomyces carlsbergensis has been found to be mediated by active transport. The yeast has two transport systems specific for different forms of the vitamin. One transport system is specific for PN and the other, specific for PA, is also capable of transporting PN. The transport system was shown to be active by its ability to concentrate PN against its concentration gradient several hundred fold as free PN and not as a metabolite (Shane & Snell, 1976). This yeast active transport system contrasts with the facilitated diffusion mechanism for transport in Salmonella typhimurium, uptake being maintained by conversion of the substrate taken up to its corresponding phosphate ester by the enzyme PA kinase (EC 2.7.1.35) (Mulligan & Snell, 1976). The same mechanism of $B_6$ uptake is used by Streptococcus faecalis (Mulligan & Snell, 1977).

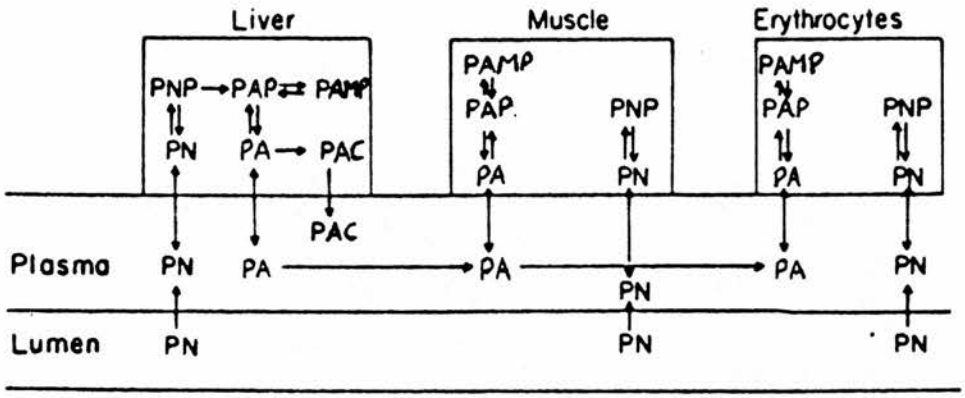
The uptake of vitamin $B_6$ by the intestinal tract of the rat has also been extensively studied. The uptake of $B_6$ by the jejunum of the rat is achieved by simple non-carrier mediated diffusion followed by metabolic trapping by pyridoxal kinase (Middleton, 1977). Uptake by the rat jejunum was shown to be non-mediated by being non-saturable, insensitive to inhibition by the pyridoxine analogue 4-deoxypyridoxine, anoxia, ouabain or removal of $Na^+$ from the medium. Uptake of PN was also shown to be relatively insensitive to temperature changes again indicating that uptake is not carrier mediated. Uptake of $B_6$ by the rat small intestine is not carrier mediated but is achieved by simple diffusion followed by metabolic trapping in the same manner as occurs in the rat jejunum (Mehansho

et al., 1978). These authors also investigated the ability of the rat intestine to take up the phosphorylated forms of $B_6$. It was found that the $B_6$ was dephosphorylated in the lumen of the intestine before being taken up by diffusion. Using perfused intestine these authors also found that the main form of $B_6$ released into the portal blood by the intestine was PA regardless of whether PA or PAP was introduced into the lumen of the perfused intestine.

Transport and metabolism of vitamin $B_6$ by the choroid plexus of rabbits and by mitochondria has also been investigated. One of the effects of $B_6$ deficiency in man and other animals is convulsions which is thought to be due to a lack of production of the neurotransmitter gamma-aminobutyric acid (GABA) which requires PAP as a co-factor in its biosynthesis. However, convulsions and abnormal behaviour patterns can also be induced by overdoses of $B_6$. For these reasons Spector(1978a & 1978b) investigated the control of entry of $B_6$ into the cerebro-spinal fluid and brain through the choroid plexus. It was found that the cells of the choroid plexus were capable of the active transport of non-phosphorylated forms of $B_6$ into the cerebro-spinal fluid although the control mechanism for regulation of transport, if one exists, remains to be elucidated. The uptake and metabolism of $B_6$ by mitochondria has been investigated by Lui et al.(1981 & 1982)who found that uptake was by simple non-carrier-mediated diffusion followed by metabolic trapping.

The transport and uptake of vitamin $B_6$ by rat and human erythrocytes has been investigated by Mehansho & Henderson(1980) who found that uptake was by passive diffusion followed by metabolic trapping by pyridoxal kinase. This is in disagreement with the results published by Yamada & Tsugi(1968 & 1970) who reported that

Figure 1·4




METABOLISM OF VITAMIN $B_6$ IN THE RAT.
(Mehansho et al., 1980)

uptake of $B_6$ by erythrocytes was mediated by active transport. These authors used long incubation periods for their uptake assays (>1 minute) and did not differentiate between the transported forms of $B_6$ and metabolites when estimating intracellular concentrations of transported $B_6$. Mehansho & Henderson(1980) used short incubation times for their assays and so measured initial rates of uptake and not rates of metabolism. These authors also distinguished between metabolised and unmetabolised $B_6$ when calculating intracellular concentrations of $B_6$ taken up. They also showed that some apparent concentration of unmetabolised PA was due to PA binding to haemoglobin.

### 1.3.2 Metabolism of Vitamin $B_6$.

The metabolism of vitamin $B_6$ by animal cells has been more thoroughly investigated than in any microorganism. The most complete picture of vitamin $B_6$ metabolism available is for metabolism in the rat. Figure 1.4 shows diagrammatically pathways for absorbtion and metabolism of vitamin $B_6$ in the rat. Vitamin $B_6$ is absorbed from the intestine by passive diffusion in the case of PN, PA and PAM. The corresponding phosphorylated forms of $B_6$ are dephosphorylated before being taken up.(Mehansho et al.,1980). The unphosphorylated forms of $B_6$ taken up by the intestinal cells are either phosphorylated or released unchanged into the portal bloodstream. The PN taken up by the intestinal mucosa cells can be converted to PA by being phosphorylated to PNP by pyridoxal kinase then oxidised to PAP by PN phosphate oxidase (EC 1.4.3.5) and subsequently dephosphorylated to PA. The vitamers released by the intestine into the portal

bloodstream are taken up by the liver again by passive diffusion followed by metabolic trapping (Mehansho et al.,1980). The vitamers PA and PN are phosphorylated to their corresponding 5-phosphate esters by pyridoxal kinase. As in the intestinal mucosa PN taken up is converted to PAP or PA via PNP. The liver can therefore take up PN and release PA. This PA released is essential for erythrocytes and muscle since these cell types lack PN phosphate oxidase. In this respect the rat erythrocyte differs from the human erythrocyte which is capable of metabolising labelled PN to labelled PA (Anderson et al.,1971). The liver also releases PAP into the bloodstream in very small amounts compared to the release of PA but PAP is found to be the most prevalent form of $B_6$ in the plasma.(Sauberlich et al,1972; Lumeng et al.,1980). This can be accounted for by the fact that other body tissues remove PA from the bloodstream but do not remove PAP which remains in the plasma complexed with serum albumin (Lumeng et al,1974; Mehansho et al.,1980).

In summary it appears that the mode of entry of vitamin $B_6$ into animal cells is by diffusion with the exception of the choroid plexus cells. The mode of entry in those bacteria which have been studied appears to be by facilitated diffusion with active transport being used for uptake by Saccharomyces carlsbergensis. To date no information on the uptake or metabolism of vitamin $B_6$ by trypanosomes has been published.

## 1.4 FUNCTIONS AND BIOSYNTHESIS OF PURINE NUCLEOTIDES.

Purine nucleotides are required as activated precursors for DNA and RNA synthesis by all cells with the exception of the mature mammalian erythrocyte. Nucleoside triphosphates are also required as energy sources to drive many biosynthetic reactions and to act as a store for energy released by the cells catabolic reactions. Adenine nucleotides are also components of four important coenzymes; $NADP^+$, $NAD^+$, FAD and co-enzyme A. Many metabolic pathways, such as glycolysis, are regulated by the intracellular concentrations of adenosine mono-, di- and triphosphates. Adenosine monophosphate is also involved in the regulation of cellular functions in the form of 3',5'-cyclic AMP which may be formed in response to an extracellular stimulus. The effect of 3',5'-cyclic AMP on metabolism may also be dependent on ATP as is exemplified by the inhibition of glycogen synthetase by 3',5'-cyclic AMP dependent protein kinases. In view of these requirements for purines, cells,if they are to reproduce and function normally, must either synthesise purines de novo or obtain them preformed from the environment via salvage pathways which are capable of converting naturally occurring purines into the forms required by the cell.

Most animal cells are capable of the de novo synthesis of the purine ring and the salvage of preformed purine compounds. However erythrocytes and lymphocytes (Scholar & Calabresi,1973) lack the enzymes of the de novo synthetic pathway but are capable of purine salvage. The de novo pathway for purine biosynthesis (Buchanan & Hartman,1959) is shown in Figure 1.5. The enzymes involved in the catalysis of steps 1 to 11 (Figure 1.5) are listed below.

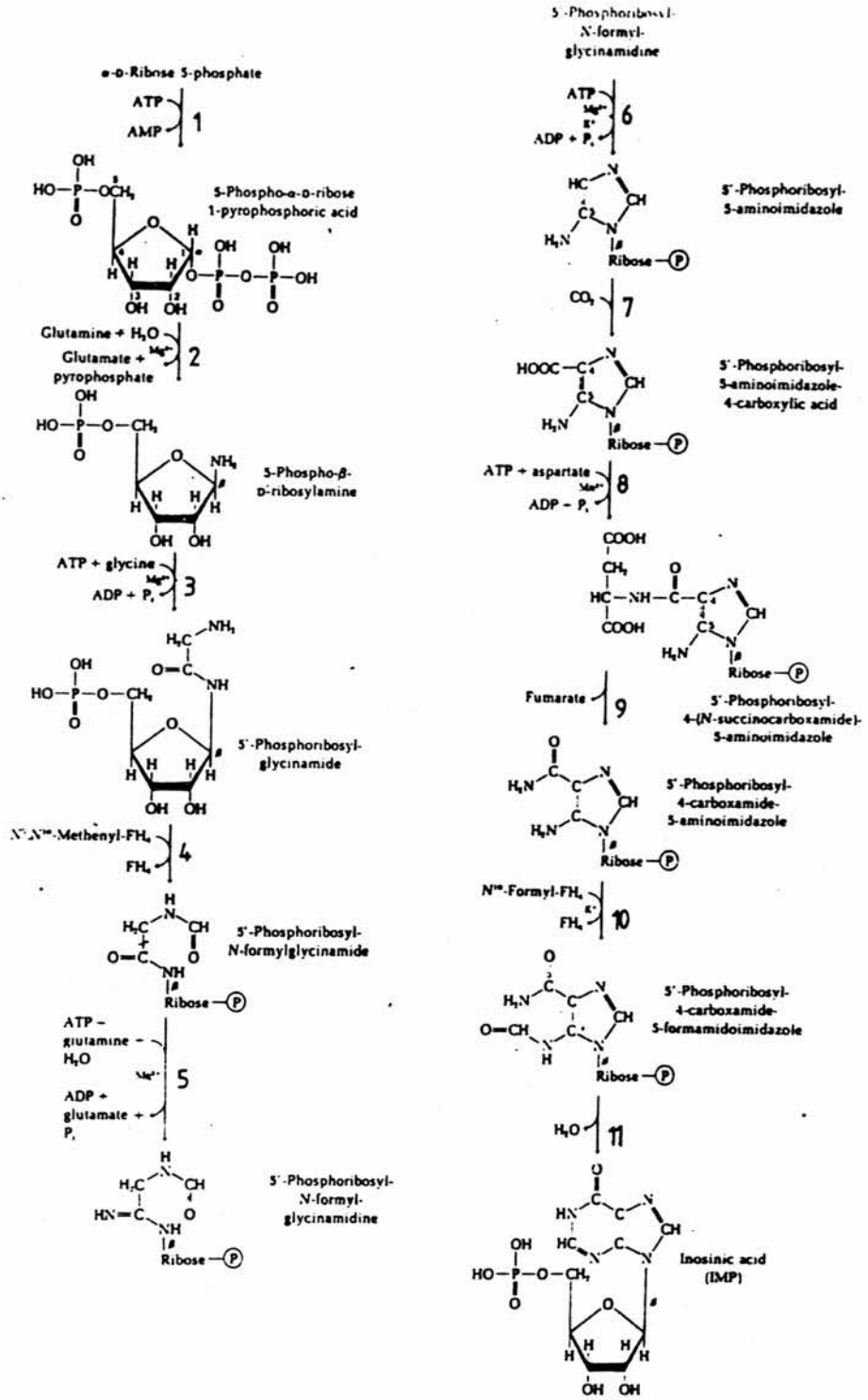
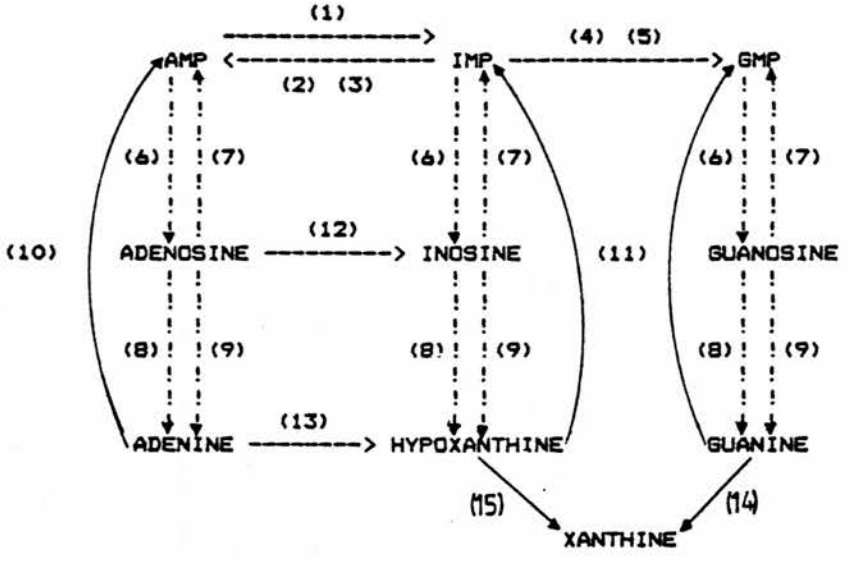

α-D-Ribose 5-phosphate
ATP
AMP
1
5-Phospho-α-D-ribose
1-pyrophosphoric acid
Glutamine + H2O
Glutamate +
pyrophosphate
2
5-Phospho-β-
D-ribosylamine
ATP + glycine
ADP + Pi
3
5'-Phosphoribosyl-
glycinamide
N5,N10-Methenyl-FH4
FH4
4
5'-Phosphoribosyl-
N-formylglycinamide
Ribose
ATP +
glutamine +
H2O
ADP +
glutamate +
Pi
5
5'-Phosphoribosyl-
N-formyl-
glycinamidine
5'-Phosphoribosyl-
N-formyl-
glycinamidine
ATP
ADP + Pi
6
5'-Phosphoribosyl-
5-aminoimidazole
CO2
7
5'-Phosphoribosyl-
5-aminoimidazole-
4-carboxylic acid
ATP + aspartate
ADP + Pi
8
Fumarate
9
5'-Phosphoribosyl-
4-(N-succinocarboxamide)-
5-aminoimidazole
5'-Phosphoribosyl-
4-carboxamide-
5-aminoimidazole
N10-Formyl-FH4
FH4
10
5'-Phosphoribosyl-
4-carboxamide-
5-formamidoimidazole
H2O
11
Inosinic acid
(IMP)

Figure 1-5

| No. | Enzyme | EC No. |
|---|---|---|
| 1 | Ribose phosphate pyrophosphokinase | 2.7.6.1 |
| 2 | Amido phosphoribosyltransferase | 2.4.2.14 |
| 3 | Phosphoribosyl-glycinamide synthetase | 6.3.1.3 |
| 4 | Phosphoribosyl-glycinamide formyltransferase | 2.1.2.2 |
| 5 | Phosphoribosyl-formylglycinamide synthetase | 6.3.5.3 |
| 6 | Phosphoribosyl-aminoimidazole synthetase | 6.3.3.1 |
| 7 | Phosphoribosyl-aminoimidazole carboxylase | 4.1.1.21 |
| 8 | Phosphoribosyl-aminoimidazole-succino carboxamide synthetase | 6.3.2.6 |
| 9 | Adenylosuccinate lyase | 4.3.2.2 |
| 10 | Phosphoribosyl-aminoimidazole carboxamide formyltransferase | 6.3.2.6 |
| 11 | IMP Cyclohydrolase | 3.5.4.10 |

The formation of IMP by the pathway outlined in Figure 1.5 is regulated by the intracellular concentrations of purine nucleotides, the ultimate end products of the pathway, via feedback inhibition to enzyme two of the pathway, amidophosphoribosyltransferase (Rowe & Wyngaarden,1968; Neirlich & Magasanik,1965). The end product (IMP) of the pathway shown is further metabolised to form GTP and ATP as the ultimate end products of purine nucleotide biosynthesis. The IMP can be converted to xanthylic acid by IMP dehydrogenase (EC 1.2.1.14), which is inhibited by GMP, and finally converted to GMP by GMP synthetase (EC 6.3.4.4) in a reaction requiring ATP. The GMP so formed can be converted to GTP by the action of nucleotide kinases. The IMP produced by the de novo pathway can also be used in the synthesis of ATP, the first step of which is catalysed by




(1) AMP DEAMINASE (EC 3.5.4.6)
(2) ADENYLOSUCCINATE SYNTHETASE (6.3.4.4)
(3) ADENYLOSUCCINATE LYASE (EC 4.3.2.2)
(4) IMP DEHYDROGENASE (EC 1.2.1.14)
(5) GMP SYNTHETASE (EC 6.3.4.1)
(6) 5'-NUCLEOTIDEASE (EC 3.1.3.5)
(7) NUCLEOSIDE KINASE (EC 2.7.1.20)
(8) PURINE NUCLEOSIDASE (EC 3.2.2.1)
(9) PURINE NUCLEOSIDE PHOSPHORYLASE (EC 2.2.4.1)
(10) ADENINE PHOSPHORIBOSYLTRANSFERASE (EC 2.4.2.7)
(11) HYPOXANTHINE/GUANINE PHOSPHORIBOSYLTRANSFERASE (EC 2.4.2.8)
(12) ADENOSINE DEAMINASE (EC 3.5.4.4)
(13) ADENINE DEAMINASE (EC 3.5.4.2)
(14) GUANINE DEAMINASE (EC 3.5.4.3)
(15) XANTHINE OXIDASE (EC 1.2.3.2)

Figure 1·6 PURINE SALVAGE PATHWAYS

adenylosuccinate synthetase (EC 6.3.4.4) in a reaction which requires GTP and is inhibited by AMP. The final reaction to form AMP is catalysed by adenylosuccinate lyase which is enzyme 9 in the de novo synthetic pathway. The dependence of the adenylosuccinate synthetase on GTP and the dependence of GMP synthetase on ATP keeps the ratio of AMP to GMP and hence the ratio of ATP to GTP in balance. The enzymes of purine biosynthesis are considered in detail in Methods in Enzymology(1978).

The pathways by which purines may be salvaged and the enzymes required are shown in Figure 1.6. These pathways and their importance in relation to transport of purines are considered below.

### 1.4.1 Uptake and Metabolism of Purine Nucleotides, Nucleosides and Bases.

The uptake and metabolism of purine nucleosides and bases have been extensively investigated in various cell types. The transport and metabolism of purine nucleosides and bases have been most thoroughly studied in the mammalian erythrocyte and bacteria.

The erythrocyte nucleoside transporter shows broad specificity towards the base moiety of nucleosides but is relatively specific towards the sugar moiety (Oliver & Paterson,1971; Cass & Paterson,1973). The single carrier hypothesis is based on the fact that many purine and pyrimidine nucleosides can cause accelerated exchange diffusion of uridine and thymidine from preloaded erythrocytes. The uptake of bases by erythrocytes appears to be mediated by a different carrier since the uptake of nucleosides is strongly inhibited by p-nitrobenzylthioinosine (NBTI) (Jarvis & Young,1980) whereas the uptake of bases such as hypoxanthine are not

inhibited (Paterson & Oliver;1971). The nucleoside and base carriers of the erythrocyte mediate uptake by facilitated diffusion followed by metabolic trapping (Cabantchik & Ginsburg,1977; Cass & Paterson,1972 & 1973). Metabolic trapping of purines by erythrocytes as nucleotides is accomplished by purine salvage enzymes. Adenosine is directly converted to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase (Agarwal & Parks,1978). The inosine so formed can be converted to free base (hypoxanthine) and ribose-1-phosphate by the action of purine nucleoside phosphorylase. The hypoxanthine is then converted to IMP by hypoxanthine/guanine phosphoribosyltransferase (HxGPRT). Adenosine does not give rise to adenine and ribose-1-phosphate since the nucleoside phosphorylase of the erythrocyte is specific for inosine and guanosine (Cabrio et al.,1956). Inosine and guanosine when transported are converted to their respective free bases and ribose-1-phosphate by nucleoside phosphorylase, the free bases being converted to IMP and GMP respectively by HxGPRT. Adenine when transported is converted to AMP by a specific adenine phosphoribosyltransferase (APRT) (Grimes,1980).

The nucleoside carrier of the erythrocyte has been identified as a band 4.5 polypeptide with a molecular weight of 45-65KD as determined by SDS gel electrophoresis. The transport protein was identified by radiolabelling with $^{14}$C-NBTI and $N^6$-(-p-azidobenzyl) adenosine (Young et al.,1983; Young & Jarvis,1983). Earlier work by Jarvis & Young(1980) resulted in estimates of 120KD for the molecular weight of the nucleoside transporter in erythrocyte ghosts as determined by radiation inactivation of the ghosts' ability to bind radiolabelled NBTI. The molecular weight of 120KD estimated for the intact nucleoside transporter suggests that the transporter exists as

a dimer in the erythrocyte membrane (Young et al.,1983).

The transport and metabolism of nucleosides and bases have been studied in other mammalian cells although in less detail than for the erythrocyte. The uptake of nucleosides by mammalian cells has been reviewed by Plagemann & Wohlhueter,1980 and Young et al.,1983. The general consensus given by the literature is that uptake of nucleosides and bases by animal cells is by facilitated diffusion followed by metabolic trapping as phosphate esters. This has been shown to be the case for Novikoff rat hepatoma cells (Marz et al.,1979), mouse leukemia cells, canine thymocytes, peripheral blood leukocytes (Lum et al.,1979) and brain tissue (Shimizu et al.,1972). When considering transport as distinct from uptake full account must be taken of metabolism as discussed in Section 1.2.1. In studies of transport of nucleosides and bases where short incubation times have been used, and metabolism of the transported substrates reduced by the use of inhibitors or mutant cell lines lacking metabolising enzymes, it has been shown that nucleoside and base concentrations come to equilibrium across the cell membrane in a matter of seconds (Lum et al.,1979; Marz et al.,1979), all further uptake being due to metabolism maintaining a diffusion gradient.

In erythrocytes all nucleosides enter the cell via a single transporter (Oliver & Paterson,1971; Cass & Paterson,1973). Initial studies of uptake by cultured cells, Novikoff rat hepatoma (Plagemann & Erbe,1974) and HeLa cells (Cass & Paterson,1977) using long incubation periods demonstrated that some nucleosides showed mutual inhibition of uptake whereas others did not suggesting the existence of transport systems specific for different groups of nucleosides. It has since been shown that these specificities were in fact a

reflection of the specificities of the metabolising enzymes rather than transport, since uptake measured over long time periods measures uptake due to metabolism rather than transport (Section 1.2.1; Taub & Berlin,1972; Mulder & Harrap,1975; Heichal et al.,1979). Information on the number of transport systems for base transport is not as clear cut as that for nucleoside transport. Hypoxanthine and guanine are apparently transported by the same system in hepatoma cells since they inhibit each others transport to the same extent (Marz et al.,1979). Nucleoside transport is inhibited by hypoxanthine and hypoxanthine transport is inhibited by nucleosides (Marz et al.,1979; Plagemann et al.,1978). These results suggest that hypoxanthine and guanine enter the cells via the nucleoside transport system. However there are distinct differences between the susceptibilities of nucleoside and base transport to inhibition by nitrobenzylthionucleosides, nucleoside transport being totally inhibited at concentrations which have no effect on base transport.(Cohen et al.,1979).

Nucleotides are not transported by mammalian cells (Plagemann & Wohlhueter,1980) due to a lack of transport systems and their high negative charge decreasing their lipid partition co-efficients which reduces the possibility of uptake by diffusion through the cell membrane. Although mammalian cells do not take up nucleotides they do release 3',5'-cyclic AMP from a variety of cell types by an active transport mechanism in response to hormonal stimulation of the cell. Davoren & Sutherland(1963) were the first to observe that the 3',5'-cyclic AMP produced by pigeon erythrocytes in response to treatment with adrenalin was released from the cell and after 120 minutes the medium concentration of 3',5'-cyclic AMP exceeded the

intracellular concentration. This release of 3',5'-cyclic AMP was not inhibited by high concentrations of 3',5'-cyclic AMP in the incubation medium before the treatment with adrenalin, suggesting the existence of an active transport system for the extrusion of 3',5'-cyclic AMP from the cell. The extrusion of 3',5'-cyclic AMP has been demonstrated in many other cell types, including rat adipose tissue in response to lipolytic hormones such as adrenalin and glucagon (Zumstein et al.,1974), isoproterenol treated mouse glioma cells (Doore et al.,1975; Rindler et al.,1978), Simian virus 40 transformed human fibroblasts after exposure to prostaglandin $E_1$ (Chalpowski et al.,1978; Rindler et al.,1978) and isoproterenol-treated perfused rat heart (O'Brien & Strange 1975). The 3',5'-cyclic AMP extrusion mechanism has been shown to be unidirectional (Schroder & Plagemann,1971; Hsie et al.,1975; Granner et al.,1975) by the inability of cells to accumulate radiolabelled 3',5'-cyclic AMP although label was rapidly accumulated. The accumulation of label was found to occur after degradation of the 3',5'-cyclic AMP by phosphodiesterase and 5'-nucleotidase located on the cell membranes or in the cell culture media used.

Purine nucleoside and base uptake has also been extensively studied in bacteria. Nucleoside transport in E.coli was first studied by Petersen & Koch(1966) and Petersen et al.(1967),who investigated the kinetics of transport, metabolism of the nucleosides taken up and the energy requirements for transport. The above authors also investigated the mutual inhibitory effects of nucleosides on transport and concluded that in E.coli there was more than one nucleoside transport system with specificities distinguishable from those of the metabolising enzymes. It has been suggested that

transport of nucleosides in E.coli and S.typhimurium occurs by a group translocation mechanism mediated by nucleoside phosphorylases (Hochstadt & Stadman,1971; Rader & Hochstadt,1976). These authors proposed that nucleosides bound to the plasma membrane of the bacteria and the ribose moiety of the nucleoside was taken up as ribose-1-phosphate during phosphorolysis of the nucleoside by nucleoside phosphorylase. They also proposed that the base moiety released by this reaction would be taken up by a second group translocation mechanism mediated by phosphoribosyltransferases (Hochstadt & Stadman,1971; Hochstadt,1974). These hypotheses were based on the observations that membrane vesicles prepared from E.coli and S.typhimurium accumulated ribose-1-phosphate and released free base into the medium when incubated with nucleosides.

Doubt has been cast on the hypothesis that nucleoside uptake in bacteria is mediated solely by group translocation by the following observations; that mutant strains of E.coli which lack purine nucleoside phosphorylase are still able to utilise inosine as a purine source (Hoffmeyer & Neuhard,1971); that mutants lacking nucleoside phosphorylase transport and concentrate nucleosides (Munch-Petersen & Mygind,1976; Mygind & Munch-Petersen,1975); and that mutants have been isolated which are incapable of nucleoside transport but have a full complement of nucleoside phosphorylases and phosphoribosyltransferases (Munch-Petersen & Mygind,1976). Two transport systems for nucleosides have been identified in E.coli designated the C and the G systems (Munch-Petersen et al.,1979). The C system is specific for cytosine and the G system specific for guanosine. The specificities of the two transport systems are indicated by the mutual inhibitory effects of various nucleosides on

each others transport. The purine specificities of the two systems are as follows. The C system transports adenosine and deoxyadenosine but will not transport guanosine, deoxyguanosine or inosine. The G system on the other hand will transport all nucleosides.

In E.coli K12, E.coli B and S.typhimurium nucleoside uptake is mediated by an active transport process as demonstrated by the ability of mutant cells, which lack nucleoside metabolising enzymes, to accumulate nucleosides to a concentration several hundred times that of the incubation medium (Munch-Petersen & Mygind,1976; Munch-Petersen & Pihl,1980). Active transport has been shown not to require ATP directly in cells containing only the G system (Munch-Petersen & Pihl,1980); however, in membrane vesicles active transport was supported by the addition of electron donors and was shown to be sensitive to inhibition by 2,4-dinitrophenol (Munch-Petersen et al.,1979) which suggests that transport is dependent on a proton gradient generated by an electron transport chain in the bacterial membrane. Similar results have been obtained for S.typhimurium (Rader & Hochstadt,1976) and E.coli B (Roy-Burman et al.,1978).

The transport systems for nucleobases in bacteria are less well defined than those for nucleoside transport. There is some dispute as to whether or not uptake of nucleobases is mediated by group translocation. The same arguments as have been presented for and against uptake of nucleosides by group translocation, as regards the use of mutants and membrane vesicles, can be applied to the problem of nucleobase transport (See Hays,1978 for a review). Investigations into the cellular localisation of purine phosphoribosyltransferases (Page & Burton,1978) indicate that these enzymes are cytoplasmic as

shown by subcellular fractionation and the observation that they are not inactivated by impermeant enzyme inhibitors in whole cells but are inactivated completely by the same reagents in a cell lysate (Kabach,1971;Futai,1974). This distribution of the phosphoribosyltransferases is consistent with a facilitated diffusion mechanism for transport followed by metabolic trapping rather than group translocation. That transport may be by facilitated diffusion is supported by the observation that experiments designed to show active transport of purine bases have given inconclusive results (Burton,1977).

In summary mammalian cells transport purine nucleosides and pyrimidine nucleosides by a facilitated diffusion mechanism followed by metabolic trapping by the purine and pyrimidine salvage enzymes. Purine bases are transported in mammalian cells by a separate carrier but by the same mechanism. In bacteria nucleosides are transported by active transport systems which appear to be dependent on a proton gradient generated by proton translocating electron transport chains in the bacterial plasma membrane. Bacteria contain at least two different transport systems which differ in their specificities for nucleosides transported. Bacteria also take up purine bases, mediated by facilitated diffusion followed by metabolic trapping. With this background in mind the transport, uptake and metabolism of purine nucleotides, nucleosides and bases by trypanosomes will be examined.

### 1.4.2 Uptake and Metabolism of Purine Nucleotides, Nucleosides and Bases by Trypanosomes.

In comparison to mammalian cells and bacteria very little is known about the uptake, transport and metabolism of purine nucleotides, nucleosides and bases by the Trypanosomatidae. The Trypanosomatidae are incapable of de novo synthesis of the purine ring as indicated by their inability to incorporate radiolabelled precursors into their nucleotides (Marr et al.,1978a; Gutteridge & Gaborak,1979, Ceron et al.,1979; Fish et al.,1982a). The lack of de novo synthesis of the purine ring is also indicated by the requirement for preformed purines for growth of trypanosomes in culture (Berens et al.,1981; Fish et al.,1982b). They therefore must obtain their purines preformed from the host organism in vivo, which requires that they have mechanisms for the uptake of purine nucleosides and bases and also purine salvage enzymes capable of forming nucleotides.

The existence of salvage pathways in trypanosomes is substantiated by the ability of single purine compounds to support the growth of various trypanosomes in culture, although not all purines are equally effective. Hypoxanthine and adenosine are equally effective in promoting the growth of Leishmania donovani with inosine only supporting growth after a lag period of two days and guanosine stimulating growth in a manner intermediate between adenosine and inosine (Marr et al.,1978a). The growth of T.gambiense is supported by adenine, hypoxanthine,guanine and xanthine in order of decreasing efficiency whereas the order for T.rhodesiense is hypoxanthine, adenine, guanine and xanthine (Schmidt et al.,1975; Fish

et al.,1982a; Fish et al.,1982b). Other orders of purine preference are possible in other species such as T.cruzi (Gutteridge & Gaborak,1979).

The individual enzymes of purine salvage have been studied in cell lysates of various trypanosomes. Hypoxanthine, guanine and adenosine phosphoribosyltransferase activities have been detected in Crithidia fasciculata (Kidder et al.,1979), L.donovani (Tuttle & Krenitsky,1980), T.cruzi (Gutteridge & Davies,1981 & 1982) and T.brucei and L.mexicana (Davies et al.,1983). In most cases the hypoxanthine and guanine phosphoribosyltransferase activities have been found to be associated with a single enzyme as is the case in mammalian tissues. However these activities have been shown to be associated with separate enzymes in C.fasciculata (Kidder et al.,1979). It has been proposed that the HxGPRT of T.cruzi and other trypanosomes is associated with the glycosome as shown by subcellular fractionation experiments (Gutteridge & Davies,1982). The enzyme APRT has been detected in the same organisms as HxGPRT activities by the same authors as given above, and in L.donovani by Looker et al.(1983). In contrast to HxGPRT the enzyme APRT appears to be a soluble cytoplasmic protein (Hammond & Gutteridge,1984). These phosphoribosyltransferase enzymes are capable of converting bases to nucleotides but trypanosomes can also use nucleosides as purine sources. Therefore, either nucleoside kinases or enzymes capable of generating free bases from nucleosides must be present. Many animal cells and bacteria use a nucleoside phosphorylase enzyme to generate free bases from nucleosides (See Section 1.4.1). Nucleoside phosphorylase activities have been detected in L.mexicana amazonensis promastigotes, T.brucei trypomastigotes and

T.cruzi amastigotes, epimastigotes and trypomastigotes (Gutteridge & Davies,1982; Davies et al.,1983) all of which contain adenosine and guanosine phosphorylase activities. These findings are in conflict with the findings of Ogbunude & Ikediobi(1983); Ogbunude et al.(1985) and the work in this thesis (Section 3.2.5). Ogbunude & Ikediobi(1983); and Ogbunude et al.(1985) were unable to detect any nucleoside phosphorylase activity in T.brucei or T.vivax but did find phosphorylase activity in T.congolense. These conflicting results may be due to differences in the strain of organism investigated. In the organisms, T.brucei and T.vivax, in which no nucleoside phosphorylase activity was detected, nucleoside hydrolases (nucleosidases) were found to be present allowing the production of free base from nucleoside. Nucleosidases have been detected in other trypanosomes (Davies et al.,1983; Looker et al.,1983) and purified from T.gambiense (Schmidt et al.,1975). Nucleosides may also be converted to nucleotides by the action of nucleoside kinases which have been detected in a variety of trypanosomes. However there is disagreement between authors as to whether or not kinase activity is present in certain species. Adenosine kinase activity has been reported to be present in extracts of L.donovani (Tuttle & Krenitsky,1980) and T.cruzi (Gutteridge & Davies,1982). Adenosine, inosine and guanosine kinases have also been reported to be present in L.m.amazonensis, T.brucei and all morphological forms of T.cruzi (Davies et al.,1983). However, adenosine kinase could not be detected in T.brucei by Ogbunude & Ikediobi(1983), Ogbunude et al.(1985) or in this thesis (Section 3.2.5). Ogbunude et al.(1985) did however detect adenosine kinase in T.vivax and T.congolense.

The ability of some trypanosomes to grow normally in culture

when supplied with only one purine base suggests that they are capable of the interconversion of purines. Aminohydrolases (deaminases) are responsible for the conversion of the adenine ring to the hypoxanthine ring. This conversion may take place as a conversion of adenine to hypoxanthine (adenine deaminase), adenosine to inosine (adenosine deaminase) or AMP to IMP (AMP deaminase). Adenine deaminase has been detected in L.donovani, L.mexicana and T.vivax (Kidder & Nolan,1979). Adenosine deaminase has been shown to be present in lysates of Leishmania spp. (Looker et al.,1983), T.cruzi (Gutteridge & Davies,1981) and T.brucei (Davies et al.,1983). However, the existence of adenosine deaminase in T.brucei has failed to be confirmed by other workers (Berens et al.,1981; Ogbunude & Ikediobi,1983; Ogbunude et al.,1985). The interconversion of purine nucleotides may be possible via the common intermediate, IMP. The enzyme AMP deaminase can form IMP from AMP and IMP can be further converted to GMP by the action of IMP dehydrogenase and GMP synthetase. This sequence of reactions can be reversed by the conversion of GMP to IMP by the enzyme GMP reductase, the IMP so formed can then be converted to AMP by adenylosuccinate synthetase and adenylosuccinate lyase. The existence of these salvage enzymes in T.cruzi and C.deani(aposymbiotic) has been shown by their ability to radiolabel guanine nucleotides when grown in medium containing labelled adenine and also their ability to label adenine nucleotides when grown in the presence of labelled guanine or hypoxanthine. However, C.deani can not incorporate label into adenine nucleotides when provided with labelled guanine but can label adenine nucleotides when provided with labelled hypoxanthine which suggests that C.deani lacks GMP reductase (Ceron et al.,1979). Similar findings have been

reported for T.gambiense (Fish et al.,1982a) and L.donovani (Spector & Jones,1982).

The inability of trypanosomes to synthesise the purine ring de novo and their reliance on the salvage pathways provides a potential target for chemotherapeutic treatment of trypanosome infections with nucleoside analogues which can inhibit enzymes of the salvage pathways. The effects of some nucleoside analogues such as allopurinol, tubericidin, coformycin, deoxycoformycin and dipyridamole have been tested for trypanocidal activity against T.cruzi (Marr et al.,1978b), C.fasciculata and L.tropica (Kidder & Nolan,1979), T.gambiense(Ogbunude & Ikediobi,1982) and T.brucei and T.congolense (James & Born,1980). Most of the above work was concerned with the effects of these analogues on the salvage enzymes; however, their effects on the uptake of nucleosides may also be of importance as regards their effectiveness as drugs.

The uptake of purines by trypanosomes has not been extensively studied and in the few cases where investigations have been carried out little distinction has been made between transport and overall uptake and metabolism (James & Born,1980). Transport of pyrimidines has also been investigated by Manjra & Dusanic(1973), who reported that T.lewisi actively transports pyrimidines. This conclusion is untenable since these authors made no distinction between uptake and transport (See Section 1.2). In other trypanosomes uptake of purines is carrier-mediated although no information is available concerning whether or not uptake is by facilitated diffusion or by active transport. That uptake is carrier-mediated has been shown by mutual inhibition of uptake amongst various purine nucleosides and bases. The existence of multiple carriers with differing specificities for

purines has been suggested by Hansen et al.(1982) in L.panamensis which may have three separate purine carriers. Multiple carriers have also been suggested for purines in T.brucei and T.congolense (James & Born,1980) and C.fasciculata (Kidder et al.,1978). Purine transport in T.vivax has been investigated by Okochi et al.(1983); however, the results presented by these authors must be regarded with some caution since they report using hypoxanthine and guanine as uptake substrates at concentrations in excess of 100mM which is not only unphysiological but almost thirty times more concentrated than a saturated aqueous solution. Sanchez et al.(1976) reported uptake of nucleotides by T.brucei and T.gambiense; however, they did not prove that the purine nucleotide was taken up intact.

In summary purine nucleosides and bases are taken up by a carrier-mediated process in those trypanosomes so far studied but no information is available as to whether or not the carriers are involved in active transport.

It appears that all trypanosomes are incapable of de novo synthesis of the purine ring and obtain all their purines preformed from their hosts. Since the trypanosomes obtain their purines from their hosts they all appear to be capable of interconversion of purines although the salvage enzymes used appear to vary from species to species and perhaps from strain to strain.

## 1.5 OBJECTIVES OF THE INVESTIGATION.

The two main objectives of this investigation were;

1) to investigate the basic biochemistry of uptake and metabolism of the enzyme co-factor, vitamin $B_6$, by T.brucei.

2) to investigate the basic biochemistry of the uptake and

metabolism of purine nucleosides and bases by T.brucei.

Both of the above investigations were carried out with a view to characterising the transport and metabolising systems in the hope of detecting differences from the host organisms which may be useful chemotherapeutic targets. An example of how basic understanding of transport systems can help in chemotherapy is given by a recent paper by Ogbunude & Ikediobi(1982) in which they demonstrate the ability of the mammalian nucleoside transport inhibitor NBTI to protect the host organism from toxic nucleoside analogues which are lethal to both host and trypanosomes in the absence of NBTI. The basis for this chemotherapeutic strategy is that NBTI is a potent inhibitor of the mammalian nucleoside transport systems but is not an effective inhibitor of the trypanosomal nucleoside transport systems. This exemplifies how a knowledge of an organism's basic transport biochemistry can lead to new strategies for the use of existing drugs, if not to new drugs.

# 2.MATERIALS AND METHODS

## 2. MATERIALS AND METHODS.

### 2.1 STRAIN OF T.BRUCEI STUDIED.

The trypanosome strain used throughout this work was T.brucei TREU 55 (Trypanosomiasis Research Edinburgh University) which is a monomorphic population of trypanosomes derived from a pleomorphic wild population. The original wild type trypanosomes were maintained by serial passage (an unknown number of times) to produce TREU 1. This line was subsequently passaged (uncloned) an unknown number of times in rats to give TREU 55. As stated above TREU 55 is an uncloned monomorphic strain consisting of long slender bloodstream forms, which cause a fulminating parasitaemia and death of the host rats within three days of inoculation with the parasites.

### 2.2 HOST AND MAINTENANCE.

Male albino Wistar rats (350g-750g) were used as host animals for the T.brucei infection throughout this investigation. Infection was established by intraperitoneal injection of $8 \times 10^7$ trypanosomes in a volume of 0.5 ml phosphate buffered saline pH 8.0. The injection was prepared by thawing a 0.5 ml stabilate of infected rat blood and diluting to a final volume of 3 ml with phosphate buffered saline. This procedure resulted in peak parasitaemia within 72 hours.

### 2.3 PREPARATION OF STABILATES.

Rats infected as described above were exsanguinated by direct cardiac puncture under light ether anaesthesia. The blood collected was immediately diluted with phosphate buffered saline pH 8.0,

containing 5 IU heparin/ml, to give 4-5 x $10^8$ trypanosomes /ml and glycerol added to a final concentration of 10% (V/V). The number of parasites was determined by haemocytometry. The diluted blood was then dispensed in 0.5ml aliquots into Eppendorf pots and frozen at -70°C. Stabilates prepared in this fashion remained infective for over one year but after six months storage a slight decrease in the yield of trypanosomes obtained was noted. Therefore, fresh stabilates were prepared as described above every six months to obtain maximum yields of trypanosomes.

**2.4 BUFFER SOLUTIONS.**

Phosphate buffered saline (PS buffer) 53mM disodium hydrogen orthophosphate, 3mM sodium dihydrogen orthophosphate, 45mM sodium chloride, pH 8.0.

Phosphate saline glucose (PSG buffer) as PS buffer plus 1% (w/v) glucose, pH 8.0 unless otherwise stated.

Tris saline glucose (TSG buffer) 49.5mM Tris, 42.3mM HCl, 85.5mM sodium chloride, glucose 1% (w/v), pH 7.4 unless otherwise stated.

Tris choline glucose (TCG buffer) as for TSG but with the 85.5mM sodium chloride replaced with 85.5mM choline chloride, pH 7.4.

Potassium phosphate buffered glucose (PSGK buffer) 53mM dipotassium hydrogen orthophosphate, 3mM potassium dihydrogen orthophosphate, 45mM potassium chloride, glucose 1% (w/v), pH 7.4 unless otherwise stated.

**2.5 ISOLATION OF TRYPANOSOMES FROM RAT BLOOD.**

Trypanosomes were isolated from infected rat blood by the method of Lanham(1968) as detailed below. Rats were exsanguinated, 72 hours

post-infection, by direct cardiac puncture under light ether anaesthesia and the blood collected into 2ml of PSG containing 5 IU heparin/ml. After centrifugation of the blood at 1000xg av. for 10 minutes at 4°C the supernatant plasma fraction was removed and the white trypanosome layer collected from the top of the erythrocyte pellet. The trypanosome layer was then resuspended in PSG and the centrifugation step repeated. The white trypanosome layer was then collected and suspended in fresh PSG before being applied to the top of a DEAE 52 cellulose column (bed volume 18-20ml) equilibrated with PSG. Due to the charge difference between the trypanosomes and the erythrocytes and platelets at pH 8.0 the latter stick to the column and allow the trypanosomes to be eluted from the column with PSG. The trypanosomes eluted from the column were stored as a dilute suspension in PSG buffer at 0°C and used within thirty minutes. The above purification method produced, in terms of packed cell volume, 2-3ml of cells per rat. The trypanosome suspension produced by this method was routinely inspected by phase contrast microscopy and only cell suspensions with >95% motile cells were used in experiments.

## 2.6 TRYPANOSOME VIABILITY.

Trypanosome viability was estimated by inspection of cell motility using phase contrast microscopy, a purely subjective parameter, and by measurement of their rate of oxygen utilisation which is more objective. The method used for the measurement of oxygen uptake is described below.

Trypanosomes (1ml at 1.5mg protein/ml) were added to a thermostatted chamber with a magnetic stirring bar containing 2ml of

PSG, pH 7.4 at 37°C. The chamber was sealed and the rate of oxygen utilisation measured with a Clark oxygen electrode which formed part of the chamber seal. The oxygen electrode was connected to a slave recorder calibrated to 100% deflection with air saturated buffer and calibrated to zero oxygen tension by the addition of sodium dithionite to the sealed chamber. The above system for measuring oxygen uptake was used to indicate any deleterious effects of compounds utilised in experiments on trypanosome transport in conjunction with inspection by phase contrast microscopy.

## 2.7 PREPARATION OF TRYPANOSOME LYSATE.

Trypanosomes were lysed by hypotonic shock as follows: the required number of trypanosomes were pelleted by centrifugation at 1000x*g* av. for 5 minutes and the cell pellet obtained lysed by the addition of 5 volumes of double distilled water. This lysate was then homogenised (five strokes) in a tight fitting Dounce homogeniser and inspected for whole cells by phase contrast microscopy. Concentrated buffer was added to this homogenate to give a final concentration of 100mM TrisHCl, 1mM DTT, 1mM benzamidine, 1mM PMSF, pH 7.4. The lysate was then dialysed against 100 volumes of this buffer (typically 10ml lysate:1 litre buffer) for 10 hours with one change of dialysis buffer. The addition of the protease inhibitors PMSF and benzamidine had no effect on any of the enzyme activities assayed in the lysate. This was determined by assaying all enzymes in an undialysed lysate in the presence and absence of the protease inhibitors. Throughout this thesis trypanosome lysate refers to a cell free homogenate prepared as described above unless otherwise stated.

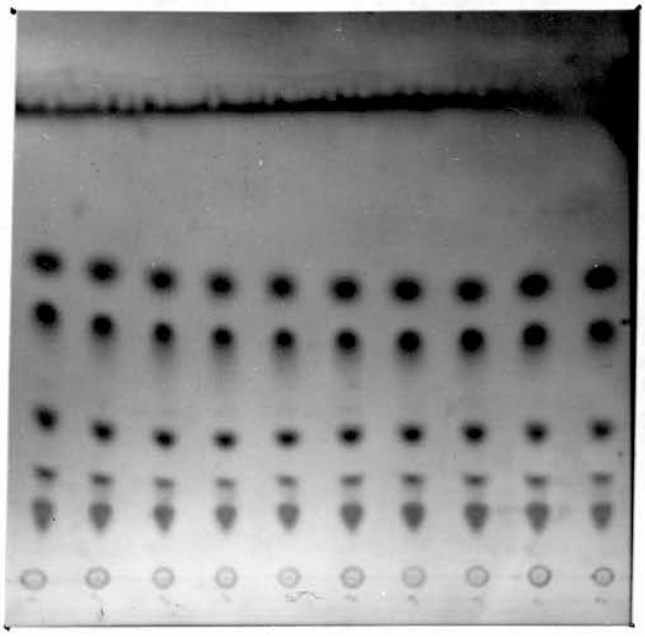
### 2.7.1 Cell Breakage Methods.

Apart from water lysis three other methods were used to disrupt the trypanosomes. These were grinding the cells with silicon carbide (Crystalon: Norton Company. Grit No. 37C 400), grinding the cells with glass beads (75-150μm diameter, Sigma Chemical Co.Ltd,) and sonication for 30 seconds by a Rapidis 150 sonicator (Ultrasonics Ltd.) in six five second bursts. The three procedures described above were carried out as follows. The disruption of the cells by grinding with silicon carbide or glass beads was achieved by grinding a paste of cells and abrasive in 0.25M sucrose 10mM HEPES, 1mM DTT, 1mM benzamidine, 1mM PMSF, pH 7.4 in a mortar and pestle at $4^{o}C$. Cells were sonicated as described above after being pelleted by centrifugation and made into a paste by the addition of a small volume of 0.25M sucrose 10mM HEPES, 1mM DTT, 1mM benzamidine, 1mM PMSF, pH 7.4.

## 2.8 THIN LAYER CHROMATOGRAPHY (TLC).

Thin layer chromatography of purine nucleosides and bases was carried out on silica coated aluminium TLC plates (Kieselgel 60 $F_{254}$, Merck Co.) impregnated with a fluorescent dye ($F_{254}$). Radioactive samples to be chromatographed were applied to the plates by calibrated capillaries (Camlab Ltd.) in neutral or slightly acid solution with 20nmoles of each of the carrier compounds; adenosine, adenine, inosine, hypoxanthine and xanthine. After air drying the applied samples and carriers the chromatograms were developed, by ascending chromatography, in the solvent system of Arch & Newsholme(1976) which consists of propan-2-ol: ethylacetate: 0.88 $NH_3$

**Figure 2.1 TLC OF PURINE NUCLEOSIDES AND BASES.**



| COMPOUND | Rf. |
|---|---|
| Inosine | 0.13 |
| Xanthine | 0.19 |
| Hypoxanthine | 0.28 |
| Adenosine | 0.49 |
| Adenine | 0.57 |

in the ratio 9:4:3 (v/v/v). Chromatography was carried out in a sealed tank in a solvent saturated atmosphere. The solvent was allowed to migrate up the plates to within 1-2cm of the top of the plates. The plates were then removed, air dried and the resolved components visualised by their quenching of the plates fluorescence under UV light. The Rf. values for the compounds listed are given in the legend to Figure 2.1 which shows a typical chromatogram photographed in UV light.

### 2.8.1 TLC of Vitamin $B_6$ and Metabolites.

Chromatography of Vitamin $B_6$ and its metabolites was carried out as described for chromatography of purine nucleosides and bases with the following exceptions. The solvent system used was chloroform:methanol in the ratio 75:25 (v/v) (Ahrens & Korytnyk,1970) and 60nmoles of each of the carriers, pyridoxine(PN), pyridoxal(PA), pyridoxic acid(PAC), pyridoxamine(PAM), pyridoxal-5-phosphate(PAP) and pyridoxamine-5-phosphate(PAMP) were chromatographed with radioactive samples to be analysed. The Rf. values for the compounds listed above are given below.

| $B_6$ Metabolite | Rf. |
|---|---|
| PN | 0.67 |
| PA | 0.55 |
| PAC | 0.20 |
| PAM | 0.05 |
| PAP | 0.00 |
| PAMP | 0.00 |

As for TLC of purine nucleosides and bases the resolved compounds were visualised by quenching of the TLC plates fluorescence under UV

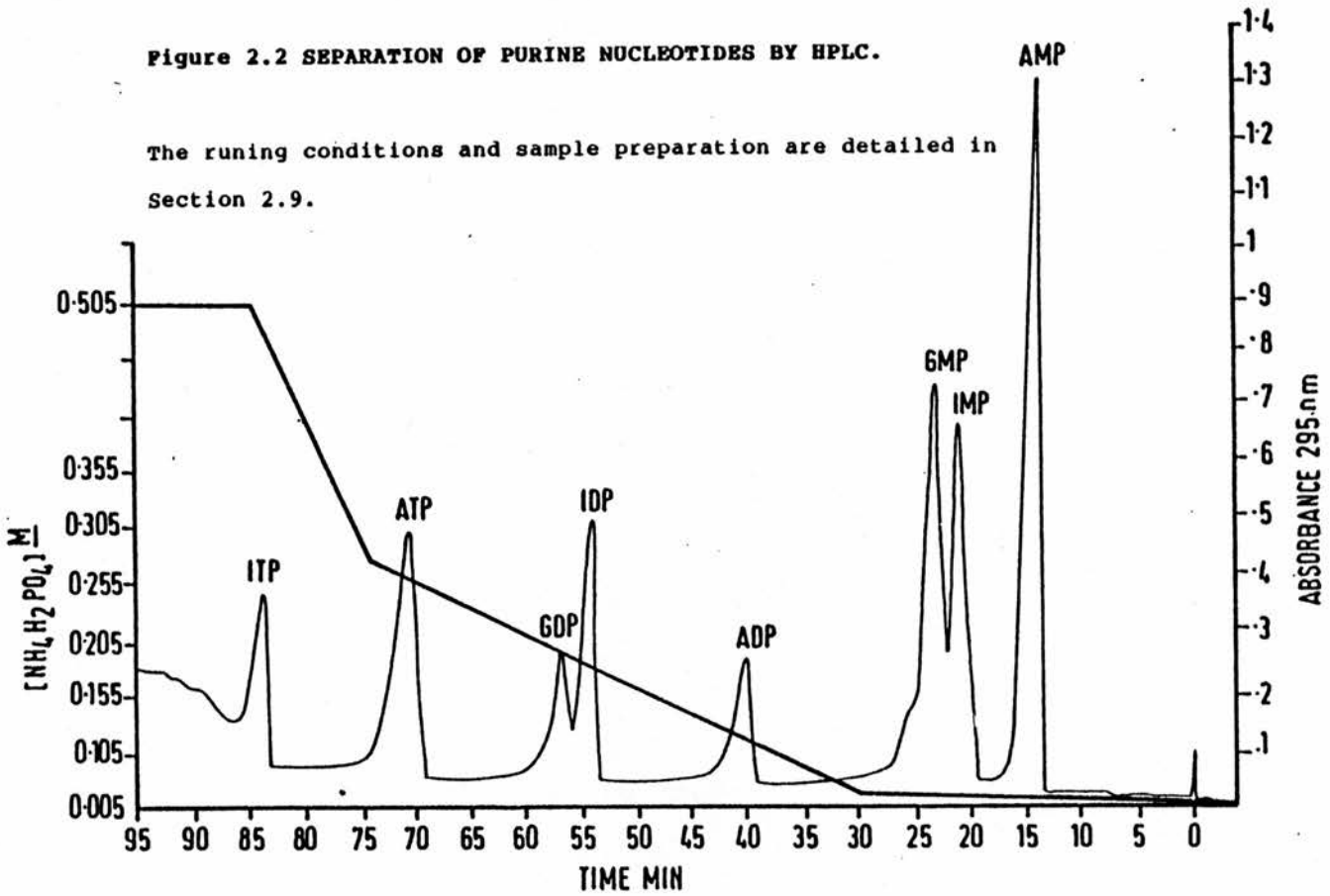
light.

## 2.9 HPLC OF PURINE NUCLEOTIDES.

The purine nucleotides AMP, ADP, ATP, IMP, IDP, ITP, GMP and GDP were separated as described by Marr et al.(1978a) on a silica based anion exchange column containing 5um spherical silica beads coated with anion exchange polymer. The "Spherisorb" 5 SAX column (length 25cm internal diameter 4.6mm) was obtained from Anachem Ltd. and used in conjunction with a guard column packed with the same material (2.5cm long with internal diameter 4.6mm). The column was connected to an Altex dual pump HPLC system with a 412 programmable controller. Column eluent was continuously monitored at 260nm by a Hitachi variable wave length spectrophotometer equipped with a flow cell (volume 0.1ml) and 0.5ml fractions collected. Samples for HPLC analysis were prepared as follows. Cells incubated in radiolabelled nucleotide precursors were separated from the incubation medium by silicon sandwich centrifugation (Section 2.11.2) with 40% formic acid as the lower layer. Samples of the formic acid layer (100µl) were removed, carrier nucleotides added (20nmoles of each) and lyophilised for 36 hours to remove the formic acid. The dried material was then redissolved in 50µl 0.005M $NH_4H_2PO_4$ pH 3.5 and passed through a 0.25µm "Millipore" filter before being applied to the column. The sample (20µl) was applied to the column and eluted with a gradient of 0.005M to 0.505M $NH_4H_2PO_4$ pH 3.5. The formation of the gradient was under the control of the 412 HPLC controller which had been programmed as follows. The sample was run into the column at a flow rate of 0.5ml/min for 1 minute at which time a gradient from 0.005M to 0.045M $NH_4H_2PO_4$ was started, reaching the higher concentration of

**Figure 2.2 SEPARATION OF PURINE NUCLEOTIDES BY HPLC.**

The runing conditions and sample preparation are detailed in Section 2.9.



buffer 30 minutes into the run. At 30.5 minutes into the run the flow rate was increased to 1ml/min and a second gradient from 0.045M to 0.280M $NH_4H_2PO_4$ was started, the higher concentration being reached 75 minutes into the run. At this point the gradient was steepened to give 0.505M $NH_4H_2PO_4$ 85 minutes into the run. After elution at this concentration for a further 10 minutes, giving a total run time of 95 minutes, the run was terminated and the column re-equilibrated with the starting buffer. The elution profile obtained with this system and the gradient used are shown in Figure 2.2. The label incorporated in each nucleotide was estimated by liquid scintillation counting of the fractions collected.

**2.10 ENZYME ASSAYS**

Adenosine hydrolase, APRT and HxPRT were assayed by measuring the production of radiolabelled adenine, AMP and IMP respectively from [2,8-$^3$H] adenosine, [8-$^3$H] adenine and [G-$^3$H] hypoxanthine, their respective substrates. The assays were carried out in 10mM TrisHCl buffer, 1mM DTT at pH 7.4 supplemented with 0.5mM PRPP and 1mM $MgCl_2$ for the assay of APRT and HxPRT. The assay mixes also contained the labelled substrates at the required concentration (specific activity 100$\mu$Ci/$\mu$mole). The assays were started by addition of trypanosome lysate and incubated at 22$^{\circ}$C for 3 minutes. The assays were terminated by pipetting samples of the assay mix into glass tubes heated to 120$^{\circ}$C which were maintained at this temperature for a further 1 minute. Precipitated protein was then removed by centrifugation and samples of the supernatants mixed with the appropriate carriers were analysed by TLC as described in Section

2.8. The production of adenine (adenosine hydrolase assay), AMP (APRT assay) and IMP (HxPRT assay) was estimated by liquid scintillation counting of the appropriate spots cut from the TLC plates. The radiolabelled purines used in these assays were obtained from Amersham International plc.

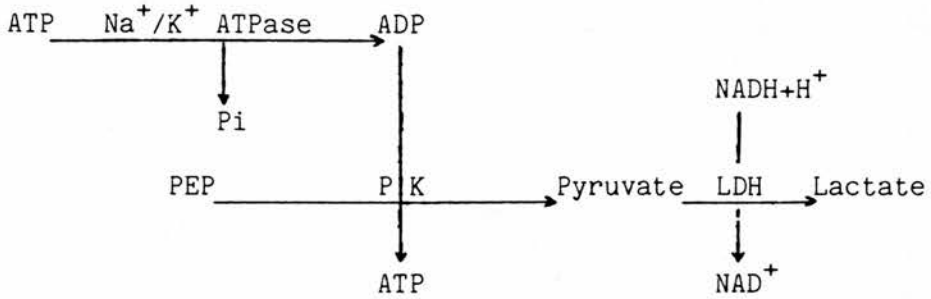
**2.10.1 ∝-Glucosidase Assay.**

The enzyme ∝-glucosidase (EC 3.2.1.20) was assayed by the fluorimetric method of Steiger et al.(1980). In this assay system the enzyme catalyses the hydrolysis of the synthetic substrate 4-methylumbelliferyl-∝-D-glucoside (Sigma Chemical Co.Ltd.) causing the release of 4-methylumbelliferone which is measured fluorimetrically. The assays were carried out in 0.1M sodium phosphate buffer (2.5ml) containing 1mM DTT, 0.1% Triton X100,pH 7.4 and 2mM substrate. The incubations were started by the addition of the enzyme source to the above incubation mix in a thermostated fluorimeter cell at 35°C. The production of 4-methylumbelliferone was measured in a Perkin Elmer 3000 fluorimeter at an excitation wave-length of 366nm and an emission wave-length of 460nm. The fluorescence was continuously monitored by a chart recorder attached to the fluorimeter. The amount of 4-methylumbelliferone produced was estimated from a standard curve.

**2.10.2 $Na^+/K^+$ Adenosine Triphosphatase Assay.**

The activity of ouabain inhibitable $Na^+/K^+$ ATPase was determined by a coupled enzyme assay which measured the production of ADP from ATP in the presence and absence of ouabain which inhibits the $Na^+/K^+$

ATPase. The use of ouabain allowed discrimination between $Na^+/K^+$ ATPase activity and general phosphatase and ATPase activity. The assay mix contained in a volume of 3ml; 6mM sodium phosphate buffer pH 7.0, 10mM KCl, 2mM $MgCl_2$, 0.03mM ATP, 0.03mM phosphoenolpyruvate(PEP), NADH 0.16mM, lactate dehydrogenase(LDH) (EC 1.1.1.27) 0.2mg (100 units) and pyruvate kinase(PK) (EC 2.7.1.40) 0.2mg (100 units) either with or without 2mM ouabain. The principle of the assay is outlined below;(assay temperature 30°C)




The disappearance of NADH was monitored at 340nm by a Pye Unicam SP8000 spectrophotometer linked to a chart recorder.

**2.10.3 Adenosine Deaminase Assay.**

The activity of adenosine deaminase (EC 3.5.4.4) was estimated using the spectrophotometric assay described by Agarwal & Parks(1978) in which the decrease in absorbance at 265nm is measured as adenosine is converted to inosine by the deaminase. The assays were carried out in thermostatted 1ml quartz cuvettes containing the protein being tested for activity, 50mM potassium phosphate buffer pH 7.4 (0.7-0.9ml), 10µl of adenosine (10mM) to give a final concentration of 0.1mM in a final volume of 1ml at 30°C. The assays were started by the addition of adenosine and the change in absorbance recorded on a chart recorder linked to an SP8000 spectrophotometer.

## 2.11 TRYPANOSOMAL UPTAKE ASSAYS.

Three methods were evaluated for the estimation of trypanosomal uptake as detailed in the following sections. The uptake of radio-labelled substrates by trypanosomes was assayed by incubating trypanosomes in PSG pH 7.4 with the required concentrations of labelled substrate, the incubations being terminated by dilution of the incubation mixture with 10 volumes of ice cold PSG followed by centrifugation at 3000xg av. to pellet the cells. The cell pellet was then resuspended in ice cold PSG and centrifuged a further three times followed by water lysis of the cells. Protein was then removed from the lysate by centrifugation after precipitation of the protein with PCA. This procedure resulted in the production of a supernatant in which the label taken up was estimated by liquid scintillation counting.

### 2.11.1 Rapid Filtration.

The uptake of labelled substrates by trypanosomes was also assayed by rapid filtration. Cells were removed from the incubating medium (PSG pH 7.4 and labelled substrate) by rapid filtration through glass fibre filters (Whatman GF-C). Samples of the incubation mixture were injected into 4 volumes of ice cold PSG pH 7.4 contained in the sample well of a "Millipore" filter tower and a vacuum immediately applied to draw the incubation mix through the filter which was followed by a further 20ml of PSG. The filters used were prewashed in high concentrations of the substrate being used for uptake to minimise background labelling of the filter. Uptake of label was then estimated by liquid scintillation counting of the filters after drying for 1 hour at 70$^{o}$C.

### 2.11.2 Silicon Sandwich Centrifugation.

Uptake of labelled substrates by trypanosomes was also estimated by the silicon sandwich centrifugation technique (Damper & Patton, 1976a & 1976b). In this method the incubation medium containing the trypanosomes was centrifuged (10,000x*g* av. for 1.5 minutes) after being layered onto a sandwich consisting of silicon oil (200µl) and 6M PCA (150µl). The oil used had a density of 1.04854 $g/cm^3$ which was obtained by mixing the silicon oils MS 550 density 1.07 $g/cm^3$ and MS 556 density 0.98 $g/cm^3$ (BDH Ltd.). The oils were weighed out on a five place balance in the ratio 3.468:1 (w/w) MS 550:MS 556. One of the drawbacks of this assay method is that some of the incubation medium is carried through the oil layer on the surface of the cells. However this can be estimated and corrections made to the estimated uptake. The carry through was estimated by measuring the amount of an impermeant radiolabel ([carboxy-$^{14}$C] inulin) in the PCA layer after centrifugation. The average carry-through was found to be 1.2±0.2µl/mg trypanosomal protein. It should be noted that throughout this thesis all results obtained using this method are shown corrected for carry-through where appropriate. Uptake of radiolabelled substrate was estimated by liquid scintillation counting of samples of the PCA layer.

### 2.12 ESTIMATION OF PROTEIN.

Throughout this work protein was estimated by the dye binding method of Bradford (1976). The dye reagent used consisted of 0.01% Serva blue dye (w/v), 4.7% ethanol (w/v) and 8.5% phosphoric acid (w/v). Samples of protein (100µl final volume) were mixed with 3ml of the reagent described above and the absorbance at 595nm of the

resultant solution measured after fifteen minutes. The amount of protein present was estimated from the absorbance measured using a standard curve with BSA fraction IV as the standard protein. The absorbance produced was found to be proportional to the amount of protein present in the range 1μg to 100μg.

## 2.13 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS.

The discontinuous SDS PAGE method used was as described by Laemmli & Favre (1973). The solutions required are listed below.

GEL BUFFER. The composition of the gel buffer was as follows: 1.5M TrisHCl, 8mM EDTA, 0.4% SDS, pH8.8. The buffer was made up as follows; 181.5g Trizma base (Sigma Chemical Co. Ltd.), 2.68g Disodium EDTA (BDH Ltd.) dissolved in distilled water and titrated to pH 8.8 with HCl (11M) before making the volume up to 1000ml. The SDS (4.0g) (BDH Ltd. Biochemical grade) was then dissolved in the buffer to prevent damage to the pH electrode.

ACRYLAMIDE/BIS-ACRYLAMIDE. The acrylamide solution used had the following composition: 30%(w/v) acrylamide and 0.9% (w/v) methylene bis-acrylamide. 60g of acrylamide and 1.6g N,N', bis-methylene acrylamide (BDH Ltd.) were dissolved in 200 ml of water and filtered through glass wool before storage in the dark at $4^{o}C$.

STACKING GEL BUFFER. The stacking gel buffer contained 0.5M Tris HCl, 8mM EDTA, 0.4% SDS, pH 6.8. The buffer was made up as follows: 12.0g Trizma base. (Sigma Chemical Co. Ltd.), 0.523g disodium EDTA were dissolved in water and titrated to pH 6.8 with HCl (11M) before the

addition of 0.8g SDS and water to a final volume of 200ml.

POLYACRYLAMIDE. The solution of polyacrylamide used consisted of polyacrylamide 1%, 1mM sodium azide and 1mM sodium fluoride. This solution was made by adding Polyacrylamide (2g) (Aldrich Chemical Co.), very slowly with vigorous stirring, to 200ml of the azide/fluoride solution and filtering through glass wool.

ELECTRODE BUFFER. The electrode buffer contained 0.050M Tris, 0.384M glycine, 2mM EDTA, 0.1% SDS which was made up as follows:
30g Tris base(BDH Ltd. general purpose.), 144g glycine (Sigma Chemical Co. Ltd. ammonia free.), 5g SDS, 3.72g disodium EDTA were dissolved in 5 l of double distilled water.

FOUR TIMES CONCENTRATED SAMPLE BUFFER. The sample buffer used was made up as follows: SDS (8g) was dissolved in 0.5M TrisHCl pH 6.5 (40ml), 0.2M EDTA pH 7.0 (4ml) and glycerol (40ml). This solution was made up to 100ml with double distilled water to give 0.2M TrisHCl, 8% SDS, 8mM EDTA, 40% glycerol, pH 6.5.

In all solutions disodium EDTA was used since using the free acid lowers the pH of the buffers resulting in poor resolution of proteins on the gel.

All samples were electrophoresed on 8% to 20%(w/v) exponential gradient gels with a 1cm stacking gel unless otherwise stated. The gels were prepared by pumping (1.5ml/min) an 8% acrylamide solution into a 20% acrylamide solution in a mixing chamber held at a constant volume. The mixed solution was then pumped (1.5ml/min) between two

chromic acid washed glass plates sealed around three edges with 1.0mm thick plastic spacers. The plates were 16cm broad and 20cm high with one plate notched (14cmx1cm) to accommodate the 'comb' mould used to form sample wells in the stacking gel. The two gel solutions which were mixed to form the gradient had the following composition. The 8% gel mix consisted of 5ml of gel buffer, 5.3ml of acrylamide/bis-acrylamide, 3.3ml of polyacrylamide, 6.4ml of water, 80µl of 10% ammonium persulphate solution and 10µl of TEMED. The 20% gel mix consisted of 2.5ml gel buffer, 6.6ml of acrylamide/bis-acrylamide solution, 1.7ml of polyacrylamide, 60µl of 10% ammonium persulphate solution and 5µl of TEMED. The ammonium persulphate and TEMED chemically polymerise the gel. The solutions were pumped until the low percentage gel chamber of the gradient forming apparatus was empty. The unpolymerised gel was then overlaid with water saturated butan-2-ol to give a flat surface to the gel. The gel was then left for 20-30 minutes to polymerise after which time the butanol overlay was removed and the gel surface washed with water and stacking gel buffer. The stacking gel (3ml stacking gel buffer, 1.8ml acrylamide/bis-acrylamide, 2.6ml polyacrylamide, 5.1ml water, 250µl ammonium persulphate and 20µl TEMED) was then poured onto the running gel and the sample 'comb' inserted between the plates before the gel polymerised. After the gel had polymerised (5 minutes) the 'comb' and bottom spacer were removed and the plates clipped to an electrophoresis tank. The top and bottom buffer reservoirs were then filled with electrophoresis buffer and the protein samples injected into the sample wells using a Terumo syringe.

Protein samples were prepared for electrophoresis by mixing the protein, four times concentrated sample buffer and water to give a

protein solution with a minimum concentration of 1mg/ml and a four fold dilution of the sample buffer. The samples were prepared with a protein concentration of 1mg/ml or greater since 100μg to 200μg of protein per sample, was required for loading onto the gel and the maximum sample volume which could be applied to the gel was 120μl. The protein concentration of the sample was reduced when dealing with purified and semipurified proteins, the concentrations quoted being applicable to whole cell lysates.

After electrophoresis the gels were fixed in a solution of 20% methanol, 10% acetic acid for 1 hour. The gels were then stained in a solution of 0.25% Coomassie brilliant blue R-250 in 25% methanol, 10% acetic acid (w/v/v) in water. Gels were destained by soaking in 25% methanol 10% acetic acid. The destaining of the gels was accelerated by heating in a shaking water bath at 45°C in the presence of polyurethane packing foam to absorb the dye washed from the gel.

The molecular weights of proteins were estimated from their mobilities relative to the marker dye, bromophenol blue, and a set of standard proteins of known molecular weight. These molecular weight markers were obtained from Sigma Chemical Co. Ltd. and are listed below.

| Protein. | Molecular Weight KD. |
|---|---|
| Myosin | 205,000 |
| β-Galactosidase | 116,000 |
| Phosphorylase b | 97,400 |
| Bovine serum albumin | 66,000 |
| Egg Albumin | 45,000 |
| Glyceraldehyde-3-phosphate dehydrogenase | 36,000 |
| Carbonic anhydrase | 29,000 |
| Trypsinogen | 24,000 |
| Trypsin inhibitor | 20,100 |
| α-Lactalbumin | 14,200 |

The molecular weights of proteins were calculated from a standard

curve. The curve was constructed by plotting $\log_{10}$ of the molecular weight of the standard proteins listed above against their mobilities relative to bromophenol blue.

## 2.14 AUTORADIOGRAPHY AND FLUOROGRAPHY OF RADIOLABELLED PROTEINS ON SDS PAGE GELS.

Gels of protein mixtures labelled with $^{32}P$ or $^{125}I$ were fixed, stained and destained as described above, then soaked in destain containing 15%(w/v) glycerol for 1 hour in order to prevent cracking of the gel during drying. Following this the gel was then dried under vacuum on a Biorad gel drier onto filter paper or acetate sheets. The dried gels were then placed in light-proof cassettes in contact with a sheet of X-ray film and exposed at $-70^{\circ}C$ for a period of time determined by the amount of radioactivity present in the gel.

Gels containing proteins labelled with $^{3}H$ were analysed by fluorography which enables detection of radio-isotopes with low emission energies such as $^{3}H$. The gels were stained and destained as described above and then soaked in a solution of 1M sodium salicylate, 15%(w/v) glycerol for 1 hour before drying. The gels were then exposed to X-ray film as for autoradiography. The distribution of $^{3}H$ labelled proteins on gels was also determined by cutting the gel into 2mm strips and heating the strips in 15%(w/v) $H_2O_2$ at $60^{\circ}C$ for 12 hours to dissolve the gel and release the label for liquid scintillation counting (Cabantchick & Rothstein, 1974). The gel must be dissolved before counting since the $\beta$-particle emitted by $^{3}H$ does not have enough energy to penetrate the gel and therefore would not be detected in intact gel slices.

## 2.15 SYNTHESIS OF $\gamma$-$^{32}$P ATP.

The synthesis of $\gamma$-$^{32}$P ATP was carried out by the exchange procedure described by Glynn & Chappell(1964) and England(1979). The reaction mixture used is listed below.

| | |
|---|---|
| 1M Tris pH 8.0 | 100µl |
| 0.1M EDTA pH 7.0 | 5µl |
| 2-mercaptoethanol | 0.3µl |
| 0.1M ATP pH 7.0 | 15µl |
| 1M $MgCl_2$ | 10µl |
| 0.2M 3-PGA | 20µl |
| 10mM $NAD^+$ | 15µl |
| Water | 735µl |
| $^{32}$Pi 10mCi/ml | 100µl |

The exchange procedure was started by the addition of a mixture of the enzymes phosphoglycerate kinase (EC 2.7.2.3) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) as 20µl of a 1:1 activity mixture supplied by The Boehringer Corporation Ltd. The reaction mixture was left to stand at room temperature for 2 hours. At the end of this incubation time the reaction mix was diluted with 1ml of water and the incorporation of the labelled phosphate into the ATP measured. Incorporation was measured by diluting 1µl of the reaction mix to 1ml and counting 5µl by liquid scintillation counting. The remainder of the diluted reaction mix was then mixed with 0.3ml of Norit activated charcoal, centrifuged and 5µl of the supernatant counted as before. Subtraction of the number of counts in the charcoal supernatant from the number of counts present before the addition of the charcoal gives an approximate measure of the incorporation of label. The incorporation of label was normally >95%.

The labelled ATP was purified from free labelled phosphate on a column of 200 mesh Dowex 1x8 ion exchange resin 1.4cm diameter by 0.5cm high. The resin was cycled 3 times with 0.1M NaOH and 0.1M HCl then washed with 1M HCl and washed to neutrality with water. The reaction mix was then applied to the column followed by 2ml of water, 3 x 2ml of 0.05M $NH_4Cl$/0.02M HCl, 2ml of water and finally 0.25M HCl collecting 0.5ml fractions. The fractions eluted in the final acid wash were assayed for labelled ATP by the charcoal binding assay. The fractions containing labelled ATP were then pooled and neutralised with 2M Tris. The labelled ATP was stored frozen at -20°C until required.

## 2.16 IODINATION OF CELL SURFACE PROTEINS WITH $^{125}I$ IODINE BY LACTOPEROXIDASE.

Trypanosomes were iodinated by a modification of the method of Marchalonais(1969) as described by Mancini et al.(1982). Approximately $1x10^9$ trypanosomes were suspended in 200µl of PSG buffer pH 7.4 containing 5mM KCl, 1mM $MgCl_2$, 1mCi of carrier free $Na^{125}I$ and 50µl of lactoperoxidase (EC 1.11.1.7) (1mg/ml, 2 Units/ml) on ice. At time zero the iodination was started by the addition of 30µl of 0.001% $H_2O_2$ in PSG. An additional 10µl of $H_2O_2$ was added 1 minute and 2 minutes after the start of the iodination. The reaction was terminated after three minutes by the addition of 10 volumes of PSG containing 10µM KI. The cells were washed by centrifugation and resuspension in this buffer four times and used immediately in cell fractionation experiments or frozen until required.

## 2.17 MEASUREMENT OF ΔpH ACROSS THE TRYPANOSOMAL PLASMA MEMBRANE.

The pH gradient across the trypanosomal plasma membrane was measured by following the distribution of [$^{14}$C] methylamine across the plasma membrane in response to the pH on either side. The principles of this technique are explained below.

In the following equations the subscripts I and O refer to inside and outside the cell respectively. The weak base, methylamine, has the dissociation constant.

$$Kd = \frac{[CH_3NH_3^+]}{[CH_3NH_2]\,[H^+]}$$

In the absence of a pH gradient and at equilibrium the total concentration of methylamine inside and out side the cell will be equal.

$$\frac{[CH_3NH_3^+]_I}{[CH_3NH_2]_I\,[H^+]_I} = \frac{[CH_3NH_3^+]_O}{[CH_3NH_2]_O\,[H^+]_O}$$

Taking logs of the above equation gives

$$\log [CH_3NH_3^+]_I - \log [CH_3NH_2]_I + pH_I$$
$$= \log [CH_3NH_3^+]_O - \log [CH_3NH_2]_O + pH_O$$

At equilibrium irrespective of the presence of a pH gradient across the plasma membrane the concentrations of the uncharged forms of methylamine must be equal. Therefore the equation simplifies to;

$$\log [CH_3NH_3^+]_I + pH_I = \log [CH_3NH_3^+]_O + pH_O$$

or

$$pH_I - pH_O = \log [CH_3NH_3^+]_O - \log [CH_3NH_3^+]_I$$

ie.

$$\Delta pH = \log \frac{[CH_3NH_3^+]_O - [CH_3NH_2]_O}{[CH_3NH_3^+]_I - [CH_3NH_2]_I}$$



Given that the pKa of methylamine is 10.6 it can be seen in the above equation the term $CH_3NH_2$ out is negligable at the pH used.

The final equation above is some-what misleading in that experimentally the total amount of methylamine inside and outside the cell is measured. However the above equation does show that any differences in internal and external concentrations of methylamine will be due to differences in pH across the plasma membrane. The experimental protocol used in measurements of $\Delta$pH across the plasma membrane is detailed in Section 3.6.8.

## 2.18 LIQUID SCINTILLATION COUNTING.

All radioisotopes were quantified by liquid scintillation counting in commercially supplied scintillant (Scintran cocktail T. BDH Chemicals Ltd.) in a programmable Packard scintillation counter. The counter automatically corrected for quenching using a quench curve held in the counter's memory. Quenching was estimated from data obtained from an external radium source and standard channel ratios.

## 2.19 TREATMENT OF KINETIC DATA.

In all cases the kinetic parameters of uptake or of an enzyme catalysed reaction were estimated from a Hanes plot ($S/\dot{V}$ against S). The line was fitted to the data points by an unweighted linear regression programme run on a Sinclair microcomputer.

The thermodynamic characteristics of transport were estimated from Arrhenius plots and the line of best fit was determined by linear regression as before. The thermodynamic parameters $\Delta H$ (enthalpy change), $\Delta G$ (Gibbs free energy) and $\Delta S$ (entropy change) were calculated from the Arrhenius plot using the equations listed below;

$\Delta H = Ea - RT$

$-\log K_{eq} = -\Delta H / 4.6 \times 1/T$

$\Delta G = -2.303\ RT\ \log K_{eq}$

$\Delta S = \Delta H - \Delta G/T$

Where R = gas constant, T = absolute temperature, Ea = activation energy and $K_{eq}$ = equilibrium constant.

## 2.20 CHEMICALS.

All enzymes used were supplied by The Boehringer Corporation Ltd. All radiochemicals were supplied by Amersham International Plc. All enzyme substrates were supplied by The Sigma Chemical Co. Ltd. unless otherwise stated. All other chemicals and solvents were of 'ANALAR' grade and supplied by BDH Chemicals Ltd. unless otherwise stated.

# 3.RESULTS

## 3.1 PYRIDOXINE UPTAKE AND METABOLISM.

Various methods were tried for estimating the amounts of radio-labelled substrates taken up by trypanosomes.

These methods were;

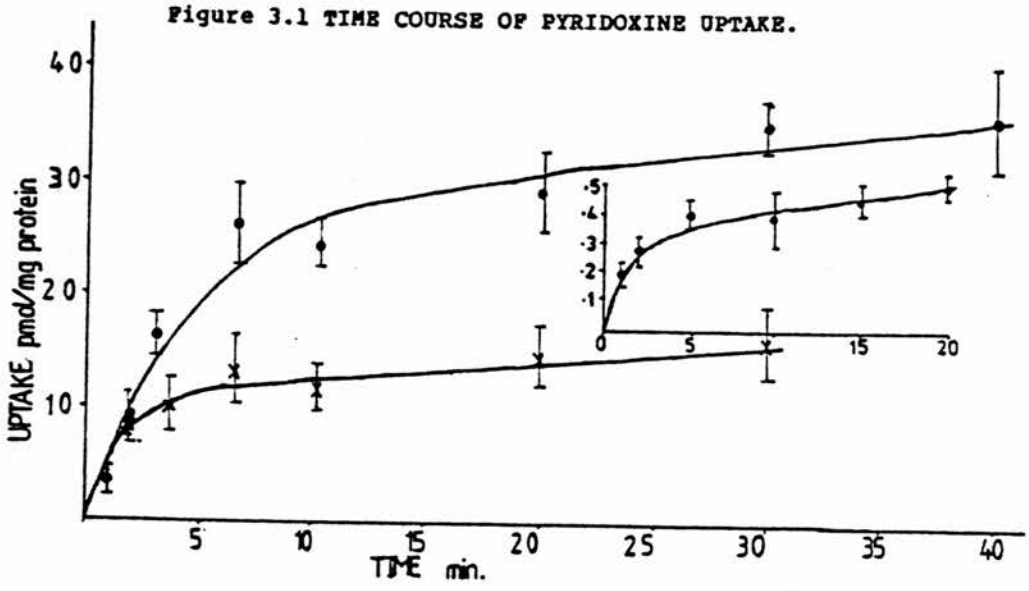
(a) Pelleting the cells by centrifugation after dilution of the incubation medium by ice cold buffer, repeatedly washing with buffer followed by water lysis of the cells. Protein could then be removed by precipitation and centrifugation leaving a supernatant fraction for the analysis of label taken up (Materials & Methods 2.11).

(b)Cells were removed from the incubating medium by rapid filtration onto glass fibre filters. Uptake of label was then estimated by liquid scintillation counting of the filters (Materials & Methods 2.11.1).

(c) Centrifugation of the cells out of the incubation medium through a silicon oil layer (silicon sandwich) into an appropriate stopping solution (Materials & Methods 2.11.2).

Method (c), silicon sandwich centrifugation, was the method finally chosen since method (a) can not easily be used to obtain short incubation periods unless the incubation medium can be diluted by a very large excess of buffer. This method also allows metabolism of material taken up to continue during washing. Even if ice cold washing buffers are used, some heating during centrifugation is unavoidable. Method (b), filtration, is suitable for use with short incubation periods but it allows metabolism to continue on the filters, metabolites are difficult to extract quantitatively for further analysis and non-specific binding of labelled substrate to

**Figure 3.1 TIME COURSE OF PYRIDOXINE UPTAKE.**




Trypanosomes at a protein concentration of 1.90mg/ml were incubated at a temperature of 22°C (•) or 2°C (x) with [G-$^{3}$H] pyridoxine (10μM; specific activity 100μCi/μmole) for the times indicated at pH 7.4 in PSG buffer. The inset shows the time course of pyridoxine uptake at a concentration of 21nM (specific activity 1.4Ci/μmole) at 22°C. Uptake was estimated by silicon sandwich centrifugation as described in the text (Section 3.1.1). The results are shown as means and range of two determinations.

the filters results in unacceptably high background counts.

The silicon sandwich centrifugation assay system allows short incubation times and metabolism is stopped at the same time as uptake when the cells hit the stop solution below the oil layer.

Metabolites of the labelled substrate can easily be recovered from the stop solution for further analysis.

### 3.1.1 Time course of Pyridoxine uptake.

Pyridoxine uptake was measured as a function of time using [G-$^{3}$H]-Pyridoxine as substrate and silicon sandwich centrifugation as described in Materials & Methods (2.11.1).

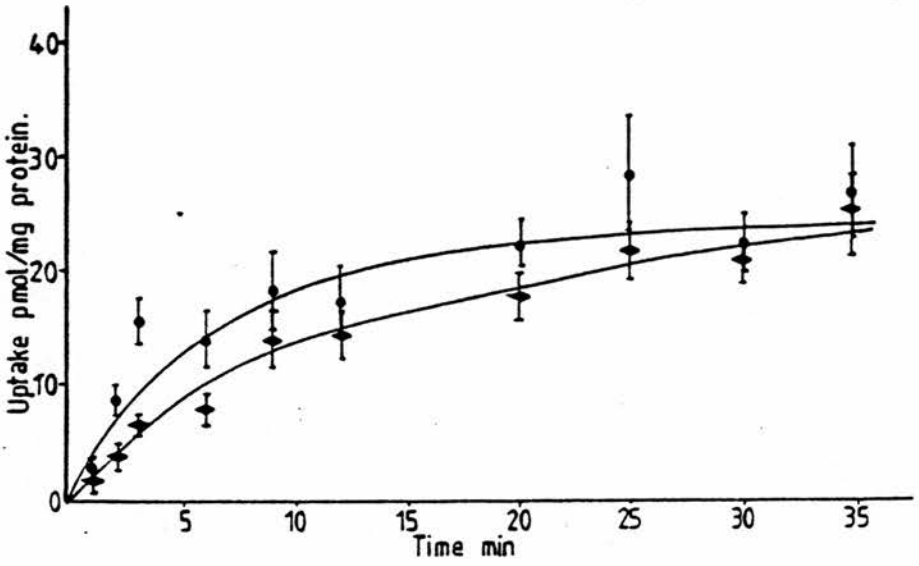
Trypanosomes were incubated with [G-$^{3}$H]-pyridoxine as described in the legend to Figure 3.1. The uptake assay was started by addition of [G-$^{3}$H]-pyridoxine to a suspension of cells. Samples were then taken and centrifuged on silicon sandwiches at the times shown in Figure 3.1.

Samples of the lower layer of the silicon sandwiches, in this case 6M perchloric acid (PCA.), were taken for liquid scintillation counting. Uptake was then calculated and expressed as pmol pyridoxine taken up/mg of protein.

Figure 3.1 shows the results obtained for the time course of pyridoxine uptake. The insert shows the time course of uptake at 22$^{o}$C at a more physiological concentration of pyridoxine (21nM). The approximate plasma levels of vitamin $B_6$ vitamers in humans are listed below;

PYRIDOXAL PHOSPHATE.....61.0nM
PYRIDOXINE PHOSPHATE.....3.4nM
PYRIDOXAL.....12.5nM
PYRIDOXINE.....30.0nM
PYRIDOXAMINE.....5.7nM

**Figure 3.2 SURFACE BINDING OF PYRIDOXINE.**




Trypanosomes at a protein concentration of 1.90mg/ml were incubated at 22°C with [G-$^{3}$H] pyridoxine (10µM; specific activity 100µCi/µmole) for the times shown above. The uptake assays were terminated by washing the cells, in either ice cold PSG (●) or ice cold PSG supplemented with 5mM pyridoxine(⊖), as described in the text (Section 3.1.2). The uptake of pyridoxine was estimated by lysing the cell pellets in water followed by liquid scintillation counting. The figure shows the mean and range of duplicate determinations.

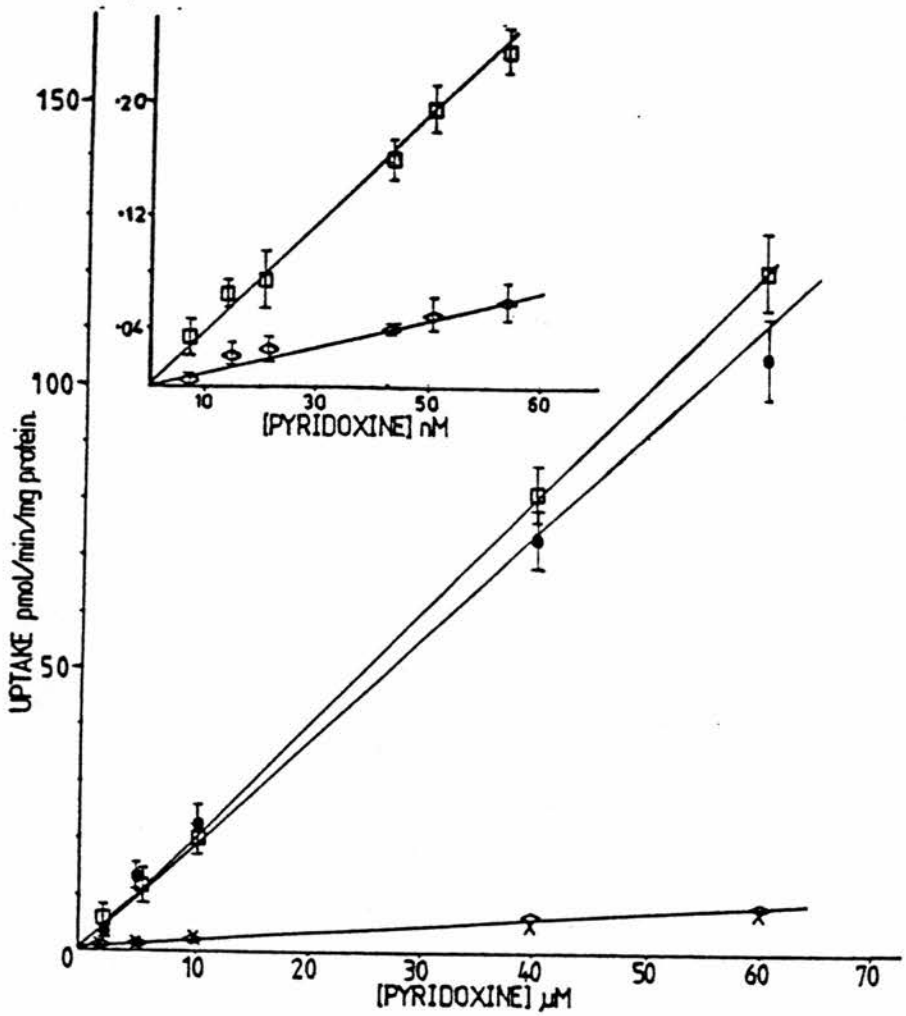
The above concentrations were calculated from data published by Lumeng et al.(1980).

Lowering the incubation temperature from 22°C to 2°C reduces the amount of pyridoxine taken up by the trypanosomes by approximately 50%. This 50% inhibition suggests that the uptake system consists of a temperature sensitive component such as metabolism and a less temperature sensitive component such as diffusion through the plasma membrane. These findings are similar to those reported for the uptake of pyridoxine by the erythrocyte (Mehansho & Henderson,1980) and by liver (Mehansho et al.,1980).

**3.1.2 Surface binding of Pyridoxine.**

It could be argued that the apparent uptake of pyridoxine at 2°C is due to non-specific binding of the [G-$^3$H] pyridoxine to the cell surface. To investigate this possibility trypanosomes were incubated with [G-$^3$H] pyridoxine as previously described (Section 3.1.1) but instead of terminating the uptake assay by silicon sandwich centrifugation the cells were pelleted by centrifugation and washed 3 times in either PSG or PSG supplemented with 5mM pyridoxine. If the observed uptake at 2°C was due to surface binding the 5mM pyridoxine ( 500 times greater concentration than substrate pyridoxine) would be expected to compete for binding sites with the [G-$^3$H] pyridoxine. This would result in a reduction of the amount of $^3$H present in the cell pellet when compared to the PSG washed cells. The results of this experiment are shown in Figure 3.2. The results show that washing the cells in PSG supplemented with 5mM pyridoxine results in a slight lowering of the amount of label present in the cell pellet. If the uptake measured at 2°C in the silicon sandwich centrifugation

**Figure 3.3 CONCENTRATION DEPENDENCE OF PYRIDOXINE UPTAKE.**




Trypanosomes (1.90mg protein/ml) were incubated at 22°C for 1.5 minutes (□) or 30 minutes (◇) or at 2°C for 1.5 minutes (●) or 30 minutes (X) over a range of pyridoxine concentrations 5-65nM (specific activity 1.4Ci/µmole) and 10-60µM (specific activity 100µCi/µmole). All incubations were carried out at pH 7.4 in PSG buffer uptake being estimated as described in the text Section 3.1.1. The results are shown as the mean and range of duplicate determinations.

experiment was due to surface binding a 50% reduction in the estimated uptake of pyridoxine would be expected in the cells washed in PSG supplemented with the 5mM pyridoxine. The small reduction in uptake observed varied with time and may be due to the exchange of free intracellular [G-$^{3}$H] pyridoxine with the extracellular unlabelled pyridoxine in the wash solution. This point will be considered in full in the discussion.

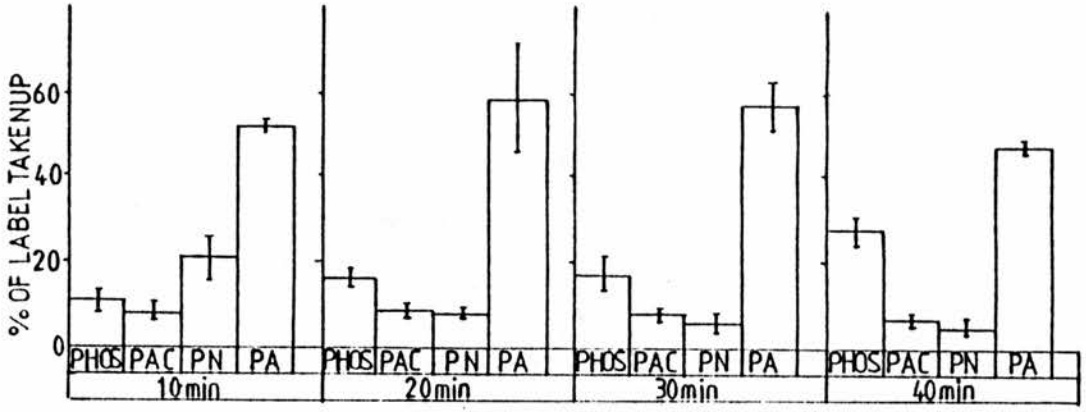
If entry of pyridoxine into the cell is by diffusion it should not be saturable, therefore the concentration dependence of uptake was investigated.

### 3.1.3 Kinetic parameters of Pyridoxine uptake.

Trypanosomes were incubated with [G-$^{3}$H]-pyridoxine at the concentrations shown in Figure 3.3. Experimental details are given in the legend to Figure 3.3. The results show that uptake is non-saturable over the substrate concentration range 5nM-60μM. This non-saturability of uptake provides further evidence that uptake is by diffusion. In this experiment lowering the temperature had no marked effect on the rates of uptake.

The concentration dependence of uptake was measured using 1.5 minute and thirty minute incubation times, both of which gave linear plots for uptake. The rates of uptake at thirty minutes are much slower than the uptake rates measured at two minutes as would be expected from consideration of the time course of uptake (Figure 3.1). The inability to saturate uptake suggests that either diffusion through the plasma membrane is the rate limiting step for uptake or that the pyridoxine metabolising enzymes have high Km's for pyridoxine and are therefore not saturated at the substrate

Figure 3.4 TIME COURSE OF [G-$^3$H] PYRIDOXINE METABOLISM IN WHOLE CELLS.




Samples (50µl) of the PCA layer from a time course of pyridoxine uptake, carried out as described in the legend to Figure 3.1 with 10µM pyridoxine as substrate (specific activity 200µCi/µmole), were neutralised with KOH (25µl; 12 molar) and centrifuged to remove precipitated potassium perchlorate. Samples (10µl) were then chromatographed, with unlabelled carriers, on silica thin layer plates as described in Materials & Methods (2.8.1). The sections of the developed chromatogram containing the $B_6$ metabolites were then cut from the plate and their content of label determined by liquid scintillation counting. The results are shown as the mean and range of duplicate determinations. (PHOS= phosphorylated forms of $B_6$)

concentrations used. These points will be considered in more detail in the Discussion (Section 4.1).

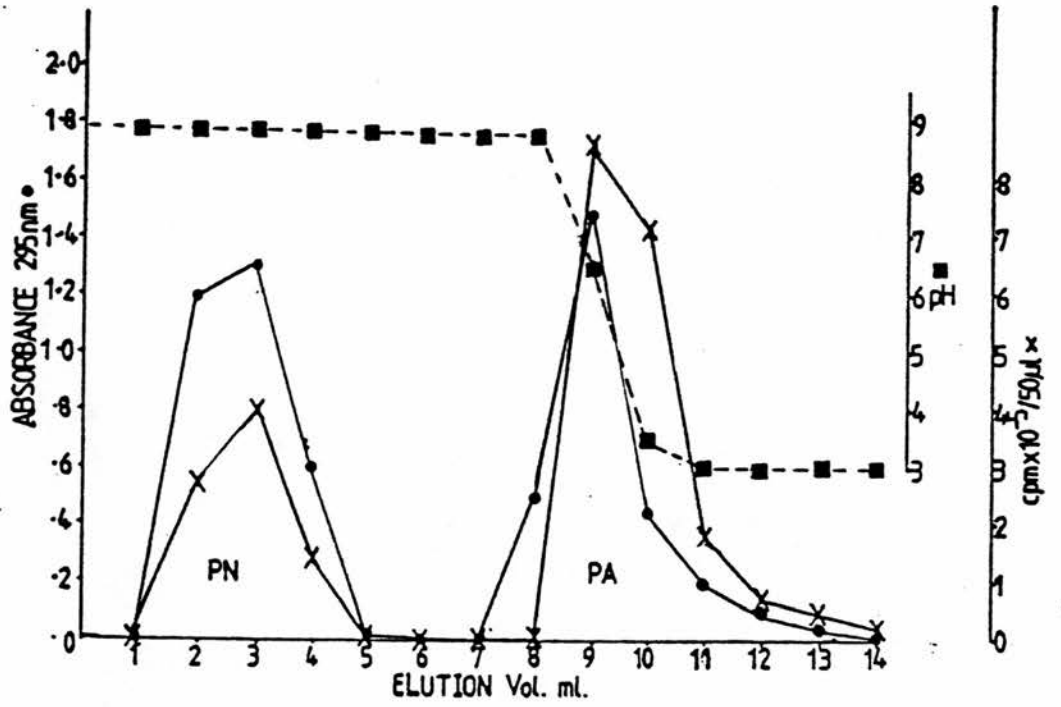
As mentioned previously uptake of pyridoxine by the erythrocyte and by the liver is by diffusion followed by metabolic trapping (Mehansho and Henderson 1980; Mehansho et al, 1980), so the metabolism of pyridoxine by whole cells was investigated.

### 3.1.4 Time Course of Pyridoxine Metabolism in Whole Cells.

Samples of the PCA layer from a time course of pyridoxine uptake were analysed for their content of vitamin $B_6$ vitamers as described in the legend to Figure 3.4.

The results shown in Figure 3.4 indicate that the pyridoxine taken up is oxidised to pyridoxal since pyridoxine levels fall as pyridoxal levels rise. The next stage in metabolism appears to be the phosphorylation of pyridoxal to pyridoxal phosphate since the levels of pyridoxal begin to fall as the levels of the phosphorylated forms of $B_6$ begin to rise. The apparent oxidation of pyridoxine to pyridoxal is unexpected since in the liver pyridoxal phosphate is formed from pyridoxine by first converting pyridoxine to pyridoxine phosphate using the enzyme pyridoxal kinase (ATP:Pyridoxal 5-phosphotransferase EC 2.7.1.35) which can phosphorylate pyridoxine or pyridoxal equally well. The pyridoxine phosphate is then oxidised by pyridoxamine phosphate oxidase (Pyridoxamine phosphate:$O_2$ oxidoreductase (deaminating) EC 1.4.3.5) to pyridoxal phosphate. The pyridoxamine phosphate oxidase has slightly lower affinity for pyridoxine phosphate than for pyridoxamine phosphate but both

Figure 3.5 PRYIDOXINE AND PYRIDOXAL PURIFICATION ON AN AMINO-ETHYL CELLULOSE COLUMN.




The above figure shows the elution profiles of a mixture of authentic pyridoxine and pyridoxal (•) and [G-$^{3}$H] pyridoxine and pyridoxal from a synthesis of [G-$^{3}$H] pyridoxine from [G-$^{3}$H] pyridoxal (X). The figure also shows the pH of the eluting buffer. The pyridoxine peak is labeled PN and the pyridoxal peak PA. All other experimental details are given in the text (Section 3.1.4).

substrates give the same Vmax value. The pyridoxamine phosphate oxidase has a very low affinity for pyridoxine which makes the direct oxidation of pyridoxine unlikely in the presence of an active pyridoxal kinase (Wada & Snell,1961;Mehansho et al.,1980). There is however another enzyme, found in E.coli, capable of direct oxidation of pyridoxine to pyridoxal, pyridoxine - 4-dehydrogenase (pyridoxine:NADP oxidoreductase EC 1.1.1.1). This enzyme produces $NADPH_2$ whereas the pyridoxamine phosphate oxidase produces $H_2O_2$ and since bloodstream forms of T.brucei have no haem proteins such as catalase for the removal of peroxides it seems likely that a dehydrogenase is responsible for the oxidation of pyridoxine to pyridoxal in the trypanosome.

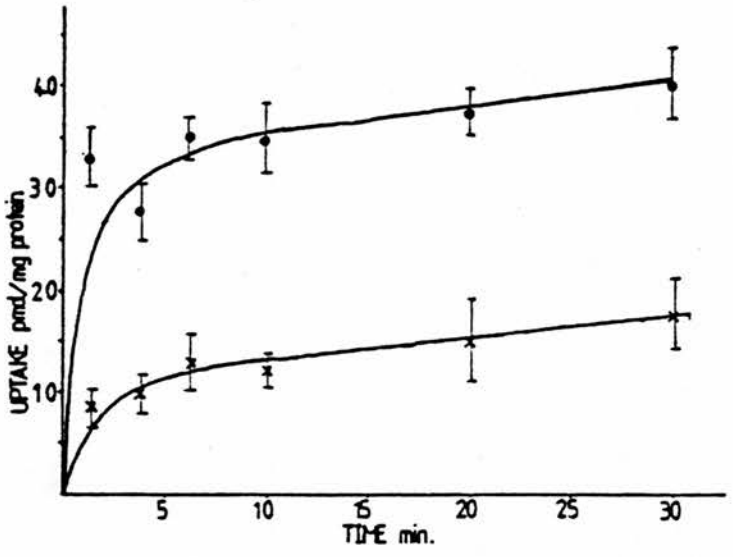
To investigate the uptake and metabolism of vitamin $B_6$ further [G-$^3$H]-pyridoxal was synthesised chemically from [G-$^3$H]-pyridoxine as [G-$^3$H]-pyridoxal is not commercially available.

### 3.1.5 Synthesis and Purification of Radiolabelled Pyridoxal from Radiolabelled Pyridoxine.

The chemical conversion of pyridoxine to pyridoxal was achieved by the oxidation of pyridoxine by potassium permanganate at 20°C. (Snell,1944). The synthesis was first carried out using unlabelled pyridoxine and a method for purifying pyridoxal from the reaction mixture was developed, which is detailed below.

The synthesis was carried out as follows;
Pyridoxine (100µl;1.4Ci/mmol) at 1mCi/ml was added to 0.2µmoles of unlabelled pyridoxine in a total volume of 120µl. Potassium permanganate (40µl of a 10mM solution) was then added giving a potassium permanganate:pyridoxine ratio of 3:2 on a molar basis. This

**Figure 3.6 TIME COURSE OF PYRIDOXAL UPTAKE.**




Trypanosomes at a protein concentration of 1.90mg/ml were incubated at a temperature of 22°C (•) or 2°C (×) with [G-$^{3}$H] pyridoxal (8.7µM; specific activity 100µCi/µmole) for the times indicated at pH 7.4 in PSG buffer. Uptake was estimated by silicon sandwich centrifugation and subsequent liquid scintillation counting of the PCA layer. The results are shown as the mean and range of duplicate determinations.

mixture was then allowed to stand in the dark for 2 hr. The precipitate of manganous dioxide which formed during the reaction was removed by centrifugation.

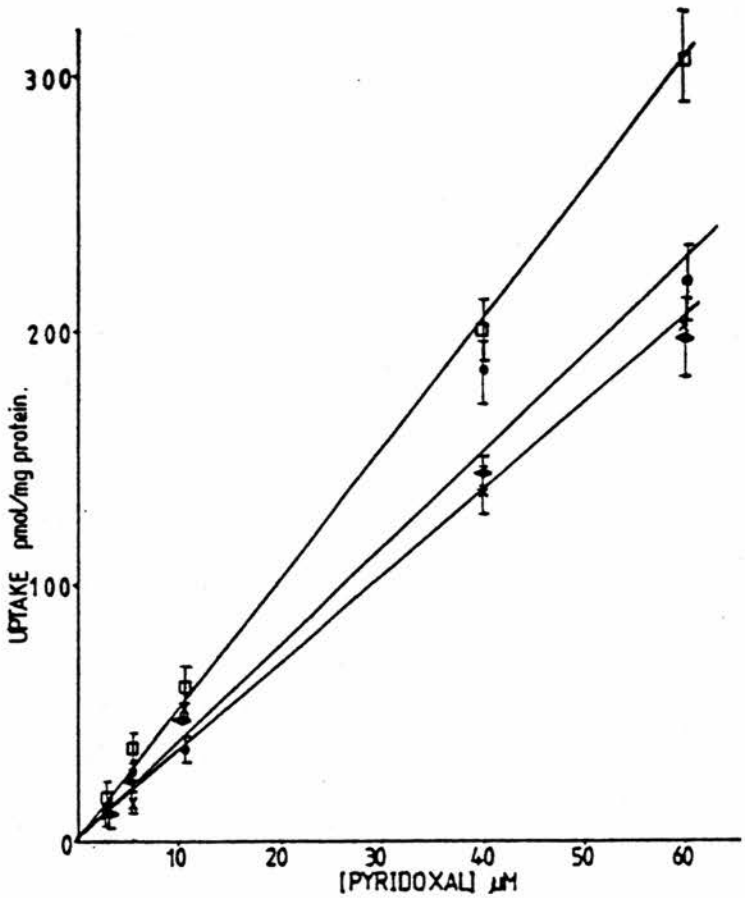
The purification of the pyridoxal was carried out on an amino-ethyl cellulose ion exchange column (0.5cm diameter x 10cm high) equilibrated with 0.05M disodium hydrogen orthophosphate at pH 9.0. The reaction mixture was then applied to the column and eluted with 8ml of the phosphate buffer followed by 8-10ml 0.1M acetic acid pH 3.0. Unreacted pyridoxine and pyridoxic acid are eluted from the column by the phosphate wash and the pyridoxal is eluted by the acid wash. When the column eluent was monitored at 295nm the elution profile shown in Figure 3.5 was obtained. The identities of the peaks were confirmed by TLC as described in Materials & Methods (Section 2.8.1) and by co-elution with authentic samples of pyridoxine and pyridoxal.

The pyridoxal peak was collected and lyophilised for 36 hours to remove the acetic acid from the sample. The pyridoxal was then redissolved and its purity checked by TLC as above. The radiochemical purity was 95% at a specific activity of 368.3μCi/μmole. The overall yield of the synthesis was 60%. This [G-$^{3}$H]-pyridoxal was used in the following experiments.

### 3.1.6 Time course of Pyridoxal uptake.

Pyridoxal uptake was measured as a function of time as described in the legend to Figure 3.6. The results of this experiment, shown in Figure 3.6, are very similar to those obtained for the time course of pyridoxine uptake. The only difference between the time courses of pyridoxine uptake and pyridoxal uptake is that the amount of

**Figure 3.7 CONCENTRATION DEPENDENCE OF PYRIDOXAL UPTAKE.**




Trypanosomes (1.90mg protein/ml) were incubated at 22°C for 1.5 minutes (•) or 30 minutes (□) or 2°C for 1.5 minutes (▬) or 30 minutes (×) over a range of pyridoxal concentrations 10-60µM (specific activity 100µCi/µmole). All incubations were carried out at pH 7.4 in PSG buffer uptake being estimated by silicon sandwich centrifugation as before.(Figure 3.6) The results are shown as the mean and range of duplicate determinations.

pyridoxal taken up is greater than the amount of pyridoxine taken up at any given time point.

The uptake of pyridoxal shows the same partial temperature dependence as pyridoxine uptake in that a drop in incubation temperature from 22°C to 2°C results in a 50% to 60% reduction in uptake. These results as outlined in Section 3.1 suggest a two component uptake system of which one component is temperature sensitive, (possibly metabolism or binding to intracellular proteins), and one component which is less temperature sensitive, possibly diffusion. As was the case with pyridoxine uptake these results prompted the investigation of the concentration dependence of uptake.

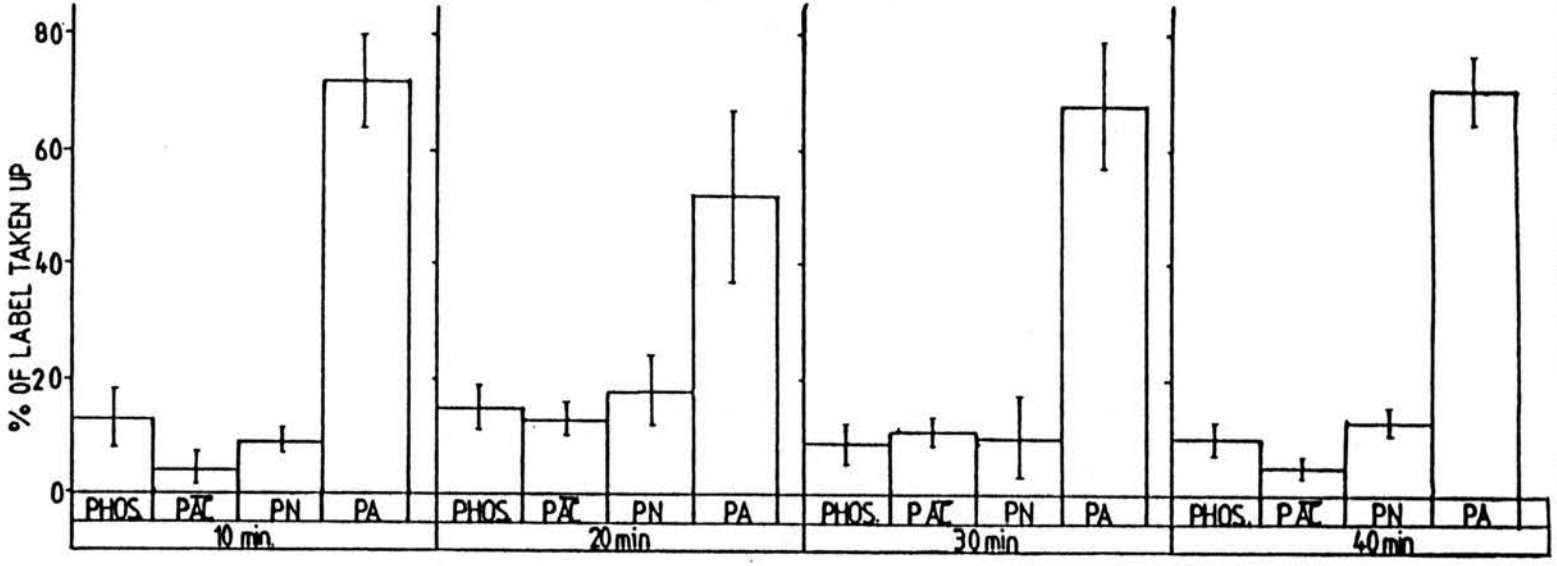
### 3.1.7 Kinetic parameters of Pyridoxal uptake.

The concentration dependence of pyridoxal uptake was determined as for pyridoxine (Section 3.1.2) with similar results, which are shown in Figure 3.7. Incubations were carried out for 1.5 minutes and for 30 minutes at both 2°C and 22°C. The results show that uptake is non-saturable and only partially temperature sensitive as was the case for pyridoxine uptake. These results suggest that the uptake of pyridoxal like that of pyridoxine is by simple diffusion.

### 3.1.8 Time Course of Pyridoxal Metabolism by Whole Cells.

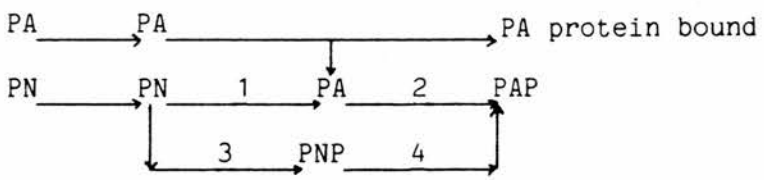
The time course of the metabolism of pyridoxal was investigated as described in the legend to Figure 3.8. The various metabolites were analysed by TLC as described in Materials & Methods (Section 2.8.1). The time course of pyridoxine metabolism (Section 3.1.4)

**Figure 3.8 TIME COURSE OF [G-$^3$H]-PYRIDOXAL METABOLISM IN WHOLE CELLS.**



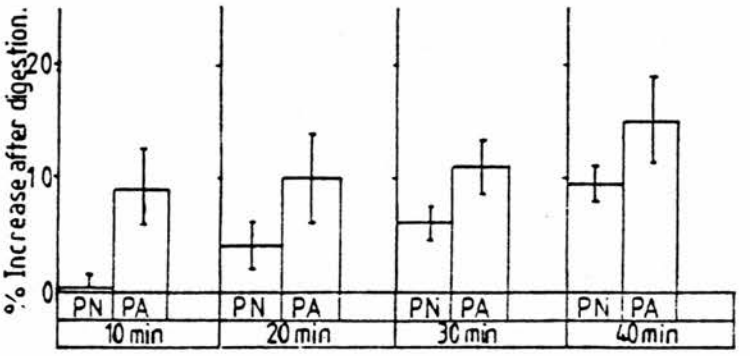

Samples (50µl) of the PCA layer from a time course of pyridoxal uptake, carried out as described in the legend to Figure 3.1 with 10µM pyridoxal as substrate (specific activity 200µCi/µmole), were neutralised with KOH (25µl; 12 molar) and centrifuged to remove precipitated potassium perchlorate. Samples (10µl) were then chromatographed, with unlabelled carriers, on silica thin layer plates as described in Materials & Methods (2.8.1). The sections of the developed chromatogram containing the $B_6$ metabolites were then cut from the plate and their content of label determined by liquid scintillation counting. The results are shown as the mean and range of duplicate determinations.

suggests that pyridoxine is oxidised to pyridoxal which is then phosphorylated to give pyridoxal phosphate. It would therefore be logical to assume that pyridoxal taken up would be metabolised to pyridoxal phosphate by the enzyme pyridoxal kinase. Examination of Figure 3.8 shows that little label is incorporated into phosphorylated forms of vitamin $B_6$. This apparent lack of metabolism of pyridoxal to pyridoxal phosphate prompted a more detailed examination of the phosphorylated forms of vitamin $B_6$ formed from the metabolism of pyridoxine. This experiment was carried out by digesting the metabolites of pyridoxine with alkaline phosphatase followed by TLC as previously described (Materials & Methods Section 2.8.1). Comparison of the amounts of pyridoxine and pyridoxal present before and after alkaline phosphatase treatment allows the estimation of the relative amounts of pyridoxine phosphate and pyridoxal phosphate formed. The results and experimental details of this experiment are given in Figure 3.9 and legend respectively. These results confirm that pyridoxine is metabolised to pyridoxal and then to pyridoxal phosphate. However, these results also show that pyridoxine can be converted to pyridoxine phosphate. These results are still not consistent with the observation that pyridoxal is not metabolised to pyridoxal phosphate at a significant rate (Figure 3.8). In order to explain this apparent inconsistency the hypothetical scheme of metabolism shown below has been developed.




If in the above hypothetical system steps 1 and 4 are fast and steps 3 and 2 are slow the observed results can be accounted for. If step 4

**Figure 3.9 PERCENTAGE INCREASE IN PYRIDOXINE AND PYRIDOXAL AFTER DIGESTION WITH ALKALINE PHOSPHATASE.**




The perchloric acid (PCA) layer from incubations identical to those described in the legend to Figure 3.4 were neutralised with KOH and the potassium perchlorate removed by centrifugation. The supernatants were lyophilised and redissolved in 0.1M sodium bicarbonate buffer pH 10 containing 2mM magnesium chloride. Alkaline phosphatase was then added to a final concentration of 3mg/ml. This mixture was then incubated for twenty minutes at 30°C. The digestion was terminated with PCA followed by neutralisation and clarification by centrifugation. The resultant supernatant was analysed by TLC and liquid scintillation counting as described in Materials and Methods Section 2.8.1. The results are shown as the mean and range of duplicate experiments.

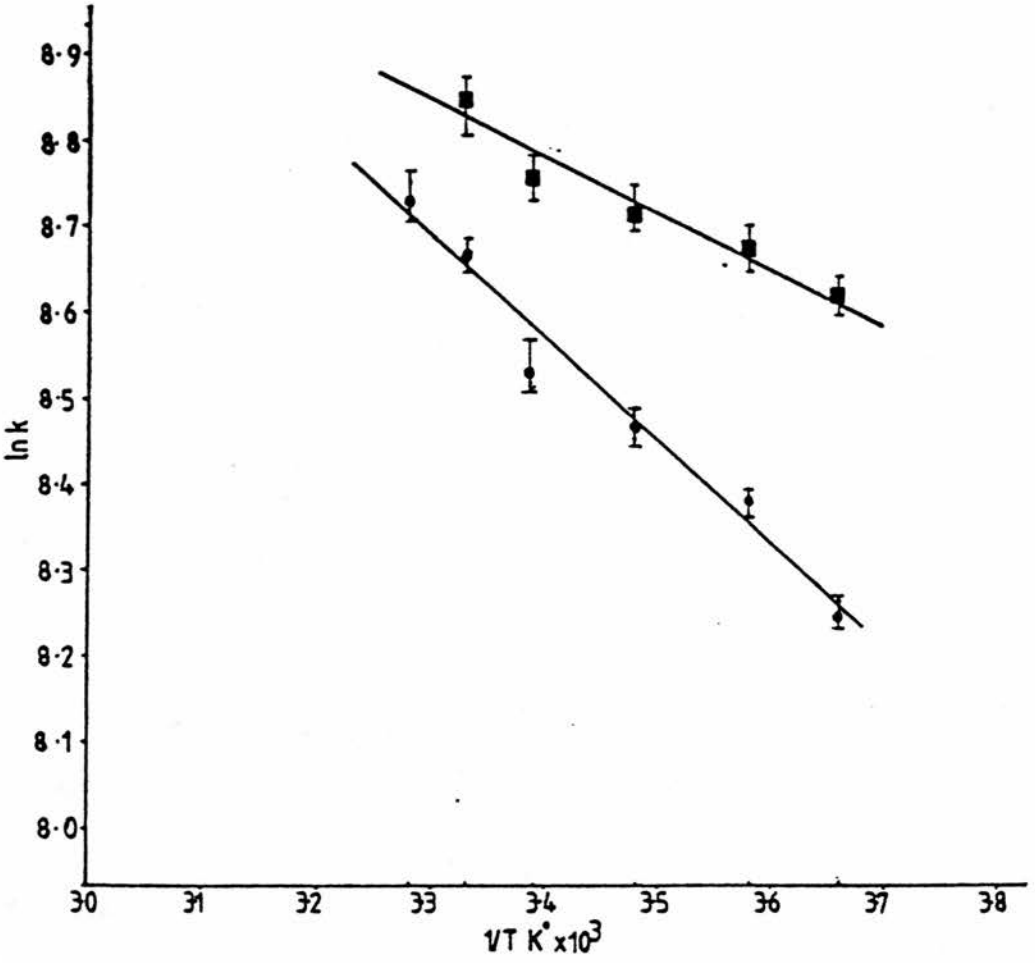
is very much faster than step 3 the apparent conversion of pyridoxine to pyridoxal phosphate shown in Figures 3.4 & 3.9 can be explained in terms of the rapid step 1 and the slower step 3 competing for the pyridoxine taken up and if step 4 is rapid then initially little pyridoxine phosphate would be detectable as it would be rapidly converted to pyridoxal phosphate. Pyridoxine phosphate levels would begin to increase as the system comes to a steady state or some feed back inhibition slows step 4.

### 3.1.9 Thermodynamic Characteristics of Pyridoxine and Pyridoxal Uptake.

The uptake of pyridoxine and pyridoxal are not as temperature dependent as would be expected if uptake was by facilitated diffusion (Kaback,1968;Stein,1967). The temperature sensitive component of uptake described (Section 3.3.1) is probably due to the temperature sensitivity of metabolism. The uptakes of both pyridoxine and pyridoxal have been shown to be non-saturable which is further evidence against uptake being mediated by facilitated diffusion. A further indication of whether a facilitated diffusion step involving a carrier is involved in the translocation of substrate across the plasma membrane can be obtained by estimation of the activation energy (Ea) of uptake. A high Ea would indicate the formation of a transition complex between the substrate and a carrier protein in the membrane as is the case for adenosine uptake (Section 3.2.3) and other uptake systems (Dixon & Webb,1964;Goldman et al.,1968). A low Ea on the other hand indicates that there is no energetic binding step required for translocation across the membrane.

Figure 3.10 shows Arrhenius plots for pyridoxine and pyridoxal

Figure 3.10 ARRHENIUS PLOTS OF PYRIDOXINE AND PYRIDOXAL UPTAKE.




Trypanosomes (1.10mg protein /ml) were incubated with either pyridoxal 1µM (specific activity 200µCi/µmole) (■) of pyridoxine 3µM (specific activity 200µCi/µmole) (●) in PSG buffer at pH 7.4. Incubations were carried out over a temperature range of 0°C to 32°C for 2 minutes. Uptake was estimated by the silicon sandwich centrifugation technique. The results are plotted as ln rate of uptake (dpm/min/mg) against the reciprocal of the absolute temperature of the incubation. The points shown are the means and range of triplicate determinations.

uptake. The experimental details are given in the legend to Figure 3.10. The information obtained from the Arrhenius plots allows the calculation of the activation energy (Ea), the enthalpy change ($\Delta$H), the entropy change ($\Delta$S) and the Gibbs free energy change of the system ($\Delta$G) for uptake as described in Materials & Methods (Section 2.19). The values calculated for these thermodynamic parameters are shown below;

| | PYRIDOXINE UPTAKE | PYRIDOXAL UPTAKE |
|---|---|---|
| Ea | 9.9 kJ/mol | 5.4 kJ/mol |
| $\Delta$H | 7.5 kJ/mol | 2.9 kJ/mol |
| $\Delta$S | 128.7 J/mol/K | 30.2 J/mol/K |
| $\Delta$G | -31.1 kJ/mol | -6.2 kJ/mol |

These values for Ea are 70% to 80% lower than those reported for uptake systems involving a membrane transport step.(Section 3.2.3; Damper & Patton,1976b; Goldman et al.,1968). In fact these activation energies are much lower than the activation energy of diffusion in free solution for a molecule the size of pyridoxine or pyridoxal. The reasons for this and consideration of $\Delta$S, $\Delta$H, and $\Delta$G will be left until the Discussion (Section 4.1).

### 3.1.10 Summary of Pyridoxine and Pyridoxal uptake and metabolism.

Pyridoxine and pyridoxal uptake processes have been shown to be non-saturable over a wide range of substrate concentrations.(Sections 3.1.2 & 3.1.6). Uptakes of pyridoxine and pyridoxal are only partially temperature sensitive and both have a very low Ea for uptake.(Section 3.1.8) These results are qualitatively similar to those reported by Mehansho & Henderson (1980) for the uptake of

pyridoxal and pyridoxine by diffusion in the rat erythrocyte.The metabolism of pyridoxine and pyridoxal will be considered in full in the Discussion (Section 4.1)

### 3.2 ADENOSINE UPTAKE AND METABOLISM.

Since the bloodstream forms of Trypanosoma brucei are incapable of the de novo synthesis of the purine ring they must obtain all their purines preformed from the host. Any differences found between host uptake and metabolism of purines and the trypanosomal system would provide potential targets for chemotherapy of trypanosome infections.

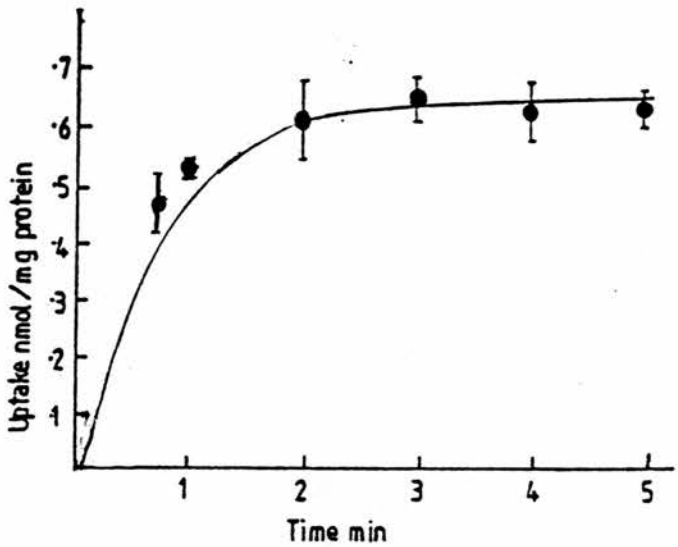
Purine nucleosides and bases are present at low concentrations in mammalian plasma, for example;

| Rat plasma Concentrations (μM). | | Source. |
|---|---|---|
| Hypoxanthine | 0.7-1.0 | Simmonds & Harkness(1981) |
| Adenosine | 1.0-1.5 | Von Borstel et al.(1982) |

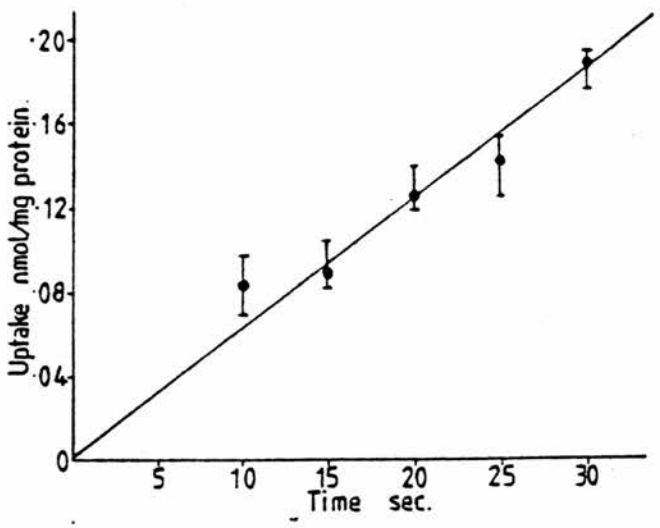
| Human plasma Concentrations (μM). | | Source. |
|---|---|---|
| Hypoxanthine | 0.5-2.8 | Taylor et al.(1980a) |
| Inosine | 0.2-1.5 | Taylor et al.(1980a) |
| Adenine | 0.1-1.4 | De Verdier et al.(1977) |
| Adenosine | 0.5-0.9 | Maurizio et al.(1982) |

Since the concentrations of purines are so low in the plasma it would be reasonable to assume that the trypanosome has some form of uptake system for purines. The erythrocyte like the trypanosome can not synthesise the purine ring de novo but has a high affinity uptake system for nucleosides.(Oliver & Paterson,1971; Cass & Paterson,1973; Cabantchik & Ginsburg,1977; Jarvis & Young,1980). The erythrocyte is a nondividing cell and so has no purine requirement for DNA synthesis or RNA synthesis since the mature erythrocyte has lost the ability to synthesise protein. However the trypanosomes are reproducing in the host's bloodstream and therefore must have a high purine requirement

Figure 3.11 TIME COURSE OF ADENOSINE UPTAKE.




Trypanosomes 0.31mg protein/ml were incubated in PSG buffer pH 7.4, 22°C with 1μM [2,8-$^{3}$H] adenosine (specific activity 50μCi/μmole). Uptake was assayed by liquid scintillation counting of samples of the PCA layer of silicon sandwiches (500μl of incubation) centrifuged at the times shown. The results are plotted as the mean and range of triplicate incubations.



for synthesis of nucleic acids. The fact that T.brucei and T.gambiense are capable of taking up purines at physiological concentrations has been shown by James & Born(1980). These authors showed that the trypanosomes had a saturable uptake system for purine nucleosides and bases but did not report on the kinetics of uptake or on the metabolism of purines after uptake.

When assaying uptake James & Born(1980) used centrifugation and repeated resuspension to remove extracellular label before estimating the uptake of labelled purine. As stated previously (section 3.1) there are drawbacks to this experimental approach which are avoided by the silicon sandwich technique. Also a complete analysis of the mechanism(s) by which purines are made available as nucleotides for the parasite, must include investigation of the kinetic and metabolic aspects of the problem. The following sections present the results of such an investigation.

### 3.2.1 Time course of Adenosine uptake.

Adenosine uptake was measured as a function of time by the silicon sandwich centrifugation technique, described in Materials and Methods (Section 2.11.2), using [2,8-$^{3}$H] adenosine as substrate. Trypanosomes were incubated with the labelled adenosine as described in the legend to Figure 3.11.

The uptake assay was started by the addition, with thorough mixing, of labelled adenosine to 5ml of a cell suspension to give a final concentration of 1μM adenosine. Samples were then taken and centrifuged on silicon sandwiches at the times shown in Figure 3.11. Adenosine uptake was estimated by liquid scintillation counting of samples of the PCA layer from the silicon sandwiches. The results of

these experiments are shown in Figure 3.11.

Figure 3.11 shows that the long term 45 seconds to 5 minutes and short term 10 to 30 second time course of adenosine uptake. The long time course shows that uptake is initially very rapid but appears to cease after 2-3 minutes. The short time course shows that uptake is linear for the first thirty seconds. These results do not agree with the results obtained by James & Born (1980) who found that uptake, although slowing down after an initial burst over 2-3 minutes, continued for up to 30 minutes. The differences in these results and results obtained for the uptake of other purines, determined in later experiments (Sections 3.2 to 3.3.5), cannot be wholly explained by differences in experimental methods but may be due to differences in the strain of trypanosome used. One major difference is that James & Born (1980) used a cloned strain whereas TREU.55 used here is monomorphic but uncloned. It may be that James & Born (1980) were studying a particular variant of the normal transport system. For these reasons any further comparisons between work presented here and that of James & Born (1980) will be considered in the discussion.

When the time course experiment was repeated at a temperature of $0^{o}C$ the uptake of adenosine was reduced to barely detectable levels. This temperature dependence suggests the existence of a transport system for adenosine at the level of the plasma membrane.

The apparent halt in uptake which occurs after three minutes (Figure 3.11) could be due to utilisation of all the substrate present in the incubation medium. This is not the case since at four minutes uptake is 0.6nmol/mg protein and the protein concentration in the incubation is 0.31mg/ml, therefore the total uptake in a 1ml incubation will be 0.31x0.6=0.186nmol. The substrate concentration

Table 3.1 EFFLUX OF ADENOSINE

| Time min. | % Total counts released in PSG | % Total counts released in PSG + Ar |
|---|---|---|
| 0·5 | 5·8 ± 1·0 | 5·0 ± 1·2 |
| 1 | 2·7 ± 0·5 | 2·7 ± 0·5 |
| 2 | 2·3 ± 0·2 | 2·5 ± 0·5 |
| 4 | 4·3 ± 0·5 | 3·0 ± 0·3 |

Trypanosomes were suspended in PSG pH 7.4 to a concentration of 0.127mg protein/ml (total volume 3ml). [2,8-$^{3}$H] Adenosine (500μC/μmol) was then added to a final concentration of 2μM and the mixture incubated at 22°C for 5 minutes. The trypanosomes were then centrifuged (8,000xg av) at 0°C and resuspended in PSG at 0°C four times. After the final wash step the cells were resuspended in either PSG or PSG supplemented with 20μM unlabelled adenosine at 22°C. After resuspension samples of cells were taken and added to liquid scintillation fluid for estimation of total uptake. Further samples were taken at the times shown in the table above and the cells removed by centrifugation. The label in the resultant supernatants was estimated by liquid scintillation counting. The results shown in the table are the means and population deviations of five experiments.

was 1μM, therefore a 1ml incubation will contain 1nmol of substrate. It is therefore obvious that lack of substrate is not the reason for the slowing of uptake since only 18.6% of the available substrate has been taken up.

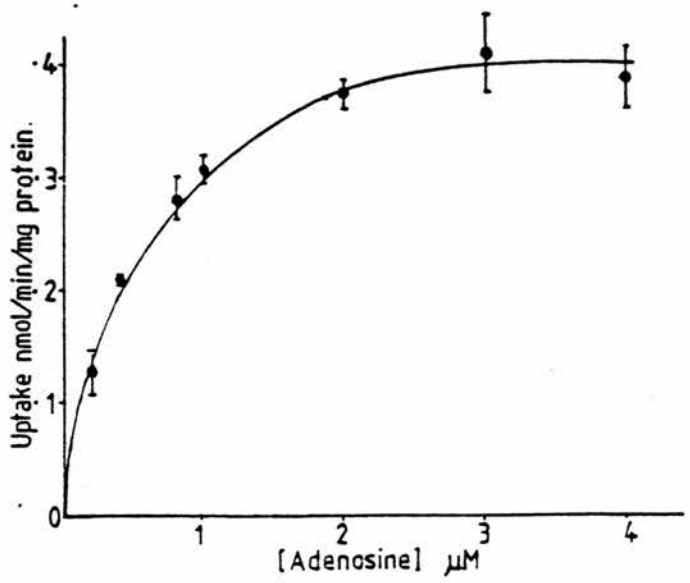
It could be argued that at 2-3 minutes the system comes to a steady state where labelled substrate is leaving the cell as quickly as it is entering. Considering the results presented in Section 3.2.4 this is unlikely since the levels of adenosine in the cell fall to barely detectable levels in five minutes. However this hypothesis can be tested experimentally by loading the cells with [2,8-$^{3}$H] adenosine, washing the cells free of extracellular adenosine at 0$^{o}$C, resuspending the cells in warm buffer with and without unlabelled adenosine and measuring any efflux of $^{3}$H. This experiment was performed as described in the legend to Table 3.1. The results presented in Table 3.1 show that very little of the $^{3}$H taken up is released on resuspension of the cells in PSG or PSG supplemented with adenosine.

The fact that addition of unlabelled adenosine does not result in an increase in label released from the cells suggests that there is very little non-specific surface binding of the $^{3}$H-adenosine since it would be released by competitive binding of the unlabelled adenosine. The small amount of label which is released may arise from cell death due to the repeated centrifugation involved in this experiment or the non-pelleting of a few cells.

### 3.2.2 Kinetic Parameters of Adenosine Uptake.

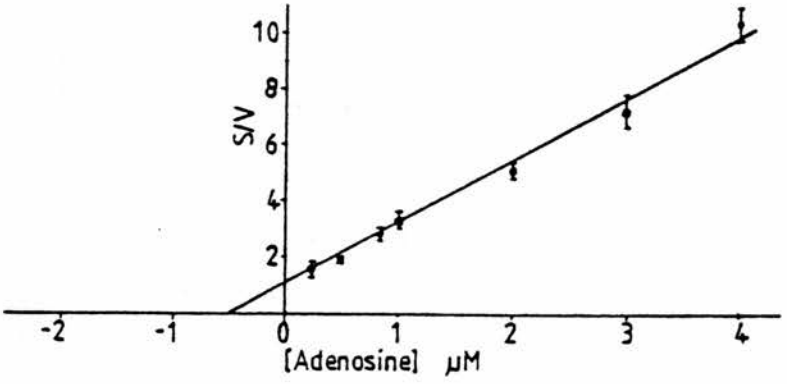
In order to determine the kinetic parameters of adenosine uptake trypanosomes were incubated with increasing concentrations of

**Figure 3.12a CONCENTRATION DEPENDENCE OF ADENOSINE UPTAKE.**




The rate of [2,8-$^{3}$H] adenosine uptake over the concentration range 0.2 to 4µM (constant specific activity 50µCi/µmole) was measured by the silicon sandwich centrifugation technique as described in the legend to Figure 3.11. All incubations were carried out at 22°C in PSG buffer at pH 7.4 for 15 seconds. The results are shown as mean and range of duplicate incubations. (Trypanosomes 0.13mg/ml incubation volume 1ml)

**Figure 3.12b HANES PLOT OF THE DATA PRESENTED IN FIGURE 3.12a.**



labelled adenosine as described in the legend to Figure 3.12a. The incubation period chosen for this experiment was 15 seconds which examination of the results of the time course experiment (Figure 3.11) shows that uptake is linear at this time point.

It should be noted that the kinetic parameters of the uptake system as a whole were measured and not just transport across the plasma membrane unless the transport step is rate limiting for uptake. In view of the above consideration the kinetic parameters obtained in this experiment can only be applied to uptake and not transport alone.

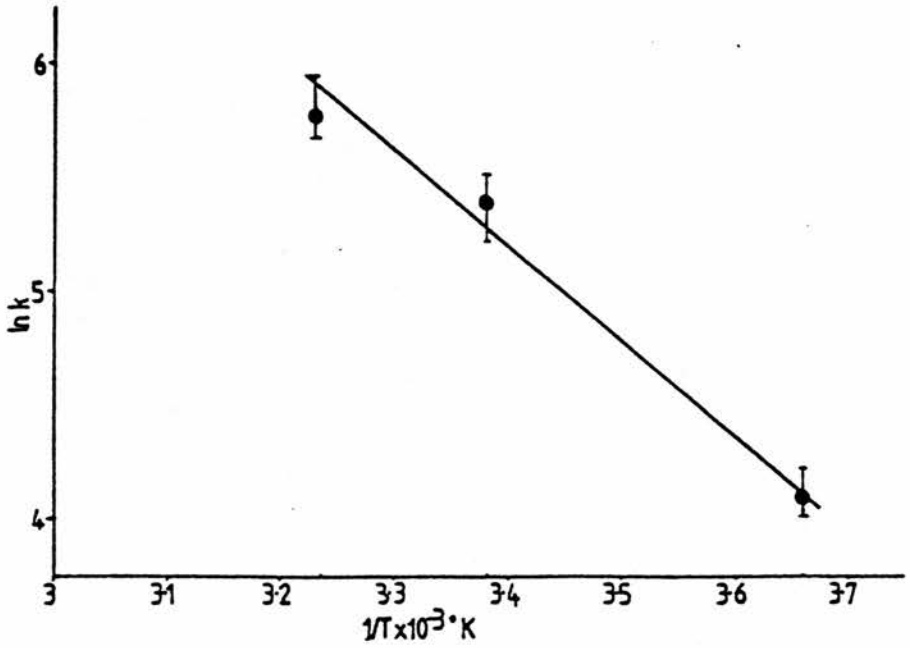
Figure 3.12a shows that the uptake of adenosine is saturable and Figure 3.12b is a Hanes plot of the uptake data presented in Figure 3.12a. From the Hanes plot the Km and Vmax can be calculated as 0.5μM and 0.50 nmol/min/mg protein respectively.

The low Km of 0.5μM is probably consistent with estimates of plasma adenosine concentration of about 1.0μM (Section 3.2). This value for plasma adenosine concentration is higher than has been quoted in the past, (<0.1μM Dobson et al.,1971 & Miyazaki et al.,1974). The reasons for this discrepancy in estimations of plasma adenosine concentrations are detailed in the Discussion (Section 4.2).

It is unlikely that adenosine is entering the trypanosome by simple diffusion across the plasma membrane since uptake is saturable and highly temperature dependent. If the adenosine is entering the cell by facilitated diffusion this might be indicated by a high activation energy (Ea) for uptake.

A high Ea would indicate the binding of adenosine to a carrier at the level of the plasma membrane, the activation energy being a measure

**Figure 3.13 ARRHENIUS PLOT OF ADENOSINE UPTAKE.**




Trypanosomes (0.5mg protein/ml) were incubated in PSG buffer at pH 7.4 with labelled adenosine 1μM (specific activity 50μCi/μmole). Incubations (30 seconds) were carried out at 0°C, 22°C and 36°C in triplicate. The results are plotted (mean and range) as ln rate of adenosine uptake against the reciprocal of the absolute temperature of the incubation medium.

of the energy required to form the transition complex.

### 3.2.3 Thermodynamic Characteristics of Adenosine Uptake.

Uptake of adenosine was assayed at various temperatures as described in the legend to Figure 3.13, which shows the results expressed as ln of the rate of adenosine uptake against the reciprocal of the absolute temperature. From this Arrhenius plot the following thermodynamic parameters can be calculated as described in Materials & Methods (Section 2.19).

Ea.....34.2 KJ/mol.

$\Delta$H.....31.7 KJ/mol.

$\Delta$S.....149.0 J/mol/K.

$\Delta$G.....-13.2 KJ/mol.

In using the Arrhenius equation the transport step across the plasma membrane is being considered as a chemical reaction. The reaction could be considered to be adenosine taken from outside the cell (reactant) to adenosine inside the cell (product).(Stein,1976).

The Ea calculated indicates that some form of transport across the membrane, other than simple diffusion, is taking place. The high value of Ea calculated above can be compared with the low value obtained for the uptake of pyridoxine and pyridoxal (Section 3.1.8) which are taken up by simple diffusion.

The possible significance of $\Delta$H, $\Delta$S and $\Delta$G will be considered in the discussion.

### 3.2.4 Metabolism of Adenosine by Whole Cells.

The uptake of adenosine may be dependent on its subsequent metabolism to maintain an adenosine gradient across the plasma

Trypanosomes (1mg protein/ml) were incubated in PSG buffer pH 7.4, 22°C with 0.5μM [2,8-$^{3}$H] adenosine (1mCi/μmole) for 45 seconds to 20 minutes. Samples of the incubation medium (500μl) were taken and centrifuged on silicon sandwiches at the times shown. Samples of the PCA layer (100μl) were neutralised by the addition of KOH (50μl) and 10 μl of the neutralised material applied to silica gel TLC plates and chromatographed with unlabelled standards as described in Materials & Methods (Secion 2.8). Radioactivity in the resolved components was estimated by liquid scintillation counting. The results are given as the mean and range of duplicate experiments. N=Nucleotide, Ir=Inosine, Hx=Hypoxanthine, Ar=Adenosine and A=Adenine.

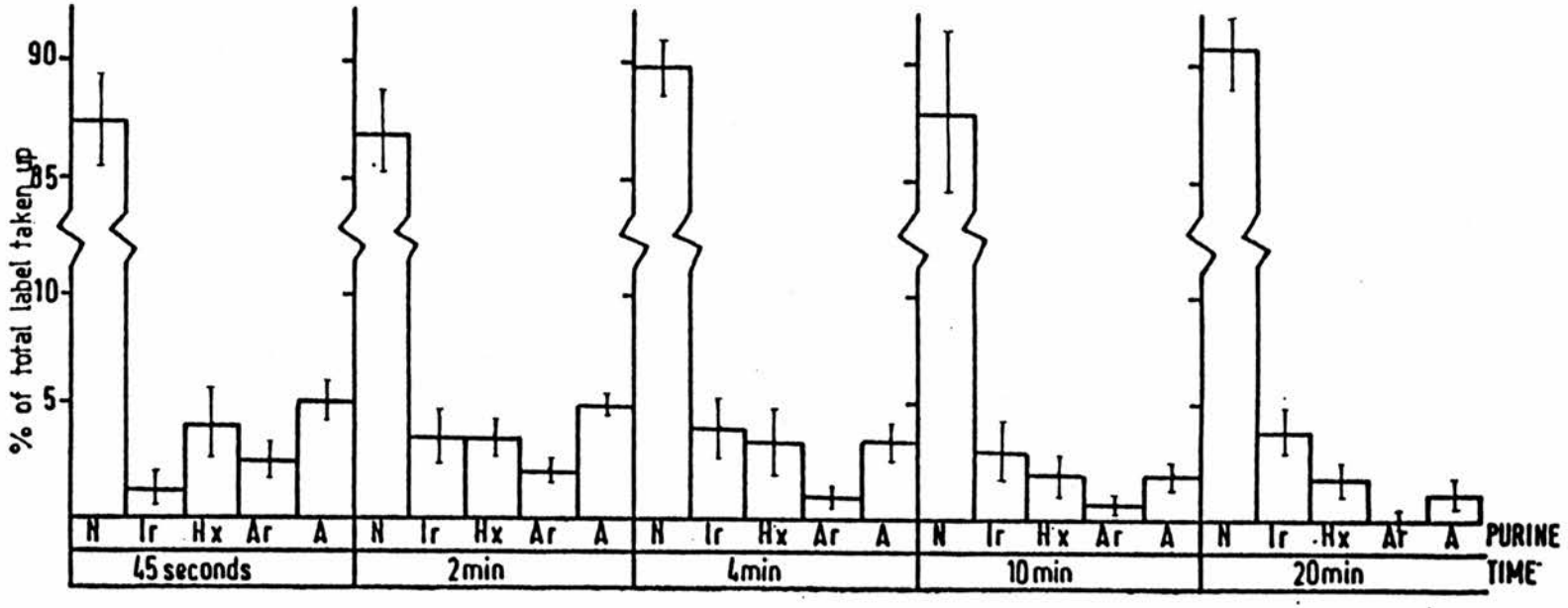



Figure 3.14 METABOLISM OF [2,8-$^{3}$H] ADENOSINE BY WHOLE CELLS.

membrane to drive facilitated diffusion. This possibility was investigated by determining the metabolic fate of [2,8-$^{3}$H] adenosine taken up and determination of the intracellular concentration of adenosine. The intracellular concentration will be dealt with in a later section (3.6) when considering the mechanism of adenosine uptake in more detail.

The experimental conditions for studying the metabolic fate of adenosine are given in the legend to Figure 3.14. Figure 3.14 shows the variation in distribution of [2,8-$^{3}$H] adenosine in various nucleotide, nucleoside and base pools as a percentage of the total label taken up.

This experiment shows that when adenosine is taken up it is very quickly metabolised to nucleotide. Over 85% of the label taken up at 45 seconds is present as nucleotide. This percentage increases to over 90% after 20 minutes. The percentage of label present as adenine falls as nucleotide levels rise, as does the percentage of the label present as adenosine. However it should be noted that the percentage of label present as adenosine is always lower than the percentage as adenine and becomes undetectable before adenine. It should also be borne in mind that uptake has almost slowed to a stop by 3 minutes (section 3.2.1), therefore all changes in the distribution of label after this time are due to metabolism. The possible significance of the levels of hypoxanthine and inosine will be considered in the discussion.

Having shown that adenosine taken up is metabolised to nucleotide the questions asked were, what intermediates are involved, and what enzymes are responsible for this metabolism ?.

Table 3.2

METABOLISM OF [2,8-$^{3}$H] ADENOSINE AND [8-$^{3}$H] ADENINE BY DIALYSED TRYPANOSOME LYSATE

| | | | | % OF TOTAL COUNTS | | |
|---|---|---|---|---|---|---|
| SUBSTRATE $^{3}$H | ATP | ATP(R) | PRPP | % NUCLEOTIDE | % ADENOSINE | % ADENINE |
| ADENOSINE | + | - | - | 5 | 4 | 90 |
| ADENOSINE | - | - | + | 87 | 7 | 4 |
| ADENOSINE | - | + | - | 3 | 5 | 91 |
| ADENOSINE | - | - | - | 0.4 | 0.4 | 99 |
| ADENINE | + | - | - | 1 | 0.8 | 98 |
| ADENINE | - | - | + | 97 | 1 | 1.2 |
| ADENINE | - | + | - | 1 | 2 | 95 |
| ADENINE | - | - | - | 2.1 | 4 | 94 |

A fresh dialysed trypanosome lysate (0.1mg protein/ml) was incubated with $^{3}$H labelled substrates at concentrations of 10μM (specific activity 50μCi/μmol) in PSG buffer pH 7.4, 30°C for 3 minutes. As shown in the table the incubations contained either ATP (1mM), PRPP (0.5mM) or ATP(R) which was ATP plus an ATP regenerating system. The incubations containing the ATP regenerating system contained 10mM KCl, 2mM $MgCl_2$, 1mM ATP, 1mM PEP, NADH 0.16mM, pyruvate kinase 100 units and lactate dehydrogenase 100 units. All other incubations were suplemented with 2mM $MgCl_2$. All concentrations given above were the final concentrations in the incubation. The incubations were terminated by the addition of PCA (1M final concentration) followed by neutralisation and TLC of 10μl samples as described in Materials & Methods (Section 2.8). Radioactivity in the separated components was estimated by liquid scintillation counting.

### 3.2.5 Metabolism of Adenosine by Dialysed Cell Lysate.

In intact mammalian brain tissue exogenous adenosine is taken up and metabolised to nucleotide by adenosine kinase (ATP:adenosine 5-phosphotransferase, EC 2.7.1.20 (Shimizu et al., 1972). In mouse leukaemia cells adenosine kinase is also responsible for phosphorylation of adenosine taken up with excess adenosine being deaminated to inosine by adenosine deaminase (Adenosine aminohydrolase, EC 3.5.4.4) (Lum et al., 1979). In the human erythrocyte phosphorylation by adenosine kinase appears to be the main route adenosine takes to nucleotide. Erythrocytes also contain the enzymes adenosine phosphorylase (Adenosine orthophosphate ribosyltransferase, EC 2.4.2.1) and adenine phosphoribosyltransferase (AMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) (APRT). However the levels of PRPP in fresh erythrocytes is very low which would limit the cell's ability to form AMP from adenine, produced by the phosphorylase, using the phosphoribosyltransferase.(Grimes, 1980).

A recent publication by Davies et al.(1983) gives a list of purine metabolising enzymes found in T.cruzi, T.brucei and L.mexicana with their relative specific activities. The results presented in this thesis are in broad agreement with those of Davies et al.(1983) with three exceptions which will be considered below.

With the above background in mind a dialysed trypanosome lysate was incubated with the substrates as listed in Table 3.2. The experimental conditions are given in detail in the legend to Table 3.2.

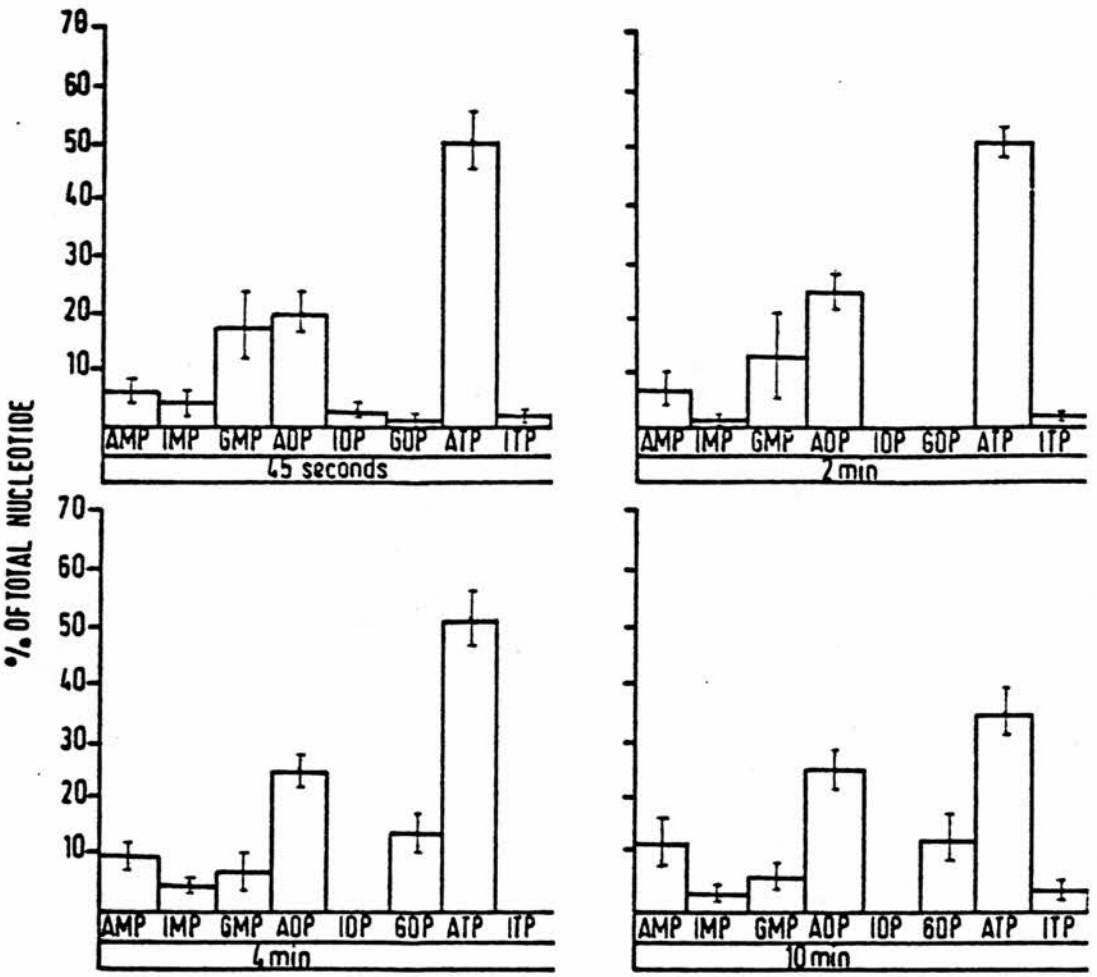
When the dialysed lysate was incubated with [2,8-$^{3}$H] adenosine, ATP and magnesium, 90% of the label was metabolised to adenine with virtually no nucleotide formed. The same result was obtained in the

presence of an ATP regenerating system. This result indicates that there is little or no adenosine kinase activity present in the lysate. When the experiment is repeated and ATP replaced by PRPP, 87% of the label is metabolised to nucleotide which demonstrates the presence of a phosphoribosyltransferase in the lysate. Adenosine alone added to the lysate results in the conversion of the adenosine to adenine. These results show that adenosine is first deribosylated to adenine and then phosphorylated by a phosphoribosyltransferase. These enzymes and their properties will be considered in section 3.4.

The metabolic steps outlined above are consistent with the results obtained for the metabolism of adenosine by whole cells (section 3.2.4). Adenine levels are higher than adenosine levels both of which fall as nucleotide is formed with adenosine levels falling to zero before adenine levels.

The absence of adenosine kinase activity is in disagreement with the results for T.brucei published by Davies et al.(1983) who found the specific activity of adenosine kinase to be 22% of the specific activity of APRT. These same authors also detected adenosine deaminase activity which was not detected (in this work) in the dialysed lysate since no labelled inosine was detected when adenosine alone was used as substrate. Adenosine deaminase could not be detected in trypanosome lysate by the spectrophotometric method described in Materials and Methods (Section 2.10.3). The lysate also failed to produce any hypoxanthine from added [8-$^{3}$H] adenine indicating the absence of adenine deaminase (Adenine aminohydrolase EC 3.5.4.2). Adenine deaminase could not be detected by the spectrophotometric method used as described in Materials and Methods (Section 2.10.3). Again the lack of these enzymes is in disagreement

**Figure 3.15 DIVERSITY OF LABELLED NUCLEOTIDE IN WHOLE CELL.**




Trypanosomes (1mg protein/ml) were incubated in PSG buffer pH 7.4, 22°C with 0.5µM labelled adenosine (1mCi/µmole) for 45 seconds to 10 minutes. Samples of the incubation medium (500µl) were taken and centrifuged on silicon sandwiches with 40% formic acid as the lower layer. Samples of the formic acid layer (100µl) taken and prepared for analysis by HPLC as described in Materials & Methods (Section 2.9).Radioactivity in the resolved components was estimated by liquid scintillation counting. The results shown are the mean and range of duplicate experiments.

with the results of Davies et al.(1983).

The nucleotide produced so rapidly by whole cells may be metabolised into diverse forms. Therefore the nucleotide content of cells metabolising [2,8-$^{3}$H] adenosine was analysed to determine the diversity of nucleotides formed.

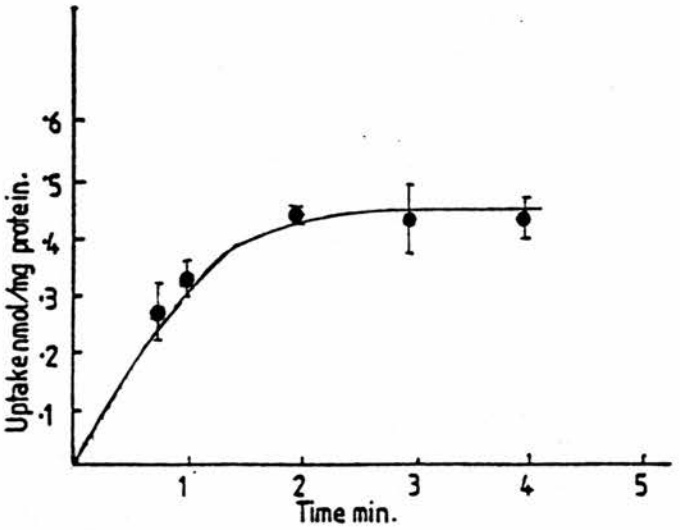
### 3.2.6 Diversity of Labelled Nucleotide in Whole Cells.

The experimental details for the analysis of labelled nucleotides, produced by the metabolism of [2,8-$^{3}$H] adenosine, in whole cells is given in the legend to Figure 3.15 and in Materials and Methods (Section 2.9).

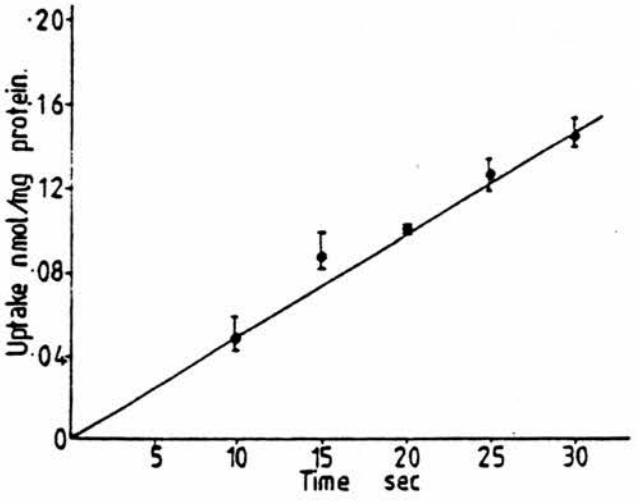
Figure 3.15 shows that the [2,8-$^{3}$H] adenosine taken up is rapidly metabolised to ATP. Initially AMP is the least significant adenine nucleotide followed by ADP then ATP. ATP levels are already at a maximum after 45 seconds of the incubation, ADP levels reach a maximum after 2 minutes and AMP levels continue to rise for the duration of the experiment (10 minutes); as the AMP and ADP levels rise ATP levels fall.

Bearing in mind that after 3 minutes uptake of adenosine has almost ceased the result could reflect a deficiency of adenine nucleotide in the cell which is made up after 3 minutes with a rapid flux of label through the synthetic pathway to ATP followed by a distribution of the label throughout the adenine nucleotide pool as general metabolism involving ATP consumption proceeds. The possible significance of the other nucleotides labelled will be considered in the discussion.

**Figure 3.16 TIME COURSE OF HYPOXANTHINE UPTAKE.**




Trypanosomes 0.6lmg protein/ml were incubated in PSG buffer pH 7.4, 22°C with 1μM [G-$^{3}$H] hypoxanthine (50μl;0.1mM,specificactivity 50μCi/μmole). Uptake was assayed by liquid scintillation counting of samples of the PCA layer of silicon sandwiches (500μl of incubation) centrifuged at the times shown. The results are given as the mean and range of triplicate determinations.



## 3.3 HYPOXANTHINE AND ADENINE UPTAKE AND METABOLISM.

The results of the previous sections have shown that adenosine is taken up and metabolised by T.brucei, but adenosine is not the only source of purine found in the plasma. Hypoxanthine and adenine are present in the plasma at almost the same concentration as adenosine (Section 3.2). Since hypoxanthine and adenine are present in the plasma it is possible that the trypanosomes could make use of these compounds as a source of preformed purine ring.

The ability of T.brucei to take up hypoxanthine and adenine was investigated using the same methods as were used in the investigation of adenosine uptake. Before any detailed investigations of hypoxanthine uptake were considered the time course of hypoxanthine uptake was determined.

### 3.3.1 Time Course of Hypoxanthine Uptake.

Hypoxanthine uptake was measured as a function of time by the silicon sandwich centrifugation technique, described in Materials and Methods (Section 2.11.2), using $^{3}$H-hypoxanthine as substrate.

The uptake assay was started by the addition, with thorough mixing, of [G-$^{3}$H] hypoxanthine to 5ml of a cell suspension to give a final concentration of 1μM hypoxanthine. Hypoxanthine uptake was estimated by liquid scintillation counting of samples of the PCA layer from the silicon sandwiches. The results of these experiments and the experimental details are given in Figure 3.16 and legend respectively.

Figure 3.16 shows the long- and short-term time courses for

Table 3.3 EFFLUX OF HYPOXANTHINE

| Time min. | % Total counts released in PSG | % Total counts released in PSG +Hx |
|---|---|---|
| 0·5 | 4·0 ±1·0 | 4·3 ±1·1 |
| 1 | 3·4±1·2 | 4·0 ±1·2 |
| 2 | 3·6 ±0·5 | 4·0 ± 0·4 |
| 4 | 3·1 ±0·2 | 4·2 ± 0·5 |

Trypanosomes were suspended in PSG pH 7.4 to a concentration of 0.127mg protein/ml (total volume 3ml). [G-$^{3}$H] Hypoxanthine (500µC/µmol) was then added to a final concentration of 2µM and the mixture incubated at 22°C for 5 minutes. The trypanosomes were then centrifuged (8,000xg av) at 0°C and resuspended in PSG at 0°C four times. After the final wash step the cells were resuspended in either PSG or PSG supplemented with 20µM unlabelled hypoxanthine at 22°C. After resuspension samples of cells were taken and added to liquid scintillation fluid for estimation of total uptake. Further samples were taken at the times shown in the table above and the cells removed by centrifugation. The label in the resultant supernatants was estimated by liquid scintillation counting. The results shown in the table are the means and population deviations of five experiments.

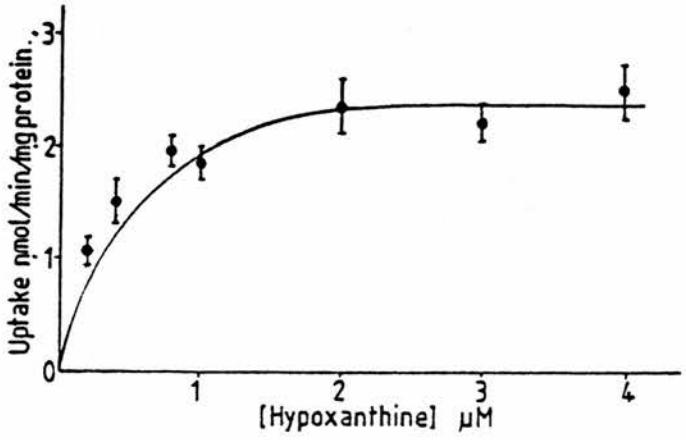
hypoxanthine uptake. The overall pattern of uptake is similar to that obtained in the investigation of adenosine uptake, the only difference being, the amount of hypoxanthine taken up is 30% less than the amount of adenosine taken up at the same time point. The uptake of hypoxanthine is initially very rapid but appears to cease after 2 minutes as was the case for adenosine uptake.

When the time course experiment was repeated at a temperature of $0^{o}C$ the uptake of hypoxanthine was reduced to barely detectable levels. This temperature dependence suggests that hypoxanthine uptake is not by simple diffusion but is a carrier mediated process.

The halt in hypoxanthine uptake is similar to that observed during the time course of adenosine uptake (Figure 3.11) in that it is not due to the cells using up all the available substrate. Calculation, as for adenosine uptake, of the amount of hypoxanthine taken up at four minutes shows that only 27% of the added hypoxanthine has been used.

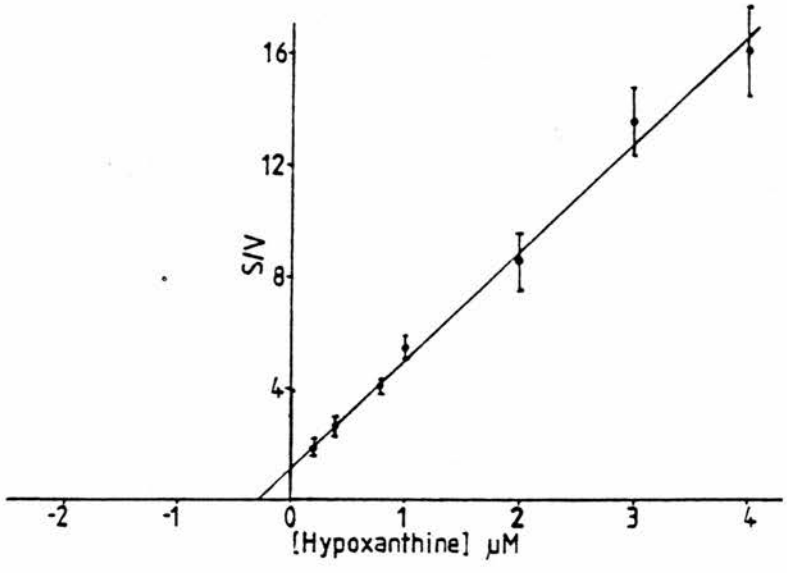
The argument that the system comes to a steady state of influx and efflux was tested in the same manner as for adenosine uptake (Section 3.2.1). The results of this experiment and the experimental details are given in Table 3.3 and legend respectively. As was the case for adenosine uptake, addition of unlabelled hypoxanthine to cells pre-loaded with [G-$^{3}H$] hypoxanthine does not cause an efflux of $^{3}H$ from the cells. This indicates that the apparent halt in the uptake of hypoxanthine is not due to the system coming to a steady state. The results also indicate that there is little or no non-specific surface binding of hypoxanthine to the cell surface as was the case for adenosine (Section 3.2.1).

**Figure 3.17a CONCENTRATION DEPENDENCE OF HYPOXANTHINE UPTAKE.**




The rate of hypoxanthine uptake was assayed over the concentration range 0.2 to 4µM (constant specific activity 50µCi/µmole). Incubations contained trypanosomes at a density of 0.60mg protein/ml in PSG buffer pH 7.4, 22°C. All incubations were for 15 seconds uptake being measured by the silicon sandwich centrifugation technique as described in the legend to Figure 3.11. The results are shown as the mean and range of duplicate experiments.

**Figure 3.17b HANES PLOT OF THE DATA PRESENTED IN FIGURE 3.16a.**



### 3.3.2 Kinetic Parameters of Hypoxanthine Uptake.

The kinetic parameters of hypoxanthine uptake were determined by incubating trypanosomes with increasing concentrations of [G-$^3$H] hypoxanthine. Full experimental details are given in the legend to Figure 3.17a. As shown for adenosine uptake (Section 3.2.1) hypoxanthine uptake is linear over the first 10 to 30 seconds as shown by Figure 3.16, therefore initial rates of uptake were measured with 15 second incubations in this experiment. It should also be borne in mind that the kinetic parameters measured are those for uptake and not for transport alone unless transport is rate limiting for uptake.

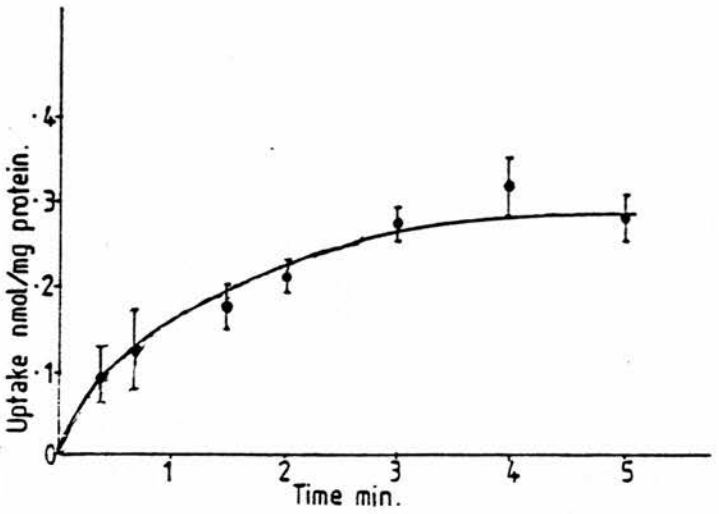
Figure 3.17a shows that the uptake of hypoxanthine is saturable and Figure 3.17b is a Hanes plot of uptake data presented in Figure 3.17a. From the Hanes plot the Km and Vmax can be calculated as 0.3μM and 0.27 nmol/min/mg protein respectively. The low Km of 0.3μM is consistent with estimates of plasma hypoxanthine concentration of 0.7-1.0μM (Section 3.2).

### 3.3.3 Time Course of Adenine Uptake.

The uptake of adenine was measured as a function of time by the silicon sandwich centrifugation technique, as described in Materials & Methods (Section 2.11.2), using [8-$^3$H] adenine as substrate. The experimental details are given in the legend to Figure 3.18.

The time course of adenine uptake (shown in Figure 3.18) was found to be similar to tht obtained for adenosine and hypoxanthine uptake with uptake being linear for the first 30 seconds.

Figure 3.18 TIME COURSE OF ADENINE UPTAKE.




Trypanosomes 0.5mg protein/ml were incubated in PSG buffer pH 7.4, 22°C with 1uM [8-$^{3}$H] adenine (50µl;0.1mM,specificactivity 50µCi/µmole). Uptake was assayed by liqud scintillation counting of samples of the PCA layer of silicon sandwiches (500µl of incubation) centrifuged at the times shown. The results are given as the mean and range of triplicate determinations.

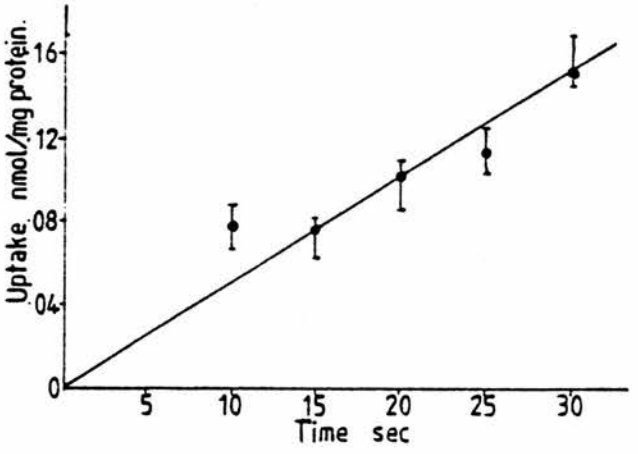
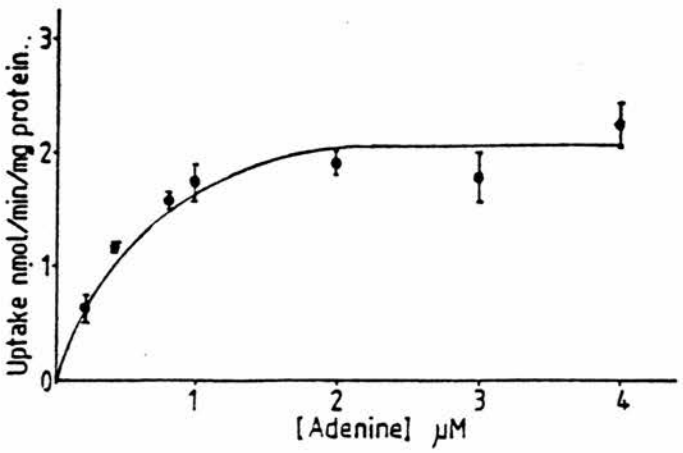
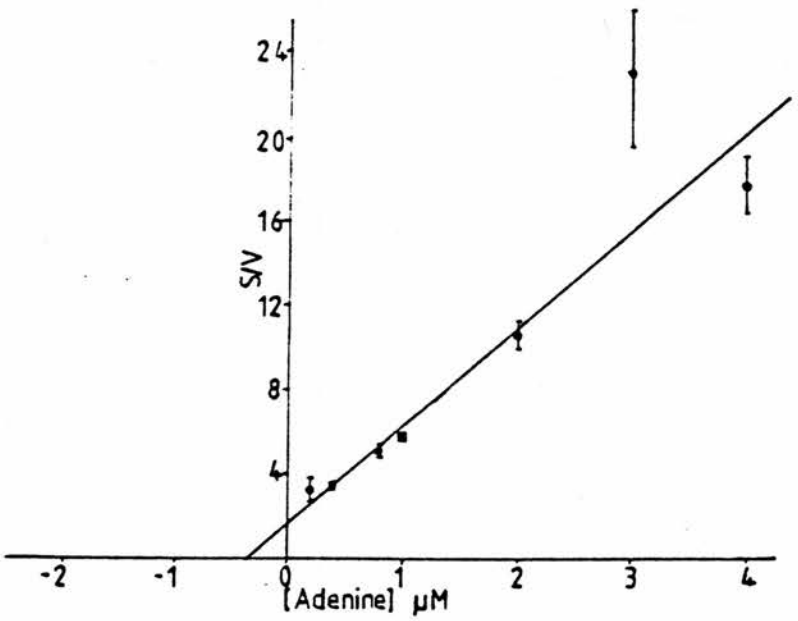


**Figure 3.19a CONCENTRATION DEPENDENCE OF ADENINE UPTAKE.**




The concentration dependence of adenine uptake was determined as described for hypoxanthine in the legend to Figure 3.17a with labelled adenine being substituted for hypoxanthine.

**Figure 3.19b HANES PLOT OF THE DATA PRESENTED IN FIGURE 3.19a.**



### 3.3.4 Kinetic Parameters of Adenine Uptake.

The kinetic parameters of adenine uptake were determined as described for hypoxanthine uptake in Section 3.3.2 with experimental details given in the legend to Figure 3.19a. The kinetic parameters measured only apply to uptake and not to transport alone for the reasons detailed in the previous sections.

Figure 3.19a shows that uptake of adenine is a saturable process and Figure 3.19b is a Hanes plot of the data in Figure 3.19a. From the Hanes plot the Km and Vmax for uptake can be calculated to be 0.35μM and 0.21nmol/min/mg protein respectively. Again the estimated Km for uptake is within the normal range of plasma adenine concentrations.

### 3.3.5 Metabolism of Hypoxanthine by Whole Cells.

As for the uptake of adenosine the uptake of hypoxanthine may be dependent on its subsequent metabolism to maintain a hypoxanthine gradient across the plasma membrane to drive facilitated diffusion. Therefore the metabolism of [G-$^{3}$H] hypoxanthine by whole cells was investigated and the intracellular concentration of hypoxanthine estimated.The intracellular concentration of hypoxanthine will be dealt with in a later Section (3.6) when considering the transport mechanisms in more detail.

The experimental conditions for studying the metabolic fate of hypoxanthine are given in the legend to Figure 3.20. Figure 3.20 shows the variation in distribution of [G-$^{3}$H] hypoxanthine in various nucleotide, nucleoside and base pools as a percentage of the total label taken up.

Trypanosomes (1mg protein/ml) were incubated in PSG buffer pH 7.4, 22°C with 0.5μM [G-$^3$H] hypoxanthine (1mCi/μmole) for 45 seconds to 20 minutes. Samples of the incubation medium (500μl) were taken and centrifuged on silicon sandwiches at the times shown. Samples of the PCA layer (100μl) were neutralised by the addition of KOH (50μl) and 10 μl of the neutralised material applied to silica gel TLC plates and chromatographed with unlabelled standards as described in Materials & Methods (Secion 2.8). Radioactivity in the resolved components was estimated by liquid scintilation counting. The results are the mean and range of duplicate experiments. N=Nucleotide, Ir=Inosine, Hx=Hypoxanthine, Ar=Adenosine and A=Adenine.

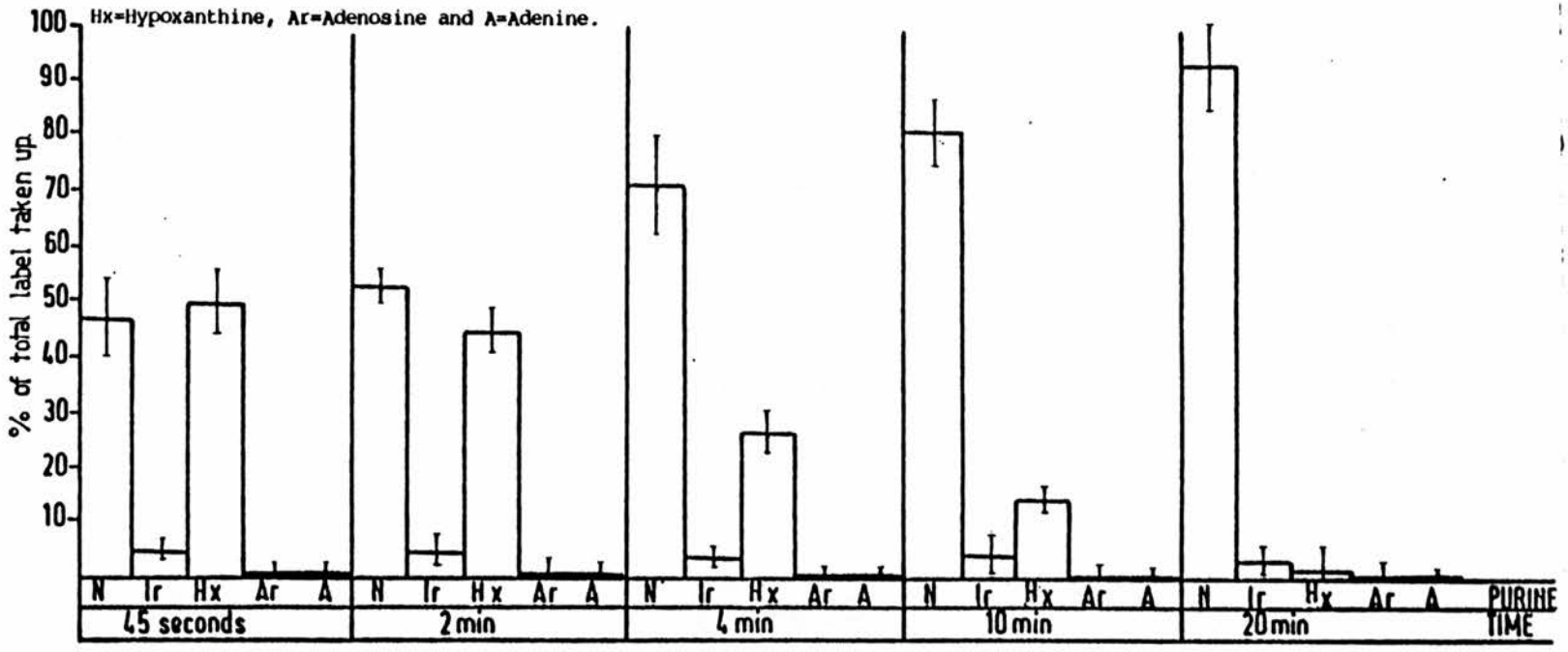



Figure 3.20 METABOLISM OF [G-$^3$H] HYPOXANTHINE BY WHOLE CELLS.

The results of these experiments (Figure 3.20) show that when hypoxanthine is taken up it is very quickly metabolised to nucleotide. The results also show that as the percentage of label present as hypoxanthine falls the percentage of label present as nucleotide rises. The only other compound labelled was inosine (Ir) which is labelled to a limited extent and is probably produced by the dephosphorylation of IMP formed from the hypoxanthine. It should be noted that uptake has almost ceased after 2 minutes (Section 3.3.1), therefore all changes in the distribution of label after this time are due to metabolism only.

The direct formation of nucleotide from hypoxanthine can only take place via the enzyme hypoxanthine phosphoribosyltransferase (IMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) (HxPRT). The assay and kinetic parameters of this enzyme are described in Section 3.4.

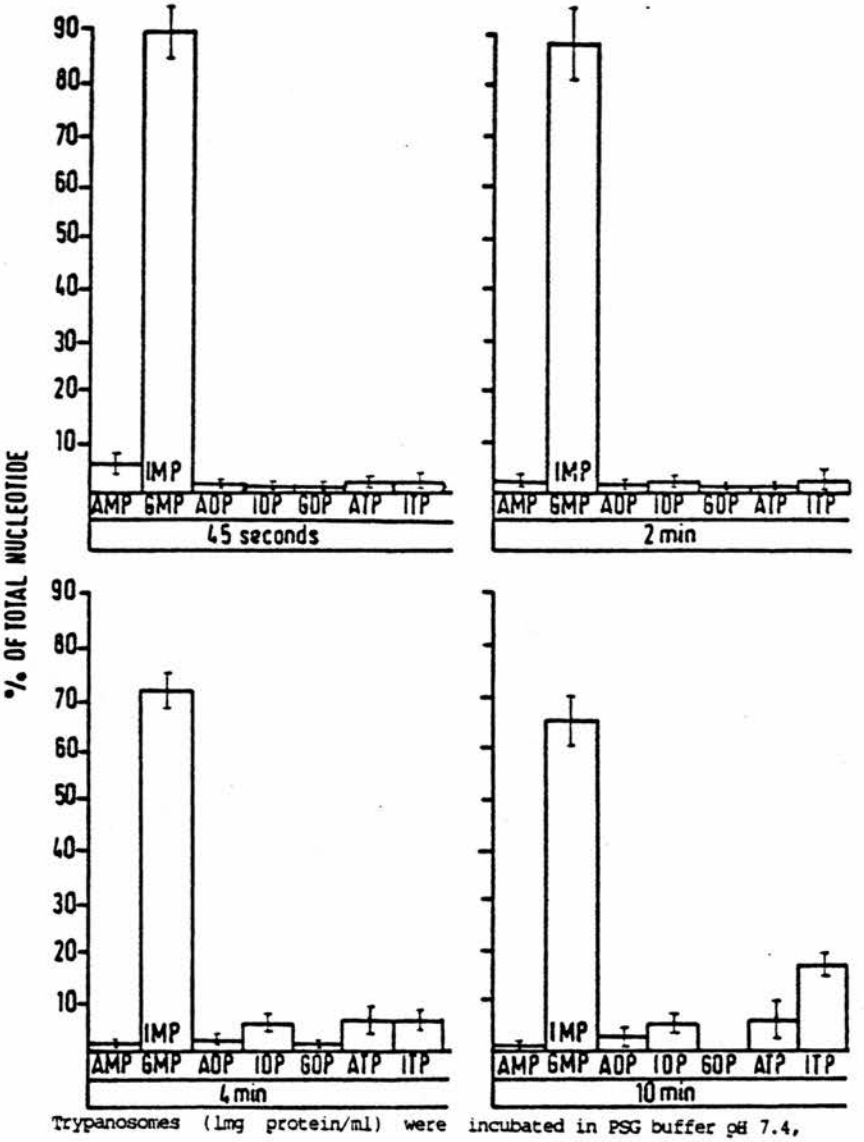
The nucleotide produced by whole cells from hypoxanthine may be metabolised into diverse forms. Therefore the nucleotide content of cells metabolising [G-$^{3}$H] hypoxanthine was analysed to determine the diversity of nucleotide formed.

### 3.3.6 Diversity of Labelled Nucleotide in Whole Cells.

The experimental details for the analysis of labelled nucleotides produced by the metabolism of [G-$^{3}$H] hypoxanthine by whole cells is given in the legend to Figure 3.21 and in Materials and Methods (Section 2.9).

Figure 3.21 shows that the [G-$^{3}$H] hypoxanthine taken up is first metabolised to IMP. As time passes the IMP is metabolised to IDP and

Figure 3.21 DIVERSITY OF LABELLED NUCLEOTIDE IN WHOLE CELLS.




Trypanosomes (1mg protein/ml) were incubated in PSG buffer pH 7.4, 22°C with 0.5µM [G-$^{3}$H] hypoxanthine (1mCi/µmole) for 45 seconds to 10 minutes. Samples of the incubation medium (500µl) were taken and centrifuged on silicon sandwiches with 40% formic acid as the lower layer. Samples of the formic acid layer (100µl) taken and prepared for analysis by HPLC as described in Materials & Methods (Section 2.9). Radioactivity in the resolved components was estimated by liquid scintillation counting. The results are the mean and range of duplicate experiments.

eventually to ITP. The formation of ITP from hypoxanthine is much slower than the formation of ATP from adenosine (Section 3.2.6) which may reflect a difference in the trypanosome's requirements for these two nucleotide's precursors. A full comparison between the metabolism of hypoxanthine and adenosine will be made in the Discussion (Section 4.2.4).

## 3.4 ENZYMES OF PURINE NUCLEOSIDE AND BASE METABOLISM.

In Sections 3.2 and 3.3 the kinetic parameters of the adenosine, adenine and hypoxanthine uptake systems were defined. The metabolic fates of these compounds were also determined (Sections 3.2.4 & 3.3.5) and indications as to the enzymes involved in this metabolism were obtained (Sections 3.2.5 & 3.3.5). The enzymes involved in metabolism of purine nucleosides and bases were studied in more detail with a view to determining their kinetic parameters.

### 3.4.1 Summary of Adenosine Metabolism and Phosphate Dependence of Adenosine Hydrolase.

The metabolic fate of [2,8-$^{3}$H] adenosine in whole cells was shown to be rapid conversion to ATP (Sections 3.2.4 & 3.2.6). A time course of this metabolism and the metabolism of [2,8-$^{3}$H] adenosine by a dialysed cell lysate (Sections 3.2.4 & 3.2.5) showed that adenosine is first deribosylated to adenine, then the adenine converted to nucleotide by APRT. No evidence of an adenosine kinase could be found. Figure 3.22 shows diagrammaticaly the possible routes by which adenosine could be metabolised. Step 3 of Figure 3.22, representing phosphorylation of adenosine by adenosine kinase, as stated previously does not take place (Section 3.2.5). The initial

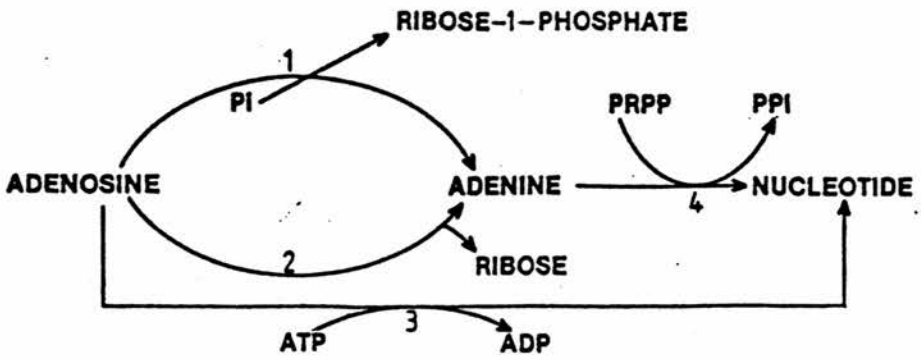



**Figure 3.22 POSSIBLE ROUTES OF ADENOSINE METABOLISM.**

(1) ADENOSINE PHOSPHORYLASE (EC 2.4.2.1)

(2) ADENOSINE HYDROLASE (EC 3.2.2.1)

(3) ADENOSINE KINASE (EC 2.7.1.20)

(4) ADENINE PHOSPHORIBOSYLTRANSFERASE (EC 2.4.2.7)

**Table 3.4 PHOSPHATE DEPENDENCE OF ADENOSINE DERIBOSYLASE ACTIVITY.**

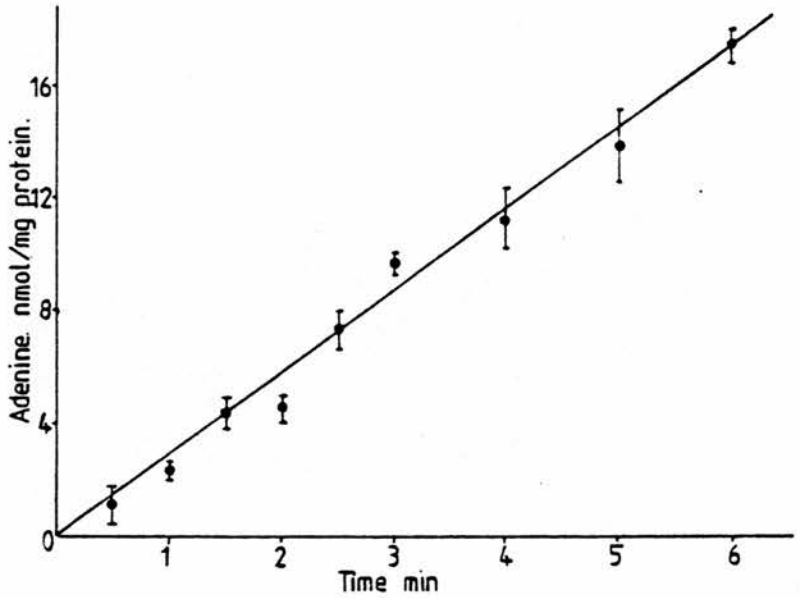
| [Pi] mM | 0 | ·0135 | ·027 | ·18 | ·25 | ·5 | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|---|
| ADENINE µmol/min/mg | ·180 ± ·002 | ·176 ± ·020 | ·174 ± ·014 | ·181 ± ·018 | ·156 ± ·034 | ·160 ± ·011 | ·172 ± ·040 | ·181 ± ·022 | ·171 ± ·031 |

[2,8-$^{3}$H] adenosine (40µM; specific activity 50µCi/umole) was incubated with dialysed trypanosome lysate (0.02mg protein/ml) in 10mM TrisHCl, 1mM DTT buffer pH 7.4 at 25°C for 2 minutes. The assay was terminated by heating samples of the incubation to 120°C as described in the text (Section 3.4.2). Samples of the incubation media were then analysed for their content of labelled adenine by TLC and liquid scintillation counting as described in Materials & Methods (Section2.8). The results are the mean and range of duplicate incubations.

deribosylation of adenosine to adenine could take place by either route 1 or route 2 as shown in Figure 3.22. The first route via adenosine phosphorylase (Section 3.2.5) is known to take place in the mammalian erythrocyte, (Tsuboi & Hudson,1957; Huennekens et al.,1956; Grimes 1980) brain (Huennekens et al.,1956) and liver (Korn & Buchanan,1955). The hydrolytic pathway, route 2, is known to operate in some bacteria (Rabinowitz & Barker,1965) and in bakers yeast (Heppel & Hilmoe,1952). The hydrolytic pathway has also been shown to exist in T.gambiense bloodstream forms (Schmidt et al.,1975). Davies et al.(1983) detected the presence of adenosine phosphorylase in T.cruzi, T.brucei and L.mexicana; these same three organisms were also found to be capable of phosphorolysis of guanosine but not inosine. However all three of the above organisms showed inosine hydrolase activity. In view of this diversity of distribution of nucleoside hydrolase and phosphorylase activities the phosphate dependence of the metabolism of adenosine to adenine was investigated.

The phosphate dependence of the metabolism of adenosine to adenine was investigated by incubation of [2,8-$^{3}$H] adenosine with exhaustively dialysed trypanosome lysate with increasing concentrations of inorganic phosphate added. The results of these experiments and experimental details are given in Table 3.4 and the legend to Table 3.4. The results show that the metabolism of adenosine to adenine by the trypanosome lysate is phosphate independent. This indicates that in the whole cell adenosine is metabolised to adenine and ribose, via route 2 (Figure 3.22), by adenosine hydrolase (Kalckar,1947; Schmidt et al.1975). Having determined the type of enzyme involved in adenosine hydrolysis its

Figure 3.23 TIME COURSE OF ADENOSINE HYDROLASE ACTIVITY.




The above figure shows the amount of adenine produced by adenosine hydrolase with time. A trypanosome lysate (0.2mg protein/ml) was incubated with [2,8-$^{3}$H] adenosine at a concentration of 50μM (specific activity 100μCi/μmol) for the times shown at 22°C. The reaction was terminated and the amount of adenine formed estimated as described in the text (Section 3.4.2). The results are given as the mean and range of duplicate experiments.

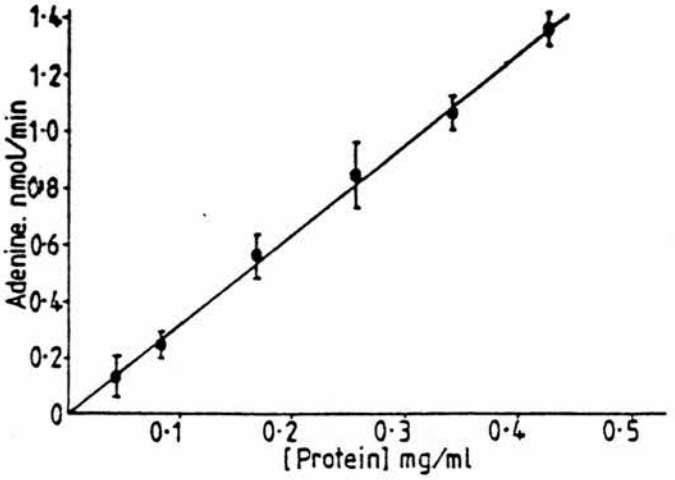
kinetic parameters were determined.

### 3.4.2 Kinetic Parameters of Adenosine Hydrolase.

Preliminary experiments were conducted to determine the optimum protein concentration and incubation duration for measuring initial rates of adenosine hydrolase activity. The results of these experiments are shown in Figures 3.23 (time course) and Figure 3.24 (protein dependence). The experimental details are given in the figure legends. For estimates of Km and Vmax the following experimental protocol was followed:

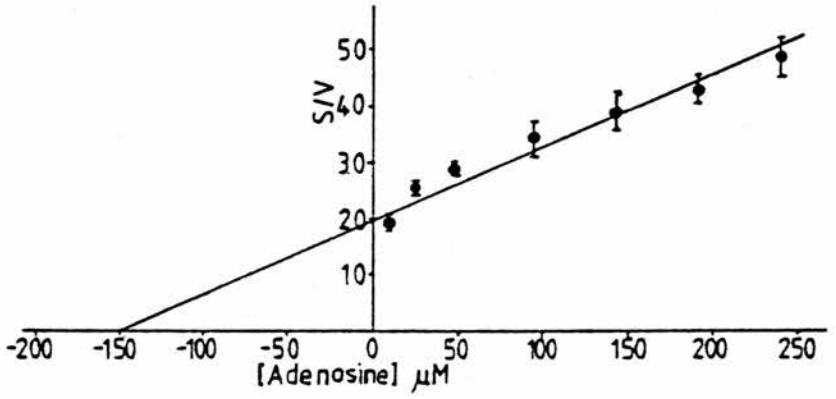
Increasing concentrations of [2,8-$^{3}$H] adenosine at a constant specific activity of 100μCi/μmol. were incubated with trypanosome lysate. The lysate was added to the assay mix to a final concentration of 0.2mg protein /ml in TrisHCl buffer 10mM, DTT 1mM at pH 7.4. The assays were started by addition of trypanosome lysate and incubated at 22$^{o}$C for 3 minutes. The assays were terminated by pipetting samples (200μl) of the assay mix into glass tubes heated to 120$^{o}$C which were maintained at this temperature for a further 1 minute. Precipitated protein was then removed by centrifugation and 10μl samples of the supernatants with unlabelled adenosine and adenine carrier added were chromatographed by TLC as described in Materials and Methods (Section 2.8). The production of adenine was estimated by cutting the resolved adenine spots from the TLC plate and estimating their content of radioactive adenine by liquid scintillation counting. No metabolism of the adenosine to any other nucleoside or base was detected since all added label was recovered as adenine or adenosine, no significant radioactivity being detected on any other sections of the TLC plate. The initial rate of adenine

Figure 3.24 PROTEIN DEPENDENCE OF ADENOSINE HYDROLASE ACTIVITY.



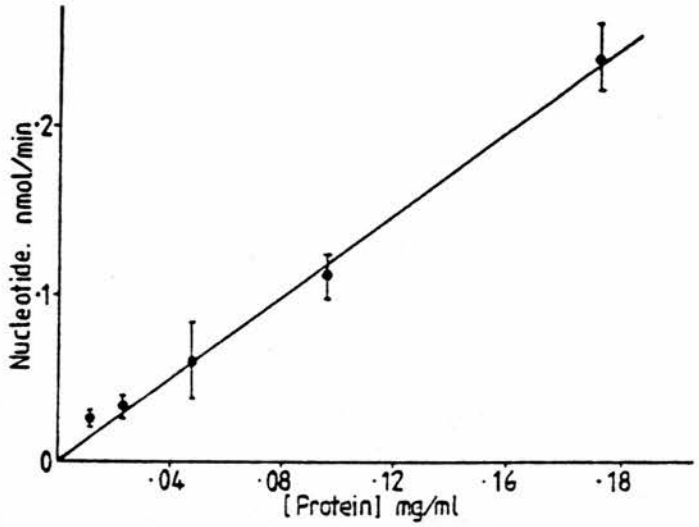

The above figure shows the dependence of adenine production on increasing protein concentration. All experimental conditions were as describes in the legend to Figure 3.23 with the exception of protein concentration. The results are given as the mean and range of duplicate experiments.

Figure 3.25 CONCENTRATION DEPENDENCE OF ADENOSINE HYDROLASE ACTIVITY.




The figure shows a Hanes plot of the initial rates of formation of adenine from adenosine over an adenosine concentration range 9.6µM to 150µM. The experimental protocol used is described in the text (section 3.4.2). The results show the mean and range of duplicate incubations.

**Figure 3.26 PROTEIN DEPENDENCE OF ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY.**




The above figure shows the dependence of the amount of nucleotide produced by adenine phosphoribosyltransferase on protein concentration. A trypanosome lysate at the protein concentrations shown was incubated with [8-$^{3}$H] adenine at a concentration of 2μM (specific activity 100μCi/μmol) for 2.5 minutes at 22°C. The reaction was terminated and the amount of adenine formed estimated as described in the text (Section 3.4.3). The results are given as the mean and range of duplicate experiments.

production was estimated in duplicate for each adenosine concentration used in the range 9.6μM to 240μM.

The results of these experiments are shown as a Hanes plot in Figure 3.25. From this plot the kinetic parameters of the adenosine hydrolase were calculated to be : Km 150μM and Vmax 7.69nmol/min/mg protein. The significance of these results will be considered in Section 3.4.7.

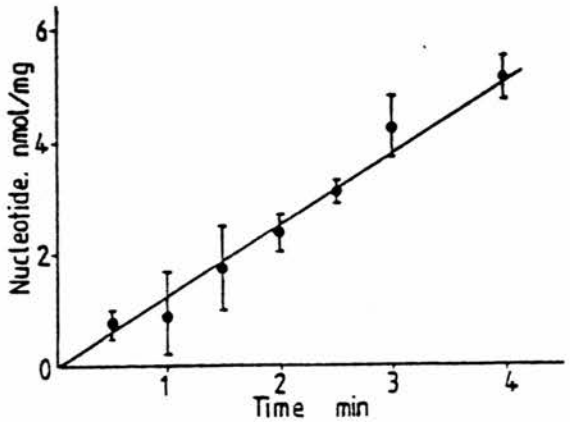
### 3.4.3 Kinetic Parameters of Adenine Phosphoribosyltransferase.

After hydrolysis of adenosine to adenine and ribose the next step in the metabolism of the purine ring is conversion to nucleotide by APRT using PRPP. The kinetic parameters of this enzyme were estimated as detailed below.

As for the estimation of the kinetic parameters of adenosine hydrolase preliminary experiments were conducted to determine optimal magnesium concentrations and a non-rate limiting concentration of PRPP for the estimation of initial rates of reaction. The optim um time and protein concentration for estimation of initial rates of reaction were also determined as shown in Figures 3.26 and 3.27. Experimental details are given in the figure legends.

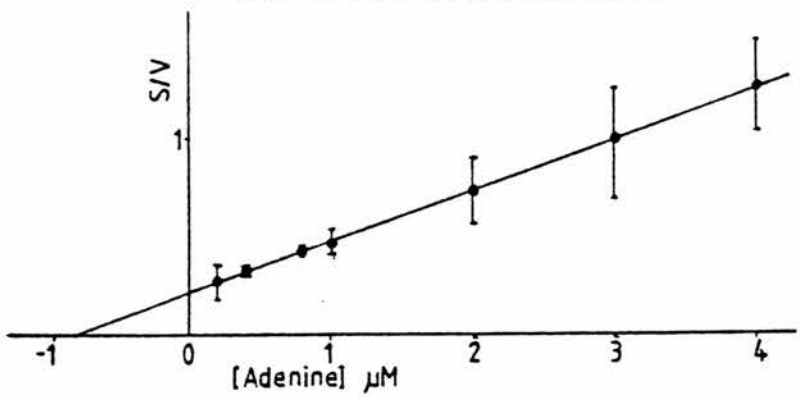
The assay mixture contained [8-$^{3}$H] adenine, at a constant specific activity of 100μCi/μmol., over a concentration range of 0.2μM to 4μM. The enzyme source was a fresh trypanosome lysate, added to the assay mix to give a final protein concentration of 0.024mg/ml. The assay mix consisted of TrisHCl buffer 10mM, DTT 1mM, $MgCl_2$ 1mM, PRPP 0.5mM at pH 7.4. The assays were started by the addition of lysate and incubated at 22$^{o}$C for 2.5 minutes. The assays were terminated by rapid heating to 120$^{o}$C of 200μl samples as described in

Figure 3.27 TIME COURSE OF ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY.



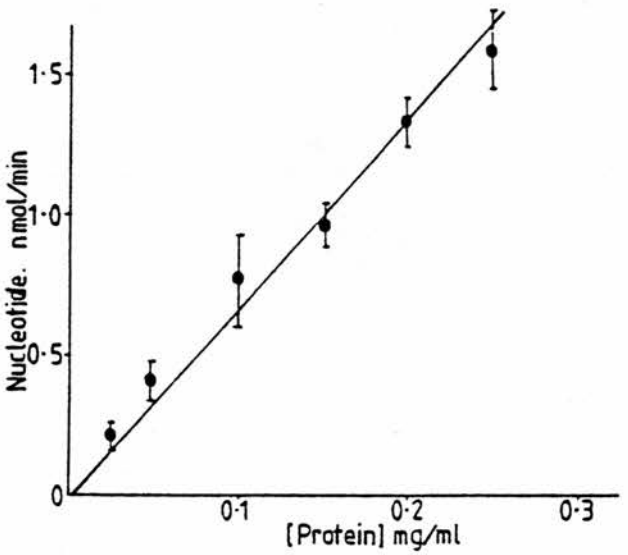

The above figure shows the dependence of the amount of nucleotide produced by adenine phosphoribosyltransferase on time. A trypanosome lysate at a protein concentration of 0.1mg/ml was incubated with [8-$^{3}$H] adenine at a concentration of 2μM (specific activity 100μCi/μmol) for the times shown at 22°C. The reaction was terminated and the amount of nucleotide formed estimated as described in the text (Section 3.4.3). The results are given as the mean and range of duplicate experiments.

Figure 3.28 CONCENTRATION DEPENDENCE OF ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY.




The figure shows a Hanes plot of the initial rates of formation of nucleotide from adenine and PRPP over an adenine concentration range 0.2 to 4μM. The experimental protocol used is described in the text (section 3.4.3). The results are given as the mean and range of duplicate experiments.

Figure 3.29 PROTEIN DEPENDENCE OF HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY.




The above figure shows the dependence of the amount of nucleotice produced by hypoxanthine phosphoriosyltransferase on protein concentration. A trypanosome lysate at the protein concentrations shown was incubated with [G-$^{3}$H] hypoxanthine at a concentration of 5μM (specific activity 100μCi/μmol) for 3 minutes at 22°C. The reaction was terminated and the amount of nucleotide formed estimated as described in the text (Section 3.4.3). The results are given as the mean and range of duplicate experiments.

Section 3.4.2. The production of nucleotide was assayed by TLC and liquid scintillation counting as described in Section 3.4.3. The rate of reaction was determined in duplicate for each adenine concentration used.

The results of these experiments are shown as a Hanes plot in Figure 3.28. The kinetic parameters of the APRT were calculated from this plot to be: Km 0.84μM and Vmax 4.20nmol/min/mg protein. The significance of these kinetic parameters along with those of uptake and other enzymes involved in purine nucleoside and base metabolism will be considered in Section 3.4.7.

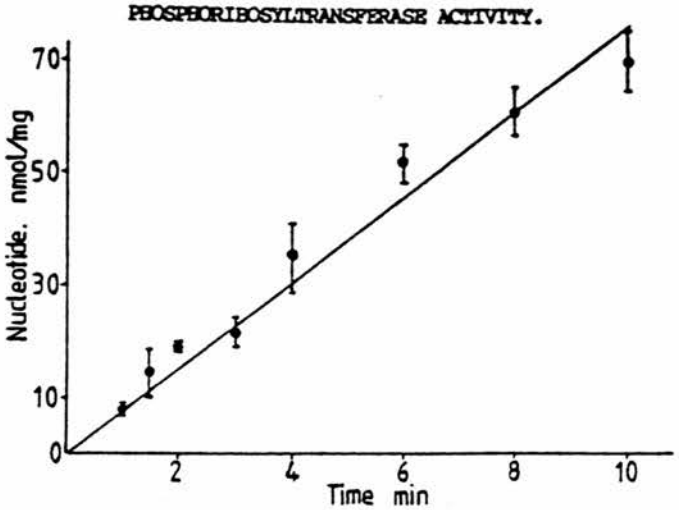
### 3.4.4 Kinetic Parameters of Hypoxanthine Phosphoribosyltransferase.

When [G-$^3$H] hypoxanthine is taken up by the trypanosomes the label appears in the nucleotide pool and no other intermediate. As described in Section 3.3.4 this conversion to nucleotide can only be achieved via HxPRT.

The kinetic parameters of HxPRT were estimated in a similar fashion to the estimation of Km and Vmax for APRT. Preliminary experiments were carried out to determine optimal assay conditions with regards to incubation duration, protein and magnesium concentration for the measurement of initial rates of reaction. The results of the protein dependence and time course experiments are shown in Figures 3.29 and 3.30 respectively. The full experimental details are given in the figure legends.

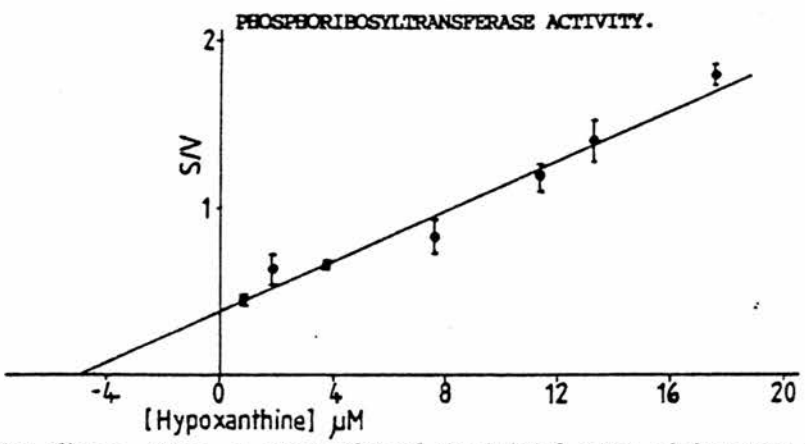
The assay mix contained TrisHCl buffer 10mM, DTT 1mM, $MgCl_2$ 1mM and PRPP 0.5mM. The concentration of [G-$^3$H] hypoxanthine, specific activity 100μCi/μmol., was varied between 0.76μM and 17.7μM. The assays were started by addition of trypanosome lysate to a final

Figure 3.30 TIME COURSE OF HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY.




The above figure shows the dependence of the amount of nucleotide produced by hypoxanthine phosphoribosyltransferase on time. A trypanosome lysate at a protein concentration of 0.1mg/ml was incubated with [G-$^{3}$H] hypoxanthine at a concentration of 5μM (specific activity 100μCi/μmol) for the times shown at 22$^{o}$C. The reaction was terminated and the amount of nucleotide formed estimated as described in the text (Section 3.4.3). The results are given as the mean and range of duplicate experiments.

Figure 3.31 CONCENTRATION DEPENDENCE OF HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY.




The figure shows a Hanes plot of the initial rates of formation of nucleotide from hypoxanthine and PRPP over a hypoxanthine concentration range 0.76 to 17.7μM. The experimental protocol used is described in the text (section 3.4.4). The results are shown as the mean and range of duplicate experiments.

concentration of 0.13mg protein /ml.

The incubation was carried out at 22°C for 3 minutes, the assay being terminated by rapid heating to 120°C for 1 minute as described in Section 3.4.2. Nucleotide formed was estimated by separation of labelled nucleotide from [G-$^{3}$H] hypoxanthine by TLC as described in Materials & Methods (Section 2.8). The rate of nucleotide formation was determined in duplicate for each concentration of hypoxanthine used.

The results obtained from these experiments are shown as a Hanes plot in Figure 3.31. From this plot the following values for Km and Vmax were calculated; Km 5.0μM and Vmax 13.05nmol/min/mg protein. These results will be considered in more detail in Section 3.4.7.

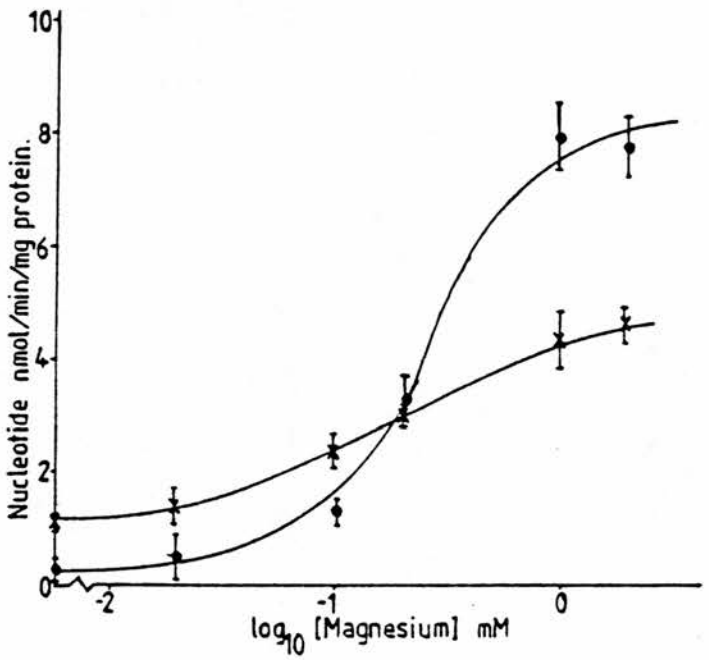
It was thought possible that both the adenine and HxPRT activities measured could be due to the presence of only one enzyme with a broad substrate specificity. Therefore the magnesium dependence and inhibition by other purines of the phosphoribosyltransferase activity were investigated to detect any differences which would indicate the presence of two enzymes.

### 3.4.5 Magnesium Dependence of Adenine and Hypoxanthine Phosphoribosyltransferase Activity.

The magnesium dependence of adenine and hypoxanthine phosphoribosyltransferase activities was investigated by assay of the respective phosphoribosyltransferase activities in a dialysed trypanosome lysate in the presence of increasing concentrations of magnesium.

The assays were carried out as described in Sections 3.4.3 & 3.4.4, with the following changes; the final protein concentration in

Figure 3.32 MAGNESIUM DEPENDENCE OF PHOSPHORIBOSYLTRANSFERASE ACTIVITY.




The figure shows the dependence of the rate of PRPP depencent phosphorylation of adenine and hypoxantnine on $Mg^{2+}$ concentration (Note log scale). The experimental details are given in the text (Section 3.4.5). The results are given as the mean and range of duplicate experiments. (● = HxPRT and x = APRT)

the assays was 0.12mg/ml, incubation time was 2 minutes at 22°C. The substrates, adenine and hypoxanthine, were both used at a concentration of 5μM (100μCi/μmol.). The assays were terminated as described previously (Sections 3.4.3 & 3.4.4). Magnesium, added as $MgCl_2$, was varied from 0.02mM to 2.0mM. Control incubations were also carried out to which no magnesium was added.

The results obtained are shown in Figure 3.32 plotted as nmol nucleotide produced/min/mg protein against $\log_{10}$ of the magnesium concentration. The results show that the two enzymic activities have different magnesium requirements, the magnesium concentration for half maximal APRT activity is 0.1mM and half maximal HxPRT activity is obtained with 0.25mM magnesium. In order to obtain more evidence for the existence of two separate enzymes the effects of inhibitors on the phosphoribosyltransferase activities were investigated.

### 3.4.6 Cross Inhibition of Adenine and Hypoxanthine Phosphoribosyltransferase activities.

The effects of adenine, guanine and hypoxanthine on the phosphoribosyltransferase activities were determined by assaying the adenine and hypoxanthine phosphoribosyltransferase activities in the presence of 20μM adenine, guanine or hypoxanthine. The assay system used was as described in Sections 3.4.3 & 3.4.4, except that adenine and hypoxanthine substrates were present at a concentration of 10μM (100μCi/μmol.) and $MgCl_2$ 1mM. The final protein concentration in the assay was 0.1mg protein /ml.

The various combinations of purine bases used in these experiments and the results, expressed as percentage inhibition of control activities, are shown in Table 3.5. These results confirm the

Table 3.5 CROSS INHIBITION OF PHOSPHORIBOSYLTRANSFERASE ACTIVITIES.

| SUBSTRATE $^3H$ | 20μM GUANINE | 20μm HYPOXANTHINE | 20μm ADENINE | NUCLEOTIDE nmol/min/mg | % INHIBITION |
|---|---|---|---|---|---|
| ADENINE | - | - | - | 0.266 | 0 |
| " | + | - | - | 0.170 | 24 |
| " | - | + | - | 0.154 | 31 |
| HYPOXANTHINE | - | - | - | 0.242 | 0 |
| " | + | - | - | 0.111 | 54 |
| " | - | - | + | 0.234 | 3 |

The table shows the results of cross inhibition of phosphoribosyltransferase activities in a trypanosome lysate by substrates and potential substrates of the enzymes. Experimental details are given in the text (Section 3.4.6)

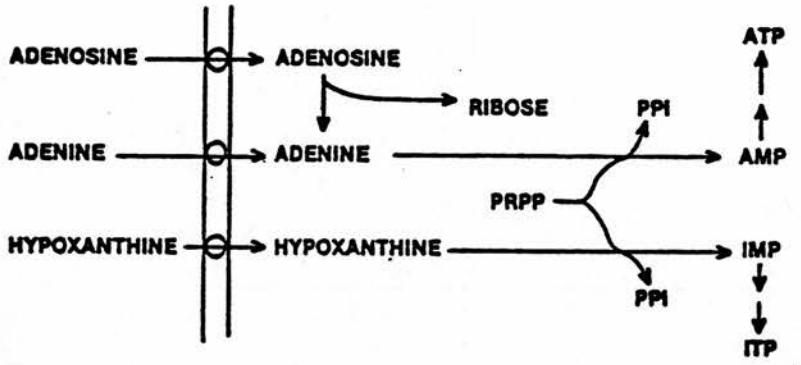
presence of two distinct enzymes. A two-fold excess of hypoxanthine or guanine over the adenine substrate inhibits APRT activity by 20% to 30% where as the same concentration of guanine inhibits HxPRT activity by 54%. Adenine has very little (3%) inhibitory activity towards HxPRT which suggests that adenine is not a substrate for this enzyme. The marked inhibition of HxPRT activity by guanine suggests that guanine may also be a substrate for this enzyme as reported by Walter & Konigk(1974) and Krenitsky et al.(1969). These differences in the enzyme activities are evidence that the two phosphoribosyltransferase activities are associated with different enzymes.

The kinetic parameters of the uptake and metabolism of adenosine, adenine and hypoxanthine so far considered individually can now be considered as a whole.

### 3.4.7 Summary of Metabolism and Kinetic Parameters of the Purine Nucleoside and Base Uptake Systems.

Figure 3.33 shows diagrammatically the proposed scheme for uptake and metabolism of adenosine, adenine and hypoxanthine by T.brucei(TREU 55) long slender bloodstream forms. The kinetic parameters of the uptake systems are consistent with the kinetic parameters estimated for metabolism of nucleosides and bases in that rates of uptake of purines appear to be less than the possible rates of metabolism. However the kinetic parameters estimated for the metabolising enzymes were estimated in a cell free system which may not be a true representation of the in vivo situation. The cell free system probably did not contain any potentially regulatory substances normally found in the cell or if they were present they would be

Figure 3.33 SUMMARY OF PURINE NUCLEOSIDE AND BASE UPTAKE AND METABOLISM.



diluted out in the preparation of the lysate. These points will be developed further in the Discussion (Section 4.2.4)

Figure 3.33 shows three separate points of entry into the cell for adenosine, adenine and hypoxanthine. The next question asked was are there one or more carriers in the plasma membrane?. In order to answer this question the specificities of uptake of the three compounds listed were determined by cross inhibition of uptake and by the effects of nucleoside analogues on uptake.

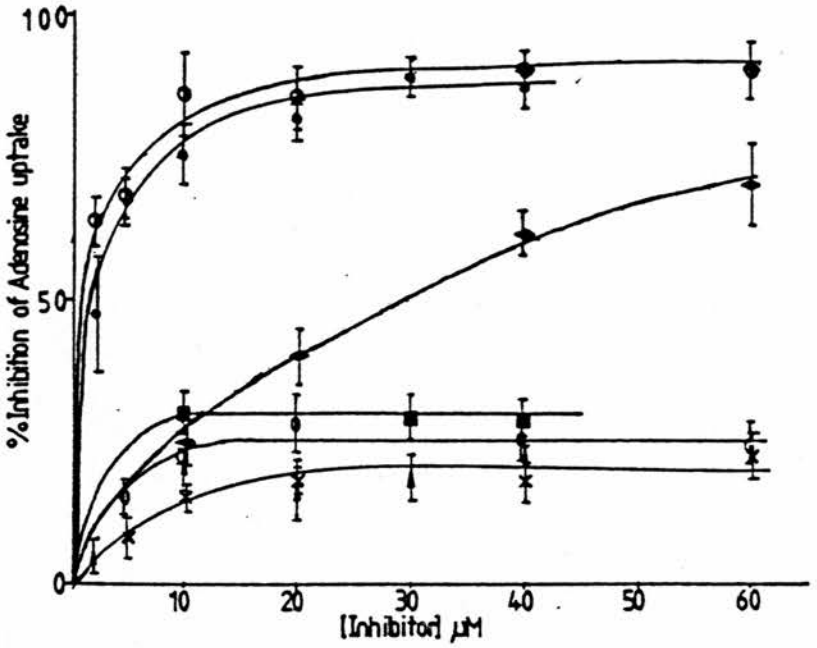
## 3.5 INHIBITORS OF NUCLEOSIDE AND BASE UPTAKE.

The effects of nucleosides, bases and analogues of nucleosides on the uptakes of adenosine, adenine and hypoxanthine were investigated. Uptake in the presence or absence of inhibitor was measured by the silicon sandwich centrifugation technique. The effects of the nucleosides adenosine, guanosine and inosine, the bases adenine, guanine and hypoxanthine and the nucleoside analogues 5'-iodo-5'-deoxyadenosine (IDA) and p-nitrobenzylthioinosine (NBTI) on the uptakes of adenosine, adenine and hypoxanthine were determined.

### 3.5.1 Inhibitors of Adenosine Uptake.

The effects of nucleosides, bases and nucleoside analogues on adenosine uptake were measured as a percentage inhibition of adenosine uptake determined in the absence of inhibitors. The uptake assay consisted of trypanosomes suspended in PSG at pH 7.4, [2,8-$^{3}$H] adenosine and the inhibitor being investigated at a range of concentrations. The uptake assays were started by the addition, with rapid mixing, of 500µl of trypanosome suspension. All other

Figure 3.34 INHIBITORS OF ADENOSINE UPTAKE.




Trypanosomes (500µl,0.72mg protein/ml in PSG pH 7.4) were incubated in the presence of labelled adenosine (10µl,0.1mM,50µCi/µmole) at a final concentration of 1µM with Inosine(○), Hypoxanthine(X), Guanine(▲), Guanosine(■), Adenine(◑), NBTI(◂) and IDA(●) in PSG pH 7.4 to give a final volume of 1ml. The uptake assays were started by addition of the trypanosomes. Incubations were carried out at 22°C;duplicate samples (400µl) were taken after mixing and layered onto silicon sandwiches which were centrifuged after 30 seconds. Uptake was estimated by liquid scintillation counting of the PCA layer.The results are presented as the mean and range of duplicate experiments.

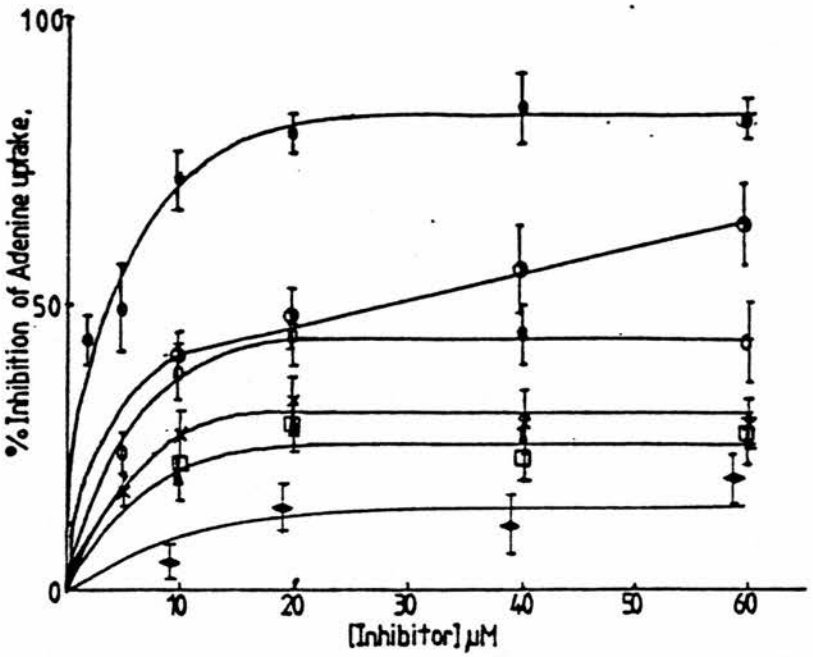
experimental details are given in the legend to Figure 3.34.

Figure 3.34 shows the effects of the compounds listed in the Figure legend on the uptake of [2,8-$^{3}$H] adenosine. The inhibitors can be classified into two main types on the basis of their maximum inhibitory activity. Iododeoxy adenosine and adenine are classified as strong inhibitors whereas hypoxanthine, guanine, inosine and guanosine are classified as weak inhibitors of adenosine uptake. Nitrobenzylthioinosine shows an intermediate type of inhibition towards adenosine uptake which will be considered later. However it should be noted that NBTI is capable of 100% inhibition of nucleoside uptake in the erythrocyte at a concentration of 17.5μM (Pickard & Paterson, 1972). The effect of NBTI shown here demonstrates that there is a difference between the erythrocyte and the trypanosomal transporter since at 20μM NBTI uptake of adenosine by the trypanosomes is only inhibited by 35%. It should also be noted that with increasing concentrations of inhibitor the strong inhibitors never achieve 100% inhibition; rather their inhibitory effects plateau at 80%-90% inhibition of uptake. The weak inhibitors of uptake show a similar plateau effect with increasing concentrations of inhibitor but this time the plateau of inhibition is reached at 20%-40% inhibition of uptake. The reasons for this plateau effect will be considered in Section 3.5.5.

### 3.5.2 Inhibitors of Adenine Uptake.

The effects of adenosine, hypoxanthine, inosine, guanine, guanosine, IDA and NBTI on the uptake of adenine were investigated by exactly the same method as described in the legend to Figure 3.34. The incubation conditions were also as described in Figure legend

Figure 3.35 INHIBITORS OF ADENINE UPTAKE.




Trypanosomes (500µl,0.50mg protein/ml in PSG pH 7.4) were incubated in the presence of labelled adenine (10µl,0.1mM,50µCi/µmole) at a final concentration of 1µM with Inosine(o), Hypoxanthine( X ), Guanine( ▲ ), Guanosine(□), Adenosine(●), NBTI(◄►) and IDA(◐) in PSG pH 7.4 to give a final volume of 1ml. The uptake assays were started by addition of the trypanosomes. Incubations were carried out at 22° C; duplicate samples (400µl) were taken after mixing and layered onto silicon sandwiches which were centrifuged after 30 seconds. Uptake was estimated by liquid scintillation counting of the PCA layer. The results are presented as the mean and range of duplicate experiments.

3.34 with [8-$^{3}$H] adenine being used as substrate in place of [2,8-$^{3}$H] adenosine. All additional details are given in the legend to Figure 3.35.

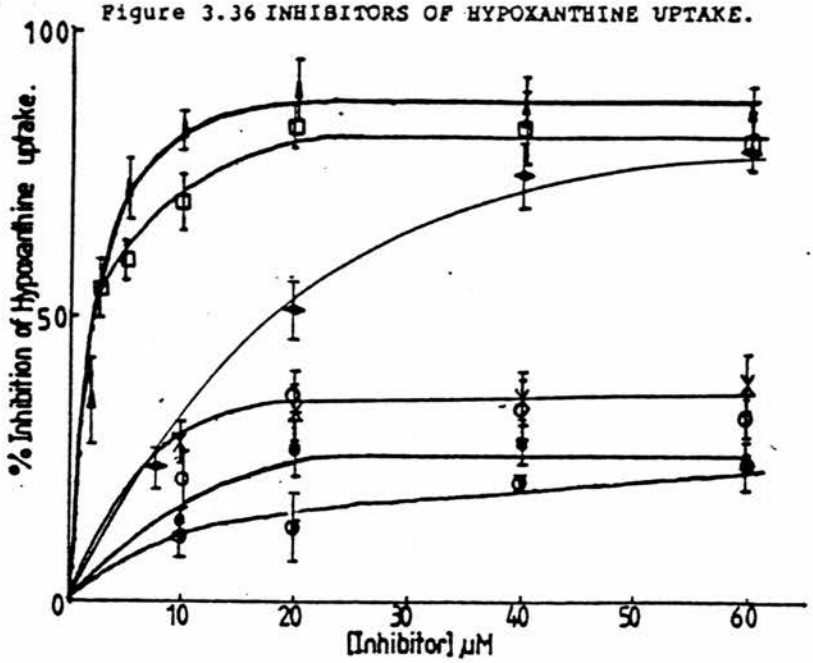
Figure 3.35 shows the effects of the compounds, listed in the legend to Figure 3.35, on the uptake of adenine. The inhibitors can again be classified into two main groups of strong and weak inhibitors. The distribution of inhibitors between these two groups is different from that obtained for the inhibition of adenosine uptake. Adenosine is a strong inhibitor of adenine uptake whereas IDA and NBTI are intermediate in their inhibitory activity with guanine, guanosine, hypoxanthine and inosine showing weak inhibitory activity. The inhibitory activities of both the strong and weak inhibitors show a plateau in activity as described in the previous section.

### 3.5.3 Inhibitors of Hypoxanthine Uptake.

The effects of adenine, adenosine, guanine, guanosine, inosine, IDA and NBTI on the uptake of hypoxanthine were investigated. The experimental methods used were identical to those described in Figure legends 3.34 & 3.35 except the labelled substrate for uptake was [G-$^{3}$H] hypoxanthine. All other parameters with respect to substrate concentration, temperature, pH and incubation time are given in the legend to Figure 3.36

The results of these experiments are shown in Figure 3.36. Again the inhibitors can be classified as being either strong; (guanosine and guanine) or weak; (adenosine, adenine, inosine, IDA and NBTI). This inhibitory pattern is different from those obtained for the inhibition of adenosine and adenine uptake. Again the plateau in inhibitory activity with increasing inhibitor concentration is

Figure 3.36 INHIBITORS OF HYPOXANTHINE UPTAKE.




Trypanosomes (500µl,0.6mg protein/ml in PSG pH 7.4) were incubated in the presence of labelled hypoxanthine (10µl,0.1mM,50µCi/µmole) at a final concentration of 1µM with Inosine(X), Guanine(▮), Guanosine(□), Adenine(○), Adenosine(◐), NBTI(◆) and IDA(●) in PSG pH 7.4 to give a final volume of 1ml. The uptake assays were started by addition of the trypanosomes. Incubations were carried out at 22°C, duplicate samples (400µl) were taken after mixing and layered onto silicon sandwiches which were centrifuged after 30 seconds. Uptake was estimated by liquid scintillation counting of the PCA layer. The results are presented as the mean and range of duplicate experiments.

observed. These results along with the results obtained for the inhibition of adenosine and adenine uptake are summarised and considered in more detail in Section 3.5.5.

The data presented so far in this chapter show the results of investigations into the effects of the purine nucleoside analogues IDA and NBTI on uptake and the effects of various purine nucleosides and bases on uptake. However it should be borne in mind that the uptake may be dependent on metabolism and that the effects observed could be due to the inhibition of metabolism rather than the inhibition of transport. Therefore the effects on metabolism of the compounds found to be inhibitors of uptake were investigated.

### 3.5.4 Effect of uptake inhibitors on Purine Nucleoside and Base Metabolising Enzymes.

The compounds which inhibited uptake in the previous experiments (Sections 3.5.1, 3.5.2 and 3.5.3) were tested for inhibitory activity towards the purine nucleoside and base metabolising enzymes. The compounds were tested for inhibitory activity towards adenosine hydrolase, APRT and HxPRT.

The activities of the above enzymes were assayed in the presence and absence of the putative inhibitors, the inhibitor:substrate ratio being 50:1 as in the uptake experiments at high inhibitor concentrations, that is at inhibitor concentrations above those giving maximal inhibition of uptake.

The assay system for adenosine hydrolase was as described in Sections 3.4.1 & 3.4.2. The [2,8-$^{3}$H] adenosine substrate (100$\mu$Ci/$\mu$mol) was present at a final concentration of 2$\mu$M either alone or with one of the putative inhibitors at a concentration of

**Table 3.6 EFFECT OF UPTAKE INHIBITORS ON PURINE NUCLEOSIDE AND BASE METABOLISING ENZYMES.**

| | % PERCENTAGE INHIBITION OF | | |
|---|---|---|---|
| INHIBITOR | ADENOSINE HYDROLASE | APRT | HxPRT |
| IODODEOXYADENOSINE | 15 | 18 | 0 |
| NBTI | 23 | 0 | 2 |
| ADENOSINE | 0 | 75 | 0 |
| ADENINE | 10 | 0 | 3 |
| INOSINE | 35 | 9 | 15 |
| HYPOXANTHINE | 0 | 31 | 0 |
| GUANINE | 0 | 24 | 34 |
| GUANOSINE | 31 | 9 | 40 |

The table shows the percentage inhibition of purine metabolising enzymes by inhibitors of uptake at an inhibitor:substrate ratio of 50:1 as used in uptake experiments. Details of the assay systems used are given in the text (Section 3.5.4)

100μM. Fresh trypanosome lysate was used as the enzyme source at a final concentration of 0.02mg protein/ml. All other assay methods and parameters were as described in Sections 3.4.1 & 3.4.2. Adenine and hypoxanthine phosphoribosyltransferase were assayed alone or in the presence of one of the putative inhibitors. The assay system used was as follows. [8-$^{3}$H] Adenine or [G-$^{3}$H] hypoxanthine substrate (both 100μCi/μmol) were present at a concentration of 2μM either alone or with one of the putative inhibitors at a concentration of 100μM. Fresh trypanosome lysate was used as the enzyme source at a final concentration of 0.1mg protein/ml. All other assay parameters and methods were as described in Sections 3.4.3 & 3.4.4.

The results of the experiments described above are shown in Table 3.6 as percentage inhibition of enzyme activity. These results when considered in conjunction with the results of inhibition of uptake provide good evidence for the presence of a carrier molecule for nucleosides and bases in the plasma membrane as detailed in the Discussion (Section 4.2.1). Inspection of the results presented in Sections 3.5.1 to 3.5.3 and Table 3.6 shows that compounds having a strong inhibitory effect on uptake are not acting by inhibition of metabolism of the labelled substrates.

In some cases the weak type of inhibition observed in Sections 3.5.1 to 3.5.3 may be due to inhibition of metabolism of the label taken up. For example guanine inhibits adenosine uptake to a maximun of 20%, has no effect on adenosine hydrolase but inhibits APRT by 24% at the same inhibitor:substrate ratio used in the uptake experiments. On the other hand IDA inhibits hypoxanthine uptake weakly by 35% yet has no inhibitory effect on HxPRT. Inspection of the results presented shows many more examples of the above two types, therefore

the inhibition of uptake observed must take into account any inhibitory effects on metabolism where appropriate.

The comparisons made between the inhibitory effects of compounds on uptake and inhibitory effects on metabolism assume that the compounds acting as inhibitors of uptake enter the cell and accumulate to the concentrations used in the in vitro assays of nucleoside and base metabolism. Whether or not the inhibitors of uptake enter the cell the strong type of inhibition still provides evidence for the existence of a membrane carrier since in these cases the inhibitors involved have very little effect on any metabolism associated with uptake.

The results presented in Table 3.6 show some other interesting points. Adenosine apparently inhibits APRT by 74% at a 50:1 adenosine:adenine ratio. This can be explained by dilution of the labelled adenine used as substrate with unlabelled adenine derived from the adenosine present. The enzyme source used in these experiments was fresh whole cell lysate which contains a very active adenosine hydrolase (Section 3.4.2) which would rapidly convert the adenosine present to adenine and ribose. The fact that this effect did not occur to the same extent when inosine was tested as an inhibitor of HxPRT suggests that inosine is not a very good substrate for adenosine hydrolase. However an alternative explanation may be that the trypanosomes contain an inosine hydrolase which is less active than the adenosine hydrolase or no inosine hydrolase at all.

The 40% inhibition of HxPRT obtained with guanosine as inhibitor is similar to the 34% inhibition by guanine which may indicate the presence of a guanosine hydrolase or that guanosine is a substrate for adenosine hydrolase. Guanosine and inosine may be poor substrates

**Table 3.7 SUMMARY OF MAXIMUM PERCENTAGE INHIBITION OF PURINE UPTAKE.**

| INHIBITOR | ADENOSINE UPTAKE MAX.% INHIBITION | HYPOXANTHINE UPTAKE MAX.% INHIBITION | ADENINE UPTAKE MAX.% INHIBITION |
|---|---|---|---|
| ADENOSINE | 0 | 25 | 85 |
| INOSINE | 25 | 35 | 45 |
| GUANOSINE | 30 | 80 | 25 |
| IDA | 90 | 25 | 65 |
| NBTI | 70 | 80 | 20 |
| ADENINE | 92 | 35 | 0 |
| GUANINE | 20 | 90 | 25 |
| HYPOXANTHINE | 20 | 0 | 30 |

The table summarises the results presented in Figures 3.34, 3.35 and 3.36.

for the adenosine hydrolase as indicated by their ability to inhibit adenosine hydrolase by 31% and 35% respectively when present in a fifty fold excess over the adenosine substrate.

### 3.5.5 Summary of Cross Inhibition of Nucleoside and Base Uptake Systems.

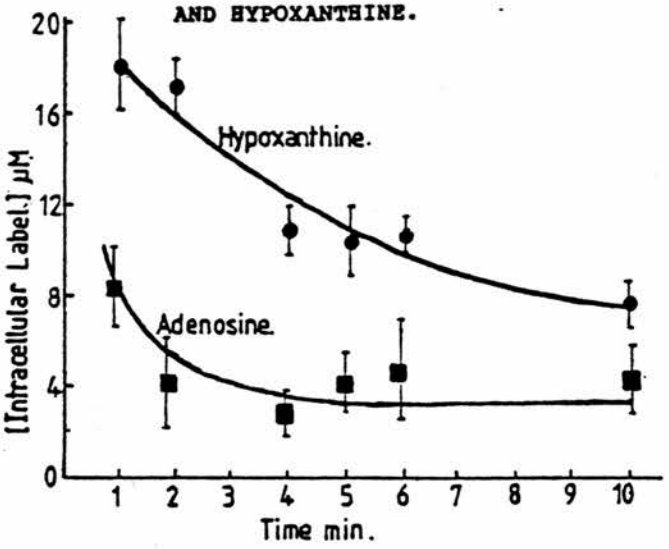
The results presented in Sections 3.5.1 to 3.5.4 show that when the concentration of an inhibitor of uptake is increased inhibition plateaus to a maximium, which can either be high, (in the range 70-90%), or low, (in the range 10-40%). This plateau effect could be explained by the presence of more than one membrane transporter. An inhibitor could block one transporter 100% but substrate for that transporter may use another transporter with different specificities which is not blocked by the inhibitor used. This explanation of the plateau effect requires the presence of at least two transport proteins in the plasma membrane. Evidence for the existence of two or more transporters is provided by the patterns of uptake inhibition obtained in Sections 3.5.1 to 3.5.4.

Table 3.7 summarises the results of the effects of inhibitors on uptake. The results are presented as maximum percentage inhibition, that is the inhibitor's plateau inhibition level described above. The significance of these results will be considered in detail in the Discussion (Section 4.2.1).

## 3.6 MECHANISM OF PURINE NUCLEOSIDE AND BASE TRANSPORT.

So far the results presented here have shown that adenosine, adenine and hypoxanthine are taken up and metabolised to nucleotide by T.brucei and that translocation of the nucleosides and bases

Figure 3.37 INTRACELLULAR CONCENTRATIONS OF ADENOSINE AND HYPOXANTHINE.




Trypanosomes ($1.53x10^8$ cells/ml, 0.63mg protein/ml) were incubated with labelled adenosine or hypoxanthine (both 1µM, specific activity 100µCi/µmol.) for 1 to 10 minutes in PSG at pH 7.4, 22°C. The incubations were terminated by silicon sandwich centrifugation with PCA as the lower layer. Samples of the PCA layer (100µl) were neutralised by potassium hydroxide (50µl), centrifuged to remove percipitated potassium perchlorate samples of supernatant (10µl) were then spotted in triplicate, with carriers, onto silica TLC plates and chromatographed as described in Materials & Methods(Section 2.8). The label present in the cell as adenosine or hypoxanthine was estimated by cutting the appropriate spots from the chromatogram followed by liquid scintillation counting. In all cases the carry through of adenosine (Materials & Methods Section 2.11.1) was corrected for as was quenching of scintillation counting by the TLC plate. The results are presented as the mean and range of duplicate experiments.

across the plasma membrane involves carrier proteins in the plasma membrane. The mechanism of this plasma membrane transport has not as yet been considered in any detail. Calculations made using the kinetic parameters measured for uptake and metabolism (Section 3.4.7) show that it is possible for metabolism to maintain a concentration gradient of substrate across the plasma membrane with transport being mediated by facilitated diffusion. As stated in Section 3.4.7 the kinetic parameters for metabolism were estimated in a cell free system and therefore may not reflect the in vivo situation. The main alternative to a facilitated diffusion mechanism for transport is active transport. Active transport would be capable of accumulating substrate in the cell against a concentration gradient. The ability of trypanosomes to accumulate adenosine and hypoxanthine against a concentration gradient was therefore investigated.

### 3.6.1 Intracellular Concentrations of Adenosine and Hypoxanthine.

In order to calculate the intracellular concentrations of adenosine and hypoxanthine the intracellular volume of the cells must be known. The intracellular volume of the long slender trypanosome has been estimated to be 80µl/$10^9$ cells (Damper & Patton, 1976b). This estimate was made by measuring the dilution of permeant and impermeant radio-labels on addition of trypanosomes to a mixture of the two labels. This method requires that very small changes in very large numbers be measured and is therefore susceptible to large errors. In view of this the intracellular volume of T. brucei Treu 55 was estimated.

The intracellular water volume of T. brucei was measured by

incubating cells ($1.412 \times 10^8$ cells/ml) with $^3H_2O$ to give a final isotope concentration of 2000dpm/µl. Duplicate incubations were set up in which [carboxy-$^{14}$C] inulin was added in place of $^3H_2O$. After 15 minutes, at which time $^3H_2O$ had distributed to equilibrium, duplicate 0.5ml samples of five separate incubations were centrifuged on silicon sandwiches. The total cell water was estimated by counting the $^3H$ found in the PCA layers and subtracting carry through as estimated from the incubations containing [carboxy-$^{14}$C] inulin. The total cell water was calculated to be $32 \pm 2.4$µl/$10^9$ cells expressed as the mean and population deviation from duplicate determinations of five experiments. This value for cell water was used in all subsequent calculations of intracellular concentrations where the same buffer composition (PSG) was used.

The intracellular concentrations of adenosine and hypoxanthine were measured as a function of time as detailed in the legend to Figure 3.37. The intracellular concentrations of adenosine and hypoxanthine were calculated assuming even distribution throughout total cell water (32µl/$10^9$ cells) as determined above. The validity of this assumption will be considered in the Discussion (Section 4.2.2).

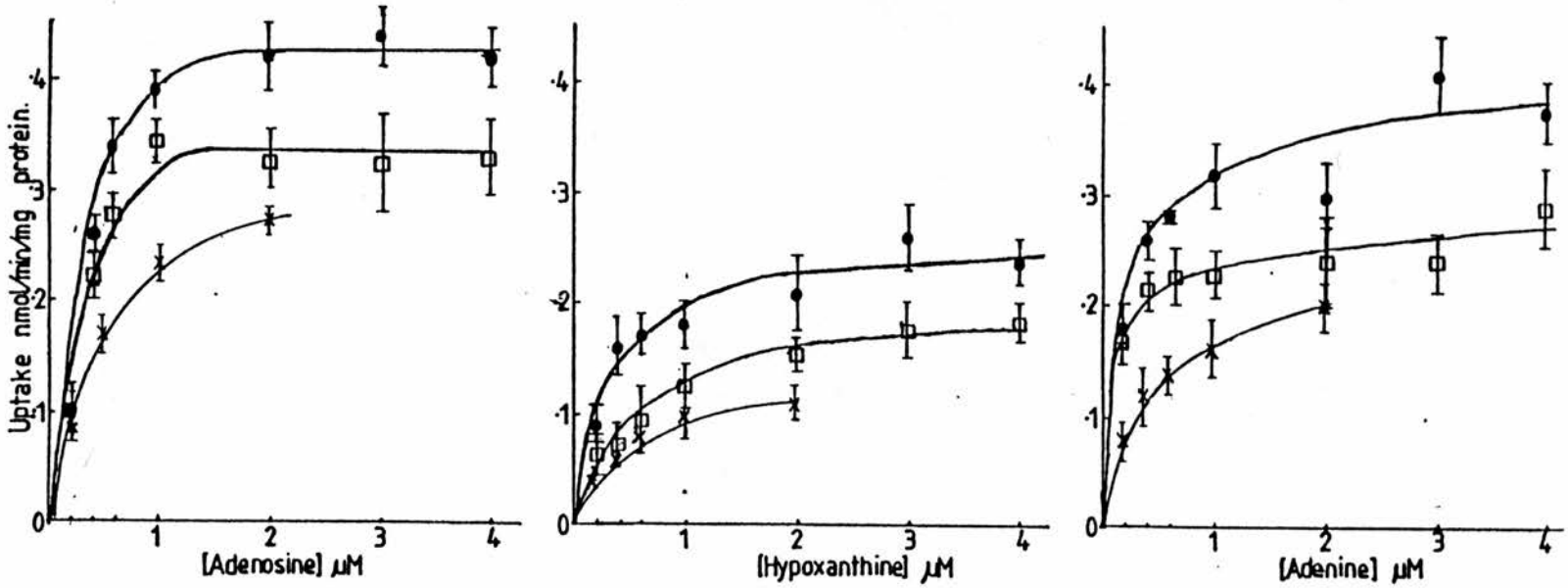
The results shown in Figure 3.37 demonstrate that hypoxanthine is accumulated against its concentration gradient as is, to a lesser extent, adenosine. These results show that initially transport is very rapid with the substrates being accumulated against their concentration gradients, then metabolism begins to decrease intracellular concentrations. This reduction in intracellular concentrations of substrates by metabolism is not surprising since it has been shown that the uptake of adenosine and hypoxanthine cease

after two minutes (Sections 3.2.1 & 3.3.1). The concentration of substrates against their concentration gradients could reflect intracellular binding without metabolism or true active transport. If the observed results are due to active transport, transport must be coupled to an energy source. This energy source could be an ion gradient established across the plasma membrane by an ion translocating ATPase such as the $Na^+/K^+$ dependent ATPase (EC 3.6.1.3) or $H^+$translocating ATPase located in the plasma membrane. (For a comprehensive review of coupled transports in bacteria see Hamilton,(1975) ). In order to differentiate between intracellular binding and active transport the effects of various ions and ionophores on uptake were investigated.

### 3.6.2 Effect of Gramicidin S and High Potassium Low Sodium Buffer on Purine Nucleoside and Base Uptake.

Gramicidin S is a cyclic peptide, produced by the bacterium Bacillus brevis, which is capable of forming dimers in a lipid bilayer resulting in the transient formation of aqueous channels across the membrane. Gramicidin shows poor selectivity in relation to which ions can pass through the channels formed. Such selectivity as it does show is as follows; $H^+ > Rb^+ > K^+ > Na^+ > Li^+$.(Pressman,1976). The addition of this ionophore to a cell suspension allows ions to move down their concentration gradients across the plasma membrane. A concentration of $\overset{\text{gramicidin S}}{\wedge}$1µg/ml is sufficient to abolish valinomycin induced uptake of $^{137}Cs^+$, which reflects a reduction in membrane potential from -129mV to -59mV (Midgley,1983). Therefore any reduction in the uptake of nucleoside or base in the presence of gramicidin would show that transport was at least partially dependent on a membrane

**Figure 3.38 EFFECTS OF GRAMICIDIN AND LOW $Na^+$ HIGH $K^+$ BUFFER ON ADENOSINE, ADENINE AND HYPOXANTHINE UPTAKE.**



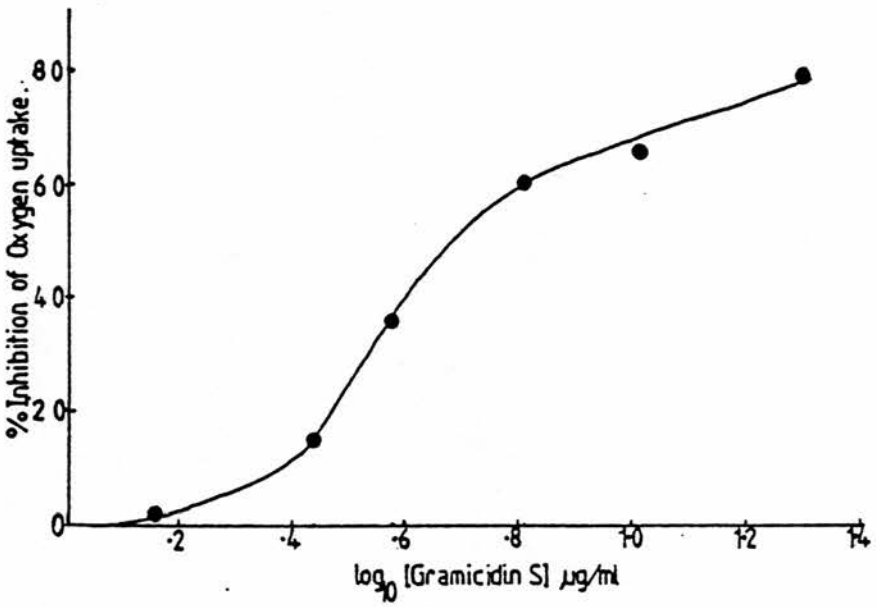

Uptakes of adenosine, adenine and hypoxanthine were assayed over a concentration range of 0.2µM to 4µM at specific activity of 100µCi/µmole in all cases. The cell density used in all incubations was 0.97 mg protein/ml. Uptake of was measured by silicon sandwich centrifugation and liquid scintillation counting of the PCA layer, duplicate 400µl samples of each incubation being assayed. Cells and substrates were incubated in PSG(●), PSG with gramicidin final concentration 1µg/ml(×) or PSG made with potassium salts throughout in place of sodium (PSGK)(□) as described in Materials & Methods (Section 2.4). All incubations were at pH 7.4, 22°C for 30 seconds. The results are presented as the mean and range of duplicate experiments.

potential. The effects of gramicidin S and low $Na^+$ high $K^+$ buffer on uptake of adenosine, adenine and hypoxanthine were assessed as described in the legend to Figure 3.38. Trypanosomes to be used in uptake experiments in PSGK were isolated in this buffer at pH 8.0 and resuspended at pH 7.4 in PSGK just before use. The assays were started by the addition of 500µl of cell suspension to 500µl of buffer containing labelled substrate and the appropriate buffer with gramicidin at 1µg/ml when required. Duplicate samples of 400µl from each incubation were layered onto silicon sandwiches and centrifuged after 30 seconds.

The results of these experiments are shown in Figure 3.38. The results show that the uptake of adenosine, adenine and hypoxanthine are inhibited by gramicidin. Adenosine uptake is inhibited at all concentrations of adenosine by 42% $\pm$ 4%, hypoxanthine uptake is inhibited by 43% $\pm$ 5% and adenine uptake is inhibited by 48% $\pm$ 8%. This suggests that uptake of all three substrates is at least partly dependent on a membrane potential. The effects of low $Na^+$/high $K^+$ buffer are also shown in Figure 3.38. The concentratiom of $Na^+$ in the PSGK buffer was calculated to be 1.7mM from the analyses given by the manufacturers of the 'Analar' reagents used in making this buffer. Figure 3.38 shows that the uptake systems for adenosine, adenine and hypoxanthine are inhibited by the PSGK buffer system possibly reflecting the dependence of uptake on a membrane potential since elevation of extracellular concentrations of $K^+$ to near intracellular concentrations of $K^+$ will abolish the $K^+$ gradient across the plasma membrane responsible for the maintainance of the membrane potential.

The effect of gramicidin could be due to cell death caused by disruption of the plasma membrane by the gramicidin and a loss of

**Figure 3.39 EFFECT OF GRAMICIDIN ON OXYGEN UPTAKE.**




The figure shows the precentage inhibition in the rate of oxygen uptake in the presence of increasing gramicidin concentrations. Trypanosomes (1ml,1.50mg protein/ml) were added to a thermostated chamber (37°C) containing 2ml of PSG pH 7.4. The chamber was sealed with a Clarke oxygen electrode and the rate of oxygen uptake measured as described in Materials & Methods (Section 2.6). Gramicidin was then added to give final concentrations im the range 1-20µg/ml.

metal ion co-factors into the incubation medium. This possibility was investigated by observing the effects of gramicidin on cell motility by phase contrast microscopy and effects on rate of oxygen uptake as measured by a Clark oxygen electrode.

### 3.6.3 Effect of Gramicidin S on Oxygen Uptake and Cell Motility.

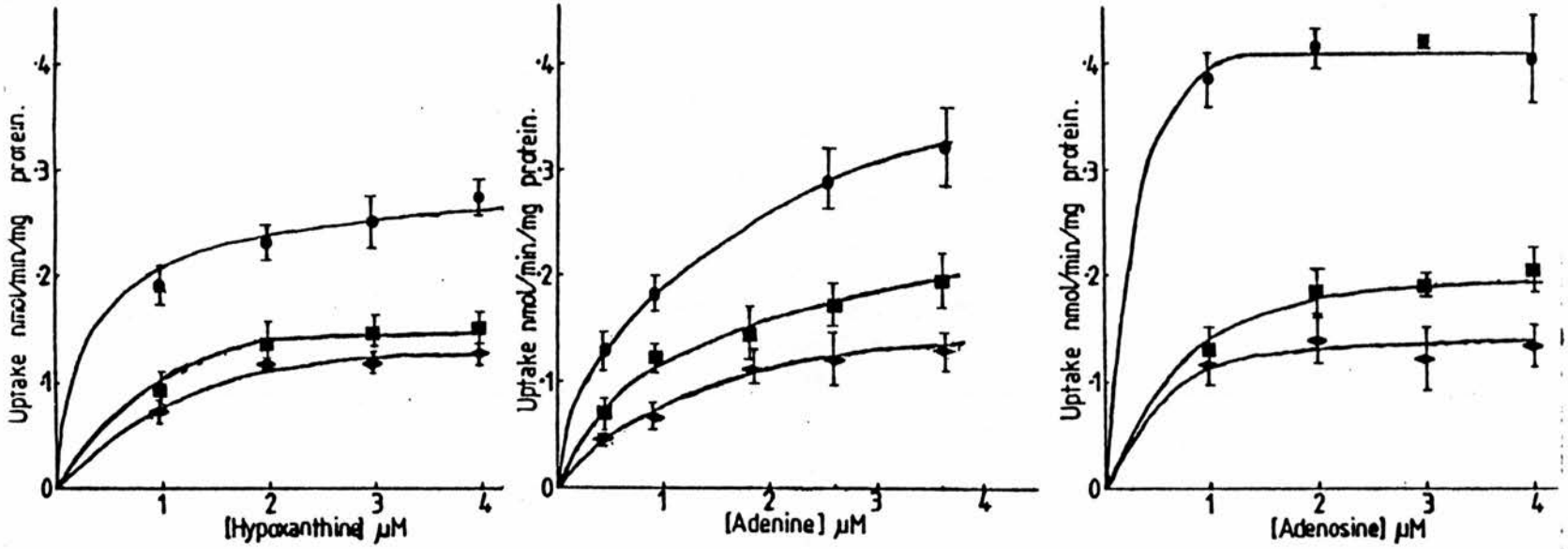
Trypanosomes were added to a thermostatted chamber with a magnetic stirring bar, containing PSG, pH 7.4 at 37°C. The chamber was sealed and the rate of oxygen utilisation measured with a Clark oxygen electrode the calibration and use of which is described in Materials & Methods (Section 2.6). All other experimental details are given in the legend to Figure 3.39.

The results obtained are shown in Figure 3.39 as percentage inhibition of oxygen uptake as a function of gramicidin concentration. At each gramicidin concentration used the decrease in rate of oxygen uptake was paralleled by a decrease in cell motility as observed by phase contrast microscopy. However in the previous experiment (Section 3.6.2) gramicidin was used at a concentration of 1μg/ml which as is shown by Figure 3.39 has no effect on the rate of oxygen uptake by the trypanosomes. Therefore the inhibitory effects of gramicidin on uptake (Figure 3.38) are not due to inhibition of general cell metabolism but more probably reflect the effect of the ionophore on the membrane potential.

### 3.6.4 Effect of Valinomycin and Valinomycin with Potassium on the Uptake of Purine Nucleosides and Bases.

Gramicidin S is a general ionophore allowing protons as well as

**Figure 3.40 EFFECT OF VALINOMYCIN AND VALINOMYCIN AND $K^+$ ON ADENOSINE, ADENINE AND HYPOXANTHINE UPTAKE.**




Uptakes of adenosine, adenine and hypoxanthine were assayed over a concentration range of 1μM to 4μM at a constant specific activity of 100μCi/μmole. The cell density used in all incubations was 0.90 mg protein/ml. Uptake of was measured by silicon sandwich centrifugation and liquid scintillation counting of the PCA layer, duplicate 400μl samples of each incubation being assayed. Cells and substrates were incubated in PSG(●), PSG with valinomycin final concentration 10μg/ml(■) or PSGK containing valinomycin 10μg/ml(◆). All cells used for uptake assays in the presence of valinomycin were preincubated in the appropriate buffer containing valinomycin for 10 minutes before use. All incubations were carried out at 22°C for 30 seconds. The results are presented as the mean and range of duplicate experiments.

monovalent cations through membranes. The observed effects of gramicidin on purine nucleoside and base uptake (Section 3.6.3) could therefore be due in part to the collapse of a proton gradient. A high extracellular concentration of $K^+$ inhibited uptake of purine nucleosides and bases (Section 3.6.3), possibly by reducing the membrane potential, but was not as effective as gramicidin. The extracellular $K^+$ concentration of 103 mM in PSGK buffer should have reduced the membrane potential to the Nernst potential (Hoffman,1982), as would the addition of gramicidin to a cell suspension. This suggests that uptake of purine nucleosides and bases is dependent on more than just the membrane potential. The contribution of the membrane potential to the uptake of adenosine, adenine and hypoxanthine was investigated further using the ionophore valinomycin.

Valinomycin is a cyclic dodecapeptide isolated from Streptomyces spp. (Brockmann & Schmidt,1955). This ionophore is specific for $K^+$ ions and has a 17,000 fold greater affinity for $K^+$ than $Na^+$(Pressman,1976). The addition of valinomycin to a suspension of trypanosomes would, by increasing the membrane's permeability to $K^+$, clamp the membrane potential or slightly hyper-polarise the cell (Bashford et al.,1985). Preliminary experiments with valinomycin showed that it had no effect on the uptake of any of the substrates tested unless the cells were incubated with valinomycin for 10 minutes before rates of purine nucleoside and base uptake were measured. The reasons for a preincubation being required before valinomycin has any effect will be dealt with in the Discussion (Section 4.2.2). The experimental protocol followed in these experiments is detailed in the legend to Figure 3.40. The effects of

valinomycin and valinomycin with $K^+$ at a concentration of 103mM were determined on the uptakes of adenosine, adenine and hypoxanthine. The incubations were started by the addition of 500µl of cell suspension, preincubated for 10 minutes in the appropriate buffer, to 500µl of buffer containing the labelled substrate. After the addition of the cells with rapid mixing duplicate samples of the incubation mix were layered on to silicon sandwiches and centrifuged after 30 seconds to terminate the uptake assay.

Valinomycin caused a reduction in the uptake of the labelled nucleoside and bases as shown in Figure 3.40. Valinomycin caused a reduction in the uptake of adenosine at all adenosine concentrations of 56% $\pm$ 6%. The uptakes of adenine and hypoxanthine were reduced by 33% $\pm$ 7% and 46% $\pm$ 4% respectively. This observed reduction in uptake may be due to the effect of valinomycin on intracellular membranes or a detergent effect on the plasma membrane. Figure 3.40 shows that high concentrations of $K^+$in the incubation medium in the presence of valinomycin is even better at inhibiting uptake of the labelled substrates than valinomycin alone or $K^+$ alone (Section 3.6.3). Adenosine uptake is inhibited by 68% $\pm$ 4%, adenine uptake is inhibited by 52% $\pm$ 9% and hypoxanthine uptake is inhibited by 55% $\pm$ 5%. These results provide further evidence for uptake of adenosine, adenine and hypoxanthine being dependent on a membrane potential. The observed effects of valinomycin on uptake are not due to any adverse effects on cellular metabolism as determined by observations of cell motility and measurements of rates of oxygen uptake after incubation of the cells with valinomycin. These results are discussed in detail in the Discussion (Section 4.2.2).

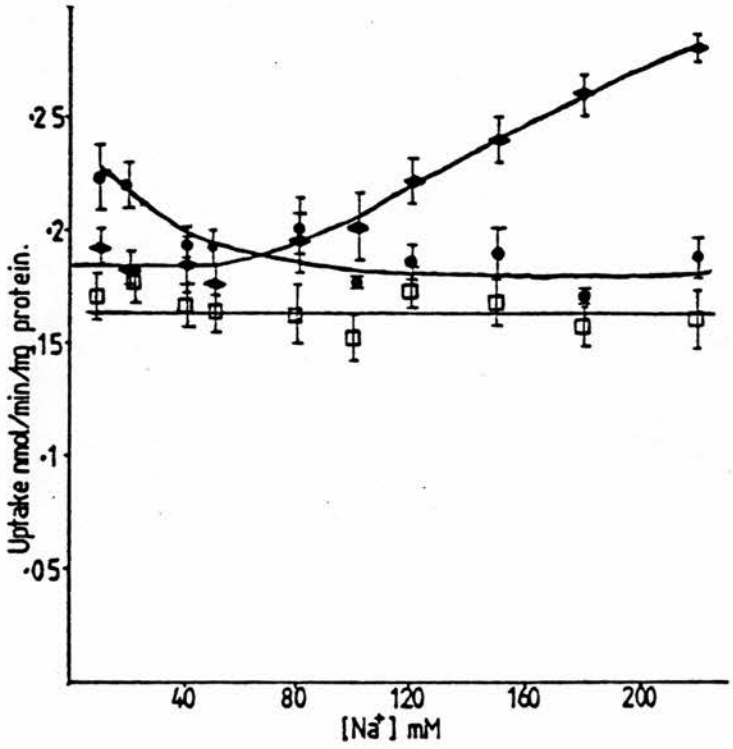
### 3.6.5 Sodium Dependence of Nucleoside and Base Uptake.

The previous experiments (Sections 3.6.2 & 3.6.3) provide some evidence that a membrane potential is required for maximal rates of nucleoside and base uptake. However in many other transport systems an ion gradient is often involved as well as a membrane potential, overall transport being driven by an electrochemical gradient composed of the ions' chemical gradient and the membrane potential. Therefore the dependence of uptake of adenosine, adenine and hypoxanthine on the extracellular concentration of $Na^+$ was investigated. As stated previously (Section 3.6.2) increasing the extracellular concentration of $K^+$ results in a decrease in membrane potential therefore any $K^+$ dependence of uptake would be masked by this effect. $K^+$ dependence of uptake was therefore not investigated further. However possible methods for investigation of $K^+$ dependence of uptake will be considered in the Discussion (Section 4.2.2). The sodium dependence of the uptake of adenosine and hypoxanthine was investigated by incubating cells with labelled substrates in the presence of increasing concentrations of NaCl as described in the legend to Figure 3.41. The NaCl concentrations in the incubations were varied between 0 and 210mM. Figure 3.41 shows the results of these experiments. The results indicate that adenosine uptake is not dependent on $Na^+$ but hypoxanthine uptake, while not dependent on the presence of $Na^+$, is stimulated by high concentrations of $Na^+$. These results will be considered in more detail in Section 3.6.8.

### 3.6.6 Effect of FCCP and pH on the Uptake of Purine Nucleosides and Bases.

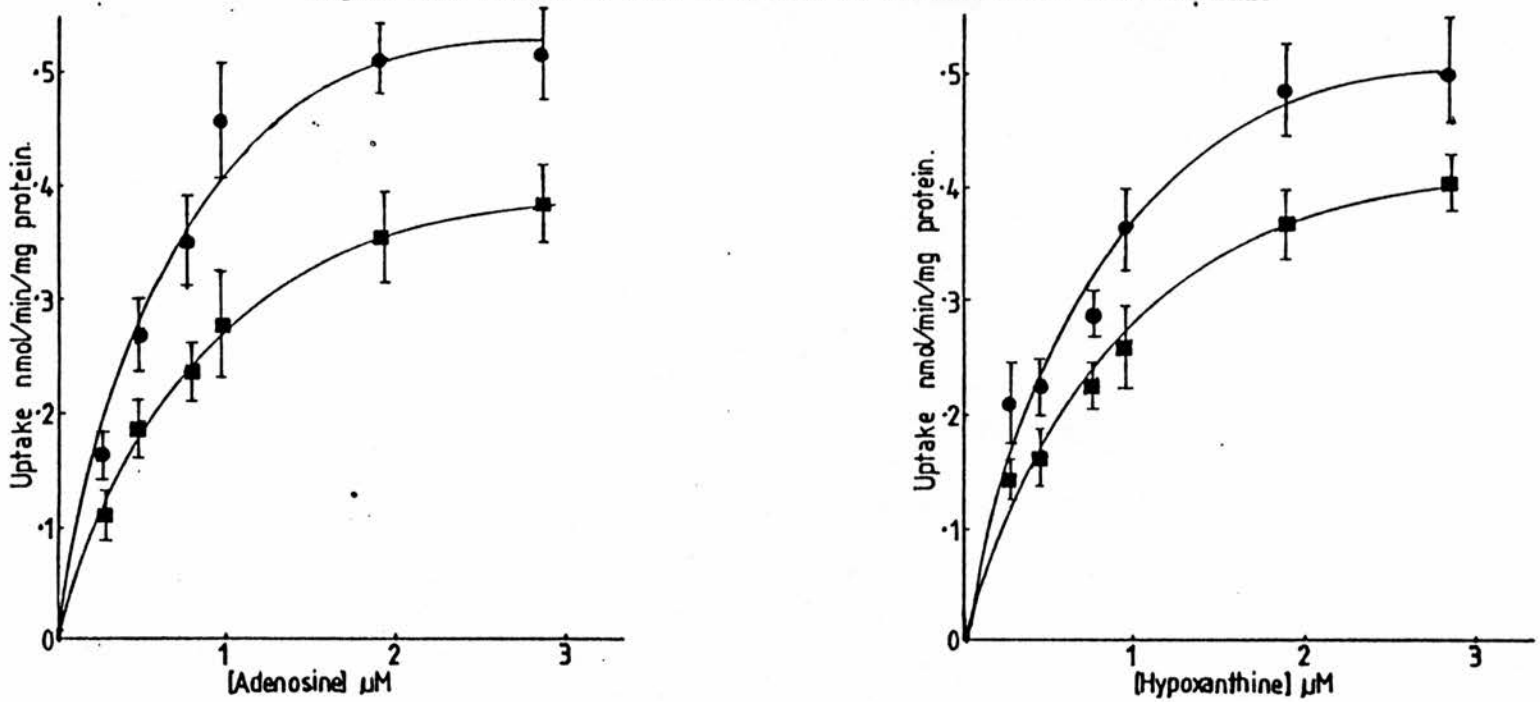
The uptake of hypoxanthine shows some slight stimulation by $Na^+$

**Figure 3.41 SODIUM DEPENDENCE OF ADENOSINE, ADENINE AND HYPOXANTHINE UPTAKE.**




Trypanosomes (1.20mg protein/ml) were incubated with adenosine( ● ), adenine( □ ) and hypoxanthine ( ◆ ) (1μM final concentration,specific activity 100μCi/μmole) in TrisHCl choline chloride glucose buffer (Materials & Methods,Section 2.4) pH 7.4 with increasing concentrations of NaCl (0-210mM). The concentration of choline cloride was reduced as the concentration of NaCl was increased so maintaining a constant osmolality. Uptake was assayed by silicon sandwich centrifugation of duplicate 400μl samples of each incubation. The results presented are the mean and population deviation of duplicate samples from two experiments.

**Figure 3.42 EFFECT OF FCCP ON UPTAKE OF ADENOSINE AND HYPOXANTHINE.**



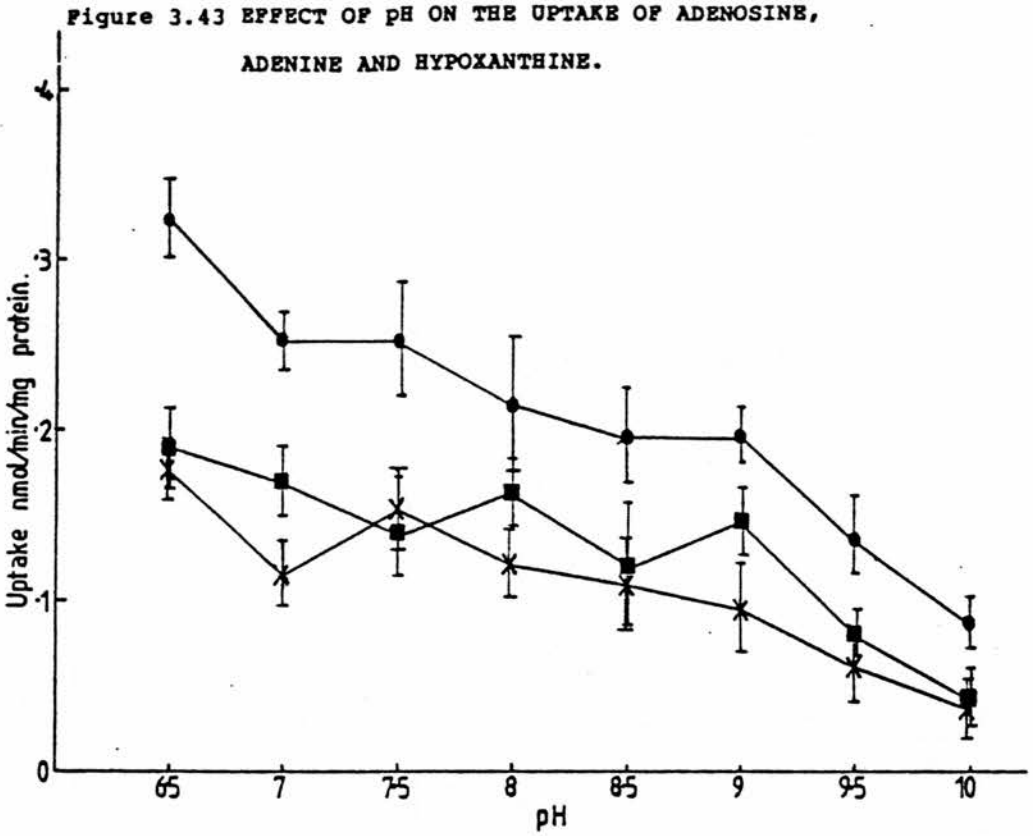

The uptake by trypanosomes (1mg protein /ml) of adenosine or hypoxanthine over the concentration range 0.2µM to 3µM (constant specific activity 100µCi/µmole) in the presence(■) or absence(●) of FCCP at a concentration of 2µM was determined. All incubations were conducted in PSG buffer pH 7.4, 22$^{o}$C for 30 seconds. Uptake being assayed by silicon sandwich centrifugation. The results are presented as the mean and range of duplicate experiments.

at high concentrations but adenosine uptake appears to be slightly inhibited by high $Na^+$ concentrations. Since uptake dependence on $K^+$ was not investigated the only other cation in the incubating media which could be involved in uptake is the proton. The uptake of nutrients such as amino acids by certain species of bacteria has been shown to be coupled to a proton gradient (MacDonald & Lanyi, 1975). The possibility that the uptake of adenosine and hypoxanthine could be coupled to an electrochemical proton gradient was investigated by measuring the uptake of labelled adenosine and hypoxanthine in the presence of protonophore (FCCP) and at various proton concentrations.

Uptake assays were carried out as described in the legends to figures 3.42 and 3.43. The protonophore FCCP was used at a concentration of 2μM which was shown to have no effect on cell motility or rates of oxygen uptake at this concentration. Increasing the pH of the incubation medium had the effect of markedly increasing the rate of oxygen consumption by the cells and their motility. The rates of oxygen uptake were measured with a Clark oxygen electrode and cell motility was observed by phase contrast microscopy as described in Materials & Methods (Section 2.6). All other assay methods and parameters were as described in Section 3.6.2.

The effects of FCCP on the uptake of labelled adenosine and hypoxanthine are shown in Figure 3.42. The uptake of adenosine is inhibited by 30% $\pm$4% and the uptake of hypoxanthine is inhibited by 25% $\pm$ 5% by FCCP. This suggests that the uptake of adenosine and hypoxanthine may be associated with the movement of protons across the plasma membrane. The effects of increasing pH (decreasing proton concentration) on the uptake of adenosine, adenine and hypoxanthine are shown in Figure 3.43. As the pH of the incubation medium is

Figure 3.43 EFFECT OF pH ON THE UPTAKE OF ADENOSINE, ADENINE AND HYPOXANTHINE.




Trypanosomes (1mg protein/ml) were incubated with adenosine(●), adenine(X) or hypoxanthine(■) (final concentration 1μM,specific activity 100μCi/μmole) in PSG buffer over a pH range of 6.5 to 10.0. All incubations were carried out at 22°C for 30 seconds uptake being measured by silicon sandwich centrifugation. The results are presented as the mean and range of duplicate experiments.

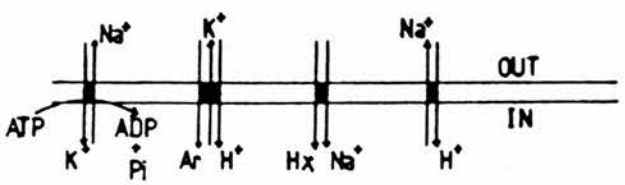
Table 3.8 EFFECT OF IONOPHORES AND ION GRADIENTS ON NUCLEOSIDE AND BASE UPTAKE

| | UPTAKE CONDITIONS | | | | | |
|---|---|---|---|---|---|---|
| SUBSTRATE | VALINOMYCIN 10.0µg/ml | GRAMICIDIN 1µg/ml | LOW $Na^+$ HIGH $K^+$ BUFFER | pH | FCCP 2µM | HIGH $Na^+$ |
| ADENOSINE | ↓56±6% | ↓42±4% | ↓20±6% | ↓HIGH pH | ↓30±7% | NO EFFECT |
| HYPOXANTHINE | ↓46±4% | ↓43±5% | ↓31±6% | NO EFFECT | ↓25±4% | STIMULATES |
| ADENINE | ↓33±7% | ↓48±8% | ↓24±3% | NO EFFECT | N.D. | NO EFFECT |

↓= INHIBITION N.D.= NOT DETERMINED

The table summarises the effects of valinomycin, gramicidin, FCCP, pH variation between pH 7 and pH 8.5, $Na^+$ and $K^+$ on the uptake of purine nucleosides and bases bases on the results presented in Sections 3.6.1 to 3.6.6.

Figure 3.44 SOME POSSIBLE MODELS FOR ACTIVE TRANSPORT OF PURINE NUCLEOSIDES AND BASES BY T.brucei.



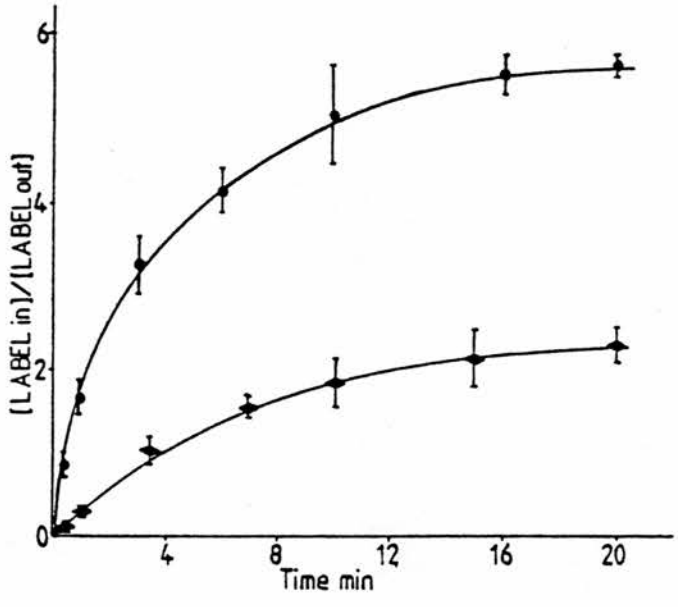
increased the rates of uptake of the labelled substrates decreases. However in the pH range 7 to 8.5 there is no effect on the uptake of adenine or hypoxanthine although adenosine uptake may be slightly inhibited by increasing pH over this range.

### 3.6.7 Summary of Ion, Ionophore and pH Effects on Purine Nucleoside and Base Uptake and a Possible Model for the Transport Mechanism.

Table 3.8 summarises the results obtained from the experiments described in Sections 3.6.2 to 3.6.6. The uptake of adenosine appears to be dependent on an electrochemical gradient as shown by the effects of the high extracellular $K^+$ and the ionophores valinomycin and gramicidin on uptake. A proton requirement for adenosine uptake is also indicated by the effects of increasing the extracellular pH. The uptake of adenine appears to have the same sensitivities as adenosine with respect to $K^+$ and the ionophores valinomycin and gramicidin. The uptake of hypoxanthine shows the same sensitivities as adenosine and adenine with respect to $K^+$ and the ionophores but is also slightly stimulated by high concentrations of extracellular sodium ion. This suggests that the uptake mechanism for hypoxanthine is different to the uptake mechanisms for adenosine and hypoxanthine.

Figure 3.44 shows diagramatically some possible models of the uptake mechanisms for adenosine, and hypoxanthine which are consistent with the results shown in Table 3.8. Since adenosine uptake shows some sensitivity to pH the existance of a pH gradient across the plasma membrane was investigated.

**Figure 3.45 DISTRIBUTION OF METHLAMINE ACROSS THE TRYPANOSOMAL PLASMA MEMBRANE.**




Trypanosomes were suspended at a cell density of $3.613 \times 10^8$ cells/ml in PSG at either pH 7.4 or pH 8.0. The [$^{14}$C] methylamine (10µl at 0.1µCi/µl) was added to suspensions of cells (5ml) at pH 7.4 and at pH 8.0. The equilibration of the label across the cell membrane was followed by taking samples of the incubations at the times shown and centrifuging on silicon sandwiches. After centrifugation the concentrations of label inside and outside the cells was estimated by counting samples of the top layer of the silicon sandwiches and the PCA layer. (●) Methylamine pH 7.4, (◂▸) Methylamine pH 8.0. The results are presented as the mean and range of duplicate experiments.

### 3.6.8 Measurement of ΔpH Across the Trypanosomal Plasma Membrane.

The detection and measurement of a pH gradient across the trypanosomal plasma menbrane was carried out by measuring the distribution across the plasma membrane of the $^{14}C$ labelled weak base methylamine at pH 7.4 and pH 8.0.

The weak base distributes across a membrane in a manner dependent on the pH on each side of the membrane. Calculation of the concentrations of the label on each side of the membrane allows calculation of the magnitude of any pH gradient present using the following equation.

$$\Delta pH = \log_{10} \frac{[CH_3NH_3^+]_{OUT}}{[CH_3NH_3^+]_{IN}}$$

The derivation of the above equation is given in Materials and Methods (Section 2.17).

The estimation of ΔpH was carried out as detailed in the legend to Figure 3.45. Distribution of the label in the cell was assumed to be even throughout the total cell water. The volume used for total cell water was 32μl/$10^9$cells as determined in Section 3.6.1. The results of these experiments are shown in Figure 3.45 expressed as the ratio of the concentration of the label extracellularly to the concentration of label intracellularly as a function of time. The results show that the label has not reached true equilibrium by twenty minutes. However the calculation of Δ pH requires the distribution of the label to be measured at equilibrium (Materials & Methods Section 2.17). The plots shown in Figure 3.45 were therefore linearised in the same manner as for a Hanes plot by plotting time divided by the ratio of label concentration inside the cell to the

Figure 3.46 LINEARISED PLOT OF THE DATA PRESENTED IN FIGURE 3.45

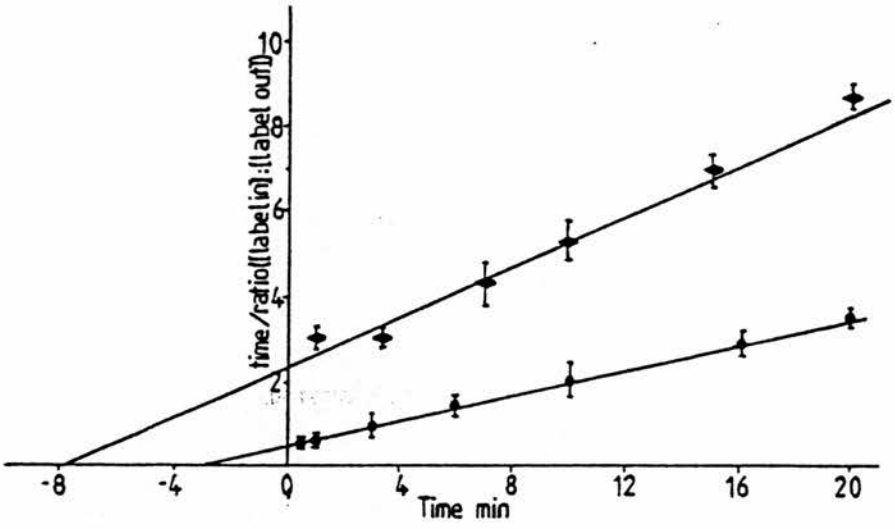



Table 3.9 $\Delta$pH ACROSS THE TRYPANOSOMAL PLASMA MEMBRANE.

| EXTERNAL pH | $pH_{in}$ METHYLAMINE | $\Delta$pH METHYLAMINE |
|---|---|---|
| 8.0 | 7.4 | -0.6 |
| 7.4 | 6.6 | -0.8 |

concentration of label outside the cell as a function of time as shown in Figure 3.46. The intercept of these plots with the y-axis is the x-axis intercept divided by the concentration ratios of label inside:label outside at equilibrium (cf. Km/Vmax). The ΔpH calculated from the equilibrium distributions of the two labels at pH 7.4 and pH 8.0 are shown in Table 3.9. These results show that at an extracellular pH of 8.0 the ΔpH is 0.6 acid inside. At an extracellular pH of 7.4 the proton gradient is larger with a ΔpH of 0.8 acid inside.The significance of these results will be considered further in the Discussion (Section 4.2.2).

**3.7 CELL FRACTIONATION.**

In order to characterise the uptake system for adenosine in more detail experiments were carried out to identify the molecules in the plasma membrane responsible for transport. The adenosine transport system was chosen for further study since labelled adenosine can be readily converted to an affinity label (Section 3.8), for use in identifying the adenosine transporter. To aid in the identification of the adenosine transporter a plasma membrane fraction was isolated from the trypanosomes to enable detection of proteins which may be present as a very low percentage of total cell protein.

Several methods have been published for the isolation of a plasma membrane enriched fraction from trypanosomes (Rovis & Baekkeskov,1980; Steiger et al.,1980; Mancini et al.,1982; Voorheis et al.,1979), which differ in the methods used for the initial disruption of the cells , the markers used to identify the plasma membrane fraction and the centrifugation techniques used to purify the plasma membrane. The main problem in purifying trypanosomal

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plasma membranes is the lack of an unequivocable marker enzyme for the membrane. However Steiger et al. (1980) showed that the enzyme $\alpha$-glucosidase (EC 3.2.1.20) was virtualy non-latent in whole cells suggesting its presence on the outer surface of the trypanosomal plasma membrane. Radioiodination of plasma membrane polypeptides, using the enzyme lactoperoxidase (EC 1.11.1.8) and $^{125}I$, was used to mark plasma membranes prior to fractionation by Mancini et al. (1982). This author also used $Na^+/K^+$ ATPase (EC 3.6.1.3) as a membrane marker as did Voorheis et al. (1979). Using ouabain inhibitable $Na^+/K^+$ ATPase activity as a plasma membrane marker suffers from the drawback that in the initial stages of the cell fractionation it can not be easily detected against the general background of phosphatase activity and the activities of other ATPases. The problem of suitable enzyme markers for the trypanosomal plasma membrane will be considered in more detail in the discussion. The plasma membrane markers chosen for use in this work were $\alpha$-glucosidase and cell surface proteins labelled with $^{125}I$. $Na^+/K^+$ ATPase was also used as a plasma membrane marker after the cell membranes had been partially purified.

### 3.7.1 Cell Breakage Techniques.

The authors listed in the previous section all used different techniques for the initial disruption of the trypanosomes. Voorheis et al. (1979) used a combination of osmotic swelling of the cells and a very tight fitting homogeniser (10-20$\mu$m clearance) to disrupt the cells whereas Mancini et al. (1982) used sonication to disrupt the cells. Nitrogen cavitation was the method used by Rovis & Baekkeskov (1980), while Steiger et al. (1980) ground the cells with silicon

**Table 3.10 METHODS OF CELL BREAKAGE AND DISTRIBUTION OF α-GLUCOSIDASE AND PROTEIN.**

| CELL BREAKAGE TECHNIQUE | % TOTAL PROTEIN | | % TOTAL α-GLUCASE. | |
|---|---|---|---|---|
| | HSS | HSP | HSS | HSP |
| SONICATION | 59% | 30% | 48% | 5% |
| WATER LYSIS | 70% | 37% | 56% | 36% |
| CARBIDE GRINDING | 40% | 25% | 48% | 24% |
| GLASS BEAD GRINDING | 58% | 27% | 46% | 54% |

The table shows the distribution of α-glucosidase activity and protein between a high speed supernatant and pellet obtained form a cell lysate produced as shown. All experimental details are given in the text (Section 3.7.1) and Materials and Methods (Section 2.7).

carbide.

Attempts to disrupt the trypanosomes by the method of Voorheis et al. (1979) failed due to the lack of a homogeniser with a small enough clearance, therefore this approach to cellular disruption was abandoned. The equipment required for carrying out cellular disruption by nitrogen cavitation was not available therefore this method was not attempted. Grinding the trypanosomes with silicon carbide caused complete disruption of the cells but recovery of material from the silicon carbide/cell paste formed during grinding was poor. However when 75μm glass beads were used for grinding in place of the silicon carbide the recovery of material from the grinding paste was much improved. The recovery of material from the grinding paste could be further improved by prewashing the glass beads in a 1mg/ml BSA solution. Sonication as used by Rovis and Baekkeskov (1980) was also successful in disrupting the trypanosomes but had some disadvantages as described below.

Four methods of cell disruption were compared; sonication, water lysis, grinding with silicon carbide and grinding with glass beads. The grinding material was removed by centrifugation at 100xg for five minutes. Table 3.10 shows the distribution of protein and the marker enzyme α-glucosidase between a high speed supernatant fraction (HSS) and a high speed pellet fraction (HSP) containing all cell membranes. The two fractions HSS and HSP were produced by centrifugation (150,000xg av. for 1 hr) of the homogenates produced by the methods listed in Table 3.10. The experimental details of the disruption methods listed in Table 3.10 are given in Materials & Methods. The results of the distribution of α-glucosidase and protein are expressed as percentages of the protein or α-glucosidase activity found in the starting cell suspension. Protein was estimated by the method of

Bradford (1976) and α-glucosidase activity was assayed by the fluorimetric method described by Steiger et al. (1980) using 4-methyl-umbelliferyl-α-D-glucoside as substrate.Full details are given in Materials & Methods (Sections 2.12 & 2.10.1 respectively).

The results presented in Table 3.10 show that sonication results in an apparent loss (47%) of α-glucosidase activity, the reasons for this loss of activity are unknown. The loss of activity observed can not be accounted for by vesicularisation of the plasma membrane depriving the enzyme of access to the substrate since the αglucosidase assay buffer contained detergent (Triton X-100 at a concentration of 1%(w/v)) to make permeable any vesicles formed. This loss of marker enzyme activity made sonication an unsuitable method for the disruption of trypanosomes.

Water lysis of the trypanosomes results in better recovery of the marker enzyme activity but caused destruction of the subcellular organelles of the trypanosomes, such as glycosomes and lysosomes, which is indicated by the high percentage of the cellular protein recovered in the high speed supernatant fraction (HSS). Destruction of the lysosomes in particular is undesirable since this will cause the release of many proteolytic enzymes into the homogenate. Water lysis was therefore discounted as a method of cellular disruption.

Grinding the trypanosomes with silicon carbide as stated above results in poor recovery of protein and αglucosidase activity from the cell grinding paste. Therefore grinding with silicon carbide was not used as a technique for cellular disruption.

Grinding the trypanosomes with 75μm diameter glass beads resulted in reasonable recovery of protein and 100% recovery of αglucosidase activity from the grinding paste. This method of cell

breakage also proved to be least harsh as indicated by 50% of the α-glucosidase remaining associated with the cell membranes. It will be shown later that α-glucosidase is only loosely associated with the plasma membrane and easily removed as was observed by Steiger et al. (1980). In view of the above results grinding with glass beads was the cell breakage method chosen for use in all subsequent fractionation experiments.

### 3.7.2 Preliminary Cell Fractionation Experiments and Estimation of Plasma Membrane Density.

Before designing a purification scheme for the routine isolation of a plasma membrane fraction the densities of the plasma membrane fragments produced by grinding the trypanosomes with glass beads was determined.

Voorheis et al. (1979) estimated the plasma membrane density to be $1.24g/cm^3$, agreeing with Steiger et al. (1980) who estimated the density to be $1.22g/cm^3$. Mancini et al. (1982) estimate the plasma membrane density to be between $1.12g/cm^3$ and $1.14g/cm^3$ which is in close agreement with Rovis & Baekkeskov (1980) who estimate the density to be $1.14g/cm^3$. The differences between these values for the plasma membrane densities could be due to the presence or absence of the subpellicular microtubules associated with the intact plasma membrane. (Anderson & Ellis,1965; Angelopoulos,1970). This point will be considered again in the discussion.

The density of the plasma membrane fragments, produced by grinding the trypanosomes with glass beads, was estimated by centrifugation of a crude membrane preparation on a continuous

sucrose density gradient from 1.018g/cm$^3$ to 1.270g/cm$^3$. All densities were estimated from the refractive index of the gradient fractions. The trypanosomes (approximately 4ml packed cell volume (PCV)) were suspended to a total of volume of 6ml in 10mM HEPES, 0.25M sucrose, 1mM EDTA, 1mM PMSF, 1mM benzamidine, at pH 7.4. Glass beads (BSA washed) were added to the cell suspension until a stiff paste of cells and beads was formed. This paste was then ground in a chilled mortar and pestle until no whole cells could be observed by phase contrast microscopy, typically after 1-2 minutes grinding. The broken cell and glass bead paste was then treated as shown in Figure 3.47. Samples of all fractions were taken for estimation of protein content and $\alpha$-glucosidase activity. The distributions of protein and $\alpha$-glucosidase activity in the fractions obtained from this experiment (Figure 3.47) are given in Table 3.11. The sucrose interface material (SI) which collected on the 2.08M (1.26g/cm$^3$) sucrose cushion was resuspended in 10mM HEPES, 0.25M sucrose, 1mM EDTA, pH 7.4 and pelleted at 100,000x$\underline{g}$ av. for 30 minutes. The pellet was resuspended in the same buffer and layered onto continuous sucrose gradients, density 1.018-1.270g/cm$^3$. The gradients were centrifuged at 150,000x$\underline{g}$ av. for 15 hours in a Beckman SW41 swinging bucket rotor. The sucrose gradients (14ml) were fractionated into 1ml fractions by upward displacement of the gradient by 2.5M sucrose being pumped into the centrifuge tube through a hole in the bottom of the tube. The refractive index of each fraction was measured to estimate the fraction's density. The membrane material in each of the fractions was collected by dilution of the fractions in 10mM Hepes, 1mM EDTA, pH 7.4 and centrifuging at 100,000x$\underline{g}$ av. for 30 minutes. The membrane pellets obtained were resuspended in the same buffer by gentle

**Figure 3.47 CELL FRACTIONATION SCHEME FOR THE ESTIMATION OF PLASMA MEMBRANE DENSITY.**

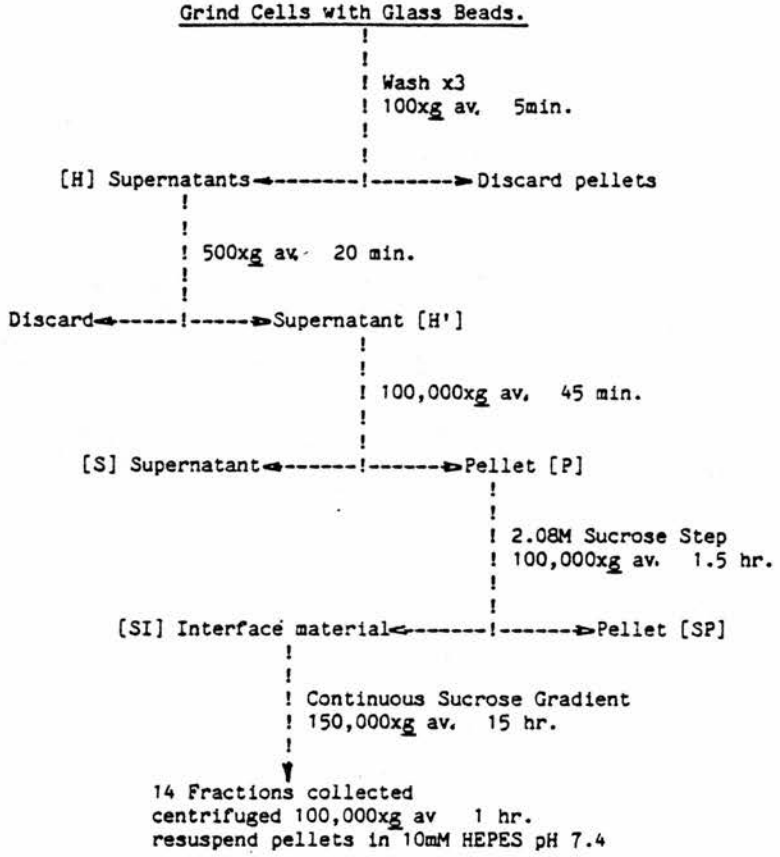
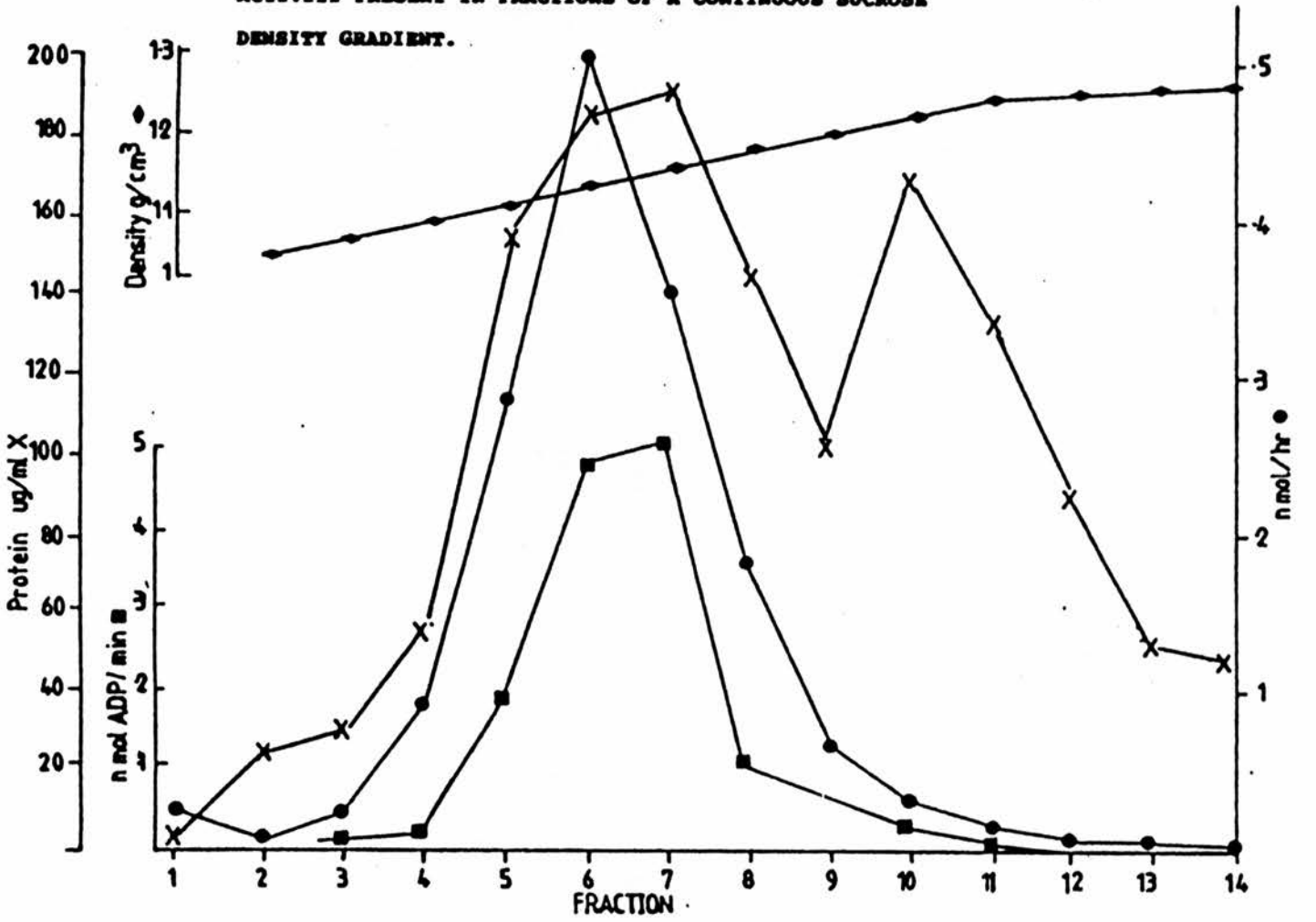



**Table 3.11 DISTRIBUTION OF α-GLUCOSIDASE ACTIVITY AND PROTEIN IN THE FRACTIONS LISTED IN FIGURE 3.47.**

| FRACTION | PROTEIN mg | α-GLUCOSIDASE TOTAL ACTIVITY |
|---|---|---|
| H | 38.2 | 2125 |
| H' | 32.1 | 1917 |
| S | 15.7 | 1293 |
| P | 7.8 | 432 |
| SI | 6.2 | 347 |
| SP | 2.6 | 46 |

Protein estimations and α-glucosidase assays were carried out as described in Materials & Methods (Section 2.12 & 2.10.1 espectively). α-glucosidase activity is given as pmol/min.

**Figure 3.48 DENSITY, PROTEIN CONTENT, $Na^+/K^+$ ATPase AND α-GLUCOSIDASE ACTIVITY PRESENT IN FRACTIONS OF A CONTINUOUS SUCROSE DENSITY GRADIENT.**



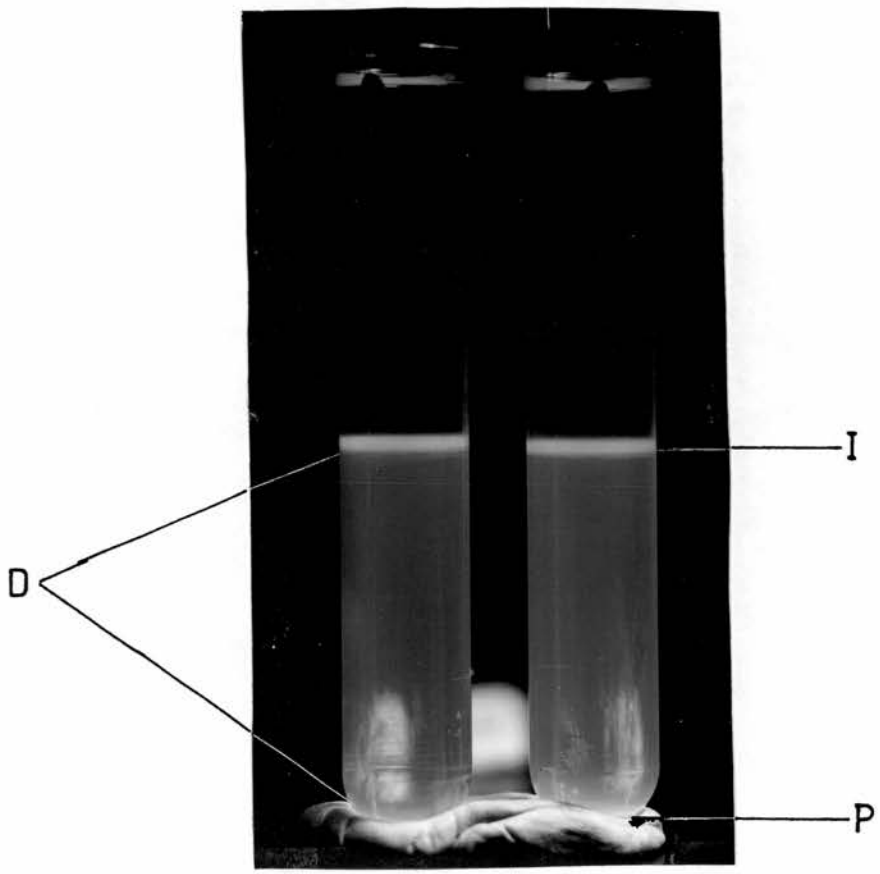

The assays used for protein and enzyme activities shown in the figure are detailed in Materials & Methods (Sections 2.10.1, 2.10.2 & 2.12). The experimental details as regards the material run on the gradient and other details are given in the text (Section 3.7.2). Density (♦), Protein (X), α-glucosidase (●) and $Na^+/K^+$ ATPase (■).

homogenisation for the determination of $\alpha$-glucosidase activity, ouabain inhibitable $Na^+/K^+$ ATPase activity and protein content. The assays used are described in detail in Materials and Methods (Sections 2.10.1, 2.10.2 & 2.12 respectively).

Figure 3.48 shows the distribution of protein, $\alpha$-glucosidase activity and ouabain inhibitable $Na^+/K^+$ ATPase in the various fractions from the continuous sucrose density gradient. The results presented in Figure 3.48 show that $\alpha$-glucosidase and ouabain inhibitable $Na^+/K^+$ ATPase band at the same densities. These two plasma membrane markers band at a density of 1.13-1.16g/cm$^3$ with a peak of activity at a density of 1.14g/cm$^3$which is in close agreement with Rovis & Baekkeskov (1980) and Mancini et al.(1982).

Rovis & Baekkeskov (1980) purified trypanosomal plasma membranes from endoplasmic reticulum and golgi membranes by dialysis of a crude microsomal membrane faction against 1mM HEPES,1mM $MgCl_2$ and protease inhibitors at pH 8.2 for 2 hours followed by centrifugation onto a plaque of 26%(w/v) dextran in the same buffer. The plasma membranes were found to form a band on top of the dextran plaque while the golgi, endoplasmic reticulum and other membranes pelleted at the bottom of the tube. This dextran centrifugation method has also been used to produce plasma membranes from adipocytes (Steck & Wallach,1970) and from Ehrlich ascites carcinoma cells (Wallach & Kamat,1964). These authors showed that the pH and magnesium ion concentration used in the dialysis step can make a marked difference in the distribution of the plasma membrane on the dextran plaque. Both of the above authors were in agreement that a high pH was required for optimum yields of plasma membrane. The optimum magnesium ion concentration used in this dextran centrifugation method appears

Figure 3.49 PHOTOGRAPH OF A DEXTRAN PLAQUE AFTER CENTRIFUGATION OF MICROSOMES IN 0.5mM $MgSO_4$.




The fractions arrowed are I interface material, D dextran plaque and P pelleted material. All other details are given in the text (Section 3.7.3)

to vary depending on the cell type being fractionated. Therefore the optimum concentration of magnesium ion for the production of plasma membranes from T.brucei TREU 55 was determined. The physical basis of this technique will be considered in the discussion.

### 3.7.3 Optimisation of Magnesium Ion Concentration for Dextran Centrifugation.

The optimum concentration of magnesium ion to be used in preparing plasma membranes by centrifugation onto a dextran plaque was determined as follows. A crude total cell membrane fraction was prepared by grinding the trypanosomes with glass beads as described previously (Section 3.7.1)and centrifuging the homogenate at 150,000xg av. for 1 hour. the pellet from this centrifugation was resuspended in 1mM HEPES,1mM PMSF, 1mM benzamidine at pH 8.6 containing magnesium sulphate at a concentration of 0.5mM, 1.0mM or 2.0mM and dialysed against 10 volumes of the same buffer for 1 hour. The dialysed material was then layered onto 26%(w/v) dextran plaques containing the appropriate concentration of magnesium sulphate and centrifuged in a swinging bucket rotor at 150,000xg av. for 1.5 hours. At the end of this centrifugation 3 fractions were collected. The sample/dextran interface material(I), the dextran plaque(D) and the material pelleted at the bottom of the tube. These fractions were resuspended and washed by resuspension and centrifugation (200,000xg av. for 30 minutes) in 10mM HEPES, 1mM EDTA at pH 7.4. The three fractions were then assayed for protein content and $\alpha$-glucosidase activity as described in Materials & Methods (Sections 2.12 & 2.10.1 respectively).

Figure 3.49 shows a photograph of the dextran plaque after

**Table 3.12**

**EFFECT OF $Mg^{2+}$ CONCENTRATION ON THE DISTRIBUTION OF THE MEMBRANE MARKER $\alpha$-GLUCOSIDASE AND PROTEIN ON A 26% DEXTRAN PLAQUE.**

| $[Mg^{2+}]$ | FRACTION | % TOTAL PROTEIN | $\alpha$-GLUCOSIDASE SPECIFIC ACTIVITY | $\alpha$-GLUCOSIDASE %TOTAL ACTIVITY |
|---|---|---|---|---|
| 0.5mM | I | 21% | 79.3 | 56% |
| | D | 22% | 25.3 | 20% |
| | P | 56% | 10.2 | 20% |
| 1.0mM | I | 20% | 47.2 | 42% |
| | D | 22% | 29.1 | 30% |
| | P | 58% | 10.1 | 26% |
| 2.0mM | I | 9% | 30.6 | 32% |
| | D | 14% | 30.8 | 51% |
| | P | 77% | 1.7 | 16% |

Experimental details for the above experiments are described in the text (Section 4.7.4). The enzyme assay used and the method of protein estimation are described in Materials & Methods (Sections 2.12 & 2.10.1). The specific activity of $\alpha$-glucosidase is given as nmol/min/mg protein.

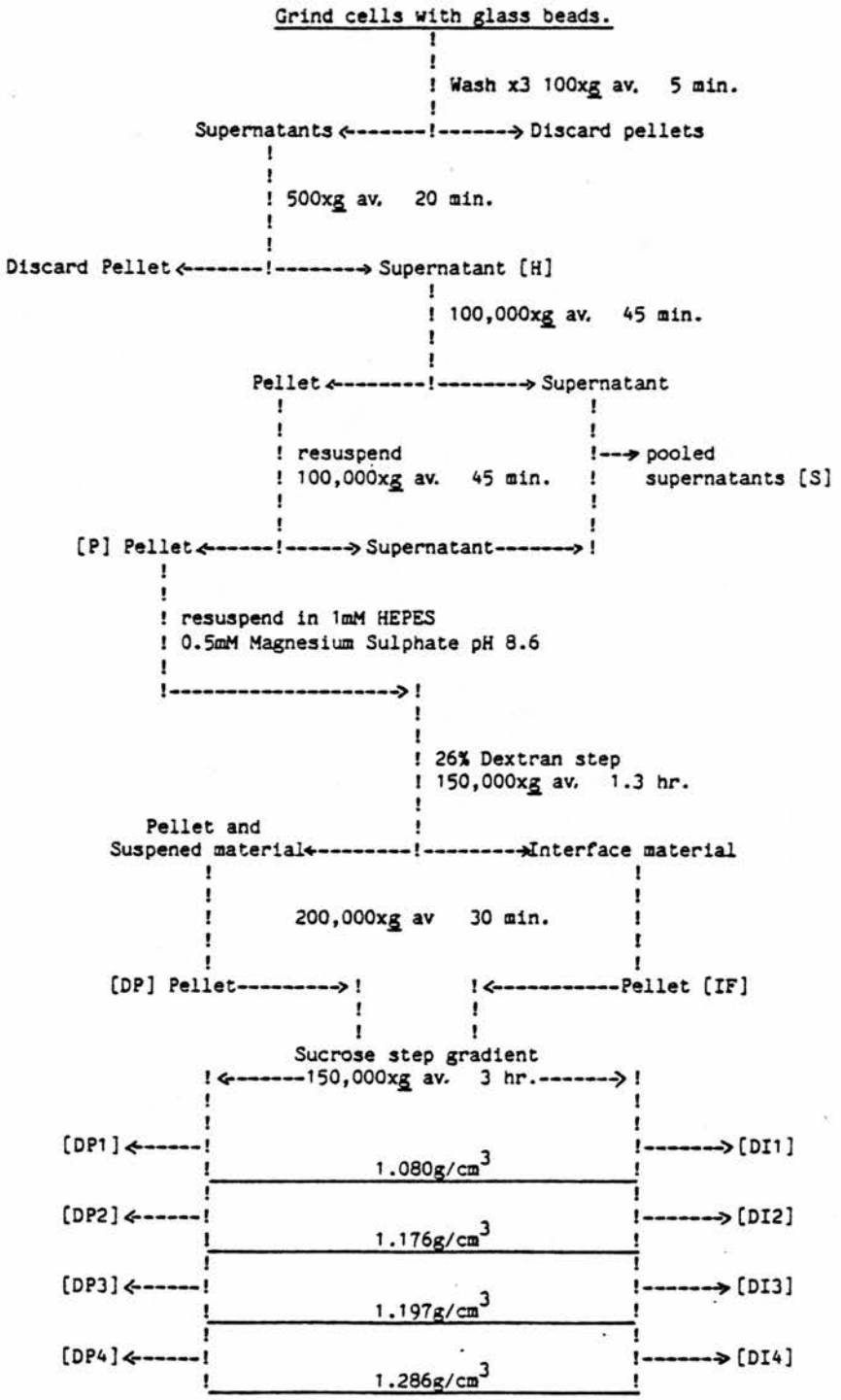
centrifugation containing 0.5mM magnesium sulphate. The three fractions collected are labelled I, D and P as described above. Table 3.12 shows how the distribution of protein and $\alpha$-glucosidase activity between these three fractions varies with changes in the magnesium ion concentration. As the magnesium ion concentration increases, the percentage of total protein in the dextran plaque and pellet increases and less is left on the sample/dextran interface. The plasma membrane marker enzyme $\alpha$-glucosidase shows this same tendency with increasing magnesium ion concentrations but the activity migrates into the dextran rather than pelleting. The optimum magnesium ion concentration for the purification of the plasma membrane appears to be 0.5mM as indicated by the highest content and specific activity of $\alpha$-glucosidase in the sample/dextran interface at this magnesium ion concentration.

### 3.7.4 Purification of Trypanosomal Plasma Membrane.

Having found a suitable method of cell breakage (Section 3.7.1), determined the density of the plasma membrane fraction by equilibrium density centrifugation (Section 3.7.2) and optimised the magnesium ion concentration for use in the dextran centrifugation purification of plasma membrane, a final purification scheme for the routine production of a plasma membrane-enriched fraction was developed.

The scheme developed for the purification of trypanosomal plasma membrane is shown in Figure 3.50. In this experiment $^{125}I$ surface labelled cells were added to the bulk of the cells prior to fractionation to act as a plasma membrane marker as well as $\alpha$-glucosidase activity. The radio-iodination of the cells was carried out as described in Materials & Methods (Section 2.16). The labelled

**Figure 3.50 CELL FRACTIONATION SCHEME FOR THE PRODUCTION OF PLASMA MEMBRANE ENRICHED FRACTION.**



cells were added to unlabelled cells (6ml PCV) which were then ground with glass beads in homogenisation buffer as described in Section 3.7.2. The broken cell debris was washed from the glass beads by repeated resuspension in homogenisation buffer and low speed centrifugation. The homogenate obtained was then centrifuged at 500x$\underline{g}$ av. for 20 minutes to remove unbroken cells and nuclei. The supernatant from this centrifugation was designated the start homogenate (H Figure 3.50). The membrane material present in this homogenate was pelleted by centrifugation at 100,000x$\underline{g}$ av. for 45 minutes and the pellet obtained washed once in homogenisation buffer by gentle homogenisation before repelleting the membranes. The first supernatant and the supernatant from the washing step were pooled (S Figure 3.50). The pellet (P Figure 3.50) was resuspended in 1mM HEPES, 0.5mM Mg $SO_4$ at pH 8.6 and dialysed against 10 volumes of this buffer for 1 hour. The dialysed material (4ml) was then layered on to dextran plaques (8ml) and centrifuged at 150,000x$\underline{g}$ av. for 1.3 hours in a SW 41 swinging bucket rotor. Two fractions were collected from the dextran centrifugation step, the sample/dextran interface material and the material suspended in the dextran along with the pelleted material. Both of these fractions were resuspended in homogenisation buffer and pelleted by centrifugation at 200,000x$\underline{g}$ av. for 30 minutes. The interface material (IF Figure 3.50), the dextran suspended material and pellet fraction (DP Figure 3.50) were further analysed by centrifugation on a sucrose discontinuous step gradient. The two fractions IF and DP were resuspended by gentle homogenisation in homogenisation buffer and layered onto the sucrose step gradients. The gradient steps were designed to collect the maximum yield of plasma membrane rather than low yield high purity. The density of the

Table 3.13 DISTRIBUTION OF MEMBRANE MARKERS IN SUBCELLULAR FRACTIONS PRODUCED BY THE FRACTIONATION SCHEME SHOWN IN FIGURE 3.50.

| FRACTION | PROTEIN mg | SPECIFIC ACTIVITY α-GLUCOSIDASE nmol/min/mg | SPECIFIC ACTIVITY $^{125}I$ CPMX$10^{-3}$/mg | PURIFICATION FACTOR αGLUCOSIDASE | PURIFICATION FACTOR $^{125}I$ |
|---|---|---|---|---|---|
| H | 117 | 31.0 | 96 | – | – |
| S | 99.5 | 22.1 | 69 | 0.7 | 0.7 |
| P | 32 | 73.3 | 212.7 | 2.4 | 2.2 |
| IF | 3.5 | 137.5 | 326.7 | 4.4 | 3.4 |
| DP | 13.4 | 54.8 | 115.1 | 1.7 | 1.2 |
| DP1 | 0.003 | N.D. | 725 | – | 7.5 |
| DP2 | 0.31 | 24.7 | 350 | 0.8 | 3.6 |
| DP3 | 0.54 | 10 | 127.7 | 0.3 | 1.3 |
| DP4 | 3.5 | 25.7 | 107.1 | 0.8 | 1.1 |
| DI1 | 0.12 | 69.6 | 511.5 | 2.2 | 5.3 |
| DI2 | 1.41 | 97.1 | 649.9 | 3.1 | 6.7 |
| DI3 | 0.011 | N.D. | 397.3 | – | 4.1 |
| DI4 | N.D. | N.D. | N.D. | – | – |

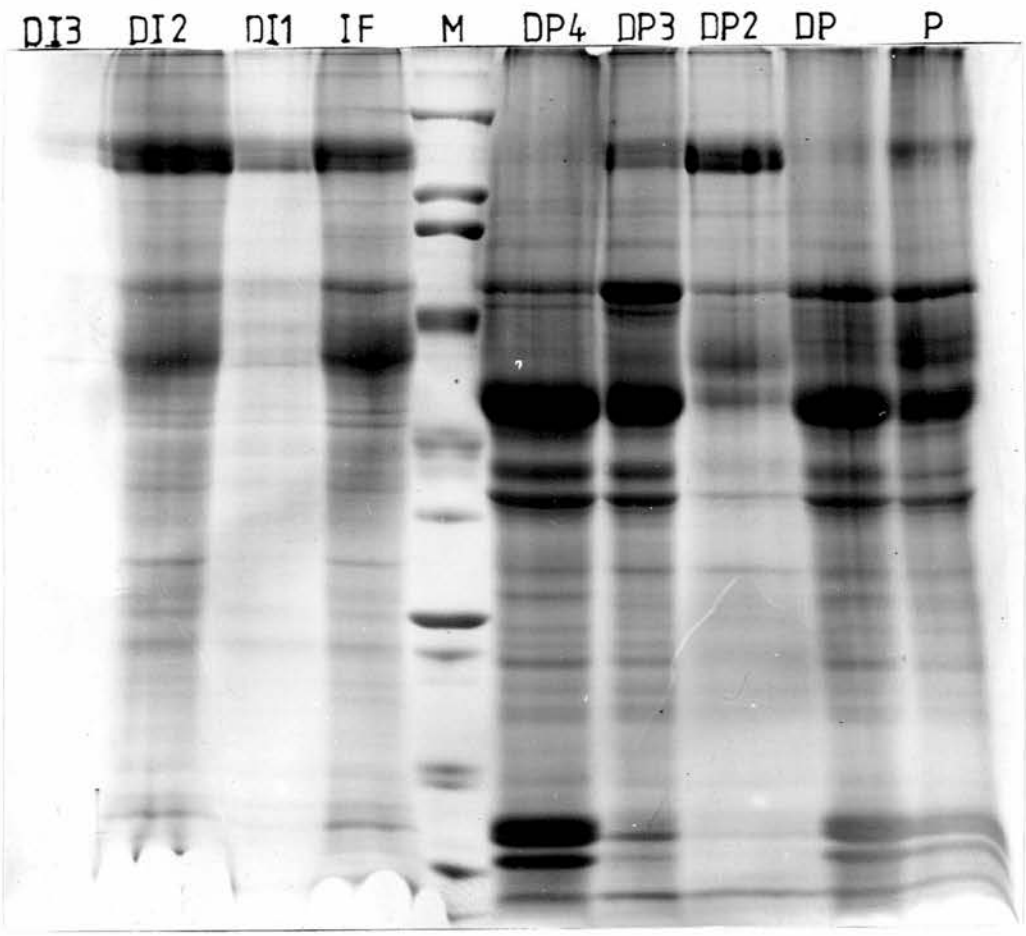
N.D. = NOT DETECTED

The fractions listed in the table were produced as described in Figure 3.50 and text (Section 3.7.4). α-Glucosidase activity was measured as described in Materials & Methods (Section 2.10.1). The $^{125}I$ content of the fractions was estimated by liquid scintillation counting.

first step was 1.080g/cm$^3$ which would allow through all the plasma membrane and denser material. The second step was composed of sucrose at a density of 1.176g/cm$^3$ which would stop all the plasma membrane material as indicated by the distribution of $\alpha$-glucosidase activity on a continuous sucrose gradient (Figure 3.39). The third step had a density of 1.197g/cm$^3$ followed by a cushion of density 1.286g/cm$^3$. After centrifugation of the gradients at 150,000x$\underline{g}$ av. for 3 hours, material from the sucrose step interfaces was collected and washed by resuspension and centrifugation before finally being resuspended in 10mM HEPES, 1mM EDTA, pH 7.4 for estimation of protein, $^{125}$I and $\alpha$-glucosidase activity. The fractions from the sucrose step gradients were named as follows; fractionated dextran interface material (IF Figure 3.50), DI1 density < 1.080g/cm$^3$, DI2 density < 1.176g/cm$^3$, DI3 density < 1.197g/cm$^3$ and DI4 density < 1.286g/cm$^3$ (Figure 3.50). The dextran suspended and pelleted material (DP Figure 3.50) separated on the sucrose step gradient was collected in identical density fractions DP1 to DP4.

Table 3.13 shows the distribution of $^{125}$I, $\alpha$-glucosidase and protein between the fractions listed in Figure 3.50. The results show that the dextran interface material (IF) contains plasma membrane material since sucrose step gradient analysis of this fraction shows only low density material is present with the highest specific activity of $\alpha$-glucosidase and $^{125}$I in fraction DI2 as would be expected from plasma membrane with a density of 1.14g/cm$^3$. Analysis of the dextran suspended material and pellet on the sucrose step gradient shows more material present at higher densities with low specific activities of $\alpha$-glucosidase and $^{125}$I-labelled proteins. The purification of the plasma membranes as indicated by increasing

Figure 3.51 SDS PAGE GEL OF THE SUBCELLULAR FRACTIONS PRODUCED BY THE FRACTIONATION SCHEME GIVEN IN FIGURE 3.50.




All lanes of the gel were loaded with 200μg of protein with the exceptions of lanes DI1 and DI3 which were loaded with 3μg and 11μg respectively being the entire protein content of these fractions. The gel electrophoresis system used is described in Materials & Methods (Section 2.13)

specific activity of $\alpha$-glucosidase parallels the increasing specific activity shown by $^{125}I$-labelled proteins until the sucrose step gradient analysis. The drop in specific activity shown by $\alpha$-glucosidase is probably due to the loss of $\alpha$-glucosidase activity, either by becoming detached from the membrane or inactivation by some other means. The fraction DP1 shows a high specific activity of $^{125}I$ which is probably due to free iodine at the top of the gradient with very little protein (0.003mg). It is likely that the specific activity of $^{125}I$ in fraction DI1 is an overestimate for the same reasons. The fractions IF, DI1, DI2, DI3, P, DP1, DP2, DP3 and DP4 were analysed by SDS polyacrylamide gel electrophoresis (SDS PAGE) to compare the distribution of proteins between fractions. Figure 3.51 shows a photograph of the Coomassie stained gel. The gel shows that the protein composition of the various fractions is markedly different. There are a great many differences between the protein compositions of the dextran interface material, IF, and the dextran suspended and pelleted material, P. However there appears to be little difference between the dextran interface material, IF, and the plasma membrane fraction, DI2, from the sucrose step gradient. Another point to notice is that fractions DI2 and IF have a very high lipid content, as would be expected of a plasma membrane fraction, indicated by the irregular banding of proteins at the bottom of the gel. This effect can be eliminated by acetone:ethanol extraction of the membrane fractions before SDS PAGE.

The fraction DI2 was used in all subsequent experiments as the plasma membrane enriched fraction which shows a 6.7 fold purification with respect to radioiodinated proteins and a 3 fold purification of $\alpha$-glucosidase although this may be an under-estimate for the reasons

stated above.

### 3.7.5 Cellular Distribution of Purine Nucleoside and Base Metabolising Enzymes.

Before attempting any further characterisation of the adenosine transport system the cellular distribution of the enzymes involved in metabolism of nucleosides and bases was investigated. The main reason for this investigation was to determine whether any of the enzymes were membrane bound which could indicate an involvement in the uptake system as in the bacterial and mould phosphotransferase systems for the uptake of sugars(Oxender,1972; Boos,1974).

The distribution of the enzymes between membranes and cytoplasm was investigated by making the cells permeable with the detergent saponin and measuring the leakage of the various enzyme activities from the cell. Trypanosomes were suspended in Tris saline glucose buffer (TSG) at pH 7.4, at a protein concentration of 1.5mg/ml, either with or without 0.1% (w/v) saponin present. The two batches of cells were then treated in the same way as described below. The cell suspensions were left on ice for 20 minutes then centrifuged at 10,000x$\underline{g}$ av. for 5 minutes to pellet the cells. The supernatants (LSS) and pellets (LSP) were then assayed for the following enzyme activities; adenosine hydrolase, APRT, HxPRT and the plasma membrane marker $\alpha$-glucosidase as described in Materials & Methods (Sections 2.10 & 2.10.1). The cell pellets were water lysed, homogenised in TSG buffer, centrifuged at 120,000x$\underline{g}$ av. for 1 hour and the supernatant (HSS) and pellet (HSP) assayed for the enzyme activities listed above. The protein content of all fractions was also determined.

The activities of the above enzymes in each fraction are shown

Table 3.14 CELLULAR LOCATION OF PURINE METABOLISING ENZYMES AND $\alpha$-GLUCOSIDASE.

| BUFFER | CELL FRACTION | % PROTEIN | % ACTIVITY APRT | % ACTIVITY HxPRT | % ACTIVITY Ar HYDROL. | % ACTIVITY $\alpha$-GLUC.ASE |
|---|---|---|---|---|---|---|
| | WHOLE CELL | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| T | LSS | 7.2 | 1.9 | 0.5 | 13.1 | 3.5 |
| R H | LSP | 85.4 | 99.0 | 57.0 | 64.4 | 86.0 |
| I C | HSS | 35.8 | 28.0 | 9.2 | 2.7 | 3.6 |
| S 1. | HSP | 56.8 | 23.0 | 19.4 | 57.2 | 79.5 |
| | WHOLE CELL | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| T + | LSS | 48.2 | 113.0 | 96.7 | 91.0 | 74.8 |
| R H S | LSP | 46.7 | 5.0 | 6.5 | 22.0 | 0.8 |
| I C A | HSS | 8.0 | 0.8 | 2.7 | 1.1 | 1.1 |
| S 1. P. | HSP | 31.4 | 0.8 | 0.0 | 22.0 | 0.0 |

The methods of producing the fractions LSS (low speed supenatant), LSP (low speed pellet). HSS (high speed supenatant) and HSP (high speed pellet) are given in the text (Section 3.4.5). The assay methods used for adenine(APRT), hypoxanthine(HxPRT) phosphoribosyltransferases, adenosine hydrolase(Ar HYDROL) and $\alpha$-glucosidase($\alpha$-GLUC.ASE) are described in Materials & Methods (Sections 2.10 & 2.10.1).

in Table 3.14 expressed as a percentage of the activity in a whole cell lysate. The low speed supernatant fraction (LSS) should contain enzymes which leaked from the cells during the 20 minute incubation on ice. The results show that in the incubation without saponin very little material has leaked from the cells as shown by the distribution of enzyme activities between the fractions LSS and LSP, most of the enzyme activities being recovered in the cell pellet fraction (LSP). The results obtained for the incubations carried out in the presence of saponin are markedly different in that half of the cell protein has been released into the incubation medium (LSS) with approaching 100% of the enzyme activities measured in whole cell lysate also appearing in this fraction. The results also show that the saponin causes release of the plasma membrane marker $\alpha$-glucosidase from the plasma membrane which is in agreement with the results obtained by Steiger et al. (1980) who showed $\alpha$-glucosidase to be loosely attached to the outer surface of the plasma membrane. In view of this result the fact that the majority of the enzyme activities measured are released from the cells by saponin may not indicate that these enzymes are soluble cytoplasmic enzymes since the detergent may solubilise them if they are not firmly bound to membranes or are not integral membrane proteins.

When the cell pellets (LSP) from the incubations without saponin were water lysed and recentrifuged at high speed the results, expressed as a percentage of the activity found in the cell pellets (LSP), show that most of the enzyme activities are associated with the pelleted membrane material (HSP). The majority of the $\alpha$-glucosidase activity pellets with the membranes as does the adenosine hydrolase activity. The distribution of the APRT and HxPRT

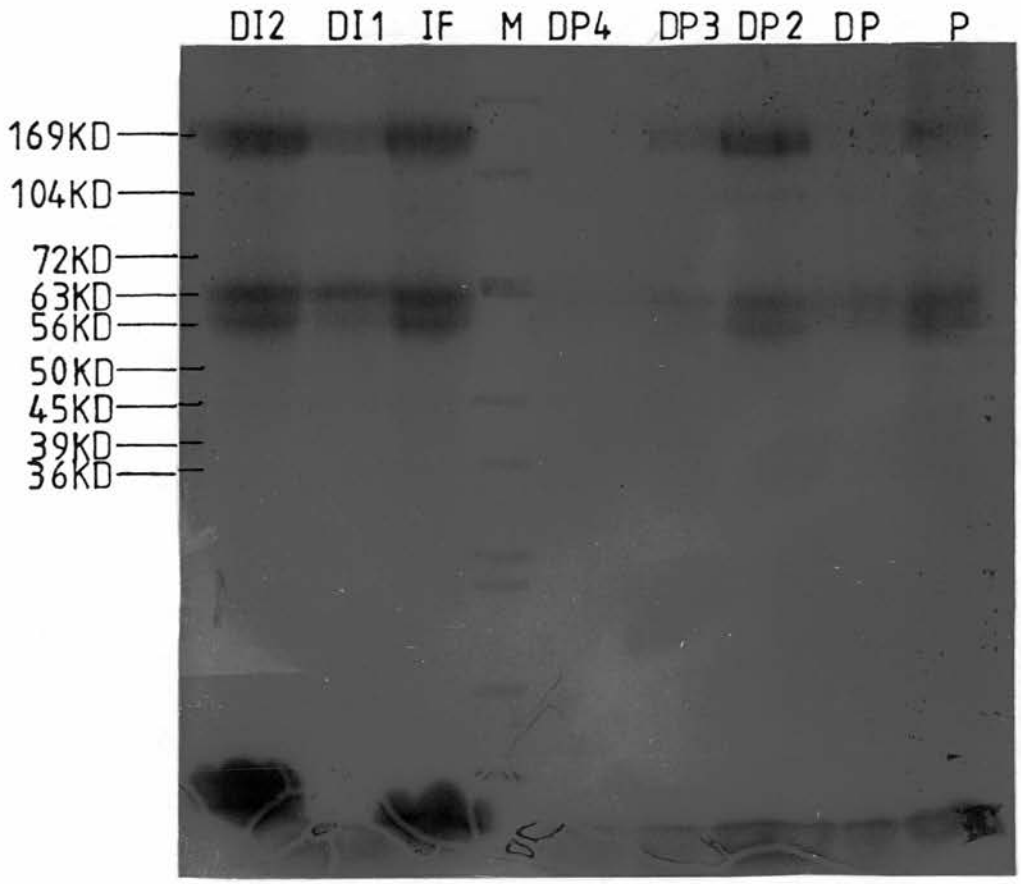
activities are less clear cut in that much of the enzyme activity is lost during homogenisation of the cell pellet (LSP) and the distribution of activities between the supernatant fraction HSS and the pellet fraction HSP are almost equal. The results for the saponin treated cells show that some of the adenosine hydrolase activity remains membrane bound even after saponin treatment.

Considered as a whole these results suggest that adenosine hydrolase and $\alpha$-glucosidase are membrane bound but easily solubilised by saponin treatment although some adenosine hydrolase activity appears to be resistant to solubilisation. The phosphoribosyltransferase activities may be soluble as indicated by the almost total release of activity from the cell in the presence of saponin and by the even distribution of detectable activity between membrane and soluble fractions in the absence of saponin. Further investigation into which subcellular fraction the adenosine hydrolase activity is associated with was not carried out. However this investigation should be carried out at a later date since as adenosine hydrolysis is the first step in the metabolism of adenosine to nucleotide, hydrolysis may be directly involved in the transport process.

### 3.7.6 Identification of Polypeptides Associated with the Outer Surface of the Trypanosomal Plasma Membrane.

The adenosine transport protein must be exposed at the cell surface to allow the binding of adenosine prior to transport across the membrane. Therefore as a preliminary to affinity labelling of the transporter, polypeptides exposed at the cell surface were identified.

Figure 3.52 AUTORADIOGRAPH OF $^{125}I$ LABELLED PROTEINS ON THE GEL SHOWN IN FIGURE 3.51.

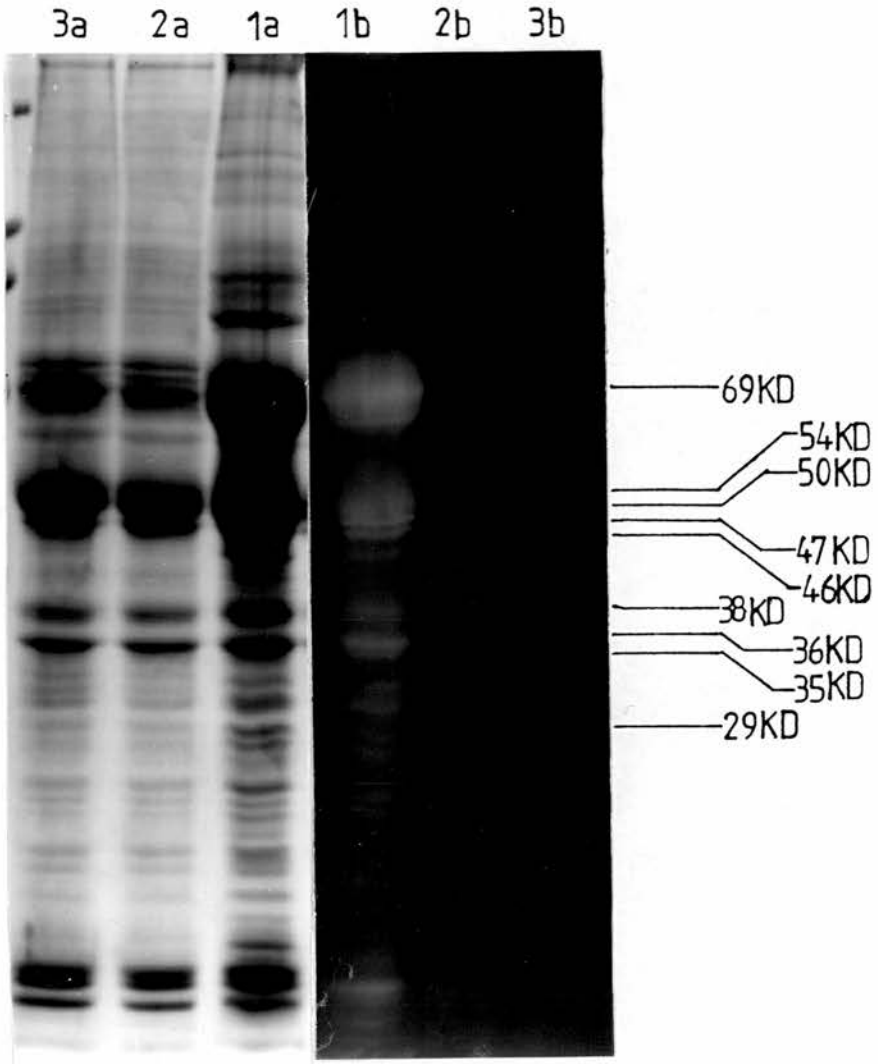



The SDS PAGE gel of the subcellular fractions shown in Figure 3.51 was prepared for autoradiography and autoradiographed as described in Materials & Methods (Section 2.14).

Two methods were used to identify proteins exposed at the cell surface. The first method used was radio iodination of the cell surface proteins by the enzyme lactoperoxidase and $^{125}I$ as described in the cell fractionation experiments (Section 3.7.4). Figure 3.52 shows an autoradiograph (Materials & Methods Section 2.14) of the SDS PAGE gel of subcellular fractions shown in Figure 3.51. This Figure shows that the fractions indicated as being rich in plasma membrane on the basis of distribution of $\alpha$-glucosidase activity (IF & DI2) also contain the majority of radiolabelled proteins. The lipid associated with these two fractions which runs at the bottom of the gel is also heavily radiolabelled. This is probably due to the reaction of free iodine, or iodine radicals produced by the lactoperoxidase, with unsaturated lipids in the plasma membrane. Figure 3.52 also shows the apparent molecular weights of the labelled proteins which were estimated from their mobilities on the gel relative to proteins of known molecular weight.

The second method used to identify cell surface proteins was labelling with fluorescein isothiocyanate (FITC). To show that only plasma membrane proteins were being labelled the labelling patterns obtained in the presence and absence of the plasma membrane barrier were compared. The fluorescent label FITC was incubated at a final concentration of 3mM (added as 500µl of a 30mM solution in 30% methanol) with 5ml of a whole cell lysate at a protein concentration of 1.5mg/ml in PSG buffer at pH 7.4. This incubation was repeated with whole cells in place of the cell lysate. The above mixtures were incubated on ice for 3 minutes. Samples of the above incubations were then prepared for SDS PAGE as follows. A sample of the cell lysate incubation was taken and prepared for electrophoresis without any

**Figure 3.53 SDS PAGE GEL OF FITC LABELED PROTEINS.**




a) Coomassie stained gel of (1a) whole cell lysate, (2a) whole cells, (3a) washed cell lysate.

b) The tracks shown 1b to 3b are identical to tracks 1a to 3a except labeled with FITC and photographed in UV light. All other experimental details are given in the text (Section 3.7.6)

further treatmemt, while a second sample was washed five times by resuspension in PSG buffer and centrifugation at 10,000x$\underline{g}$ av. for 5 minutes as were the whole cells. After centrifugation samples were prepared for electrophoresis as described in Materials & Methods (Section 2.13). The three samples, whole cells, whole cell lysate and washed cell lysate were loaded on to an 8%-20% exponential polyacrylamide gradient gel (200$\mu$g protein/sample) in duplicate. After electrophoresis the gel was divided into two portions one of which was stained for proteins by Coomassie blue dye and the other was soaked in 50% methanol and photographed under UV. light to visualise the proteins labelled with FITC.

Figure 3.53 shows a composite photograph of the Coomassie stained half of the gel (1a to 3a) and the half of the gel photographed under UV. light (1b to 3b). The lanes shown are 1 cell lysate, 2 whole cell and 3 washed cell lysate. The labelling patterns shown indicate that only plasma membrane proteins are being labelled. The whole cell lysate (1b) shows all the proteins in the cell which can be labellled by FITC, lane 2b shows which proteins are labelled in the presence of an intact plasma membrane acting as a barrier to the FITC. Lane 3b shows the membrane proteins labelled in a cell lysate once the soluble proteins and microsomal membranes have been removed by centrifugal washing. The labelling pattern shown in lanes 2b and 3b are identical which indicates that in the whole cell (2b) FITC does not label microsomal membranes or soluble cytoplasmic proteins. Figure 3.53 also shows the apparent molecular weights of the proteins labelled by FITC in whole cells as estimated by their relative mobilities on the gel compared to proteins of known molecular weight. The molecular weights of the proteins labelled by

FITC and $^{125}I$/lactoperoxidase in whole cells are summarised in Table 3.16.(Opposite page 143)

## 3.8 AFFINITY LABELLING OF THE ADENOSINE TRANSPORTER.

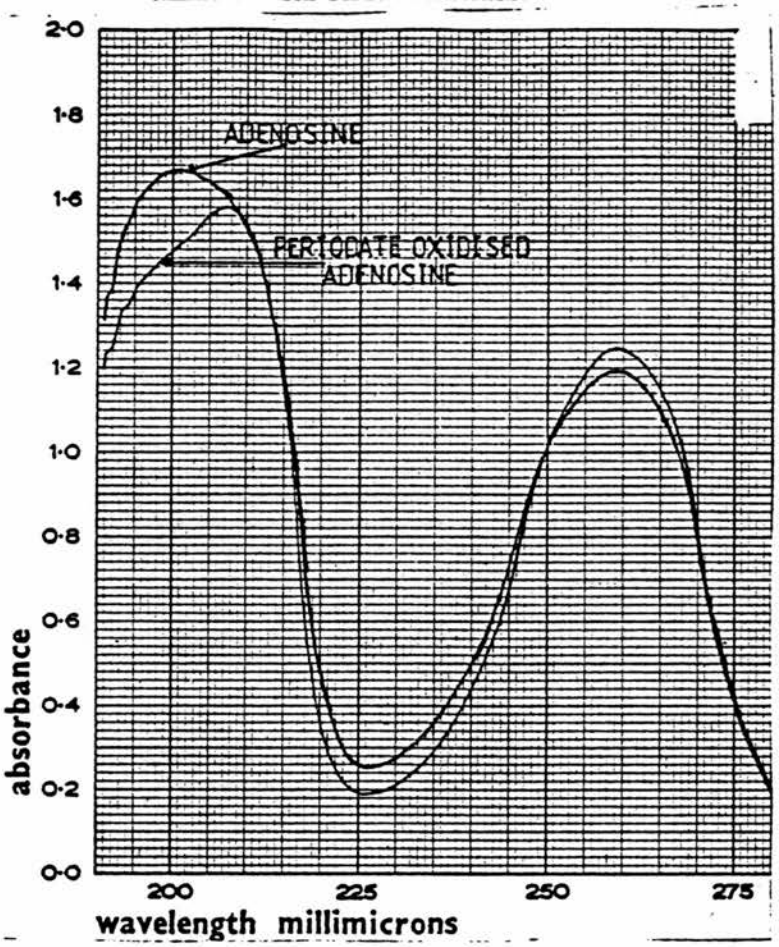
As stated in Section 3.7.1 [2,8-$^{3}H$] adenosine can be readily converted to an affinity label (2',3' dialdehyde adenosine) for use in identification of the plasma membrane transport protein. This involves oxidation of the ribose moiety of the adenosine by sodium periodate which splits the carbon bond between carbons 2 and 3 of the ribose resulting in the formation of two aldehyde groups. It is these two reactive aldehyde groups which allow the label to form covalent bonds to proteins which can be stabilised by a reducing agent such as sodium cyanoborohydride.

### 3.8.1 Synthesis of [2,8-$^{3}H$] 2',3' Dialdehyde Adenosine from Labelled Adenosine.

[2,8-$^{3}H$] Dialdehyde adenosine was prepared by essentially the same method as that described for the synthesis of dialdehyde ATP by Easterbrook-Smith et al. (1976) and Kumar et al. (1979) with [2,8-$^{3}H$] adenosine being used in place of ATP. The synthesis was carried out using the same ratios of reactants as detailed by the above authors but at a lower concentration due to the lower solubility of adenosine compared to ATP. The synthesis was first carried out using unlabelled adenosine.

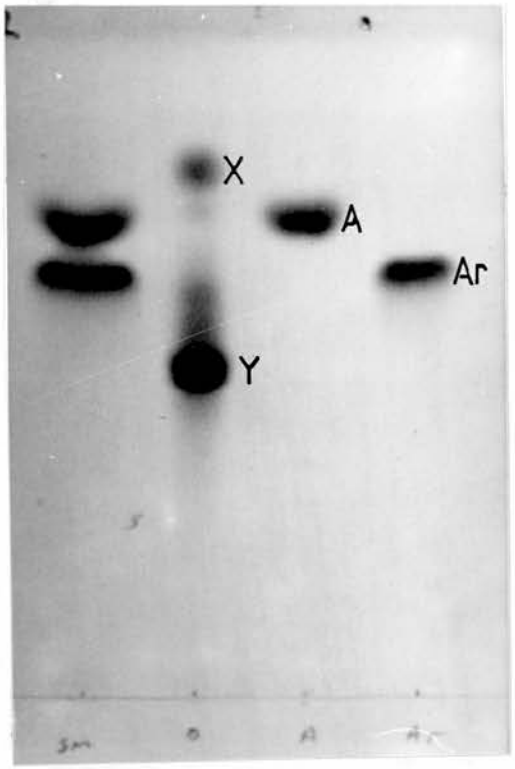
An adenosine solution (10ml) containing 0.123 mmoles of adenosine in water at pH 7.0 was mixed with 0.136 mmoles of freshly prepared sodium periodate solution (1ml) again at pH 7.0 giving a periodate:adenosine ratio of 1.1:1 on a molar basis. This reaction

Figure 3.54 ABSORBTION SPECTRUM OF ADENOSINE BEFORE AND AFTER OXIDATION WITH SODIUM PERIODATE.



mixture was stored in the dark at 4°C for 1 hr. The reaction was then stopped by the addition of 4µl of ethanediol to react with any remaining periodate. The reaction mix was then applied to a 'Sephadex' G 10 gel filtration column (2cm diameter X 50cm in length) and eluted with distilled water. The absorbance of the eluate was monitored at 259nm and the fractions (1ml) absorbing at this wave length were pooled. The pooled fractions were then lyophilised and redissolved in 5ml of distilled water. This product was then subjected to further analysis. The melting point of the product was not determined since it charred at a temperature of 240°C which is in contrast to the sharp melting point obtained for adenosine at 234°C. The UV. absorbance spectra of adenosine and the product (Figure 3.54) both show a maximum at 259nm but at shorter wavelengths (<255nm) the products spectrum differs from that of authentic adenosine. The product formed a phenylhydrazone compound when reacted with an excess of phenylhydrazine at pH 2.0 indicating that the product was indeed an aldehyde. This was also indicated by a positive reaction with Tollens reagent (0.1M $AgNO_3$ in ammonium hydroxide solution) which reacts with aldehydes but not ketones whereas phenylhydrazine reacts with both aldehydes and ketones. The product was also analysed by thin layer chromatography on silica gel plates using the solvent system for nucleosides and bases described in Materials & Methods (Section 2.8). Figure 3.55 shows a photograph of the thin layer analysis, the spots being visualised by quenching of the fluorescent silica, which shows that no adenosine is present in the product. The thin layer analysis shows that there are two compounds present in the product; the product which migrates faster than adenine (X Figure 3.55) is a breakdown product from the dialdehyde adenosine produced

**Figure 3.55 TLC OF ADENOSINE, ADENINE AND SODIUM PERIODATE OXIDISED ADENOSINE.**




The photograph of the TLC plate was taken under UV light and shows adenine(A), adenosine(Ar), the major product of periodate oxidation of adenosine(Y) and an unknown compound(X). TLC was carried out as described in Materials & Methods (Section 2.8).

by $\beta$-elimination as first described by Khym & Cohn (1961). The major slow migrating component of the product (Y Figure 3.55) reacts with phenylhydrazine to give a yellow spot, when the TLC plate is sprayed with a 10% (w/v) acid phenylhydrazine solution and warmed, indicating that the compound is an aldehyde. The identity of the product as 2',3' dialdehyde adenosine was confirmed when the Sigma Chemical Co. introduced periodate oxidised adenosine as a new product which had the same Rf. value as the major product spot (Y) shown in Figure 3.55.

The above synthesis was repeated using [2,8-$^3$H] adenosine at a specific activity of 500$\mu$Ci/$\mu$mole with the adenosine:periodate ratio 1:1.1 as above. The [2,8-$^3$H] dialdehyde adenosine eluted from the gel filtration column was further purified by concentration and TLC chromatography, the product being eluted from the TLC plate with distilled water. The [2,8-$^3$H] dialdehyde adenosine was then stored at -20$^o$C. It was found that storage in this manner or in solution at 4$^o$C resulted in decomposition of the dialdehyde adenosine to the contaminant labelled (X) in Figure 3.55.

In view of the instability of the dialdehyde all affinity labelling experiments were carried out by synthesising the dialdehyde adenosine <u>in situ</u>. Typically 100$\mu$Ci of [2,8-$^3$H] adenosine (10$\mu$l;21Ci/mmol) was added to 10$\mu$moles of sodium periodate (10$\mu$l) and incubated in the dark at 4$^o$C for 1hr. The excess periodate was removed by the addition of PSG buffer (250-500$\mu$l), the $\alpha$-glucose reacting with the periodate. The dialdehyde adenosine was then used as an affinity label. Analysis of the reaction mix by TLC as described above showed that only 5% of the label present in the incubation mix remained as adenosine the remainder being present as

Table 3.15 EFFECT OF DIALDEHYDE ADENOSINE (SYNTHESISED IN SITU AND PRESYNTHESISED) ON ADENOSINE UPTAKE IN THE PRESENCE AND ABSENCE OF A REDUCING AGENT.

| $NaCNBH_3$ | Dialdehyde Adenosine(PS) | Dialdehyde Adenosine(ISS) | % Inhibition of Adenosine Uptake |
|---|---|---|---|
| - | - | - | 0 |
| + | - | + | 36 |
| - | - | + | 24 |
| + | + | - | 18 |
| - | + | - | 0 |
| + | - | - | 0 |

The table shows the effects of dialdehyde adenosine synthesised in situ (ISS) and presynthesised (PS) on adenosine uptake in the presence and absence of the reducing agent Sodium Cyanoborohydride ($Na\ CNBH_3$). All other experimental details are given in the text (Section 3.8.2)

dialdehyde adenosine(80%) and the breakdown product X (Figure 3.55) (15%).

**3.8.2 Dialdehyde Adenosine as an Affinity Label.**

In the previous Section (3.8.1) it was stated that dialdehyde adenosine synthesised and stored before use spontaneously decomposed to unidentified compounds. This was shown by TLC as previously described (Section 3.8.1) and by the following experiment in which the effect of dialdehyde adenosine, presynthesised and in situ synthesised, on adenosine uptake was determined. The effect of a reducing agent on the reaction was also determined since the reducing agent (sodium cyanoborohydride) reduces any Schiff bases formed between the dialdehyde adenosine and proteins forming a stable bond. These experiments were carried out to show that dialdehyde adenosine is capable of reacting with the adenosine transport protein, therefore acting as an affinity label.

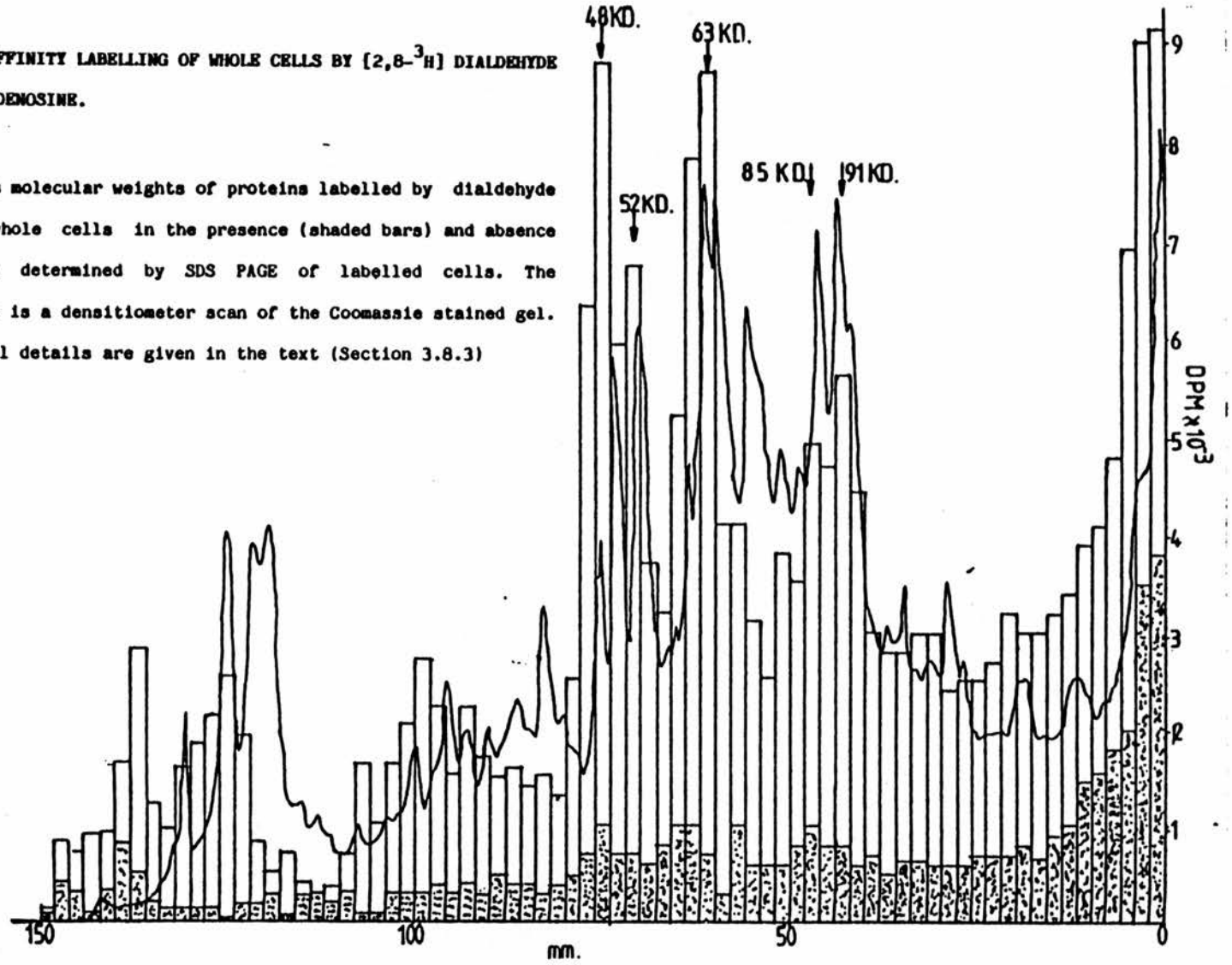
Dialdehyde adenosine was synthesised and purified as described above (Section 3.8.1) and used at a final concentration of 20μM in 5ml of trypanosome suspension (1.5mg protein/ml in PSG pH 7.4) either with or without 1mM $NaCNBH_3$. Dialdehyde adenosine was also synthesised in situ by mixing 0.1μmoles of adenosine with 10μmoles of sodium periodate in a total volume of 200μl of phosphate saline buffer at pH 7.4. This reaction mixture was then stored dark at $4^{o}C$ for 1 hour. At the end of this time 500μl of PSG pH 7.4 was added and the reaction mix stored at $4^{o}C$ for a further 20 minutes. Trypanosomes were then added to this mixture to give a final volume of 5ml (1.5mg protein/ml) either with or without 1mM $NaCNBH_3$. The four reaction mixtures, 1) presynthesised dialdehyde adenosine, 2) presynthesised dialdehyde adenosine with $NaCNBH_3$, 3) in situ synthesised dialdehyde

adenosine and 4) in situ synthesised dialdehyde adenosine with $NaCNBH_3$ were then incubated at 22°C for 5 minutes. The trypanosomes were then washed three times by centrifugation and resuspension, in 10ml of PSG pH 7.4, and finally resuspended in 5ml pf PSG pH 7.4. The abilities of the four cell suspensions to take up [2,8-$^3$H] adenosine were then determined. Uptake of adenosine was measured by adding 900ul of one of the four cell suspensions to 100ul of [2,8-$^3$H] adenosine (0.01mM; 50uCi/umol.) giving a final adenosine concentration of 1uM. Samples (400ul) were then taken and layered onto silicon sandwiches and centrifuged after 45 seconds. Uptake of [2,8-$^3$H] adenosine was then estimated by liquid scintillation counting of the PCA layer.

The results of the above experiments, Table 3.15, show that presynthesised dialdehyde adenosine blocks less adenosine transport than the in situ synthesised dialdehyde adenosine. This is due to decomposition of the dialdehyde adenosine on storage as stated previously. The results also show that including $NaCNBH_3$ in the incubations enhances the blocking effect of the dialdehyde adenosine whether presynthesised or synthesised in situ. The formation of a stable covalent bond with the transport protein rather than a Schiff base means that the dialdehyde adenosine can not be removed by washing the cells, although washing the cells does not appear to have had too much effect on the binding of the dialdehyde adenosine to the transporter as indicated by only an 18% difference in inhibition of adenosine uptake between the cells incubated with and without $NaCNBH_3$. The results in Table 3.15 also show that the reducing agent alone had no effect on the uptake of adenosine. Having shown that dialdehyde adenosine is capable of binding to the adenosine transport

**Figure 3.56 AFFINITY LABELLING OF WHOLE CELLS BY [2,8-$^3$H] DIALDEHYDE ADENOSINE.**

The figure shows molecular weights of proteins labelled by dialdehyde adenosine in whole cells in the presence (shaded bars) and absence (bars) of IDA as determined by SDS PAGE of labelled cells. The continuous line is a densitiometer scan of the Coomassie stained gel. Full experimental details are given in the text (Section 3.8.3)



protein identification of the transport protein in whole cells was attempted using $^{3}H$-dialdehyde adenosine as an affinity label.

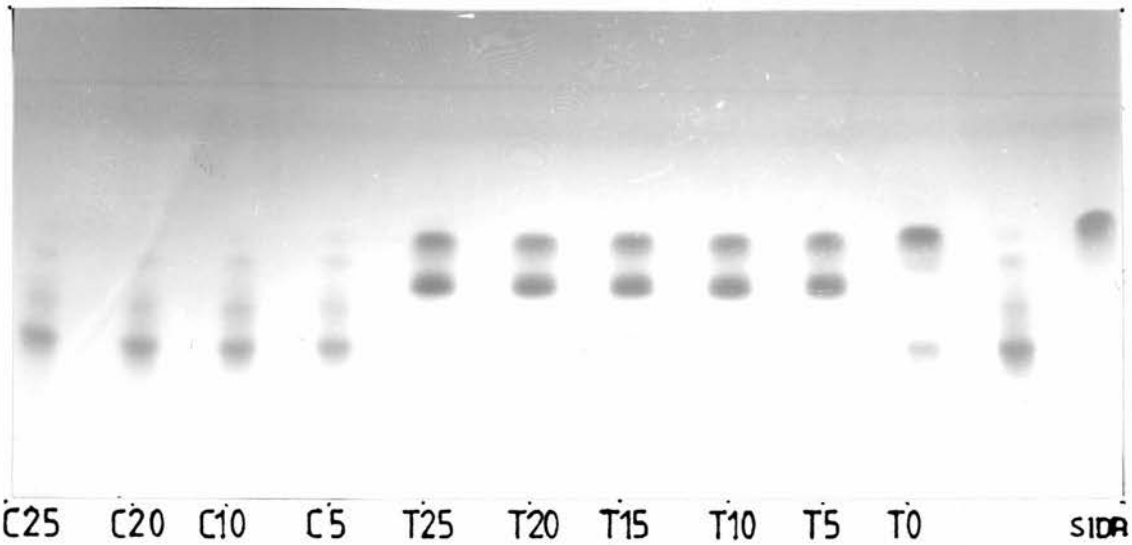
### 3.8.3 Labelling of Whole Cells with Dialdehyde Adenosine.

Radiolabelled dialdehyde adenosine was prepared by mixing 100μCi of [2,8-$^{3}H$] adenosine (10μl; 21Ci/mmol) with 10μmoles of sodium periodate (10μl) and storing in the dark at 4°C for 1 hour followed by the addition of PSG buffer pH 7.4 (250μl) and a further incubation for 20 minutes. The above synthesis was carried out in duplicate. Trypanosomes (750μl; 1.5mg protein/ml) either with or without IDA (20μM) and in the presence of 1mM $NaCNBH_3$ were then added to the dialdehyde adenosine mixture and incubated at 22°C for 30 minutes. The cells were then washed 3 times by centrifugation and resuspension in PSG pH 7.4. The cells were then prepared for electrophoresis on an 8-20% exponential polyacrylamide gel in the presence of SDS as described in Materials & Methods (Section 2.13).

After electrophoresis the gel was fixed and stained with Coomassie blue dye. The protein banding patterns on the gel given by the cells incubated in the presence or absence of IDA were identical. The gel was then cut into strips and densitometer traces made of the banding pattern produced by each sample and standard molecular weight marker proteins. The gel strips (150mm long) were then sliced into 2mm sections and prepared for liquid scintillation counting by digestion of the gel in 15% hydrogen peroxide at 60°C for 12 hours (Materials & Methods Section 2.14).

Figure 3.56 shows a typical densitometer trace obtained from the gel and also the amount of radioactivity in each 2mm gel slice for the cells incubated in the presence of IDA and those incubated with

**Figure 3.57a REACTION OF DIALDEHYDE ADENOSINE WITH IDA IN THE PRESENCE OF A REDUCING AGENT.**




Dialdehyde adenosine (1mM) was mixed with IDA (1mM) in PSG pH 7.4 $CNBH_3$ was then added to a final concnetration of 1mM. A sample of the reaction mix was taken immediately on the addition of the $CNBH_3$ and at five minute intervals. The reaction was quenched by adding the samples taken to 0.1M ethanediol. The samples were then subjected to analysis by TLC as described in Materials & Methods (Section 2.8) Figure 3.57a shows a time course of the reaction of IDA and dialdehyde adenosine (T0-T25), the figure also shows the analysis of samples of dialdehyde adenosine in solution alone (C5-C25). IDA alone is shown as a standard (SIDA).

**Figure 3.57b REACTION OF DIALDEHYDE ADENOSINE WITH A REDUCING AGENT.**

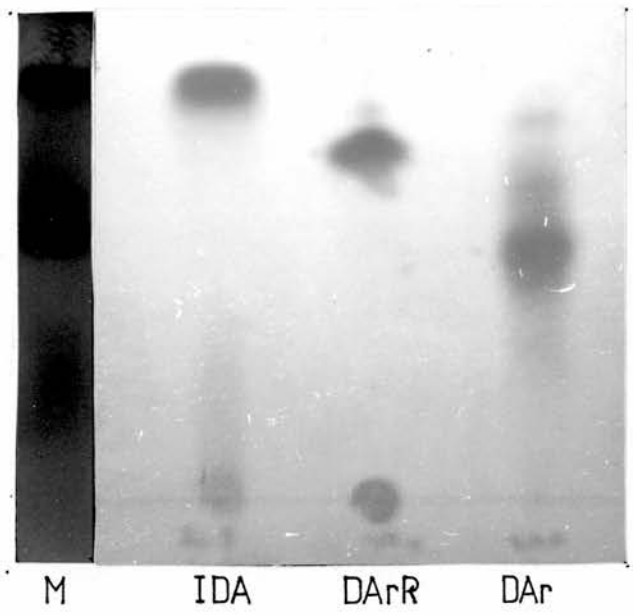



Figure 3.57b shows the reaction of dialdehyde adenosine with $NaCNBH_3$and the reaction of dialdehyde adenosine and iododeoxyadenosine in the absence of $NaCNBH_3$. Dialdehyde adenosine and $NaCNBH_3$ were incubated in PSG pH 7.4 at a final concentration of 1mM for 5 minutes then analysed by TLC as described in the legend to Figure 3.57a. Dialdehyde adenosine and iododeoxyadenosine were incubated in PSG pH 7.4 at a final concentration of 1mM for 5 minutes then analysed by TLC as described in the legend to Figure 3.57a. The figure shows IDA as a marker (IDA), dialdehyde adenosine reacted with $NaCNBH_3$ (DArR), unreacted dialdehyde adenosine (DAr) and IDA with dialdehyde adenosine (M).

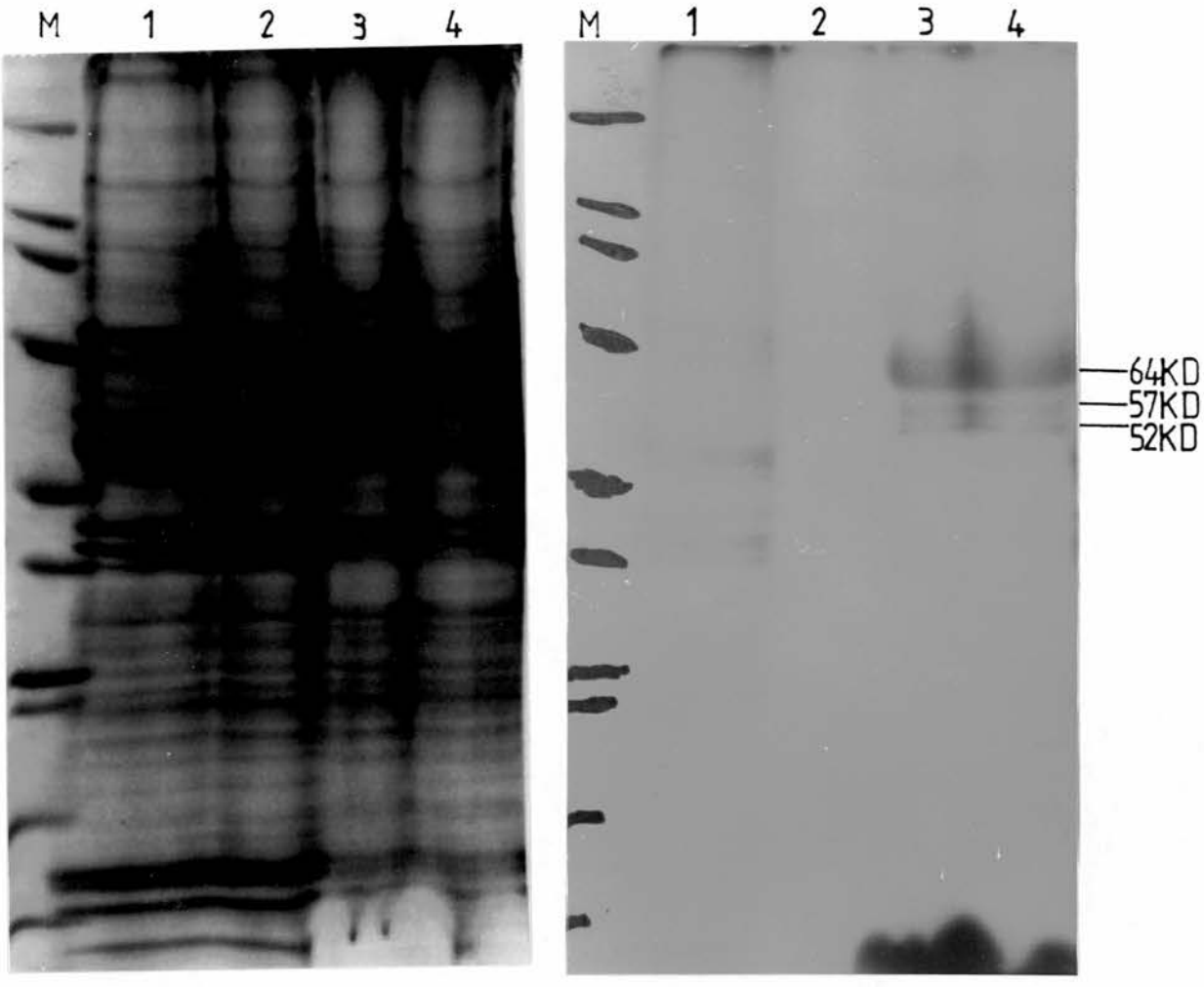
the labelled dialdehyde adenosine alone. The results show that the presence of IDA in the incubation inhibits labelling of any trypanosomal proteins. However five proteins are significantly labelled by the dialdeyde adenosine alone as indicated by arrows and their apparent molecular weights in Figure 3.56. It could be argued that the inhibition, by IDA, of labelling with dialdehyde adenosine is due to IDA reacting with the dialdehyde adenosine. This is not the case as shown by the results presented in Figures 3.57a and 3.57b. Dialdehyde adenosine was mixed with IDA in the presence of $NaCNBH_3$ as described in the legend to Figure 3.57a. The results show that dialdehyde adenosine breaks down in the presence of $NaCNBH_3$ but does not react with the IDA as shown by the constant intensity of the IDA spot during the course of the incubation. Figure 3.57b confirms that only $NaCNBH_3$ is required to cause the observed shift in position of the dialdehyde adenosine spot. These results will be considered in conjunction with other labelling experiments in Section 3.8.5.

### 3.8.4 Labelling of Plasma Membranes by Dialdehyde Adenosine.

When whole cells were incubated with [2,8-$^3$H] dialdehyde adenosine five proteins were shown to be strongly labelled after SDS PAGE (Section 3.8.3). Some of these proteins may be intracellular enzymes or proteins which have an adenosine binding site but are not directly involved in transport of adenosine. In order to simplify the labelling pattern obtained purified plasma membranes were used in place of whole cells in an affinity labelling experiment.

[2,8-$^3$H] 2',3' dialdehyde adenosine was prepared from [2,8-$^3$H] adenosine as described in the previous Section (3.8.4) with a specific activity of 21Ci/mmol. A plasma membrane enriched fraction

Figure 3.58 LABELING OF PLASMA MEMBRANES WITH [2,8-$^{3}$H] DIALDEHYDE ADENOSINE.




The figure shows an autoradiograph and Coomassie stained gel of plasma membranes and whole cells labelled with dialdehyde adenosine in the presence and absence of IDA. 1) Whole cells labelled with dialdehyde adenosine, 2) Whole cells labelled in the presence of IDA. 3) Plasma membranes labelled with dialdehyde adenosine and 4) Plasma membranes labelled in the presence of IDA. The experimental protocol is given in the text (section 3.8.4).

was prepared as described in Section 3.7.4 and resuspended in PSG buffer pH 7.4 at a protein concentration of 1.5mg/ml. The labelling reaction was started by the addition of the plasma membranes to the newly synthesised dialdehyde adenosine. The labelling was carried out in the presence of 1mM $NaCNBH_3$ both with and without 20μM IDA present. The membranes were incubated at 22°C for 2 hours in the above reaction mixtures and then washed 3 times by centrifugation and resuspension in PSG pH 7.4 at 100,000x*g* av. for 30 minutes. The membranes were then solubilised in SDS sample buffer for SDS PAGE as described in Materials & Methods (Section 2.13). The labelled membranes were then subjected to electrophoresis on 8% to 20% exponential gradient polyacrylamide gels. Affinity labelled whole cells from the previous experiment (Section 3.8.4) were also run on the same gel for comparison. After electrophoresis the gel was stained, photographed and prepared for fluorography as described in Materials & Methods (Section3.14) and x-ray film exposed to the fluorograph for two months.

Figure 3.58 shows a photograph of the Coomassie stained gel and the autoradiograph. The labelling patterns obtained for the whole cell and the purified membranes are markedly different in that the proteins labelled in the plasma membrane fraction do not appear to be labelled in the whole cell. This is possible if the proteins labelled in the plasma membrane fraction are only present as a very small percentage of the total cellular protein which would make their detection in the whole cell difficult. Figure 3.48 also shows the apparent molecular weights of the labelled proteins as calculated from their mobilities on the gel relative to marker proteins of known molecular weight. These results together with the results of other

Table 3.16 SUMMARY OF MOLECULAR WEIGHTS OF PROTEINS LABELLED BY $^{125}I$, FITC AND [2,8-$^{3}H$] DIALDEHYDE ADENOSINE IN WHOLE CELLS AND PLASMA MEMBRANES.

| $^{125}I$-LACTOPEROXIDASE LABELLED PROTEINS WHOLE CELLS | $^{3}H$-O-ADENOSINE LABELLED PROTEINS WHOLE CELL | $^{3}H$-O-ADENOSINE LABELLED PROTEINS PLASMA MEMBRANE | FITC. LABELLED PROTEINS WHOLE CELL |
|---|---|---|---|
| 169 KD | 144 KD | - | - |
| 104 KD | 91 KD | - | - |
| 72 KD | 72 KD | - | - |
| 63 KD | 66 KD | 64 KD | 69 KD |
| 56 KD | 56 KD | 57 KD | 54 KD |
| 50 KD | 52 KD | 52 KD | 50 KD |
| 45 KD | 48 KD | - | 47 KD |
| - | 43 KD | - | 46 KD |
| 39 KD | 40 KD | - | 38 KD |
| 36 KD | 38 KD | - | 36 KD |
| - | - | - | 35 KD |
| - | - | - | 29 KD |

labelling expeiments will now be considered as a whole.

### 3.8.5 Summary of Membrane Labelling Experiments.

Table 3.16 summarises the results of all surface labelling and affinity labelling experiments carried out. This includes labelling of the cell surface with $^{125}I$ and FITC (Sections 3.7.4 & 3.7.6) also affinity labelling of the adenosine transporter with [2,8-$^{3}H$] dialdehyde adenosine in whole cells and purified plasma membrane (Sections 3.8.3 & 3.8.4). It should be borne in mind that the molecular weights for the labelled proteins quoted in Table 3.16 are apparent molecular weights since some of these proteins will probably be glycosylated and will therefore not bind SDS in the normal ratio of 1.4g/g. This results in a slower migration through the gel which gives an over estimate of the protein's molecular weight. Estimating the molecular weights of proteins from their mobilities on a gel using a standard curve constructed from the molecular weights and relative mobilities of known proteins involves measurement errors which result in a cumulative error of $\pm$4% in all molecular weight estimates. Bearing this in mind the results presented in Table 3.16 show that the adenosine transporter is likely to be a 57 or 52 KDa polypeptide since proteins of this molecular weight are labelled by [2,8-$^{3}H$] dialdehyde adenosine in whole cells and in purified plasma membranes. Additional evidence that they are plasma membrane proteins is provided by the fact that they are also labelled by $^{125}I$ and FITC in whole cells. The many other bands labelled by [2,8-$^{3}H$] dialdehyde adenosine in whole cells are probably intracellular proteins although some are also labelled by the cell surface labels. The fact that they do not appear to be labelled in the plasma membrane may indicate

that they are only loosely associated with the plasma membrane and are lost during the purification of the plasma membranes. If this is the case it is very unlikely that they are involved in transport since a transporter would be an integral membrane protein and not easily removed from the membrane without the use of a detergent. The proteins of molecular weights quoted as 63, 66, 64 and 69 KDa labelled by all the labelling methods and in all fractions is probably the trypanosomes surface glycoprotein coat protein since it is labelled by the cell surface labels and runs on the SDS gels as a broad diffuse band, which is a characteristic of glycoproteins.

Having identified the polypeptides most likely to be constituents of the adenosine transporter further analysis of the mechanism of action of the transport system could be undertaken by purifing these proteins and attempting to reconstitute the transport system in liposomes. This would allow investigation into the mechanism of energy coupling to active transport without the problems associated with using metabolic inhibitors on the whole cell system such as maintaining membrane integrity in metabolically poisoned cells. However these investigations must be left until some later date.

## 3.9 REGULATION OF UPTAKE AND METABOLISM OF PURINE NUCLEOSIDES AND BASES.

The time courses of the uptake of adenosine and hypoxanthine presented in Sections 3.2.1 & 3.3.1 show that uptake slows to a stop after two to three minutes. The results presented in Sections 3.6.1 to 3.6.8 indicate that uptake is probably by active transport and so should be independent of metabolism of the nucleoside or base taken

up. This is supported by the observation that metabolism of the nucleosides and bases taken up continues after the cessation of uptake.(Sections 3.2.4 & 3.3.4) Consideration of the above results suggests that the transport system is under some form of control by the cell.

Regulation of the transport step could be by feed back inhibiton of uptake by intracellular levels of nucleoside or base but this is unlikely since the levels of nucleoside and base in the cell continue to fall after uptake has stopped (Sections 3.2.4 & 3.3.4). The transporter could be controlled by the levels of intracellular nucleotide since the nucleosides and bases are metabolised to nucleotide after they have been transported. As stated above any feed back effects of nucleotide levels on metabolism of the nucleosides and bases taken up would be unlikely to affect transport since transport is an active process independent of further metabolism of the transported substrate. Another method the trypanosome could use to control the activity of the transport system is phosphorylation. Phosphorylation has recently been implicated in the control of glucose transport in the erythrocyte. Witters et al. (1985) have shown that a protein kinase C stimulated by phorbol esters phosphorylates the glucose transporter both in vivo and in vitro. This correlates with the observation that phorbol esters stimulate glucose uptake in the erythrocyte. Therefore it is not unreasonable to suppose that transport of substrates across the trypanosomal plasma membrane may be regulated by phosphorylation.

The above possibilities for regulation of purine nucleoside and base transport were investigated with the exception of the effects of free intracellular nucleosides and bases on transport since there is

Table 3.17 EFFECT OF NUCLEOTIDES AND ADENOSINE ON THE ACTIVITIES OF PURINE METABOLISING ENZYMES.

| NUCLEOTIDE BASE OR 1mM NUCLEOSIDE | Ar HYDROLASE umol/min/mg | APRT nmol/min/mg | HxPRT nmol/min/mg |
|---|---|---|---|
| --- | 0.18 | 5.0 | 9.3 |
| AMP | 0.18 | 2.9 | 9.2 |
| IMP | 0.18 | 5.0 | 8.1 |
| ATP | 0.17 | 5.1 | 6.9 |
| AMP + ATP | 0.17 | 2.9 | 7.8 |
| IMP + ATP | 0.18 | 5.1 | 6.1 |
| ADENOSINE | 0.18 | 1.3 | 8.9 |
| HYPOXANTHINE | 0.12 | 5.0 | 9.3 |

The table shows the effects of various nucleotides, combinations of nucleotides, hypoxanthine and adenosine on the activities of adenine(APRT), hypoxanthine(HxPRT) phosphoribosyltransferases and adenosine hydrolase(Ar hydrolase). Experimental details are given in the text (Section 3.9.1).

no way to control the required parameters, such as intracellular concentrations of nucleoside or base, in the whole cell.

### 3.9.1 Effect of Nucleotides,Nucleosides and Bases on Enzymes of Purine Metabolism.

Although it is unlikely that transport of nucleosides and bases is affected by any regulation of metabolism, for the reasons stated above,the effects of nucleotides and bases on some of the purine metabolising enzymes were investigated. The effects of 1mM AMP, IMP, ATP, adenosine, hypoxanthine, AMP with ATP and IMP with ATP on the enzymes adenosine hydrolase, APRT and HxPRT were investigated as detailed below. The enzyme activities were assayed as described in Materials & Methods (Section 2.10). The labelled substrates were present at a specific activity of 100μCi/μmol. and the following concentrations; adenosine 50μM, adenine 2μM and hypoxanthine 5μM. The enzyme source for the assays was fresh dialysed trypanosome lysate at a final protein concentration of 0.02mg/ml for the adenosine hydrolase assay and 0.1mg/ml for the adenine and HxPRT assays. All assays were carried out at a temperature of 22°C in TrisHCl buffer at pH 7.4. The assays were started by the addition of the trypanosome lysate and incubated for 3 minutes.

The results of the above experiments are shown in Table 3.17. Adenosine hydrolase activity does not appear to be affected by any of the additions of nucleotide but does show some slight inhibition in the presence of 1mM hypoxanthine. This inhibition has no immediate significance with respect to regulation of transport. The combinations of a nucleoside monophosphate with ATP were included in this experiment to try and detect any nucleotide monophosphate

effects on the enzyme mediated by a protein kinase.

The activity of APRT appears to be sensitive to product inhibition by AMP but this observed inhibition could be due to dilution of the labelled adenine substrate by adenine derived from the added AMP via phosphatase activity and adenosine hydrolase. The inhibition of APRT activity by adenosine is almost certainly due to this effect.

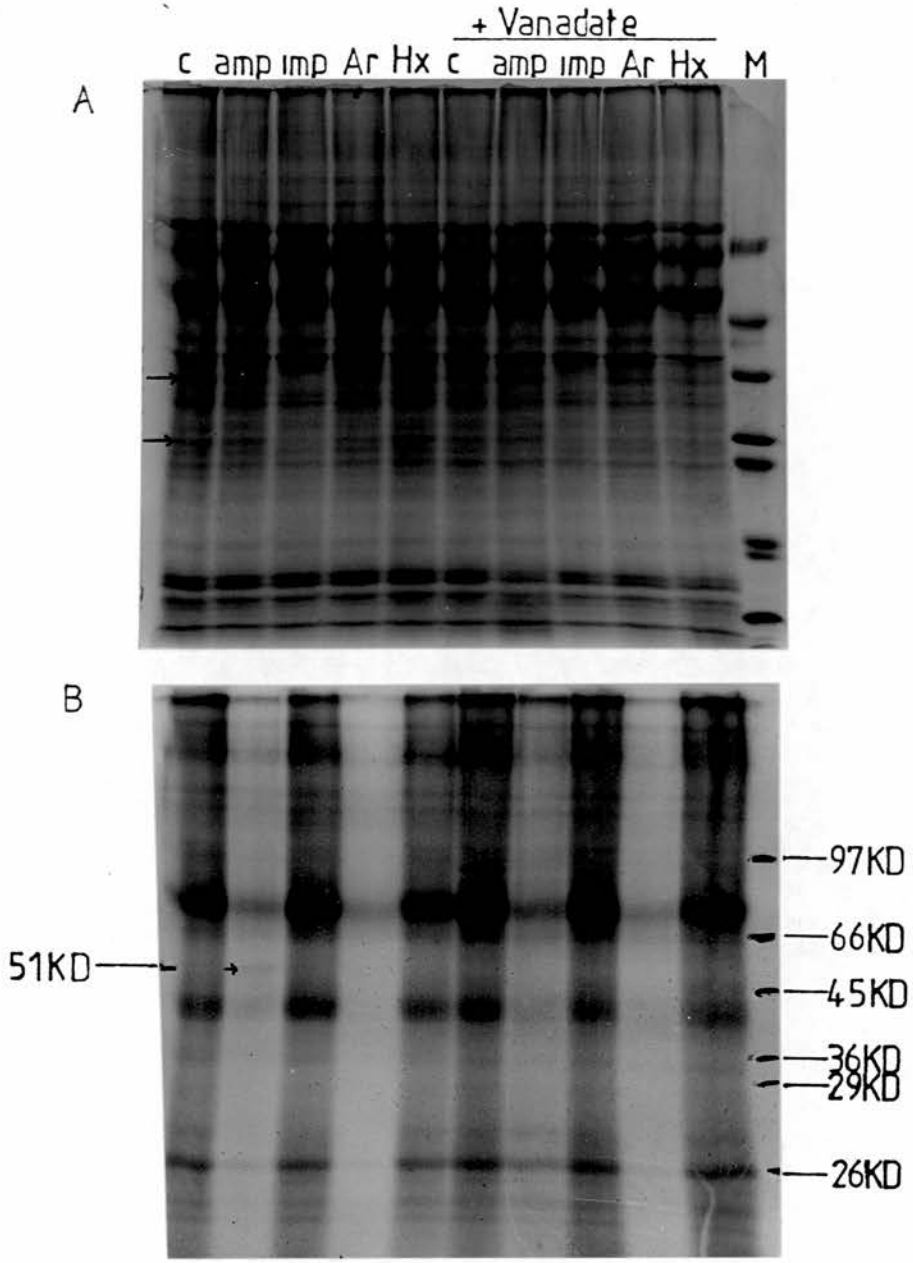
The activity of HxPRT does not appear to be sensitive to product inhibition at a concentration of 1mM IMP but does appear to be inhibited by 1mM ATP. The results presented above have little significance with regards to transport regulation but may be significant with respect to control of metabolism of purine nucleosides and bases once taken up and would be worth investigating in more detail.

### 3.9.2 Effect of Purine Nucleotides, Nucleosides and Bases on Phosphorylation of Trypanosomal Proteins in a Whole Cell Lysate and a Crude Membrane Fraction.

The possible regulation of transport by phosphorylation was investigated by examining which proteins were phosphorylated in a whole cell lysate and in a crude membrane fraction, when incubated with $\gamma$-$^{32}$P ATP and various purine nucleotides,nucleosides and bases.

The $\gamma$-$^{32}$P ATP used in the following experiments was synthesised from $^{32}$Pi phosphate and unlabelled ATP using an enzymatic exchange system which is described in detail in Materials & Methods (Section 2.15). The phosphorylation of proteins was carried out by incubating either whole cell lysate or membranes prepared from a whole cell lysate, by centrifugation at 100,000x*g* av. for 1 hour. The standard

**Figure 3.59 EFFECT OF NUCLEOTIDES AND NUCLEOSIDES ON PROTEIN PHOSPHORYLATION OF CRUDE MEMBRANE PROTEINS.**

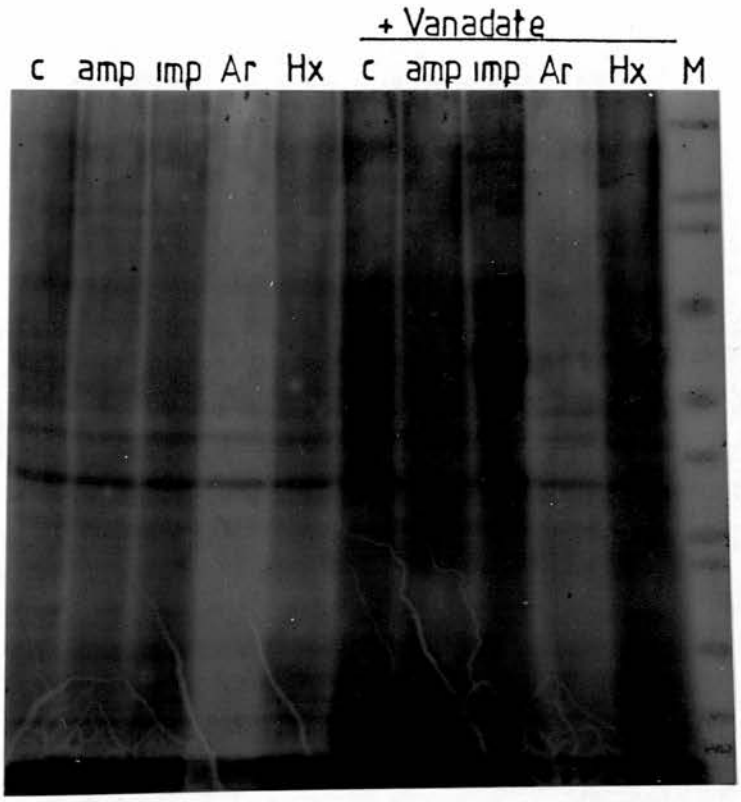



The figure shows the effects of AMP, IMP, adenosine(Ar) and hypoxanthine(Hx) on phosphorylation of trypanosomal membrane proteins in the presence and absence of sodium ortho vanadate. Part b) is an auto radiograph of the Coomassie stained gel of $^{32}P$ labelled proteins shown in part a). The full experimental protocol is given in the text (Section 3.9.2).

reaction mixture contained 0.2mg of the appropriate trypanosome protein susupension (60µl) to give a final protein concentration of 1mg/ml. The incubation mixture also contained 5mM (final concentration) $MgCl_2$ (25µl), TrisHCl buffer pH 7.4 to a final concentration of 10mM (20µl), $\gamma$-$^{32}P$ ATP(specific activity 80mCi/µmol.) to a final concentration of 75µM (10µl) and one of AMP, IMP, adenosine or hypoxanthine at a final concentration of 1mM (20µl) and water to give a final volume of 200µl. All incubations were repeated with 2mM sodium orthovanadate present to inhibit general ATPase activity. The reaction mixtures described above were incubated at 30°C for 10 minutes. The incubations were then terminated by the addition of 10 volumes of acetone:ethanol (1:1) at a temperature of -18°C. This step precipitated the protein and removed lipid before preparing the proteins for SDS PAGE as described in Materials & Methods (Section 2.13). After electrophoresis the gels were stained for protein and photographed prior to autoradiography by exposure to x-ray film for 2 days at -70°C.

The results of these experiments are shown in Figures 3.59a, 3.59b and 3.60. Figure 3.59a shows the gel of the crude membrane preparation stained for protein. The incubation which contained IMP shows a different banding pattern to the other incubations since the bands marked with arrows in the control track are absent. The effect is independent of the presence of sodium orthovanadate in the incubation and does not correlate with any changes in phosphorylation pattern as is shown in Figure 3.59b which is an autoradiograph of the gel shown in Figure 3.59a. The significance of these results is unknown and requires further investigation. The autoradiograph of the gel of the phosphorylated crude membranes shows that AMP is a general

Figure 3.60 EFFECT OF NUCLEOTIDES AND NUCLEOSIDES ON PROTEIN PHOSPHORYLATION IN A WHOLE CELL LYSATE.




The figure shows an autoradiograph of $^{32}P$ labelled proteins in a whole cell lysate in the presence of AMP, IMP, adenosine(Ar) and hypoxanthine(Hx) both in the presence and absence of sodium ortho vanadate. The full experimental protocol is given in the text (Section 3.9.2).

inhibitor of the protein kinase present as is adenosine but the AMP stimulates phosphorylation of one band at an apparent molecular weight of 51 KDa. This is close to the molecular weight estimated for the adenosine transport protein by affinity labelling experiments (Section 3.8.5). This specific phosphorylation appears to be inhibited by the presence of sodium orthovanadate in the incubation which may indicate that the protein kinase involved is inhibited by vanadate ions. Adenosine appears to be a good inhibitor of all protein kinase activity which is in disagreement with the results of other authors. Full consideration of this result will be given in the Discussion (Section 4.5). Figure 3.60 shows the autoradiograph of phosphorylated proteins in a whole cell lysate. The results are much the same as for the crude membrane fraction with the following exceptions. The sodium orthovanadate has had a more marked effect on the overall phosphorylation. This probably reflects the presence of vanadate sensitive ATPases in the lysate which were not associated with the membranes. The AMP again reduces the amount of phosphorylation observed but this time the 51 KDa band is phosphorylated in the other incubations except those containing vanadate ion and adenosine. This may indicate that the protein kinase responsible for this phosphorylation is a soluble cytoplasmic protein which was mostly removed from the crude membrane preparation. The 51 KDa band is also much fainter in the whole cell lysate providing more evidence that it is membrane associated since semi-purifing the cell membranes intensifies the band.

### 3.9.3 Summary of Regulation of Purine Nucleoside and Base Transport and Metabolism.

The results presented in the previous two sections provide some evidence for regulation of adenosine transport by phosphorylation of the transport protein. This result is by no means conclusive but further work with a reconstituted transport system and purified trypanosomal protein kinases could settle the question. The trypanosomal protein kinases, their purine nucleoside, nucleotide and base dependencies and subcellular distributions will be considered in the Discussion (Section 4.5).

Regulation of metabolism of purine nucleosides and bases taken up has been considered here only briefly in order to determine if there was any possibility of metabolism having any regulatory effect on the transport system. The results presented make this possibility highly unlikely but any further work on the sensitivities of the metabolising enzymes to regulation should be carried out on purified enzymes since the effects of adenosine are difficult to determine in the presence of adenosine hydrolase activity.

# 4.DISCUSSION

## 4. DISCUSSION.

The results which have been presented in this thesis will now be compared with the results of other authors where available. The results presented will also be discussed against the background of the general literature as regards general transport and more specifically the transport of purines and vitamin $B_6$.

### 4.1 PYRIDOXINE AND PYRIDOXAL UPTAKE AND METABOLISM.

The results presented in Sections 3.1 to 3.1.10 provide some evidence that the uptake of pyridoxine and pyridoxal by T.brucei is mediated by simple diffusion followed by metabolic trapping as pyridoxine phosphate and pyridoxal phosphate. These conclusions are supported by the observations that the pyridoxine and pyridoxal uptake systems are non-saturable at physiological substrate levels and above, are only partially temperature sensitive and have low energies of activation.

Calculation of the intracellular concentrations of pyridoxine and pyridoxal shows that these compounds are not accumulated against their concentration gradients. This, in conjunction with the nonsaturability of uptake, suggests that diffusion through the plasma membrane may be the rate limiting step for the uptake of pyridoxine and pyridoxal, or that the metabolising enzymes involved in the conversion of the compounds taken up to pyridoxal phosphate have high Km's for their substrates. The kinetic parameters of the T.brucei vitamin $B_6$ metabolising enzymes are unknown but the table below lists the Km values of pyridoxal kinase with pyridoxal or pyridoxine as substrate in other organisms.

| ENZYME SOURCE | SUBSTRATE | Km (mM) |
|---|---|---|
| E.coli | PA | 0.3 |
| E.coli | PN | 0.008 |
| Rat liver | PA | 0.015 |
| Rat liver | PN | 0.025 |
| Beef brain | PA | 0.050 |
| Beef brain | PN | 0.020 |

(Snell,1944)

The maximum concentration of pyridoxine or pyridoxal used in the uptake assays carried out in this work was 0.06mM. It therfore would appear that diffusion across the membrane may be the rate limiting step for uptake since at a concentration of 0.06mM, pyridoxine or pyridoxal would be beginning to show some signs of saturating the metabolising enzymes assuming that the trypanosomal enzymes are similar to those found in other organisms.

The thermodynamic parameters of uptake calculated from Arrhenius plots of the uptake of pyridoxine and pyridoxal indicate that uptake is by simple diffusion, by virtue of the low activation energies calculated for pyridoxal and pyridoxine uptake (Dixon & Webb,1964; Goldman et al.,1968; Damper & Patton,1976b).

The calculated activation energies for the uptake of pyridoxine and pyridoxal, 9.9 kJ/mol and 5.4 kJ/mol respectively, are much lower than would be expected for a molecule of the same size in free solution. However, it should be noted that the Arrhenius plot is a plot of ln rate of uptake against reciprocal temperature and the rate being measured is not the rate of diffusion in free solution but the rate of diffusion through the plasma membrane. The rate of diffusion in free solution can be disregarded if the pore size of the membrane is close to, or smaller than ( in the case of partition into the membrane), the size of the diffusing molecule (Bull, 1964; Kedem & Katchalsky, 1958). Diffusion through a membrane cannot be accurately

described by Fick's law of diffusion. The diffusion coefficient must be replaced by a constant which takes into account the interactions between solute and membrane as well as membrane solvent interactions. As stated above the interactions of solute and solvent become less significant as the effective pore size of the membrane decreases (Kedem & Katchalsky,1958). When these additional factors are taken into account the diffusion coefficient is replaced by the coefficient of permeability. If pyridoxine and pyridoxal partition into the trypanosomal plasma membrane in the process of entering the cell this will have a marked effect on the permeability coefficient. In most respects the permeability coefficient behaves in the same way as the diffusion coefficient in Ficks law in that as temperature increases the diffusion coefficient increases therefore the permeability coefficient will increase and hence the rate of diffusion will increase. As the temperature increases the partition coefficient will also change affecting the permeability coefficient. This may result in a reduction of the effect of temperature on the permeability coefficient and hence reduce the slope of the Arrhenius plot and the calculated Ea. This hypothesis, although speculative, is supported by the fact that pyridoxal and pyridoxine both migrate more quickly than other forms of $B_6$ in the chloroform : methanol solvent system described in Materials & Methods (Section 2.8.1). Since this is a completely organic solvent with no aqueous component both pyridoxine and pyridoxal must be appreciably lipophilic. The Arrhenius plots obtained for pyridoxine and pyridoxal uptake were linear and showed no sharp changes in slope which would have indicated a change in membrane fluidity affecting uptake (Plagemann & Erbe,1975; Stein & Rosengurt,1975; Zylka & Plagemann,1975) as has been shown to occur

for the uptake of pyridoxine and pyridoxal by the erythrocyte (Mehansho & Henderson, 1980). The other parameters calculated from the Arrhenius plot, $\Delta H$ and $\Delta S$, are difficult to interpret with regard to uptake or transport systems. The $\Delta H$ calculated can be regarded as a measure of the degree of deformation of structure involved in the uptake process (Cornish-Bowden, 1979), a large $\Delta H$ indicating extensive deformation of structure in either the substrate, the enzyme or the carrier protein. The calculated $\Delta S$ can be regarded as a measure of the probability of the transition state forming. However, the above only applies to enzyme catalysed reactions or carrier-mediated transport which is not the case for pyridoxine or pyridoxal. The only significance which can be attached to the negative $\Delta G$ values obtained is that the formation of the transition state, if one exists, is favoured energetically. A comparison of the thermodynamic parameters obtained for uptake of pyridoxine and pyridoxal by simple diffusion and the adenosine active transport system is considered in Section 4.2.1. In general the results obtained from the investigations of pyridoxine and pyridoxal uptake by T.brucei are in agreement with results obtained in studies of vitamin $B_6$ uptake by other eukaryotic cells such as rat jejunum cells (Middleton, 1977), intestinal mucosa cells (Mehansho et al., 1978) and the rat and human erythrocyte (Mehansho & Henderson, 1980). There are two exceptions to this general statement, the yeast Saccharomyces carlsbergensis takes up pyridoxine by an active transport mechanism (Shane & Snell, 1976) and the choroid plexus of the rabbit actively transports vitamin $B_6$ (Spector, 1978a & 1978b). Uptake of pyridoxine and pyridoxal by bacteria appears to be mediated by facilitated diffusion as shown for Salmonella typhimurium (Mulligan and Snell, 1976) and Streptococcus faecalis (Mulligan &

Snell,1977). Active transport of pyridoxal by erythrocytes has been reported by Yamada & Taugi,(1968 & 1970); however, these authors did not differentiate between the transported form of pyridoxal and its metabolites when estimating intracellular concentrations of pyridoxal.

Investigations into the metabolism of pyridoxine and pyridoxal by T.brucei (Sections 3.1.4 to 3.1.8) show that both pyridoxine and pyridoxal are converted into phosphorylated compounds within the cell. The results presented in Section 3.1.4 show that pyridoxine is oxidised to pyridoxal and may be slowly phosphorylated by pyridoxal kinase to pyridoxal phosphate. This route to pyridoxal phosphate differs from that of mammalian cells (eg. intestinal mucosal cells, Mehansho et al.,1979); liver cells (Mehansho et al.,1980)) which use pyridoxal kinase to phosphorylate pyridoxine to pyridoxine phosphate and then oxidise pyridoxine phosphate to pyridoxal phosphate using the enzyme pyridoxine phosphate oxidase. However, the model for metabolism presented in Section 3.1.8 requires that pyridoxine be metabolised as in mammalian systems. The oxidation of pyridoxine to pyridoxal in T.brucei is probably carried out by pyridoxine dehydrogenase which, as stated in Section 3.1.4, produces NADPH rather than $H_2O_2$ which is produced by pyridoxine phosphate oxidase. The use of pyridoxine dehydrogenase would appear to be to the trypanosome's advantage since the bloodstream forms of the organism are reported to contain no catalase activity for the removal of $H_2O_2$. In this work no catalase activity could be detected in trypanosome lysate using an oxygen electrode or a fluorometric assay to detect the production of oxygen from hydrogen peroxide (results not shown). The most prevalent usable form of vitamin $B_6$ found in the mammalian

bloodstream is pyridoxine. Pyridoxal is also released by the liver for utilisation by muscle cells since they cannot form pyridoxal from pyridoxine as they lack the enzyme pyridoxine phosphate oxidase (Anderson et al., 1971). This supply of pyridoxine may be the main source of $B_6$ utilised by the trypanosomes if the proposed model for metabolism is correct. Pyridoxal released by the liver may also be utilised to a lesser extent. Pyridoxal phosphate may also be a source of pyridoxal if it is degraded to pyridoxal by a phosphatase before being taken up since it is present in the bloodstream in high concentration for the reasons detailed in Section 1.3.2. The metabolism of $B_6$ in this organism requires futher investigation in order to verify or disprove the model presented.

## 4.2 PURINE NUCLEOSIDE AND BASE TRANSPORT AND METABOLISM.

The study of purine nucleoside and base uptake, transport and metabolism presented in Sections 3.2 to 3.6 will be considered as three separate components, overall uptake, mechanism of transport and metabolism.

### 4.2.1 Uptake of Adenosine, Adenine and Hypoxanthine.

The uptakes of adenosine, adenine and hypoxanthine are carrier mediated processes and in the case of adenosine and hypoxanthine some evidence has been presented which indicates that uptake is mediated by an active transport process. The evidence for these conclusions is considered below.

The plasma concentrations of purines in the rat and in the human, and the values obtained for the kinetic parameters of

adenosine, adenine and hypoxanthine uptake (Sections 3.2.2, 3.3.2 and 3.3.4 respectively) are shown below.

| Rat plasma Concentrations (μM). | | Source. |
|---|---|---|
| Hypoxanthine | 0.7-1.0 | Simmonds & Harkness (1981) |
| Adenosine | 1.0-1.5 | Von Borstel et al. (1982) |
| **Human plasma Concentrations (μM).** | | **Source.** |
| Hypoxanthine | 0.5-2.8 | Taylor et al. (1980a) |
| Inosine | 0.2-1.5 | Taylor et al. (1980a) |
| Adenine | 0.1-1.4 | De Verdier et al. (1977) |
| Adenosine | 0.5-0.9 | Maurizio et al. (1982) |

| SUBSTRATE | UPTAKE Km | UPTAKE Vmax |
|---|---|---|
| Adenosine | 0.50μM | 0.50nmol/min/mg protein |
| Adenine | 0.30μM | 0.27nmol/min/mg protein |
| Hypoxanthine | 0.35μM | 0.21nmol/min/mg protein |

As stated in the results, the values quoted for the plasma concentrations of adenosine are higher than those reported by authors before 1980. This discrepancy is due to the fact that no precautions were taken, previous to 1980, to prevent the destruction of adenosine by plasma adenosine deaminase which gave high estimates for plasma inosine concentrations and low estimates for adenosine concentrations. The estimates for plasma adenosine levels quoted were obtained from blood samples taken into anticoagulant with the potent adenosine deaminase inhibitor 2-deoxycoformycin present (Capogrossi et al.,1982). It should be noted that the kinetic parameters shown apply only to overall uptake for the reasons stated in Section 3.2.2. Comparison of the data presented in the two tables above shows that the uptake systems for adenosine, adenine and hypoxanthine will be at least 50% saturated at the mean plasma concentrations of their substrates. The inability of the bloodstream forms of trypanosomes to synthesise the purine ring de novo (Marr et al.,1978a; Gutteridge & Gaborak,1979; Ceron et al.,1979; Berens et al.,1981; Fish et al.,1982a) suggests that they must obtain all of their purine

from the host bloodstream. The following calculations, based on the kinetic parameters and plasma purine concentrations listed above, show that the adenosine transport is capable of meeting the cell's demand for adenosine. All assumptions made below are biased towards over estimating the amount of purine required by the cells.

At a plasma adenosine concentration of 1.25μM (mean rat plasma concentration) the rate of adenosine uptake would be 0.36nmol/min/mg protein. The DNA and RNA content of T.brucei will be taken to be 881μg/$10^{10}$ cells and 6430μg/$10^{10}$ cells respectively. These values are based on the values reported by Voorheis (1979) and Rovis & Baekkeskov (1980) who are in close agreement as regards the amount of DNA present but differ as to the amount of RNA present by several orders of magnitude: 11.6μg/$10^{10}$ cells (Rovis & Baekkeskov, 1980) and 6430μg/$10^{10}$ cells (Voorheis, 1979). The value of Voorheis, 1979 wil be used in the following calculations. The reason for this vast discrepancy is probably due to degradation by endogenous nucleases. The adenosine/adenine requirement of the cells can be calculated as follows.

Assume DNA is 25% adenine nucleotide and cell numbers double from $10^{10}$ cells to $2x10^{10}$ cells in eight hours.

This would require 881/4 = 220μg of adenine nucleotide

= $6.37x10^{-7}$ moles of AMP/$10^{10}$ cells

Assume RNA is 35% adenine nucleotide (including poly A tails of messenger RNA) doubling from $10^{10}$ cells to $2x10^{10}$ cells as before

requires 6430/100x35 = 2250μg of adenine nucleotide

= $3.49x10^{-6}$ moles of AMP/$10^{10}$ cells

The adenine nucleotide requirement for $10^{10}$ cells doubling in eight hours is therefore 3.83μmoles. This is equivalent to a requirement of

7.9nmoles/min/$10^{10}$ cells. The rate of uptake of adenosine at a plasma concentration of 1.25μM is 0.36nmol/min/mg protein which is equivalent to 0.36nmol/min/$1.96 \times 10^{8}$ cells. Therefore, the rate of uptake for $10^{10}$ cells would be 18nmol/min which is aproximately 2× the rate required. However, if the total purine content of rat plasma is estimated using the highest estimates for plasma purine concentrations, a calculated value of 216nmole is obtained (assuming a blood volume of 40ml in a 250g rat) which if utilised at a rate of 7.9nmol/min, as calculated above, will be cleared from the rat's plasma 17 times in the course of 8 hours. The rat must therefore release purine into the bloodstream to maintain plasma levels or the trypanosomes may cause destruction of host cells resulting in the release of purine into the bloodstream.

The Km and Vmax values quoted above are in disagreement with those obtained by James & Born, 1980, who estimated the Km for adenosine uptake to be 1.8μM as opposed to 0.5μM. However, these authors made no attempt to differentiate between uptake and transport since they used methods to measure uptake which do not give estimates of initial rates. The method used to estimate uptake consisted of a 3 minute incubation at 37°C in the presence of labelled substrate followed by centrifugation for 30 seconds and repeated centrifugation in cold buffer to remove extracellular label. This procedure would not result in estimates of initial rates of uptake being measured but would measure initial rates of reaction of the metabolising enzymes and accumulation of metabolised label. This view is supported by the results presented in Section 3.2.4 which show that after 45 seconds exposure to labelled adenosine, over 85% of the label taken up is converted to nucleotide. The use of incubation times greater than one

minute has been shown to result in the misinterpretation of the results of uptake experiments in several cell types (Wohlhueter et al.,1976 & 1979; Plagemann,1971b; Lum et al.,1979; Marz et al.,1979; Cybulski,1982). The methodologies applicable to the study of transport systems are given in detail in the Introduction (Section 1.2.2)

It should also be noted that James & Born (1980) found that adenosine uptake continued for up to 20 minutes whereas adenosine and hypoxanthine uptake ceased after two minutes in the time course studies carried out in this investigation(Sections 3.2.1 & 3.3.1 respectively). This discrepancy may be due to differences in the strain of trypanosomes used, or, since James & Born used a cloned strain of trypanosomes their clone may have been an atypical representative of the original uncloned population. The observed cessation in uptake of adenosine and hypoxanthine suggests the existence of some form of regulation of uptake as will be discussed later.

Evidence for adenosine, adenine and hypoxanthine uptake being mediated by a carrier or carriers comes from estimations of the thermodynamic parameters of adenosine uptake, (in particular the activation energy Section 3.2.3) and experiments investigating the effect of nucleoside analogues on uptake. The existence of a carrier, or carriers, in the plasma membrane is also supported by the observations that the uptake systems for adenosine, adenine and hypoxanthine are all saturable and highly temperature dependent. (Sections 3.2.1 to 3.2.2 and 3.3.1 to 3.3.4 respectively.)

The thermodynamic parameters calculated from an Arrhenius plot of adenosine uptake (Section 3.2.3) are shown below along with the parameters calculated for pyridoxine and pyridoxal for comparison.

| Parameter | Ar Uptake | PN Uptake | PA Uptake |
|---|---|---|---|
| Ea | 34.2 kJ/mol | 9.9 kJ/mol | 5.4 KJ/mol |
| $\Delta H$ | 31.7 kJ/mol | 7.5 kJ/mol | 2.9 kJ/mol |
| $\Delta S$ | 149.0 J/mol/K | 128.0 J/mol/K | 30.0 J/mol/K |
| $\Delta G$ | -13.2 kJ/mol | -31.1 kJ/mol | -6.2 kJ/mol |

As stated in the previous Section (4.1) a high activation energy is associated with a carrier mediated process. The results listed above show that the activation energy for adenosine uptake is 3 to 6 times greater than the Ea for pyridoxine or pyridoxal uptake which appears to be mediated by simple diffusion and not by a carrier. The value of $\Delta H$ for adenosine uptake is also greater than $\Delta H$ for pyridoxine or pyridoxal uptake as would be expected if $\Delta H$ is a measure of the degree of bond stretching involved in a binding process as suggested by Cornish-Bowden (1979). The binding of the adenosine to the carrier molecule is likely to involve some deformation of the adenosine, the transport protein, or both. These steps would not be involved in simple diffusion where a lower $\Delta H$ would be expected as is in fact the case.

The results presented in Sections 3.5.1 to 3.5.5 show the effects of nucleoside analogues and other nucleosides and bases on the uptake of adenosine (Section 3.5.1), adenine (Section 3.5.2) and hypoxanthine (Section 3.5.3). The results presented not only prove that uptake is carrier mediated but also that more than one carrier is present. The evidence for the existence of a carrier molecule in the plasma membrane comes from consideration of the results presented in Tables 3.6 & 3.7. At a 50:1 inhibitor:substrate concentration ratio IDA inhibits adenosine hydrolase activity by 15%, APRT activity

by 18% and has no effect on HxPRT activity, whereas at the same inhibitor:substrate ratio IDA inhibits adenosine uptake by 90%, adenine uptake by 65% and hypoxanthine uptake by 25%. This suggests that the IDA is inhibitng uptake at some point other than metabolism, such as a plasma membrane carrier. The same arguments can be applied to the results obtained for the effects of NBTI since it inhibits adenosine hydrolase by 23%, has no effect on APRT and inhibits HxPRT by only 2% while at the same inhibitor:substrate ratio it inhibits adenosine uptake by 70% and hypoxanthine uptake by 80%, again suggesting inhibition of some step in uptake before metabolism.

As stated in Section 3.5.1 inhibitors can be classified as strong (giving 70% to 90% inhibition) or weak (giving 10% to 40% inhibition). The percentage inhibitions of uptake quoted are the plateau inhibition values described previously (Section 3.5.1). This plateau effect may reflect a multiplicity of transport proteins in that any one inhibitor may block one transporter 100% but not the others for which the substrate being studied may have a low affinity. The evidence for more than one transporter in T.brucei is supported by the markedly different inhibition of adenosine uptake and hypoxanthine uptake by IDA, adenosine uptake being inhibited by 90% and hypoxanthine uptake being inhibited by 25% (Table 3.7). This difference is not due to any differential inhibition of metabolism since IDA has very little effect on the activity of the purine metabolising enzymes (Table 3.6). The same arguments can be applied to the results obtained using NBTI as an uptake inhibitor since NBTI like IDA has little effect on the activity of the purine metabolising enzymes. NBTI inhibits the uptake of adenosine by 70% and hypoxanthine uptake by 80% suggesting that hypoxanthine and adenosine

may use the same transporter. However, when the inhibitions of uptake by NBTI and IDA are considered together with the inhibition of adenine uptake which NBTI inhibits by 20% and IDA inhibits by 65%, the existence of three separate transport systems is suggested. One transport system specific for adenosine, one for hypoxanthine and one for adenine, IDA being a good inhibitor of the adenosine and adenine carriers and NBTI being a good inhibitor of the adenosine and hypoxanthine carriers. Table 3.7 summarises the results of the effects of inhibitors on uptake. The results are presented as maximum percentage inhibition, that is the inhibitor's plateau inhibition level described above.The results show that there is one transporter which appears to recognise 6-amino substituted purine nucleosides and bases such as adenosine, adenine and IDA. This is shown by adenosine uptake being inhibited by adenine and IDA and adenine uptake being inhibited by adenosine and IDA, whereas compounds lacking the 6-amino substitution such as hypoxanthine, guanine and guanosine are not strong inhibitors of either adenine or adenosine uptake.

There also appears to be a transporter which recognises 6-hydroxy substitutions as in hypoxanthine, guanine and guanosine. This is shown by inhibition of hypoxanthine uptake by guanosine and guanine, while adenosine, adenine and IDA have very little effect. The inhibitory patterns obtained with inosine and NBTI as inhibitors do not fit the simple specificities described above. This may be due to one of the transporters having some specificity towards the ribose moiety of nucleosides or the existence of three transporters. Further information on the exact number of transport proteins present or their specificities must await further investigation with a greater range of structural analogues or

purification of the proteins themselves.

### 4.2.2 Mechanism of Purine Nucleoside and Base Transport.

The uptake of adenosine and hypoxanthine by T.brucei appears to be mediated by active transport since they are accumulated by the cells against a concentration gradient as free adenosine and hypoxanthine. This is shown by the results presented in Figure 3.37 (Section 3.6.1). The intracellular concentrations of adenosine and hypoxanthine shown in Figure 3.37 were calculated assuming distribution of the label throughout total cell water which is unlikely considering the presence of intracellular organelles such as the glycosomes and the kinetoplast. As a result the values given for the cytosolic concentrations of adenosine and hypoxanthine are likely to be underestimates. The variation of intracellular concentration with time (Figure 3.37) shows that metabolism rapidly decreases the intracellular concentrations of adenosine and hypoxanthine, the metabolism of adenosine being more rapid than the metabolism of hypoxanthine. In both cases it should be borne in mind that uptake stops after 2-3 minutes and at this point the intracellular concentrations of adenosine and hypoxanthine are still greater than the external concentration. It should also be noted that the results presented in Tables 3.1 and 3.3 show that intracellular and extracellular adenosine and hypoxanthine do not exchange, indicating a one way transport system. The concentrations of intracellular adenosine and hypoxanthine quoted above refer only to the concentrations of labelled substrate; no account is taken of any endogenous pools of unlabelled purines. However, considering the efficiency with which the labelled adenosine and hypoxanthine are

metabolised, it seems unlikely that any significant endogenous pools of adenosine or hypoxanthine are present within the cell. It could be argued that the unmetabolised adenosine and hypoxanthine measured are not free in the cell but bound intracellularly in which case transport need not be an active process. Further evidence that active transport of adenosine and hypoxanthine occurs comes from investigations into the dependence of uptake on a membrane potential and ion gradients since active transport must be coupled to some energy source.

The dependence of adenosine and hypoxanthine uptake systems on a membrane potential was investigated by perturbing the membrane potential with ionophores and changing the composition of the incubation media. The ionophore gramicidin S allows all monovalent cations to pass through a lipid bilayer by forming aqueous pores through the membrane. This ionophore shows little specificity and also allows protons to pass through the membrane. The results of uptake assays conducted in the presence of gramicidin S are shown in Figure 3.38 as is the effect of high concentrations of $K^+$ in the incubation medium. Gramicidin S inhibits the uptake of adenosine, adenine and hypoxanthine as do high concentrations of $K^+$(103mM) in the incubation medium. The presence of gramicidin S at a concentration of 1μg/ml has been shown by Midgley (1983b) to reduce the membrane potential of T.brucei by 54%, from -129mV to -59mV, which equals the Nernst potential which would be obtained across a membrane permeable to ions but not to macromolecules. Similar effects of gramicidin S on the membrane potential of lymphocytes have been reported by Rink et al. (1980). Midgley (1983a), when using fluorescent probes of membrane potential found that gramicidin S

caused a depolarisation of the membrane reflected by inhibition of uptake of the fluorescent probe. In this paper Midgley acknowledges that gramicidin S may have an effect on any intracellular membrane potentials. However, Montecucco et al. (1979) showed that in lymphocytes gramicidin S did not partition into the mitochondrial membranes in whole cells. Gramicidin S is a potent uncoupler of mitochondrial oxidative phosphorylation (Montecucco et al., 1979) and would be expected to have the same effect in whole cells if the ionophore entered the cells intracellular membranes. Montecucco et al. (1979) showed that there was no significant difference in the ATP levels of lymphocytes treated with gramicidin S and controls. These results contrast with the effect of valinomycin on intracellular membranes. Davis et al. (1985) and Wilson et al. (1985) used valinomycin in combination with variations in extracellular $K^+$ concentrations to estimate the contribution of mitochondria to the uptake of fluorescent probes of membrane potential. High extracellular concentrations of $K^+$ depolarise the plasma membrane but not the mitochondrial membrane. However, in the presence of valinomycin, which partitions into intracellular membranes, and high concentrations of $K^+$ the mitochondria are depolarised. Comparison of the results obtained under these different conditions allows estimation of the contribution of the mitochondrial membrane potential to the measured plasma membrane potential using fluorescent probes.

In the case of T. brucei TREU 55 any contribution to a plasma membrane potential from the mitochondrion is unlikely to be significant since in the long slender bloodstream form the trypanosomal mitochondrion is virtually inactive. The oligomycin

sensitive ATPase reported to be present in some lines of long slender bloodstream forms of T. brucei (Opperdoes et al., 1976 & 1977b) which may be able to generate a mitochondrial membrane potential is not present in T.brucei TREU 55 (Flynn I.W. personal communication). This observation is consistent with the fact that TREU 55 is unable to multiply in culture or generate short stumpy forms which is a characteristic of trypanosome lines lacking the oligomycin sensitive ATPase (Opperdoes et al., 1976 & 1977b). In view of the above information it is unlikely that any of the results obtained with ionophores in this work are affected by ionophore effects on the mitochondrion.

The high external $K^+$ medium (Figure 3.38) also reduces the membrane potential by reducing the $K^+$ gradient across the membrane which is responsible for the membrane potential. The reduction in uptake of all three purines by lowering the membrane potential indicates that transport is at least partially dependent on a membrane potential or an ion gradient. A high extracellular $K^+$ concentration is less effective in inhibiting uptake than gramicidin S which will allow the equilibration of protons and $Na^+$ and $K^+$ across the membrane. This may indicate that uptake is also dependent on an ion gradient as well as a membrane potential. It should be noted that all ionophores were tested for trypanocidal activity and were found to have no effect on cell motility or rates of oxygen uptake at the concentrations used. This is exemplified by the results of the investigation into the effects of gramicidin S on oxygen uptake by the trypanosomes (Section 3.6.3). All the ionophores used were tested in the same manner.

Valinomycin, a $K^+$ specific ionophore, had no effect on

adenosine, adenine or hypoxanthine uptake unless the cells were preincubated with the ionophore before uptake of substrates was assayed. The observation that valinomycin had no effect on uptake in the absence of a preincubation period is as would be expected, since valinomycin, in the absence of extracellular $K^+$, does not depolarise plasma membranes and may even cause a slight hyperpolarisation (Bashford et al., 1985). The inhibition of uptake of adenosine, adenine and hypoxanthine by valinomycin after a preincubation period may be due to non-specific interaction with membrane proteins after it has partitioned into the plasma membrane. The possibility that the preincubation is required for the valinomycin to have an effect on the mitochondrion is unlikely in view of the lack of an oligomycin sensitive ATPase as discussed above.

Incubating cells in the presence of $K^+$ (103mM) and valinomycin resulted in even greater inhibition of uptake than that caused by either $K^+$ or valinomycin alone, indicating that, in the presence of $K^+$, valinomycin may be causing a depolarisation of the membrane over and above its effect in the absence of $K^+$.

The $Na^+$ dependence of adenosine and hypoxanthine uptake was determined as described in Section 3.6.5. It was found that while neither adenosine uptake nor hypoxanthine uptake was dependent on $Na^+$, hypoxanthine uptake was stimulated by high concentrations of $Na^+$. The significance of this result will be considered later. The $K^+$ dependence of uptake was not investigated since changing the extracellular $K^+$ concentration would affect the membrane potential which has been shown to have an effect on uptake. Therefore any direct dependence of uptake on $K^+$ concentrations would be masked by this effect. Investigations of the $K^+$ dependence of uptake could be

conducted using phospholipid vesicles into which the transport system could be incorporated and $K^+$ gradients established across the vesicle membrane.

The pH dependence of adenosine, adenine and hypoxanthine uptake was investigated as detailed in Section 3.6.6. The results of this investigation (Figure 3.43;Section 3.6.6) showed that as the pH of the incubating medium was increased the rate of uptake of adenosine, adenine and hypoxanthine decreased. This observed decrease in uptake is probably due to effects on cell motility and metabolism since the rate of oxygen uptake and cell motility increased markedly as the pH of the medium was increased. It should be noted that adenosine, adenine and hypoxanthine have no pK's in the pH range used in these experiments. In the pH range 7-8.5 the uptake of adenine and hypoxanthine appears to be unaffected by changes in pH. The uptake of adenosine does appear to be slightly inhibited by increasing the pH from pH 7 to 8.5. It should be noted that in the pH range 7-8.5 no significant changes in rates of oxygen utilisation or cell motility were observed. Figure 3.42 (Section 3.6.6) shows the effect of the protonophore FCCP on the uptake of adenosine and hypoxanthine. Both adenosine and hypoxanthine uptake are partially inhibited by FCCP. The addition of FCCP to the cells may be resulting in a slight increase in the magnitude of a proton gradient across the plasma membrane in a fashion similar to the hyperpolarisation of a membrane in the presence of valinomycin. Increasing the membrane permeability to $H^+$ would allow the protons to distribute across the membrane according to the membrane potential which in the case of trypanosomes is negative inside (Midgley, 1983b). Considering the effect of FCCP on uptake it seemed possible that a proton gradient could be involved

in the mechanism of purine uptake. The existence of a pH gradient across the plasma membrane is shown by the results presented in Figure 3.45, Figure 3.46 and Table 3.9. It was found that a proton gradient exists with the inside of the cell acid relative to the outside.

The results of the investigations into the dependence of uptake on a membrane potential and its dependence on extracellular ions are summarised in Table 3.8. A speculative model for the uptake mechanisms of adenosine, and hypoxanthine is shown in Figure 3.44.

The model for adenosine uptake shown in Figure 3.44, although highly speculative, is consistent with the results presented in Section 3.6. Adenosine uptake is inhibited by the addition of the ionophore gramicidin S to the incubation which will reduce any membrane potential by allowing potassium to leave and sodium to enter the cells. This suggests that adenosine uptake is dependent on a membrane potential or iongradient. Further evidence for adenosine uptake being dependent on a membrane potential is provided by the observation that high concentrations of extracellular potassium, which reduce any potassium membrane potential, also inhibit adenosine uptake. The inhibition of adenosine uptake by valinomycin has been discussed above. The model for adenosine uptake presented in Figure 3.44 in which the substrate enters the cell in exchange for a potassium ion and entry of a proton, was first suggested by Riggs et al.(1958) and later by Crane (1977). This is consistent with the results presented in Section 3.6 in that the efflux of potassium would be inhibited by increasing the extracellular potassium concentration or this pathway for potassium efflux may be by-passed

in the presence of high concentrations of valinomycin. Reduction of the membrane potential by high extracellular concentrations of potassium or by gramicidin S would inhibit the influx of protons since the proton is entering the cell against a proton gradient (Section 3.6.8). The inhibition of adenosine uptake by FCCP is also consistent with this model since the addition of FCCP to the cells makes their membrane more permeable to protons so that the proton gradient across the membrane may be increased as protons enter the cell in response to the membrane potential. A similar effect is observed as regards the membrane potential when valinomycin is added to cells in the absence of potassium, as discussed above. The slight inhibition of adenosine uptake caused by increasing the extracellular pH is also consistent with this model in that reducing the extracellular proton concentration effectively creates a greater gradient of protons across the membrane. The potassium lost from the cell in this model could be reclaimed by the plasma membrane $Na^+/K^+$ ATPase.

The model proposed for the uptake of hypoxanthine (Figure 3.44) shows the entry of hypoxanthine by a sodium symport mechanism with the sodium being removed from the cell either by the membrane $Na^+/K^+$ ATPase or sodium/proton antiport. The observation, that hypoxanthine uptake is stimulated by high extracellular concentrations of sodium, is consistent with the proposed model. Inhibition of uptake by factors reducing membrane potential (discussed above) could be due to reducing the rate of entry of the protons in the proton/sodium antiport system in response to the membrane potential. The membrane potential may also have an effect on the rate of entry of the sodium ions in the hypoxanthine/sodium symport system. This type of active

transport has been reviewed by Eddy (1978). In conclusion it should be borne in mind that the models presented are speculative, but the sensitivity of uptake of hypoxanthine and adenosine to factors known to affect membrane potentials and cellular ion gradients suggests some form of dependence on a membrane potential. The active transport hypothesis is also supported by the observation that both adenosine and hypoxanthine are accumulated against their concentration gradients by the cell.

Having discussed the results obtained from the work carried out, and some of the conclusions which can be drawn from the data, these results will now be considered in conjunction with what is known about purine nucleoside and base transport in other organisms.

### 4.2.3 Comparison of Transport of Purine Nucleosides and Bases in T.brucei and other Organisms

The results discussed in the previous section give a strong indication that the uptake of the purines adenosine, adenine and hypoxanthine by T.brucei is mediated by an active transport system. This active transport contrasts with the facilitated diffusion mechanisms prevalent in other eukaryotic cells. Evidence has also been presented indicating the presence of more than one transporter with differing specificities which again appears to differ from other eukaryotic cells.

The uptake of nucleosides by the mammalian erythrocyte has been extensively studied and found to be mediated by facilitated diffusion (Cabantchik & Ginsburg,1977; Cass & Paterson,1972 & 1973) using a single carrier which shows little specificity towards the base portion of the nucleoside but is relatively specific for the ribose moiety (Oliver & Paterson,1971; Cass & Paterson.1973). The uptake of

bases by the erythrocyte appears to occur via a different carrier since the uptake of nucleosides is strongly inhibited by NBTI (Jarvis & Young,1980) whereas the uptake of bases is not (Paterson & Oliver,1971). The uptake of nucleosides by mammalian cells has been investigated in great detail and recently reviewed by Plagemman & Wohlhueter (1980) and Young et al. (1983). The general opinion given in these reviews is that in animal cells the uptake of nucleosides is mediated by facilitated diffusion followed by metabolic trapping. This has been shown to be the case for uptake of nucleosides by Novikoff rat hepatoma cells (Marz et al.,1979), mouse leukemia cells , canine thymocytes, canine peripheral blood leukocytes (Lum et al.,1979) and brain tissue (Shimizu et al.,1972). Lum et al. (1979) and Marz et al. (1979) showed that the nucleoside and base carriers allow equilibration of nucleosides and bases across the plasma membrane in a few seconds, all further uptake being due to metabolism of the nucleosides and bases maintaining a concentration gradient across the membrane. In animal cells other than erythrocytes several carriers of differing specificites were thought to exist as indicated by cross inhibition studies of nucleoside and base uptake in rat hepatoma cells (Plagemann & Erbe,1974) and HeLa cells (Cass & Paterson,1977). This apparent specificity was later shown to be due to the specificity of the nucleoside and base metabolising enzymes and not the membrane carriers. This is caused by the cross inhibition experiments being conducted with incubation periods greater than 1 minute in which the measured uptake is due to metabolism rather than the initial rapid transport step (Section 1.2.1; Taube & Berlin,1972; Mulder & Harrap,1975; Heichal et al.,1979).

The results of cross inhibition studies using various

nucleosides and bases to inhibit each other's uptake must be considered with caution since even with incubation periods as short as 30 seconds a substantial amount of the nucleoside and base taken up is metabolised to nucleotide and some of the nucleosides and bases used in the cross inhibition experiments are substrates for the metabolising enzymes. However, guanine inhibits HxPRT activity by 34% at a guanine:hypoxanthine concentration ratio of 50:1 but at the same concentration ratio guanine inhibits hypoxanthine uptake by 90% indicating that the observed inhibition is not due only to the inhibition of HxPRT.

It should also be noted that the percentage inhibitions of uptake quoted are the maximum inhibition levels which could be obtained as described in Section 3.5.1. The inhibitions of uptake plateau at certain fixed percentage inhibitions which do not increase with increasing inhibitor concentration. If uptake was being inhibited by inhibition of metabolism then inhibition of uptake would be expected to continue to increase with increasing inhibitor concentration. The plateau effect is probably a reflection of the presence of multiple carriers since any given inhibitor may block completely the substrate's normal carrier, but allow uptake of the substrate via one of the other carriers for which the substrate may have a low affinity.

The number of nucleoside and base transporters in rat hepatoma cells has been investigated using nucleoside analogues in the same manner as described above (Cohen et al.,1979). The results of these investigations suggested the existence of distinct nucleoside and base transports. The uptake systems of T.brucei appear to be more complex than those of mammalian cells in that there does not appear

to be a simple distinction between a nucleoside transporter and a base transporter, but rather some specificity towards the purine base moiety as opposed to the presence or absence of ribose (Section 4.2.1).

Purine nucleoside and base uptake by bacteria has also been extensively studied. Peterson & Koch (1966) and Peterson et al. (1967) investigated the mutual inhibitory effects of nucleosides and bases on each other's uptake and concluded that in E.coli there was more than one transport system distinguishable from metabolism. A group translocation mechanism for the uptake of purine nucleosides and bases by E.coli and S.typhimurium has been proposed by Hochstadt & Stadman (1972) and Rader & Hochstadt (1976). However, by the use of mutants deficient in nucleoside phosphorylase and phosphoribosyl transferase enzymes required for the proposed group translocation system it has been shown in E.coli that uptake is independent of these enzymes, the mutant bacteria still being capable of accumulating nucleoside against a concentration gradient (Hoffmeyer & Neuhard,1971; Mygind & Munch-Petersen,1975;Munch-Petersen & Mygind,1976). Two uptake systems have been identified in E.coli designated the C and G systems, the C system being specific for cytosine and the G system being specific for guanosine (Munch-Petersen et al.,1979). The C system has also been found to transport adenosine and deoxyadenosine but no other purine nucleosides whereas the G system will transport all nucleosides, whether purine or pyrimidine.

As stated in the Introduction (Section 1.4.2) little information is available on the purine transport systems of trypanosomes. In the few cases where the uptake of purines by trypanosomes has been

investigated little effort has been made to distinguish between transport and overall uptake (James & Born,1980). Uptake of purines by L.panamensis has been shown to be carrier mediated (Hansen et al.,1982) with three separate carriers involved as determined by studies of mutual inhibition of uptake amongst purine nucleosides and bases. Multiple carriers have also been suggested for purines in T.brucei, T.congolense (James & Born,1980) and C.fasciculata (Kidder et al.,1978). Although the results reported by James & Born (1980) agree in part with the results presented here, the long incubation times used by these authors mean that in many cases the reported inhibitions could be due to inhibition of metabolism and therefore overall uptake, and not inhibition of transport. Active transport of pyrimidines by T.lewisi has been reported by Manjra & Dusanic (1973); however these authors made no distinction between transport and uptake (Section 1.2) and did not show accumulation of unmetabolised pyrimidines. As a result, their conclusion that uptake was mediated by active transport is invalid. Active transport of purine nucleosides or bases has not been shown to occur in any mammalian cells studied to date but has been shown to occur in E.coli K12, E.coli B and S.typhimurium. Active transport was demonstrated by the ability of mutant cells, lacking purine metabolising enzymes, to concentrate purine nucleosides and bases several hundred fold. (Munch-Petersen & Mygind,1976; Munch-Petersen & Pihl,1980). The active transport process was shown to be dependent on a proton gradient by its sensitivity to the uncoupler 2,4-dinitrophenol. In membrane vesicles transport was supported by the addition of electron donors suggesting that the proton gradient is generated by a proton-pumping electron transport chain (Munch-Petersen et al.,1979).

Similar results have been obtained in studies on S.typhimurium (Rader & Hochstadt,1976) and E.coli B (Roy-Burman et al.,1978).

The results presented in this work are indicative of an active transport system for the uptake of purine nucleosides and bases by T.brucei. This is supported by the observations that adenosine and hypoxanthine are accumulated against their concentration gradients (Section 3.6.1) and the sensitivity of uptake to reduction of the cell membrane potential (Sections 3.6.2 & 3.6.4). The sensitivity of uptake to inhibition by the uncoupler FCCP and, in the case of adenosine, high external pH (Section 3.6.6) suggests that a movement of protons is involved in the uptake mechanism. The points above are discussed in detail in Section 4.2.2.

The existence of active transport systems in trypanosomes is not unknown, as demonstrated by the examples given below. The transport of glucose by L.donovani is an active process as indicated by the organism's ability to accumulate glucose and the partially metabolisable glucose analogue 2-deoxyglucose against their concentration gradients (Zilberstein & Dwyer,1984). This active transport process was also found to be sensitive to inhibition by the uncoupler 2,4-dinitrophenol suggesting the involvement of a proton gradient in the transport process. The uptake of threonine by T.brucei is mediated by active transport (Voorheis, 1971; Fricker et al.,1984) but the energy source to which transport is coupled has not been conclusively identified. The transport is independent of $Na^+$ concentration (Voorheis, 1971; Fricker et al.,1984) and independent of a proton gradient (Voorheis,1979). Fricker et al. (1984) proposed that transport is coupled to an exchange of alanine which is produced from pyruvate by transamination and accumulated to high

concentrations in the cell (Chappell et al.,1971); however no direct evidence for this hypothesis is available. Voorheis (1980) provided some evidence that a glycolytic intermediate from before the aldolase reaction was required for transport, but this hypothesis does not appear to have been investigated further. In contrast to the findings of the above authors, Hansen (1979) found threonine uptake in T.brucei to be $Na^+$ dependent and inhibitable by ouabain, suggesting that a $Na^+/K^+$ ATPase is responsible for the maintenance of the required $Na^+$ gradient and the membrane potential. The requirement for a membrane potential for active transport by C.fasciculata has also been demonstrated, in that an electrochemical proton gradient was found to be required for the uptake of γ-aminobutyric acid by a proton symport mechanism (Midgley 1978; Midgley & Stephenson,1980).

The proton gradient across the trypanosomal plasma membrane (Section 3.6.8) may be generated by a proton pumping ATPase or by an antiport with another ion such as $K^+$. A $K^+/H^+$ antiport working to expel protons is known to operate in $K^+$ depleted yeast cells (Ryan & Ryan, 1972). It has also been proposed that the efflux of lactate in fermentative bacteria can be used to establish a proton gradient across the cell membrane by a symport mechanism (Konings,1985). The possibility that the trypanosomal proton gradient may also be generated by the efflux of the metabolic end product pyruvic acid should be investigated since the bloodstream forms of the African trypanosomes produce large amounts of pyruvic acid from glycolysis. The mechanism of generating the proton gradient would differ from that found in bacteria since the direction of the gradient would be reversed. This could be achieved by expelling pyruvate as an anion in either antiport or symport with a cation.

### 4.2.4 Metabolism of Purine Nucleosides and Bases by *T.brucei*.

The effect of the nucleoside analogues NBTI and IDA on the purine metabolising enzymes APRT, HxPRT and adenosine hydrolase have been discussed (Section 4.2.1) in relation to inhibition of uptake as distinct from metabolism. That these three enzymes are involved in the initial stages of salvage of purine nucleosides and bases is shown by the results presented in Sections 3.2.4 & 3.2.5. When trypanosomes were incubated with radiolabelled adenosine for varying periods of time it was found that over 85% of the label taken up after 45 seconds was present in the cell as nucleotide. At later time points it was noted that the levels of nucleotide slowly increased while the levels of free adenosine and adenine fell. It was also noted that the levels of labelled adenosine in the cell fell below detectable levels more rapidly than adenine. This suggests that adenosine is deribosylated to adenine before being converted to nucleotide. Experiments in which a dialysed cell lysate was incubated with labelled adenosine showed that the lysate contained deribosylase activity as it rapidly deribosylated adenosine to adenine. This activity was shown to be adenosine hydrolase as opposed to adenosine phosphorylase by the enzymes ability to function in phosphate free media with no loss of activity (Section 3.4.1). The conversion of adenosine to nucleotide was shown not to occur when a lysate was supplied with adenosine, ATP and $Mg^{2+}$*, but nucleotide was formed when the lysate was supplied with adenosine, PRPP and $Mg^{2+}$ (Section 3.2.5). These results lead to the conclusion that adenosine when taken up is first hydrolysed to adenine and ribose before being converted to AMP by APRT.

* or with an ATP regeneration system present.

A time course of hypoxanthine metabolism (Section 3.3.4) showed that hypoxanthine entered the cell as hypoxanthine and was subsequently metabolised to nucleotide but at a much slower rate than observed for the conversion of adenosine to nucleotide. An interesting observation from the time courses of adenosine and hypoxanthine metabolism by whole cells is that metabolism of the label taken up continues for up to 20 minutes but uptake ceases after 2 minutes as shown by the time courses of adenosine and hypoxanthine uptake (Sections 3.2.1 & 3.3.1). This observation will be discussed in detail later. The only known pathways for the conversion of hypoxanthine to nucleotide are via the enzymes HxPRT or HxGPRT.

The kinetic parameters of these three enzymes, adenosine hydrolase, APRT and HxPRT were estimated (Sections 3.4.2, 3.4.3 & 3.4.4 respectively) for comparison with the kinetic parameters estimated for overall uptake of adenosine, adenine and hypoxanthine. The kinetic parameters of the uptake systems are listed in Section 4.2.1. These data show that in all cases the Km for uptake is lower than the Km for the enzymes involved in metabolism. Observed saturability of uptake must therefore be due to saturation of transport rather than metabolism, since saturation of uptake occurs at a substrate concentration below that needed to saturate any of the metabolic steps. Calculation of the rates of uptake at a substrate concentration of 1μM, when compared to the rates of reaction of the metabolising enzymes (estimated in cell lysates) at the same substrate concentration, show that metabolism is capable of much higher reaction rates than uptake. This calculation assumes that intracellular and extracellular concentrations are equal which may not be the case if active transport is occurring. Also this ignores

the fact that the kinetic parameters calculated for the metabolising enzymes apply only to a cell free situation. In the cell these enzymes could be under many forms of regulation which would affect their kinetic characteristics making any calculations based on the experimentally obtained kinetic parameters invalid. The simple calculation carried out deals with only one static time point which is a poor representation of the in vivo situation where the intracellular concentration of transported substrate will increase rapidly consequently increasing the rate of metabolism. Any meaningful calculations to determine the rate limiting step in uptake would require the construction of a computer model of the system for which much more data would be required regarding possible regulation of transport, metabolism and also the intracellular concentrations of other substrates such as PRPP.

In other systems, as will be discussed later, the HxPRT may also utilise guanine as substrate. This is indicated by the results presented in Section 3.4.6 which show that guanine is a good inhibitor of HxPRT activity and therefore may be a substrate. The results presented also show that the APRT and HxPRT activities are associated with separate enzymes since the two enzyme activities have different $Mg^{2+}$ dependence curves (Section 3.4.5) and HxPRT activity is not inhibited by adenine and inhibited 50% guanine (Section 3.4.6).

Figure 3.33 summarises the uptake and metabolism of adenosine, adenine and hypoxanthine by T.brucei with the exception of the interconversion of nucleotides. The data presented in Sections 3.2.6 & 3.3.5 shows the diversity of nucleotides labelled during a 10 minute incubation of whole cells with labelled adenosine and

hypoxanthine respectively. The results presented in Figure 3.15 (Section 3.2.6) show that adenosine when taken up is rapidly incorporated into the cell's ATP pool and then distributed through the ADP and AMP pools as metabolism proceeds. The formation of labelled GMP and GDP indicates the presence of AMP deaminase, IMP dehydrogenase and GMP synthetase which would convert AMP to IMP, IMP to XMP and XMP to GMP respectively. The results of a similar experiment using labelled hypoxanthine as substrate for the uptake system are presented in Figure 3.21 (Section 3.3.5). The results show the incorporation of label into IMP, and as time progresses, incorporation of label into IDP and ITP. If, as stated above, the enzymes IMP dehydrogenase and GMP synthetase are present, labelling of GMP and GDP would be expected. From the data shown it is not possible to tell if GMP is labelled but very little labelled GDP was detected. However the formation of GMP by GMP synthetase requires ATP and if, as suggested by the rapid incorporation of exogenous labelled adenosine into the cell's ATP pool, the cells are deficient in ATP this reaction would not occur at a significant rate. The little label incorporated into ATP after ten minutes incubation may reflect the presence of adenylosuccinate synthetase and adenylosuccinate lyase which can convert IMP to AMP. The metabolism of purines in T.brucei will now be compared with what is known of purine metabolism in other organisms.

### 4.2.5 Comparison of Purine Nucleotide,Nucleoside and Base Metabolism in T.brucei and other Organisms.

The metabolism of purine nucleosides and bases by T.brucei, as determined in this work, has been discussed in Section 4.2.4 and will

now be considered in relation to the metabolism of purine nucleosides and bases in other organisms. The results discussed in Section 4.2.4 indicate that when adenosine is taken up it is deribosylated to adenine and ribose by hydrolysis as opposed to phosphorolysis. The apparent inhibition of APRT by adenosine can be explained by dilution of the labelled adenine used as substrate with unlabelled adenine derived from the adenosine present due to the action of adenosine hydrolase (Section 3.5.4). The fact that this effect was not observed to occur to the same extent when inosine was tested as an inhibitor of HxPRT suggests that inosine is not a good substrate for adenosine hydrolase. However the existance of an inosine hydrolase cannot be ruled out since the inosine hydrolase may be much less active than adenosine hydrolase.

The inhibition of HxPRT obtained with guanosine as inhibitor is similar to the inhibition by guanine (Section 3.5.4) which may indicate the presence of a guanosine hydrolase or that guanosine is a substrate for adenosine hydrolase. Alternatively guanosine and inosine may be poor substrates for the adenosine hydrolase as indicated by their ability to inhibit adenosine hydrolase by 31% and 35% respectively when present in a fifty-fold excess over the adenosine substrate.

Adenosine phosphorylase (Section 3.2.5) is known to be the enzyme responsible for deribosylation of adenosine in the mammalian erythrocyte, (Tsuboi & Hudson,1957; Huennekens et al.,1956; Grimes 1980) brain (Huennekens et al.,1956) and liver (Korn & Buchanan,1955). The hydrolytic pathway is known to operate in some bacteria (Rabinowitz & Barker,1965) and in bakers yeast (Heppel & Hilmoe,1952). Nucleoside phosphorylase activities have been detected

in L.mexicana amazonensis promastigotes, T.brucei trypomastigotes and T.cruzi amastigotes, epimastigotes and trypomastigotes (Gutteridge & Davies,1982; Davies et al.,1983) all of which contain adenosine and guanosine phosphorylase activities. These findings are in conflict with the findings of Ogbunude & Ikediobi (1983), Ogbunude et al. (1985) and this work Section 3.2.5. Ogbunude & Ikediobi (1983) and Ogbunude et al. (1985) did not detect any nucleoside phosphorylase activity in T.brucei or T.vivax but did find activity in T.congolense. In the organisms, T.brucei and T.vivax, in which no nucleoside phosphorylase activity was detected, nucleoside hydrolases (nucleosidases) were found to be present allowing the production of free base from nucleoside. Nucleosidases have also been detected in other trypanosomes (Davies et al.,1983; Looker et al.,1983) and purified from T.gambiense (Schmidt et al.,1975).

Nucleosides may also be converted to nucleotides, without conversion to free bases, by the action of nucleoside kinases which have been detected in a variety of trypanosomes. Adenosine kinase activity has been reported to be present in extracts of L.donovani (Tuttle & Krenitsky,1980) and T.cruzi (Gutteridge & Davies,1982). Adenosine, inosine and guanosine kinases have also been reported to be present in L.m.amazonensis, T.brucei and all morphological forms of T.cruzi (Davies et al.,1983). However adenosine kinase could not be detected in T.brucei by Ogbunude & Ikediobi (1983), Ogbunude et al. (1985) or this work (Section 3.2.5). Ogbunude et al. (1985) did however detect adenosine kinase in T.vivax and T.congolense. Nucleoside kinases have also been detected in mammalian systems such as the erythrocyte (Agarwal & Parks,1978), brain (Shimizu et al.,1972), and mouse leukemia cells (Lum et al.,1979).

Hypoxanthine, guanine and adenine phosphoribosyltransferase activities have been detected in Crithidia fasciculata (Kidder et al.,1979), L.donovani (Tuttle & Krenitsky,1980), T.cruzi (Gutteridge & Davies,1981 & 1982) and T.brucei and L.mexicana (Davies et al.,1983). In most cases the hypoxanthine and guanine phosphoribosyltransferase activities have been found to be associated with the same enzyme, as occurs in mammalian tissues, which is in agreement with the results obtained in this investigation. However these activities have been shown to be associated with separate enzymes in C.fasciculata (Kidder et al.,1979).

The presence of adenosine deaminase in trypanosomes is a matter of debate in that Davies et al. (1983) reports that adenosine deaminase is present in T.brucei and T.cruzi but not present in L.mexicana whereas Ogbunude et al. (1985) found no adenosine deaminase in T.brucei, T.vivax or T.congolense. Experiments carried out during this investigation also failed to detect any adenosine deaminase activity in T.brucei. The lack of adenosine deaminase precludes the interconversion of adenine and hypoxanthine. Therefore, any interconversion of bases must take place at the level of the nucleotides. This must be the case since single purines are capable of supporting the growth of trypanosomes in culture (Marr et al.,1978; Schmidt et al.,1975; Fish et al.,1982a & 1982b; Gutteridge & Gaborak,1979). The formation of labelled GMP and GDP from labelled adenosine (Section 3.2.6) indicates the presence of AMP deaminase, IMP dehydrogenase and GMP synthetase which would convert AMP to IMP, IMP to XMP and XMP to GMP respectively. Using labelled hypoxanthine as the substrate for uptake, label is incorporated into IMP and as time progresses IDP and ITP are labelled. If, as stated

above, the enzymes IMP dehydrogenase and GMP synthetase are present, GMP and GDP would be expected to be labelled. From the data obtained it is not possible to tell if GMP is labelled but very little labelled GDP was detected. The little label incorporated into ATP after ten minutes incubation may reflect the presence of adenylosuccinate synthetase and adenylosuccinate lyase which can convert IMP to AMP. The enzymes adenylosuccinate synthetase and adenylosuccinate lyase have been shown to be present in T.gambiense and T.rhodesiense along with IMP dehydrogenase and GMP synthetase (Fish et al.,1982b). These enzymes have also been shown to be present in L.donovani and L.braziliensis (Marr et al.,1978)

**4.3 PREPARATION OF PLASMA MEMBRANE ENRICHED FRACTION.**

In order to investigate the adenosine transporter in more detail a purification scheme for the preparation of a plasma membrane enriched fraction was developed. It was hoped that this would aid in the detection of proteins present in the membrane as a low percentage of the total protein.

As previously stated in Section 3.7 several methods for the purification of trypanosomal plasma membranes have been published (Rovis & Baekkeskov,1980; Steiger et al.,1980; Mancini et al.,1982; Voorheis et al.,1979). One of the main differences between the methods published by these authors was the method of disrupting the cells before fractionation. The method of osmotic swelling and homogenisation used by Voorheis et al. (1979) required a precision homogeniser which was not available during this investigation. Mancini et al. (1982) used sonication to disrupt the cells which in this investigation was found to destroy marker enzyme activity and so

was not used. Rovis & Baekkeskov (1980) used nitrogen cavitation to disrupt the cells; however the equipment required for this technique was not available during this investigation. Steiger et al. (1980) ground the cells with silicon carbide to disrupt the plasma membranes. Where possible these methods plus water lysis were evaluated for the production of a cell homogenate as described in Section 3.7.1. The method finally chosen for the disruption of the cells was a modification of the silicon carbide grinding method of Steiger et al. (1980) in which 75μm diameter glass beads were substituted for the silicon carbide. The use of glass beads was found to enable greater recovery of material from the grinding paste.

One of the main problems encountered in attempting purification of the trypanosomal plasma membrane was the lack of suitable plasma membrane marker enzymes. However Steiger et al. (1980) showed that the enzyme $\alpha$-glucosidase is located on the outer surface of the plasma membrane of T.brucei and is therefore suitable as a plasma membrane marker enzyme. The observation that this enzyme is located on the outer surface of the plasma membrane suggests that it may be involved in processing the glycosylated residues of the glycoprotein coat to their mature forms after insertion of the glycoprotein into the membrane. Ouabain inhibitable $Na^+/K^+$ ATPase has been used as a plasma membrane marker in T.brucei subcellular fractionations (Voorheis et al. (1979) but as stated in Section 3.7.1 this marker was only found to be useful in semi-purified fractions due to the high levels of non-specific phosphatase activity and the activities of other ATPases found in the initial homogenate. The presence of phosphatases makes difficult the use of 5'-nucleotidase activity as a marker. The enzyme 5'-nucleotidase is one of the most frequently used

plasma membrane marker enzymes in fractionations of mammalian cells (Solyom & Tranns,1972). However this enzyme was not detected in T.brucei by Rovis & Baekkeskov (1980). Plasma membranes have been artificially marked to aid in the identification and purification of membrane proteins (Mancini et al.,1982). The cell surface proteins were marked by labelling the cell surface with $^{125}I$ and by incubation of the cells with [$^{3}H$]-mannose which was incorporated into the cell surface glycoproteins. Purification of the membranes was then followed by monitoring the purification of the radiolabelled proteins. The markers used in this investigation were $\alpha$-glucosidase, $^{125}I$ iodinated surface proteins and ouabain inhibitable $Na^{+}/K^{+}$ ATPase in some semi-purified fractions.

The density of the plasma membrane enriched fraction (Section 3.7.2) was found to be 1.14g/cm$^3$ which is in agreement with the densities estimated by Mancini et al. (1982) and Rovis & Baekkeskov (1980) but is much lighter than 1.22 -1.24g/cm$^3$ as estimated by Steiger et al. (1980) and Voorheis et al. (1979). This difference between the densities estimated by these authors could be due to the presence or absence of microtubules attached to the plasma membrane. If the tubules remain attached to the membrane during fractionation they would be expected to markedly increase the apparent density of the membrane since the subpellicular microtubular array of trypanosomes is quite extensive (Anderson & Ellis,1965; Angelopoulous,1970). Whether or not the microtubules remain associated with the plasma membrane appears to depend on the homogenisation buffer used in the cell breakage procedure. It has been found that the equilibrium density of the plasma membrane marker $\alpha$-glucosidase shifts from 1.22g/cm$^3$ to 1.17g/cm$^3$ if the initial

disruption of the cells is carried out in Tris buffer rather than imidazole buffer (Walter & Opperdoes, 1982). Voorheis et al. (1979) and Steiger et al. (1980) used TES and imidazole buffers respectively during homogenisation and, as stated previously, estimated the plasma membrane equilibrium density to be 1.22g/cm$^3$ whereas the buffers used by Mancini et al. (1982) and Rovis & Baekkeskov were Tris and HEPES* respectively. These authors estimated the equilibrium density of the plasma membrane to be 1.14g/cm$^3$. These comparisons show that detailed consideration must be given to experimental method when comparing results obtained by different authors.

The purification scheme finaly adopted for the production of a plasma membrane enriched fraction is given in Section 3.7.4. The purification consists of simple differential centrifugation steps and a sucrose step gradient centrifugation. One step of interest is the collection of the plasma membranes by centrifugation onto a dextran plaque in a high pH and low osmotic strength buffer. The physical basis of this procedure is explained in detail by Wallach & Kamat (1964) and Avruch & Wallach (1971). The principle of the method is described briefly below. The fractionation of microsomal and plasma membrane vesicles occurs due to differential swelling in a hypo-osmotic medium. Due to the fixed charge on the inner surface of the membrane vesicles an assymmetric distribution of permeant ions occurs as determined by the Gibbs-Donnan equilibrium this in turn causes an osmotic swelling of the vesicles. The osmotic effect of the assymmetric distribution of ions is insignificant in a medium of high osmotic activity (eg. sucrose ) or high ionic strength (eg. CsCl). However this osmotic effect becomes significant in media of low osmotic activity such as high molecular weight dextran. In this case

* HEPES was used in this work.

the vesicles will swell until ionic-osmotic equilibrium is reached which markedly reduces their buoyant density. The magnitude of this effect can be manipulated by changing the ionic strength of the medium and titration of the fixed charges on the membrane by changing the pH of the medium. The separation of the microsomal and plasma membranes in this investigation was achieved due to the greater negative charge of the plasma membrane pulling in more ions and swelling the vesicles to a point where their buoyant density was less than the dextran and much less than the microsomal vesicles.

The $\alpha$-glucosidase activity found in the microsomal fraction after dextran centrifugation may have been due to fragments of plasma membrane which did not form vesicles. The effect of changing the concentration of the permeant ions on the separation of plasma membranes and microsomal membranes on a dextran plaque is shown in the Section 3.7.3. The effect of pH and ionic composition on the relative buoyant densities of endoplasmic reticulum and plasma membrane is demonstrated in a series of experiments conducted by Avruch & Wallach (1971) in which the degree of separation of the two fractions was measured over a broad pH range and at different ionic strengths giving varying degrees of purification and different yields of the two fractions.

### 4.3.1 Cellular Distribution of Purine Nucleoside and Base Metabolising Enzymes.

While conducting preliminary cell fractionation experiments the cellular distribution of the purine metabolising enzymes adenosine hydrolase, APRT and HxPRT was determined. The results presented in Section 3.7.5 indicate that adenosine hydrolase is probably membrane

bound and that APRT and HxPRT are soluble cytoplasmic enzymes. However it has been proposed that the HxPRT of T.cruzi is associated with the glycosome (Gutteridge & Davies,1982). The enzyme APRT appears to be a soluble cytoplasmic enzyme in a variety of trypanosomal species (Hammond & Gutteridge,1984).

The observation that adenosine hydrolase appears to be membrane bound may be significant in consideration of the mechanism of adenosine transport since hydrolysis of adenosine to adenine is the first step in its metabolism to nucleotide. It is possible to envision a system where adenosine is hydrolysed to adenine and the adenine transported instead of adenosine, with the ribose being taken up by another transport system. However this is unlikely since transport of adenosine can be inhibited by IDA without inhibition of adenosine hydrolase activity.

**4.3.2 Identification of Plasma Membrane Proteins.**

As described in Section 3.7.6 cell surface proteins were labelled with $^{125}I$ and FITC and subjected to analysis by SDS PAGE from which the apparent molecular weights of the labelled proteins were estimated. The molecular weights of the proteins labelled by these methods are summarised in Table 3.16. These results show that many proteins appear to be labelled by both $^{125}I$ and FITC. The reasons for the differences between the labelling patterns obtained with the two labels is unknown although the differences could be due to differing availability or accessibility of the required chemical groups for reaction with the labels, which would be tyrosine residues in the case of the $^{125}I$ label and primary amino groups for FITC. These results will be discussed further in conjunction with the results of experiments in which the adenosine transport protein was

affinity labelled.

**4.4 AFFINITY LABELLING OF THE ADENOSINE TRANSPORTER.**

The adenosine transport protein was covalently labelled with [2,8-$^{3}$H] 2',3'-dialdehydeadenosine in whole cells and in the plasma membrane enriched fraction as described in Sections 3.8.3 & 3.8.4. Before attempting to affinity label the transporter with dialdehyde adenosine it was shown that the dialdehyde adenosine was capable of irreversible inhibition of adenosine transport (Results Section 3.8.2).

Table 3.16 summarises the results of all surface labelling and affinity labelling experiments carried out. This includes labelling of the cell surface with $^{125}$I and FITC (Sections 3.7.4 & 3.7.6); also affinity labelling of the adenosine transporter with dialdehyde adenosine in whole cells and purified plasma membrane (Sections 3.8.3 & 3.8.4). It should be borne in mind that the molecular wieghts for the labelled proteins quoted in Table 3.16 are apparent molecular weights since some of these proteins will probably be glycosylated and will therefore not bind SDS in the normal ratio of 1.4g/g. This results in a slower migration through the gel which gives an over estimate of the proteins' molecular weight. Estimating the molecular weights of proteins from their mobilities on a gel using a standard curve constructed from the molecular weights and relative mobilities of known proteins involves measurement errors which result in a cumulative error of $\pm$5% in all molecular weight estimates. Bearing this in mind the results presented in Table 3.16 show that the adenosine transporter is likely to be a 57 or 52 KDa polypeptide since proteins of this molecular weight are labelled by dialdehyde

adenosine in whole cells and in purified plasma membranes. Additional evidence that they are plasma membrane proteins is provided by the fact that they are also labelled by $^{125}I$ and FITC in whole cells. The many other bands labelled by dialdehyde adenosine in whole cells are probably intracellular proteins although some are also labelled by the cell surface labels. The fact that they do not appear to be labelled in the plasma membrane may indicate that they are only loosely associated with the plasma membrane and are lost during the purification of the plasma membranes. If this is the case it is very unlikely that they are involved in transport since a transporter would be an integral membrane protein and not easily removed from the membrane without the use of a detergent. The proteins of molecular weights quoted as 63, 66, 64 and 69 KDa labelled by all the labelling methods and in all fractions is probably the trypanosomes surface glycoprotein coat protein since it is labelled by the cell surface labels and runs on the SDS gels as a broad diffuse band, which is a characteristic of glycoproteins. Alternatively these may be separate proteins since the glycoprotein coat band on SDS gels masks other bands beneath.

Having identified the polypeptides most likely to be constituents of the adenosine transporter further analysis of the mechanism of action of the transport system could be undertaken by purifing these proteins and attempting to reconstitute the transport system in liposomes. This would allow investigation into the mechanism of energy coupling to active transport without the problems associated with using metabolic inhibitors on the whole cell system, such as maintaining membrane integrity in metabolicaly poisoned cells. However these investigations must be left until some later

date.

The estimated molecular weights (52 and 57KDa) of the proteins likely to be the transport proteins compare with estimates of 45-65 KDa for the nucleoside transporter of the erythrocyte estimated by affinity labelling with $^{14}$C-NBTI followed by analysis by SDS PAGE (Young et al.,1983; Young & Jarvis,1983). Estimates have also been made of the molecular weight of the erythrocyte nucleoside transport protein in its native state in the membrane by radiation inactivation analysis (Jarvis et al.,1980) which indicated that the transporter may exist as a dimer with a molecular weight of 120KDa. No comparable information is available for the nucleoside transporters of other cell types.

**4.5 REGULATION OF PURINE NUCLEOSIDE AND BASE TRANSPORT.**

The time courses of adenosine and hypoxanthine uptake show that uptake ceases after two minutes. This is not due to metabolism of the purines being switched off since metabolism of the labelled purines taken up continues for up to 10 minutes (Results Sections 3.2.4 & 3.3.4). The results presented in Sections 3.6.1 to 3.6.8 indicate that adenosine and hypoxanthine are probably taken up by an active transport process which is independent of the metabolism of the transported substrates. This suggests that regulation of transport must occur at the level of the transport process itself.

Transport could be regulated by simple feed-back inhibition of transport when the transported substrates reach a certain intracellular concentration. This is unlikely since nucleoside and base concentrations within the cell fall very rapidly after uptake stops but transport does not restart. An alternative feed-back to the

transport system could be mediated by the levels of intracellular nucleotide. The effects of nucleotide concentrations on the activities of the purine metabolising enzymes should not affect transport since this is an active process independent of the metabolism of the transported substrates. Transport may also be regulated by phosphorylation of the transport protein as has been shown for glucose transport in the erythrocyte (Witters et al.,1985). These authors have shown that a protein kinase C stimulated by phorbol esters phosphorylates the glucose transporter both in vivo and in vitro. This correlates with the observation that phorbol esters stimulate glucose uptake in the erythrocyte. It does not seem unreasonable to assume that in other systems phosphorylation of a transport protein may be inhibitory.

Where possible these mechanisms of regulation were investigated. The regulation of transport by high intracellular nucleotide levels was not investigated since there is no way to control the intracellular concentrations of nucleotides in a live cell. Although as stated above it is unlikely that regulation of purine metabolism would have any effect on transport the effects of nucleosides and nucleotides on the activities of these enzymes was investigated.

The results of investigations into the regulation of the purine metabolising enzymes adenosine hydrolase, APRT and HxPRT are shown in Section 3.9.1. Adenosine hydrolase activity does not appear to be affected by any of the mononucleotides added or mononucleotides with ATP added but does show some slight inhibition in the presence of 1mM hypoxanthine. This inhibition has no immediate significance with respect to regulation of transport. The incubations in which ATP was included with the mononucleotide were carried out in case regulation

of any of the enzymes was mediated by a protein kinase stimulated by mononucleotide since nucleoside dependent protein kinases have been reported to be present in T.gambiense (Walter,1976 & 1978)

The APRT activity appears to be sensitive to product inhibition by AMP but this observed inhibition could be due to dilution of the labelled adenine substrate by adenine derived from the added AMP via phosphatase activity and adenosine hydrolase. The inhibition of APRT activity by adenosine is almost certainly due to this effect.

The activity of HxPRT does not appear to be sensitive to product inhibition at a concentration of 1mM IMP but does appear to be inhibited by 1mM ATP. The results presented above have little significance with regards to transport regulation but may be significant with respect to control of metabolism of purine nucleosides and bases.

The possible regulation of transport by phosphorylation of the transport protein was investigated as detailed in Section 3.9.2. The results of these experiments are shown in Figures 3.59a, 3.59b and 3.60. Figure 3.59a shows the gel of the crude membrane preparation stained for protein. The incubation which contained IMP shows a different banding pattern to the other incubations since the bands marked with arrows in the control track are absent. This effect was found to be independent of the presence of sodium ortho-vanadate in the incubation and does not correlate with any changes in phosphorylation. The significance of this result is unknown and requires further investigation. AMP was found to be a general inhibitor of the protein kinases present as was adenosine, but the AMP stimulated phosphorylation of one band at an apparent molecular weight of 51KDa. This ,considering the errors involved in the

estimations of molecular weight, is the same molecular weight estimated for one of the proteins affinity labelled by dialdehyde adenosine in the plasma membrane enriched fraction at 52KDa. This specific phosphorylation is inhibited by the presence of sodium ortho-vanadate in the incubation which may indicate that the protein kinase involved is inhibited by vanadate ions. Adenosine appears to be a good inhibitor of all protein kinase activity which is in disagreement with the results of Walter (1965 & 1978) who found protein kinases in trypanosomes to be stimulated by nucleosides and inhibited by nucleotides. Figure 3.60 shows the autoradiograph of phosphorylated proteins in a whole cell lysate. The results are much the same as for the crude membrane fraction with the exception that sodium ortho-vanadate has a more marked effect on the overall phosphorylation. This probably reflects the presence of vanadate sensitive ATPases in the lysate which were not associated with the membranes. The AMP again reduces the amount of phosphorylation observed but this time the 51 KDa band is phosphorylated in all incubations except those containing vanadate ion. This may indicate that the protein kinase responsible for this phosphorylation is a soluble cytoplasmic protein which was mostly removed from the crude membrane preparation. The 51 KDa band is also much fainter in the whole cell lysate providing more evidence that it is membrane associated since semi-purifying the cell membranes intensifies the band. It is interesting to note that Walter & Opperdoes (1982) have determined the subcellular distribution of protein kinases in T.brucei and found a soluble cytoplasmic nucleoside stimulated protein kinase and a suramin-sensitive protein kinase associated with the plasma membrane.

## 4.6 GENERAL SUMMARY

In summary the work presented in this thesis will be considered in three parts, the transport of the compounds studied, their subsequent metabolism and finally the identification of the transport system.

The results presented show that T.brucei (TREU 55) is capable of absorbing vitamin $B_6$ from the environment. The results obtained indicate that this process is mediated by simple diffusion followed by metabolic trapping the details of which are given below. That vitamin $B_6$ is taken up by simple diffusion is indicated by the non-saturability of uptake (Sections 3.1.3 & 3.1.7) and the low activation energy estimated for uptake (Section 3.1.9).

In contrast to this simple mechanism for the uptake of vitamin $B_6$ the trypanosome has active transport systems for the uptake of adenosine, adenine and hypoxanthine. The transport of adenosine, adenine and hypoxanthine were shown to be active processes by the ability of the trypanosomes to accumulate unmetabolised adenosine and hypoxanthine against their concentration gradients (Section 3.6.1) and the sensitivity of uptake to ionophores capable of destroying the membrane potential (Sections 3.6.2 to 3.6.4). Also evidence was obtained implicating the movement of protons in the transport process (Section 3.6.6). The existance of at least two and possibly three separate transporters is indicated by the sensitivities of the uptake of adenosine, adenine and hypoxanthine to inhibition by nucleoside analogues (see Section 3.5.5 for summary) which have no significant effect on the intracellular metabolism of the purines (Section 3.5.4).

The metabolism of the transported compounds studied is summarised below. The metabolism of vitamin $B_6$ by the trypanosomes results in the production of the enzymic co-factor pyridoxal-5-phosphate.The trypanosome does not appear to phosphorylate pyridoxal to pyridoxal phosphate at a significant rate but rapidly converts pyridoxine to pyridoxal. The production of pyridoxal phosphate from pyridoxine appears to take place by the same route as in mammalian systems.

The metabolism of adenosine, adenine and hypoxanthine to nucleotide by the trypanosomes was found to be carried out by phosphoribosyltransferases in the case of adenine and hyoxanthine with adenosine first being hydrolysed to adenine before being phosphorylated (Sections 3.4.1 & 3.4.7). The distribution of labelled purines through the nucleotide pool (Sections 3.2.6 & 3.3.5) indicated that little interconversion of nucleotides takes place but the incorporation of labelled adenosine into ATP was very rapid as indicated by the observation that most of the label taken up appeared as ATP at the earliest time points obtainable by the experimental technique used. The metabolism of hypoxanthine to ITP was found to be much slower since most of the label taken up appeared as IMP with only small amounts of ITP being formed by the end of the experiment.

The identification of the adenosine transport protein was attempted by affinity labelling with dialdehyde adenosine in whole cells and semipurified plasma membranes (Sections 3.8.3 & 3.8.4). The results obtained from these experiments and labelling cell surface proteins (Sections 3.7.6) allowed the adenosine transport protein to be tentatively identified as either a 57 or 51 KDa protein or it may be that both these proteins are part of the transport system (Section

3.8.5).

The observed control of adenosine uptake by the trypanosomes may be mediated by phosphorylation of the 51 KDa protein as indicated by the results presented in Sections 3.9 to 3.9.3.

## REFERENCES

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