STUDIES ON ACID PHOSPHATASES OF *TRYPANOSOMA CONGOLENSE*

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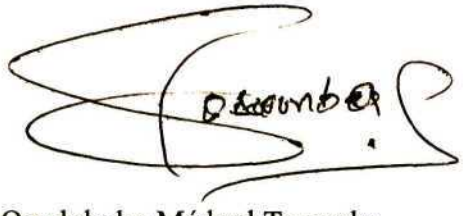
PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg and the International Livestock Research Institute, Nairobi, Kenya, from November 1991 to November 1996 under the supervision of Dr. Theresa H.T. Coetzer and co-supervision of Prof. John D. Lonsdale-Eccles.

These studies represent original work by the author and have not been submitted in any other form for any degree or diploma to another university. Where use was made of the work of others it has been duly acknowledged in the text.



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ABSTRACT

Bloodstream forms of African trypanosomes, which endocytose macromolecules exclusively through their flagellar pockets, contain an acid phosphatase (AcP) activity in this organelle. In the present thesis, AcP activity was demonstrated cytochemically in some intracellular vesicles and on the surface of *Trypanosoma congolense* as well as in the flagellar pocket. Unlike other trypanosomatids such as *Leishmania* spp. and *Trichomonas* spp., these trypanosomes, while viable, did not release this enzyme into the surrounding medium.

In contrast to mammalian cells, the AcP in *T. congolense* was shown by cell fractionation to be a non-lysosomal enzyme. The enzyme was mostly recovered in the microsomal and cytosolic fractions which had 52.7% and 44.4% of the total activity, respectively. Further separation of the microsomal fraction showed an association of AcP activity with vesicles derived from the plasma membrane, Golgi apparatus and endoplasmic reticulum.

After ammonium sulfate precipitation and chromatography on a succession of columns containing Sephacryl S-300, DEAE-cellulose and Sephadex G-75, two acid phosphatases (AcP_1 and AcP_2) were produced from the cytosolic fraction. A membrane-bound acid phosphatase (AcP_3) was isolated from the microsomal pellets extracted with Triton X-114 and subjected to the above chromatographic procedures. The molecular mass of AcP_1 was higher than 700 kDa. It had an isoelectric point of 4.7. AcP_2 (pI 5.3) and AcP_3 (pI 6.5) had molecular masses of 33 and 320 kDa, respectively. AcP_1 and AcP_3 were strongly inhibited by vanadate while AcP_2 was strongly inhibited by p-chloromercuribenzoate. None of the enzymes was inhibited by tartrate but all were inhibited by NaF. The K_m values for each of the various substrates differed widely between the three AcPs indicating that the binding site of each enzyme was distinct. The best of all the substrates tested was para-nitrophenyl phosphate.

On non-denaturing gels the enzymes exhibited very high molecular masses but on denaturing SDS-PAGE, two similar bands of activity, localised at 62 and 65 kDa, were observed in all three AcP preparations. Thus the three isolated enzymes may be derived from the same base 62 and 65 kDa units. Differences between enzymes may be derived from differential processing of the isoenzymes for different functions at different locations.

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ABBREVIATIONS

A	absorbance
A_{280}	absorbance at 280 nm
A_{405}	absorbance at 405 nm
AcP	acid phosphatase
ATP	adenosine triphosphate
BC	basic copy
BSA	bovine serum albumin
CKII	casein kinase II
Cy	cytosolic fraction
CycR	cytochrome c reductase
CysP	cysteine protease
Da	daltons
kDa	kilodaltons
DEAE	diethyl aminoethyl
DFMO	difluoro-methyl-ornithine
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E	extinction coefficient
E-64	L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane
EDTA	ethylene diamine tetra-acetic acid
g	relative centrifugal force
G6P	glucose-6-phosphate
β-GLOP	beta-glycerophosphate
GPI	glycosylphosphatidylinositol
h	hour
HDL	high density lipoprotein
dH_2O	distilled water
IEF	isoelectric focusing
ILRAD	now called ILRI (International Livestock Research Institute)
L.	*Leishmania*
LDL	low density lipoprotein

LG	large granule fraction
MEE	microsomal enzyme extract
min	minute
MI	microsomal fraction
M_r	relative molecular mass
NAD	nicotinamide-adenine dinucleotide (unspecified state of oxidation)
NAD^+	nicotinamide-adenine dinucleotide (oxidised form)
NADH	reduced NAD
NHMec	7-amino-4-methyl coumarin
α-NPP	alpha-naphthyl phosphate
p-NPP	para-nitrophenyl phosphate
NU	crude nuclear fraction
p65	protein of M_r 65 000 Da
pp52	phosphoprotein of M_r 52 000 Da
PAGE	polyacrylamide gel electrophoresis
PARP	procyclic acid repetitive protein
pI	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
PNS	post nuclear supernatant
PSG	phosphate-buffered saline glucose
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SG	small granule fraction
T.	*Trypanosoma*
TCA	trichloracetic acid
TEMED	N, N, N' ,N'-tetramethyl ethylene diamine
V_o	void volume
VSG	variable specific surface glycoprotein

CHAPTER ONE
LITERATURE REVIEW

1.1 - African trypanosomiasis

1.1.1 - Introduction

African trypanosomiasis is endemic to a large portion of sub-Saharan Africa. It is estimated that some 50 million people and comparable numbers of cattle and small ruminants, within an area of 10 million square kilometres and representing 37 countries, are exposed to the risk of infection with African trypanosomes (ILRAD, 1991; Kuzoe, 1993).

Within this area, human trypanosomiasis continues to remain a health problem despite efforts to control the disease. Additionally, the corresponding livestock disease leads to significant economical losses. Not only is there a direct loss in meat and milk, but tractive power and waste products that provide both natural fertiliser and fuel are diminished as well. Roughly 7 million square kilometres of grazing land are unused because of animal trypanosomiasis, or, "nagana", and approximately 6-7 million cattle die each year of the disease (Hajduk et al., 1994). In order to compensate for the lack of animal protein, affected countries have to import meat and milk products at high cost, thus contributing to keep them in an untenable situation of financial debt (Wissocq, 1986).

Many approaches have been attempted to eliminate, or at least control trypanosomiasis, such as the administration of drugs to treat or prevent the disease. The use of methods to reduce populations of tsetse flies that transmit the parasites, and thus spread the disease, as well as the keeping of trypanotolerant livestock breeds have also been tried with varying degrees of success. The control of trypanosomiasis is a multifaceted operation that must also take into account the control on wildlife. Although these animals are important for the tourism industry, they constitute a natural source of infection for tsetse flies, which thereafter infect domestic animals and humans.

Despite efforts over many years, trypanosomiasis is an increasingly important health problem in many African countries. In my home country, the Republic of Zaire, the optimism of the late 1950s concerning eventual eradication of trypanosomiasis was premature.

A resurgence of trypanosomiasis has occurred in humans as well as in animals. With its land area of 2 345 thousand square kilometres, the Republic of Zaire is known to contain several species of tsetse fly that transmit human and animal trypanosomiasis. The major tsetse fly groups are *Glossina fusca* (55.5%), *G. palpalis* (30%) and *G. morsitans* (16.5%) (Makumyaviri, 1986). Human trypanosomiasis which is caused by *Trypanosoma brucei gambiense*, has been on the increase for the last five years with more than 5 000 new cases reported each year (D.S. Molisho, pers. commun.). Due to inadequate reporting over the whole country, the true increase is likely to be much higher. The Republic of Zaire also contains about 40% of the African equatorial forest in which wildlife animals constitute a natural reserve for tsetse flies. These tsetse flies thereafter continue to infect humans as well as domestic animals, thereby perpetuating the disease. Consequently, the incidence of trypanosomiasis remains high and many patients need treatment for trypanosomiasis each year.

Animal trypanosomiasis which is caused chiefly by *T. vivax*, *T. congolense* and *T.b. brucei,* is also a serious obstacle to human welfare because of the severe nutritional and economic problems it causes. Except for Kivu province, cattle breeding in Zaire was virtually unknown until the beginning of this century. Initially, the cattle population consisted of non-resistant imported breeds (Payne, 1964) and later trypanotolerant cattle were introduced into the country with the N'Dama group representing one of the most important trypanotolerant breeds. At present, the livestock population is concentrated in the eastern and south-eastern highlands which represents 70% of the country's cattle, 75% of the sheep, and 57% of the goats (ILCA, 1979).

1.1.2 - Classification of African trypanosomes

The organisms that cause African trypanosomiasis are unicellular eukaryotic cells belonging to the family *Trypanosomatidae* (Fig. 1). They share with other *Sarcomastigophora* the characteristic mode of locomotion by flagella. The flagella of trypanosomes are attached to the body of the cell by means of an undulating membrane but may extend beyond the body of the parasite where the flagellum is said to be free. African trypanosomes are also classified in the superclass *Mastigophora* which contains specifically those protozoans which move by flagella only. Since they do not possess chlorophyll, African trypanosomes are placed in the class *Zoomastigophora*. They fall into the order *Kinetoplastida* because they possess a

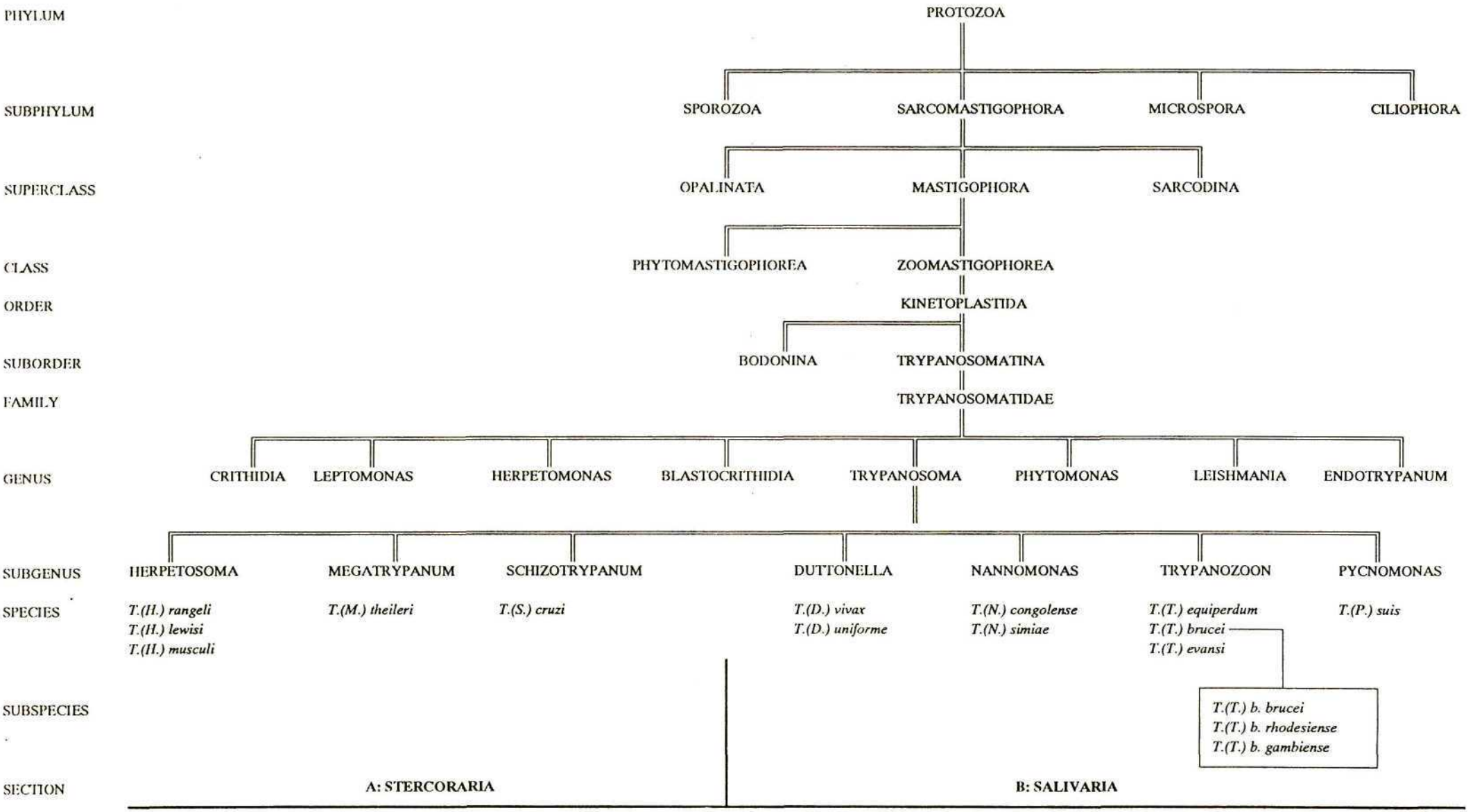

PHYLUM
PROTOZOA
SUBPHYLUM
SPOROZOA
SARCOMASTIGOPHORA
MICROSPORA
CILIOPHORA
SUPERCLASS
OPALINATA
MASTIGOPHORA
SARCODINA
CLASS
PHYTOMASTIGOPHOREA
ZOOMASTIGOPHOREA
ORDER
KINETOPLASTIDA
SUBORDER
BODONINA
TRYPANOSOMATINA
FAMILY
TRYPANOSOMATIDAE
GENUS
CRITHIDIA
LEPTOMONAS
HERPETOMONAS
BLASTOCRITHIDIA
TRYPANOSOMA
PHYTOMONAS
LEISHMANIA
ENDOTRYPANUM
SUBGENUS
HERPETOSOMA
MEGATRYPANUM
SCHIZOTRYPANUM
DUTTONELLA
NANNOMONAS
TRYPANOZOON
PYCNOMONAS
SPECIES
T.(H.) rangeli
T.(H.) lewisi
T.(H.) musculi
T.(M.) theileri
T.(S.) cruzi
T.(D.) vivax
T.(D.) uniforme
T.(N.) congolense
T.(N.) simiae
T.(T.) equiperdum
T.(T.) brucei
T.(T.) evansi
T.(P.) suis
SUBSPECIES
T.(T.) b. brucei
T.(T.) b. rhodesiense
T.(T.) b. gambiense
SECTION
A: STERCORARIA
B: SALIVARIA

Figure 1 - Classification of the genus *Trypanosoma* (Hoare, 1972; Baker et al., 1978).

kinetoplast, a dense organelle at the base of the flagella which contains the DNA of the organism's mitochondrion. The suborder *Trypanosomatida* is composed of those *Kinetoplastida* with only one flagellum and all members appear to be parasitic. African trypanosomes belong to the genus *Trypanosoma* which is divided into two groups or sections (stercoraria and salivaria). These two sections differ in the site where the parasites develop in their vectors and in the mode of transmission of the infections (Hoare, 1972).

The stercoraria section consists of those species in which parasite development is completed in the terminal gut (posterior position) of the vector and where transmission occurs via the faeces of the vector through skin wounds in the host. *T. cruzi*, the American trypanosome which causes Chagas'disease, is the best known species in this section. The stercorarian trypanosomes have a free flagellum extending beyond the posterior end of the parasite. The posterior end of the body is pointed and the anterior rounded. Except for *T. cruzi*, most of the stercorarian trypanosomes, although not parasitic to man, are not pathogenic (Hoare and Wallace, 1966; Bowman, 1974).

The salivaria section comprises species in which parasite development is completed in the anterior part of the vector's digestive tract and where transmission is via vector saliva during feeding. Among this group are the causative agents of African human sleeping sickness (*T.b. gambiense* and *T.b. rhodesiense*). The corresponding agents in animal trypanosomiasis, *T.b. brucei*, *T. congolense* and *T. vivax*, also belong to this section. The salivarian trypanosomes may or may not have a free flagellum. The kinetoplast may be located in a terminal or sub-terminal portion of the parasite, and the posterior end of the body is usually blunt. Salivarian trypanosomes are usually pathogenic.

1.1.3 - The life cycle of African trypanosomes

African trypanosomes are parasites with life cycles alternating between a vertebrate host and the digestive tracts of arthropod or leech vectors. The life cycle of *T. congolense* is illustrated in Fig. 2. The infection in the mammalian host is initiated by the injection into dermal connective tissue of metacyclic trypanosomes contained in the saliva of tsetse flies.

A localised inflammatory reaction called "the chancre", develops in the skin at the site of the bite where trypanosomes multiply by binary fission. After several days, the parasites invade the bloodstream and lymphatic fluid. Thereafter, fluctuating waves of parasitaemia

characterise the infections. These parasitaemia waves are associated with different parasite populations which have altered antigenic properties (discussed later).

In the case of *T. brucei*, two distinct morphological forms of trypanosomes are seen in the bloodstream. Slender (dividing) forms predominate early in the infection while stumpy (non-dividing) forms are seen at declining parasitaemia (Vickerman, 1965; Hecker et al., 1973). The later stumpy forms are able to continue their life cycle in the vector. Morphological distinctions in the case of *T. congolense* are less apparent than those shown by *T. brucei* in early or late stages of infection.

Trypanosomes in the blood are ingested by tsetse flies during feeding. In the midgut of the fly they transform into procyclic forms and multiply. They then migrate via the proventriculus to the salivary glands where they differentiate into the epimastigote forms. Epimastigote forms are characterised by the position of their nucleus being posterior to the flagellar pocket. Finally, the epimastigote forms transform into (non-dividing) metacyclic forms. These mammal infectious forms are injected into the bloodstream when the fly feeds again, thus allowing the life cycle to restart.

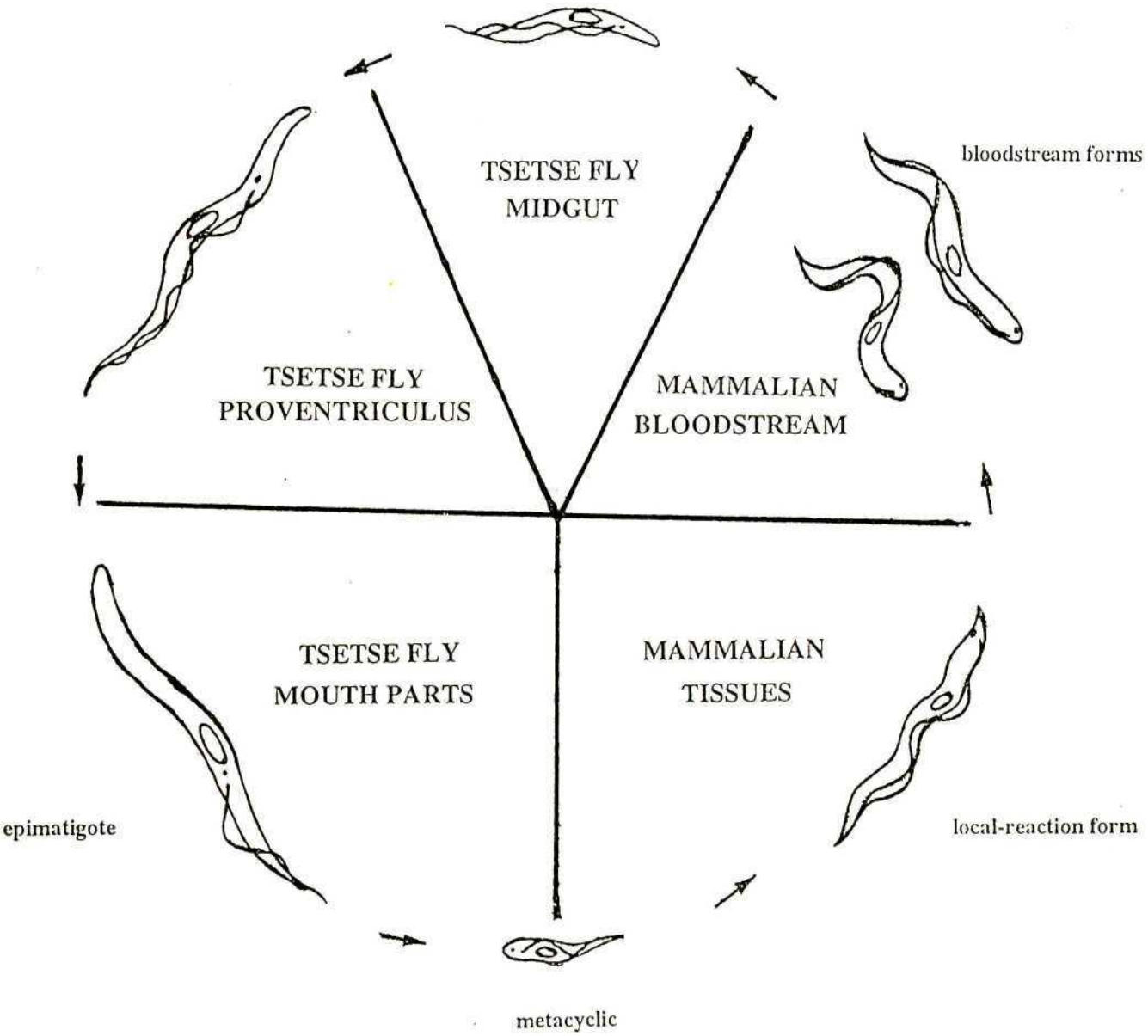

TSETSE FLY
MIDGUT
bloodstream forms
TSETSE FLY
PROVENTRICULUS
MAMMALIAN
BLOODSTREAM
TSETSE FLY
MOUTH PARTS
MAMMALIAN
TISSUES
epimatigote
local-reaction form
metacyclic

Figure 2 - Schematic diagram showing the life cycle of *Trypanosoma congolense*, one of the pathogenic African trypanosomes in animals (adapted from ILRAD annual Report, 1988).

1.1.4 - Pathology of trypanosomiasis

In Africa, two forms of human trypanosomiasis occur: West African or Gambian sleeping sickness caused by *T.b. gambiense* and East African or Rhodesian sleeping sickness caused by *T.b. rhodesiense*. Both trypanosomes cause the same type of clinical disease, but the time scale of its development differs. Rhodesian sleeping sickness, which is much more acute than Gambian sleeping sickness, has an incubation period of 2-3 weeks and a course of several weeks, involving the brain after 3-4 weeks. Gambian sleeping sickness has an incubation period of several weeks to months and may not involve the brain for months or even years. Trypanosome infections are generally characterised by anaemia, leucopenia, thrombocytopenia, as well as biochemical aberrations such as hypoglycaemia, elevated blood urea nitrogen, hypoalbuminaemia, and hypogammaglobulinaenia (Anosa, 1988).

When the intradermal incubation period is completed and the organisms have disseminated into the bloodstream, intermittent fevers develop. Rashes, particularly erythema multiform, occur in some patients and lymphadenopathy, especially of the posterior cervical nodes, are

common. Invasion of the central nervous system is heralded by headaches, neck stiffness, sleep disturbances, and depression. Progressive mental deterioration follows and eventually, coma develops before the patient dies.

Closely related to human trypanosomiasis is "nagana" in livestock. Nagana is characterised by anaemia and cachexia leading to weakness and an inability to forage for food which causes most deaths. In infections with *T.b. brucei*, the central nervous system may be invaded. The two main livestock pathogens, *T. congolense* and *T. vivax* rarely invade the central nervous system.

1.1.5 - *Trypanosoma congolense* parasite

Trypanosoma congolense consists of a single cell varying in length from 9 to 22 μm. This parasite is pathogenic for domestic livestock, especially cattle, and causes extensive losses in animal production throughout the African continent. Infected animals show severe signs of organ damage, anaemia, weight loss, abortion and infertility (Molyneux and Ashford, 1983). Once infection has occurred, the rapidly dividing trypanosomes circulate within the bloodstream of mammalian hosts. They are protected from the host's immune response by

their variant surface glycoprotein coat (Boothroyd, 1985; Vickerman, 1985). It has been reported that *T. congolense* shed surface proteins in order to evade complement lysis by the host (Frevert and Reinwald, 1990), but the mechanism by which the shedding occurs, has not been elucidated yet. It is also possible that *T. congolense* may be able to clear immune complexes from its surface by endocytosis as has been suggested by Russo et al. (1994).

T. congolense, in contrast to other trypanosomatids such as *T.b. brucei* or *T. evansi,* does not invade tissues. Nor, unlike the South American *T. cruzi,* does it invade cells. Although *T. congolense* is generally regarded as a true extracellular blood parasite, it should be stated that *T. congolense* does bind by their flagella to erythrocytes and to endothelial cells of microvasculature (Banks, 1980).

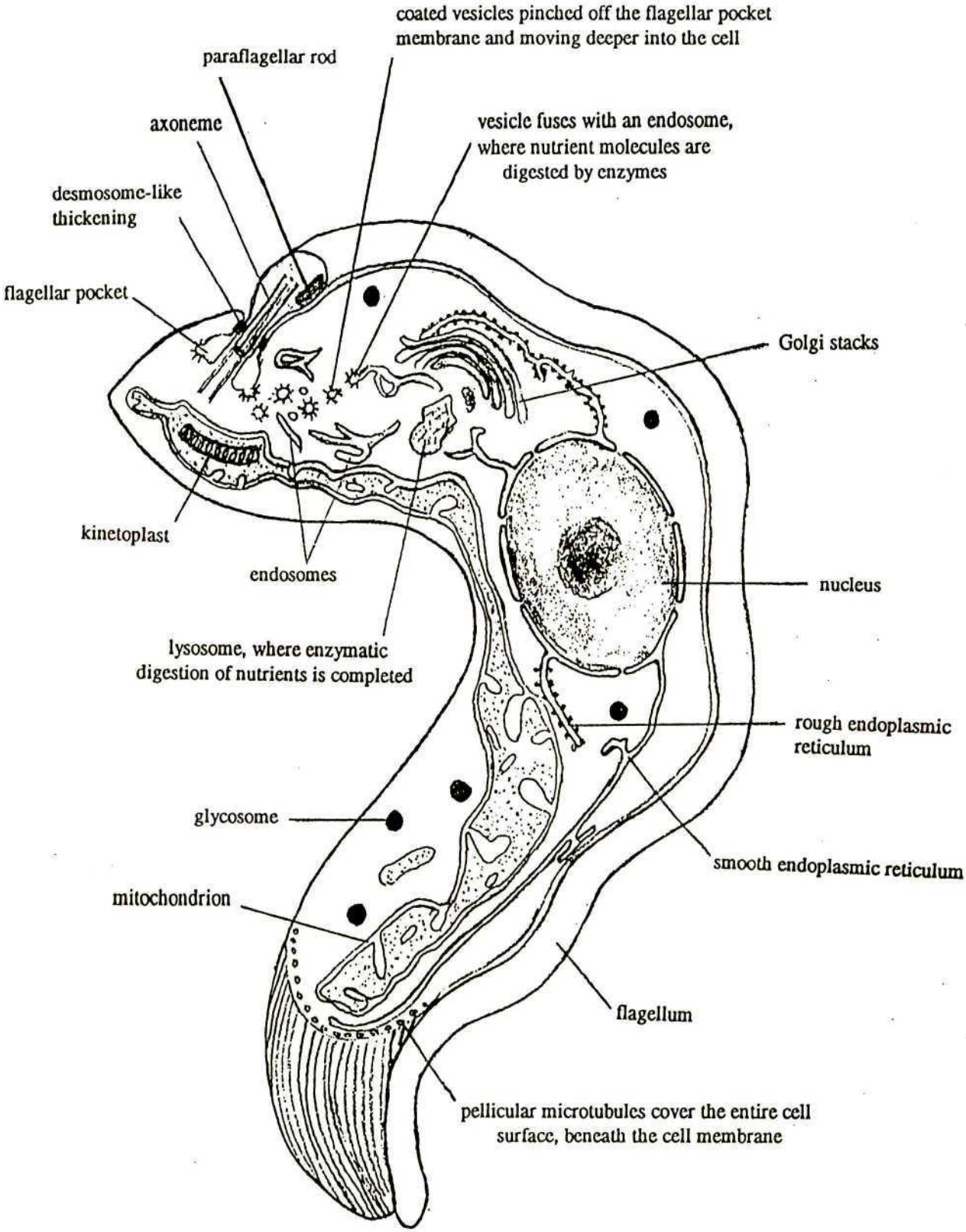
1.1.6 - Trypanosome cellular organisation

T. congolense was first identified by Broden (1904) and is one of the most important agents of African animal trypanosomiasis. A schematic diagram of *T. congolense* is given in Fig. 3. Like other single cells (unicellulars), *T. congolense* has a variety of organelles some of which are discussed in this section.

1.1.6.1 - Cell surface

Bloodstream and metacyclic forms of the African trypanosomes are covered by a protein coat composed of a monomolecular layer of specific glycoprotein called "variant surface glycoprotein" (VSG). The replacement of one layer of this glycoprotein by another in trypanosomes is called antigenic variation (see later) and it enables trypanosomes to avoid the immune responses of their mammalian host.

Biochemical analysis indicated that the VSG coat is homogenous and is composed of approximately 10^7 identical glycoprotein molecules (Cross, 1990). A VSG molecule consists of a single polypeptide chain of approximately 450-480 amino acid residues and two or more carbohydrate units.



ANTERIOR END
(defined relative to the direction of motion)

Figure 3 - Schematic diagram of *Trypanosoma congolense* showing the major organelles (adapted from Vickerman, 1969; Clayton et al., 1995).

The N-terminal end of the protein is exposed on the surface of the parasite and covers the carbohydrate molecules. The C-terminus of the protein is attached to the trypanosome plasma membrane via a glycosyl phosphatidyl inositol (GPI) structure (Englund, 1993) which contains ethanolamine, phosphate, inositol, galactose, mannose, glycerol, and the fatty acid myristate (Ferguson et al., 1988).

VSG molecules have been shown to vary from 50 to 65 kilodaltons in size. The approximate molecular mass of a VSG from *T. congolense* is approximately 50 kDa (Fish et al., 1989) while that from *T.b. brucei* is 65 kDa (Vickerman and Luckins, 1969). In *T. vivax*, VSG molecules are 8-14 kDa smaller than in *T.b. brucei* (Gardiner, 1989). The VSG on the surface of *T.b. brucei* and *T. congolense* is compact and dense whereas in *T. vivax* it appears to be diffuse.

When the trypanosome enters the midgut of the tsetse fly, or are cultured *in vitro* at 26°C, the VSG coat is rapidly replaced by a different abundant surface glycoprotein called procyclin or procyclic acid repetitive protein (PARP) (Mowatt and Clayton, 1987; Roditi et al., 1987). The function of PARP has not yet been established. It may simply be protective, shielding the parasite while in the insect gut. Additionally, it may serve as a means to direct attachment to epithelial membranes (Vickerman et al., 1988).

Recently, an equally abundant and immunodominant surface glycoprotein termed GARP (glutamic acid/alanine - rich protein) has been characterised in *T. congolense* (Bayne et al., 1993; Beecroft et al., 1993). Although the *T.b. brucei* procyclins and GARP share no sequence similarity or antigenic properties, they are structurally similar and are believed to be functional analogs of each other.

T. congolense differs from other trypanosomatids in that its flagellum does not protrude extensively from the anterior end of the cell body (Vickerman, 1969; Vickerman and Tetley, 1990). Nevertheless, this organelle appears to play a key role in the cell adhesion events which occur at different life-cycle stages within the insect and in the animal hosts (Banks, 1980). In infected animals, *T. congolense* are found to adhere by their flagella to erythrocytes and to endothelial cells of microvasculature (Banks, 1980). Although the direct effects of this adhesion are not yet entirely understood, Hemphill and co-workers (1994) provided data suggesting that *T. congolense* possess lectin-like domains localised on the flagella surface

which interact with sialic acid residues of endothelial plasma membranes and erythrocyte surfaces. Thus, a non-VSG component of the *T. congolense* plasma membrane constitutes the receptor interacting with sialic acid residues and this binding of trypanosomes has been shown to be restricted exclusively to the anterior two thirds of the flagellum.

1.1.6.2 - Microtubules, Flagellar Pocket and Flagellum

Microtubules are fibrillar structures common to most eukaryotic cells. They are polymers consisting of one major protein called tubulin and a variety of minor components known as microtubule-associated proteins (MAPs). MAPs are divided into two types, the architectural or structural and the energy-transducing or dynamic (MacRae, 1992; Zhang and MacRae, 1994). The first group of MAPs either modulates tubulin polymerisation or its members link microtubules to one another or to other organelles, with both of these activities involved in the extension of cell processs and the establishment of morphology. The second group includes the microtubule-based mechanochemical enzymes, such as kinesin, kinesin-like proteins and cytoplasmic dynein. Their role is to direct movement of subcellular components, including chromosomes and membrane-bound vesicles, tasks accomplished by the hydrolysis of either ATP or other nucleotides. In summary, microtubules participate in a wide variety of cellular events such as the maintenance of cell shape, particle and organelle movements, the separation of chromosomes on the mitotic spindle, and the movement of eukaryotic cilia and flagella.

Beneath the plasma membrane in trypanosomes is a series of pellicular microtubules that are believed to have a cytoskeletal function. The microtubules are arranged longitudinally in a single row around the trypanosome's body and, until recently, the flagellar pocket was considered to be the only portion of the cell membrane not associated with microtubules. However, Hemphill and co-workers (1991) found that the microtubular array also remains opened ended at the posterior end of *T.b. brucei.* The microtubular array of trypanosomes is very stable and stays intact even after the plasma membrane is removed by detergent treatment (Sherwin and Gull, 1989). It maintains the asymmetric shape of trypanosomes (broad end, posterior; narrow end, anterior). In *T.b. brucei*, individual microtubules also appear to have intrinsic structural polarity due to the asymmetric arrangement of tubilin dimers. Their morphogenesis has been reported to be central to cell cycle events such as organelle positioning, segregation, mitosis, and cytokinesis (Robinson et al., 1995).

The outer face of the pellicular microtubules is covered with an amorphous coating of protein. Some of the microtubule-associated proteins appear to function as cross-linkers between neighbouring microtubules. The microtubule-associated proteins orientated toward the cell membrane may be involved in the interaction between microtubules and the cell membrane which suggests that both organelles may form a single functional entity.

The flagellar pocket is a small invagination of the plasma membrane through the microtubular array out of which the flagellum emerges. The membrane lining the flagellar pocket has no associated microtubules and is considered to be the only site for endocytosis and exocytosis in trypanosomes. It comprises 0.2% of the total cellular membrane area (Webster, 1989). Several different enzymes have been associated with the flagellar pocket including cysteine proteases and acid phosphatases (Langreth and Balber, 1975; Venkatesan et al., 1977; Steiger et al., 1980).

The flagellum emerges from the flagellar pocket toward the surface of the cell body by penetrating through an opening in the microtubular array. As the flagellum penetrates through this opening, it remains connected to the microtubular array by numerous fine filaments of unknown functional significance. The trypanosome flagellum contains two major structural components: the axoneme and the paraflagellar rod (Schlaeppi et al., 1989).

The axoneme is formed by the characteristic 9 + 2 associated microtubules structure. It consists of nine outer doublet microtubules held in a ring by interdoublet linkages termed nexin links. This ring surrounds a central core of two singlet microtubules. In contrast to the rough appearance of pellicular microtubules, the surface of the microtubules of the axoneme seems devoid of associated proteins. The axoneme also contains regularly spaced dyneins. Dyneins are the inner and outer arm structures present on the outer doublet microtubules of ciliary and flagellar axonemes. These arms contain ATPases responsible for generating the sliding movements between microtubules that underlay ciliary and flagellar motility (Porter and Johnson, 1989).

Besides the microtubular axoneme, the trypanosomal flagellum contains the paraflagellar rod, a conspicuous hexagonal fiber network. From its lower periphery, numerous fibers apparently form connections with the pellicular microtubules. The paraflagellar rod is connected to the

axoneme by a regular array of linkers. In an effort to develop an effective vaccine against the South American trypanosomiasis, the paraflagellar rod proteins present in the flagellum of *Trypanosoma cruzi* were reported to induce an immune response capable of reducing the level of circulating parasites in the bloodstream and protecting mice against an otherwise lethal inoculum of *T. cruzi* trypomastigotes (Wrightsman et al., 1995). This indicate the increasing interest of nonvariant, trypanosome proteins in the development of vaccine against sleeping sickness.

1.1.6.3 - Mitochondrion

In trypanosomes, the mitochondrion is unusual among eukaryotes in that it exists in the form of a single elongated tubular structure that extends along the length of the cell. It is continuous with the kinetoplast, a discrete physical structure which constitutes the mitochondrial genome. It undergoes functional and morphological changes during the life cycle of trypanosomes.

Bloodstream forms of the parasites have completely suppressed many of their mitochondrial enzyme activities and functions; they are deficient in cytochromes and Krebs cycle enzymes. Consequently, their ATP-generating mechanism depends exclusively on glycolysis (Vickerman et al., 1988) and thus the aerobic conversion of glucose to pyruvate. In contrast, when the parasites are in the insect vector, there is a switch to a more efficient utilisation of available respiratory substrates and a reactivation of Krebs cycle enzymes (Bienen et al., 1981). This enables pyruvate to be oxidised, with the production of more ATP per glucose molecule. The switch is accompanied by structural changes in subcellular organelles such as the increase in the number of mitochondrial cristae and an expansion of the entire mitochondrion. The reverse change occurs in the lumen of the salivary glands where the mitochondrion and cristae of the metacyclic trypomastigotes regress.

1.1.6.4 - Glycosomes

Morphologically, the glycosomes are round or ellipsoid in shape and homogenous in size with an average diameter of 0.3 μm. They contain an electron-dense matrix which is surrounded by a single membrane and they resemble other microbodies such as the peroxisomes and plant glyoxysomes (Borst, 1986). While peroxisomes are rich in enzymes that form and utilize hydrogen peroxide, glycosomes of *T.b. brucei*, *T. cruzi* and *Leishmania* spp. have been shown

to contain several enzymes of glycolysis and glycerol metabolism as well as enzymes involved in purine salvage, pyrimidine biosynthesis, carbon-dioxide fixation, ether-lipid biosynthesis and β-oxidation of fatty acids (Opperdoes, 1987). Each trypanosome has between 200 - 300 glycosomes.

One major difference between mammalian and trypanosome energy generating systems is in the location of the glycolytic enzymes. Whereas in mammalian systems the glycolytic enzymes are located in the cytosol, in trypanosomes they are located in the glycosome. In *T.b. brucei*, most of the glycosomal proteins display isoelectric points several pH units higher than those of their cytosolic or mammalian counterparts (Misset et al., 1986), which has led to the suggestion that the glycolytic enzymes in African trypanosomes may be potential targets for development of new trypanocidal drugs (Michels, 1988; Sommer and Wang, 1994).

1.1.6.5 - Lysosomes

Lysosomes are irregularly shaped acid organelles with diameters of 0.2-0.8 μm and are bound by a single membrane which encloses a dense granular matrix. Amongst lysosomal enzymes, proteolytic enzymes (or proteases) have been studied extensively during the last decade in many parasites including *Entamoeba histolytica* (Reed et al., 1989), *Leishmania* spp. (Chang et al., 1990), *Plasmodium* spp. (Rosenthal and Nelson, 1992) and *T. cruzi* (Ashall, 1990). In the latter parasite, several functions for cysteine proteases have also been proposed, including host cell invasion, intracellular metabolism and adhesion to target cells (Calderon et al., 1989; Souto-Padrón et al., 1990).

In bloodstream forms of African trypanosomes, lysosomal cysteine proteases have been reported (Lonsdale-Eccles and Grab, 1987; Lonsdale-Eccles, 1991; North, 1991) and have been proposed to play a role in the changing of the surface coat (Russo et al., 1993). Host immune responses against these proteases have also been correlated with the enhanced resistance against the trypanosomes (Authié et al., 1992). Therefore, cysteine proteases appear to be promising targets for the development of potential vaccines or new drugs in number of parasites (Rosenthal et al., 1991; Mbawa et al., 1991).

Lysosomal enzymes are also implicated in the nutrition of trypanosomes which depend on external food supply. Electron-microscope studies on *T. cruzi* (Soares and de Souza, 1991),

T. congolense (Webster, 1989) and *T.b. brucei* (Langreth and Balber, 1975; Webster, 1989) have demonstrated that trypanosomes are able to endocytose extracellular materials. Once inside the cell, these materials are found in small vesicles, large tubular networks similar to the endosomes of mammalian cells (Webster, 1989) and in large lysosome-like vesicles in which the digestion is believed to occur. In bloodstream and metacyclic trypanosomes, the uptake of low density lipoprotein (LDL) and transferrin (carriers of cholesterol and iron respectively) have been shown to occur through the process of receptor-mediated endocytosis, with the formation of coated vesicles in the flagellar pocket region (Coppens et al., 1987; 1988; Webster and Grab, 1988; Webster and Russel, 1993). In contrast, the uptake of albumin and peroxidase occurs through fluid-phase pinocytosis (Fairlamb and Bowman, 1980).

Procyclic trypanosomes lack coated pits at the flagellar pocket membrane and coated endocytic vesicles. They are also deficient in lysosomal cysteine proteases (Mbawa et al., 1991). Therefore, procyclic trypanosomes must take up and digest macromolecules by a mechanism different from that used by the bloodstream and metacyclic trypanosomes (Langreth and Balber, 1975; Vickerman, 1985).

Despite the extensive knowledge of some of the lysosomal hydrolytic enzymes, much less is known about the components of characteristic lysosomal membrane glycoproteins. No specific function has been assigned to these glycosylated proteins, although they may function in protecting lysosomal membranes from hydrolytic enzymes present in the lumen of these organelles (Fukuda, 1991).

1.1.7 - Molecular aspects of Trypanosomes.

1.1.7.1 - Nuclear genome organisation

T.b. brucei's nuclear genome has been studied extensively and is organised into approximately 20 large chromosomes and 100 minichromosomes. The large chromosomes, which range in size from approximately 200 kb to 5.7 Mb, contain most of the coding information of the genome. The minichromosomes, which range in size from 50 to 100 kb, have been extensively studied (Weiden et al., 1991). They have been found to be mainly composed of a tandem array of 177-bp G-C rich direct repeats (> 90% of the sequence) which are separated by bent helical A-T rich regions, and a VSG gene. Minichromosomes are found only in trypanosome

species that undergo antigenic variation, raising the possibility that minichromosomes might have a role in antigenic variation. While most of the minichromosomes have a VSG gene, these are not transcribed. The function of the minichromosomal VSG genes may be to serve as a source of VSG genes for translocation to expression sites at the telomeres of the large chromosomes. The termini of the large chromosomes as well as the minichromosomes have the same telomeric repeat sequence (GGGTTA)n.

1.1.7.2 - Antigenic Variation

As mentioned above, both bloodstream and metacyclic forms of the African trypanosomes are covered by a VSG coat. African trypanosomes have evolved a way of avoiding the immune responses of their mammalian host through a process of antigenic variation which involves the replacement of one layer of glycoproteins by another biochemically and antigenically distinct coat. The process of antigenic variation occurs spontaneously without induction by the host's immune system or other factors in the bloodstream of the mammalian host (Pays and Steinert, 1988; Van der Ploeg et al., 1992).

During infection of a mammalian host, trypanosomes alter the composition of the VSG coat by the selective expression of one of several hundred VSG genes. Trypanosomes possess a large repertoire of silent VSG genes termed the basic copies which are located at internal sites on the approximately 20 large chromosomes. These genes are not transcribed. In order for these basic copy genes to be transcribed, they must first be duplicated and transposed to a telomeric expression site on the chromosome. Despite the presence of many VSG genes within telomeric expression sites, only one VSG gene is transcribed at a time, leading to the appearance of a new VSG coat on the surface of the parasite. Antigenic switching from one VSG coat to another can occur by either one of two general mechanisms. In the first, a previously silent basic copy VSG gene is translocated into the transcriptionally active VSG gene expression site, leading to the expression of a new VSG coat. The second mechanism relies on the differential transcriptional control of the expression sites. A switch to the expression of a new VSG gene can occur by the *in situ* inactivation of one expression site, with activation of the transcription of a second previously inactive expression site (Majiwa et al., 1982; Pays, 1991; Myler, 1993; Vanhamme and Pays, 1995).

The elucidation of the molecular mechanisms responsible for the controlled duplication and transposition of the VSG genes and the factors triggering the spontaneous switch remain a challenge. Once these processes are understood, it may be possible to design inhibitors which would specifically block antigenic switching and leave the trypanosome vulnerable to the host immune response.

1.1.7.3 - Kinetoplast

The kinetoplast of trypanosomes is situated at the base of the flagellum close to the basal body from which the flagellum arises. It is continuous with the mitochondrial membrane and contains an unusual complex, concatenated network of circular DNA molecules. This DNA network consists of two types of circular DNA molecules, minicircles and maxicircles.

The kinetoplast DNA (kDNA) is composed of 5 000-10 000 minicircles. They represent about 95% of the kDNA and their sizes vary from 0.5 to 2.8 kilobases amongst species of kinetoplastids. Although their function is unknown, the minicircles have been shown to code for a small RNA (Rohrer et al., 1987). The minicircles form a planar, interlocked network (like a monolayer of chain-nail) which condenses to form a disc with a replisome. This complex contains topoisomerases on either side of the disc. The replisomes are only present during replicating stages of the parasites (i.e. they are present during the S-phase but not during the G_1-phase). The kDNA is also composed of 20-50 maxicircles which are structurally and functionally similar to the mitochondrial DNA of most other eukaryotes. The maxicircles comprise 5% of kDNA and their sizes vary from 20 to 37 kilobases amongst different species of kinetoplastids. Maxicircles encode mitochondrial enzymes and were shown to contain genes essential for mitochondrial biogenesis (Englund et al., 1982; Simpson, 1986).

Studies of kDNA organisation and expression in kinetoplastids led to the discovery of a form of RNA processing called RNA editing in which the nucleotide sequence of an mRNA molecule was found to be modified extensively by the addition or deletion of uridine residues from that encoded by the gene (Benne, 1990; Stuart, 1991). The presence of both edited and unedited sequences on the same molecules of RNA suggests that editing is a post-transcriptional process and proceeds in the 3' to 5' direction. This ability to modify their

transcripted RNA may provide trypanosomes with an adaptive flexibility necessary to undergo the dramatic shifts in respiration metabolism during their life cycle.

1.1.8 - Chemotherapy

In the absence of a suitable vaccine against African trypanosomiasis, the treatment of the disease by drugs becomes extremely important. There are only a handful drugs available today for treating African trypanosomiasis. These drugs are plagued by various problems, ranging from oral inabsorption, acute toxicities, short durations of action, and low efficacious to the emergence of trypanosomal resistance (Wang, 1995).

The following commercially available trypanocides are known to be effective for the treatment of African trypanosomiasis in humans: suramin (Bayer AG.), pentamidine, melarsoprol, and difluoromethylornithine (DFMO) (Merrell Dow). For the treatment of the disease in animals, homodium bromide (FBC Limited), diminazene aceturate (Hoechst AG., E.R. Squibb and Sons Ltd), isometamidium chloride, homodium chloride and quinapyramine sulphate (May & Baker Ltd, Rhône-Mérieux), are currently used.

For early bloodstream stages of the disease, suramin is an effective trypanocide. The mode of action of suramin, a sulfated naphthylamine developed more than 70 years ago, is poorly understood, as it inhibits numerous enzymes, including L-α-glycerophosphate oxidase (Fairlamb and Bowman, 1977; Gutteridge, 1985), RNA polymerase (Hawking, 1978) and many others that probably have no relation to its trypanocidal effect e.g. hyaluronidase, urease, hexokinase, fumarase and trypsin-like proteolytic activities. In the bloodstream suramin is bound to serum albumin (Fairlamb and Bowman, 1980) and LDL (Vansterkenburg et al., 1993). These macromolecules are believed to enter the parasite through the flagellar pocket when they are endocytosed.

DFMO is another probant trypanocide that inhibits ornithine decarboxylase which catalyses the conversion of ornithine to putrescine, the first and rate-limiting step in the biosynthesis of the polyamines spermidine and spermine (Schechter and Sjoerdsma, 1986). Although DFMO is effective against the Gambian form of sleeping sickness, it was found ineffective against the Rhodesian form of sleeping sickness (Bacchi et al., 1993).

Once the central nervous system is invaded by trypanosomes, the only effective drug currently available is melarsoprol since it has the ability to penetrate the blood-brain barrier. Melarsoprol is a highly toxic arsenical drug. The cellular target for melarsoprol has recently been shown to be trypanothione [N^1,N^8-bis(glutathionyl) spermidine] (Fairlamb et al., 1989). Trypanothione, which is not found in mammalian cells, is synthesised form glutathione and spermidine. Melarsoprol interferes with trypanothione by inactivating its sulfhydryl groups. Because of its sulfhydryl groups, trypanothione is involved in the defence of trypanosomatids against damage by oxidants such as H_2O_2 and certain heavy metals and possibly xenobiotics (Fairlamb and Cerami, 1992). Melarsoprol has adverse side-effects causing reactive encephalopathy in 5-10% of patients treated, with a fatal outcome in 1 to 5% (Kuzoe, 1993).

1.2 - Acid Phosphatases

1.2.1 - General characteristics

1.2.1.1 - Classification

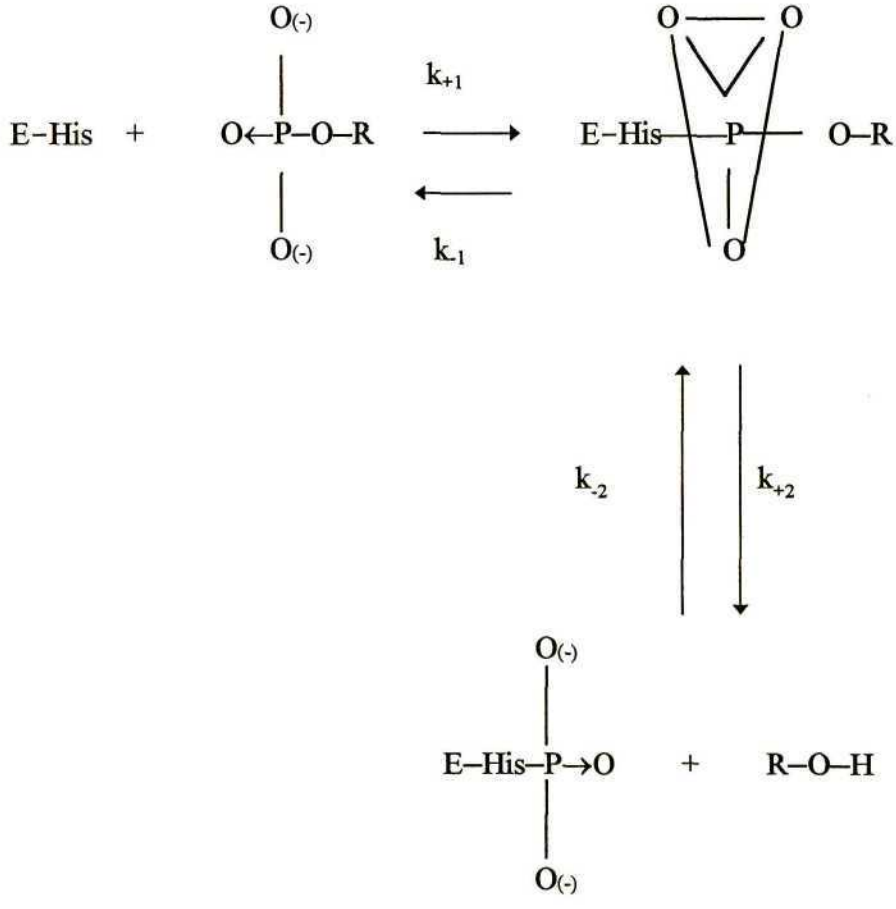
Phosphatases or phosphomonesterases (ortho-phosphoric-monoester phosphohydrolases, EC.3.1.3) is a group of enzymes which are responsible of the hydrolysis of phosphate monoesters and oxygen exchange from water to inorganic phosphate (see scheme in Section 1.2.1.2). These enzymes achieve the biological hydrolysis of single phosphate groups from a variety of substrates in a thermodynamically favourable process ($\Delta G^{\circ\prime} \leq -9$ kJ $mole^{-1}$) (Vincent et al, 1992). Traditionally, they have been classified as being acid or alkaline phosphatases according to whether their optimal pH for catalysis is below or above pH 7.0. At least five classes of phosphatases have been extensively characterised: (i) alkaline phosphatases; (ii) purple acid phosphatases; (iii) low molecular mass acid phosphatases; (iv) high molecular mass acid phosphatases; and (v) protein phosphatases. In general, phosphatases can be distinguished from other enzymes which act on phosphate groups, such as phosphodiesterases, pyrophosphatases and ATPases, in that one of the products of the reaction is free inorganic phosphate and the other is a dephosphorylated organic molecule.

1.2.1.2 - Mechanism of action.

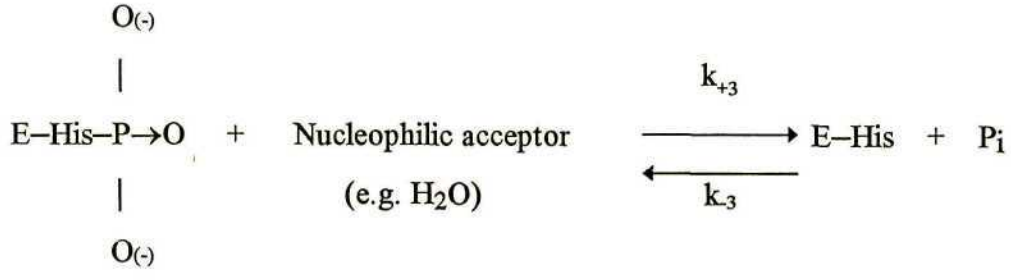
In many (and perhaps all) cases, kinetic studies on acid phosphatases have indicated that the reaction proceeds by a two-step transfer mechanism, where each of the transfer reactions gives

an inversion of configuration, resulting in net retention of configuration at phosphorus for the overall reaction (Saini et al., 1981). The chemical pathway of phosphoric-ester hydrolysis by acid phosphatases in mammalian metabolism can be summarised in the following scheme:

Step I:



Step II:



In the first step of the reaction, the enzyme reacts with the phosphate ester to form the Michaelis complex. A nucleophilic attack by an enzyme histidine group (McTigue and Van Etten, 1978) on the phosphate group leads to the formation of a covalent phosphohistidyl enzyme intermediate (McTigue and Van Etten, 1978; Zhang and Van Etten, 1991) and the alcohol is released, probably assisted by the deprotonation of the leaving carboxyl group on the enzyme.

In the second step, the phosphate group is transferred to water or some other nucleophilic acceptor and the phosphohistidyl enzyme intermediate is hydrolysed forming the non-covalent enzyme/phosphate complex. The transition state for the breakdown of the phosphoryl intermediate has been suggested to be a trigonal bipyramidal species with the nucleophilic enzyme group and the attacking water molecule at the axial positions (Van Etten et al., 1974). Kinetic data and trapping of phosphoenzyme intermediate suggests that the rate-limiting step in the overall reaction is the dephosphorylation of the phosphoenzyme (Step II) when the phosphate group is transferred to water or some other nucleophilic acceptor.

Isotope exchange experiments where various phosphate esters are cleaved in the presence of H_2O^{18} were carried out. It was shown that acid phosphatases catalyse the rupture of an oxygen-phosphorus bond and that oxygen is exchanged between H_2O^{18} and phosphate (Van Etten and Risley, 1978; Saini et al., 1981). The theoretical amount of oxygen isotope is recovered in the liberated orthophosphate, and none is found in the alcohol moiety of the substrate.

1.2.2 - Occurrence and cellular location

Acid phosphatases are ubiquitous and occur in mammalian body fluids and tissues. It has been reported in different mammalian tissues including bone, kidney, placenta and spleen (Hollander, 1971; Bodansky, 1972; Yam, 1974). The two main sources of acid phosphatases in humans are the prostate gland and red cells. Acid phosphatases are also distributed in plants (Duff et al., 1994), as well as in a variety of micro-organisms (Table 1).

Table 1 - Occurrence of acid phosphatases in different micro-organisms.

Source	References
Crithidia sp.	McLaughlin et al., 1976
Entamoeba. histolytica	Müller et al., 1988
Herpetomonas muscarum	Coombs et al., 1987
Leishmania spp.	Avila et al., 1989; Menz et al., 1991; Singla et al., 1992
Leptomonas collosoma	Hunt and Ellar, 1974
Myxococcus coralloides D.	González et al., 1994
Saccharomyeces cerevisiae	Schönholzer et al., 1985
Tetrahymena pyriformis	Banno et al., 1987
Trichomonas vaginalis	Lockwood et al., 1988
Trichomonas foetus	Lockwood et al., 1988
T. b. brucei	Grab et al., 1987; Schell et al., 1990
T.b. rhodesiense	McLaughlin, 1986
T.b. gambiense	Seed et al., 1967
T. cruzi	Lettelier et al., 1986

Acid phosphatases have been found in the cytosol, lysosomes and microsomal fractions of mammalian cells with acid phosphatases frequently being used as specific markers for the lysosomes. In plants, acid phosphatases have traditionally been considered to be vacuole enzymes (Nishimura and Bevers, 1978; Duff et al., 1994). In addition, however, surface membrane acid phosphatases have been localised within the cell wall (Kaneko et al., 1990; Duff et al., 1991) or released by the root / suspension cells into the surrounding media (LeBansky et al., 1992; Miernyk, 1992).

In contrast to mammalian cells, the purification of potential lysosomal acid phosphatases has not been achieved in trypanosomatids. Cytochemical studies have demonstrated acid phosphatase activities in the flagellar pockets and cytoplasmic vesicles of these parasites (Seed

et al., 1967; Langreth and Balber, 1975; McLaughlin *et al.* 1976; Venkatesan *et al.*, 1977). In addition, the presence of surface membrane and/or secreted acid phosphatases has been detected in a number of micro-organisms but their subcellular origin and biological functions have not been elucidated. Although acid phosphatases have been shown to be secreted by some trypanosomatids, neither surface membrane nor secreted acid phosphatases have been purified from African trypanosomes. However, subcellular fractionation studies suggested that in addition to the flagellar pocket enzyme there may be an acid phosphatase associated with the surface membrane of bloodstream forms of *T.b. rhodesiense* (McLaughlin, 1986).

1.2.3 - Properties

1.2.3.1 - Physical properties

Acid phosphatases exhibit considerable heterogeneity with regards to their native molecular mass, subunit structrure, and pI as shown in Table 2. In mammalian tissues, acid phosphatases occur in multiple forms and can be categorised into three groups according to their molecular masses, i.e. > 200 kDa, approximately 100 kDa and 35 kDa or less. The very large molecular mass forms are membrane bound while the 100 kDa forms are known to be either secretory or lysosomal enzymes. The smaller enzymes are soluble cytoplasmic proteins. The plant acid phosphatase counterparts also display a similar heterogeneity with regards to their molecular masses. They may also exhibit subunit molecular masses of approximately 50 to 60 kDa (Duff et al., 1994). The better-characterised acid phosphatases in mammals, plants and micro-organisms, have been demonstrated to be monomeric or dimeric glycoproteins and may have some structural similarities.

An amino acid sequence Arg-His-Gly-X-Arg-X-Pro (where X is any amino acid) has been shown to be a conserved motif in most acid phosphatases (Roiko et al., 1990; Van Etten et al., 1991). In the site-directed mutagenesis studies of acid phosphatase from *Escherichia coli*, this conserved sequence motif was shown to be part of the active site of the enzyme (Ostamin and Van Etten, 1993).

The majority of acid phosphatases shows acidic optimal pH values ranging from 4.5 to 6.5, although considerable variation has been observed between various preparations. This may reflect cofactor requirements (if any) as well as enzyme purity (Table 2).

Table 2 - Physical properties and protein complex forms of various acid phosphatases.

Acid phosphatase source	Native M_r (kDa)	Subunit M_r (kDa)	pI	Optimal pH	Glycoprotein	References
Bovine spleen	35			5.3-5.9	yes	Dietrich et al., 1991
Bovine liver	118		7.4-7.5	6.0-6.2	no	Panara, 1986
Bovine heart	18		7.0	5.3-6.0		Zhang and Van Etten, 1990
Human spleen	174.4	67	3.8-4.1	4.5	yes	Robinson and Glew, 1980
Human spleen	34			5.3	yes	Ketcham et al., 1985
Human prostate	100			4.0-6.0	yes	Lin and Clinton, 1986
Human prostate	100-105	50	5.05-5.35			Lee et al., 1991
Human red cells	17-18			5.5-6.0	no	Dissing and Svensmark, 1990
Human placenta	17		7.2	5.0-6.0		Waheed et al., 1988
Potato tuber	100	55, 57		5.8		Gellatly et al., 1994
Black mustard	60	59	4.5	5.6	yes	Duff et al., 1991
Soya bean	55	53	6.7			Ullah and Gibson, 1988
Myxococcus c.D.	320-330	150		4.5		González et al., 1994
L. donovani [a]	>700	110-130		4.5-5.0	yes	Bates and Dwyer, 1987
L. donovani [b]	128	65-68	4.1	5.5		Remaley et al., 1985
	108		5.4	5.0-6.0		
	133		7.1	5.0-6.0		
T.b. brucei	70		6.5, 8	5.0	yes	Schell et al., 1990
T.b. rhodesiense				4.5, 6.0		McLaughlin, 1986

[a] Extracellular enzyme.
[b] Intracellular enzymes.

1.2.3.2 - Enzymatic properties

Para-nitrophenyl phosphate (p-NPP), a non-physiological phosphomonoester, is the most commonly used substrate for the *in vitro* estimation of acid phosphatase activity because it is relatively sensitive and easy to use in routine assays. Kinetic parameters for several acid phosphatases are shown in Table 3. Although the use of artificial substrates has made it possible to rapidly analyse the kinetic properties of acid phosphatases, their ease of use limits our understanding of the naturally occurring substrates. Therefore, for enzymes with such broad specificity as acid phosphatases, it is perhaps appropriate to study the kinetics of P_i release from various key metabolites of intermediary metabolism such as adenosine triphosphate (ATP), inorganic pyrophosphate (PP_i), 3-phospho-glycerate (3-PGA), and phospho-enol-pyruvate (PEP). Moreover, the K_m values of acid phosphatases for these metabolites are frequently in the μM range, suggesting that these compounds could serve as substrates for the acid phosphatase *in vivo*. However, the identification and characterisation of physiological relevant substrates for acid phosphatases remains a major challenge in this field.

Most acid phosphatases are considered to be non-specific phosphomonoesterases since they hydrolyse phosphate monoesters, with the general formula R-O-P, at similar rates regardless of the size or chemical nature of the R group. This suggests that these enzymes have no specific binding pocket for the alcoholic or phenolic portion of the substrate. Nevertheless, they exhibit their highest activity against artificial substrates that are 0-phosphoric esters of aromatic compounds such as p-nitrophenyl phosphate or α-naphthyl phosphate rather than against phosphoric esters of aliphatic alcohols such as PEP and β-glycerophosphate. Acid phophatases were also found to be able to hydrolyse only O-substituted monoesters (R-O-P; e.g. O-4-nitrophenyl thiophosphate) while S-substituted monoesters (R-S-P; e.g. cysteamine-S-phosphate), cannot be hydrolysed (Hollander, 1971). This suggests that an oxygen linkage is required for the hydrolysis of the substrate and can not be substituted by sulfur.

Acid phosphatases undergo inactivation on exposure to various compounds such as fluoride, orthovanadate and molybdate. Fluoride and tartrate differentially inhibit certain acid phosphatases and may be used to discriminate between acid phosphatases.

Some acid phosphatases which have been isolated from mammalian and plant sources are metalloproteins. They are readily distinguished from other acid phosphatases by their purple colour in solution and are called purple acid phosphatases (Table 3). This purple colour is due to the presence of a binuclear iron centre (in the mammalian enzymes) or an iron-zinc centre (in the plant enzymes). However, the mammalian enzymes exist in two forms: an oxidised, purple form containing an Fe(III)-Fe(III) centre, which exhibits little if any catalytic activity, and a pink, reduced form containing a mixed-valent Fe(III)-Fe(II) centre, which is the enzymatically active species (Dietrich et al., 1991). Purple acid phosphatases are also distinguished by their resistance to inhibition by tartrate (Vincent and Averill, 1991).

Table 3 - Enzymatic properties of various acid phosphatases.

Acid phosphatase	p-NPP		Best non-synthetic substrate			Cofactor	Inhibitor	References
source	K_m (mM)	V_{max} (U/mg)	substrate	K_m (mM)	V_{max} (U/mg)			
Bovine spleen	0.2-0.6					Fe	V, Mo, W	Dietrich et al., 1991
Bovine liver	0.38					Zn	Hg, Ag, pCMB	Panara, 1986
Bovine heart	0.38	0.114	P-Tyr	14	0.086		V, Mo	Zhang and Van Etten, 1990
Human spleen	0.044[(a)]						F, Mo, Tartrate	Robinson and Glew, 1980
Human spleen	0.75-3					Fe	Mo	Ketcham et al., 1985
Human prostate		0.00027					V, F	Lin and Clinton, 1986
Human prostate	0.00059	0.00005					F, Tartrate	Lee et al., 1991
Human red cells	0.043						pABP	Dissing and Svensmark, 1990
Human placenta			P-Tyr	1.6			V, Mo, Zn	Waheed et al., 1988
Potato tuber	1.10	1250	PTyr	0.99	1917	Mg	V, Mo, Zn	Gellatly et al., 1994
Black mustard	0.29	1225					Mo, F, P_i	Duff et al., 1991
Soya bean	0.07	153						Ullah and Gibson, 1988
Myxococcus c.D.	1.5						F	González et al., 1994
L. donovani (a)								Bates and Dwyer, 1987
L. donovani (b)	0.35						F	Remaley et al., 1985
(c)	1.7						Mo	
(d)	0.44						Mo	
T.b. brucei								Schell et al., 1990
T.b. rhodesiense	2.1						Tartrate	McLaughlin, 1986
	2.3							

Abbreviations: p-NPP, p-nitrophenyl phosphate; P-Tyr, O-phospho-L-tyrosine; p-ABP, p-aminobenzylphosphonic acid; p-CMB, p-chloromercuribenzoate; P_i, inorganic phosphate.

1.2.3.3 - Acid phosphatases in African trypanosomes

In African trypanosomes, evidence for acid phosphatase activity has been obtained by cytochemistry and by enzymatic analysis of subcellular fractions (Langreth and Balber, 1975; Steiger et al., 1980). Although the acid phosphatase located in the flagellar pocket of various trypanosomes has not previously been purified, it is thought that this enzyme may be used as a marker for the flagellar pocket. However, other data obtained from subcellular fractionation studies indicated the predominant localisation of acid phosphatases in the Golgi apparatus rather than in lysosomes as found in mammalian cells (Grab et al., 1987).

Two membrane-bound acid phosphatase activities were separated by isoelectric focusing from bloodstream forms of *T.b. brucei* (Schell et al., 1990). The tartrate-sensitive enzyme had a pI of about 6.5 while the tartrate-resistant enzyme had a pI of about 8.0. The tartrate-sensitive enzyme was purified to homogeneity by affinity chromatography and its estimated molecular mass was 70 kDa. This glycoprotein had a K_m of 0.4 mM for p-nitrophenyl phosphate and showed a broad pH optimum around pH 5. It was inhibited by 50% in the presence of 15 μM L-(+)-tartrate while the tartrate-resistant activity was not inhibited at a concentration as high as 100 mM.

Studies on the substrate specificity of an acid phosphatase from *T.b. brucei* have shown considerable differences in affinity of this enzyme for various substrates. The enzyme has a K_m of 0.055 mM for methylumbelliferyl phosphate (MUP), 0.23 mM for p-nitrophenyl phosphate (pNPP) and 7.84 mM for β-glycerophosphate (Opperdoes et al., 1987). Although this acid phosphatase had high affinity for MUP and pNPP substrates, the V_{max} in a crude homogenate was found to be highest when assayed against β-glycerophosphate (100 nmol . min^{-1} .mg $protein^{-1}$) and only 50% of that with the other two substrates.

1.2.4 - Functions

Despite their widespread distribution and abundance in nature, the physiological functions of acid phosphatases are not known with certainty. Their roles can be broadly characterised as digestive and nutritive. As with other phosphatases, the two main roles of acid phosphatases seem to be either in removing the phosphate group from the substrate so that the product can then be used in other ways, for example as biosynthetic precursor, or in providing a source of

free phosphate itself (Bates, 1991). In the latter case, the acid phosphatases may play a vital role in the regulation of physiological levels of inorganic phosphate and phosphorylated metabolites. The secreted acid phosphatases reported in some micro-organisms such as *L. donovani* (Lovelace and Gottlieb, 1986), *E. histolytica* (Müller et al., 1988), *Trichomonas vaginalis* and *T. foetus* Lockwood et al., 1988), may play a role in the hydrolysis of external phosphate esters found in the host and may facilitate the penetration of the parasite into a host.

1.3 - Objectives

Electron-microscope studies on *T. cruzi* (Jadin, 1971), *T. congolense* (Webster, 1989) and *T.b. brucei* (Langreth and Balber, 1975; Webster, 1989) have demonstrated that these protozoan parasites are capable of the uptake of molecular materials by endocytosis. The flagellar pocket, small vesicles, large tubular networks and large lysosome-like vesicles are all involved in the endocytic process.

Amongst several other hydrolases, acid phosphatases have been localised cytochemically in bloodstream forms of *T.b. brucei* (Langreth and Balber, 1975; Steiger et al., 1980), *T.b. gambiense* (Seed et al., 1967) and *T.b. rhodesiense* (Venkatesan et al., 1977). Subcellular fractionation studies have also revealed the presence of acid phosphatase activities predominantly in fractions associated to Golgi apparatus and endoplasmic reticulum. Whereas, a relatively small number of studies have been done on the acid phosphatases of the *T. brucei* group, none have been done on *T. congolense* which is also an important pathogen for livestock in Africa. To date no evidence has been reported indicating that the acid phosphatases found in the above mentioned organelles in *T. congolense* are the same or distinct, nor whether they are involved in endocytosis.

In keeping with our goal of understanding the possible role acid phosphatases may have in the endocytosis process of bloodstream forms of *T. congolense* and to devise a strategy to inhibit these enzymes in order to control the disease, this work will involve:

(1) - studies with live trypanosomes in order to determine the potential release of acid phosphatases into the surrounding medium;

(2) - localisation of acid phosphatases in trypanosomes by subcellular fractionation and by cytochemistry;

(3) - isolation of distinct acid phosphatases from trypanosome lysates;

(4) - characterisation of acid phosphatases, including kinetic parameters.

CHAPTER TWO
MATERIALS AND METHODS

2.1 - Introduction

The techniques used in this study were mainly in the area of cell culture, protein purification and electron microscopy. This chapter details the more fundamental biochemical techniques common to the different areas covered in this study. In cases where individual methods pertained to a specific area, they will be described in the relevant chapters.

2.2 - Reagents

General reagents were of analytical grade or of the highest available purity and the source of specialised products used in this study will be described here. Dephosphorylated casein, dithiothreitol (DTT), p-nitrophenyl phosphate (p-NPP), β-glycerophosphate (β-GLOP), Trypan Blue Stain and Fast Red TR salt, were purchased from Sigma Chemical Co. (St Louis, MO, USA). Diethylaminoethyl (DE-52)-cellulose was obtained from Whatman Ltd (Maidstone, Kent, UK). Antipain, L-trans-epoxysuccinyl-leucylamide-[4-guanidino]-butane {E-64}, chymostatin and leupeptin, were purchased from Cambridge Research Biochemicals (UK). Sephacryl S-300 and Percoll, were purchased from Pharmacia Fine Chemical LKB (Uppsala, Sweden). Medium 199 was purchased from Gibco (Grand Island, New York, USA). [γ^{32}]ATP (10 Ci per mmol) was obtained from Radiochemical Centre Limited (Amersham, UK). Aquasol was obtained from New England Nuclear, (Boston, MA, USA). Acrylamide, N,N'-methylene bis acrylamide, ampholytes (Servalyt) were purchased from Serva F.Gmbh Co. (Heidelberg, Germany). Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* was bought from Boehringer Mannheim Gmbh (Mannheim, Germany).

2.3 - General Buffers

General buffers used repetitively in the methodology of this thesis are detailed here.

PSG [phosphate saline glucose, pH 8.0]. Na_2HPO_4 (8.088 g), NaH_2PO_4 (0.359 g), NaCl (2.5 g) and glucose (10 g) were dissolved in approximately 800 ml of dH_2O, adjusted to pH 8.0 with NaOH and made up to 1000 ml. The recommended ionic strength for isolation of trypanosomes from blood of rats is 0.217 (Lanham and Godfrey, 1970).

Buffer A [10 mM Hepes, 145 mM NaCl, 5 g/l glucose, pH 7.2]. Hepes (2.383 g), NaCl (8.474 g) and glucose (5 g) were dissolved in approximately 800 ml dH_2O, the solution titrated to pH 7.2 with dilute HCl, and made up to 1000 ml with dH_2O in a volumetric flask.

Buffer B [100 mM sodium cacodylate, 200 mM sucrose, pH 7.2]. A 100 mM sodium cacodylate solution was prepared by mixing 10 ml of the 1 M sodium cacodylate stock solution (Section 4.3.2.1), 6.846 g sucrose and approximately 80 ml dH_2O. The pH was adjusted to pH 7.2 with dilute sodium hydroxide and made up to 100 ml with dH_2O in a volumetric flask.

Buffer C [20 mM Na acetate, 200 mM sucrose, pH 5.0]. Glacial acetic acid (0.114 ml) and sucrose (6.846 g) were dissolved in approximately 80 ml dH_2O, the solution titrated to pH 5.0 with dilute NaOH, and made up to 100 ml with dH_2O in a volumetric flask.

Buffer D [20 mM Tris-HCl, 1 mM DTT, 250 mM sucrose, pH 7.4, and 40 µg per ml of each of the following protease inhibitors: antipain, chymostatin, E-64 and leupeptin]. Tris (0.606 g), DTT (0.039 g), sucrose (21.394 g) were dissolved in approximately 200 ml dH_2O, titrated to pH 7.4 with diluted HCl and made up to 250 ml dH_2O. All protease inhibitors (4 mg each) were dissolved in 2.5 ml DMSO that constituted a stock solution kept at -20°C. Prior to use, 625 µl of this stock solution was mixed to 25 ml buffer.

Buffer E [40 mM Tris-acetate, 1 mM EDTA, pH 8.0]. Tris (0.484 g) and Na_2EDTA (0.037 g) were dissolved in approximately 80 ml dH_2O, the solution titrated to pH 8.0 with acetic acid and made up to 100 ml with dH_2O in a volumetric flask.

Buffer F [50 mM Hepes, 250 mM sucrose, 25 mM KCl, 1 mM EDTA, pH 7.4]. Hepes (1.192 g), sucrose (8.558 g), KCl (0.186 g) and Na_2EDTA (0.037 g) were dissolved in approximately 80 ml dH_2O, the solution titrated to pH 7.4 with dilute HCl, and made up to 100 ml with dH_2O in a volumetric flask.

Buffer G [20 mM Tris-HCl, 5 mM EDTA, pH 7.4]. Tris (0.242 g) and Na_2EDTA (0.186 g) were dissolved in approximately 80 ml dH_2O, titrated to pH 7.4 with dilute HCl and made up to 100 ml with dH_2O in a volumetric flask.

Buffer H [75 mM Tris-HCl, 140 mM NaCl, 11 mM KCl, pH 7.4, and 40 µg per ml of each of the following protease inhibitors: antipain, chymostatin, E-64 and leupeptin]. Tris (0.908 g), NaCl (0.818 g) and KCl (0.082 g) were dissolved in approximately 80 ml dH_2O, titrated to pH 7.4 with dilute HCl and made up to 100 ml dH_2O. All protease inhibitors (4 mg each) were dissolved in 2.5 ml DMSO that constituted a stock solution kept at -20°C. Prior to use, 625 µl of this stock solution was mixed to 25 ml buffer

Buffer I [20 mM Tris-HCl, containing 5% (v/v) glycerol, pH 8.0]. Tris (2.422 g) and glycerol (50 ml) were dissolved in approximately 800 ml dH_2O, titrated to pH 8.0 with dilute HCl and made up to 1000 ml with dH_2O.

Buffer J [buffer I containing 100 mM NaCl]. Tris (2.422 g), NaCl (5.844 g) and glycerol (50 ml) were dissolved in approximately 800 ml dH_2O, titrated to pH 8.0 with dilute HCl and made up to 1000 ml with dH_2O.

2.4 - Experimental Animals and Parasites

2.4.1 - Rats

Inbred adult male Sprague-Dawley rats were obtained from breeding colonies at either ILRAD (now called ILRI, Nairobi, Kenya) or the University of Natal (Department of Biochemistry, Pietermaritzburg, South Africa). All rats weighted between 200-300 g and were 3-4 months old. The rats were fed *ad libitum* on a diet of maize based mice pencils (Unga Limited, Nairobi, Kenya and Epol Foods, Pietermaritzburg, South Africa) and drinking water was continuously available.

2.4.2 - Trypanosomes

The clone IL3 000 of *T. congolense*, derived from the Trans Mara I strain of *T. congolense*, was used throughout the present studies. This strain was originally isolated in 1966 from an infected cow in the "Trans-Mara" area of the Kenya-Tanzania border (Wellde et al., 1974). This clone exhibits good growth characteristics both *in vitro* and *in vivo*. The clone was kept

as frozen stabilates in liquid nitrogen. When necessary, trypanosomes were propagated in lethally irradiated (600-900 rads total body irradiation) Sprague-Dawley rats at ILRI (Nairobi, Kenya) or non irradiated rats at the University of Natal (Department of Biochemistry, Pietermaritzburg, South Africa). Appropriate concentrations of trypanosome stabilates used for inoculation were prepared using PSG.

2.5 - Growth conditions and isolation of trypanosomes

2.5.1 - Preparation of Percoll solution

Percoll is a suitable medium for density gradient centrifugation of cells, viruses and Subcellular particles. It is composed of colloidal silica coated with polyvinylpyrrolidone, which renders the material non-toxic and appropriate for use with biological material. Percoll which provides a self forming gradient at high speed centrifugation, was used in the process of separating trypanosomes from blood cells.

Sucrose (17.1 g) and glucose (4.0 g) were dissolved in approximately 170 ml Percoll, adjusted to pH 7.4 with solid Hepes and made up to 200 ml with Percoll (Pertoft et al., 1978; Grab and Bwayo, 1982).

2.5.2 - Growth conditions and isolation of trypanosomes

Stabilates of the bloodstream form of *T. congolense* (clone IL3 000 stored in liquid nitrogen) were thawed and 1 ml of inoculum containing 1×10^5 parasites was prepared from the stabilates using PSG. Each rat was administrated intraperitoneally with 1 ml inoculum. The levels of parasitaemia in the infected rats were monitored by counting the number of parasites in a drop of blood obtained from the rat tail, in a haemocytometer. When the number of parasites per ml of blood was between 3×10^8 and 1×10^9, which usually occurred 5-7 days post-inoculation, the rats were anaesthetised with ether and the infected blood was collected by cardiac puncture with a 10 ml syringe containing 1 ml of 2% sodium citrate (m/v) as anticoagulant. The anticoagulant was made up in PSG. Trypanosomes were isolated from blood cells by isopycnic density centrifugation on Percoll gradients (Grab and Bwayo, 1982). The infected rat blood was mixed 1:1 (v/v) with Percoll solution and centrifuged (30 000 x g; 10°C; 30 min). The resultant layer of trypanosomes was carefully pipetted off and then diluted with an equal volume of PSG. The trypanosomes were centrifuged (3 000 x g; 10°C; 20 min). The pelleted trypanosomes were resuspended in a minimum volume of PSG which was

adjusted to pH 8.0 with solid Tris base. The trypanosomes were layered on top of a column of DEAE-cellulose equilibrated in PSG. Under these conditions, trypanosomes do not adhere to the anion exchanger and were eluted from the column with PSG as described by Lanham and Godfrey (1970). The eluted trypanosomes were pelleted by centrifugation (1 000 x g; 10°C; 10 min) and washed twice by resuspension in PSG and recentrifugation. Trypanosomes remained viable for 2-4 hours in PSG kept on ice (cf. Section 3.2.2). Glucose is essential for the survival of trypanosomes *in vitro* conditions. Pelleted trypanosomes were stored at -70°C for 3 months until required.

2.6 - Acid phosphatase assays

Unless otherwise stated, one unit of activity was defined as the amount of enzyme that catalysed the formation of 1 µmole of product per minute at 37°C.

2.6.1 - Para-nitrophenyl phosphate

2.6.1.1 - Reagents

Assay buffer [100 mM Na acetate buffer, pH 5.0]. Glacial acetic acid (2.86 ml) was dissolved in 450 ml of dH_2O, adjusted to pH 5.0 with NaOH and made up to 500 ml with dH_2O.

Para-nitrophenyl phosphate [50 mM p-NPP disodium]. Para-nitrophenyl phosphate (131.55 mg) was dissolved in 9 ml of assay buffer, transferred into a 10 ml volumetric flask and made up to 10 ml with the assay buffer. This solution was distributed into 2 ml aliquots and kept at -20°C until required.

Stopping solution [10 mM NaOH]. NaOH (1.0 g) was dissolved in 230 ml of dH_2O, transferred into a 250 ml volumetric flask and the volume adjusted to 250 ml with dH_2O.

2.6.1.2 - Procedure

The reaction mixture consisted of 20 µl of assay buffer, 5 µl of 50 mM p-NPP and 25 µl of enzyme solution (10-20 µg protein) in a total volume of 50 µl made up in a 96-wells microplate. Two wells representing blanks were prepared by substituting the enzyme solution with buffer. The microplate was then incubated in water bath for 60 min at 37°C with gentle shaking (50 oscillations/min). After incubation, the reaction was stopped by the addition of

200 µl of stopping solution. The amount of liberated p-nitrophenol was immediately determined spectrophotometerically at 405 nm using an automated microplate reader, Model EL 312 (Bio-Tek Instruments Inc., Winooski, USA). A molar extinction coefficient of 1.465 $\mu mole^{-1}$. ml . cm^{-1} was used to determine the number of µmoles of p-nitrophenol released. The procedure used for the determination of AcP activity was adapted from several sources (Helwig *et al.* 1977; Gottlieb and Dwyer 1981; Lettelier *et al.* 1985).

2.6.2 - Beta-glycerophosphate

2.6.2.1 - Reagents

Assay buffer [100 mM Na acetate buffer, pH 5.0]. Glacial acetic acid (2.86 ml) were dissolved in 450 ml of dH_2O, adjusted to pH 5.0 with NaOH and made up to 500 ml with dH_2O.

50% (m/v) TCA. TCA (25 g) was dissolved in 50 ml of dH_2O.

Beta-glycerophosphate [100 mM β-GLOP disodium salt]. Beta-glycerophosphate (216 mg) was dissolved in 9 ml of assay buffer, transferred into a 10 ml volumetric flask and made up to 10 ml with the assay buffer.

Ascorbic-molybdate solution [2% (m/v) $C_8H_8O_8$, 0.42% (m/v) $(NH_4)_6Mo_7O_{24}.4H_2O$, 1 N H_2SO_4]. Ammonium molybdate (0.42 g) and conc. H_2SO_4 (2.86 ml) were dissolved in 80 ml of dH_2O, transferred in a 100 ml volumetric flask and the volume adjusted to 100 ml with dH_2O. This solution is stable at room temperature. The needed volume of ascorbic-molybdate solution was made 2% ascorbic acid (the reducing agent) just before use (Ames, 1966).

2.6.2.2 - Procedure

Acid phosphatase activity against β-GLOP was measured in 250 µl reaction mixtures containing 75 µl of assay buffer, 150 µl of 100 mM β-GLOP solution and 25 µl of enzyme solution (10 - 20 µg protein). Incubations were carried out at 37°C with gentle shaking (50 oscillations/min) for 60 min. The reactions were stopped by the addition of 5 µl of 50% TCA. Blanks were prepared in separate microfuge tubes by either replacing enzyme solution aliquots

with buffer and assaying as normal or by stopping the reaction at time zero with 5 µl of 50% TCA. After centrifugation (15 000 x g; 25°C; 5 min) in a microfuge to remove any precipitate, 200 µl of the clear supernatant was mixed with 800 µl of ascorbic-molybdate solution and incubated 20 min at 45°C or 1 hour at 37°C (Ames, 1966). The inorganic phosphate was measured by monitoring absorbance at 820 nm using a Pharmacia LKB Ultrospec III Spectrophotometer. An extinction coefficient of 6.05 $\mu mole^{-1}$. ml . cm^{-1} was used to determine the number of µmoles of the inorganic phosphate released.

2.7 - Determination of protein concentration

Protein concentration was determined by the Bradford dye-binding assay (Bradford, 1976). This assay provides a sensitive method (microgram level). It was used routinely for protein determination since it is a rapid quantitative method. It is also devoid of interference by common laboratory reagents, with the exception of relatively high concentrations (≥1%) of detergents, flavenoids and basic buffers. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from the cationic red form at 465 nm to the anionic blue form at 595 nm when binding to protein occurs (Compton and Jones, 1985). Coomassie brilliant blue G-250 dye binds to basic amino acid side chains, mainly arginine, and to a lesser extent to histidine and lysine and the aromatic aminoacid side chains tyrosine, tryptophan and phenylalanine. A micro-protein assay (1-20 µg) was sufficient for the amounts of protein measured in this study.

2.7.1 - Reagents

Dye Reagent. The Bradford dye reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G-250 in 50 ml of 95% ethanol. Concentrated O-phosphoric acid (100 ml) was added followed by distilled water to a final volume of 200 ml. This mixture was stirred overnight and filtered through Whatman N° 1 filter paper. This solution was stored in a brown bottle at 4°C and was stable for at least 6 months. If precipitation was visible, the reagent was filtered and re-calibrated before use.

Standard protein solution. Bovine serum albumin stock solution (1.4 mg/ml) was prepared by dissolving 14 mg of protein in 10 ml dH_2O and aliquots of 2 ml solution were stored at -20°C

until required. This solution was further diluted to yield solutions ranging from 1.4-14 μg protein/ml for the calibration curve in a micro-assay.

2.7.2 - Procedure

To determine the protein concentration, dye reagent (0.2 ml) was mixed with 0.8 ml of sample (the sample was prepared at an appropriate dilution in distilled water). The mixtures were gently homogenised and the colour was allowed to develop at room temperature for 10 min. The absorbance was then measured at 595 nm in a plastic cuvette using a Pharmacia LKB Ultrospec III Spectrophotometer. Several dilutions of bovine serum albumin (1.4-14 μg protein/ml) were used to construct a standard curve. Unknown protein concentrations were determined using equations generated by linear regression analysis of the calibration curve. All assays, including blanks without protein aliquots, were done in triplicate.

2.8 - Concentration of samples

Protein samples obtained throughout this study often required to be concentrated before subjecting them to further purification, or for SDS-PAGE analyses. Protein samples were concentrated by ultrafiltration using Centriprep-10 concentrators (Amicon Ltd, Upper Mill, Stonehouse, UK). The Centriprep-10 concentrator was found to be sufficiently mild to retain enzyme activity and offer some advantages over concentration techniques such as chemical precipitation, evaporation and lyophilisation.

A Centriprep concentrator consists of a sample container with a twist-lock cap, a filtrate collector containing an Amicon YM membrane, plus an air-seal cap for sample isolation. Concentration by these Centriprep devices is based on the pressure differential between the sample solution and filtrate containers during the centrifugation. This hydrostatic pressure is created by the raised sample solution level in the sample container which exerts an upward buoyancy force on the membrane at the filtrate collector bottom. During centrifugation, the pressure increases and forces low molecular mass materials and solvent through the membrane into the filtrate collector. Subsequently, solutes with molecular masses above the membrane cut-off (retentate) remain in the sample container and become increasingly concentrated as the operation continues. The filtration stops when the hydrostatic pressure difference between the sample meniscus and the filtrate meniscus reaches an equilibrium.

The protein sample (maximum volume, 15 ml) was placed in the sample container and the Centriprep-10 concentrator was assembled as recommended in the Manufacturer's manual. The assembled concentrator was centrifuged (1000 x g, 4°C) until the desired volume was obtained in the sample container. Once the sample had been sufficiently concentrated (usually 5 to 10 fold in 2-4 h), the concentrator was disassembled and the sample withdrew into a suitable container. Both retentate and filtrate were kept separately and assayed either for AcP activity or protein concentration as described in Sections 2.6 and 2.7 to assess the solute recovery.

2.9 - Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to assess the protein composition of fractions from purification steps, to visualise the AcP activity and to evaluate the molecular masses of AcPs and their subunits. Proteins were resolved by discontinuous polyacrylamide gel electrophoresis in the presence or absence of SDS as described by Laemmli (1970).

The anionic detergent, SDS, binds tightly to most proteins in a ratio of 1.4 g per g protein, thereby converting them from globular native amphoteric proteins into highly negatively charged rodlike complexes, the length of which varies with the molecular mass of the protein moiety of the detergent-protein complex (Reynolds and Tanford, 1970). Concomitant treatment with a disulfide reducing agent, such as 2-mercaptoethanol, will break the proteins down into their constituent subunits. This opens up the protein structure, allowing SDS to bind more effectively, giving a truer estimate of M_r. These negatively charged detergent-polypetide complexes will have similar charge-to-mass ratios, resulting in size dependent anodal migration in an electrical field. Since an inverse relationship exists between the logarithm of the M_r of a protein and the distance migrated in a gel, a standard curve can be generated for M_r estimation by running standard proteins, of known M_r, alongside the polypeptides to be characterised.

2.9.1 - Reagents

The following stock solutions were prepared and filtered through Whatman N° 4 filter paper before storage at 4°C. Solution D was stored at RT since it crystallises at 4°C.

Solution A: Monomer Solution [30% (m/v) acrylamide, 1% (m/v) bis-acrylamide]. Acrylamide (60 g) and bis-acrylamide (2 g) were dissolved in approximately 170 ml of dH_2O and made up to 200 ml with dH_2O. The solution was stored in an amber coloured bottle.

Solution B: Running Gel Buffer (1.5 M Tris-HCl, pH 8.8). Tris (45.38 g) was dissolved in approximately 200 ml of dH_2O, adjusted to pH 8.3 with HCl and made up to 250 ml.

Solution C: Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8). Tris (6 g) was dissolved in approximately 80 ml dH_2O, adjusted with HCl to pH 6.8 and made up to 100 ml. This buffer was made up monthly, because, as a result of its poor buffering capacity at 2.1 pH units below its pKa at 4°C (Pharmacia products catalogue), pH drift led to anomalous running patterns in non-reducing SDS-PAGE.

Solution D: 10% (m/v) SDS. SDS (10 g) was dissolved in 100 ml dH_2O and stored at room temperature.

Solution E: Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was dissolved in 1 ml dH_2O. The solution was replaced with fresh solution after seven days.

Solution F: Tank buffer [0.25 M Tris-HCl, 0.192 M glycine, 0.1% (m/v) SDS, pH 8.3]. Tris (15 g) and glycine (72 g) were dissolved and made up to 5 l with dH_2O. Prior to use, 5.0 ml of SDS stock (solution D) was added and made up a 500 ml total.

Solution G: Reducing Treatment Buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dH_2O.

Solution H: Non-reducing Treatment Buffer [125 mM Tris-HCl, 2% (m/v) SDS, 20% (v/v) glycerol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (2 ml) (solution D) and glycerol (2 ml) were made up to 10 ml with dH_2O.

2.9.2 - Procedure

Unless otherwise stated, 5 - 20% polyacrylamide gradient gels (1.5 mm thickness) with a 3% stacking gel were used. SDS-PAGE was performed as described by Laemmli (1970) using the Protean II electrophoresis apparatus (Bio-Rad, Richmond, Canada).

For reducing SDS-PAGE, protein samples (20-50 µg) were mixed with an equal volume of the reducing treatment buffer, heated at 100°C for 1 min and cooled before loading. Electrophoresis was carried out at 40 mA for 1 hour followed by 80 mA for 4 hours in the Protean II electrophoresis apparatus. When the bromophenol blue tracking dye reached the bottom of the gel, electrophoresis was terminated. Gels were stained according to the procedures outlined below with either Coomassie brilliant blue R250 dye (Section 2.11.1) or silver (Section 2.11.2).

Table 4 - SDS-PAGE Formulations for the preparation of separating and stacking gels.

Reagent	Running Gel							Stacking Gel	
	5 %	7.5 %	10 %	12.5 %	15 %	17.5 %	20 %	3 %	5 %
ml A	5	7.5	10	12.5	15	17.5	20	1.8	3.0
ml B	7.5	7.5	7.5	7.5	7.5	7.5	7.5		
ml dH_2O	17.5	14.65	12.15	9.65	(7.05)	4.55	2.05	10.96	10.26
ml C								4.5	4.5
ml D	0.6	0.6	0.6	0.6	0.6	0.6	0.6	180	180
µl TEMED	10	10	10	10	10	10	10	9	9
µl E	50	50	50	50	50	50	50	60	60

For electrophoresis carried out under non reducing conditions, the method was adapted from Thaller et al. (1995). Each protein sample (20-50 µg) was mixed with an equal volume of the non-reducing treatment buffer before loading onto the gel. Electrophoresis was carried out at

40 mA for 1 hour and 80 mA for 5 hours and stopped when the bromophenol blue tracking dye reached the bottom of the gel.

PAGE of native proteins was performed as for SDS-PAGE except that SDS was omitted from gels and buffers.

2.10 - Detection of AcP activity in gels

For detection of AcP enzyme activity run on a SDS-PAGE, the gel was washed for 16 hours at RT in several changes of renaturation buffer to achieve renaturation of the enzyme (Thaller et al., 1995). Renaturation buffer constituted 100 mM Na acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100. After the renaturation treatment, the gel was rinsed 4 x 15 min in 100 mM Na acetate buffer (pH 5.0), and then incubated at 37°C in 100 mM Na acetate buffer (pH 5) containing 1 mg/ml α-naphthyl phosphate and 1 mg/ml Fast Red TR salt.
For PAGE of native proteins, gels were rinsed 2 x 15 min in cold 100 mM Na acetate buffer (pH 4) and once in 100 mM Na acetate buffer (pH 5) as described by Katakura and Kobayashi (1988). For detection of acid phosphatase activity, gels were then incubated at 37°C in 100 mM Na acetate buffer (pH 5) containing 1 mg/ml α-naphthyl phosphate and 1 mg/ml Fast Red TR salt.

2.11 - Protein Stains

After electrophoresis, polyacrylamide gels were stained for proteins either with Coomassie Brilliant Blue or with Silver as described below.

2.11.1 - Coomassie Brilliant Blue R-250 Staining

Coomassie Brilliant Blue R-250 staining is designed to detect proteins at μg per single protein band. Methanol was included in the staining solution to allow the dye to penetrate the gel and acetic acid served to fix the protein in the gel. After the staining procedure was complete, the gel was destained by placing it in methanol which contained acetic acid to maintain the protein in its fixed form.

2.11.1.1 - Reagents

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (2.0 g) was dissolved in a total volume of 200 ml dH_2O, stirred (60 min) using a magnetic stirrer and filtered through Whatman N° 4 filter paper.

Staining solution [0.125% (v/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock solution (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml) and made up to 500 ml with dH_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) and acetic acid (100 ml) were mixed and made up to 1 l with dH_2O.

Destaining solution II [5% (v/v) methanol, 7% (v/v) acetic acid]. Methanol (100 ml) and acetic acid (140 ml) were mixed and made up to 2 l with dH_2O.

2.11.1.2 - Procedure

After electrophoresis, the gel was placed in staining solution (4 hours), transferred to destain I (16 hours) and rinsed several times in destain II until the background was clear.

2.11.2 - Silver Staining

This method appeared to be the method of choice for protein visualisation in gels as it is a very sensitive technique and can detect small amounts of protein (nanogram level). The method was derived from that of Merril et al. (1981). Because of time taken to remove all the oxidiser ($K_2Cr_2O_7$) from gels, this solution was replaced by sodium thiosulphite ($Na_2S_2O_3.5H_2O$). This chemical appears to prevent unspecific background staining during image development (Blum et al., 1987). All steps were carried out on an orbital shaker (50 rpm; 25°C) and in scrupulously clean glass containers to minimise background staining.

2.11.2.1 - Reagents

Fixative I [40% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (240 ml) and acetic acid (60 ml) were made up to 600 ml with dH_2O.

Fixative II [10% (v/v) ethanol, 5% (v/v) acetic acid]. Ethanol (60 ml) and acetic acid (30 ml) were made up to 600 ml with dH_2O.

Oxidiser [0.02% (m/v) sodium thiosulphate]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in dH_2O to a final volume of 200 ml.

Silver solution [12 mM silver nitrate]. $AgNO_3$ (2.05 g) was dissolved in 1 l of dH_2O.

Developer [280 mM sodium carbonate, 0.0004% (m/v) sodium thiosulphate, 0.5 % (v/v) formaldehyde]. Na_2CO_3 (29.7 g), oxidiser solution (20 ml) and 37% (v/v) formaldehyde (0.5 ml) were made up to 1 litre with dH_2O. This reagent was prepared in a fume hood

Stop reagent [5% (v/v) acetic acid]. Acetic acid (25 ml) was made up to 500 ml with dH_2O.

2.11.2.2 - Procedure

The polyacrylamide gel was soaked in fixative I (200 ml, 3 x 20 min) immediately after electrophoresis and in fixative II (200 ml, 2 x 30 min) to remove SDS. The gel was then incubated in oxidiser (200 ml, 1 x 1 min), washed (3 x 20 sec), immersed in 10% (v/v) silver reagent (200 ml, 1 x 30 min) and washed briefly in dH_2O (200 ml, 2 x 2 min). Silver stained bands were developed in three changes of developer solution (600 ml, 1 x 30 sec and 2 x 5 min) with the changes being done whenever the solution turned yellow or until a brown smokey precipitate appeared. When the desired intensity of the bands was obtained, the development was stopped with stop reagent (200 ml, 2 x 15 min) and the gel stored in dH_2O.

2.12 - Glycoprotein stains

The detection of the carbohydrate moiety of glycoproteins was performed by the periodic acid-Schiff reagent (PAS). The method is based on the oxidation of hexose vicinal 1,2-diol groups to aldehydes using periodate with subsequent staining by Schiff base (Jay et al., 1990).

2.12.1 - Reagents

Fixative [50% methanol]. Methanol (200 ml) was mixed with 200 ml of dH_2O.

2% (m/v) Periodic acid. H_5IO_6 (4.0 g) was dissolved in 200 ml of dH_2O.

Schiff's Reagent [0.01% (m/v) pararosaniline and 3.4% (m/v) potassium metabisulfite in 250 mM HCl]. This reagent was prepared in a fume hood. Conc. HCl (1.572 ml) was diluted in approximately 180 ml of dH_2O. Pararosaniline (20 mg) and $K_2S_2O_5$ (6.8 g) were added to the acid solution, stirred until dissolved and made up to 200 ml with dH_2O. The mixture was filtered through Whatman N° 4 filter paper before use.

2% (m/v) Sodium metabisulfite. $Na_2S_2O_5$ (10 g) was dissolved in 500 ml of dH_2O.

2.12.2 - Procedure

After electrophoresis, the gel was soaked in fixative (200 ml, 25°C, 16 hours), followed by one change in the same fixative (200 ml, 25°C, 1 x 60 min) with gentle agitation to remove SDS. The gel was washed in dH_2O (200 ml, 25°C, 3 x 20 min) to allow swelling of the gel. The water was then replaced with 2% periodic acid for 60 min. After two brief washes with dH_2O (200 ml, 2 x 2 min), the gel was immersed in Schiff's Reagent in a fume hood until the gel turned magenta (~ 60 min). The gel was washed twice with dH_2O (200 ml, RT, 2 x 2 min) and the reduction was performed by incubation in several volumes of 2% (m/v) sodium metabisulfite until the gel showed a clear background. Lengthly reduction with metabisulfite appeared critical in removing unreacted pararosaniline. After metabisulfite reduction, the gel was rinsed with several changes of dH_2O until the water remained clear, indicating complete removal of unreacted pararosaniline. The gel was photographed to record the staining result.

2.13- Isoelectric focusing

Isoelectric focusing electrophoresis (IEF) is a technique that separates proteins according to their isoelectric point (pI). The pI of a protein is defined as the pH at which a particular protein has no net overall charge and will therefore concentrate at this point as migration ceases. The separation is accomplished by placing the protein in a pH gradient generated through the distribution of poly-amino poly- carboxyl ampholytes in an electric field. Resolution is improved when a high voltage gradient is applied with an efficient cooling system. Isoelectric focusing was performed using a 110 ml capacity LKB 8100 Electrofocusing column (Ui, 1971).

2.13.1 - Reagents

Ampholyte mixture [60% (v/v) ampholyte(pH 3.0-10), 20% (v/v) ampholyte(pH 4.0-6.0), 20% (v/v) ampholyte(pH 5.0-7.0)]. The ampholyte mixture was prepared by mixing the wide range of ampholyte (3 ml) and the two overlapping ranges of ampholyte (1 ml each).

Dense gradient solution [50.9% (m/v) sucrose, 3.8% (m/v) ampholyte mixture]. Sucrose (27.0 g), ampholyte mixture (2.0 ml) and sample (~ 20 ml) were mixed, the volume made up to 53 ml with dH_2O and degassed.

Light gradient solution [5.09% (m/v) sucrose, 1.5% (m/v) ampholyte].. Sucrose (2.7 g), ampholyte mixture (0.8 ml) and sample (~ 6 ml if any available) were mixed, the volume made up to 53 ml with dH_2O and degassed.

Anode solution [16 mM HCl]. Sucrose (15.0 g), 1 M HCl (4.0 ml) were dissolved in 12 ml of dH_2O and degassed.

Cathode solution [250 mM NaOH]. 1 M NaOH (2.5 ml) was dissolved in 7.5 ml of dH_2O.

2.13.2 -Procedure

The column was filled with a linear sucrose gradient ranging from 5 to 50% using a peristaltic pump. Electrofocusing was carried out for 20 h at 4°C with a constant voltage of 1600 V. On completion of the run, 3 ml fractions were collected by draining the column from the bottom with the aid of a peristaltic pump and assayed for AcP activity (Section 2.6.1). The pH and absorbance at 280 nm of the collected fractions were also measured.

CHAPTER THREE

STUDIES WITH LIVE *T. CONGOLENSE.*

3.1 - Introduction

Various hydrolytic enzymes, including acid phosphatases and proteases, have been reported in many species of protozoa such as *Leishmania* spp. (Pupkis et al., 1986; Coombs et al., 1987); *Paramecium caudatum* and *Paramecium tetraurelia* (Fok and Paeste, 1982); *Trypanosoma* spp. (Lonsdale-Eccles and Grab, 1987; Ashall, 1990). Some species were found to release hydrolytic enzymes such as acid phosphatase (AcP) into the extracellular medium (Bates and Dwyer 1987). In African trypanosomes, AcP has been localised in subcellular organelles, but the possibility that this enzyme could be secreted has rarely been studied. Experiments that were performed using live trypanosomes to address this issue will be described in this chapter.

3.2 - Methods

Trypanosomes (1 - 2 x 10^9 cells) were harvested as described in Section 2.6.2. The isolated cells were washed twice in ice-cold buffer A and resuspended in the same buffer at 4 x 10^8 cells/ml. All procedures were performed on ice unless otherwise stated.

3.2.1 - Determination of AcP secretion by living parasites (*T. congolense* and *L. donovani*)

In order to determine if the parasites secrete (or otherwise release) AcP into their surrounding medium, 4 x 10^8 freshly isolated cells/ml were incubated at 26°C in buffer A. At appropriate time intervals, 100 µl aliquots of the cell suspension were transferred into 1.5 ml microfuge tubes and centrifuged for 15 sec at 15 000 x g. Ninety µl of supernatant was removed and recentrifuged for 2 min to pellet any residual cells. The final supernatant (80 µl) was assayed for the presence of AcP activity using p-NPP (Section 2.7.1).

In order to compare these results with other trypanosomatids that are known to secrete AcP, *L. donovani* promastigotes were studied for their ability to secrete AcP. *L. donovani* promastigotes used were a gift from Dr J. Olobo (The Institute of Primate Research, Nairobi, Kenya). These cells were cultivated in sterile 50 ml scewcap-tubes containing 15 - 20 ml of medium 199 supplemented with 25 mM Hepes buffer and 20% (v/v) heat-inactivated fetal bovine serum. The pH of the medium was adjusted to 7.4 with 1 M NaOH prior to addition

of the serum and filtered through a 0.45 μm Millipore filter. When required, these parasites were pelleted by centrifugation (1000 x g, 10 min) and washed twice in buffer A. Pelleted *L. donovani* cells were resuspended in the same buffer (4 x 10^8 cells/ml) and used under the identical conditions as described above for *T. congolense* to determine whether AcP was secreted.

3.2.2 - Viability of trypanosomes

In addition to measuring the AcP content of the supernatants, each of the pellets obtained in the AcP secretion experiments (Section 3.2.1) were separately resuspended in 1 ml of PSG and the viability of trypanosomes monitored by the Trypan blue exclusion test. This test is based on the principle that live (viable) cells do not take up the dye, whereas dead (non-viable) cells do.

3.2.2.1 - Reagents

Trypan Blue Stain stock solution 0.4% (m/v). Trypan Blue Stain (0.4 g) was dissolved in 100 ml of PSG, stirred (60 min) using a magnetic stirrer and filtered through Whatman N° 4 filter paper.

Hanks'Balanced salts solution. NaCl (0.80 g), KCl (0.04 g), glucose (0.10 g), KH_2PO_4 (6 mg) and Na_2HPO_4 (47 mg) were dissolved in approximately 70 ml of dH_2O and made up to 100 ml with dH_2O.

3.2.2.2 - Procedure

Trypan blue solution (0.5 ml) was mixed with Hank's balanced salts' solution (0.3 ml) and the resuspended cell pellet (0.2 ml). After mixing thoroughly, cells were left 5 minutes to stain. With the cover-slip in place, a small amount of the Trypan blue-cell suspension mixture was transferred into a Neubauer haemocytometer chamber using a Pasteur pipette. With an Erma hand-counter, stained and unstained cells were counted and viable cells estimated.

Cell viability (%) = [total viable cells (unstained) ÷ total cells (stained and unstained)]x100

3.2.3 - Hydrolysis of p-nitrophenyl phosphate by living trypanosomes

To investigate whether living trypanosomes could hydrolyse p-nitrophenyl phosphate, the parasites (4 x 10^8 cells/ml) were incubated at 26°C in buffer A containing p-nitrophenyl phosphate (5 mM). At various time intervals, 200 µl aliquots were transferred into 1.5 ml microfuge and centrifuged (15 000 x g, 15 sec). The supernatant (180 µl) was recentrifuged for 2 min to pellet any residual cells. The final supernatant (50 µl) was mixed with 200 µl of 10 mM NaOH and the liberated p-nitrophenol was immediately estimated at 405 nm using a Pharmacia LKB Ultrospec III Spectrophotometer (Section 2.7.1).

3.3 - Results

3.3.1 - Determination of AcP secreted by live trypanosomes

In agreement with earlier studies (Bates et al., 1989), cultured *L. donovani* promastigotes were shown to release (or to secrete) AcP into the surrounding medium. In contrast, over the same period of 60 min freshly isolated *T. congolense* did not show substantial release of AcP activity into the supernatant fluids (Fig. 4). It therefore appears that *T. congolense* and *L. donovani* differ in this property.

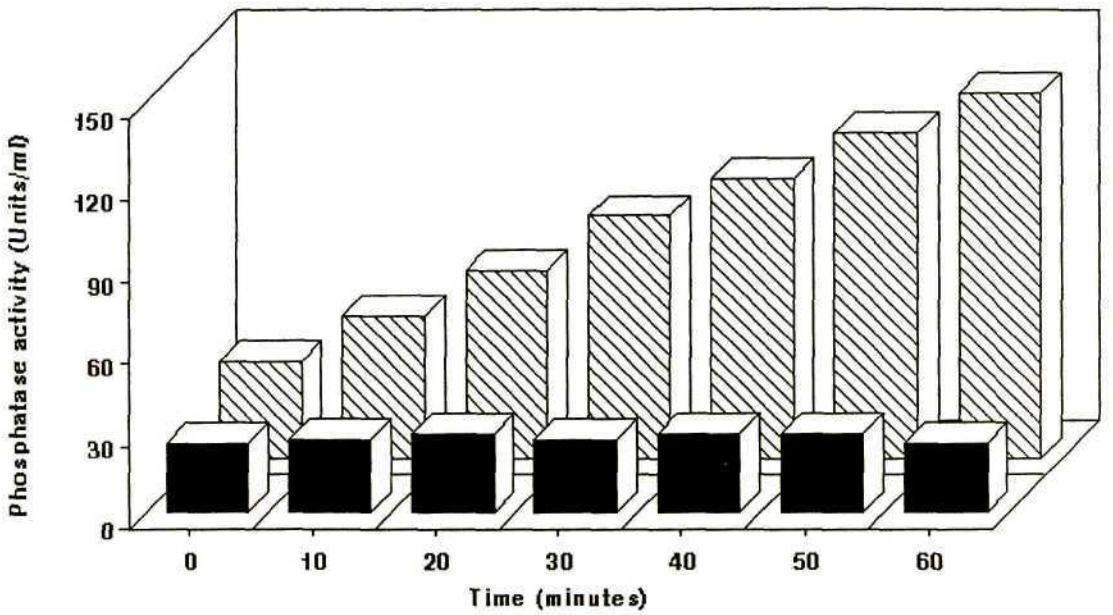


Figure 4 - Time course of secretion of AcP by live *T. congolense* and live *L. donovani.*

T. congolense and *L. donovani* ($4x10^8$ cells per ml) were incubated separately at 26 °C in 10 mM Hepes buffer (pH 7.2) containing 145 mM NaCl and 5 g glucose per l. At required time intervals, aliquots of the cell suspension were centrifuged for 15 seconds at 15 000 x g. Supernatants were assayed for AcP activity as described in Section 3.2.1. Solid black boxes indicate *T. congolense* and hatched boxes indicate *L. donovani.*

3.3.2 - Viability of trypanosomes

Although *T. congolense* showed no significant release of AcP when incubated for up to 1 hour (Fig. 4), additional experiments over longer incubation periods showed increasing amounts of activity in the supernatant (Fig. 5). During the initial period, trypanosomes remain motile (as assessed by microscopic examination) and viable (as assessed by the Trypan blue exclusion test) but after 1 hour, steadily increasing numbers of parasites were observed to have died. Thus, the enhanced AcP activity found in the *T. congolense* supernatants after 60 min appears to be associated with cell death.

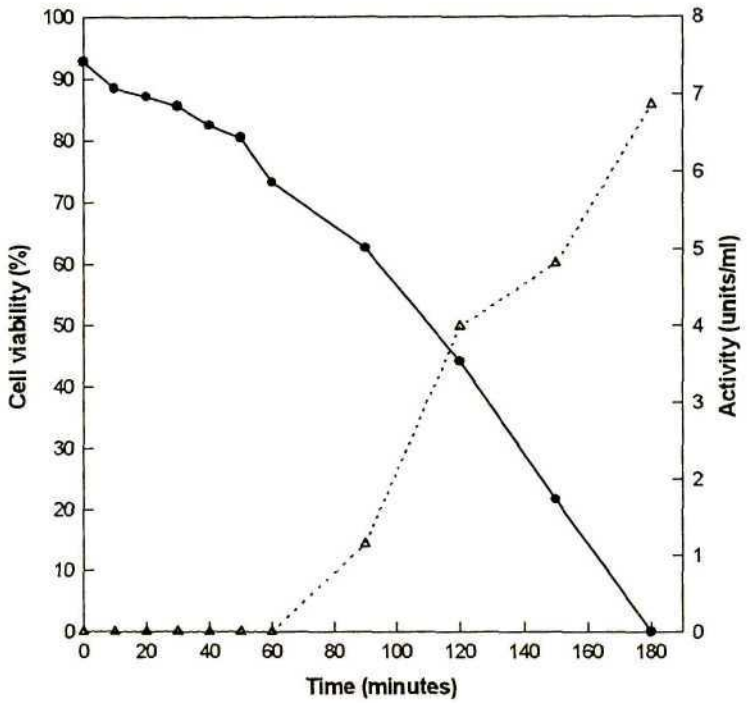


Figure 5 - Viability of live *T. congolense* as assessed by the Trypan blue exclusion test.

Cell viability (●) corresponds to the total viable cells (unstained) compared to the total amount of cells (stained and unstained) expressed in percentage. The AcP activity (△) was estimated using p-nitrophenyl phosphate assay (Section 2.5.1).

3.3.3 - Hydrolysis of p-nitrophenyl phosphate by live trypanosomes

Athough little or no AcP was secreted by live *T. congolense*, in order to ascertain whether or not there might be AcP associated with, or bound to, the surface of the parasites, the ability of live trypanosomes to hydrolyse p-NPP was examined. Considerable hydrolysis was observed when live cells were incubated at different temperatures (0-37°C) in the presence of p-NPP (Fig. 6). As expected, p-NPP hydrolysis was more rapid at elevated temperatures. Nevertheless, hydrolysis was observed at all temperatures, including 0 and 4°C where endocytosis should be halted. When examined by light microscopy, the parasites incubated at 42°C were found to be dying and so these results were excluded.

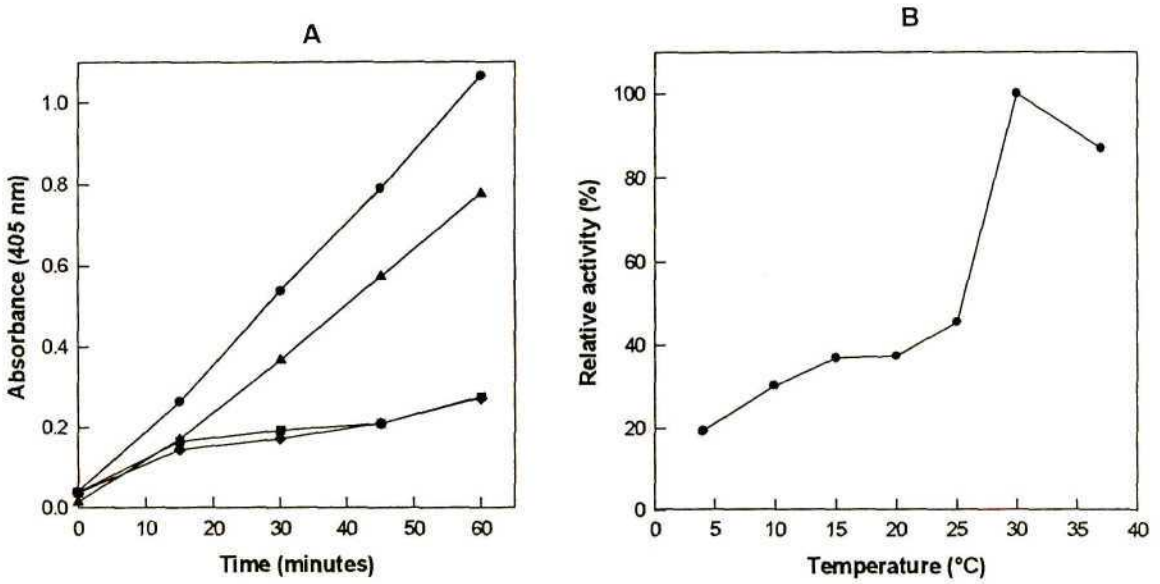


Figure 6 - The hydrolysis of p-nitrophenyl phosphate by live *T. congolense* at different temperatures.

Trypanosomes (4 x 10^7 cells) were incubated at the indicated temperature values in the presence of 5 mM of p-NPP. Either at each of the indicated time intervals (panel **A**) or after a single 30 min incubation period (panel **B**), the cells were centrifuged (15 000 x g, 15 sec) and the supernatants assayed for p-nitophenol released (Section 3.2.3). In the results shown in panel A, trypanosomes were incubated at 26°C (●), 15°C (▲), 4°C (■) and on ice (◆). The results are represntative of four separate experiments (panel **A**). On panel **B** the data are the means and the S.D. for three or more values obtained in separate experiments.

3.3.4 - Effect of pH and of phosphatase inhibitors on the hydrolysis of p-NPP by live trypanosomes

Although the previous experiment (Section 3.3.3) showed that phosphatase activity was probably associated with the surface of the parasite, it gave no indication as to whether the enzyme might be an acid or alkaline phosphatase. When live trypanosomes were incubated at different pH values, phosphatase activity increased steadily as the pH decreased (Fig. 7). Thus the enzyme activity was probably due to an AcP rather than an alkaline phosphatase. This was

confirmed by repeating the experiments in the presence the AcP inhibitor, NaF (10 mM). The release of p-nitrophenol from the substrate was almost completely inhibited at all pH values in the presence of NaF. In contrast, parasites that were incubated in the same substrate and buffers containing 3 mM tetramisole (a known alkaline phosphatase inhibitor; Van Belle, 1972) showed no inhibition of the hydrolysis of p-NPP. Indeed, tetramisole appeared to increase the activity slightly. Interestingly, light microscopic examination showed that the parasites were immediately rendered immobile in the presence of NaF whereas those in the presence of tetramisole were still motile.

3.3.5 - Distribution of AcP and surface-bound iodine in Percoll gradient

The AcP activity, radioactivity and absorbance at 280 nm profiles obtained with radiolabelled plasma membranes isolated on Percoll gradients are depicted in Fig. 8. AcP activity was concentrated in the 25-28.8 % Percoll interface, in a well defined peak. The migration of this enzyme was coincident with a proportion of the labelled iodine. The radioactivity recovered from this peak represents about 20 % of the 123 000 x g pellet, indicating that a substantial proportion of the membrane co-sediments with other heavier subcellular fractions that represent the major peak of radioactivity (Fig. 8). Nevertheless, radioactivity was clearly associated with phosphatase activity.

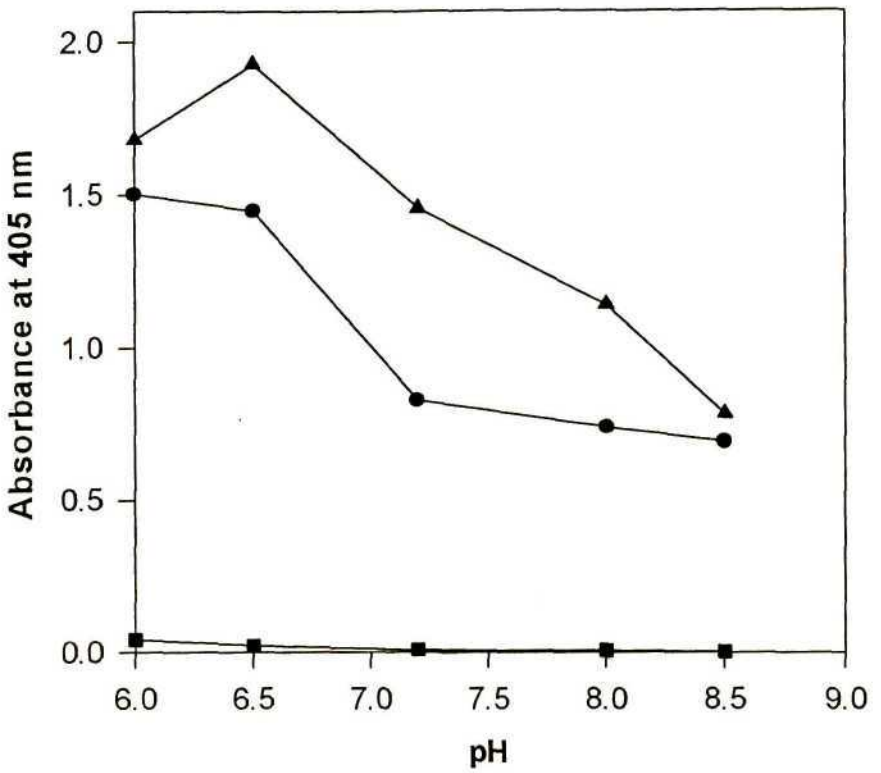


Figure 7 - Effect of pH and phosphatase inhibitors on the hydrolysis of p-nitrophenyl phosphate by live *T. congolense*.

Trypanosomes (4 x 10^7 cells) were prepared in 10 mM Hepes buffer (at the indicated pH values) containing 145 mM NaCl, 5 g/l glucose and 5 mM of p-NPP alone (control, ●) or supplemented with 10 mM of NaF (■) or 3 mM of tetramisole (▲). After incubation of trypanosomes at 37 °C for 30 min, samples were centrifuged (15 000 x g, 15 seconds) and the p-nitrophenol released from p-NPP measured (Section 3.2.3). Data are means ± S.D. for four values obtained in separate experiments.

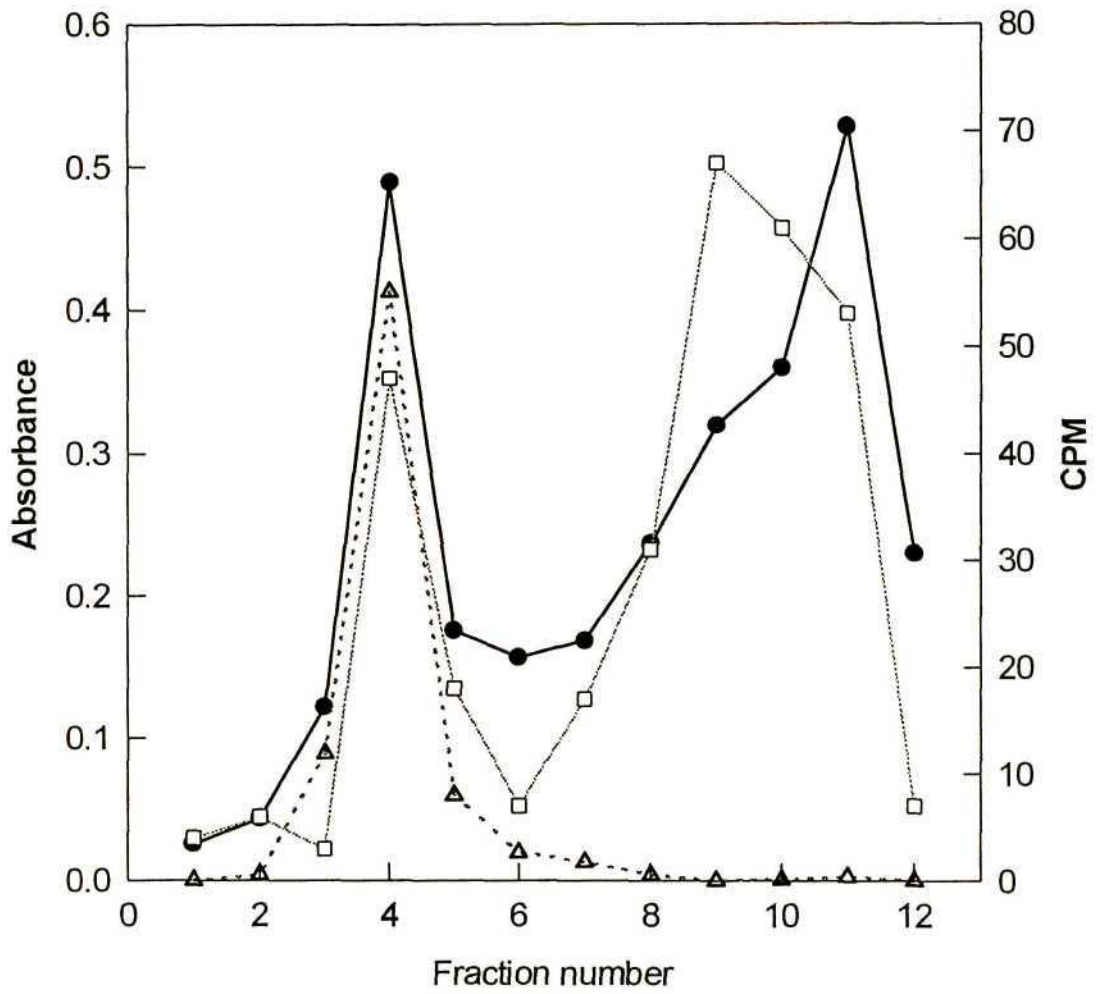


Figure 8 - Centrifugation of the 123 000 x g pellet in Percoll gradients.

This pellet obtained as described in Section 3.2.5 was centrifuged in Percoll gradients. The A_{280} nm profile (●), as well as the distribution of AcP (▲) and bound iodine (□) were analysed. The top of the gradients is on the left. The results are representative of two separate experiments.

3.4 - Discussion

In contrast to other trypanosomatids such as *Leishmania* sp. (Bates and Dwyer, 1987) and *Trichomonas* spp. (Lockwood *et al.*, 1988), it is generally accepted that African trypanosomes do not release AcP(s) into their extracellular medium. This was confirmed by the present

studies obtained with living bloodstream forms of *T. congolense*. These trypanosomes did not show any substantial release of AcP into the supernatant fluids assayed for as long as the parasites were viable. However, living *T. congolense* parasites that were incubated in the presence of the phosphatase substrate p-NPP were able to hydrolyse this substrate. This observation indicated that an AcP may be associated with the cell surface. This is in agreement with the report of the presence of an AcP associated with the surface membrane-containing fractions of *T. rhodesiense* (McLaughlin, 1986).

Alternatively, the hydrolysis of p-NPP may have resulted from endocytosed p-NPP. However, the observed hydrolysis of this substrate by *T. congolense* at low temperatures does not support this contention because temperature is among the factors that affect endocytosis. For endocytosis to occur in African trypanosomes the temperature of the incubation medium must exceed a critical threshold value of about 6-8°C (Lonsdale-Eccles *et al.*, 1993). Similarly, Brickman *et al.* (1995) also observed no endocytosis by *T. b. rhodesiense* at 4°C, although delivery to collecting tubules was observed at 12°C. In mammalian cells endocytosis is also blocked below a critical threshold value of about 10°C (Dunn *et al.*, 1980). In the present study, *T. congolense* was shown to hydrolyse p-NPP readily on ice and at 4°C, albeit at expected lower rates than those observed at higher temperatures. These results suggest that the AcP activity observed with living parasites is not a consequence of endocytic processes but rather that the activity is located on the surfaces of the parasites.

In order to characterise the nature of the surface phosphatase, inhibition and pH studies were conducted. The inhibitors used were NaF, a widely recognised AcP inhibitor, and tetramisole, an alkaline phosphatase inhibitor (Van Belle, 1972). The cell surface phosphatase activity in *T. congolense* was unaffected by tetramisole but was inhibited by NaF. (The NaF also caused the parasites to stop moving). These observations indicated that the phosphatase activity associated with the living parasites is an AcP. This conclusion was supported by pH activity profile studies showing increased activity at lower pH values and diminished activity at higher pH values. It is not clear what function this enzyme may have on the surface of the parasites but its location at this site may add to the possible immunologic and/or drug targets for future therapeutic intervention.

CHAPTER FOUR
LOCALISATION OF ACID PHOSPHATASES IN *T. CONGOLENSE.*

4.1 - General introduction

Acid phosphatase (AcP) has been considered to be a lysosomal enzyme in mammalian cells. In African trypanosomes, AcP has been localised in flagellar pockets and the Golgi apparatus of number of parasites (Seed et al., 1967; Venkatesan et al., 1977; Langreth and Balber, 1975; Steiger et al., 1980; Grab et al., 1987). In addition to the above mentioned locations, the likely presence of a surface-bound AcP activity has recently been demonstrated in bloodstream forms of *T. congolense* (Tosomba et al., 1996; Chapter 3). This suggests a heterogeneous distribution of AcPs in trypanosomes. The purpose of this Chapter was to determine the subcellular location of AcPs in bloodstream forms of *T. congolense*. The localisation of AcPs in these parasites were evaluated by two different techniques. Firstly, trypanosomes were disrupted by the French press technique and the cell homogenate was fractionated by differential centrifugation. The subcellular fractions obtained were screened for the presence of AcP activity. The efficiency of organelle separation was ascertained with specific organelle markers. A second approach to localising AcP in *T. congolense* involved cytochemistry at the electron microscope level. In this technique, the enzymatic reaction product was visualised by the formation of a lead phosphate precipitate at the site of substrate hydrolysis (Gomori, 1952).

4.2 - Subcellular distribution of acid phosphatases in *T. congolense*

4.2.1 - Differential centrifugation

T. congolense parasites were purified (Section 2.6.2), washed twice by centrifugation in ice-cold buffer D (750 x g, 4°C, 5 min) and resuspended in the same buffer at 5 x 10^6 cells/ml. Cells were disrupted by passage through a pre-cooled French Pressure Cell under a chamber pressure of 2 500 psi. The homogenate was subjected to differential centrifugation as indicated in the flow diagram shown in Fig. 9. Unbroken cells, nuclei and cell debris were sedimented (750 x g, 4°C, 15 min). The resulting pellet was called the crude nuclear fraction (NU). The supernatant was centrifuged (3 000 x g, 4°C, 15 min)

Trypanosoma congolense resuspended at 5 x 10^6 cells/ml in 20 mM Tris-HCl (pH 7.4) containing 1 mM DTT, 250 mM sucrose and 40 µg/ml of each the protease inhibitors antipain, chymostatin, E-64 and leupeptin. Resuspended cells were passed through a French Pressure Cell (2500 psi).

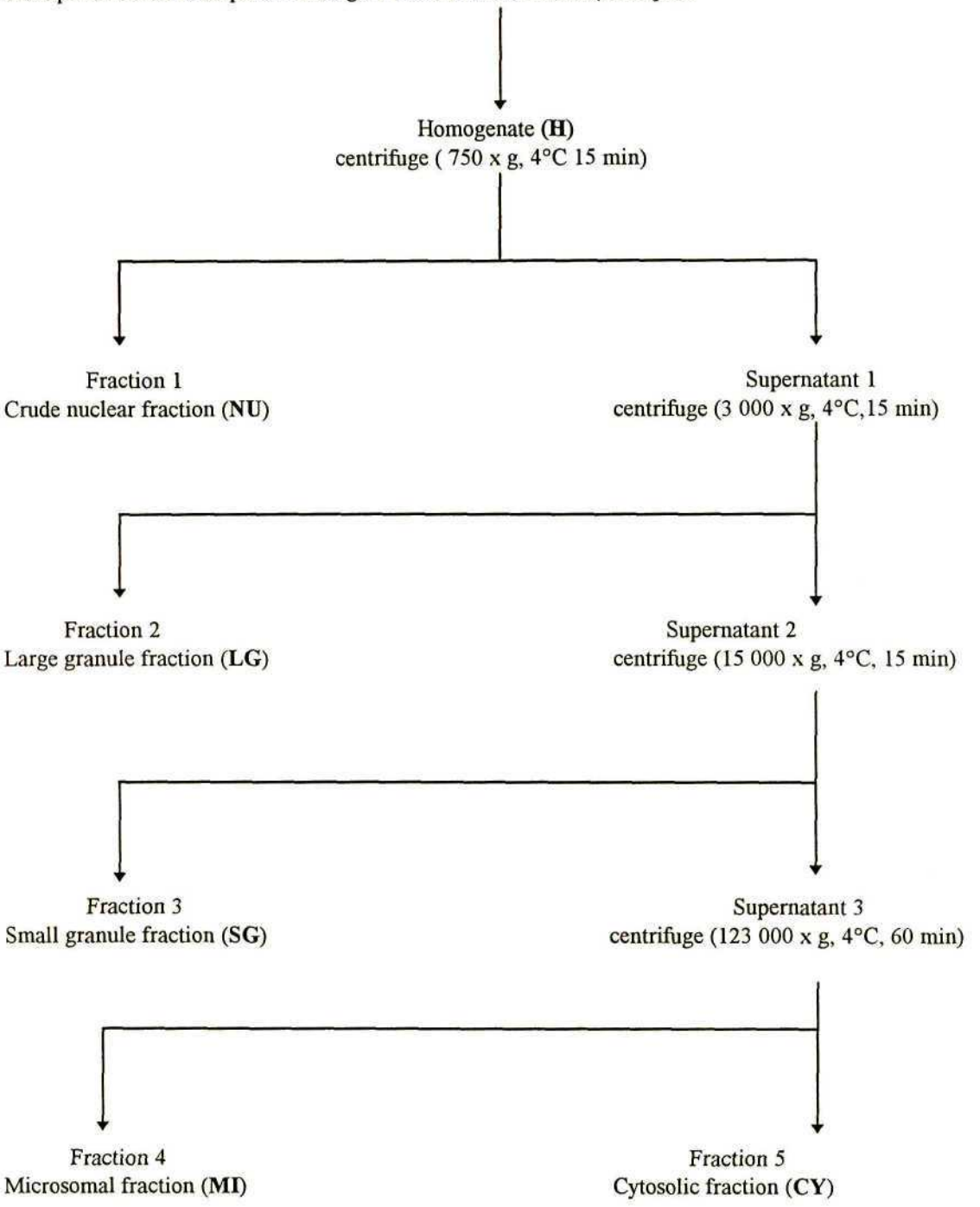


Figure 9 - Flow diagram showing the cell fractionation procedure employed for isolating *T. congolense* subcellular fractions.

to sediment a large granule fraction (LG). The small granule fraction (SG) was obtained by centrifuging the supernatant from the LG fraction (14 600 x g, 4°C,15 min). Centrifugation of the SG supernatant (123 000 x g, 4°C, 60 min) yielded the microsomal pellet (MI) and the cytosolic fraction (CY). Except for the microsomal pellet, pellets of all fractionation steps were washed once in the buffer D and the appropriate supernatants recombined. All pellets were also resuspended in the same buffer D for biochemical analysis.

4.2.2 - Characterisation of subcellular fractions with biochemical markers

4.2.2.1 - Acid phosphatase

Acid phosphatase (EC 3.1.3.2) activity was measured by the release of p-nitrophenol from p-NPP under the conditions described in Section 2.6.1.

4.2.2.2 - Cysteine protease

Cysteine protease activity was measured fluorometrically at 37°C by following the liberation of amidomethylcoumarin (NHMec) (excitation 360 nm, emission 460 nm) in a 7600 Microplate Fluorometer (Cambridge Technology, Inc., Watertown, MA, USA). The method used was an assay adapted from that of Mbawa et al. (1991). The reaction mixture (90 µl) containing 10 µM benzyloxycarbonyl-phenylalanyl-arginyl-7-amido-methylcoumarin (Z-Phe-Arg-NHMec), 100 mM Mes buffer (pH 6.0), 2 mM EDTA, and 6 mM dithiothreitol was pre-incubated at 37°C for 5 min prior to adding the enzyme solution (2-5 µg protein) that was also pre-incubated at 37°C for the same period of time. The release of the fluorometric NHMec was monitored for 15 min. Solutions of NHMec ranging from 20-800 nM were prepared from a stock solution (1 µM) to construct a calibration curve for calculation of the enzyme activity.

4.2.2.3 - Cytochrome c reductase

Cytochrome c reductase (EC.1.6.2.1) activity was measured spectrophotometrically at 37°C by following the reduction of cytochrome c at 550 nm by NADH (Sottocasa et al., 1967). The assay mixture (0.9 ml) consisted of 0.1 mM NADH, 0.1 mM cytochrome c, 0.3 mM KCN, 10-20 µg enzyme protein and 50 mM phosphate buffer (pH 7.5). After incubation for 30 min at 37°C, the reaction was stopped by addition of 0.1 ml of 50% (m/v) TCA aqueous solution and the absorbance read at 550 nm. The cytochrome c was reduced chemically with $Na_2S_2O_4$

to obtain a calibration curve as described by Van Gelder and Slater (1962). A molar extinction coefficient of 131.5 $\mu mole^{-1}$. ml . cm^{-1} was used to determine the number of μmoles of the cytochrome c reduced.

4.2.2.4 - Glucose-6-phosphatase

Glucose-6-phosphatase (EC.3.1.3.9) activity was assayed spectrophotometrically by determining the inorganic phosphate liberated after incubation for 30 min at 37°C as described by Ames (1966). The reaction mixture (220 μl) consisted of 100 mM Tricine (pH 6.0), 2 mM EDTA, 10 mM NaF, 10 mM glucose-6-phosphate and 10-20 μg enzyme protein. The amount of inorganic phosphate liberated was assayed as described in Section 2.6.2.

4.2.2.5 - Malate dehydrogenase

Malate dehydrogenase (EC.1.1.1.37) activity was measured spectrophotometically by following the reduction of NAD^+ in an assay adapted from Opperdoes et al., (1977). The reaction mixture (0.1 ml) was composed of 100 mM Tris-HCl (pH 7.5), 0.1 mM NAD^-, 0.5 mM malate, 1 mM EDTA and 10-20 μg enzyme protein. The reaction mixture was incubated at 25°C for 15 min and the enzyme reaction stopped by the addition of 100 μl of 50% (m/v) TCA. The amount of reduced NAD (NADH) was then estimated at 340 nm using a Pharmacia LKB Ultrospec III Spectrophotometer. A molar extinction coefficient of 1.109 $mmole^{-1}$. ml . cm^{-1} was used to determine the number of μmoles NAD reduced.

4.2.2.6 - Ribonucleic acids

The RNA content of subcellular fractions was measured by the Schmidt-Thannhäuser method as described by Evans (1979). In this method, each sample containing about 5 mg of protein was precipitated by mixing with 2 ml of ice-cold 300 mM perchloric acid for 15 min. Solutions were centrifuged (10 000 rpm, 4°C, 5 min) and the pellets were washed twice by centrifugation in 2 ml of 300 mM perchloric acid. Each pellet was then digested in 1 ml of 300 mM KOH at 37°C for 60 min. Following digestion, 1 ml of 600 mM perchloric acid was added to each pellet. This final precipitate was collected by centrifugation and retained for DNA analysis as described in Section 4.2.2.7. The absorbance of the supernatant was determined at 260 and 275 nm. The concentration of RNA was calculated using the following formula:

RNA (μg /ml) = $(125 \times A_{260}) - (109 \times A_{275})$.

4.2.2.7 - Deoxyribonucleic acid

The DNA content in subcellular fractions was determined by the diphenylamine reaction as described by Evans (1979). The final precipitate obtained during preparation for the RNA determination (Section 4.2.2.6) was mixed with 1.5 ml of 500 mM perchloric acid and the solution was incubated at 70°C for 30 min. The remaining insoluble material was removed by centrifugation and 1 ml of the supernatant was added to 3 ml of Burton's reagent (see below). The sample was then incubated at 30°C for 16 hours. Tubes containing known amounts of standard DNA (Calf Thymus gland, Sigma Co.) and a blank containing 500 mM perchloric acid, but no DNA, were treated in a similar manner. The absorbance at 600 nm was then determined and DNA content calculated by reference to a standard curve constructed using 0-18 μg DNA.

The Burton's reagent was prepared by dissolving diphenylamine (1.5 g) in glacial acetic acid (100 ml) containing 0.5 ml of acetaldehyde solution (16 mg/ml). This mixture was filtered through Whatman N° 4 filter paper and kept at RT.

4.2.3 - Detection of nucleic acids in subcellular fractions by ethidium bromide staining

The distribution of nucleic acids within the subcellular fractions was also analysed by ethidium bromide staining performed on 2 % (w/v) agarose gels. The gel solution was prepared by boiling agarose in buffer E and then cooled to 50 - 60°C before casting. The gel was cast in a 5 cm x 7.5 cm horizontal format plastic tray that could be installed on the platform of the MINNIE Submarine Agarose gel unit Model HE 33 (Hoefer Scientific Instruments, San Francisco, USA). After solidification, the gel was placed in the electrophoresis tank and covered with sufficient buffer E containing 0.5 μg per ml ethidium bromide (Sambrook et al., 1989). Each sample (15-20 μl, representing 10-15 μg protein) was mixed with 5 μl of the gel-loading buffer (0.025 % bromophenol blue, 50 % glycerol and 100 mM EDTA) and slowly loaded into the sample slot of the submerged gel. Electrophoresis was carried out at 100 V for 1.1 hour at room temperature. On completion of electrophoresis, gels were viewed on a Fotodyne incorporated UV light box and photographed.

4.2.4 - Isopycnic centrifugation

Prior to subjecting the sample to sucrose gradient centrifugation, the microsomal (MI) fraction was further fractionated by isopycnic centrifugation in Percoll by a procedure adapted from Grab et al. (1987). The MI fraction was made 57.6 % (v/v) with respect to Percoll in buffer F.

This suspension (3 ml) was overlayered sequentially with 3 ml each of 43.2, 28.8 and 20.3 % Percoll solutions. The gradient was centrifuged (25 000 x g, 4°C, 60 min). Fractions (1 ml) were collected by draining the tube from the top with the aid of a peristalic pump. The absorbance of the collected fractions was measured at 280 nm and the AcP activity assayed as described in Section 2.7.1.

After centrifugation in Percoll, the recovered AcP activity was diluted (1:5) in buffer G and layered on top of a 5-55 % (w/w) linear sucrose gradient prepared in the same buffer. This gradient was layered on top of a cushion of 65 % (w/w) sucrose (1 ml). The gradient was centrifuged (120 000 x g, 4°C, 14 h) using the slow acceleration mode. After centrifugation, gradient fractions (1 ml) were collected starting from the top as above. Fractions were assayed for AcP activity and the absorbance at 280 nm was measured. The purified organelles were resuspended in buffer G, pelleted at 120 000 x g for 1 h and finally resuspended in the same buffer at a protein concentration of 5-10 mg/ml, frozen and stored at -70 °C until use.

4.2.5 - Results

The subcellular distribution of AcP was evaluated using the protocol illustrated in Figure 9.

4.2.5.1 - Distribution of acid phosphatase in subcellular fractions

The five subcellular fractions obtained by differential centrifugation were evaluated for their ability to hydrolyse p-NPP. The distribution of AcP activity over the 5 fractions is presented in Table 5.

Most AcP activity was found predominantly distributed in fraction F_4 which corresponds to the microsomal fraction (Table 5). This was in agreement with previously published results obtained on *T. brucei* (Rovis and Baekkeskov, 1980; Steiger et al., 1980; Opperdoes and Steiger, 1981). However, the cytosolic fraction (F_5) of *T. congolense* appeared to contain a high level of total enzyme activity when compared to the distribution reported for *T. brucei*. This may be related to the cell disruption methods, i.e., French pressure cell (in the present study), nitrogen cavitation used by Rovis and Baekkeskov (1980) and grinding with silicon carbide abrasive grain in the case of Steiger et al.(1980). Although fractions F_4 and F_5 were both enriched with AcP activity, the specific activity was three-fold higher in fraction F_4 than in fraction F_5.

Table 5 - Distribution of acid phosphatase activity in subcellular fractions of *T. congolense*.

Fractions[a]	Centrifugal force (x g)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Distribution[b] (%)
F_1 (NU)	750	0.34 ± 0.09	0.022 ± 0.006	0.065	0.4
F_2 (LG)	3 000	0.59 ± 0.38	0.052 ± 0.007	0.088	1.0
F_3 (SG)	15 000	0.92 ± 0.23	0.073 ± 0.027	0.079	1.4
F_4 (MI)	123 000	10.98 ± 1.69	2.711 ± 0.323	0.247	52.7
F_5 (CY)	>123 000	32.87 ± 7.92	2.282 ± 0.282	0.069	44.4

[a]Fractions F_1 - F_5 correspond to the subcellular fractions described by Grab et al. (1987) and called NU, nuclear; LG, large granule; SG, small granule; MI, microsomal; and CY, cytosolic fraction.
[b]Distribution is expressed as percentages of the ratios of activity in the fraction over the sum of the five fractions obtained. Values refer to the means of three representative experiments.

4.2.5.2 - Distribution of biochemical markers in subcellular fractions

The five subcellular fractions obtained from *T. congolense* homogenates were tested for the presence of various biochemical markers specific to different cellular organelles. These biochemical markers permit assessment of the efficiency of the cell fractionation procedure developed in this study. Cysteine protease, cytochrome c reductase, malate dehydrogenase, glucose-6-phosphatase, DNA and RNA were selected as markers for lysosomes, mitochondria, glycosomes, endoplasmic reticulum, nuclei and ribosomes respectively. The distribution of selected biochemical markers over the five fractions are given in Table 6.

In the present study, the microsomal fraction was found to have the highest level of AcP activity (52.7 %). It contains only 2.4 % cysteine protease (Table 6) which is known to be a lysosomal enzyme in *T. congolense* (Mbawa et al., 1991). The cysteine protease activity was found in substantial amounts in the large and small granule fractions. The lack of significant overlap between the cysteine protease and AcP activities suggests that the two enzymes are not located in the same organelle. Therefore, AcP could not be considered to be a lysosomal enzyme in *T. congolense*, as has been established in mammalian cells (Lemansky et al., 1985).

Table 6 - Distribution of biochemical markers in various fractions obtained after differential centrifugation of *T. congolense* homogenates.

Fractions[a]	Distribution (%)[b]							
	Protein[c]	AcP	CysP	CycR	G6P	MDH	RNA	DNA
F_1 (NU)	0.7	11.9	2.5	27.4	6.8	17.2	28.1	35.6
F_2 (LG)	1.3	16.0	34.7	25.3	18.9	43.1	13.2	24.4
F_3 (SG)	2.0	14.4	59.7	21.3	11.5	21.5	14.6	24.6
F_4 (MI)	24.1	45.1	2.4	10.9	14.4	5.8	30.7	12.5
F_5 (CY)	71.9	12.6	0.6	15.3	48.1	12.5	13.5	3.9

[a]Fractions F_1 - F_5 (see Table 5 for explanation).
[b]Distribution of AcP, acid phosphatase; CysP, cysteine protease; CycR, cytochrome c reductase; G6P, glucose-6-phosophatase and MDH, malate dehydrogenase are expressed as the percentage of specific activity in the fraction over the sum of the five fractions.
[c]Total protein in mg determined as described Section 2.8.
Results are expressed as the mean of triplicate determinations of three separate experiments outlined in Section 4.2.2

Cytochrome c reductase activity was found to be distributed over the first three fractions. The wide distribution of this mitochondrial marker suggests either damage of the mitochondria or, alternatively that this enzyme may be easily detached from the mitochondria. However, malate dehydrogenase which is also located in glycosomes and mitochondria of *T. cruzi and T. brucei* (Cazzulo, 1992) was found mostly in the large granule fraction. This implies that the large granule fraction was enriched in mitochondria.

The total microsomal fraction consists of fragments of the endoplasmic reticulum and ribosomes plus other membrane fragments of similar sedimentation characteristics. This fraction has been shown to contain glucose-6-phosphatase activity, a classical marker for endoplasmic reticulum in mammalian cells (Morré, 1971). Surprisingly, the highest glucose-6-phosphatase activity was found in the cytosolic fraction obtained from

T. congolense homogenates, whereas in *T. brucei*, Rovis and Baekkeskov (1980) localised this enzyme in the large granule fraction.

Analysis of nucleic acid distribution showed the DNA (35.6 %) to be concentrated in the nuclear fraction as expected. This was also confirmed on agarose gels where nuclear fraction samples were digested by DNase I (Fig. 10, lane c). In addition, 30.7 % of RNA located in the microsomal fraction may be associated with the presence of ribosomes. However, microsomal fraction samples digested by RNase A did not indicate any significant degradation as analysed on agarose gels (Fig. 10, lane g). Nevertheless, contamination of nuclear DNA in the microsomal samples could be observed on the agarose gel.

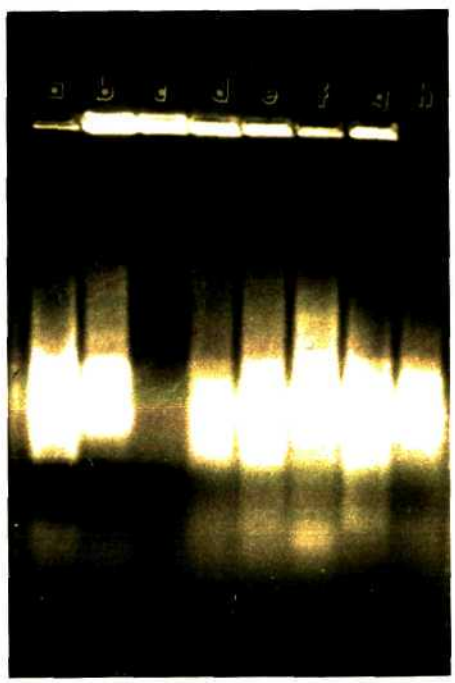


Figure 10 - Agarose gel electrophoresis of subcellular fractions examined for nucleic acid by ethidium bromide staining.

Subcellular fractions (10-15 μg proteins) were analysed on agarose gels (2%) as described in Section 4.2.3. Fractions F_1 - F_5 (see Table 5)corresponding to the subcellular fractions described by Grab et al. (1987) were analysed. Homogenate (lane a), NU (lane b), NU treated with DNase I (lane c), LG (lane d), SG (lane e), MI (lane f), MI treated with RNase A (lane g) and CY (Lane h).

4.2.5.3 - Isopycnic centrifugation of microsomal membranes

In order to achieve further purification of the AcP detected in the microsomal fraction, samples were centrifuged under the conditions described in Section 4.2.4. These samples were centrifuged in discontinuous Percoll gradients prior to applying them onto a linear sucrose gradient. The density equilibration profiles of protein and AcP are depicted in Figure 11.

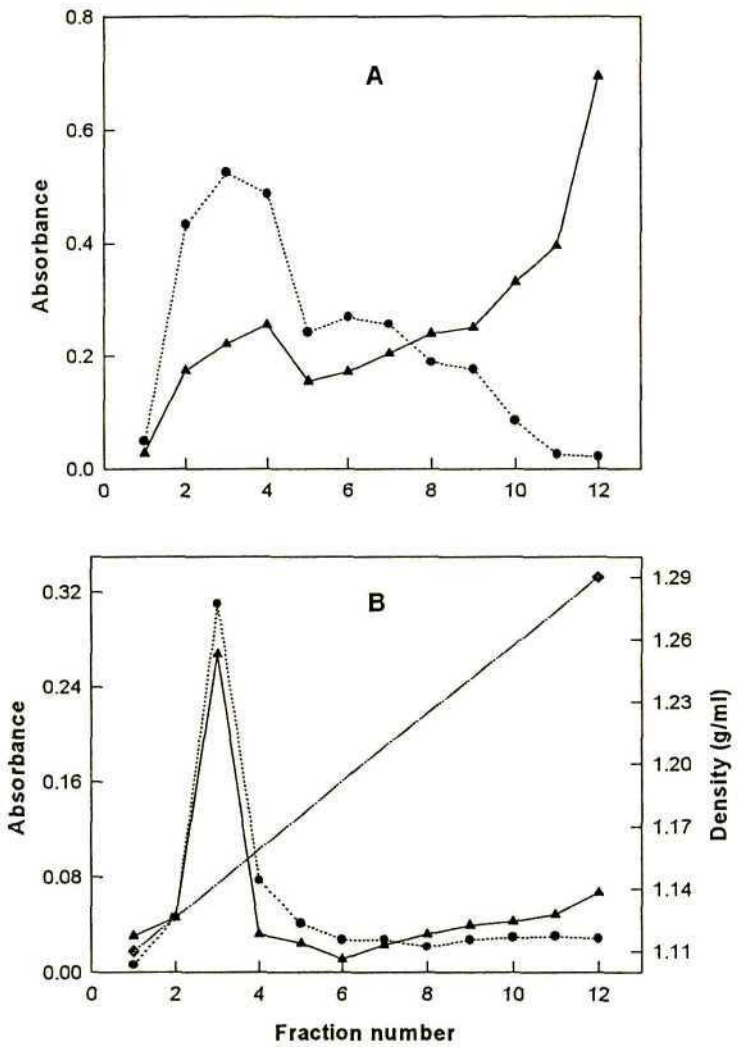


Figure 11 - Isopycnic distribution of the microsomal fraction (F_4) in Percoll (A) and in linear sucrose (B) gradients.

The microsomal fraction was centrifuged in Percoll and the peak containing AcP activity (fractions 2-4) was then loaded onto a linear sucrose gradient. Fraction number 1 represents the top of the gradients. The A_{405} (●), A_{280} (▲) and sucrose density (◆) were analysed.

Acid phosphatase activity could be detected in a concentrated, well defined peak in the 36% (w/w) sucrose layer (density ~1.15g/ml). It is tentatively concluded that the enzyme is associated either with endoplasmic reticular membranes or with the Golgi apparatus since these cell constituents all equilibrate at densities between 1.14 and 1.15 g/ml as demonstrated in *T. brucei* (Steiger et al., 1980; Opperdoes and Steiger, 1981).

This peak of AcP activity obtained from the sucrose gradient was diluted with 3 vol. of 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, and centrifuged at 6000 rpm in a microfuge for 5 min. The pellet recovered was analysed by electron microscopy (see later, Figure 19).

4.2.6 - Discussion and conclusions

In mammalian cells, AcP has been localised principally in lysosomes (Bainton, 1981; Lemansky et al., 1985) and is widely considered to be a lysosomal marker. In contrast, AcP has been localised in the lumen of the flagellar pocket of bloodstream forms of African trypanosomes. The enzyme was found to be partly associated with intracellular membranes comprising structures such as the endoplasmic reticulum, Golgi apparatus and endosomal-like system. This localisation is based on biochemical evidence (Rovis and Baekkeskov, 1980; Steiger et al., 1980; McLaughlin, 1982; Grab et al., 1987) as well as cytochemical evidence (Langreth and Balber, 1975). More recently, Grab and co-workers (1997) have localised AcP in a subfraction of Golgi preparations form *T. brucei*. The enzyme has characteristics of being part of the trans-Golgi network.

In *T. congolense*, AcP was found predominantly in the microsomal (F_4) and cytosolic fractions (F_5). The microsomal AcP activity did not overlap significantly with cysteine protease activity. The cysteine protease activity, known to be a lysosomal marker (Mbawa et al, 1991), was found predominantly in fraction F_3 (small granules). Thus, AcP did not appear to be associated with lysosomes. This is in agreement with observations of Grab et al. (1987) who localised AcP in the Golgi apparatus of *T. brucei*.

The microsomal fraction subjected to isopycnic centrifugation showed that the AcP activity of *T. congolense* sediment at a density of ~1.15 g/ml in sucrose gradients. Therefore, it was tentatively concluded that AcP is associated either with endoplasmic reticulum membranes or with the Golgi apparatus since these cell constituents all sediment at densities between

1.14 and 1.15 g/ml as demonstrated in *T. brucei* (Steiger et al., 1980; Opperdoes and Steiger, 1981). The cysteine protease activity of *T. brucei* sediment at a density of ~1.17 g/ml which is in a similar range to that of other lysosomal enzymes such as α-mannosidase (Steiger et al., 1980). These results provide additional evidence that cysteine protease and AcP activities do not share the same subcellular location.

Cytochrome c reductase was distributed throughout the first three fractions F1, F2 and F3 (crude nuclear, large granule and small granule fractions). Although it is a reliable marker enzyme for the mitochondria, this enzyme could not serve as an unequivocal mitochondria marker. Cytochrome c reductase was shown to be present in both bovine mitochondria and endoplasmic reticulum (Amar-Costesec et al., 1974).

The glucose-6-phosphatase activity, which is a classical marker for endoplasmic reticulum in mammalian cells, was found to be the highest in the cytosolic fraction of *T. congolense*. This enzyme was shown to be predominant in the large granule fraction of *T. brucei* (Rovis and Baekkeskov, 1980). The discrepancy between our results and those of Rovis and Baekkeskov (1980) may be explained by a difference in the method of cell breakage. Alternatively, this may reflect a difference between the two species of trypanosomes.

Malate dehydrogenase was predominantly recovered in the large-granule fraction from *T. congolense*. However, because it has been reported to be located in glycosome and mitochondria (Cazzulo, 1992) this enzyme was not considered to be an unequivocal enzyme marker for glycosomes.

Based on the results of the present study, it is reasonable to assume that the microsomal fraction is the most probable subcellular localisation site of AcP. The precise organelles within this fraction containing the AcP enzyme are still to be elucidated. Since AcP activity was found to co-sediment with a substantial proportion of radiolabelled plasma membranes (Chapter 3), it is assumed that part of the AcP activity contained in the microsomal fraction is originating from the surface of trypanosomes.

Finally, the microsomal AcP activity did not overlap significantly with cysteine protease activity (a lysosomal marker in trypanosomes). Consequently, AcP did not appear to be associated with lysosomes like in mammalian cells.

4.3 - Cytochemical localisation of acid phosphatase activities

4.3.1 - Introduction

Multiple localisations of AcP activity in different organelles of African trypanosomes have been reported previously (Seed et al., 1967; Venkatesan et al., 1977; Langreth and Balber, 1975). The heterogeneity of AcP localisation in the *Trypanosomatidae* family appeared to be a common phenomenon as it has also been reported in *Leishmania* sp. (Remaley et al., 1985; Avila et al., 1989). Moreover, studies of a related insect flagellate, *Crithidia* sp., confirmed the heterogeneous localisation of AcP, as evidenced by differences in enzyme latency and sedimentation as well as in the kinetic properties of isoenzymes (McLaughlin et al., 1976).

Subcellular studies on bloodstream forms of *T. congolense* using differential centrifugation of trypanosome homogenates have revealed the presence of two fractions with high AcP activity. These activities were associated with the microsomal and cytosolic fractions. Earlier, studies performed on living trypanosomes indicated the association of AcP activity with the surface of the parasite (Tosomba et al., 1996). These results suggest the existence of multiple AcP activities in *T. congolense*. Therefore, the localisation of AcP was examined at the ultrastructural level using a combination of light microscopy and transmission electron microscopy.

4.3.2 - Experimental procedures

4.3.2.1 - Reagents

Cacodylate stock solution (1 M). Sodium cacodylate (10.7 g) was dissolved in 45 ml of dH_2O, titrated to pH 7.2 with sodium hydroxide, and made up to 50 ml. For use, the stock solution was diluted to 100 mM, and the pH rechecked.

100 mM sodium cacodylate (pH 7.2), 200 mM sucrose (buffer B). A 100 mM sodium cacodylate solution was prepared by mixing 10 ml of the 1 M sodium cacodylate stock

solution, 6.846 g sucrose and approximately 80 ml dH_2O. The pH was adjusted to pH 7.2 with dilute sodium hydroxide and made up to 100 ml with dH_2O.

20 mM Na acetate (pH 5.0) containing 200 mM sucrose (buffer C). Glacial acetic acid (0.114 ml) and sucrose (6.846 g) were dissolved in approximately 80 ml dH_2O, the solution titrated to pH 5.0 with dilute NaOH, and made up to 100 ml with dH_2O.

Paraformaldehyde stock solution (16%). Paraformaldehyde (1.6 g) was dissolved in dH_2O (100 ml), warmed to 60°C and cleared with a minimum amount of 1 M NaOH. The solution was stored at -20°C until required.

Glutaraldehyde (1%). 25% glutaraldehyde (1 ml) was diluted to 25 ml with 100 mM sodium cacodylate buffer, pH 7.2.

Fixative [2% paraformaldehyde, 1% glutaraldehyde]. 16% Paraformaldehyde stock solution (1.25 ml) and 25% glutaraldehyde (0.4 ml) were made up to 10 ml with buffer B. The final pH was checked and adjusted to pH 7.2, if necessary.

Epon-Araldite resin. EPON 812 (1 part), Araldite CY212 (1 part), Dodecyl succinic anhydride (DDSA) (3 parts) and 2,4,6,-tridimethylaminomethyl phenol (DMP-30) (1.drop/ml).

4.3.2.2 - Fixation

The cytochemical procedure for the detection of AcP was adapted from Langreth and Balber (1975), Miyayama (1975) and Jones and Bowen (1993). Cytochemical reactions were carried out in 1.5 ml microcentrifuge tubes unless otherwise stated. Purified trypanosomes (5×10^6 cells) were washed twice in ice-cold buffer B by centrifugation (2 x 3 min at 1 000 x g). The cells were then resuspended in fixative solution and fixation carried out for 16 h at 4°C. The fixed trypanosomes were washed in three changes of buffer B, followed by three changes of buffer C. The trypanosomes were preincubated in buffer C containing 2 mM $Pb(NO_3)_2$ at 37°C for 10 min and then incubated at the same temperature for 60 min in buffer C containing 2 mM $Pb(NO_3)_2$ and either p-NPP (5 mM or greater) or β-glycerophosphate (25 mM). Control samples were incubated and treated under the same conditions either in the absence of substrate or in a medium containing both substrate and NaF (10 mM). After incubation,

trypanosomes were rinsed in three changes of buffer B and refixed for 60 min in 2 % (v/v) glutaraldehyde in buffer B. Cells were again washed in three changes of buffer B.

4.3.2.3 - Light Microscopic AcP Cytochemistry

For light microscopy, fixed trypanosomes (Section 4.3.2) were resuspended in 5 mM ammonium sulphide for 15 min, washed 3 times in 10 mM phosphate buffer (pH 7.2), and resuspended in this buffer. Smears were prepared from these suspensions onto gelatin-coated glass slides which were then lightly flamed to aid adhesion. The smears were stained with Giemsa stain for 1 hour and thereafter all the stain was washed off with 10 mM phosphate buffer (pH 7.2). The specimen was allow to dry at room temperature and examined under an oil-immersion lens with an Olympus BH-2 microscope equiped with an Olympus C-35 ADA camera. Alternatively, images were enlarged by using a Kontron Vidas digital image processing unit, system 2.1.

4.3.2.4 - Electron Microscopic AcP Cytochemistry

Fixed trypanosomes (Section 4.3.2.2) were post-fixed in ice-cold buffer B containing 1% OsO_4 for 1 hour. After washing three times with distilled water, cells were stained with 2% (w/v) aqueous uranyl acetate for 4 hours at room temperature and then washed twice with distilled water. The samples were dehydrated in a graded series of acetone washes (40, 60, 80 and 100 % for 10 min each wash), cleared in 100 % propylene oxide (2 x 10 min), embedded as pellets in freshly prepared propylene oxide - Epon-Araldite resin (3:1, 1:1, 1:3) for 2 hours in each mixture and then in 100 % epoxy resin (overnight) at room temperature. Finally, samples were embedded in 100 % resin at 70°C for 48 hours. Ultrathin sections were cut on an ultracut E-ultramicrotome with a diamond knife, transferred onto 200 mesh copper grids, stained for 10 min with lead citrate and washed with distilled water. Micrographs were taken on a Joel 100CX transmission electron microscope operating at 80 kV.

4.3.3 - Results and discussion

4.3.3.1 - Light microscopic observations

At the light microscope level, the localisation of AcP activity was performed with β-glycerophosphate and p-NPP substrates. In this technique, the reaction product was visualised by the formation of a black lead sulfide deposit.

Trypanosomes appeared morphologically intact, with no apparent signs of cytoplasmic degeneration. However, it was difficult to determine any perceptible reaction product of Pb sulfide either within the cytoplasm or on the surface of cells. (Fig. 12a-c,). A Kontron Vidas digital image processing unit (system 2.1) was used to enlarge images (Fig. 12d). Because of the relatively low level of the resolution given by light microscopy, it was impossible to localise the reaction products precisely in trypanosomes. Extending the incubation time up to 2 h did not greatly enhance the staining pattern to allow any localisation at the ultrastructral level.

4.3.5.2 - Electron microscopic observations

Acid phosphatase was localised ultrastructurally by electron scattering of the lead phosphate reaction product. Observations of ultra-thin sections of *T. congolense* used in control experiment samples lacking either p-NPP (Fig. 13a) or β-glycerophosphate (Fig. 13b) displayed general ultrastructural aspects. Micrographs confirmed that the general morphology of trypanosomes was maintained during glutaraldehyde fixation as observed before using light microscopy. Cells incubated in the presence of 10 mM NaF, an AcP inhibitor, did not show the reaction product with either 5 mM p-NPP (Fig. 13c) or 25 mM β-glycerophosphate (Fig 13d). This would suggest that the localisation observed (see Figs. 14-17) is truly due to enzymatic activity. It also confirms that the phosphatase activity is due to an acid rather than an alkaline phosphatase.

In trypanosomes that were incubated in the presence of either 25 mM β-glycerophosphate (Fig. 14a) or 5 mM p-NPP (Fig. 14b), the lead phosphate deposits were observed primarily in the flagellar pocket region (arrowheads). The localisation of AcP in the flagellar pocket agrees with that reported in other African trypanosomes (Seed et al., 1967; Langreth and Balber, 1975; Venkatesan et al., 1977). In some cases, discrete black spherical deposits of lead phosphate were observed adjacent to the flagellar pocket (Fig. 15, arrows) which suggests the presence of AcP in these vesicles. Therefore, this enzyme may be involved in the endocytosis process in which nutrients are taken to the lysosome for further degradation by the endosome-like system.

Lead phosphate depositions were not detected either on the Golgi apparatus (Fig.16a) or on the endoplasmic reticulum (Fig. 16b). However, AcP activity was found associated with the

microsomal particles purified by isopycnic centrifugation (Section 4.2.5.3). This observation would indicate that the substrate is probably not accessible to the enzyme in these cytochemical studies.

In Chapter 3, studies performed with living trypanosomes suggested the presence of an AcP activity associated with the surface of the parasites. However, in some preparations of trypanosomes that had been incubated with 10 mM or higher concentration of p-NPP, an electron-dense precipitate of lead phosphate was observed uniformly distributed over the surface of parasites (Fig17a) in addition to the enzyme reaction product observed in the flagellar pocket region and in some vesicles. An identical localisation of AcP on the surface membrane had been reported in an other trypanosomatid, *Leishmania donovani* (Gottlieb and Dwyer, 1981). It is important to point out that not all cells were completely labelled (Fig. 17b). Occasionally, the electron-dense precipitates of lead phosphate that were not associated with cells were also observed (Fig. 17c). This phenomenon may occur during the experimental procedure of obtaining ultrathin sections of parasites. *T. congolense* is also reported to be able to remove a limited amount of potentially lytic immune complexes from its surface by filopodia formation (Frevert and Reinwald, 1990). Possibly this phenomenon may have occurred even though no antibody was used in this experiment.

4.3.5.3 - Energy dispersive X-Ray microanalysis

In order to ascertain whether the electron dense precipitate distributed over the trypanosomes'surface was due to the lead phosphate complex, an energy dispersive x-ray microanalysis (EDX) was performed on ultrathin sections of *T. congolense* that were unstained with lead citrate. A Link exl II EDX ATW2 detector attached to a Joel 100CX transmission electron microscope was used to analyse the lead content of the surface labelling. Significant peaks of copper (from the grid), iron (from the pole piece) and molybdenum (from the aperture) were obtained together with lead resulting from the specimens (Fig. 18).

4.3.5.4 - Morphology of microsomal pellets

A preparation of microsomal pellets obtained by differential centrifugation were fixed and analysed by electron microscopy. These microsomal pellets proved to be very heterogenous and consisted of free flagella (arrows), cell membrane fragments, various spherical inclusions and vesicles (Fig. 19).

In addition, analysis of preparations of microsomal pellets purified further by isopycnic centrifugation (Section 4.2.4) at the electron microscope level revealed that the purified pellets consist of closed, single-walled vesicles which did not contain attached microtubules (Fig. 19c, d). Therefore, these preparations could not be ascribed to either inverted plasma membranes or single plasma membranes as obtained in some trypanosomatids (Gottlieb and Dwyer, 1981; Hunt and Ellar, 1974).

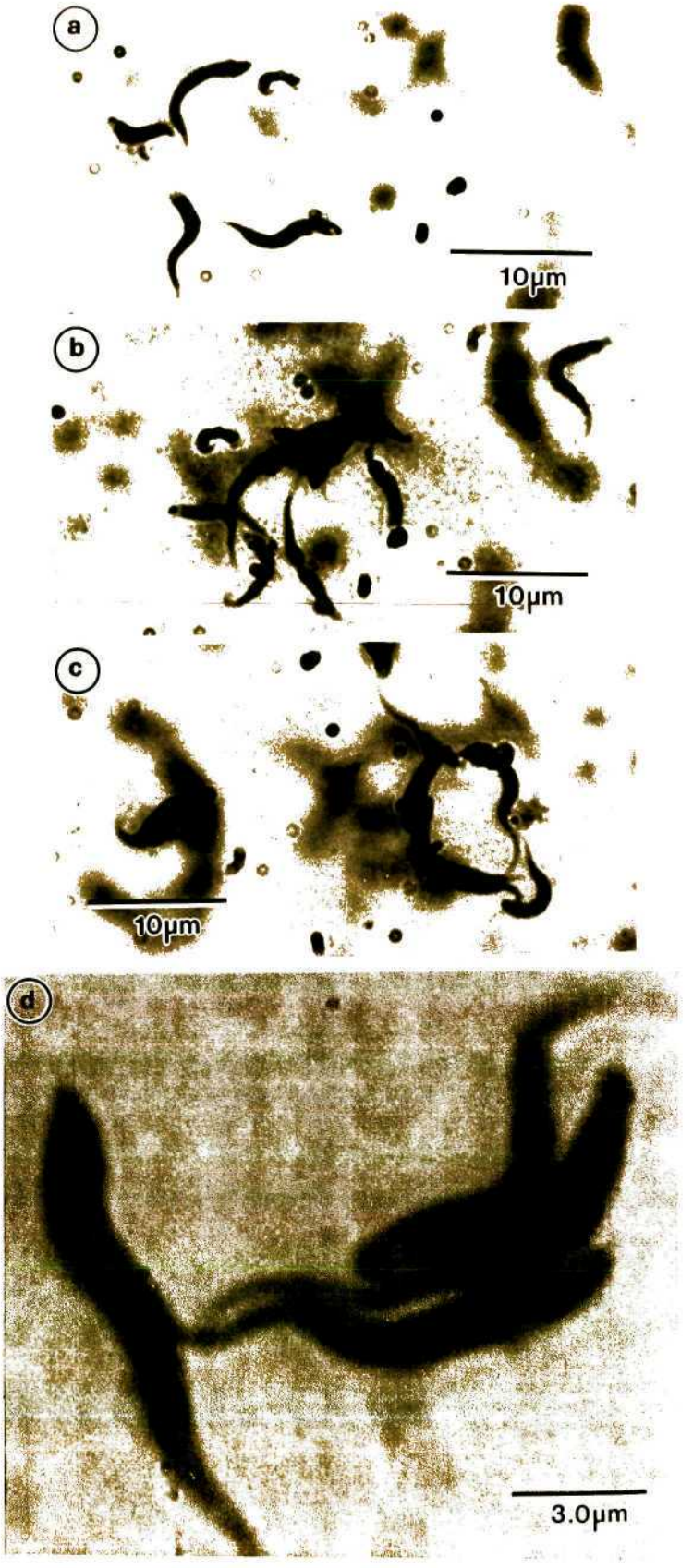

a
10μm
b
10μm
c
10μm
d
3.0μm

Figure 12 - Light microscopic enzyme cytochemistry of acid phosphatase in *T. congolense.*

Control cells that were incubated without any substrate (a); cells incubated with 25 mM β-glycerophosphate (b); or 5 mM p-NPP (c) as substrates, were analysed by light microscopy. An example of the enlarged image of control cells enhanced by using a Kontron Vidas digital image processing unit, system 2.1 is shown in (d).

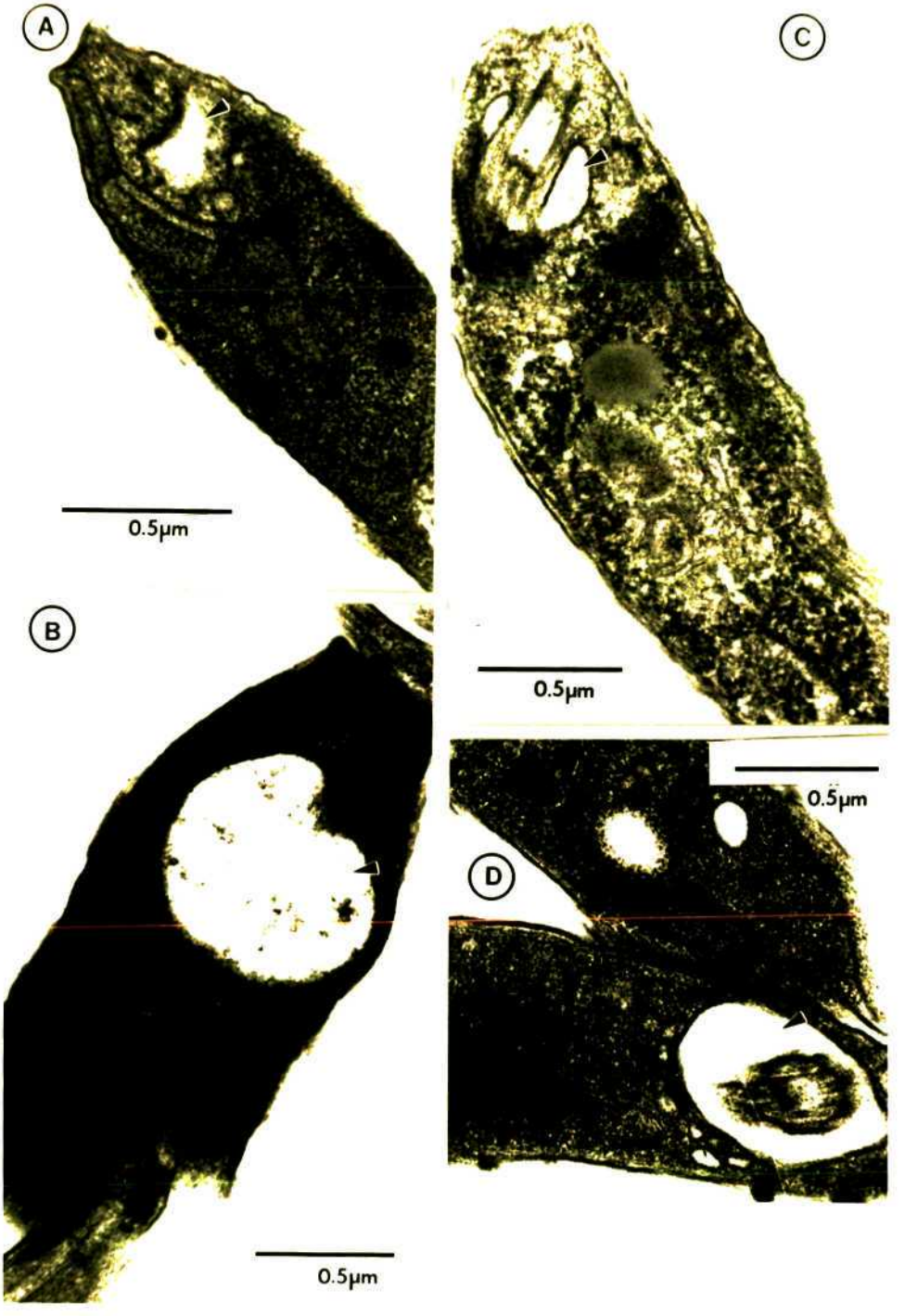

A
0.5µm
C
0.5µm
B
0.5µm
0.5µm
D

Figure 13 - Transmission electron micrograph of *T. congolense* showing general ultrastructure.

Glutaraldehyde-fixed trypanosomes were incubated in 100 mM Na acetate buffer (pH 5.0) in the absence of either 5 mM of p-NPP (A) or 25 mM of β-glycerophosphate (B) as described in Section 4.3.2. Alternatively, trypanosomes were incubated in the above buffer containing both 10 mM NaF and either p-NPP (C) or β-glycerophosphate (D). In both cases (C) and (D), no reaction product was depicted in thin-sections examined for AcP activity. The general ultrastructure of trypanosomes was maintained by this fixation method. Arrow heads indicate flagellar pocket region.

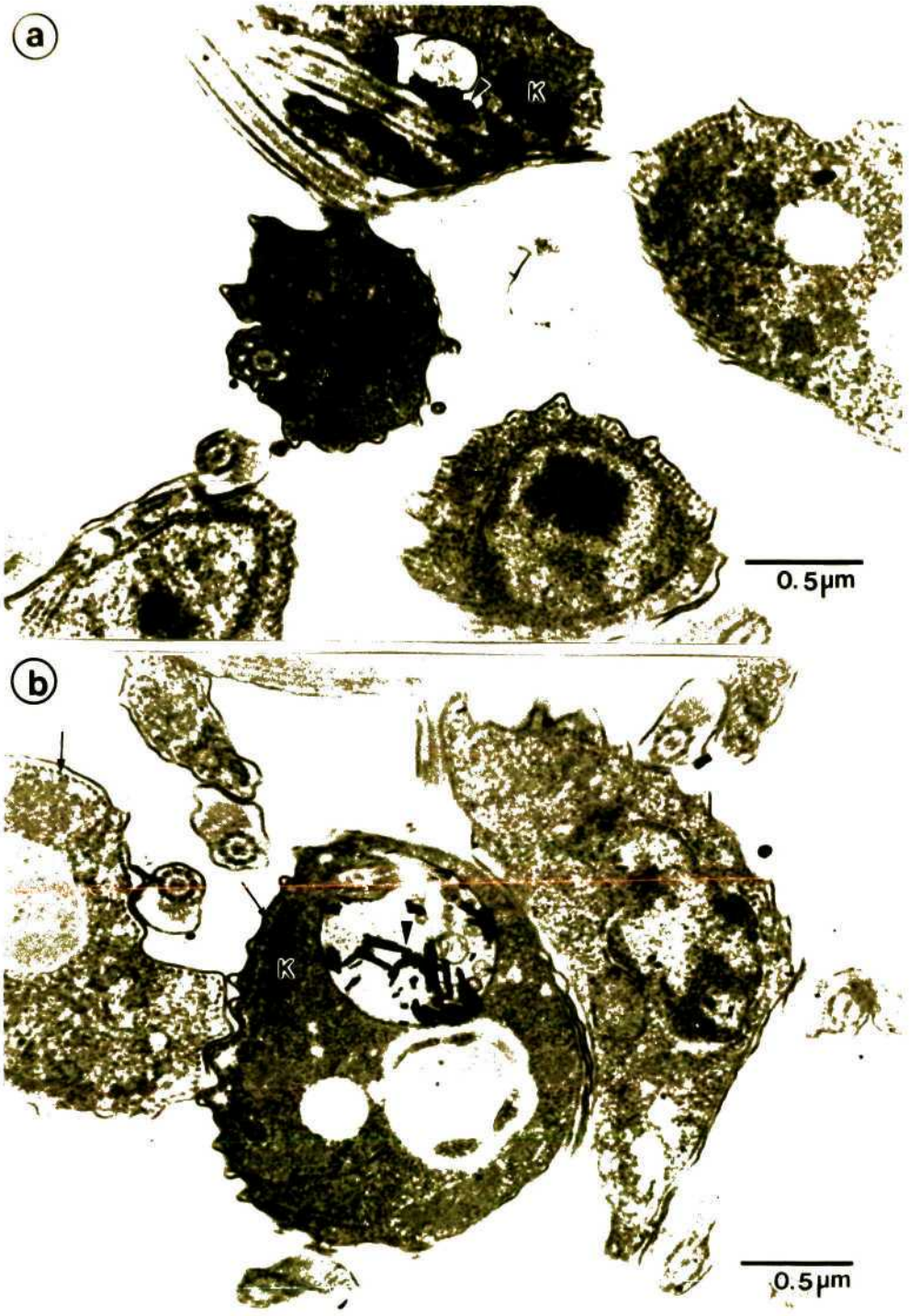

a
K
0.5μm
b
K
0.5μm

Figure 14 - Transmission electron micrograph of *T. congolense* stained for AcP activity.

Glutaraldehyde-fixed trypanosomes were incubated in 100 mM Na acetate buffer (pH 5.0) in the presence of:

(a) β-glycerophosphate (25 mM). Accumulation of lead phosphate particles is observed in the flagellar pocket region (arrowhead) where the flagellum extends from the pocket. The kinetoplast (K), which contains the cell's mitochondrial DNA, is also observed nearby.

(b) Para-nitrophenyl phosphate (5 mM). AcP reaction product is seen in the flagellar pocket region (arrowhead) in a cross section through the pocket. Subpellicular microtubules attached to the inner face of the plasma membrane also appear in this cross section as electron-dense circles (arrows).

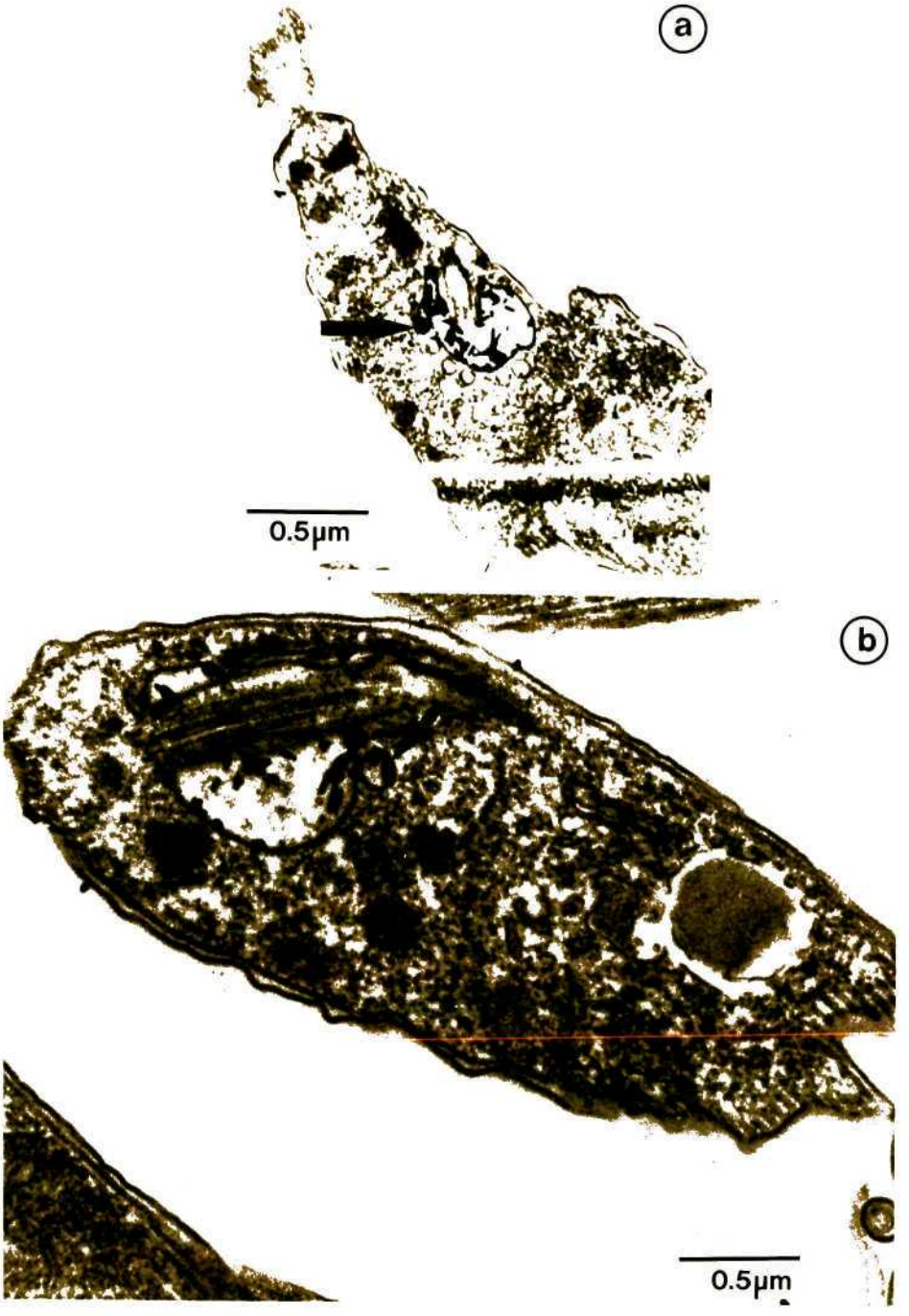

a
0.5µm
b
0.5µm

Figure 15 - Localisation of AcP in the falgellar pocket and adjacent vesicles in bloodstream forms of *T. congolense*.

Glutaraldehyde-fixed trypanosomes were incubated in 100 mM Na acetate buffer (pH 5.0) in the presence of 25 mM β-glycerophosphate (a) or 5 mM of p-NPP (b) as described in Section 4.3.2.4.

The reaction product is found in the flagellar pocket, but is also present in some vesicles (arrows) adjacent to the flagellar pocket.

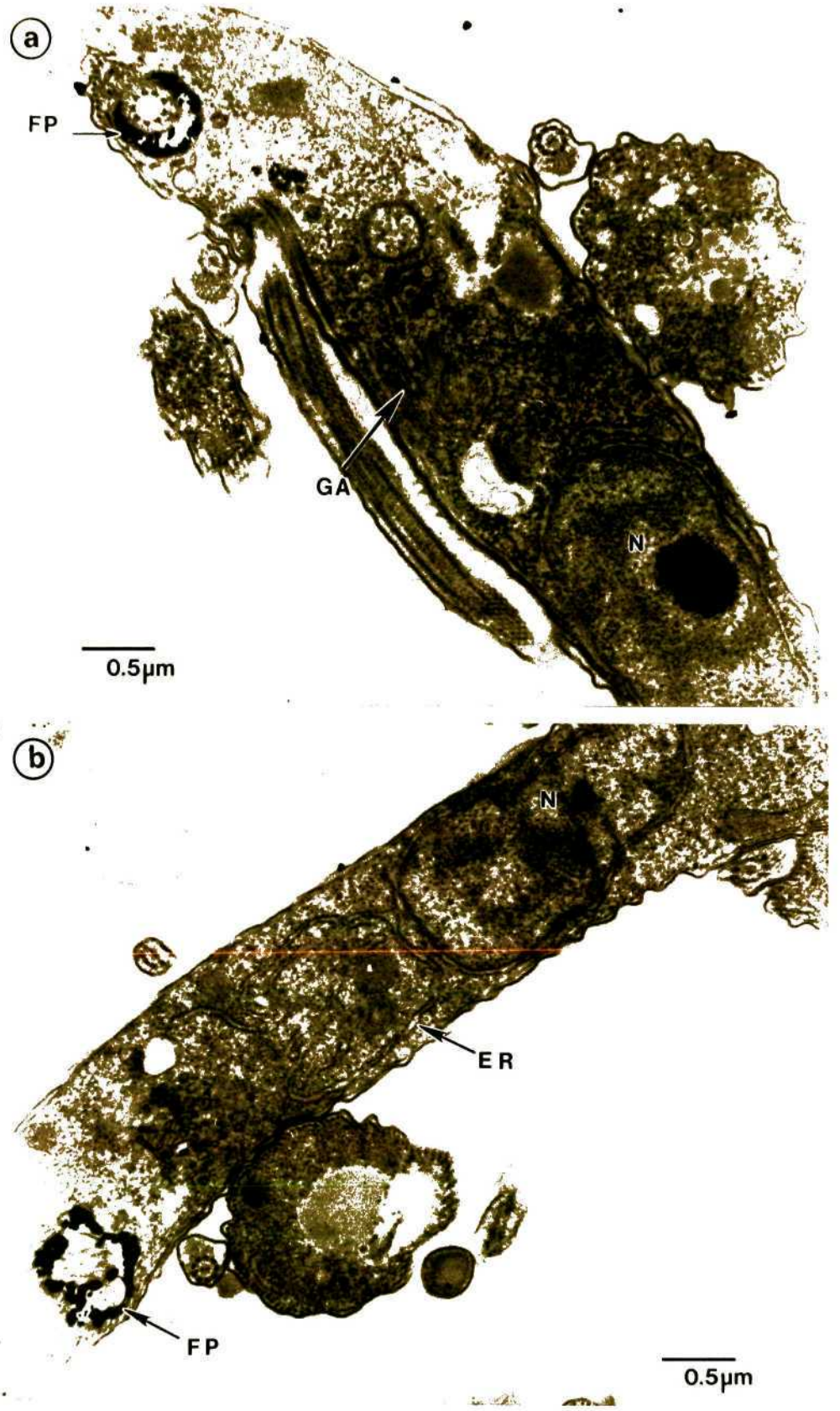

a
FP
GA
N
0.5μm
b
N
ER
FP
0.5μm

Figure 16 - Longitudinal sections of intact *T. congolense* showing the Golgi apparatus and endoplasmic reticulum devoid of lead phosphate deposits.

Glutaraldehyde-fixed trypanosomes were incubated in 100 mM Na acetate buffer (pH 5.0) in the presence of 25 mM β-glycerophosphate (a) or 5 mM of p-NPP (b) as described in Section 4.3.2.4. The reaction product is present in the flagellar pocket (FP), but was neither found in Golgi apparatus (GA, panel a) nor in endoplasmic reticulum (ER, panel b). N, nucleus.

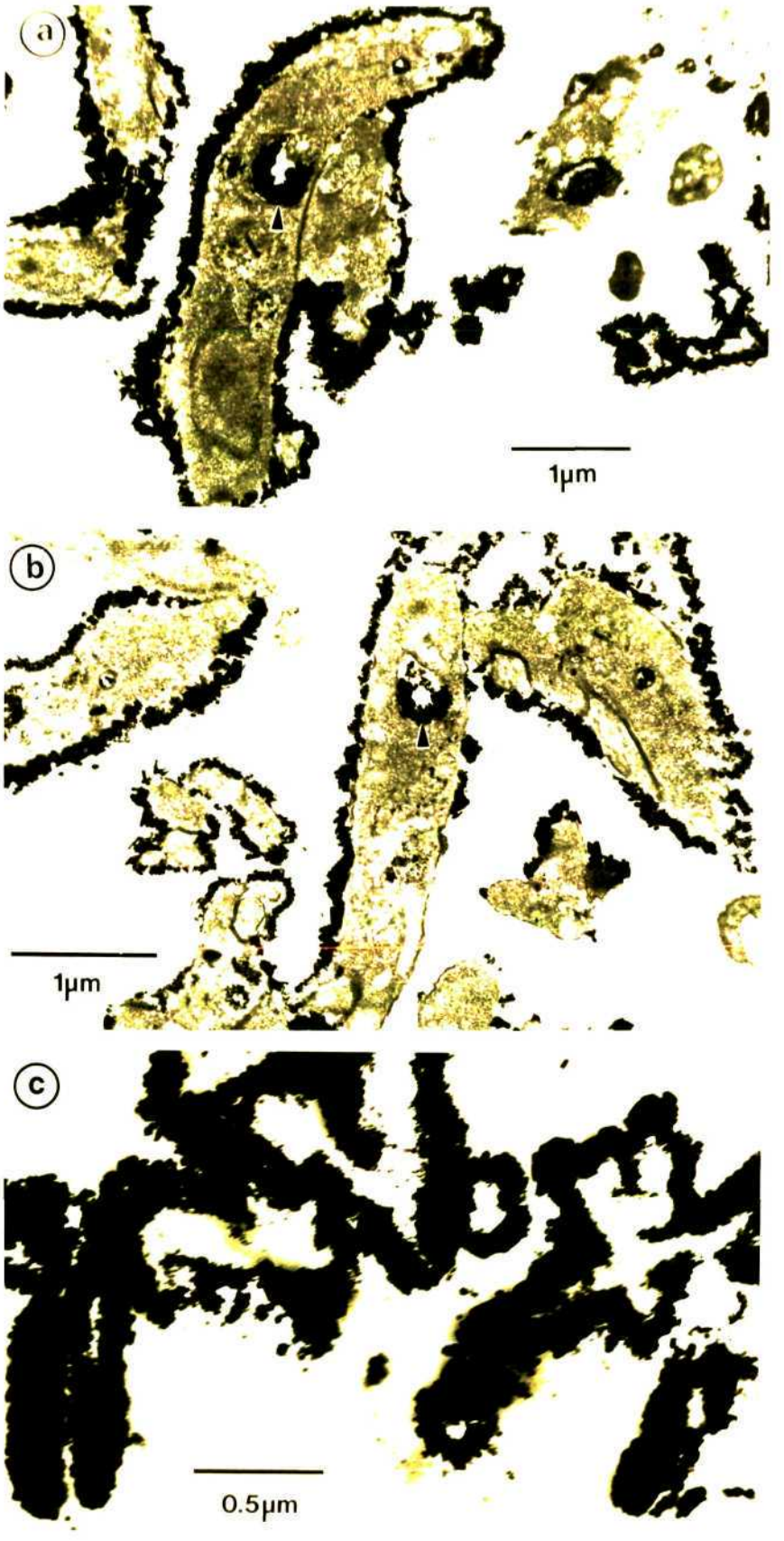

a
1µm
b
1µm
c
0.5µm

Figure 17 - Localisation of surface membrane-associated acid phosphatase activity in intact bloodstream forms of *T. congolense*.

In trypanosomes that had been incubated with 10 mM or higher concentration of p-NPP, an electron-dense of lead phosphate reaction product indicative of AcP activity was observed on the cell surface and also in the flagellar region (arrowheads) and in some vesicles (a & b). Occasionally, some incompletely labelled cells were observed (b). Sometimes, electron-dense precipitates of lead phosphate that were not associated with cells were found (c).

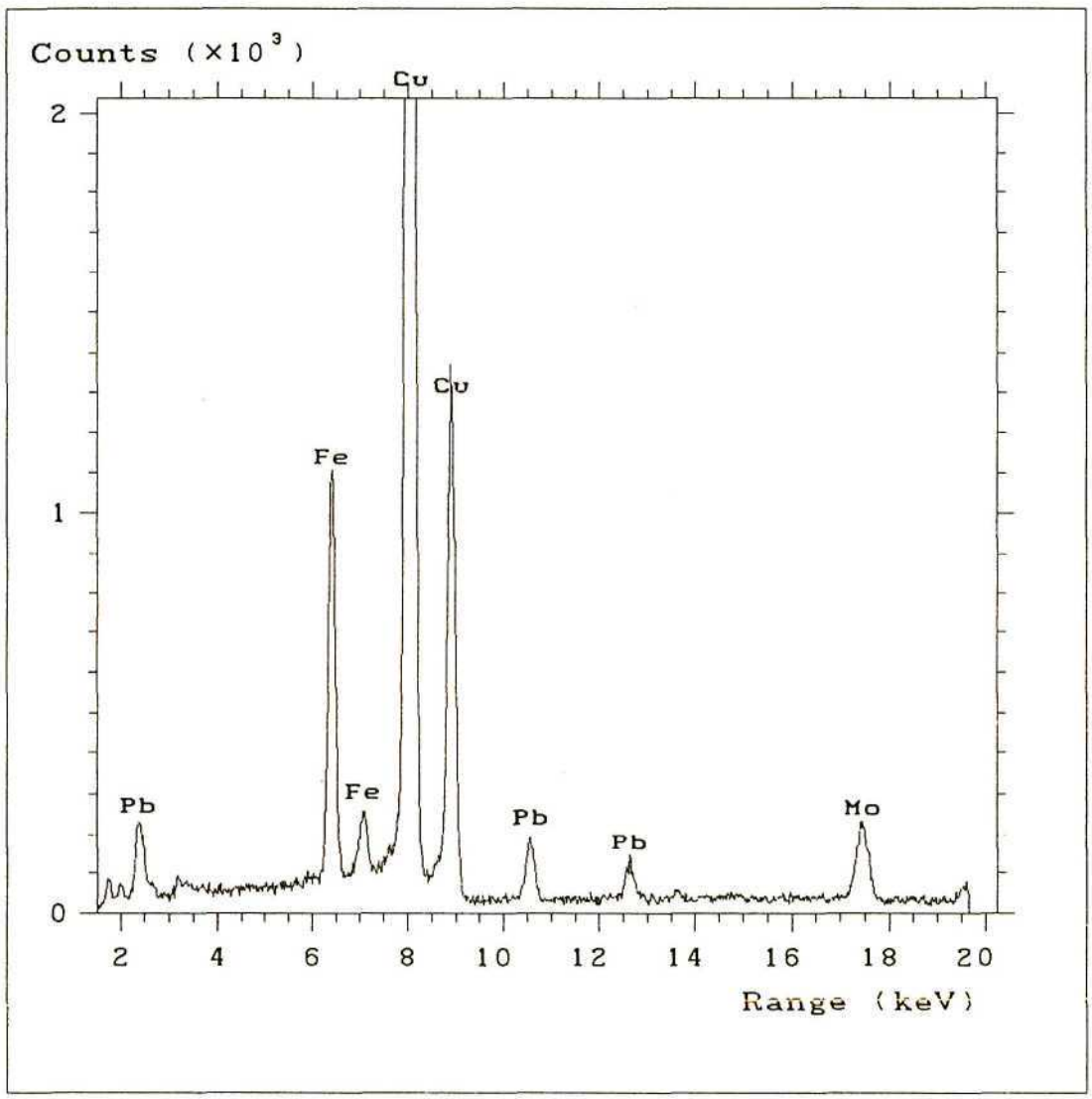


Figure 18 - An energy dispersive X-ray spectrum taken from ultrathin sections of *T. congolense* that were unstained with lead citrate.

In order to ascertain whether the electron dense precipitate distributed over the trypanosomes'surface was due to the lead phosphate complex, an energy dispersive x-ray microanalysis (EDX) was performed on ultrathin sections of *T. congolense* that were unstained with lead citrate (Section 4.3.5.3). Significant peaks of copper (grid), iron (pole piece) and molybdenum (aperture) were obtained simultaneously with lead (specimen).

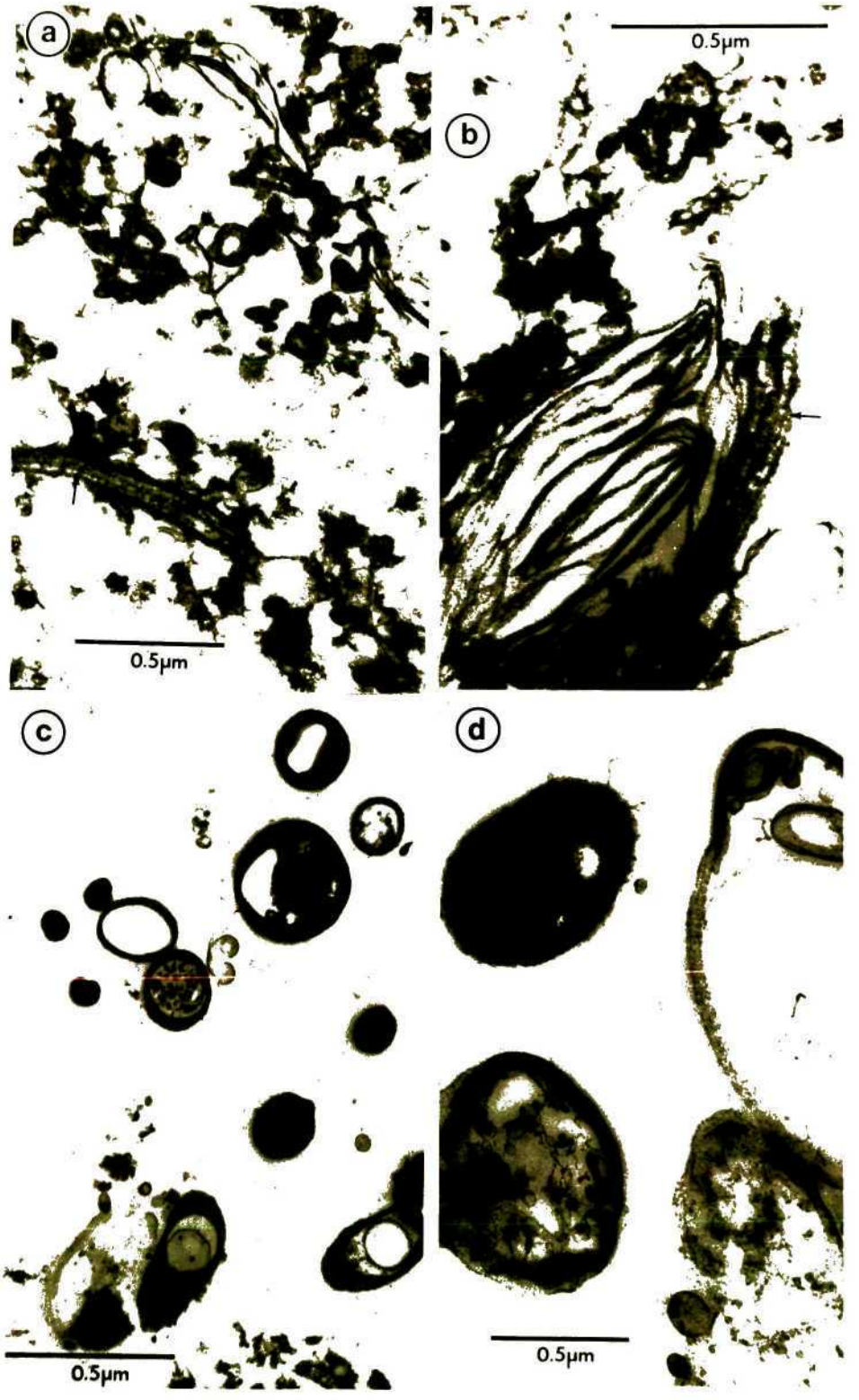

a
0.5μm
b
0.5μm
c
0.5μm
d
0.5μm

Figure 19 - Electron micrograph of thin sections of microsomal fraction derived from *T. congolense* homogenates.

Microsomal pellets prepared by differential centrifugation (Section 4.2.1), glutaraldehyde-fixed (Section 4.3.2.2) and analysed by electron microscope. This fraction was found to contain free flagella (arrow), cell membrane fragments, various spherical inclusions and vesicles. Low magnification (a) and high magnification (b). Further purification of this fraction achieved by means of isopycnic centrifugation (Section 4.2.4). Morphologically, the purified microsomal fractions consist of closed, single- walled vesicles which did not contain attached microtubules (c) and (d).

4.4 - Overview of results and conclusions

The aim of this Chapter was to determine the subcellular location of AcP activity in *T. congolense*. In the first approach, trypanosome homogenates were examined by differential centrifugation. The subcellular fractions obtained indicated that the highest AcP activities, measured under standard conditions, were recovered in the microsomal (52.7%) and cytosolic fractions (44.4%).

Biochemical markers were used to screen the prepared subcellular fractions in order to determine the presence of AcP in specific organelles. The cysteine protease, a known lysosomal enzyme of *T. congolense* (Mbawa et al., 1991), was located in the small granules fraction while AcP activity was highest in the microsomal fraction. The distribution of the two markers did not show any significant overlap between cysteine protease and AcP. This suggested that cysteine protease and AcP do not share the same subcellular location.

Further separation of the microsomal fraction by isopycnic centrifugation showed that AcP activity was concentrated in a well defined peak in the 36% (w/w) sucrose layer (density ~1.15g/ml). This peak appears to contain vesicles as analysed at the electron microscope level. Lysosomes of *T. brucei* have been shown to equilibrate at a density of ~ 1.20 g/ml in sucrose gradients (Steiger et al., 1980; Opperdoes and Steiger, 1981) and 1.082 g/ml in Percoll (Grab et al., 1987). Endoplasmic reticular membranes or Golgi apparatus of the same parasite have also been demonstrated to equilibrate between sucrose densities of 1.14 and 1.15 g/ml. Since AcP equilibrated at a similar density to that of endoplasmic reticular membranes or Golgi apparatus, it was tentatively concluded that AcP is associated with microsomal membranes derived from the endoplasmic reticulum or Golgi apparatus rather than lysosomes. These results support the view that AcP is not a lysosomal enzyme in trypanosomes and agree with Grab et al. (1987) who localised AcP in Golgi apparatus of *T. brucei*. Thus, another major difference between mammalian cells and trypanosomes is in the location of AcP which makes it a potential target for the development of new trypanocidal drugs.

Previous cytochemical studies carried out on various African trypanosomes (Seed et al., 1967; Langreth and Balber, 1975; Venkatesan et al., 1977; Steiger et al., 1980), have revealed the AcP activity within the flagellar pocket and in some vesicles located near the flagellar pocket.

Cytochemical data obtained here on bloodstream forms of *T. congolense* extends the same observation to this trypanosomatid. More interestingly, some vesicles adjacent to the flagellar pocket, contained lead phosphate deposits. These results suggest that the vesicles bud from the flagellar pocket membrane and nutrients are taken to the endosome-lysosome system for further digestion. Thus, AcP is an enzyme that is involved in the nutrition of trypanosomes.

In comparison to *Leishmania* sp. and *Trichomonas* sp., *T. congolense* was shown not to secrete AcP into their immediate environment (Chapter 3). However, cytochemical studies have shown lead phosphate deposits uniformly distributed over the surface of gluteraldehyde fixed trypanosomes in addition to the enzyme reaction product observed in the flagellar pocket region and in some vesicles. These results support the view that an AcP activity is associated with the surface of trypanosomes; a point of view already developed in Chapter 3. Attempts to prepare plasma membranes from [^{125}I]iodinated trypanosomes demonstrated that at least 20% of [^{125}I]iodine co-sedimented with AcP activity. Within this peak of activity, vesicles and membranes were observed at the electron microscope level. The localisation of AcP activity on the cell surface is an important result and interesting in the context of parasite-host interaction. This membrane bound AcP may indicate a possible protective role of the so-called common (structural) antigens to which little attention has been drawn in study on the antigenic properties of African trypanosomes rather than focusing almost exclusively on the VSG coat.

CHAPTER FIVE

PHYSICO-CHEMICAL CHARACTERISATION OF ACID PHOSPHATASES

5.1 - General introduction

The association of AcP activity with the surface membrane of live *T. congolense* has recently been demonstrated (Tosomba et al., 1996; Chapter 3). Additionally, AcP activity has been detected cytochemically in the parasite's flagellar pocket and in some adjacent vesicles. Subcellular fractionation studies showed that AcP activity was mainly distributed between the microsomal (MI) fraction (52.7%) and the cytosolic (CY) fraction (44.4%). This distribution suggests the presence of multiple forms and multiple functions of AcP in *T. congolense*. The objectives of this chapter were firstly, to separate distinct molecular forms of AcP contained in the cytosolic and microsomal fractions of bloodstream forms of *T. congolense* and, secondly, to characterise different forms of AcP isolated.

5.2 - Preparation of trypanosome cytosolic and microsomal fractions

Purified *T. congolense* IL3 000 (Section 2.5) were washed twice with buffer D and resuspended in the same buffer at a concentration of 4×10^8 cells per ml. Trypanosomes were then disrupted in a French Pressure Cell (Aminco, Silver Springs, USA) at 2 500 psi and the homogenate centrifuged (14 600 x g, 4°C, 15 min) to yield a supernatant termed post nuclear supernatant (PNS). Thereafter, the PNS fraction was centrifuged (123 000 x g, 4°C, 60 min) to produce a cytosolic fraction (CY) and a microsomal pellet (MI).

5.3 - Extraction of AcP from the microsomal pellet

Since proteins contained in the pellet are not soluble in aqueous solutions used for preparing the cytosolic fractions, a method of solubilising them with preserved biological activity was explored. Effective solubilisation of membrane proteins involves both the selection of a suitable detergent and appropriate solubilisation conditions. This Section outlines the optimisation of solubilisation methods for the isolation of the membrane-bound AcP from MI fractions. The detergents Triton X–100 or Triton X–114, were compared either alone or in combination with the delipidation agent butanol. Enzymatic extraction using

phosphatidylinositol-specific phospholipase C was attempted to elucidate the mode of anchoring of AcP in the membrane.

5.3.1 - Solubilisation methods

In preliminary experiments, the capability of some detergents (Triton X-100, Triton X-114, octyl-β-glucoside and sodium cholate) to extract AcP from the microsomal pellet was explored. Protein samples were solubilised with various concentrations of detergents ranging from 0.1 to 3.5% (v/v) using similar procedures as those outlined in this Section. After centrifugation, each pellet was resuspended in 100 mM Na acetate buffer, pH 5.0. Extracts and suspensions of pellet residues were separately assayed for AcP activity using p-NPP (Section 2.6.1). By plotting AcP activity versus detergent concentrations, activities in extracts were compared to the residual activities left in the pellets residues. The concentrations of detergent reported in the paragraphs which follow correspond to those producing the highest release of AcP activity.

5.3.1.1 - Triton X–100

Triton X–100 is widely used to solubilise membrane proteins. Intergral membrane proteins are, by definition, proteins which have hydrophobic domains that anchor them in the lipid bilayer. Non-ionic detergents with polyoxyethylene head groups can displace and replace much of the normal lipid environment around these proteins; hydrophilic proteins bind very little detergent (Helenius and Simons, 1975). The detergent monomers partition into the membrane. As the detergent concentration increases, which leads to lysis, the membrane is solubilised first in the form of detergent-protein-lipid complexes and then progressively solubilised even further to give detergent-protein complexes and detergent-lipid complexes (Neugebauer, 1988).

MI pellet fractions prepared as described in Section 5.2, were extracted for at least 6 to 16 h by gentle stirring in 3 ml of 20 mM Tris-HCl (pH 7.4) containing 2% (v/v) Triton X–100. The Triton X–100 extracts were centrifuged (123 000 x g, 4°C, 60 min) and the resultant supernatants were retained for PAGE analysis (Section 2.10) by following the method of Katakura and Kobayashi (1988).

5.3.1.2 - Triton X–114 phase separation

In order to determine whether the AcP present in the MI pellet is a member of the integral or peripheral membrane proteins, the MI fraction was subjected to phase partitioning in the detergent Triton X–114 as described by Bordier (1981). The protein samples (0.2 to 1.0 mg/ml) were taken up in 1.8 ml of 20 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl. These samples were mixed with 0.2 ml of 10% (v/v) Triton X–114 at 4°C. The microsomal pellet was solubilised for 15 min at 4°C under gentle shaking. The clear samples were then centrifuged (12 000 x g, 4°C, 3 min) and the pellet was extracted again as described above. Supernatants were combined and incubated in conical microfuge tubes at 37°C for 10 min with thorough mixing after 5 min of incubation. During the last 5 min, clouding of the solutions occurred. The two phases were separated by centrifugation (12 000 x g, 37 °C, 3 min). After centrifugation the detergent phase constituted an oily droplet at the bottom of the tube. The upper aqueous phase was removed from the tube and made up 0.5% (v/v) Triton X-114. After dissolution of the detergent, the mixture was incubated (10 min, 37°C) to allow condensation and centrifuged (12 000 x g, 37°C, 3 min). At the end of the separation, all detergent phases were combined and the volume made up to that of the aqueous phase in order to obtain approximately the same salt and detergent content for both phases. Samples were assayed for AcP activity as described in Section 2.6.1, except that it was necessary to add 5 µl of a 1% (m/v) sodium deoxycholate solution to the samples before measuring absorbance in order to reduce turbidity due to the presence of Triton X-114.

5.3.1.3 - Enzymatic extraction by phosphatidylinositol-specific phospholipase C

Glycosylphosphatidylinositol (GPI)-anchored proteins occur widely, perhaps universally, on the surface of animal cells, where they perform a variety of important functions. The possibility that the membrane-bound AcP may be a GPI-anchored protein was examined here. MI fractions were resuspended in 0.2 ml of 20 mM Tris-HCl (pH 7.4) containing 5 mU phosphatidylinositol-specific phospholipase C (PI-PLC). Mixtures were incubated for 30, 60, 120 min and 16 h at 37°C. After incubation, samples were centrifuged and assayed for AcP activity (Section 2.6.1). For samples that were incubated for 16 h, 0.1 ml of non-reducing treatment buffer (Section 2.9.1) was added and samples analysed by PAGE (Section 2.9.2) as adapted from the method of Katakura and Kobayashi (1988).

5.3.1.4 - Delipidation of membranes with Butanol

In order to achieve further enzyme extraction from MI pellet fractions, MI pellets were delipidated by 0.2% (v/v) butanol in combination with the solubilising detergent, either Triton X–100 (Section) or Triton X–114 (Section) using the same procedures described in Sections 5.3.1.1 and 5.3.1.2 respectively. The extracts and delipidated MI pellets were analysed by PAGE (Section 2.9.2) as adapted from the method of Katakura and Kobayashi (1988).

5.3.2 - Results and discussion

Microsomal pellet fractions from French Press lysed *T. congolense* contained 52% of the total AcP activity. The solubilisation of this AcP activity from the pellet by Triton X–100, Triton X–114, enzymatic hydrolysis with PI-PLC and delipidation of membranes with butanol, were compared. PAGE analyses of the success of various solubilisation methods in releasing membrane-bound AcP from the MI pellet are presented in Fig. 20. Both detergents extracted AcP from the microsomal pellets. However, Triton X-114 appeared more effective in that only 35% protein was left in the detergent enriched phase due to partitioning. This detergent enriched phase constituted the crude membrane enzyme extract during the subsequent purification procedure (Section 5.5.1.2).

The released AcP was found almost exclusively in the relatively hydrophobic, Triton X–114 enriched phase (Fig. 20, lanes 5 and 7). The finding that most of the AcP activity tended to partition into the detergent-rich phase, suggests that this enzyme activity is an integral membrane protein which could be solubilised at 0-4°C. Therefore, the detergent extracted AcP is likely to be a membrane-bound enzyme. By definition, peripheral proteins species are almost always found in the aqueous phase.

Incubation of the microsomal pellet fraction with PI-PLC did not cause the expected release of the membrane-bound AcP after 30, 60 and 120 min as well as 16 hours (lane 8). The AcP activity remained insoluble and could be observed in the stacking gel similarly to that observed in lane 1 (untreated pellet). This AcP activity appears to be resistant to enzymatic release since the increase of the concentration of PI-PLC (approximately 10 fold) did not have a substantial effect on the amount of AcP that could be released by PI-PLC (lane 9).

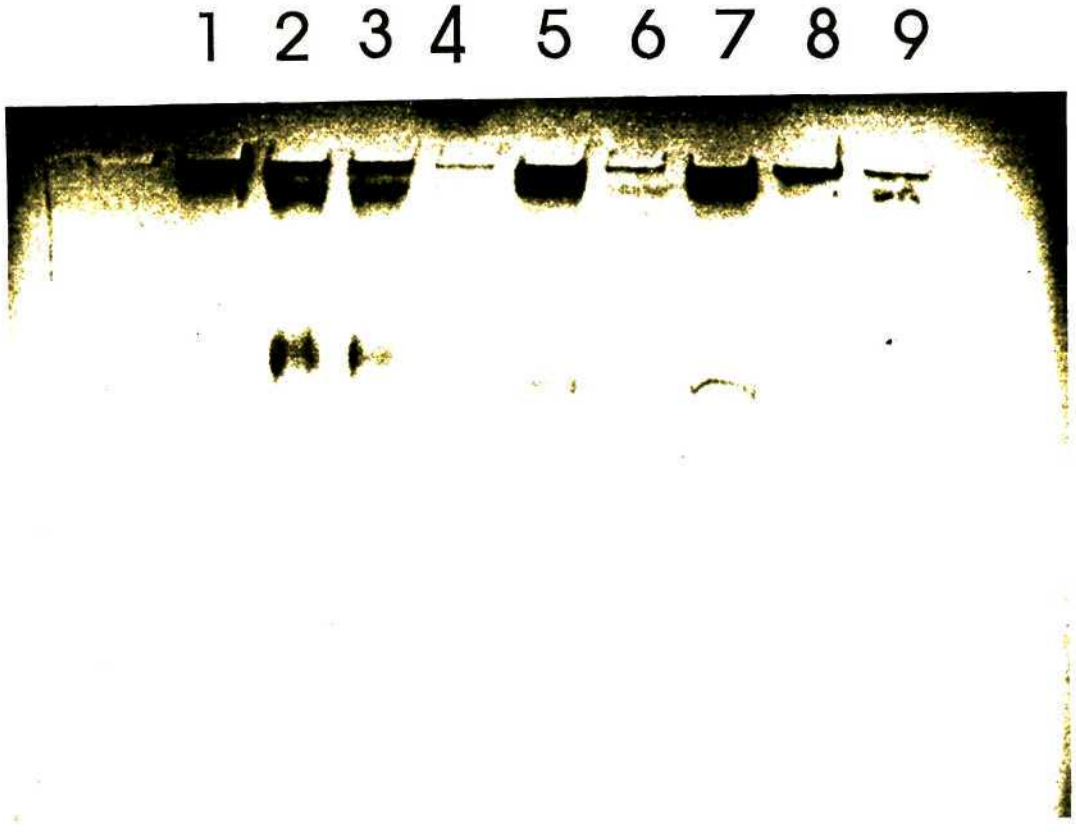


Figure 20 - Non-denaturing PAGE showing the effects of various solubilisation treatments on membrane-bound AcP from *T. congolense.*

Approximately 263 μg protein (microsomal pellet fraction), obtained from different solubilisation methods, were analysed by non-denaturing PAGE carried out in a 5-15% gradient polyacrylamide gel. Gels were stained for AcP activity with α-naphthyl phosphate and Fast Red salt at pH 5 (Section 5.4.3.1). Lane 1, untreated sample; lane 2, 2% Triton X–100 extracted sample; lane 3, 2% Triton X–100 extracted sample in the presence of 0.2% butanol; lane 4, aqueous phase of 2% Triton X–114 extracted sample; lane 5, organic phase of 2% Triton X-114 extracted sample; lane 6, aqueous phase of 2% Triton X-114 extracted sample in the presence of 0.2% butanol; lane 7, organic phase of 2% Triton X-114 extracted sample in the presence of 0.2% butanol; lane 8, sample treated with 5 mU PI-PLC; lane 9, sample treated with 50mU of PI-PLC.

In order to enhance the extraction of AcP from microsomal pellet fractions, butanol was included in extraction media. When compared to control samples (lanes 2 and 5), the inclusion of butanol did not markedly improve the amount of AcP released in association with either Triton X–100 (lane 3) or Triton X–114 (lane 6). The expected delipidation effect of butanol could not be observed at this concentration of butanol.

The solubilisation of microsomal pellets with Triton X–114 indicated that most of the AcP activity tend to partition into the detergent-rich phase. It can therefore be concluded that the AcP activity extracted from microsomal pellets of *T. congolense* is an integral membrane protein, soluble at 0-4°C. Triton X–114 appeared to be more effective than Triton X–100 in solubilising AcP activity. Since the enzyme could not be extracted with PI-PLC, it is not likely to be a GPI-anchored protein.

5.4 - Investigation of protein phosphatase activity in the microsomal pellet

5.4.1 - Introduction

Several integral membrane proteins are themselves protein kinases or protein phosphatases. They have been shown to be able to phosphorylate or dephosphorylate proteins on the cytoplasmic side of the plasma membrane. The receptor-linked tyrosine kinases and tyrosine phosphatases are well-known examples (Ullrich and Schlessinger, 1990; Fischer et al., 1991). In addition, the existence of a novel class of protein kinases and protein phosphatases, termed ecto-protein kinases and ecto-protein phosphatases, have been localised at the external surface of the plasma membrane where they exert their catalytic activity (Chen and Lo, 1991; Naik et al., 1991). Therefore, numerous physiological processes are tightly regulated by the reversible process of phosphorylation/dephosphorylation of proteins.

Live *T. congolense* parasites have been shown to be able to hydrolyse p-NPP, which structurally resembles phospho-tyrosine, extracellularly. Since the membrane-bound AcP in MI appeared to be an integral membrane protein that also hydrolyses p-NPP, the possibility that this membrane-bound AcP might be a phosphoprotein phosphatase was investigated here. The objectives of this part of the investigation were firstly the identification of a potential endogenous phosphoprotein substrate for the membrane-bound AcP form of *T. congolense*

and, secondly the detection of the phosphoprotein phosphatase activity that may be ascribed to this membrane-bound AcP.

5.4.2 - Methods

5.4.2.1 - Purification of casein kinase II from *T. congolense*

The procedure used to purify the casein kinase II was a modification of the method described by Hathaway and Traugh (1979). Cytosolic fractions of *T. congolense* were subjected successively to DE-52 (column, 1.5 x 150 cm) and heparin-agarose (column, 1.5 x 6 cm) chromatography. The purified casein kinase II (CKII) produced by this protocol was a gift from Miss Rozmin T.K. Janoo (ILRI, Nairobi, Kenya). The purified enzyme was assayed as described by Roskoski (1983) and kept at –70°C until required.

5.4.2.2 - Preparation of ^{32}P-labelled phosphoproteins

For the preparation of ^{32}P-labelled phosphoproteins, endogenous phosphorylated proteins were isolated from microsomal and cytosolic fractions obtained from trypanosome lysates (Section 5.2) using the method of Aboagye-Kwarteng et al. (1991). Prior to the use of the microsomal pellets in the ^{32}P-labelling procedure, they were solubilised for 30 min with 0.1% Triton X-100.

Larger quantities of phosphoproteins of interest were obtained by preparing 10 times the following reaction assay. Reaction mixtures (0.15 ml) contained 50 mM Tris-HCl buffer (pH 7.2), 10 mM $MgCl_2$, 150 mM KCl, 40 µg of substrate, 10 µg of CKII, 5 µM ATP and 10 µCi of [γ-^{32}P]ATP (10 Ci per mmol, Amersham). Incubations were carried out on ice for 10 min. The reactions were stopped by adding 50 µl of SDS sample treatment buffer (Section 2.9.1). The samples were heated at 100°C for 5 min prior to electrophoresis. Proteins were then separated by SDS-PAGE according to Laemmli (1970) using 7.5-15 % polyacrylamide separating gels and 5 % stacking gels (Section 2.9.1). In preliminary experiments, gels were stained with Coomassie brillant blue R-250 and destained before subjecting them to autoradiography. In subsequent experiments, each wet gel was placed on a square of Whatman 3MM paper after electrophoresis, sealed in a polythene bag and exposed to Fuji RX film at –70 °C for one day in the presence of an intensifying screen. Proteins of interest were identified by overlaying the gel with the autoradiograph, excised and

electroeluted from the gel strips into 50 mM NH_4HCO_3, 1 mM DTT and 0.1 % SDS using an Electroeluter-concentrator (CBS Scientific Co.). More than 95% of the radiolabelled proteins were eluted by applying a current of 12 mA per cell for 18 h. The phosphoproteins were dialysed extensively against several changes of 100 mM Na acetate buffer (pH 5.0) to remove SDS from the protein solutions. For comparison, α-casein was subjected to the same ^{32}P-labelling phosphorylation procedure.

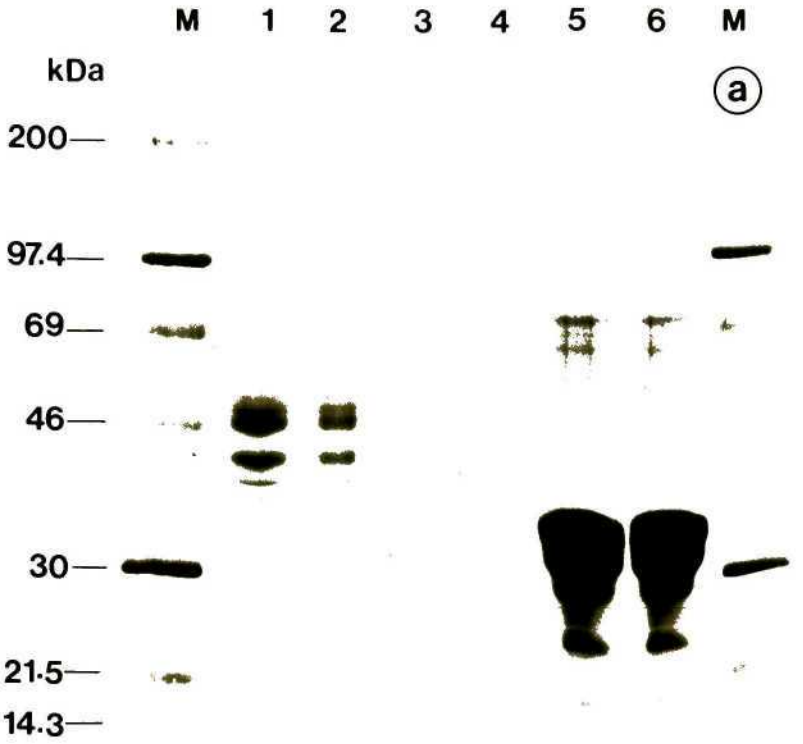
5.4.2.3 - Protein dephosphorylation

The AcP preparation used in this experiment constituted microsomal pellets extracted with Triton X-114 and then partially purified on a Sephacryl S-300 gel filtration column (1 x 96 cm) (Section 5.5.1.1). The dephosphorylation assay was carried out in a reaction volume of 0.95 ml containing 100 mM Na acetate (pH 5.0), 100 cmp/min ^{32}P-phosphoprotein and 100 μg of membrane-bound AcP. At the indicated time intervals, aliquots (190 μl) were removed and mixed with 50 μl of SDS sample treatment buffer (Section 2.11.1) and boiled for 5 min at 100°C. For the determination of the release of ^{32}P, proteins were separated by SDS-PAGE according to Laemmli (1970) using 7.5-15% polyacrylamide separating gels and 5% stacking gels. For autoradiography, each wet gel was placed on a square of Whatman 3MM paper, sealed in a polythene bag and exposed to Fuji RX film at -70 °C in the presence of an intensifying screen until bands appeared on the autoradiograph.

5.4.3 - Results and conclusions

Reducing SDS-PAGE analysis of the phosphorylation of the cytosolic and microsomal fractions of trypanosomes and α-casein is presented in Figure 21a. The Coomassie blue stained proteins were well separated and could be correlated with the profile of phosphorylated proteins obtained by autoradiography (Fig. 21b).

Whereas the 52 and 56 kDa proteins were specifically phosphorylated in the cytosolic fraction of trypanosomes (Fig. 21b, lanes 1-2), proteins at 41 and 70 kDa were phosphorylated in the casein solution (Fig. 21b, lanes 5-6). This indicated that the exogenously added [γ-^{32}P]ATP could be used by the casein kinase II from *T. congolense* to phosphorylate α-casein and other cytosolic proteins. In contrast, no significant phosphoproteins were revealed by autoradiography in a microsomal preparation solubilised in Triton X–100 (Fig. 21b, lanes 3-4).


M 1 2 3 4 5 6 M
kDa
a
200
97.4
69
46
30
21.5
14.3

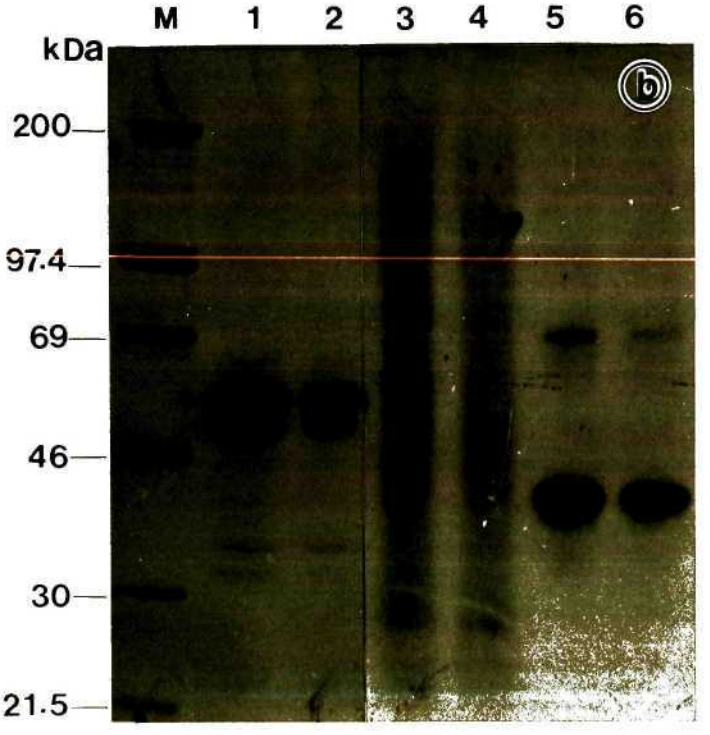

M 1 2 3 4 5 6
kDa
b
200
97.4
69
46
30
21.5

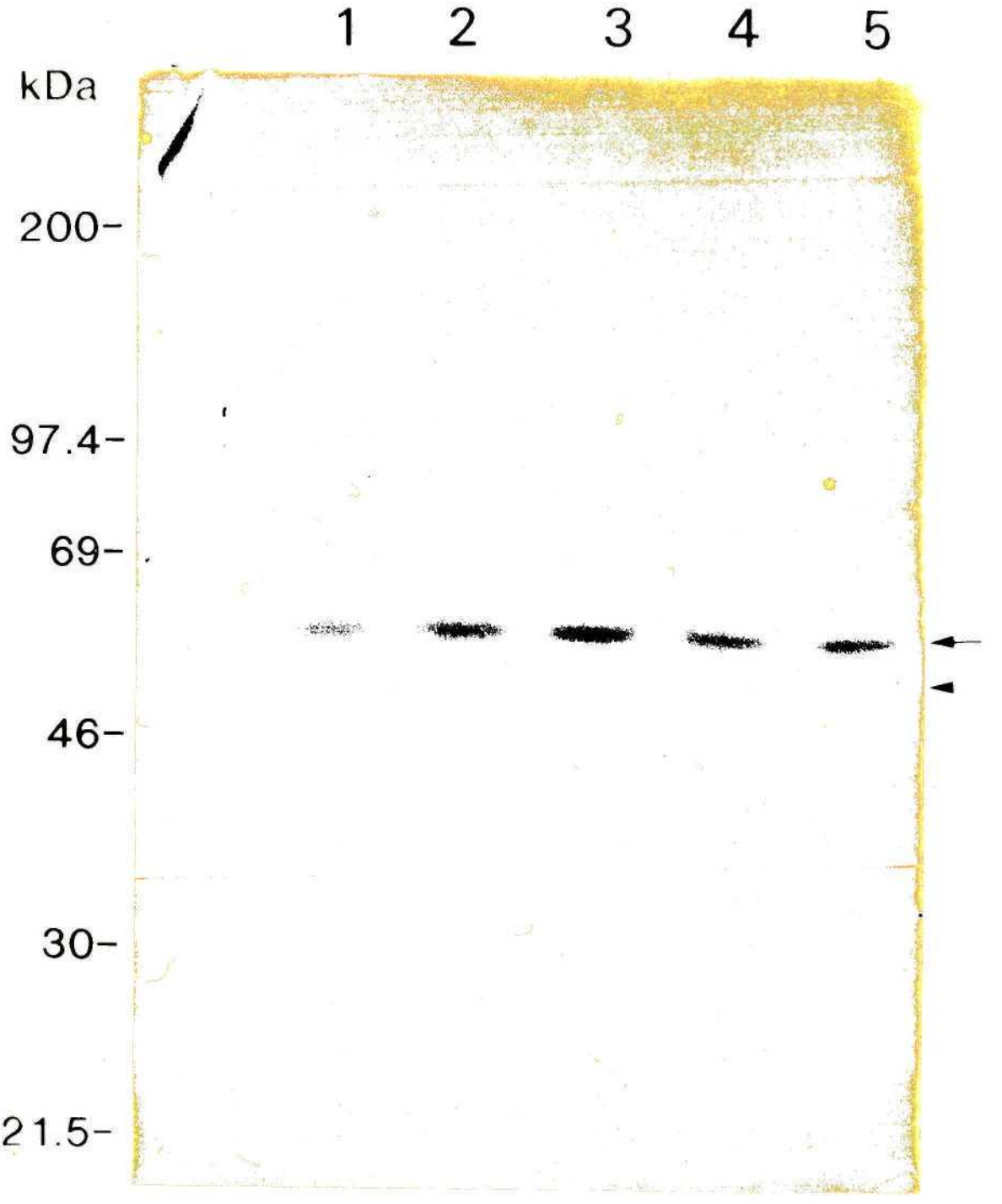
Figure 21 - Phosphorylation of endogenous and exogenous protein substrates by casein kinase II from *T. congolense*

Cytosolic (lanes 1-2) and microsomal (lanes 3-4) extracts of trypanosomes, and α-casein (lanes 5-6), were phosphorylated *in vitro* with [γ-^{32}P]ATP and separated by SDS-PAGE on a 7.5%-15% (w/v) polyacrylamide gradient gel, stained with Coomassie Blue (**a**) and autoradiographed (**b**). The M_r positions of the following standard proteins are shown on the left: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).

After electrophoresis and autoradiography, a 1 cm horizontal section was cut from the gel in the 52–56 kDa region. Phosphoproteins pp52 and pp56 were electroeluted and used in the dephosphorylation experiment. On the autoradiograph (Fig. 22, shown on the left page), no significant decrease in radioactivity was observed in the samples analysed at the different time intervals. The results indicated that the membrane-bound AcP is not a phosphoprotein phosphatase. The data obtained may also suggest that the phosphoproteins pp52 and pp56 are not phosphotyrosyl proteins towards which this enzyme may be specific. The effect of membrane-bound AcP on phosphorylated proteins from α-casein was not investigated since α-casein was used as the phosphorylation positive control.

Figure 22 - Dephosphorylation of pp52 and pp56 phosphoproteins by membrane-bound AcP from *T. congolense*

Phosphoproteins were incubated at 37°C with 100 μg of AcP in a total volume of 950 μl. At the indicated times (see below), 190 μl of the reaction mixture was removed, added to the reducing treatment buffer (Section 2.9.1) , boiled, subjected to SDS-PAGE and autoradiographed. Lanes 1-5, reactions at 2, 4, 8, 16 and 32 min. Arrow and arrowhead indicate the positions of phosphoproteins pp56 and pp52 respectively.


1
2
3
4
5
kDa
200-
97.4-
69-
46-
30-
21.5-

5.5 - Partial purification and characterisation of acid phosphatases

5.5.1 - Methods

All the purification steps were carried out at 4°C. Fractions collected in the chromatographic steps were assayed for activity with p-NPP (Section 2.6.1). Protein profiles were obtained by reading absorbances at 280 nm. The active enzyme fractions were pooled and concentrated by ultrafiltration in Amicon centriprep-10 concentrators (Section 2.8). Protein concentrations of pooled fractions were determined by the method of Bradford (1976) as described in Section 2.7.

5.5.1.1 - Purification of acid phosphatases from the cytosolic fraction

Step 1 - Preparation of crude cytosolic AcP

The crude cytosolic AcP was prepared by a protocol derived from the differential centrifugation experiments (Section 4.2.1.1) and modified as described in Section 5.2.1.

Step 2 - Ammonium sulfate precipitation

Solid $(NH_4)_2SO_4$ was added to the crude enzyme preparation to 40% saturation. The enzyme solution was gently stirred for 10 min and centrifuged (12 000 x g, 10°C, 15 min). The 0-40% ammonium sulfate pellet was dissolved in a volume of buffer J equivalent to 2.0% of the bed volume of the Sephacryl S-300 column which was used in the next purification step.

Step 3 - Sephacryl S-300 chromatography

The concentrated preparation of AcP obtained from the $(NH_4)_2SO_4$ precipitation step was subjected to gel filtration on a Sephacryl S-300 column (1 x 96 cm). The column was pre-equilibrated with buffer J and eluted with the same buffer at a flow rate of 30 ml/hour. Fractions (4 ml) were collected and fractions containing enzyme activity were pooled and dialysed against buffer I.

The Sephacryl S-300 column was calibrated in a separate experiment using both low and high molecular mass protein standards namely ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa). Blue dextran 2000 was used to determine the

void volume. A calibration curve was constructed by plotting the partition coefficients (Reiland, 1971) versus the logs of the molecular masses of the standard proteins from which the molecular masses of native acid phosphatases were determined.

The Stokes radius of a protein, which includes its bound water and other bound components, is defined in terms of the frictional coefficient and the Stokes radius of an equivalent spherical protein in a solvent of determined viscosity. It is a useful parameter which in conjunction with other parameters such as M_r, can provide some information on the shape of the protein. For comparison of the different AcPs, the equation of Ackers was selected for plotting the chromatographic data in order to estimate Stokes radii of different AcPs (Ackers, 1967; Wong et al., 1985):

$$R_s = a_o + b_o \, erf^{-1}(1-K_{av}) ,$$

where R_s is the Stokes radius of the protein, which includes its bound water and other components; a_o and b_o are two constants; K_{av} is the coefficient of partition of the protein as defined by Laurent and Killander (1964) and $erf^{-1}(1-K_{av})$ is the inverse error function of the value in parentheses. According to this equation a plot of R_s as a function of $erf^{-1}(1-K_{av})$ should be linear. A calibration curve was constructed by plotting the R_s versus the $erf^{-1}(1-K_{av})$ of the protein standards from which the Stokes radii of native AcPs were determined. The following protein standards were used namely ribonuclease A (16.4 Å), chymotrypsinogen A (20.9 Å), ovalbumin (30.5 Å), albumin (35.5 Å), aldolase (48.1 Å), catalase (52.2 Å), ferritin (61.0 Å) and thyroglobulin (85.0 Å).

Step 4 - DEAE-cellulose chromatography

Pooled fractions corresponding to the peak of enzyme activity from the gel filtration step were applied onto a DEAE-cellulose column (1.2 x 60 cm) pre-equilibrated with buffer I at a flow rate of 30 ml/hour. The column was washed extensively with the equilibrating buffer until the absorbance at 280 nm returned to baseline. The adsorbed proteins were eluted with a 0-0.6 M NaCl gradient in buffer I. Fractions (8 ml) were collected and assayed for AcP activity using p-NPP (Section 2.6.1). Fractions that contained the unbound AcP activity were pooled and termed AcP_1. Bound AcP activities which eluted in two peaks were pooled separately, concentrated and termed AcP_2 and AcP_2' according to their order of elution from the column.

The active enzyme fractions from each chromatography column were pooled, concentrated by Amicon centriprep P-10 and stored at –20°C.

Step 5 - Sephadex G-75 Chromatography

Prior to applying AcP containing fractions obtained from anion exchange chromatography to the gel filtration column, each fraction was concentrated to 2.5% of the Sephadex G-75 bed volume by ultrafiltration using Amicon centriprep P-10 concentrators. The concentrated samples were fractionated on a Sephadex G-75 column (1 x 96 cm) pre-equilibrated with buffer I. The column was eluted with the same buffer at a flow rate of 30 ml/hour. Fractions with AcP activity from each gel filtration experiment were pooled, concentrated by Amicon centriprep P-10 and stored at –20°C until required.

5.5.1.2 - Purification of an acid phosphatase from the microsomal fraction

Step 1 - Preparation of a crude extract

The microsomal fraction was resuspended in buffer I containing 1% (v/v) Triton X–114 and extracted as described in Section 5.3.1.2. After extraction, the detergent enriched phases were combined and referred to thereafter as membrane enzyme extract (MEE).

Chromatography Steps

Fractions of MEE were purified successively by Sephacryl S-300, DEAE-cellulose and Sephadex G-75 Chromatography under identical conditions to those used for the cytosolic enzyme (Section 5.5.1.1).

5.5.1.3 - Determination of pH optima of AcPs

The activity of the isolated AcP enzymes was assayed in triplicate over a pH range of 3.0 to 7.0. Reaction mixtures contained 20 µl buffer (prepared as described by McIlvaine, 1986) at the indicated pH, 5 µl of 50 mM p-NPP and 25 µl of the appropriate enzyme solution. Mixtures were incubated for 60 min at 37°C and p-nitrophenol liberated assayed as described in Section 2.6.1.

5.5.1.4 - Determination of temperature optimum and thermostability of isolated AcPs

To determine the temperature optimum of different AcPs, aliquots (25 µl) of each Sephadex G-75 derived enzyme preparation were separately incubated at different temperatures ranging from 20 to 60°C for 60 min in the presence of 5 mM p-NPP. The amount of p-nitrophenol liberated was determined as described in Section 2.6.1.

The thermostability of different AcPs was determined by incubating 100 µl of each Sephadex G-75 derived enzyme preparation without any substrate for a period of 0-10 days at a temperature of –20°C. The activity was then measured in triplicate (25 µl enzyme, each) under the standard test conditions (Section 2.6.1). Relative activities were calculated by relating the mean activity obtained at each temperature to the highest activity for the same enzyme preparation.

5.5.2 - Results

5.5.2.1 - Chromatography steps

As shown in Figure 23A, a single peak (Peak I) of AcP activity was obtained from Sephacryl S-300 chromatography of the redissolved, active 0-40% $(NH_4)_2SO_4$ precipitate. Since this peak co-purified within the void volume, the apparent M_r of this enzyme could not be estimated by this technique.

A similar procedure was employed to filter the Triton X–114 extracted microsomal pellet (MEE) through the same Sephacryl S-300 column. Figure 23B shows the gel filtration pattern of the active peak obtained from this chromatography run. A single peak (Peak II) of AcP activity was detected with an apparent M_r of 320 kDa. The active fractions of peaks I and II were pooled separately and each of them was further purified by DEAE-cellulose and Sephadex G-75 column chromatography.

Chromatography of peak I on a DEAE-cellulose column yielded a peak of activity which did not bind to the column and a major and a minor active peak which were eluted at 200 and 320 mM NaCl, respectively (Fig. 24A). The major peak (fractions 32-33) and the minor peak (fractions 39-40) were pooled separately, concentrated, and filtered through a Sephadex G-75

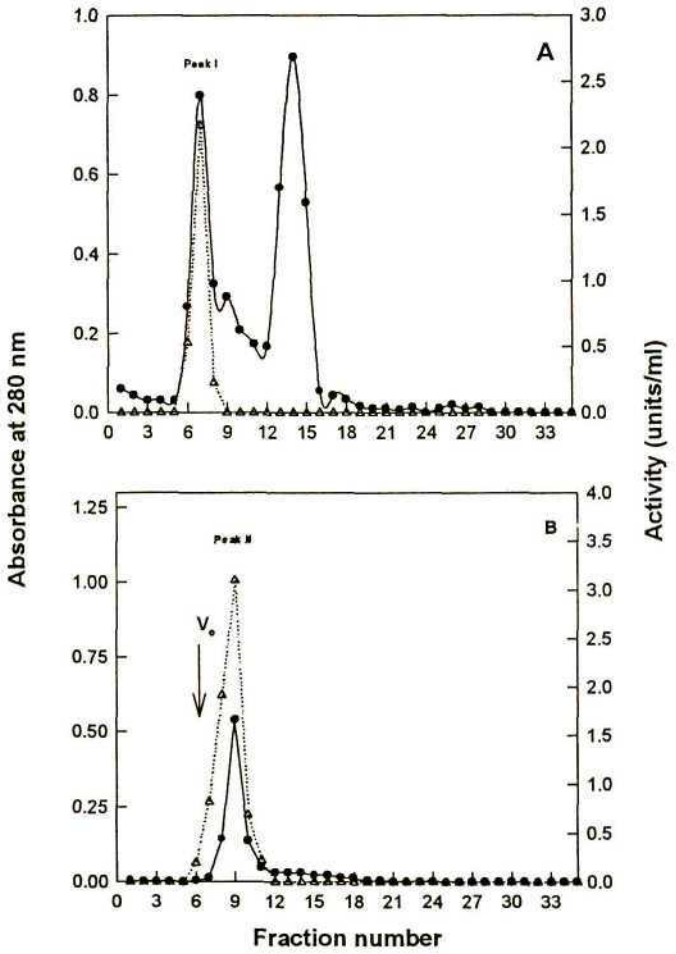


Figure 23 - Gel filtration of acid phosphatases on a Sephacryl S-300 column.

The $(NH_4)_2SO_4$ precipitated cytosolic enzyme preparation (**A**) and the microsomal enzyme extract (**B**) were subjected separately to gel filtration on a Sephacryl S-300 column (1 x 96 cm) equilibrated in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. Fractions (4 ml) were collected and (△) AcP activity against p-NPP, was determined with aliquots from the indicated fractions. (●) A_{280}. The void volume (V_o) of the column was estimated with blue dextran 2000.

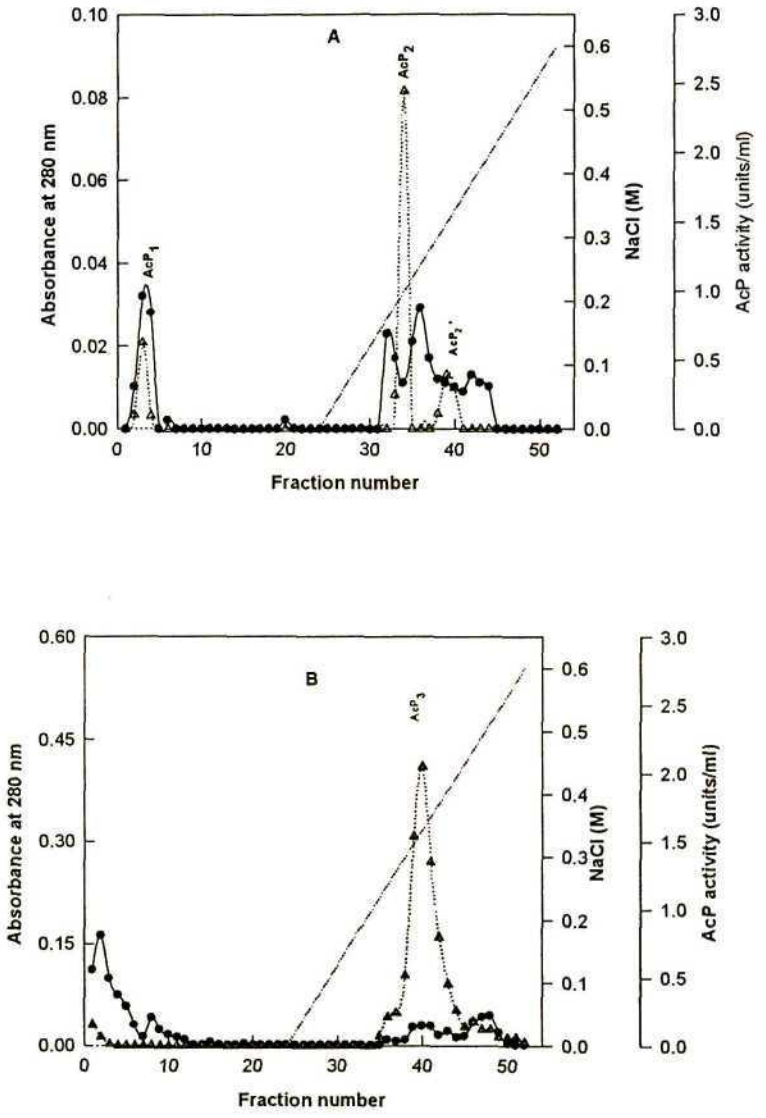


Figure 24 - Ion-exchange chromatography of acid phosphatases from *T. congolense* on DEAE-cellulose.

(**A**) The Peak I enzyme obtained from the Sephacryl S-300 column, was applied onto a DEAE-cellulose column (1.2 x 60 cm) equilibrated in 20 mM Tris-HCl, pH 8.0 at a flow rate of 30 ml/h. The column was washed with 240 ml of the same buffer before application of a NaCl gradient. All fractions were assayed for AcP activity in a p-NPP assay (Section 2.6.1). The unadsorbed AcP activity eluted in the washes was called AcP_1. The adsorbed enzymes (AcP_2 and AcP_2') were eluted with a 180 ml linear NaCl gradient (0-0.6 M).

(**B**) The Peak II enzyme obtained from the Sephacryl S-300 column, was applied onto the same column in a separate experiment. The chromatography conditions were identical to those described in (**A**). The adsorbed enzyme (AcP_3) was eluted with a 180 ml linear NaCl gradient (0-0.6 M). AcP activity (△) against p-NPP was determined with aliquots from the indicated fractions. (●) Absorbance at 280 nm and (--) NaCl gradient.

column. The activity which did not adsorb to the DEAE-cellulose resin was designated AcP_1 while the major and the minor active peaks were designated AcP_2 and AcP_2', respectively. AcP_2 had an estimated molecular mass of 33 kDa and a Stokes'radius of 25 Å. AcP_1 and AcP_2' eluted in the void volume of Sephacryl S-300 column. Their Stokes'radii could not be estimated and appeared to be greater than 85 Å.

Similar protocols were employed for further purification of peak II obtained from the Sephacryl S-300 column (Fig. 23B). As shown in Fig. 24B, a single active peak (termed AcP_3) was eluted at 320-380 mM NaCl from the DEAE-cellulose column. The Stokes' radius of this enzyme derived from peak II was estimated as 58 Å.

Stokes radii of different AcPs obtained were estimated by using the Ackers'equation as described previously (Section 5.5.1.1, step 3). The plot that was observed from the Sephacryl S-300 column used was not linear. This lack of linearity has also been previously observed by Le Maire et al., (1980) and has been attributed to the existence of a heterogeneous distribution of the sizes of the chromatography gel pores.

5.5.2.2 - Purification scheme

A major difficulty encountered in the purification of AcPs was that of a very low yield. Although 5% (v/v) glycerol appeared to be stabilising AcPs, its inclusion in the purification buffers did not increase the yield considerably. Other chromatography procedures such as carboxymethyl-cellulose (CM-52), con A, hydroxylapatite, N-(6-aminohexyl)tartramic acid AffiGel 10, phenyl Sepharose, p-aminobenzylphosphonic acid-Sepharose and p-hydroxymercuribenzoate-agarose resins, were attempted and proved to be unsuccessful.

The experimental scheme that permitted the partial purification of AcPs from cytosolic and microsomal fractions of *T. congolense* is summarised in Table 7. DEAE-cellulose chromatography of the gel filtration peak I enzyme fraction permitted the separation of three forms of AcP, namely AcP_1, AcP_2 and AcP_2'. The total recovery of AcP activity from the DE-52 column was 23 %. The elution of AcP_2 and AcP_2' from the Sephadex G-75 column gave a total yield of 27%. The AcP_1 activity was too little to be subjected to this gel filtration

Table 7 - Summary of the purification of the acid phosphatases AcP_1, AcP_2 and AcP_3, from *T. congolense*.

Purification step	*Total activity*	*Total protein*	*Specific activity*	*Purification*	*Yield*
	units[a]	mg	units / mg	fold	%
123 000 x g supernatant:					
Crude fraction	1575	37.2	42.3	1	100
$(NH_4)_2SO_4$ precipitation (0-40%)	1052	14.4	73.1	1.73	66.8
Sephacryl S-300 chromatography (Peak I)	504	7.4	67.7	1.60	32
DE-52 chromatography					
(a) AcP_1	101	0.01	10631.6	251.1	6.4
(b) AcP_2	124	0.72	172.2	4.1	7.9
(c) AcP_2'	132	0.85	155.3	3.1	8.4
Sephadex G-75 chromatography					
(a) AcP_1	ND	ND	ND	ND	ND
(b) AcP_2	175	0.35	500	11.8	11.1
(c) AcP_2'	264	0.40	660	15.6	16.8
123 000 x g pellet (AcP_3):					
Triton X-114 extract (MEE)	9798	3.38	2900.5	1	100
Sephacryl S-300 chromatography (Peak II)	1630	2.64	617.42	0.21	16.6
DEAE-cellulose chromatography	65	0.836	77.8	0.03	0.7
Sephadex G-75 chromatography	54	0.058	931	0.32	0.6

[a]One unit = 1 nmoles of p-nitrophenyl phosphate hydrolysed per min at 37°C. ND = Not determined.

step. In contrast, the crude membrane-derived AcP preparation was resolved into a single peak of AcP activity by the chromatography steps listed in Table 7.

5.5.2.3 - Effect of pH on acid phosphatase activities

The isolated AcPs hydrolysed p-NPP optimally between pH 3.0 and pH 7.0 (Figure 25). All isolated phosphatases were found to be true AcPs in that they exhibited maximum activity below pH 7.0 when assayed using p-NPP as substrate. They were optimally active within the broad pH range of 4.0 - 6.0. AcP_1 and AcP_3 had very similar pH profiles which showed a plateau between pH 4.7 and 5.8, while AcP_2 showed maximal activity at pH 5.3. There was no evidence of alkaline phosphatase activity.

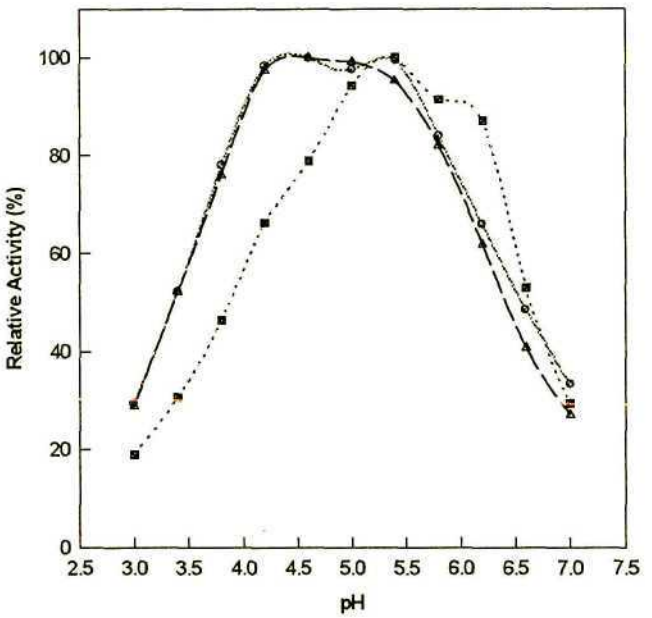


Figure 25 - The effect of pH on the activity of the three isolated AcPs from *T. congolense*.

The hydrolysis of p-NPP by AcP_1, AcP_2 and AcP_3 was determined over the pH range 3-7 as described in Section 5.5.1.3. Data were obtained with the DEAE-cellulose enzyme fractions. Values are the means of triplicate samples for each pH tested, expressed as the percentage of the activity obtained at the indicated pH divided by the optimum activity. AcP_1 (●), AcP_2 (■) and AcP_3 (△).

5.5.2.4 - Effect of temperature on AcP activities

The activity of the three isolated AcPs was measured over a temperature range from 20 to 60°C using a 60 min incubation period under p-NPP assay conditions (Section 2.6.1). Temperature optima were found to be about 30°C for AcP_1 and 37°C for AcP_2 as illustrated in Figure 26A. Although 37°C was routinely used for assaying AcP_3, this enzyme appeared to be thermoresistant in that its temperature optimum was above 42°C. This was confirmed by Arrhenius plots from where an estimated optimum temperature appeared to be about 48°C. This appeared exceptionally high and would indicate the breaking of a large number of weak bonds, such as hydrogen bonds, in the denaturation of this enzyme.

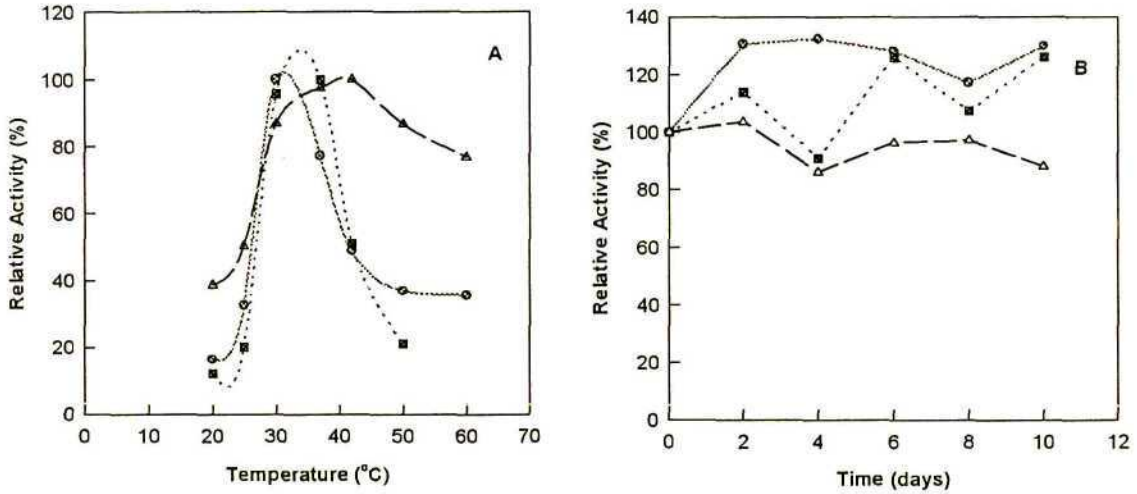


Figure 26 – Temperature dependence of acid phosphatase activities.

(**A**) Enzymes were incubated separately at various temperatures across the range of 20 to 60°C for 60 min in the presence of p-NPP. The liberated p-nitrophenol was determined and the activity calculated as described in Section 2.6.1. (**B**) The thermostability of acid phosphatases was determined by incubating aliquots of each enzyme separately in the absence of substrate for a period of 0-10 days at a temperature of –20°C. The activity was then measured under the standard test conditions (Section 2.6.1). Relative activities were calculated by relating the mean activity at each temperature to the highest activity for the same enzyme preparation. AcP_1 (●), AcP_2 (■) and AcP_3 (△).

The high optimum temperature is of interest since a high optimum temperature for a critical enzyme may ensure the survival of the parasite during temperature fluctuations resulting from infection of the host. The effect of temperature on reaction rate performed using p-NPP as substrate, permitted the calculation of an energy of activation of 8.9 kcal/deg/mol for AcP_3. Its temperature coefficient, Q_{10}, was estimated to be 1.0. This represents the factor by which the reaction rate is increased on raising the temperature by 10°C and is within the range (1-2) normally associated with the catalysed reactions. Basically, the Arrhenius equation is an empirical expression, valuable for the representation of experimental data but limiting in interpretations of the numerical values of the parameters which it allows. However for a better interpretation of data, the 'transition state' or 'absolute reaction-rate' theory which is beyond the scope of this study, should be considered.

To determine optimal storage conditions, 100 μg of each cap was preincubated at −20°C in 20 mM Tris-HCl buffer, pH 8.0, containing 5% glycerol and then assayed for AcP activity (Section 2.6.1) at the indicated time intervals (Fig. 26B). All AcPs appeared to be relatively stable at −20°C over a period of 10 days. However, AcP_1 and AcP_2 showed an increase in activity during the experimental period of 10 days whereas AcP_3 was inactivated by 15% during the same period.

5.5.2.5 - Isoelectric points

Each AcP preparation was subjected to IEF at 4°C according to the procedure described in Section 2.13. IEF profiles of the AcPs applied onto the sucrose gradient are presented in Figure 27. AcP_3 had the lowest isoelectric point (4.7) while AcP_1 and AcP_2 had isoeletric points of 6.5 and 5.3, respectively. It must be noted that only a 15-26% of the AcP activity was recovered. The remainder of the enzyme was inactivated and therefore undetected.

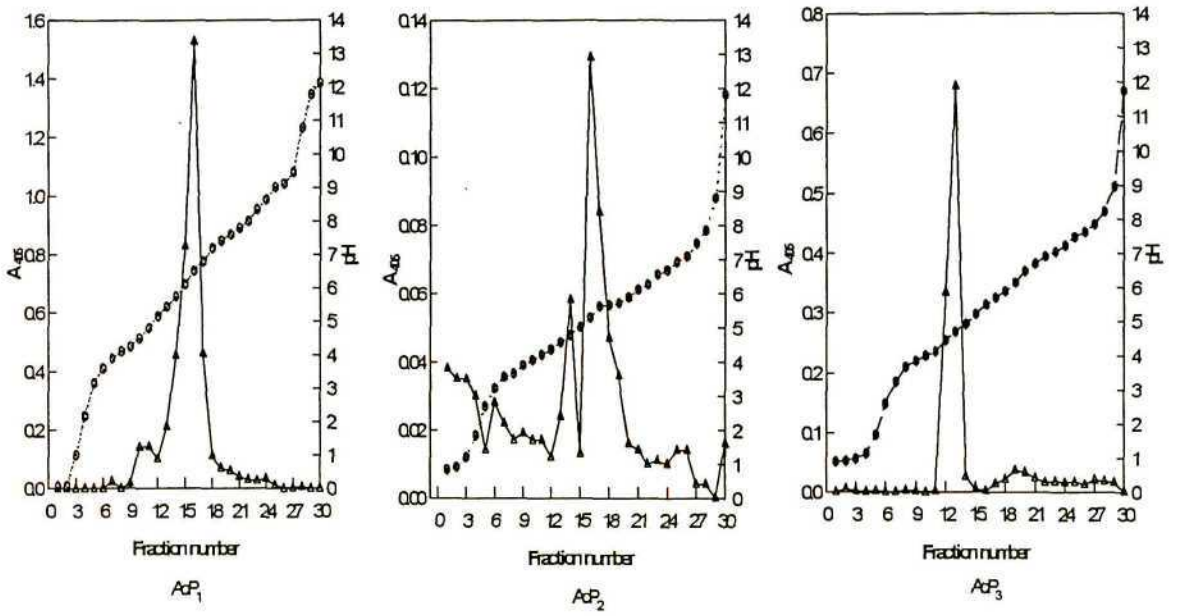


Figure 27 – Isoelectric focusing profiles of *T. congolense* AcPs.

IEF of the DEAE-cellulose AcPs was performed on a 5-50% (m/v) sucrose gradient LKB-Bromma column as described in Section 2.13. (△) A_{405} represents AcP activity of each fraction collected (3 ml) and assayed using p-NPP (Section 2.6.1), and (●) the pH measured.

5.5.2.6 - Kinetic properties

Characterisation of an enzyme usually includes determination of maximum reaction velocity (V_{max}) and the Michaelis constant (K_m) for each substrate. Knowledge of V_{max} and K_m is useful for a number of biochemical purposes such as the estimation of intracellular reaction rates, detection of metabolic control points, comparison of isoenzymes from different tissues or organisms, determination of the molecular events of catalysis, determination of turnover numbers, quantitative comparison of alternative substrates, and for definition of the potency of inhibitors or activators.

Table 8 lists K_m and V_{max} values, along with relative specificity constants (V_{max}/K_m) for the compounds which had been used as substrates for the isolated AcPs. The kinetic parameters were calculated using the ENZFITTER computer programme (Elsevier-Biosoft, Cambridge, U.K.). The isolated AcPs showed typical Michaelis-Menten kinetics for the hydrolysis of various phosphate compounds tested.

Table 8 - Kinetic parameters of acid phosphatases from *T. congolense*

	AcP_1			AcP_2			AcP_3		
Substrate	[(a)]K_m (mM)	[(a)]V_{max} (U/mg)	Ratio[(b)]	[(a)]K_m (mM)	[(a)]V_{max} (U/mg)	Ratio[(b)]	[(a)]K_m (mM)	[(a)]V_{max} (U/mg)	Ratio[(b)]
p-NPP	0.72 ± 0.04	588 ± 15	816.7	0.31 ± 0.04	100 ± 6	322.6	0.23 ± 0.06	311 ± 24	1352.2
β-GlOP	1.83 ± 0.45	263 ± 26	143.7	8.22 ± 3.08	27 ± 6	3.3	3.70 ± 0.81	52 ± 5	14.1
α-NPP	1.49 ± 0.35	70 ± 10	47.0	4.08 ± 0.61	78 ± 9	19.5	0.10 ± 0.02	26 ± 2	260
G-6-P	19.47 ± 1.14	5616 ± 284	288.4	19.83 ± 9.83	ND	ND	12.84 ± 1.98	1699 ± 210	132.3

A unit of enzyme activity = 1nmol of substrate consumed/min at 37°C. Abbreviations: p-NPP, p-nitrophenyl phosphate; β-GlOP, β-glycerophosphate; α-NPP, α-naphthyl phosphate and G-6-P, glucose-6-phosphate. [(a)]Mean value of the three experiments ± SE except for G-6-P (one determination). Kinetic parameters were determined within the range of $K_m/3$ and $3 \times K_m$. [(B)] Ratio(relative specificity constant): V_{max}/K_m

All three enzymes exhibited the highest K_m value for G-6-P which indicates that this substrate is the least good for each of the enzymes. The lowest K_m values of 0.72 mM and 0.31 mM toward p-NPP were obtained for AcP_1 and AcP_2, respectively. This indicated that p-NPP is the preferred substrate for both enzymes. Moreover, the K_m value for AcP_2 toward p-NPP was half that of AcP_1, suggesting that AcP_2 has a high affinity for p-NPP than AcP_1. In contrast to AcP_1 and AcP_2, AcP_3 which had the highest K_m value (12.84 mM) for G-6-P, appeared to prefer another non-physiological substrate, α-NPP displaying the lowest K_m value (0.10 mM) of the three AcP forms. Comparison of K_m values thus provided further evidence that the three isolated enzymes represent distinct forms of AcP.

Considering a simple reaction

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\text{-}S \xrightarrow{k_{+2}} E + P \quad ,$$

where k_{+1}, k_{-1} and k_{+2} represent the velocity constants for the individual reactions,

$V_o = k_{+2}\ [E_o].[S].\{[S] + K_m\}^{-1}$ where $[E_o]$ can be substituted using $[E_o] = [E] + [E\text{-}S]$ and $[E\text{-}S]$ in turn using $[E].[S].[E\text{-}S]^{-1} = K_m$. This gives $V_o = \{k_{+2}/ K_m\}.[E].[S]$ or

$V_o = \{k_{cat}/K_m\}.[E].[S]$ where the term k_{cat}/K_m is the catalytic efficiency which can be used to compare the specificity of an enzyme for a particular substrate. However, k_{cat}/K_m could not be used to compare AcP_1, AcP_2 and AcP_3 because the enzymes were only partially purified whereas k_{cat} refers to a pure enzyme solution.. Nevertheless, since in the relationship $k_{cat} = V_{max}.[E_o]^{-1}$, the term $[E_o]^{-1}$ is constant for a particular enzyme, V_{max} may replace k_{cat} in the term k_{cat}/K_m to give the relative specificity of an enzyme for alternative substrates. In the present study, the relative specificity constant (V_{max}/K_m) of each of the AcPs enzymes were compared for the substrates p-NPP, β-GlOP, α-NPP and G-6-P.

Such a comparison of relative specificity constants for the different substrates has also shown differences which indicated that the isolated AcPs are distinct although relatively nonspecific. The best substrate is that which has the highest relative specificity constant. With regard to this , p-NPP appeared to be the best substrate for all three enzymes. Although AcP_3 had the lowest K_m value for α-NPP, the relative specificity constant indicates that p-NPP was the best substrate for this enzyme rather than α-NPP. The apparent discrepancy may suggest a mixture of positive and negative cooperativity in the binding of the substrate in the presence of the Fast Red product that complexes the α-naphthol released into a diazonium salt. Although this assumption is based on the V_{max} values, the hypothesis requires further investigation with the use of Hill's equation. It also appeared from a comparison of V_{max}/K_m values (Table 8) that all three isolated AcPs exhibited their highest activity against non-physiological substrates (p-NPP or α-NPP) rather than against natural phosphoric esters of aliphatic alcohols such as β-glycerophosphate.

5.5.2.7 - Effect of various compounds on acid phosphatase activities

Table 9 shows the effect of different compounds on the hydrolysis of p-NPP by isolated AcPs. Vanadate (0.6 mM) appeared to be the most effective inhibitor of AcP_1 and AcP_3 producing 91% and 97% inhibition, respectively. In contrast to AcP_1 and AcP_3, p-chloromercuribenzoate (40 mM) was a potent inhibitor of AcP_2. Tartrate up to 20 mM had very little effect on AcPs of *T. congolense* while AcP was 50% inhibited at 1.8 mM in

T. rhodesiense (McLaughlin, 1986). Therefore, these enzymes appeared to be tartrate-resistant. AcPs were not activated by either Mg^{2+} or Mn^{2+}.

Table 9 - Effect of various compounds on acid phosphatase activities form *T. congolense.*

Compound	Concentration (mM)	Relative activity (%)		
		AcP_1	AcP_2	AcP_3
None	0.0	100	100	100
NaF	20.0	22	43	ND
$NaVO_3$	0.6	9	38	3
$CaCl_2$	40.0	105	25	100
$CuSO_4$	40.0	14	31	14
$MgCl_2$	40.0	99	96	103
$MnCl_2$	40.0	93	86	100
Na/K tartrate	20.0	77	102	74
Na dodecylsulphate	40.0	33	28	6
p-Chloromercuribenzoate	40.0	17	0	37

5.5.2.8 - Electrophoretic analysis of the acid phosphatases

In order to assess the degree of purity of the enzyme preparations following the purification procedures summarised in Table 7, aliquots of recovered proteins at various steps were subjected to electrophoresis under nondissociating conditions (Section 2.9) and stained with α-naphthyl phosphate for enzyme activity (Section 2.10). The results obtained are presented in Fig. 28. Enzyme activities appeared as red bands.

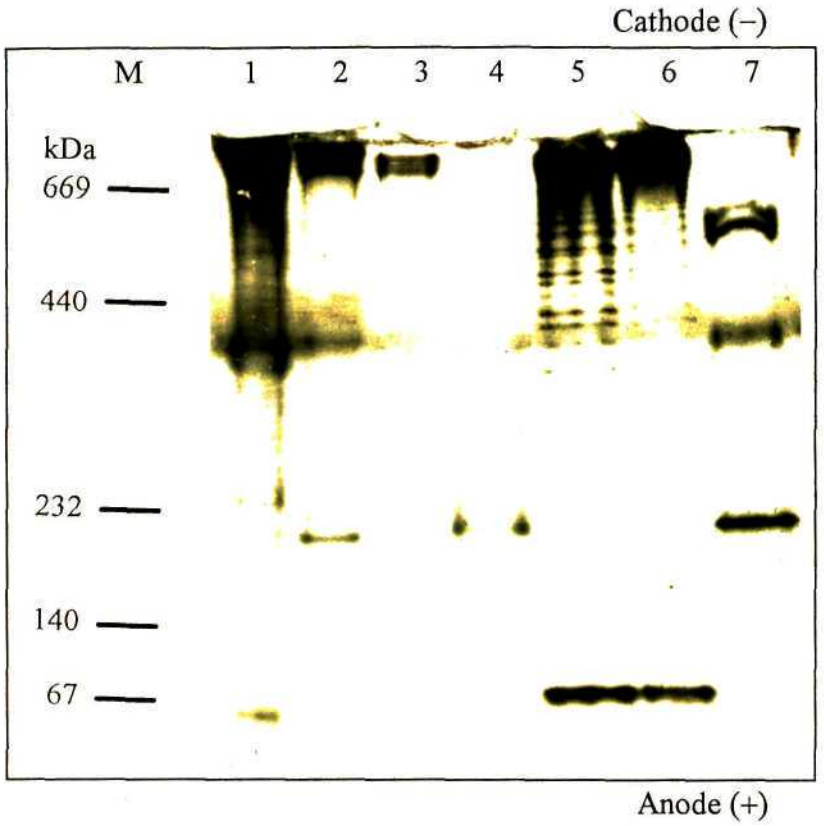


Figure 28 - Nondenaturing PAGE of endogenous AcPs of *T. congolense*.

Approximately 50 μg of each protein sample was loaded onto the gel. Electrophoresis was carried out using a 5-20% polyacrylamide gel and stained with α-naphthyl phosphate and Red Fast salt at pH 5.0 (Section 2.10). Lane 1, Crude fraction (supernatant); lane 2, $(NH_4)_2SO_4$ precipitation fraction; lane 3, AcP_1 from DEAE-cellulose; lane 4, AcP_2 from Sephadex G75; Lanes 5-6, two different preparations of AcP_2' obtained from Sephadex G75; lane 7, AcP_3 from Sephadex G75. Molecular mass markers, thyroglobulin (699 kDa), ferritin (440, kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

AcP_1 which did not bind to DEAE-cellulose at pH 8.0 had the lowest anodal mobility (lane 3). Its M_r which appeared higher than 700 kDa in gel filtration could also not be estimated by electrophoresis. Analysis of AcP_2' on PAGE showed in addition to a ladder of multiple bands of activity, a prominent band of activity with very little anodal migration comparable to AcP_1. The characterisation of this enzyme falls beyond the scope of this thesis and promises to be an interesting topic for future investigation. Native proteins separated by PAGE gels often show tailling profiles or a ladder of multiple bands because their overall charges are not uniformly distributed. Although a 40 μg/ml protease inhibitor cocktail (Section 2.3) was added to

protein samples with the aim of decreasing the suspected enzymatic degradation, the general pattern of bands of activity did not change considerably. AcP_3 appeared to possess more than two bands of activity (lane 7). The AcP_3 preparation analysed by native PAGE stained for activity by means of the α-NPP/Fast red reaction showed a doublet of bands of activity at approximately 600 kDa and an additional band of activity at 230 kDa. The 230 kDa band appeared to be similar to the major band of activity in the AcP_2 preparation which also showed additional faintly stained bands within the 240-350 kDa region.

Upon electrophoresis in the presence of SDS and in the absence of any reducing agents, two closely migrating bands (the 65 and 62 kDa proteins) appeared to be common in all the AcP preparations (Fig. 29, arrows).

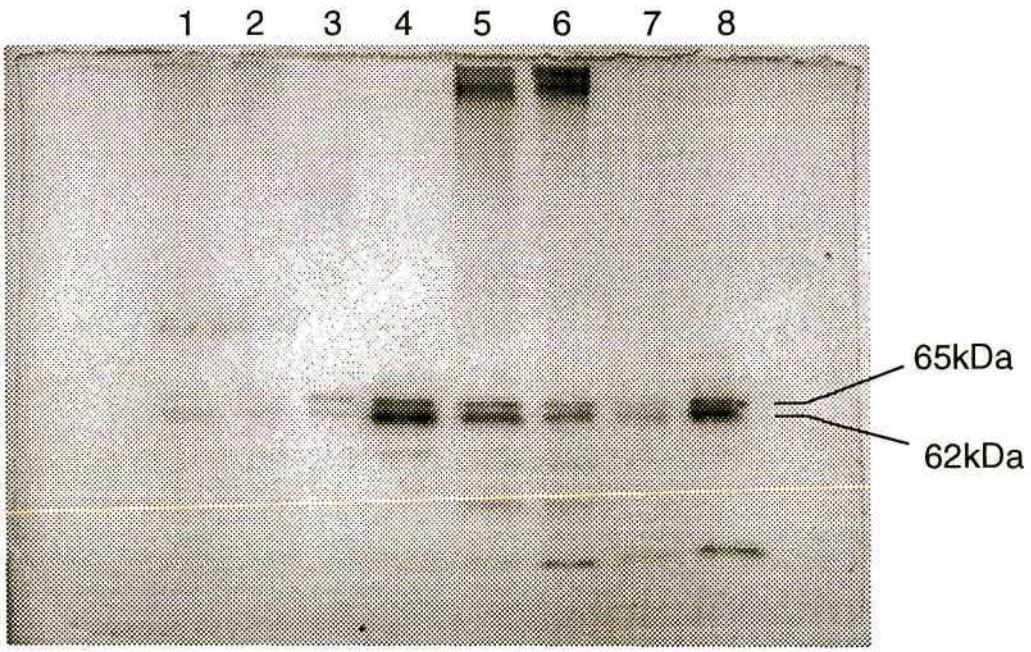


Figure 29 - SDS-PAGE of endogenous AcPs purified from *T. congolense.*

Approximately 30 μg of each protein sample was applied onto the SDS-PAGE gel. Electrophoresis was carried out on a 5-20% polyacrylamide gel stained with α-naphthyl phosphate and Red Fast salt at pH 5.0 (Section 2.10). Lane 1, crude fraction (supernatant); lane 2, $(NH_4)_2SO_4$ precipitation fraction; lane 3, AcP_1 from DEAE-cellulose; lane 4, AcP_2 from Sephadex G75; Lanes 5-6, two different preparations of AcP_2' obtained from Sephadex G75; lane 7, MEE; lane 8, AcP_3 from Sephadex G75. Reduced standard proteins (20μl): thyroglobulin (330 kDa) kDa), ferritin (half unit 220 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa), albumin (67 kDa) and ferritin (18.5 kDa).

Another band in the 30 kDa region appeared to be common in both AcP_2' and AcP_3 preparations. Interestingly, this is a typical example of hybrid proteins designated isoenzymes which catalyse the same reaction although having different K_m values. However, further investigations are required to establish if these AcPs are true isoenzymes, whose synthesis is controlled by distinct genes, or whether they are secondary isoenzymes derived by post-translational modification of a single gene product.

The detection of the carbohydrate moieties of glycoproteins was performed by the periodic acid-Schiff (PAS) reagent as described in Section 2.12. Although specific binding of AcPs to ConA was not obtained, PAS staining indicated that AcP_2' is glycosylated. Nondissociating gels stained by the PAS technique showed glycosylation patterns corresponding to those of enzyme activity. However, the enzyme was stained very faintly and sufficient contrast could not be obtained in order to produce a photograph to be shown in this thesis.

5.5.3 - Discussion

Hydrolysis of phosphate esters is an important process in biological systems involved in energy metabolism, regulation and cellular signal transduction. The present Section described, for the first time, the purification and characterisation of AcPs from *T. congolense*.

The four-step purification procedure summarised in Table 7, consists of $(NH_4)_2SO_4$ fractionation or Triton X-114 extraction, gel filtration and anion-exchange chromatography. Two AcPs designated AcP_1 and AcP_2, were isolated from cytosolic fractions while a membrane-bound, AcP_3, was purified from microsomal pellets. The molecular mass of AcP_1 could not be estimated either by gel filtration or by PAGE stained for AcP activity by α-NPP/Fast red reaction (Section 2.10). This enzyme appeared to have a molecular mass higher than 700 kDa and a Rs > 85 Å estimated by gel filtration. Although the characterisation of AcP_2' was beyond the scope of this study, the analysis of this enzyme on PAGE showed a ladder of multiple bands of activity. The prominent band with very little anodal migration on PAGE appeared to be comparable to AcP_1 with respect to their molecular masses. Since the AcP_2' prominent band was tested positive for glycoproteins by the PAS reaction, AcP_1 would appear to be a similar complex that might had been deglycosylated either during the purification procedure or by endogenous glycosidases. From this point of view,

AcP_1 and AcP_2' may therefore be products of separate genes derived from a common ancestor and which underwent different post-translational modifications.

Furthermore, analysis of the three AcPs by PAGE conducted in the presence of SDS revealed the presence of two closely migrating bands of activity at 62 and 65 kDa. This would suggest that AcPs may be proteins derived from separate genes that have arisen by duplication from a common ancestor, followed by independent variation. Because of additional minor bands of activity observed on both PAGE and SDS-PAGE, AcP preparations appeared to be heterogenous. Further purification is therefore still required. Amongst the partially purified AcPs, AcP_1 showed the highest level of purity and a specific activity of 10631.6 units/mg protein.

Unfortunately, the AcPs were isolated in relatively low yields and even the gentle procedure of gel filtration by Sephacryl S-300 chromatography showed a significant loss of activity for all the AcPs. In order to address this issue, either the ammonium sulfate precipitated cytosolic enzyme or MEE were separately chromatographed on DEAE resin. However, the yield still remained very low for all the three AcPs. Although enzymes appeared to be stable at pH 8.0 in the presence of 5% glycerol, the use of such a buffer did not appear to improve the yield of the enzyme. Moreover, a 40 μg/ml protease inhibitor cocktail (Section 2.3) was also added to protein samples and buffers with the aim of diminishing the suspected enzymatic degradation during purification. The general pattern did not change considerably. Therefore, the inactivation of AcPs may result from the lost of a cofactor leaving the inactive apo-enzyme. However, such cofactor has not yet been identified. This hypothesis is also supported by the attempts to purify the enzymes by a procedure involving IEF as described by Schell et al. (1990). These purifications were unsuccessful because of the inactivation of the AcPs. It was observed that only 15 - 26% of the activity which had been applied to the pH gradient was eventually recovered. This suggests that the remainder of the enzyme was inactivated and therefore remained undetected..

Although physical properties, e.g. those shown by analysis on electrophoresis gels, were sufficient to distinguish the isolated AcPs from each other. Inhibition and substrate specificity studies provided further support for three different AcP activities in *T. congolense*. Vanadate

was the most effective inhibitor for AcP_1 and AcP_3 while p-chloromercuribenzoate appeared to be a very good inhibitor for AcP_2. In comparison to *T. brucei* (Schell et al., 1990) and *T. rhodesiense* (McLaughlin, 1986) where tartrate-sensitive as well as tartrate-insensitive AcPs have been found, AcP_1 and AcP_3 are inhibited slightly by tartrate, albeit only weakly. Furthermore, the AcPs from *T. congolense* were all fluoride-sensitive which may indicate that the cytosolic enzymes originated from the microsomal pool of AcP.

The enzymes revealed considerable differences in their affinity for the various substrates tested. K_m values ranging from 0.23 to 0.72 mM for p-NPP have been obtained for these enzymes (Table 8) and were comparable to K_m values of 0.23 and 0.4 mM for p-NPP reported by Opperdoes et al. (1987) and Schell et al. (1990) in *T. brucei*. In *T. rhodesiense*, the K_m values of 1.7 and 3.0 mM for β-glycerophosphate reported by McLaughlin appeared to be similar to those determined for the same substrate in *T. congolense*. Overall, p-NPP appeared to be the best non-physiological substrate whereas G-6-P was the best physiological substrate.

In conclusion, a comparison of various physico-chemical and kinetic features of the three major AcPs of *T. congolense* support the idea of three distinct enzymes co-existing in these parasites. Significantly, analysis of these enzymes on SDS-PAGE indicated that these enzymes bear certain structural homology. Further studies are necessary to determine the causes of the inactivation observed during the purification of these AcPs so that higher levels of the purified AcPs may become available. Such purified enzymes will facilitate further chemical characterisation of the enzymes, as well as allow continued studies of their biosynthesis and mechanisms and their possible roles in pathology. It hoped that these results will contribute significantly to a greater understanding of the biochemistry of this parasite.

CHAPTER SIX
GENERAL DISCUSSION

Protozoans of the *Trypanosomatidae* family such as *Crithidia* (McLaughlin et al., 1975), *Leishmania* (Gottlieb and Dwyer, 1981), *Leptomonas* (Hunt and Ellar, 1974), *Trypanosoma cruzi* (Avila et al., 1979) and salvarian trypanosomes (Langreth and Balber, 1975; Venkatesan et al., 1977) harbour an acid phosphatase (AcP) in their flagellar pockets. This region of these organisms is considered to be the only site for endocytosis and exocytosis In some of these protozoa AcP has also been localised on the surface of some parasites (Gottlieb and Dwyer, 1981; Nagakura et al., 1985). Although its physiological role is not yet known, because of its accessible locations, AcP may be an antigen that can be exploited to overcome trypanosomiasis. In the flagellar pocket the enzyme may play a role in the acquisition of nutrients by trypanosomes, whereas its location on the surface may be important in parasite-host interactions. This dual location makes AcP an attractive target for trypanocidal drug design to overcome trypanosomiasis, thereby enhancing the control of this disease of importance in tropical areas. These considerations governed specific aspects examined in the present study on AcPs from *T. congolense*.

Several trypanosomatids such as *Leishmania* sp. (Bates and Dwyer, 1987) and *Trichomonas* spp. (Lockwood et al., 1988) have been shown to release AcPs into their surrounding medium. In the present study, experiments were conducted to test whether African trypanosomes are similar to, or differ from *Leishmania* and *Trichomonas* in that they do not release AcP(s) into their extracellular medium. The present studies showed that as long as the bloodstream forms of *T. congolense* were viable and healthy, no AcPs were released. Thereafter, AcP was found to be released when cells were started dying,.

Although viable *T. congolense* parasites did not show any substantial release of AcP into their surrounding medium, the live parasites were able to hydrolyse the phosphatase substrate p-NPP when incubated in the presence of this substrate. This observation suggested that an AcP may be

associated with the cell surface of the parasites. This is in agreement with the report indicating that an AcP may be associated with the surface membrane of *T. rhodesiense* (McLaughlin, 1986).

It is possible that the hydrolysis of p-NPP by the living parasites may have resulted from the hydrolysis of endocytosed p-NPP. However, this seems unlikely for two reasons. Firstly, because of its spectroscopic properties, yellow coloured reaction product (p-nitrophenol) should not be visible in any of the acidic compartments of the endosomal/lysosomal system where the p-NPP or p-nitrophenol might be expected to be delivered. Secondly, and more importantly, because the hydrolysis of this substrate by *T. congolense* also occurred at low temperatures (0-4°C) where endocytosis is known to be blocked (Lonsdale-Eccles et al., 1993; Brickman et al., 1995). Endocytosis only occurs in African trypanosomes when the temperature of the incubation medium exceeds 6-8°C although delivery to collecting tubules was observed at 12°C (Brickman et al., 1995). In mammalian cells endocytosis is blocked below a critical threshold value of about 10°C (Dunn et al., 1980). In the present study, *T. congolense* was found to hydrolyse p-NPP steadily, although slowly, on ice and at 4°C. These results suggest that the AcP activity observed in experiments with live parasites is not a consequence of the endocytic process, but rather that the activity is associated with an accessible enzyme, possibly located on the surfaces of the parasites.

The p-NPP-hydrolysing activity on the surfaces of trypanosomes could result from either alkaline or acid phosphatases. In order to characterise the activity involved, inhibition and pH studies were conducted. The effect of NaF, a well known AcP inhibitor, on the hydrolysis of p-NPP was compared to that of tetramisole, an alkaline phosphatase inhibitor (Van Belle, 1972). The inhibition studies showed that the phosphatase activity on the surface of *T. congolense* was not affected by tetramisole, but was inhibited by NaF. Moreover, NaF also inhibited the parasites motility. These findings suggested that the phosphatase activity associated with the live parasites is probably an AcP. This conclusion was supported by pH activity profile studies which showed the increase of activity at acid pH values and the decrease of activity at alkaline pH values.

In mammalian cells, AcP is localised in lysosomes (Bainton, 1981; Lemansky et al., 1985). In *T. brucei*, the enzyme is reported to be partly associated with intracellular membranes. These membranes comprise structures such as the endoplasmic reticulum, Golgi apparatus and

endosomal-like system. This localisation is based on biochemical (Rovis and Baekkeskov, 1980; Steiger et al., 1980; McLaughlin, 1982; Grab et., 1987) as well as cytochemical evidence (Langreth and Balber, 1975). The present thesis describes the first report of the distribution of AcP activity in *T. congolense* at subcellular level. Data obtained resulted from differential centrifugation studies, the screening for AcP activity in the resulting subcellular fractions as well as screening for specific organelle markers which permit assessment of the efficiency of organelles separation. AcP was found predominantly in the microsomal (F_4) and cytosolic (F_5) fractions. However, the specific activity was three-fold higher in fraction F_4 than in fraction F_5.

The distribution of the lysosomal enzyme marker, cysteine protease, (Mbawa et al, 1991b) provided first evidence that AcP activity is not a lysosomal enzyme in *T. congolense*. Cysteine protease activity was found predominantly in fraction F_3 (small granules) rather than in the microsomal fraction thus clearly showing that cysteine protease and AcP activities are not isolated from the same subcellular fraction. This agreed with observations reported by Grab et al. (1987) localising AcP in the Golgi apparatus of *T. brucei*. More recently, Grab et al., (1997) have shown by free-flow electrophoresis that the Golgi-associated AcP of *T. brucei* is specifically associated with the trans-Golgi network.

Further fractionation of the microsomal pellet by isopycnic sucrose gradient centrifugation indicated that the AcP activity of *T. congolense* sedimented at a density of 1.15 g/ml in sucrose gradients. Lysosomes of *T. rhodesiense* are reported to equilibrate at a density of 1.18 g/ml (McLaughlin, 1982). Since endoplasmic reticulum and Golgi apparatus membranes of *T. brucei* equilibrate at densities between 1.14 and 1.15 g/ml (Steiger et al.,1980; Opperdoes and Steiger, 1981), it was assumed that the AcP observed in microsomal fractions from *T. congolense* is associated with fractions containing these membranes. To confirm this, the localisation of the endoplasmic reticulum was assessed by screening for glucose-6-phosphatase activity, a classical marker for endoplasmic reticulum in mammalian cells (De Ceuster et al., 1995). Curiously, this enzyme marker was predominant in cytosolic fractions of *T. congolense*. These results disagreed with those of Rovis and Baekkeskov (1980) which localised this enzyme to the large granules fraction of *T. brucei*. The discrepancy between the present data and those of Rovis and

Baekkeskov (1980) may be explained by a difference in the method used for breaking the cells. Alternatively, this may reflect a difference between the two species of trypanosomes.

Cytochrome c reductase was distributed throughout the first three fractions F_1, F_2 and F_3 (crude nuclear, large granules and small granules fractions). Although considered to be a enzyme marker for mitochondria (Sottocasa et al., 1967), cytochrome c reductase has been shown to be present in both bovine mitochondria and endoplasmic reticulum (Amar-Costesec et al., 1974). Therefore, this enzyme should not serve as an unequivocal marker for mitochondria, a conclusion borne out by our observations in *T. congolense*.

In order to localise glycosomes, another important organelle in trypanosomes, malate dehydrogenase was selected. This enzyme was predominantly recovered in the large-granules fraction of *T. congolense* while Opperdoes et al. (1977) and Grab et al. (1987) found this enzyme largely in the cytosolic fraction of *T. brucei*. Despite the difference between the two localisations of glycosomes, AcP may not be associated with this organelle as the large granules fraction represented only 1% of the total AcP activity. Moreover, another enzyme marker such as glycerol-3-phosphate dehydrogenase would be recommended for glycosome localisation because malate dehydrogenase has been reported to be located in glycosomes and mitochondria of *T. brucei* and *T. cruzi* (Cazzulo, 1992).

In conclusion, the most probable subcellular localisation site of AcP is within the microsomal fraction. However, the precise organelles containing the AcP enzyme within this fraction still needs to be elucidated. In separate studies, the AcP activity was found to co-sediment with a substantial proportion of radiolabelled material derived from the plasma membranes of live parasites. Therefore, a proportion of the AcP activity recovered in the microsomal fraction may well originate from the surface of the trypanosomes.

Cytochemical techniques have permitted localisation of AcP in different organelles of African trypanosomes (Seed et al., 1967; Venkatesan et al., 1977; Langreth and Balber, 1975). These multiple locations of the AcP appears to be a common phenomenon in the *Trypanosomatidae* family as it was also reported in *Leishmania* sp. (Remaley et al., 1985; Avila et al., 1989).

Moreover, studies on a related insect flagellate, *Crithidia* sp., confirmed the heterogeneous localisation of AcP, as evidenced by differences in enzyme latency and sedimentation as well as in the kinetic properties of isoenzymes (McLaughlin et al., 1976). In order to establish if a similar situation of multiple AcP locations occurs in *T. congolense*, cytochemical techniques were performed on bloodstream forms of the parasite. AcP activity was located in the flagellar pocket and in some vesicles comprising coated vesicles and endosome structures. This was in agreement with observations made with other African trypanosomes (Seed et al., 1967; Langreth and Balber, 1975; Venkatesan et al., 1977; Steiger et al., 1980). In addition, the lead phosphate deposits, which are indicative of enzyme activity, were also observed in association with the plasma membrane enclosing the body of *T. congolense*. This confirmed the surface localisation of AcP already indicated in studies with live trypanosomes (Tosomba et al., 1996). Thus, *T. congolense* possess AcP activity on the surface membrane, as well as in the flagellar pocket and intracellular compartments (tubules, vesicles, endoplasmic reticulum and/or Golgi apparatus).

The intensity of the lead phosphate deposits on surfaces of positive cells was variable and requires further investigation. Although no antibody was employed in the experiments developed in this thesis, the *T. congolense* parasite is known to be able to remove a limited amount of potentially lytic immune complexes from its surface by filopodia formation (Frevert and Reinwald, 1990). This phenomenon might have occurred because the *T. congolense*-infected rats that were used to grow the parasites were not irradiated and were thus able to mount an immune response against the infecting parasites. This would explain differential formation of lead phosphate on the surface of *T. congolense* parasites as well as the presence of isolated filopodia-like material in the samples.

Because the phosphorylation and dephosphorylation of certain host enzymes result in the alteration of their activities (Cohen, 1982), the possibility that the membrane-bound AcP may be a phosphoprotein phosphatase was investigated and an attempt was made at finding an endogenous substrate for the enzyme. Two endogenous phosphoproteins pp52 and pp56 were therefore isolated and used as substrates to test for possible dephosphorylation activity of the membrane-bound AcP. Results indicated that this enzyme does not dephosphorylate either pp 52

or pp56 phosphoproteins. However, the data obtained may also suggest that the phosphoproteins pp52 and pp56 are not the specific substrates for this enzyme.

It was shown for the first time in the present study that at least three phosphatase activities can be isolated from *T. congolense* homogenates. All three have pH optima below 7.0 which clearly distinguish them from alkaline phosphatases. In contrast to the AcPs of *T. brucei* (Schell et al., 1990) and *T. rhodesiense* (McLaughlin, 1986), all AcPs from *T. congolense* were tartrate-resistant. One enzyme was isolated from the microsomal fraction and two further enzymes from the cytosolic fraction of *T. congolense*. Their respective enzymatic and physical properties clearly distinguished them from one another. For instance, AcP_1 and AcP_2 which were derived from the cytosolic fraction, had pI values of 6.5 and 5.3 respectively while AcP_3 isolated from the microsomal fraction had the lowest pI value of 4.7. However, such differences do not address the question of whether the three activities are distinct enzymes or are merely isoenzymes. Evidence supporting the distinctiveness of the enzymes came from inhibition and substrate specificity studies. Whereas vanadate inhibited AcP_1 and AcP_3 more strongly than AcP_2, p-chloromercuribenzoate inhibited AcP_2 more effectively than AcP_1 and AcP_3. Also, although p-NPP was the best of all the substrates tested, the K_m values for each of the various substrates, which are approximate indicators of the binding affinities of the enzymes for the substrates, differed widely between the three AcPs. Nevertheless, p-NPP was the best of all the substrates tested. Finally, analysis of AcPs on these non-denaturing PAGE gels showed further differences between the enzymes. AcP_1 had low anodal mobility and a M_r in excess of than 700 kDa. This was in agreement with the conclusion made using gel filtration where the M_r AcP_1 could no be estimated since it eluted with the void volume. AcP_2 and AcP_3 presented distinct electrophoretic bands at 600 kDa. Two different preparations of AcP_2' run under the same nondenaturing conditions showed a similar ladder of multiple bands which may represent a major slower-migrating band which degenerated into fast migrating bands over time. Thus each of the enzymes appears to be distinct.

However, in contrast to the nondenaturing gels, analysis of the three AcPs separated by denaturing SDS-PAGE showed the presence of two similar bands of activity in all three AcP preparations. Some minor bands of activity were also present within each preparation. The two

major bands of activity had apparent molecular masses localised at 62 and 65 kDa and were designated p62 and p65 respectively were present in all three AcP preparations besides minor bands of activity within each preparation. Because these two proteins were present in each of the three fractions (AcP_1, AcP_2 and AcP_3), hence each of these activities may be derived from the same base 62 and 65 kDa units. If this is the case, then the subsequent differences must be derived from differential processing of the isoenzymes for different functions at different locations. Although these two proteins p62 and p65 have not yet been characterised, it is clearly important that these proteins and their associated molecules be identified and characterised. Interestingly, preliminary results indicated that these two common proteins can be further separated on 10% SDS-PAGE and recovered after electroelution. Therefore, they are useful tools for either protein sequencing analysis or immunological studies.

Sequencing of the two common AcP proteins p62 and p65 will facilitate sequencing of the genes that encode these two proteins. A comparison of their gene sequences should reveal whether these AcP proteins (p62 and p65) are encoded by the same or different genes. It must be pointed out that different genes might have arisen from a common ancestor by gene duplication. Therefore, by undergoing independent variations, the genes could produce different proteins with different properties allowing them to function at different locations in the parasite. The amino acid sequences not only constitute a foot-bridge to genetic studies but will also permit determination of the structures of AcPs. This information could be used to determine the distribution of hydrophobic and/or hydrophilic regions that could explain the fragility of AcPs. This may also explain the low yield obtained during the purification of AcPs.

During early stages of this study, each of the MEE doublet bands of activity which as a doublet in the 600 kDa range on native PAGE (Figure 28, lane 7) were excised and separately used to immunise rabbits. Western blot analysis following PAGE showed that the antibodies targeted proteins located in 230 kDa (one band) and around 60-70 kDa (two bands) regions. Repeat experiments also did not give any indication of an immune reaction at 700 kDa which is the size of the immunogen. At that stage, there was no obvious explanation for these results. In the light of more recent findings it is suggested that the high molecular mass AcP complex dissociates into

several bands which results in a ladder of multiple bands on PAGE. As a result of this, the antibodies targeted components of lower M_r than those of the immunogen..

Since the hydrolysis of p-NPP by viable live trypanosomes and AcP_1 showed similar sensitivity to NaF and vanadate, it was concluded that AcP_1 is likely to be the AcP that is associated with the parasites'surfaces. Its recovery in the cytosolic fraction would indicate that this enzyme is easily removable from the surface membrane. AcP_1 was not found to be neither a glycoprotein nor a GPI-anchor protein. This enzyme may be a surface glycolipid-protein termed lipophosphoglycan AcP as demonstrated in related trypanosomatids such as *L. mexicana* (Ilg et al., 1994)

This work has laid the groundwork toward the ultimate goals of a better understanding of the patho-physiological role of AcPs in trypanosomes. Using cell culture techniques, this work has demonstrated that *T. congolense*, in contrast to many other trypanosomatids, but in common with other African trypanosomes, do not secrete AcP into its surrounding medium. These studies also showed for the first time, the localisation of a surface membrane-bound AcP in *T. congolense*. This is a feature shared by many trypanosomatids and may be related to some physiological adaptation necessary for survival within the host. Ultrastructural studies also permitted the demonstration of other common locations of AcP within *T. congolense* that are similar to other African trypanosomes, i.e. in the flagellar pocket. The use of analytical cell fractionation procedures in the present study clearly demonstrated that AcPs are not lysosomal enzymes like in mammalian cells. Using specific enzyme characterisation techniques, these studies have also shown the presence of at least three distinct acid phosphatases, one in the microsomal pellets and two in the cytosol based on differences in temperature and pH optima, molecular masses, substrate specificity, and inhibitory effects of certain compounds. From the light of these studies, it appeared that only one of these AcPs, AcP1, is found on the surface of the parasites. This conclusion is based upon the observations that viable live trypanosomes were 50% inhibited by 0.15 mM NaF while AcP1 was 50% inhibited by 0.2 mM NaF. In contrast, AcP_2 and AcP_3 were 50% inhibited by 10 and 0.02 mM NaF, respectively, which is outside the range of that observed with the live parasites. Immunolocalisation studies will be needed to confirm this conclusion. Finally, all these studies are expected to be complemented by immunological and genetic studies in order to unravel the role of AcPs in *T. congolense*.

Although much of the groundwork in the study of AcPs in *T. congolense* has been laid, new challenges still confront the investigator. Some of these have arisen from the findings of this thesis. With advances in genetic and immunologic studies, it is hoped that information provided in this thesis to other workers will permit effective and genetically-based strategies toward the elucidation of the patho-physiological role of AcPs in trypanosomes.

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O.M. Tosomba, T.H.T. Coetzer and J.D. Lonsdale-Eccles. Characterisation of acid phosphatases in bloodstream forms of *Trypanosoma congolense*. (Submitted to Comp. Biochem. Physiol.).

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Localisation of Acid Phosphatase Activity on the Surface of Bloodstream Forms of *Trypanosoma congolense*

OMALOKOHO M. TOSOMBA*,† THERESA H. T. COETZER,* AND JOHN D. LONSDALE-ECCLES*,[1]

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TOSOMBA, O. M., COETZER, T. H. T., AND LONSDALE-ECCLES, J. D. 1996. Localisation of acid phosphatase activity on the surface of bloodstream forms of *Trypanosoma congolense*. *Experimental Parasitology* **84**, 429–438. *In vitro*, living bloodstream forms of *Trypanosoma congolense* were shown to hydrolyse *p*-nitrophenyl phosphate, a substrate for phosphatases. This activity appears to be from an acid phosphatase because it was enhanced at low pH values, was inhibited by the acid phosphatase inhibitor sodium fluoride, and was not inhibited by the alkaline phosphatase inhibitor tetramisole. The activity did not appear to be secreted into the surrounding medium by the living parasites although phosphatase activity could be detected in the surrounding medium when dead or dying parasites were present. Studies at various temperatures indicated that at least some of this acid phosphatase activity may be associated with the surface of the parasites, rather than with endocytic or intracellular systems. This was supported by subcellular fractionation of radiolabelled parasites which showed some cosedimentation of acid phosphatase activity with radiolabelled iodine. Histochemical studies of the parasites also supported this conclusion. Electron microscopical examination of trypanosomes incubated with lead nitrate and *p*-nitrophenyl phosphate showed lead phosphate deposits on the surface of the parasites in addition to the expected localisation in the flagellar pocket. We conclude that *Trypanosoma congolense* possesses a surface-bound acid phosphatase. © 1996 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Trypanosoma congolense; Leishmania donovani;* trypanosome, surface membrane enzymes; flagellar pocket; AcP, acid phosphatase (EC 3.1.3.2); E-64 [L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane]; p-NPP, *p*-nitrophenyl phosphate; PSG, phosphate-buffered saline glucose.

INTRODUCTION

Acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) has been demonstrated in various parasitic protozoa of the family *Trypanosomatidae* such as *Crithidia* spp. (McLaughlin *et al.* 1976), *Trypanosoma cruzi* (Lettelier *et al.* 1985), *Leishmania donovani* (Gottlieb and Dwyer 1981), and salivarian trypanosomes (Seed *et al.* 1967; Langreth and Balber 1975; McLaughlin 1986). Some of these parasites have been found to secrete acid phosphatase (AcP) into the surrounding medium, and evidence of AcP activity associated with the surface membrane has been demonstrated in *T. cruzi* (Nagakura *et al.* 1985), *L. donovani* (Gottlieb and Dwyer 1981), and *Leptomonas collosoma* (Hunt and Ellar 1974).

In African trypanosomes, AcP activity is located in the Golgi apparatus, in the flagellar pocket, and in the endosomal system, thus indicating that the enzyme may play a role in the nutrition of trypanosomes. Furthermore, McLaughlin (1986) reported that bloodstream forms of *T. rhodesiense* contain a tartrate-sensitive and a tartrate-resistant AcP which was suggested to be distributed between the flagellar pocket and the surface membrane. Although this may be related to some physiological adaptation necessary for the parasite's survival within the host, the location of AcP activity on the surface membrane of African trypanosomes has not yet been extensively studied. In the present paper, evidence is provided for an association of AcP with the surface of bloodstream forms of *Trypanosoma congolense*.

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Materials and Methods

Reagents. All chemicals were of the highest purity available. Cacodylic acid, lactoperoxidase, p-NPP, Percoll, and tetramisole were purchased from Sigma Chemical Co. (St Louis, MO). Diethylaminoethyl (DEAE)-52 cellulose was obtained from Whatman Ltd. (Maidstone, Kent, UK). Antipain, chymostatin, E-64, and leupeptin were from Cambridge Research Biochemicals (Cambridge, UK). Hepes was obtained from Boehringer-Mannheim (Mannheim, Germany). Medium 199 was purchased from GIBCO (Grand Island, NY). Iodine-125 was purchased from Amersham International plc (Buckinghamshire, England).

Parasites. Trypanosoma congolense (clone IL 3000) were grown from cryopreserved stabilates in rats. The parasites were isolated from the infected blood using isopycnic Percoll gradients (Grab and Bwayo 1982). Briefly, infected blood was mixed 1:1 with Percoll containing 2% (w/v) glucose, 8.6% (w/v) sucrose (buffered to pH 7.4 with solid Hepes) and centrifuged at 31,000 *g* for 30 min. The resultant layer of trypanosomes was carefully removed by aspiration and diluted with an equal volume of PSG, pH 8.0. The trypanosomes were centrifuged at 3,000 *g* for 20 min. The pelleted trypanosomes were resuspended in a minimum volume of PSG and the pH was adjusted to 8.0 with 1 *M* Tris-base. The parasites were then subjected to chromatography on a column of DEAE-cellulose (equilibrated in PSG, pH 8) and eluted with the same buffer as described by Lanham and Godfrey (1970).

Enzyme assay. The procedure used for the determination of AcP activity was adapted from several sources (Helwig *et al.* 1977; Gottlieb and Dwyer 1981; Letelier *et al.* 1985). The reaction mixture consisted of 20 μl of 100 m*M* sodium acetate buffer, pH 5.0, 5 μl of 50 m*M* p-NPP solution made up in 100 m*M* sodium acetate buffer, pH 5.0, and 10–20 μg enzyme protein solution in a total volume of 50 μl. Incubations were carried out for 60 min at 37°C with gentle shaking (50 oscillations per minute). The reaction was stopped by the addition of 200 μl of 10 m*M* NaOH. The amount of *p*-nitrophenol liberated was determined spectrophotometrically at 405 nm using a BIO-TEK EL 312 microtitre plate reader. One unit enzyme was defined as the amount liberating 1 μmole of *p*-nitrophenol per minute under the above conditions. An extinction coefficient of 1.465 $\mu mole^{-1} \cdot ml \cdot cm^{-1}$ was used to determine the number of units of the *p*-nitrophenol.

Determination of AcP secretion by living parasites (T. congolense *and* L. donovani). Freshly isolated *T. congolense* IL 3000 (4×10^8 cells/ml) were washed twice in buffer A (10 m*M* Hepes, pH 7.2, containing 145 m*M* NaCl and 5 g/litre glucose) and incubated at 26°C. At appropriate time intervals, 100-μl aliquots of the cell suspension were transferred into 1.5-ml microfuge tubes and centrifuged for 15 sec at 15,000 *g*. Ninety microlitres of supernatant was removed and recentrifuged for 2 min to pellet any residual cells. The final supernatant (80 μl) was assayed for the presence of AcP activity using p-NPP as described above.

The *L. donovani* promastigotes, which were used in control studies, had been cultivated in sterile 50-ml screw-cap tubes containing 15–20 ml of medium 199 supplemented with 25 m*M* Hepes buffer and 20% (v/v) heat-inactivated foetal bovine serum. The pH of the medium was adjusted to 7.4 with 1 *M* NaOH prior to addition of the serum and filtered through a 0.4-μm Millipore filter. Before the AcP secreted was measured, the *L. donovani* promastigotes were washed in buffer A. The AcP assay was performed under the identical conditions described above for the bloodstream-form trypanosomes.

In addition to measuring the AcP content of the supernatants, each of the pellets obtained in the AcP secretion experiments was separately resuspended in 1 ml of PSG and the viability of trypanosomes monitored by the trypan blue exclusion test. Trypan blue (0.5 ml of 0.4% (m/v) trypan blue solution prepared in PSG), 0.3 ml of Hank's balanced salts' solution (8.0 g/litre NaCl, 0.4 g/litre KCl, 1.0 g/litre glucose, 0.06 g/litre KH_2PO_4, 0.047 g/litre Na_2HPO_4), and 0.2 ml of the resuspended cell pellet were mixed thoroughly and cells were left for 5 min to stain. Stained and unstained cells were counted using a Neubauer haemocytometer and an Erma hand-counter, and viable cells were estimated.

Hydrolysis of p-nitrophenyl phosphate by living trypanosomes. To investigate whether living trypanosomes could hydrolyse *p*-nitrophenyl phosphate directly, the parasites (4×10^8 cells/ml) were incubated at 26°C in buffer A containing *p*-nitrophenyl phosphate (5 m*M*). At various time intervals, 200-μl aliquots were centrifuged (15 sec at 15,000 *g*). The supernatant (180 μl) was recentrifuged for 2 min to pellet any residual cells and 50 μl of the final supernatant mixed with 200 μl of 10 m*M* NaOH. The liberated *p*-nitrophenol was immediately estimated at 405 nm.

Enzymatic radioiodination of live trypanosomes. The iodination procedure was adapted from Zingales *et al.* (1979) and Urbina *et al.* (1988). Purified trypanosomes were washed twice in buffer B (75 m*M* Tris–HCl, pH 7.4; 140 m*M* NaCl, 11 m*M* KCl) and resuspended to a final cell concentration of 1×10^9 cells/ml in the incubation medium. The incubation medium consisted of buffer B containing 50 μCi ^{125}I per millilitre, 25 μ*M* KI, 8 μ*M* H_2O_2 and 0.2 mg lactoperoxidase per millilitre. Cells were incubated for 15 min on ice with gentle agitation and the reaction was stopped by the addition of $Na_2S_2O_3$ to a final concentration of 100 m*M* in buffer B. The cells were washed four times with ice-cold buffer B supplemented with 5 m*M* KI. Cell viability of labelled cells was assessed using trypan blue.

Isolation of plasma membranes. Radio-iodinated trypanosomes were resuspended at 0°C in 20 m*M* Tris–HCl, pH 7.4; 1 m*M* EDTA at 4×10^8 cells/ml and disrupted by passage through a French pressure cell under a chamber pressure of 2500 psi. The homogenate was centrifuged at 14,600 *g* for 15 min and the pellet washed once in the same buffer. The supernatant fractions were then recentrifuged at 123,000 *g* for 60 min. In order to achieve further isolation of the radiolabelled plasma membranes, the 123,000 *g* pellet was fractionated by isopycnic centrifugation in Percoll (Grab *et al.* 1987). Briefly, the pellet was made 57.6% (v/v)

with respect to Percoll in 50 m*M* Hepes, 250 m*M* sucrose, 25 m*M* KCl, 1 m*M* EDTA, pH 7.4. This suspension (3 ml) was overlaid sequentially with 2 ml each of 43.2, 28.8, 25, and 20.3% Percoll solutions. The gradient was centrifuged for 40–45 min at 120,000 *g*, after which fractions (1 ml) were collected by draining the tube from the top with the aid of a peristalic pump. The absorbance at 280 nm of the collected fractions was measured as was the acid phosphatase activity. The radioactivity of the fractions was determined in a Packard 1500 TRI-CARB liquid scintillation analyser.

Cytochemistry. The cytochemical procedure for the detection of AcP was adapted from Langreth and Balber (1975) and Miyayama (1975). Briefly, the trypanosomes were fixed overnight at 4°C in 1% (v/v) glutaraldehyde, 2% (m/v) paraformaldehyde (grade I; Sigma Co.) contained in buffer C (100 m*M* cacodylate buffer, pH 7.2, 200 m*M* sucrose). Thereafter they were rinsed three times in buffer C and then three times in 100 m*M* Na acetate buffer, pH 5.0. Washed cells were preincubated in sodium acetate buffer containing 2 m*M* $Pb(NO_3)_2$ for 15 min at 37°C and then incubated in the same buffer containing 2 m*M* $Pb(NO_3)_2$, 10 m*M* p-NPP, for 60 min at 37°C. Control cells were incubated in a reaction medium without the substrate or, alternatively, in the reaction medium containing 10 m*M* sodium fluoride as an enzyme inhibitor. After incubation in the cytochemical medium, the cells were rinsed with buffer C, fixed again for 1 hr in 2.5% glutaraldehyde made up in buffer C, rinsed with buffer C and then postfixed in 1% OsO_4 for 1 hr at 4°C. Cells were washed with water, stained with 2% (w/v) aqueous uranyl acetate, dehydrated with a graded series of acetone solutions, and embedded in Epon. Ultrathin sections were obtained with a LKB Ultratome III ultramicrotome. Sections were stained with lead citrate and observed in a Joel 100CX transmission electron microscope operating at 80 kV. For the EDX scanning experiments the final staining with lead citrate was omitted.

Results

Determination of AcP Secreted by Live Trypanosomes

In agreement with earlier studies (Bates *et al.* 1989), cultured *L. donovani* promastigotes were found to release (or to secrete) AcP into the surrounding medium. In contrast, over the same period of 60 min freshly isolated *T. congolense* did not show substantial release of AcP activity into the supernatant fluids (Fig. 1). It therefore appears that *T. congolense* and *L. donovani* differ in this property.

Viability of Trypanosomes

Although *T. congolense* showed no significant release of AcP when incubated for up to 1 hr (Fig. 1), additional experiments over longer incubation periods showed increasing amounts of activity in the supernatant (Fig. 2). During

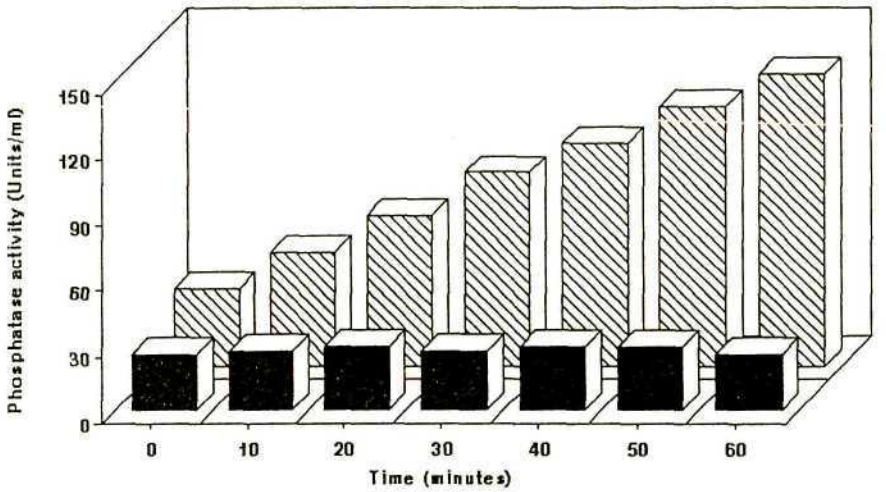

FIG. 1. Time course of secretion of AcP by living parasites (*T. congolense* and *L. donovani*). *T. congolense* and *L. donovani* (4×10^8 cells/ml) were incubated separately at 26°C in 10 m*M* Hepes buffer (pH 7.2) containing 145 m*M* NaCl and 5 g/litre glucose. At specific time intervals, aliquots of the cell suspension were centrifuged for 15 sec at 15,000 *g*. Supernatants were assayed for AcP activity as described under Materials and Methods. Solid black boxes indicate *T. congolense* and hatched boxes indicate *L. donovani* AcP activity. The results are representative of three separate experiments.

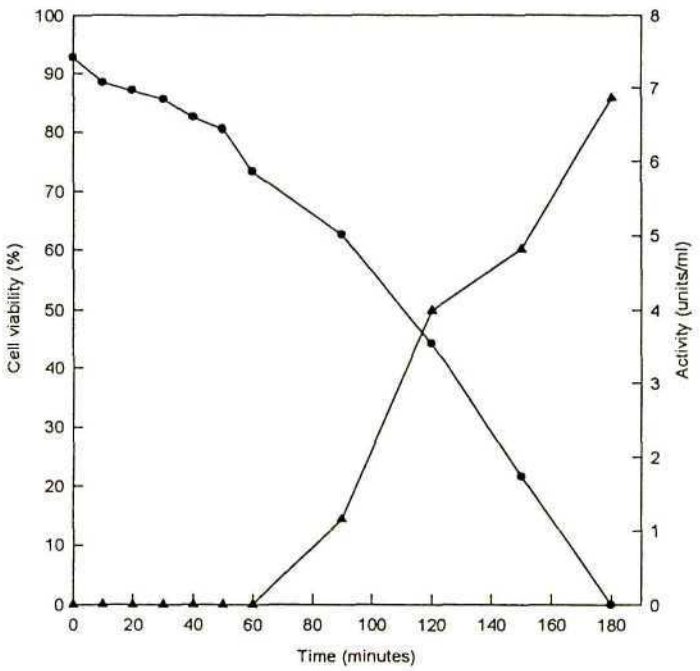

FIG. 2. Viability of live *T. congolense* as assessed by the Trypan blue exclusion test. Cell viability (●) corresponds to the total viable cells (unstained) compared to the total amount of cells (stained and unstained) expressed in percentage. The AcP activity (▲) was estimated using *p*-nitrophenol phosphate as described under Materials and Methods. The results are representative of three separate experiments.

the initial period, trypanosomes remained motile (as assessed by microscopic examination) and viable (as assessed by the Trypan blue exclusion test) but, after 1 hr, steadily increasing numbers of parasites were observed to have died. Thus, the enhanced AcP activity found in the *T. congolense* supernatants after 1 hr appears to be associated with cell death.

Hydrolysis of p-NPP by Live Trypanosomes

Although little or no AcP was secreted by live *T. congolense,* in order to ascertain whether or not there might be AcP associated with, or bound to, the surface of the parasites, the ability of live trypanosomes to hydrolyse p-NPP was examined. Considerable hydrolysis was observed when live cells were incubated at different temperatures (0–37°C) in the presence of p-NPP (Fig. 3). As expected, p-NPP hydrolysis was more rapid at elevated temperatures. Nevertheless, hydrolysis was observed at all temperatures, including 0 and 4°C where endocytosis should be halted. When examined by light microscopy, the parasites incubated at 42°C were found to be dying and so these results were excluded.

Effect of pH and Inhibitors on the Hydrolysis of p-NPP by Living Trypanosomes

When living trypanosomes were incubated at different pH values, phosphatase activity increased steadily as the pH decreased (Fig. 4). Thus the enzyme activity was probably due to an acid phosphatase rather than an alkaline phosphatase. This was confirmed by repeating the experiments in the presence of the acid phosphatase inhibitor NaF (10 m*M*). The release of *p*-nitrophenol from the substrate was almost completely inhibited at all pH values in the presence of NaF. In contrast, parasites that were incubated in the same substrate and buffers containing 3 m*M* tetramisole (an alkaline phosphatase inhibitor; Van Belle 1972) showed no inhibition of the hydrolysis of p-NPP. In-

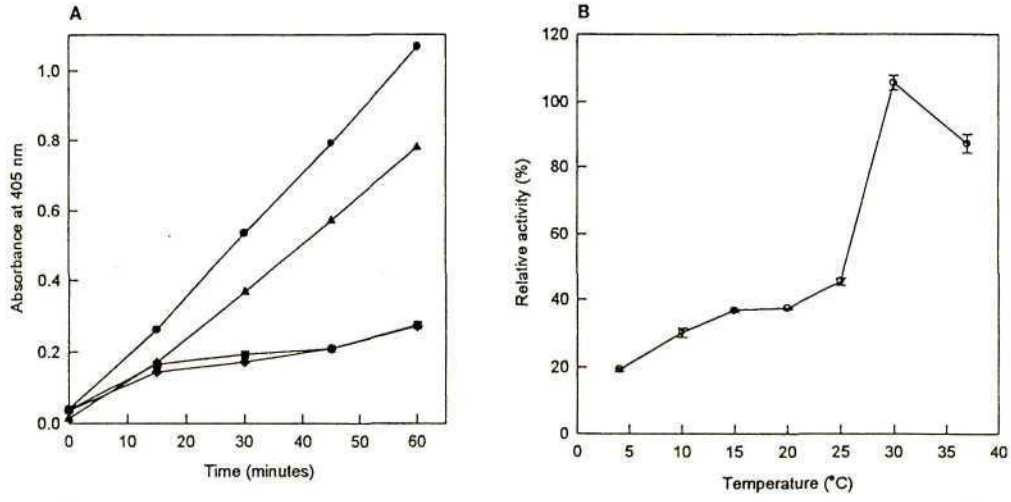

FIG. 3. The hydrolysis of p-NPP by living *T. congolense* at different temperatures. Trypanosomes (4×10^7 cells) were incubated at the indicated temperature values in the presence of 5 m*M* p-NPP. Either at each of the indicated times (A) or after a single 30-min incubation period (B) the cells were centrifuged for 15 sec at 15,000 *g* in a microfuge and the supernatants assayed for *p*-nitrophenol released as described under Materials and Methods. In the results shown in A, trypanosomes had been incubated at 26°C (●), 15°C (▲), 4°C (■), and on ice (♦). The results are representative of three separate experiments (A). In (B) the data are the means and SD for three or more values obtained in separate experiments.

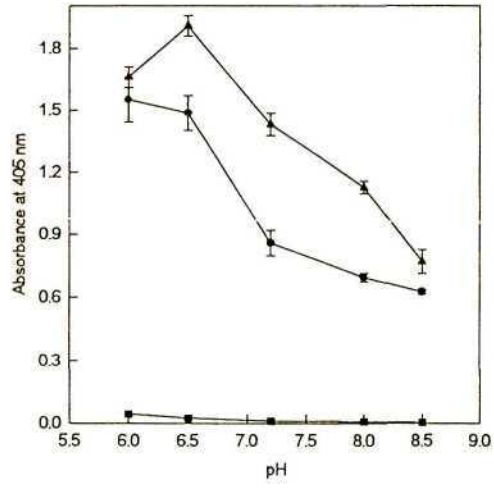

FIG. 4. Effect of pH and phosphatase inhibitors on the hydrolysis of p-NPP by live *T. congolense*. Trypanosomes (4×10^7 cells) were prepared in 10 m*M* Hepes buffer (at the indicated pH values) containing 145 m*M* NaCl, 5 g/litre glucose and 5 m*M* p-NPP alone (control, ●) or supplemented with 10 m*M* NaF (■) or 3 m*M* tetramisole (▲). After incubation of trypanosomes at 37°C for 30 min, samples were centrifuged for 15 sec at 15,000 *g* in a microfuge and the *p*-nitrophenol released from p-NPP measured as described under Materials and Methods. Data are means ± SD for four values obtained in separate experiments.

deed, tetramisole appeared to increase the activity slightly. Interestingly, light microscopic examination showed that the parasites were immediately rendered immobile in the presence of

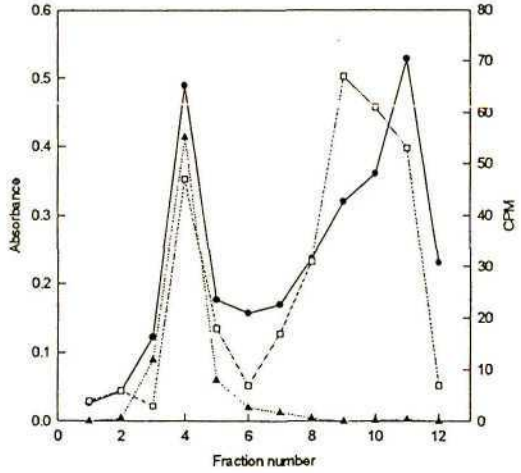

FIG. 5. Centrifugation of the 123,000 *g* pellet in Percoll gradients. This pellet obtained as described under Materials and Methods was centrifuged in Percoll gradients. The A_{280} nm profile (●), as well as the distribution of acid phosphatase (▲) and bound iodine (□) are analysed. The top of the gradients is on the left. The results are representative of two separate experiments.

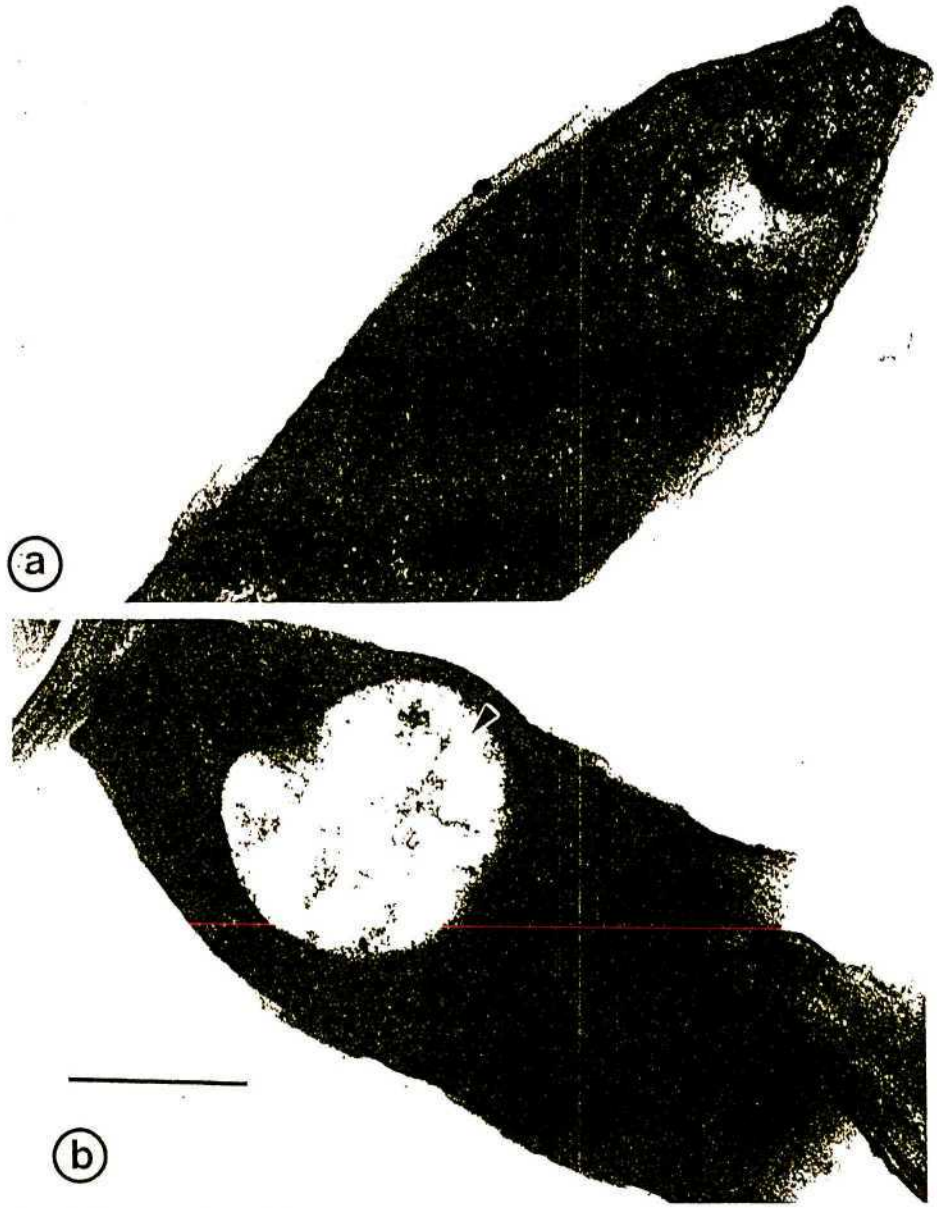

FIG. 6. Bloodstream forms of *T. congolense* prepared for cytochemical study. (a) Trypanosomes incubated in the absence of the substrate (pNPP) showed no Pb phosphate deposition in the flagellar pocket nor over the surface. (b) Trypanosomes incubated in the presence of the substrate and the inhibitor (NaF) also showed no Pb phosphate deposition in the flagellar pocket (arrowhead) nor over the surface. The magnification of a and b is the same. Bar, 0.5 μm. (c) In trypanosomes incubated in the presence of the substrate, the dense deposits of Pb phosphate were observed on the cell surface and in the flagellar pocket (arrowhead). Occasionally, some incompletely labeled cells were observed (open arrows). Bar, 1 μm. (d) Occasionally electron-dense precipitates of lead phosphate that were not associated with cells were found (arrow). Bar, 1 μm.

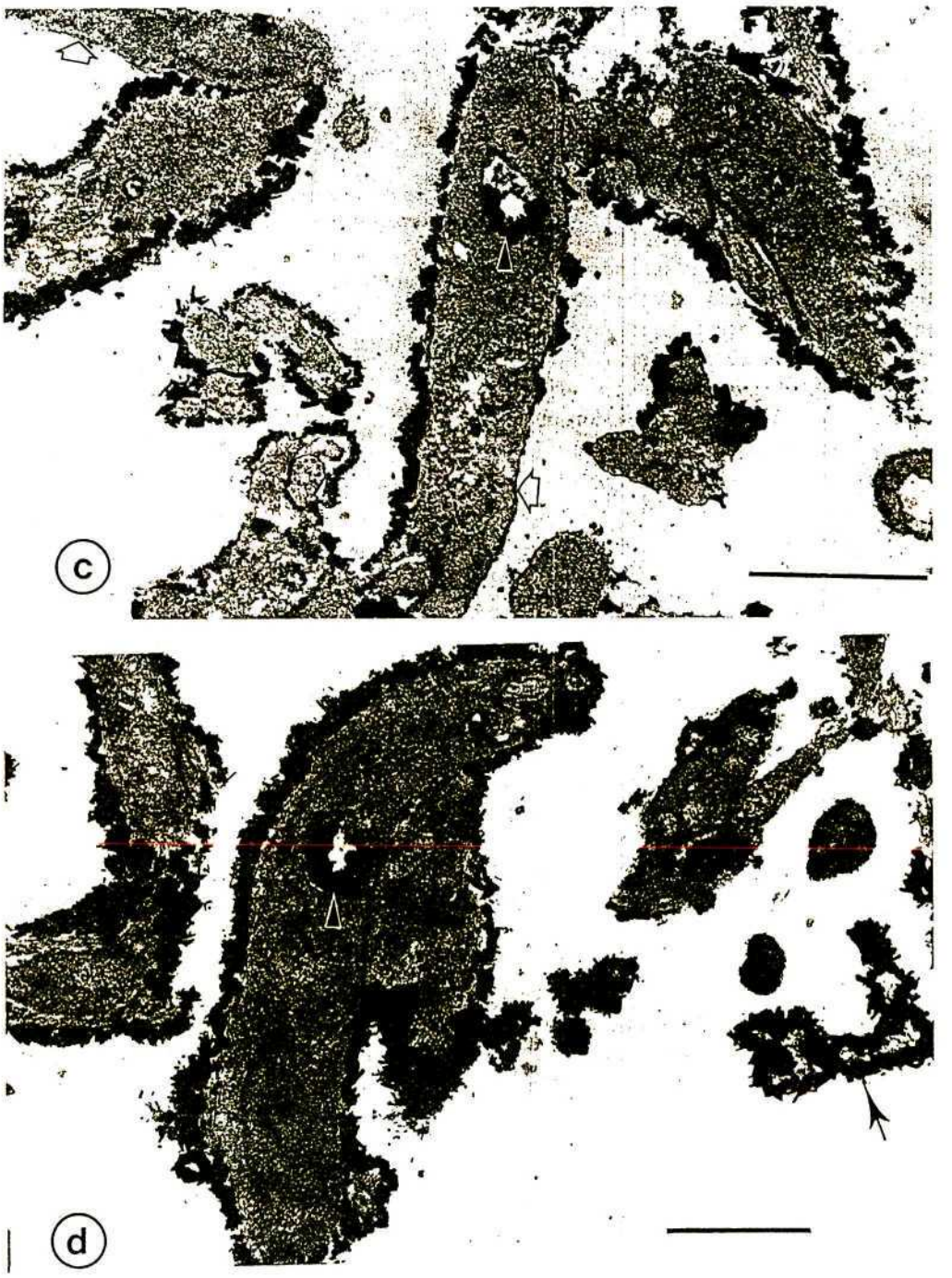

FIG. 6—*Continued.*

NaF whereas those in the presence of tetramisole were still motile.

Percoll Gradient Distribution of AcP and Surface-Bound Iodine

The AcP activity, radioactivity, and absorbance at 280 nm profiles obtained with radiolabelled plasma membranes isolated on Percoll gradients are depicted in Fig. 5. AcP activity was concentrated in the 25–28.8% Percoll interface, in a well-defined peak. The migration of this enzyme was coincident with a proportion of the labelled iodine. The radioactivity recovered from this peak represents about 20% of the 123,000 *g* pellet, indicating that a substantial proportion of the membrane cosediments with other heavier subcellular fractions that represent the major peak of radioactivity (Fig. 5). Nevertheless, radioactivity was clearly associated with phosphatase activity.

Acid Phosphatase Cytochemistry

In control experiments, ultrathin sections of *T. congolense* that had been incubated in the absence of p-NPP (Fig. 6a) or in the presence of p-NPP and NaF (Fig. 6b) show that the general morphology of the trypanosomes was maintained. In trypanosomes that had been incubated with p-NPP (10 m*M*), an electron-dense precipitate of lead phosphate was observed to be widely distributed over the surface (Figs. 6c and 6d). Occasionally, some incompletely labelled cells were observed (Fig. 6c; open arrow). Electron-dense precipitates of lead phosphate were also found apart from the cells (Fig. 6d; closed arrow). In addition to the strong cell surface labelling, trypanosomes also displayed strong AcP reactivity in the flagellar pocket (Figs. 6c and 6d; arrowheads).

In order to ascertain whether the electron-dense precipitate distributed over the trypanosomes' surface was due to a lead phosphate complex, an energy dispersive X-ray microanalysis (EDX) was performed on ultrathin sections of *T. congolense*. A Link exl II EDX ATW2 detector attached to a Joel 100CX transmission electron microscope was used to analyse the lead content of the surface labelling. Significant peaks of copper (from the grid), iron (from the pole piece), and molybdenum (from the aperture) were obtained together with lead resulting from the specimens (Fig. 7).

Discussion

In contrast to other trypanosomatids such as *Leishmania* sp. (Bates and Dwyer 1987) and *Trichomonas* spp. (Lockwood *et al.* 1988), it is generally accepted that African trypanosomes do not release acid phosphatase(s) into their extracellular medium. This was confirmed by the present studies obtained with living bloodstream forms of *T. congolense*. These trypanosomes did not show any substantial release of acid phosphatase into the supernatant fluids assayed for as long as the parasites were viable. However, living *T. congolense* parasites that were incubated in the presence of the phosphatase substrate p-NPP were able to hydrolyse this substrate. This observation indicated that an acid phosphatase may be associated with the cell surface. This is in agreement with the report of the presence of an acid phosphatase associated with the surface membrane-containing fractions of *T. rhodesiense* (McLaughlin 1986).

Alternatively, the hydrolysis of p-NPP may have resulted from endocytosed p-NPP. However, the observed hydrolysis of this substrate by *T. congolense* at low temperatures does not support this contention because temperature is among the factors that affect endocytosis. For endocytosis to occur in African trypanosomes the temperature of the incubation medium must exceed a critical threshold value of about 6–8°C (Lonsdale-Eccles *et al.* 1993). Similarly, Brickman *et al.* (1995) also observed no endocytosis by *T. b. rhodesiense* at 4°C, although delivery to collecting tubules was observed at 12°C. In mammalian cells endocytosis is also blocked below a critical threshold value of about 10°C (Dunn *et al.* 1980). In the present study, *T. congolense* was shown to hydrolyse *p*-nitrophenyl phosphate readily on ice and at 4°C, albeit at lower rates than those observed at higher temperatures. These results suggest that the AcP

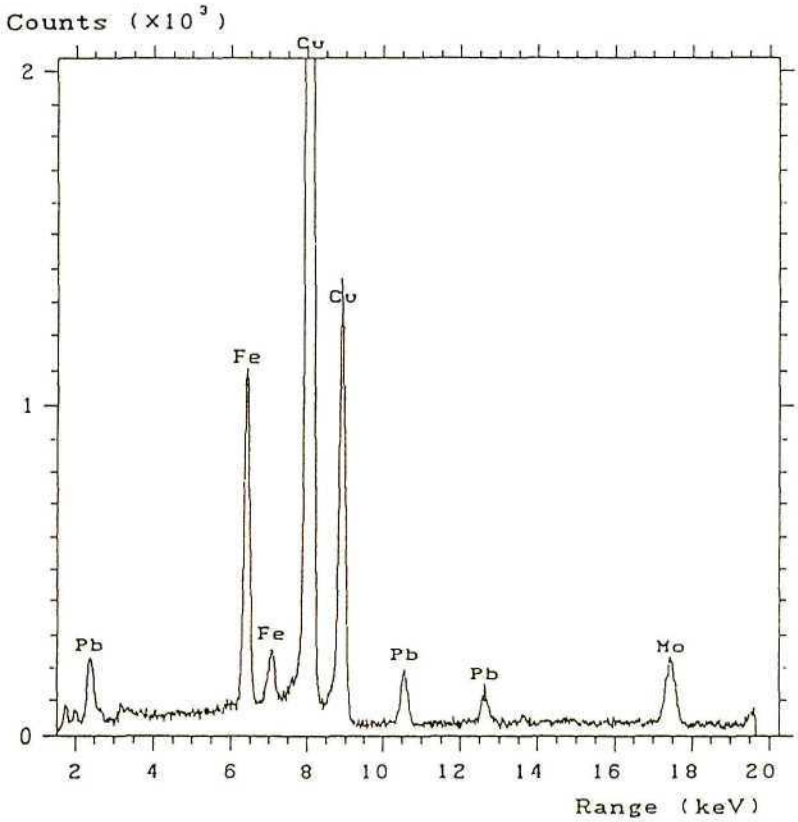

FIG. 7. An energy dispersive X-ray spectrum taken from ultrathin sections of *T. congolense* that were unstained with lead citrate. Significant peaks of copper (grid), iron (pole piece), and molybdenum (aperture) were obtained simultaneously with lead (specimen).

activity observed with living parasites is not a consequence of endocytic processes but rather that the activity is located on the surface of the parasites.

The electron microscopic detection of AcP activity on the surface of the parasites supports the biochemical data described above. The trypanosomes show strong p-NPP hydrolytic reactivity on the surface as well as within the flagellar pocket of the parasites. Similarly, the results from the subcellular fractionation studies indicated a cosedimentation of AcP and surface-bound radioactive iodine. Thus the biochemical studies, histochemical studies, and subcellular fraction studies are all consistent with the presence of a surface located phosphatase on *T. congolense.*

In order to characterise the nature of the surface phosphatase, inhibition and pH studies were conducted. The inhibitors used were NaF, a widely recognised acid phosphatase inhibitor, and tetramisole, an alkaline phosphatase inhibitor (Van Belle 1972). The cell surface phosphatase activity in *T. congolense* was unaffected by tetramisole but was inhibited by NaF. (The NaF also caused the parasites to stop moving.) These observations indicated that the phosphatase activity associated with the living parasites is an acid phosphatase. This conclusion was supported by pH activity profile studies showing increased activity at lower pH values and diminished activity at higher pH values. It is not clear what function this enzyme may have on the surface of the parasites but its location at this site may add to the possible immunologic and/or drug targets for future therapeutic intervention.

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