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Studies on the glycosome of

Trypanosoma brucei

by

Rashid Abdi Aman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Glycosomes (microbodies) have been purified from bloodstream form *Trypanosoma brucei* by an improved procedure involving freezing and thawing live organisms in 15% glycerol prior to cell disruption. Highly purified organelles of bloodstream form *T. brucei* contain 11 major proteins of which 8 tentatively identified glycolytic enzymes make up about 90% of the total glycosomal protein. Treatment of these intact isolated organelles with the bisimidoester dimethylsuberimide (DMSI) resulted in crosslinking of all glycosomal proteins into a large complex suggestive of juxtapositioning of the glycosomal proteins. The crosslinked complex was capable of catalyzing the multienzyme conversion of glucose to glycerol-3-phosphate but did not possess any special kinetic features different from those of the unaggregated enzymes represented by solubilized glycosomes. The multienzyme reaction had a lag phase associated with it and [^{14}C]-glucose label incorporation into sugar phosphate intermediates was effectively competed by unlabeled intermediates. Glucose-derived fructose-1,6-diphosphate was equally accessible to exogenous fructose-1,6-diphosphatase in both systems. 2-deoxyglucose and *in situ* generated 2-deoxyglucose-6-phosphate both were weak inhibitors of the multienzyme reaction with no preferential inhibition of the crosslinked complex-catalyzed reaction. All data indicate that substrate channeling plays no role in the glycolytic process in the glycosome of bloodstream form *T. brucei*. Glycosomes were also purified from culture form *T. brucei* by several different procedures. Comparision of highly purified organelles from the two different life stages of the organism showed reduced specific activities and contents of the early glycolytic enzymes in organelles from the culture form with a decrease from 87% to 35% of the contribution of glycolytic enzymes to the total glycosomal

protein. 18% of the protein in culture form glycosomes is contributed by four new polypeptides. Two polypeptides of subunit M_r 24.8 and 23.2 kD have been identified as integral membrane proteins. In the bloodstream form organelles, these two proteins account for 7% of the total glycosomal protein and are the only integral membrane proteins. Glycosomal membrane fractions from organelles of the culture form of the parasite contained two additional proteins whose precise subglycosomal location is uncertain at this point. Purified bloodstream form organelles lack external peripheral membrane proteins with most of the major glycosomal proteins being cisternal or matrix proteins.

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My parents and family for their endless support of all forms

My brother Mohamed and cousin Mohamud for their friendship and support

Fardouse Adan for giving new meaning to my life

Dedication

To my loving parents

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Abbreviations

ALD :	Aldolase
2DG :	2-Deoxyglucose
2DG6P :	2-Deoxyglucose-6-phosphate
DHAP :	Dihydroxyacetone phosphate
DMSI :	Dimethyl suberimide
DTBP :	Dimethyl-3,3'-dithiobispropionimide
FDP :	Fructose-1,6-diphosphate
FDPase :	Fructose-1,6-diphosphatase
F6P :	Fructose-6-phosphate
G :	Glucose
GAP :	Glyceraldehyde-3-phosphate
GAPDH :	Glyceraldehyde-3-phosphate dehydrogenase
G3P :	Glycerol-3-phosphate
GDH :	Glycerol-3-phosphate dehydrogenase
G6P :	Glucose-6-phosphate
GK :	Glycerol kinase
HK :	Hexokinase
MDH :	Malate dehydrogenase
PEP :	Phosphoenolpyruvate
PEPCK :	Phosphoenolpyruvate carboxykinase
1,3-PGA :	1,3-Phosphoglycerate
2-PGA :	2-Phosphoglycerate
3-PGA :	3-Phosphoglycerate
PFK :	Phosphofructose kinase
PGI :	Phosphoglucose isomerase
PGK :	Phosphoglycerate kinase

SHAM : **Salicyl hydroxamic acid**
TCA : **Trichloroacetic acid**
TPI : **Triose phosphate isomerase**

CHAPTER I.

INTRODUCTION : AFRICAN TRYPANOSOMES

Medical and Veterinary Importance, Distribution and Epidemiology ¶

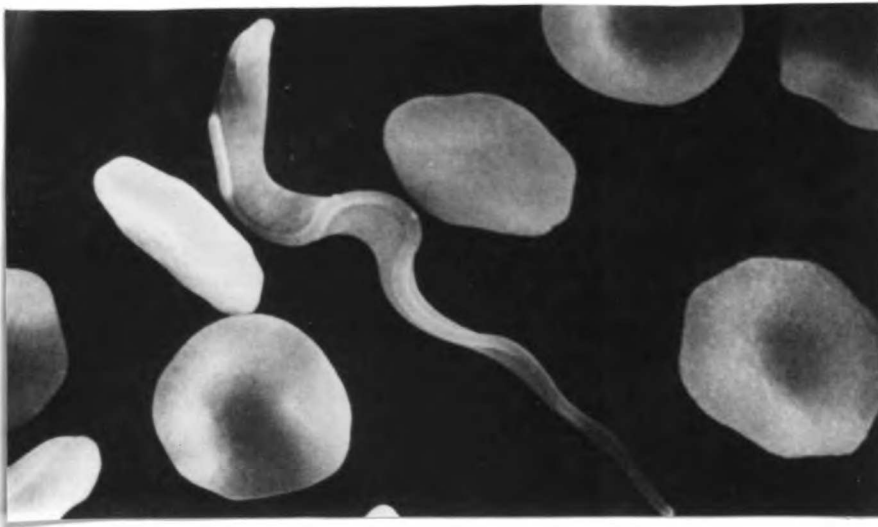
Trypanosomes are flagellate unicellular organisms (see Fig. 1) parasitizing a whole range of vertebrate hosts. The mammalian trypanosomes are important in that they are responsible for serious disease in man and his domesticated animals (1-9). Trypanosomiasis, as the diseases caused by these protozoan parasites are called, are a group of serious and often fatal diseases that prevail in large areas of Africa, Latin America, Middle East and Asia. Various species of the genus *Trypanosoma* infect cattle, sheep, goats, pigs, horses, donkeys, camels, many wildlife species and man. Two broad classes of trypanosomiasis are generally recognized: the African trypanosomiasis and the American or more appropriately South American trypanosomiasis. This distinction is based not primarily on the geographic location as the names would suggest but on the mode of transmission of the parasite as determined by the anatomical location of infective forms in the insect vector (see below).

African trypanosomiasis is still one of the most serious threats to the health of man and a serious obstacle to the agricultural industry in sub-Saharan Africa (10,11). Human African trypanosomiasis or sleeping sickness is caused by the two closely related subspecies *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. Another close relative of these two subspecies is *Trypanosoma brucei brucei* which together with *Trypanosoma congolense* and *Trypanosoma vivax* are

¶ Detailed accounts of the biology, epidemiology and the medical and veterinary importance of African trypanosomes are given in refs. 1,2,10,11.

FIGURE 1

Scanning electron micrograph of a long slender bloodstream form trypomastigote of *Trypanosoma brucei brucei* among erythrocytes. The cells have been enlarged 5200 times.[¶]



[¶]Reproduced from ref. 40.

the most important species which infect domestic ruminants, notably cattle in Africa.

Human trypanosomiasis represents a continuous serious threat to the health of many communities in Africa. Recent epidemiological studies indicate that at least 45 million people are exposed to risk of infection (11). The actual number of documented cases of sleeping sickness has increased from the pre-1979 values of 10,000 cases per year to approximately 20,000 cases a year, largely due to the recent epidemic outbreaks in Cameroon, Sudan and Uganda (8, 11). Still, this value is considered to be an underestimate given the difficulty of diagnosis of the disease especially the chronic type disease (see below) and the poor accessibility to health-care facilities in many parts of Africa. The need for constant vigilance and maintenance of effective diagnostic and surveillance facilities is important because of the propensity of the disease to rapidly spread to epidemic proportions anytime that vector control and containment efforts are compromised either due to war or mass migration as is exemplified by the recent outbreak in Uganda (12,13).

The human disease occurs throughout a large tract of sub-Saharan Africa covering some 4 million square miles or roughly one-third of the continent's land mass. (Figure 2) (11). In this belt are found 34 or so species and subspecies of the tsetse fly vector. Despite this wide distribution, the disease is usually associated with well defined endemic foci which can be the centres of epidemic outbreak at any given time. Classically, human trypanosomiasis in Africa is regarded as having two distinct forms. The acute rhodesiense form is caused by *Trypanosoma brucei rhodesiense* which is found from Ethiopia south to Botswana.

FIGURE 2

Tsetse fly-infested zone of sub-Saharan Africa.¶



¶Reproduced and modified from ref. 40.

In Central and West Africa is found the chronic form of the disease caused by *Trypanosoma brucei gambiense*. While there may be overlap of the two forms in some parts of Africa, it is certain that the acute form predominates in East Africa where the duration of the disease is weeks or months. Infected individuals of the chronic gambiense form may sometimes be asymptomatic carriers for several years. The two forms of the disease, though quite distinct clinically and epidemiologically, have the same essential pathology and the principles of treatment are the same. Both are fatal if left untreated. The economically important animal trypanosomiasis is found throughout the tsetse-infested belt and is responsible for the loss of some 3 million head of cattle each year (10). Only one-eighth of the total cattle population of about 160 million in Africa is located in this tsetse infested zone representing a third of the land mass of the continent, and most of which is potential rangeland (14). 20 million head of cattle are continuously exposed to the risk of infection. It has been estimated that effective control of trypanosomiasis would make it possible to increase the cattle rearing capacity of this region by 600% ! and provide an additional 1.5 million tons of meat a year, without creating any undue environmental stress (14). The far reaching consequences of animal trypanosomiasis in Africa could be an important factor in the crisis facing the continent today. The exclusion of cattle rearing from this large area of land certainly contributes to the overgrazing and land erosion problems as well as to the protein malnourishment in many communities in Africa.

Taxonomic Classification(2,15,16,17)

The genus *Trypanosoma* is a member of the order Kinetoplastida. Members of this order, which includes the important human pathogens of the genus

Leishmania, are characterized by the presence of an unusual self-replicating mitochondrial DNA-containing organelle or kinetoplast found at one end of the large single mitochondrion. The complete taxonomic classification of trypanosomes is summarized in Table 1. Mammalian trypanosomes are generally divided into seven subgenera (15) (Tables 1&2). Members of these subgenera fall into one of two sections. The Stercoraria are those trypanosomes which complete their developmental cycle in the insect vector in the hind gut of the insect. Infective metacyclics are therefore found in the faeces and transmission is contaminative. When the insect vector (Triatomid bugs) bites a human, it defaecates at the same time and subsequent scratching of the site and touching of mucuous membranes such as those of the eye lead to spread and systemic penetration of the parasites. The Stercorarian trypanosomes are found mostly in Latin America and therefore commonly referred to as the South American trypanosomes. Of the South American trypanosomes, *Trypanosoma cruzi* is the single most important human pathogen causing the untreatable and debilitating 'Chaga's disease'. The Salivarian trypanosomes encompass members of four subgenera all of which are important pathogens of man and domestic animals. Various species of the tsetse fly (genus *Glossina*) are vectors in which the developmental cycle of the parasite is completed in the mouth parts or salivary glands of these insects. Since infective metacyclic forms are present in the saliva of the fly, transmission of the disease is by inoculation during a blood meal. Salivarian trypanosomes are found largely in sub-Saharan Africa although some species, such as *Trypanosoma equiperdum* (transmission during coitus in horses) and subspecies of *Trypanosoma vivax* adapted to noncyclic transmission by biting Diptera, are found in the Middle East, Asia and Latin America. Table 3 presents some features of

TABLE 1 ¶

Systematic position of the genus Trypanosoma and the seven subgenera that infect mammals

Phylum	Protozoa	
Subphylum	Sarcomastigophora	
Superclass	Mastigophora	
Class	Zoomastigophorea	
Order	Kinetoplastida	
Suborder	Trypanosomatina	
Family	Trypanosomatidae	
Genus	<i>Trypanosoma</i>	
Subgenera		<i>Herpetesoma</i> <i>Megatrypanum</i> } <i>Stercoraria</i> <i>Schizotrypanum</i> <i>Duttonella</i> <i>Nannomonas</i> } <i>Salivaria</i> <i>Trypanozoon</i> <i>Pcynomonas</i>

¶ Adapted from 36.

TABLE 2 ¶

Some features of the subgenera of mammalian trypanosomes

Subgenus	Type species	Disease produced	Vector
Megatrypanum	<i>T. theileri</i>	None	Tabanids and hippoboscids
Herpetesoma	<i>T. lewisi</i>	None	Fleas (and triatomid bugs for <i>T. rangeli</i>)
Schizotrypanum	<i>T. cruzi</i>	Chagas' disease in man	Triatomid bugs
Duttonella	<i>T. vivax</i>	Sourra in cattle	Tsetse fly
Nannomonas	<i>T. congolense</i>	Nagana in cattle	Tsetse fly
Trypanozoon	<i>T. b. brucei</i>	Nagana in cattle	Tsetse fly
	<i>T. b. gambiense</i>	Sleeping sickness in man	Tsetse fly
	<i>T. b. rhodesiense</i>		
Pycnomonas	<i>T. suis</i>	Chronic porcine trypanosomiasis	Tsetse fly

¶ Adapted from 36.

TABLE 3 ¶**Specific and subspecific classification of salivarian trypanosomes**

Trypanozoon	Duttonella	Nannomonas	Pycnomonas
T. brucei brucei †	T. vivax vivax	T. congolense	T. suis
T. brucei rhodesiense	T. vivax viennei		
T. brucei gambiense		T. simiae	
	T. uniforme		
T. evansi evansi			
T. evansi equiperdum			
T. equinum			

¶ Adapted from 36.

† Although the correct taxonomic classification requires that these sub-species be referred to by their full names as in this Table, it is common practice to use the simple species name e.g. *T. brucei* instead of *T. brucei brucei*. In this work, the two names are used interchangeably.

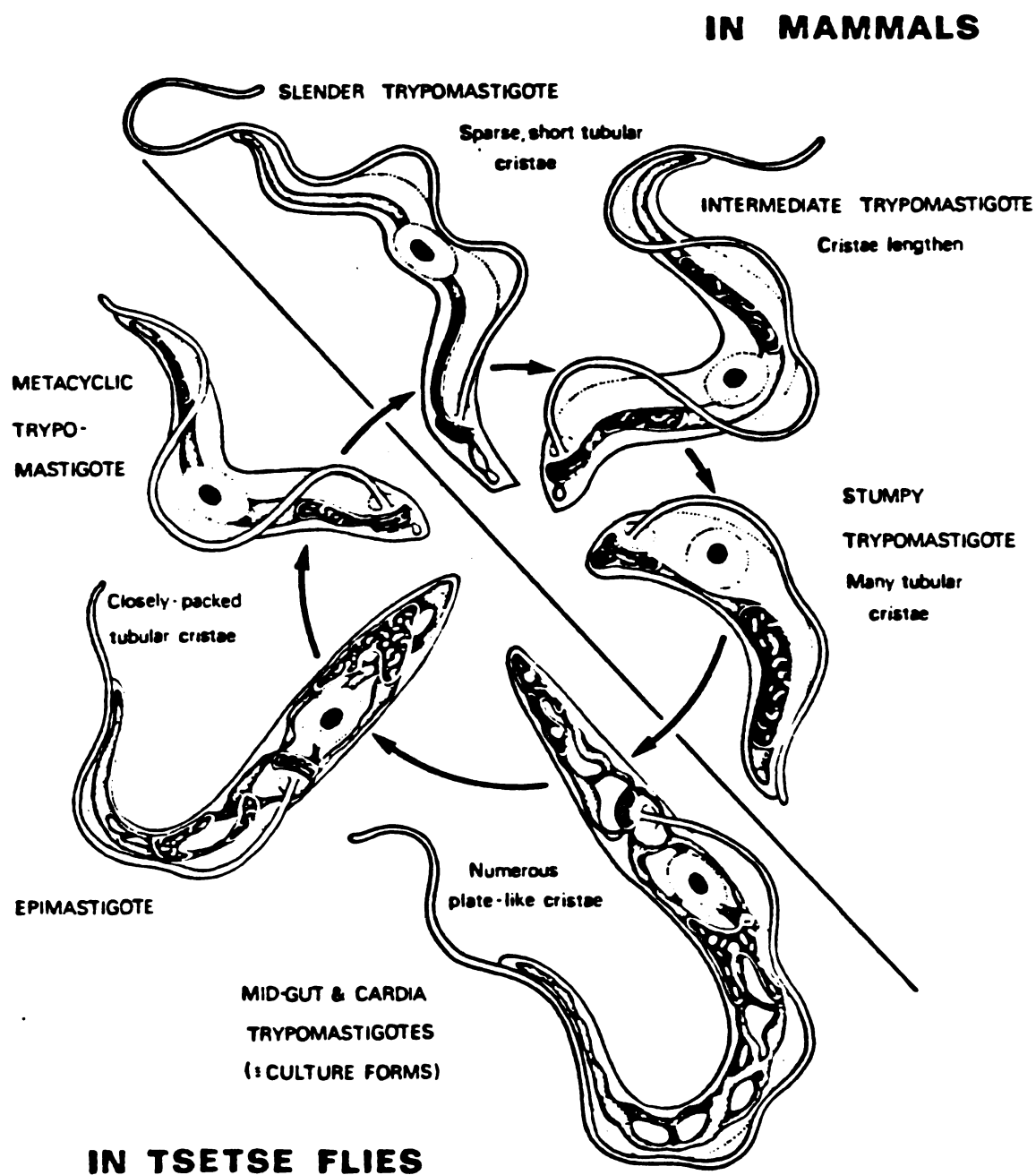
the mammalian trypanosomes with prototype species of each subgenera. Within each subgenus of the Salivarian trypanosomes, the classification into subspecies is not clearly defined but is based mainly on differences in epidemiology, host range and virulence. *Trypanosoma brucei brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are all subspecies of the *brucei* species and may be considered as 'nosodemes' or clinical races that are morphologically indistinguishable and biochemically very similar but differ only in host range and the virulence of the disease caused. For example, *T. b. brucei* infects cattle whereas *T. b. gambiense* and *T. b. rhodesiense* are human pathogens, the former causing the chronic form of sleeping sickness in West Africa and the latter the acute form of the disease in Eastern Africa. In other cases, a major difference may be in the mode of transmission. For example, *Trypanosoma evansi evansi* which is transmitted mechanically (noncyclically) by biting Diptera is believed to be an evolutionary adaptation of *T. brucei*. Subspecies *Trypanosoma evansi equiperdum* is morphologically indistinguishable from *T. e. evansi* but differs from it in that its transmission requires no arthropod vector and is by venereal means in horses. *T. evansi equinum* on the other hand is distinguished from *T. e. evansi* by its lack of a kinetoplast.

Life Cycle(11,16,17,18)

African trypanosomes develop and multiply in the mammalian bloodstream. The parasites are transmitted cyclically by various species of *Glossina* although noncyclic or mechanical transmission of some types of trypanosomes by other biting flies and venereal transmission have been described (see above). The life cycle chart of *T. brucei brucei* shown in Figure 3 is applicable to all other cyclically transmitted African

trypanosomes. When a tsetse fly feeds on an infected mammal, it ingests parasites along with the blood meal. Parasites ingested by the insect undergo essentially three stages of development within the insect before they transform to infective metacyclic forms. There is the initial establishment and multiplication within the endoperitrophic cavity of the midgut. As the parasites differentiate to the procyclic form, they proceed on to colonize the ectoperitrophic space and then the proventriculus. From here they migrate to the salivary glands where they establish themselves as infective metacyclic forms. These developmental changes in the fly take from six to thirty days to complete. The developmental changes are associated with both morphological and biochemical alterations. Morphological changes include changes in cell size, flagellum size and location of the kinetoplast. Midgut forms have their kinetoplast located halfway between the nucleus and the posterior end (trypomastigote forms) whereas the early salivary gland forms have their kinetoplast adjacent and just anterior to the nucleus (epimastigote forms). The final infective metacyclic forms found in the salivary glands are morphologically similar to the mammalian bloodstream forms, possessing a posteriorly positioned kinetoplast (trypomastigote forms). Only the infective metacyclic parasites of the insect-stage forms possess the important glycoprotein surface coat found in all mammalian-stage forms.

When the fly next feeds on a mammalian host, the metacyclic forms are introduced subcutaneously into the host. The parasites divide within the interstitial space of the new host triggering an immune response from the host that results in a hard swelling or chancre at the site of the bite. From this site, the parasites eventually gain entrance to the bloodstream via

FIGURE 3**Life cycle chart of *Trypanosoma brucei***

invasion of the lymphatic system. The parasites reside extracellularly in the bloodstream and tissue spaces and fluids throughout the infection. Two forms of the parasite occur in the bloodstream: long slender forms (20-40 micron) and short stumpy forms (15-25 micron). Occasionally forms that are intermediate between these two are seen. The long slender bloodstream forms have a long, free flagellum, up to 6 micron, and a subterminal kinetoplast whereas the short stumpy forms have a short (1 micron) free flagellum and a posteriorly located kinetoplast. During rising parasitaemias, the slender forms predominate and divide by binary fission. The stumpy forms are non-dividing and are believed to arise from the slender forms under the influence of the host's immune response (17). The degree of mitochondrial development and metabolic potential of these stumpy forms suggests that they may be preadapted to life in the insect and are probably the forms that are infective to the insect. The polymorphism of the blood forms into slender, stumpy and intermediate forms is seen in natural infections characterized by successive waves of parasitaemia. Continued passage of parasites in laboratory animals results in monomorphic forms, namely long and slender, of enhanced virulence.

Arthropod Vector(10,11,14,17)

At least 34 species and subspecies of the genus *Glossina* have been described in the tsetse-infested zone. Despite this large vector pool, only a few species are important in the transmission of African trypanosomiasis. Both male and female flies transmit the parasites. The high mobility and wide variety of habitat of these disease-transmitting species presents considerable challenge to vector control programs. The acute form of human sleeping sickness caused by *T. b. rhodesiense* is transmitted by members of the *Glossina morsitans* group comprising *G. morsitans*, *G. pallidipes* and *G. swynnertoni*. These species of *Glossina* are found in the low woodland and thicket habitats of the East African savannah and lake shore areas. Chronic human trypanosomiasis caused by *T. b. gambiense* is transmitted by riverine species *G. palpalis*, *G. fuscipes* and *G. tachinoides*. *G. fuscipes*, a riverine lacustrine species, can also transmit infections by *T. b. rhodesiense*. The animal trypanosomiasis caused by *T. brucei brucei*, *T. congolense* and *T. vivax* are transmitted to a large extent by the *Glossina morsitans* group. Although parasites are known to occur in the salivary glands of infected *Glossina*, dissection in the field reveals only a low rate of infection of about 0.03%. Much higher rates of infection are obtained, however, if the infectivity of randomly collected flies is tested on susceptible hosts. This discrepancy may be partly explained by the observation that an infected fly needs multiple probes for one average feeding dose.

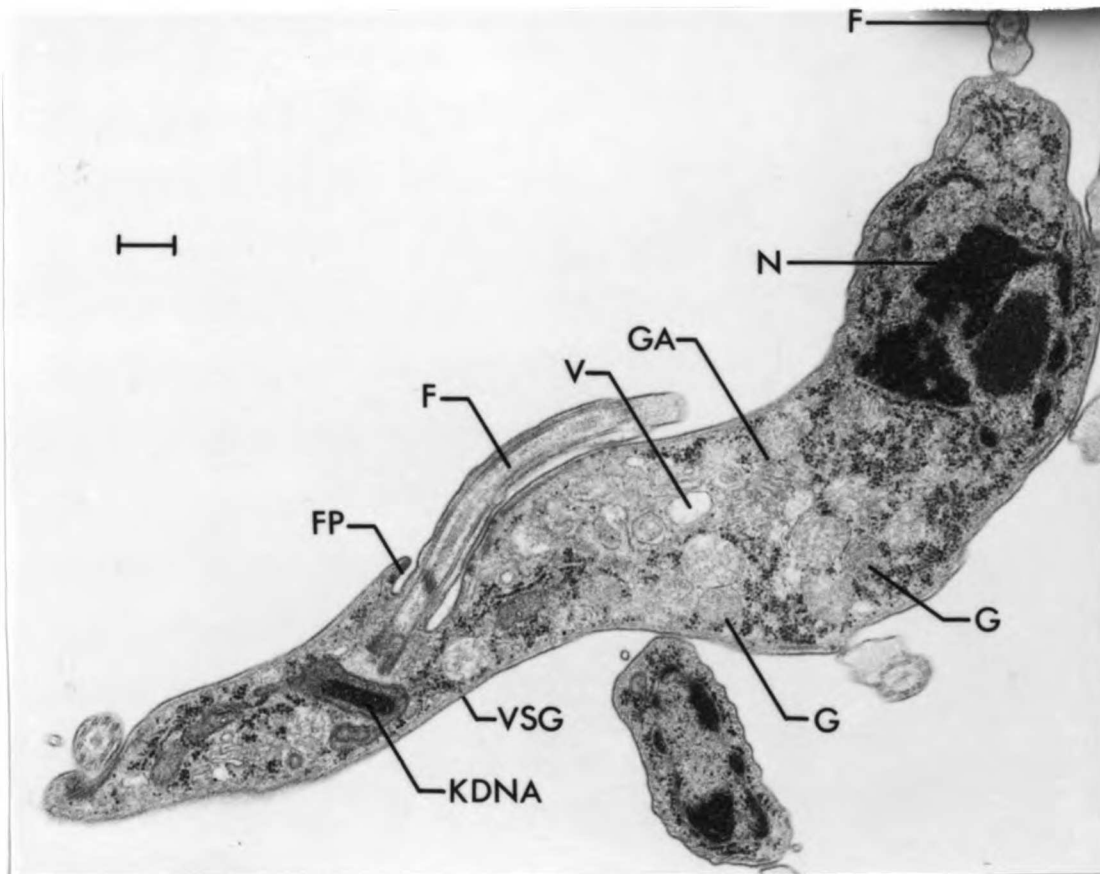
Morphology and Ultrastructure of the Parasite(17,19)

T. b. brucei, *T. b. gambiense* and *T. b. rhodesiense* are morphologically indistinguishable. Because it is non-infective to man and relatively easy to grow in laboratory animals, *T. b. brucei* is the species that has been most

studied. The description that follows is of *T. b. brucei* bloodstream stages but is equally applicable to other species of African trypanosomes. The ultrastructure of the various stages of the parasite is basically the same except for changes in kinetoplast location, flagellum length, mitochondrial development and presence or absence of a variant glycoprotein surface coat. The parasite is a unicellular protozoan flagellate ranging in size from 15 to 30 micron long (long slender forms) (Figure 4). The spindle-shaped cell retains its form by means of a skeleton of parallel microtubules just below the surface of the plasma membrane (subpellicular microtubule array). These structures are readily identified in Figure 4 which shows the detailed ultrastructure of the parasite as revealed by transmission electron microscopy. The vigorous motility of the trypanosome is activated by a single flagellum originating from the basal body near the kinetoplast. The kinetoplast is a densely staining complex of extranuclear DNA occurring in the single mitochondrion. An invagination of the cell surface at the point where the flagellum emerges from the cell body forms the flagellar pocket. It is at this enclosed part of the cell surface that most of the excretion and pinocytosis takes place. The flagellum is firmly secured to the cell pellicle by a string of attachments, the maculae adherens, creating a fold of cell membrane often referred to as an undulating membrane. Long slender forms have a subterminal kinetoplast and a free flagellum (ca. 6 micron) extending beyond the anterior end. The free flagellum in short stumpy forms is usually less than 1 micron in length or completely absent, while the kinetoplast is located near the posterior end of the cell. The flagellum core or axoneme has the canonical "9+2" arrangement of microtubules found in almost all cilia and

FIGURE 4

Ultrastructure of a bloodstream form *T. brucei* as revealed by transmission electron microscopy. Abbreviations are: F, flagellum; FP, flagellar pocket; G, glycosome; GA, golgi apparatus; KDNA, kinetoplast DNA; V, vacuole; VSG, variant surface glycoprotein. The scale bar corresponds to 0.4 micron. The sample was prepared according to the method described in ref. 20.



eukaryotic flagella. The other prominent component of the flagellum is the paraxial rod which is essentially a network of actin fibres which extends along the axoneme and stays in close contact with it. The subpellicular microtubule array consists of a single layer of microtubules situated immediately beneath the surface membrane providing a high degree of flexibility to the cells and presumably are involved in motility as well. A central or slightly posterior nucleus as well as other eukaryotic features such as Golgi complex, endoplasmic reticulum and a single mitochondrion are present. The degree of development of the mitochondrion and consequently the metabolic potential depends on the life cycle stage of the parasite. The long slender bloodstream forms have a poorly developed mitochondrion devoid of cristae and often reduced to a peripheral tube. Short stumpy forms have better developed mitochondrial structure but only in the insect stages is mitochondrial development complete. Important cytoplasmic organelles present include lysosomes and glycosomes. Secretory sacs and lipid vacuoles may be seen. A most notable feature of the ultrastructure of the parasite is the presence of a thick surface coat covering the entire surface and flagellum of the organism. This 15-20 nm thick surface coat is composed of a single glycoprotein and is found exclusively in mammalian stage forms and the infective metacyclic insect stage form (20). Changes in this surface coat glycoprotein form the basis of the antigenic variation (see below) displayed by the organism.

Clinical Symptoms and Pathology of the Disease (11,17)

When an infected tsetse fly bites a mammalian host, infective metacyclic forms are introduced into the subcutaneous tissue where they multiply and eventually change into long slender forms. At the site of the bite, a local skin

reaction occurs and a swelling or 'chancre' develops within 2-3 days after inoculation. The chancre or primary lesion represents the host's first response to trypanosome infection and develops probably as a result of an interaction between the antigens deposited in the subcutaneous tissue and the first circulating antibodies. In man, the chancre presents as a circumscribed rubbery induration, adherent to the skin, extending underneath and around the visible swelling and can easily be mistaken for an innocent boil except by the very wary in a highly endemic area. Usually the chancre subsides in 2-3 weeks. The primary lesion appears to be more common in acute trypanosomiasis caused by *T. b. rhodesiense*. In animals, chancre development and size appears to be related to the susceptibility of the host to infections (14). Between the fifth and twelfth day after infection, trypanosomes become detectable in the blood and a series of parasitaemic waves (see Antigenic Variation) occurring at intervals of about 10 days begins. In *T. b. rhodesiense* infections there may be as many as 10^5 parasites per millilitre of blood at the height of the parasitaemic wave whereas in *T. b. gambiense* infections, parasites are scanty from the beginning.

The symptoms associated with parasitaemia are irregular and remittant fever, soft lymphadenopathy typically located in the position of the posterior cervical triangle and peripheral oedema. The patient's history consists of periodic malaise with major complaints being the nonspecific symptoms of headache, dizziness, pain in the joints and limbs, loss of weight, general itching often located in the sternal region, etc. At this stage, the disease is easily taken for 'flu' or malaria. The disease progresses to a second stage marked by organ lesions primarily in the brain and heart. The lesions consist of perivascular infiltration of plasma and mononuclear cells into brain and cardiac tissues. The onset of this stage is marked by anomalies of the

cerebro-spinal fluid (CSF) and appearance of parasites in this fluid.

Symptoms of irritability, paraesthesia, insomnia and hyperreflexia are due to lesions in the central nervous system (CNS). Late stages of the disease is characterized by severe headache and papilloedema resulting from cerebral oedema, one of the marked features of the brain pathology. Further signs may manifest as epileptic attacks, psychotic changes like euphoria, convulsions, somnolence (hence the term 'sleeping sickness') and eventually coma. Functional abnormalities are mainly due to cardiac failure and consist of peripheral oedema and lung oedema. Because of the gross disturbances in organ function, secondary infections is common with pneumonia being a cause of death in over 50% of the fatal cases. The prognosis of untreated *T. b. rhodesiense* infections is between a few weeks and nine months and in the case of *T. b. gambiense* from months to years.

In animals the disease follows essentially the same course as in man with intermittent fever, anaemia and wasting being the most common manifestations. The disease in cattle may be acute, subacute or chronic. A severe degree of anaemia is rapidly produced and as the disease progresses, impairment of bodily functions becomes prominent and the animals lay down and die. Some animals may recover from the infection but relapses, even after a few years are not uncommon.

Pathogenesis of the Disease (10,17,21,22,23)

Much of the pathology observed is immunological in nature although important physiological alterations are also involved in the pathogenesis of the disease. Pronounced immune changes marked by a noticeable increase in the activity of the mononuclear phagocytic system occurs. Lymph nodes and the spleen are remarkably reactive, showing a preponderance of plasma cells in the acute phase of the disease. However during late stages, depletion of lymphoid cells of the immune system may occur. The levels of circulating immunoglobulins are increased with IgM being the most elevated species. The antibody response is directed against trypanosome surface antigens, both variable and somatic, but there is also an autoimmune response in which antibodies against various self-tissues such as the liver and blood elements (fibrin and fibrinogen) are detected. The direct significance of these auto-antibodies for the pathogenesis of sleeping sickness is not clear. Circulating and tissue-located immune complexes in association with complement have been demonstrated in infected laboratory animals. Immune complexes are deposited in the kidneys, heart tissues and around blood vessels of the myocardium. These immune complexes are likely to have wide ranging pathological effects including induction of anaemia, complement activation, tissue damage and interference with both induction and effector mechanisms of the immune response. There is a noticeable alteration in the complement system manifested as decreased levels of the complement components and complement deposition in tissues. Complement and immune-complex activation of the kinin system results in increased blood kinin levels. These pharmacologically active substances cause enhanced permeability of the blood vessels and probably contribute to the state of anaemia which is a major component of the pathology of the disease both in man and animals.

Several mechanisms such as an expanded mononuclear phagocytic system, haemolytic factor(s) produced by the trypanosome, fever and disseminated intravascular coagulation may be operative in the cause of the haemolytic anaemia observed. Anaemia persists throughout the course of the disease and undoubtedly contributes to the tissue damage observed. This damage is often found in association with granular immunoglobulin deposits and with a marked cellular infiltration into the tissues of cells of the immune system. In both man and animals, the heart, the skeletal muscles, liver and kidneys are usually involved. Damage to the meninges of the brain resulting in meningo-encephalitis dominates the pathological picture during late stages of the disease. In addition, metabolic disturbances, metabolite pool depletion and generation of toxic metabolites by the parasite may play a role in the pathogenesis of the disease.

Chemotherapy (10,17,24)

Treatment of sleeping sickness requires hospitalization for an average of six weeks in early stage infections. This is necessitated by the dangerous side effects and toxicity of the drugs used. If at the time of diagnosis the CSF is normal, the disease can be treated quite safely by suramin and pentamidine, the former being the drug of choice for rhodesiense infection and the latter for the early stages of gambiense infection. Suramin, a derivative of trypan red developed by Bayer in 1916, is effective against both *T. rhodesiense* and *T. gambiense*. However for early stage *T. gambiense* infections, pentamidine is preferred because of its advantage of a shorter course of treatment (see Table 4). Both these drugs are effective only in early stages of infection when CNS involvement has not occurred since they cannot penetrate the blood-brain barrier. Suramin has the disadvantages that it

requires treatment courses of at least 30 days and it may cause toxic nephrosis. Although the cure rates of pentamidine are possibly lower than with suramin, it has the advantage that treatment lasts only one week. Pentamidine is often not active against *T. b. rhodesiense*. Both suramin and pentamidine have been used as chemoprophylactics. Because of the serious risk of masking the disease in its early stages when it is most easily curable, chemoprophylaxis is discouraged especially in *T. b. rhodesiense* areas. Where used for this purpose, pentamidine is preferred over suramin because of its longer period of protection and greater ease of administration. During the late stages of the disease, drugs that can penetrate into the CSF are indispensable. The only drug available for late stage sleeping sickness is melarsoprol (Mel B; Arsobal). This drug, introduced in 1949 is a combination of melarsen oxide and dimercaprol (see Figure 5). Although very effective, this compound has serious side effects in 5-10% of treated cases causing reactive encephalopathy and 2-5% die from this reaction. Because of this fatal side-effect, the drug must be used only when there are strict indications that pathological changes in the CSF have occurred. This restriction means a lumbar puncture in each patient found to have sleeping sickness and consequently seriously complicates the work of survey teams and personnel in the rural medical centres. Furthermore since melarsoprol is administered as an intravenous injection in propylene glycol, a highly irritant solvent, expert administration of the injection is required to avoid severe local tissue damage (Table 4).

Nitrofurazone has been used in melarsoprol resistant cases but it is not suitable for regular use because of its toxicity. The veterinary drug berenil (diaminazine aceturate) has shown promising results against the human illness as well, but is still undergoing clinical trials for human use.

Supplementation of chemotherapy with nutritional therapy to improve the anaemia and restore the fluid balance is important for raising the chances of cure.

Control of animal trypanosomiasis requires curative and preventive use of five main trypanocidal drugs. These are homidium, quinapyramine, diminazene, pyrithidium and isometadium salts. These compounds afford effective treatment of the different animal trypanosomiasis. Pyrithidium and isometadium salts are often used for prophylactic purposes in animals exerting a preventive action that can last from two to four months.

Occurrence of drug resistance to most trypanocides is common, particularly to those that exert preventive action and the choice and condition of use of these agents must be a judicious one to minimize this problem. Table 4 summarizes the pertinent information about the three mainstay drugs of human trypanosomiasis. The chemical structures of these agents are presented in Figure 5. For in-depth accounts of the chemotherapy of African trypanosomiasis, the reader is referred to the articles by Meshnick (25) and Holmes and Scott (26).

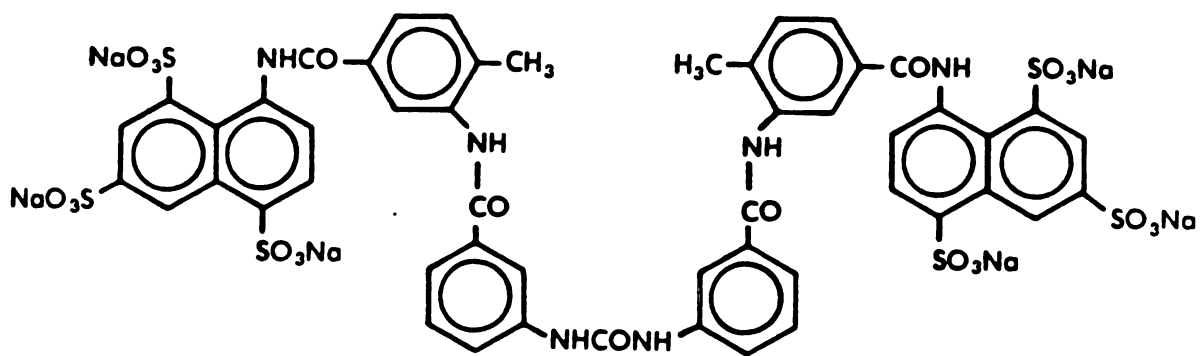
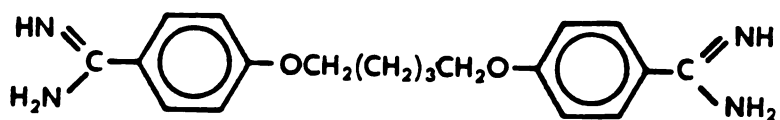
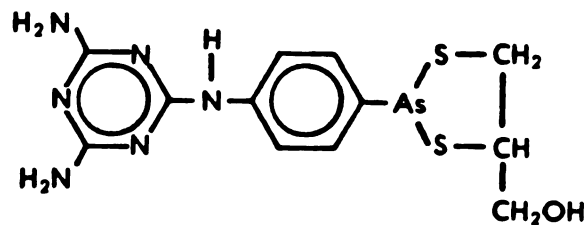
TABLE 4 ¶**Chemotherapy of Human Trypanosomiasis**

	SURAMIN	PENTAMIDINE	MELARSOPOL
<u>INDICATIONS</u>	Extremely effective in early stages of both <i>T. gambiense</i> and <i>T. rhodesiense</i>	Very effective in early stages of <i>T. gambiense</i> infections	Drug of choice in late stage infections of both <i>T. gambiense</i> and <i>T. rhodesiense</i>
<u>DOSAGE AND ADMINISTRATION</u>	20mg/kg body wt. Treatment requires 5 injections given every 5 or 7 days	3-4mg/kg body wt. Total of 7-10 injections given as single injections daily or on alternate days	Max. dose/injection is 3.6mg/kg body wt. Usually course comprises of 3 series of 3 daily injections (1 injection X 3 days) each separated by at least one week
	Administered intravenously	Administered intramuscularly	Drug is dissolved in propylene glycol, a highly irritant solvent. Injections are intravenous and technique of injection must be impeccable
<u>SIDE EFFECTS</u>	Pyrexial reaction, pupular irritable rash, pain in joints and soles of feet, nephrotoxicity	Hypotensive effect, nausea and vomiting, sometimes peripheral neuritis and hypoglycaemia	Leakage during injection results in an intense local reaction at site. Reactive encephalopathy at 3rd injection or beginning of 2nd course. Jaundice, severe diarrhoea and conjunctival infection may occur

¶ Modified from ref. 10.

FIGURE 5

Chemical structures of the clinically important trypanocidal drugs

**Suramin Sodium****Pentamidine****Melarsoprol**

Antigenic Variation in African Trypanosomes

As early as the first decade of this century, it was observed that a natural infection with African trypanosomes was characterized by successive waves of parasitaemia occurring every tenth day or so and coinciding with the recurrent fever often seen in patients (27,28). Not long thereafter, it became clear that each parasitaemic wave represented a population of trypanosomes that was antigenically distinct from preceeding or succeeding wave populations. Antisera specific against one form would react with homologous but not heterologous populations of trypanosomes (29). The critical observation that extensive antigenic variation occurred in experimental infections using a cloned population of trypanosomes as inoculum suggested that the parasites have evolved a novel mechanism for altering their antigens, presumably on the surface, for purposes of evading the immune system of the host (30). An understanding of antigenic variation at the molecular level has come about only in recent years. In 1969, Keith Vickerman described the presence of a surface coat revealed, by electron microscopy, as an electron dense 12-15 nm thick surface layer covering the entire surface and flagellum of the organism (31). This surface coat is found on all mammalian stages of the parasite as well as in infective metacyclic forms from the tsetse salivary gland but is absent in the procyclic forms present in the fly midgut. The biochemical characterization of the surface coat, reported in 1975, showed it to be composed of a single glycoprotein of subunit molecular weight of about 65,000 daltons and present on the cell surface probably as a monolayer of ca. 10^7 molecules (20). This glycoprotein which may be the only protein exposed on the cell surface is characteristic for a given variant and carries all the antigenic determinants of the living trypanosome which give rise to variant-specific antibodies. Antigenic variation in the African trypanosome

therefore involves shedding of the coat, in the face of the host immune response, and replacing it with one composed of a new and different glycoprotein. Indeed the surface glycoproteins isolated from different variants differ dramatically in amino acid sequence (32,33). The trypanosomes appear to have a large repertoire of variable antigen types or VATS as exemplified by one often-cited case in which a *T. equiperdum* infection of an experimental rabbit resulted in the successive expression of over 100 different antigenic types (34).

The genetic basis of this interesting phenomenon has been a subject of intense study in recent years and even a cursory mention of the mechanism(s) would not do the subject justice in this medium. The reader is referred to several in-depth reviews of the subject that are continually being updated as new information emerges(35,36,37,38,39,40,41,42,43,44,45). Suffice it to say that the complexity of this phenomenon has made the realization of a vaccine to control the disease an almost unachievable goal. For the moment therefore, the classical methods of chemotherapy and vector control programs still remain the only methods of combating the disease.

CARBOHYDRATE METABOLISM

Glucose is the most important substrate of bloodstream forms of members of the Trypanozoon subgenus of the African trypanosomes although other hexoses, notably mannose and fructose as well as glycerol can be utilized (46,47). Bloodstream forms of African trypanosomes require an exogenous supply of carbohydrate for survival (48,49) since they do not have storage polysaccharides (50) and they lack cytochromes of the respiratory chain or enzymes of the tricarboxylic acid (TCA) cycle (46,48,51,52). Removal of exogenous carbohydrate results in total and rapid loss of respiratory activity and cellular motility (48).

Sugar Transport

Mechanisms by which substrates are transported into the cell are poorly understood (53-59). However, evidence suggests that uptake of sugars is not energy coupled and that mediated systems are involved. In long slender forms of *T. b. brucei*, D-glucose permeation appears to be by a carrier-mediated process driven by the downhill concentration gradient of free substrate maintained by the continual removal of sugar by the highly active glycolytic enzymes (60). Permeation of glucose rather than its subsequent phosphorylation appears to be the rate-limiting step in glucose consumption.

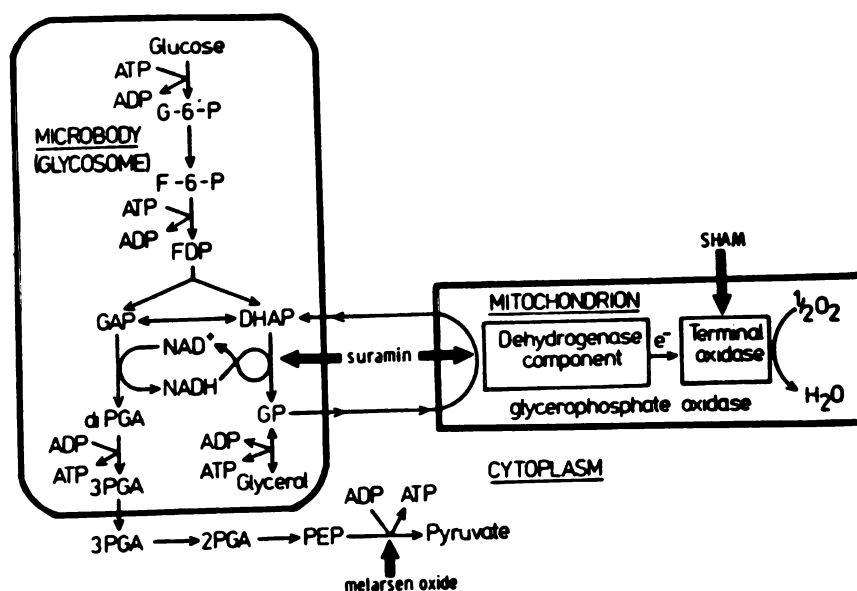
Oxidative Metabolism

Bloodstream form Trypanozoon species have an enormously high glucose-supported respiration rate that is about 50 times that of mammalian tissues (46). This respiration is insensitive to massive concentrations of cyanide (up to 10mM) or carbon monoxide (46,48,52). Cytochromes are absent and several enzymes of the TCA cycle are absent or present to a quantitatively insignificant extent (48,52). The catabolism of glucose via the Embden-

Meyerhof pathway (glycolysis) is therefore the only energy trapping pathway for bloodstream forms of Trypanozoon species. Under aerobic conditions, long slender bloodstream forms of *T. b. brucei* metabolize glucose exclusively to pyruvate since lactate dehydrogenase is also lacking (61). Reoxidation of the NADH produced during this process is accomplished by a glycerol-3-phosphate dehydrogenase and a novel terminal oxidase, the glycerol-3-phosphate oxidase (GPO) complex (50,62). This cyanide insensitive oxidase, now known to be located in the regressed mitochondrion (63), mediates the indirect transfer of electrons from NADH to molecular oxygen via a glycerol-3-phosphate: dihydroxyacetone shuttle (Figure 6). Although some strains of trypanosomes contain an oligomycin-sensitive ATPase (64), electron transport does not appear to be coupled to oxidative phosphorylation. A state of anaerobiosis can be induced in the parasite by the action of various hydroxamic acids such as m-chlorobenzhydroxamic acid (65) and salicylhydroxamic acid (SHAM) (66,67,68,69,70) which inhibit glycerol-3-phosphate oxidase. Glucose is quantitatively converted to pyruvate under aerobic conditions with the concomitant net production, by substrate level phosphorylation, of 2 moles of ATP per mole of glucose. Under anaerobic conditions, glucose catabolism proceeds at the same rate with the production of equimolar pyruvate and glycerol (48,71) plus one mole of net ATP synthesis (72). The net production of one mole of ATP per mole of glucose utilized under anaerobic conditions is achieved by the operation of trypanosomal glycerol kinase in the reverse direction (see below). For each mole of triose phosphate converted to pyruvate, a mole of NAD^+ is reduced to NADH, which, in turn, is reoxidized by conversion of a mole of DHAP to glycerol-3-phosphate.

FIGURE 6

Compartmentation of glycolysis in long slender bloodstream forms of *T. brucei*. Bold arrows denote the principal sites of inhibition by trypanocidal drugs. Abbreviations are: G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; GP, glycerol-3-phosphate; diPGA, 1,3-diphosphoglycerate; 3PGA and 2PGA, 3- and 2-phosphoglycerate respectively; PEP, phosphoenolpyruvate; SHAM, salicylhydroxamic acid. Reproduced from ref. 102.



Further conversion of glycerol-3-phosphate to glycerol by glycerol kinase is coupled to ATP production (see below).

Glycolysis

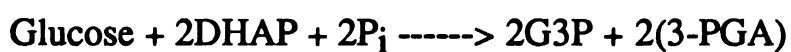
As mentioned earlier, the long slender bloodstream forms of the African trypanosomes are entirely dependent on glycolysis for their energy needs. This restriction is imposed by the lack of cytochromes of the mitochondrial respiratory chain and the absence of functional enzymes of Krebs's cycle. The single mitochondrion, although present, has very few poorly developed tubular cristae and the entire structure has been reduced to a peripheral canal somewhat resembling the promitochondrion of anaerobically grown yeast (73).

Glucose is the preferred energy source even though fructose, mannose and glycerol can also support motility and respiration. Glycolysis in *T. b. brucei* proceeds at an extremely high rate of 85 nmoles glucose/min/mg protein and this is coupled directly to the rate of ATP production (74). Such a high rate of glycolysis, equivalent to the consumption of glucose equal to their own dry weight every hour, is necessary to enable the trypanosome to divide every 7 hours (75).

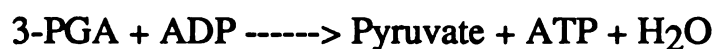
The Glycosome

Several salient features distinguish glycolysis in bloodstream forms of *T. b. brucei* from glycolysis in other eukaryotes. Perhaps the most distinguishing feature of the glycolytic apparatus in trypanosomes is that in contrast to other eukaryotic systems, most of the glycolytic enzymes are particulate in nature being localized in microbody-like subcellular organelles (64). It was during investigations on the intracellular location of the unusual glycerophosphate oxidase in bloodstream form *T. brucei* that Opperdoes et al noted the difference in the sucrose sedimentation profiles of this enzyme and NAD⁺-linked glycerophosphate dehydrogenase, which acts in concert with the oxidase to reoxidize NADH generated during glycolysis (the glycerophosphate:DHAP shuttle) (63). Whereas the oxidase was located in the mitochondrion, the NAD⁺-linked glycerophosphate dehydrogenase was found to sediment in sucrose density centrifugation at a density characteristic of microbodies. This finding prompted the search for other dehydrogenases in the microbodies of bloodstream *T. brucei* to explain the maintenance of a NAD⁺/NADH ratio inside these organelles given the fact that they are most likely impermeable to these pyridine nucleotide coenzymes. In 1977, Opperdoes and Borst described the particulate nature of the first seven enzymes of glycolysis in the bloodstream forms of *T. brucei* i.e. hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, triose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (64). These enzymes along with glycerol kinase were shown to band at the same density in isopycnic sucrose gradients as the microbody located NAD⁺-linked glycerophosphate dehydrogenase. The activities of all nine enzymes were markedly increased by the detergent Triton X-100 supporting the concept that they were present within a

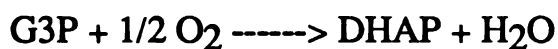
microbody-like organelle which was impermeable to the phosphorylated glycolytic intermediates and cofactors. However, typical microbody and peroxisome marker enzymes such as catalase, peroxidase or urate oxidase were not present in these organelles from the long slender bloodstream forms of *T. brucei*. Because of their content of glycolytic enzymes, they were named glycosomes, although several other non-glycolytic enzymes have since been described in these organelles. This complement of glycolytic enzymes permits the following reactions to occur inside the glycosome:



In both overall reactions, ATP utilization is balanced by ATP synthesis and NAD reduction by NADH oxidation. Net ATP synthesis occurs at the pyruvate kinase-catalysed step in the cytoplasm where this enzyme and the two preceeding glycolytic enzymes i.e. 3-phosphoglycerate mutase and enolase are located. Thus 3-PGA exits the glycosome and partakes in the following overall reaction:



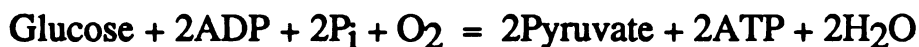
G3P, the other product of glycosome metabolism also leaves the glycosome to become oxidized to DHAP in the mitochondrion by the action of the glycerophosphate oxidase with the concomitant transfer of electrons to molecular oxygen in the following overall reaction:



Entry of DHAP into the glycosome completes the shuttle and reoxidation of NADH. The features of this proposed model of glycolysis in *T. brucei* are represented schematically in Figure 6. To reconcile features of this model with the fact that all the enzymes in the glycosome exhibit high latency indicative of impermeability of glycolytic intermediates and cofactors,

Opperdoes and Borst proposed that the entry of inorganic phosphate and DHAP into the glycosome and the exit of G3P and 3-PGA were probably mediated by specific translocators (Figure 6). A translocator exchanging cytosolic DHAP for glycosomal G3P seemed favorable because it also provided a simple explanation for the mechanism of anaerobic glycolysis in *T. brucei*. Anaerobiosis renders the glycerophosphate oxidase inactive leading to a rapid depletion of cytoplasmic DHAP. The ensuing cessation of the exchange of G3P and DHAP through the glycosomal membrane results in an accumulation of G3P within the glycosome thereby setting up conditions favourable for the reverse reaction of glycerol kinase.

Since lactate dehydrogenase is absent, the reducing equivalents generated in glycolysis are reoxidized indirectly by molecular oxygen through the participation of the glycerol-3-phosphate:DHAP shuttle and the mitochondrial glycerol-3-phosphate oxidase. When this shuttle is operational (aerobic conditions), excreted pyruvate is the only end-product of glycolysis accounting for nearly 100% of the glucose carbon (67) and there is net synthesis of 2 moles of ATP per mole of glucose utilized, represented stoichiometrically as :



When the terminal oxidase is completely inhibited by anaerobiosis or salicylhydroxamic acid (SHAM) (72), the bloodstream trypanosomes convert glucose into equimolar amounts of pyruvate and glycerol. This dismutation of glucose proceeds with net synthesis of one mole of ATP as a result of the reversal of glycerol kinase within the glycosome allowing the following reaction to take place:



Although this reaction is thermodynamically unfavourable, special conditions

existing inside the glycosome under anaerobiosis permit the phosphorylation of ADP to take place. Under anaerobic conditions, the intracellular level of glycerophosphate in bloodstream forms of *T. b. brucei* has been shown to increase rather drastically whereas levels of other glycolytic intermediates do not change much when aerobic metabolism is switched to an anaerobic one by the addition of SHAM (76). Furthermore, labeling experiments under anaerobic conditions indicate that glycerophosphate is the direct precursor of glycerol (77,78), the conversion being mediated by the reversed reaction of glycerol kinase (79,80,81). In the light of the discovery of the glycosome, it is now clear that the high levels of glycerophosphate in the glycosome under anaerobiosis, and restricted within this compartment due to impermeability of the glycosomal membrane, causes glycerol kinase to operate in the reverse direction with net production of ATP. That the reversal of glycerol kinase is crucial for anaerobic glycolysis is evident from the earlier observations of the lethal effect of a combination of SHAM and glycerol on the bloodstream parasite (66,67,69,70). Anaerobic glycolysis can only be sustained by the production of glycerol through the reversed glycerol kinase reaction. Blockage of this reaction by the mass action of exogenous glycerol leads to a complete inhibition of anaerobic glycolysis and the subsequent death of the trypanosome.

The important finding of the particulate nature of the glycolytic enzymes in bloodstream forms of *T. brucei* was soon corroborated by other workers (82,83) and it seemed attractive to suppose that the glycosome was a special form of adaptation of *T. brucei* to its stay in the glucose-rich bloodstream of its host. This notion was quickly discarded when glycosomes were described in insect stages of *T. brucei* (84,85) and in all major representatives of the family Trypanosomatidae such as the insect

trypanosomes of the genus *Crithidia* (82,86), *Trypanosoma cruzi* (82,87) and in all clinically important species of *Leishmania* (89). The presence of glycosomes is therefore a general property of all Trypanosomatids as appears to be their lack of marker enzymes of other eukaryotic microbodies and peroxisomes. In addition to the nine enzymes of glycolysis and glycerol metabolism described above, other enzymes that have since been shown to be present in the glycosomes of Trypanosomatids include adenylate kinase (84,90,94), the last two enzymes of the pyrimidine biosynthetic pathway namely orotate phosphoribosyl transferase and orotidine decarboxylase (91,92), phosphoenolpyruvate carboxykinase (87,88,89,90,93), malate dehydrogenase (84,90), key enzymes of ether-lipid biosynthesis (DHAP acyltransferase, 1-acyl G3P:NADP⁺ oxidoreductase and acyl-CoA reductase) (89,95) and at least one enzyme involved in beta-oxidation of fatty acids, 3-beta-hydroxybutyryl CoA dehydrogenase (89). Table 5 presents a summary of the various enzymes reported in the glycosomes of several members of the Trypanosomatidae together with the source references. In *T. brucei*, both phosphoenolpyruvate carboxykinase and malate dehydrogenase are stage-specific being found only in the glycosomes of the insect-stage culture form parasites (84,88,90,93). Another notable difference in culture form *T. brucei* is that the glycolytic enzyme phosphoglycerate kinase is no longer glycosomal but is found in the soluble fraction (cytosolic) instead (85,90). It appears therefore, at least in *T. brucei*, that transformation from the bloodstream form to the insect-stage or culture form is attended by a reorganization of the glycosomal components perhaps in response to the new environment and metabolic requirements. Notable in this regard is the reduced levels of activity of most of the glycolytic enzymes in the glycosomes of culture form *T. brucei* (90) in which glycolysis is no

TABLE 5

Enzymes reported to be present in the glycosomes of various members of the family Trypanosomatidae

ENZYMES	DEMONSTRATED IN:	REFERENCE
GLYCOLYTIC ENZYMES (First seven + GDH and GK)	<i>T. brucei</i> (BSF) <i>T. brucei</i> (CF) <i>T. cruzi</i> <i>L. m. amazonensis</i> <i>L. major</i> , <i>L. donovani</i> , <i>L. m. mexicana</i> } <i>L. b. braziliensis</i> <i>Crithidia fasciculata</i>	64, 82, 83, 90 84, 85, 90 82 91, 92 89 82
ADENYLATE KINASE	<i>T. brucei</i> (BSF) <i>T. brucei</i> (CF) <i>T. rhodesiense</i> (BSF) Leishmania (various spp.)	90 84 94 89
PEPCK	<i>T. brucei</i> (CF) <i>T. cruzi</i> Leishmania (various spp.) <i>Crithidia fasciculata</i>	90, 93 87 89 87
MDH	<i>T. brucei</i> (CF) Leishmania (various spp.)	84, 90, 93 89
OPRTase	<i>T. brucei</i> (BSF) <i>T. cruzi</i> <i>L. m. amazonensis</i>	91, 92 91, 92 91, 92
HGPRTase	<i>T. brucei</i> (BSF)	81
ETHER-LIPID BIOSYNTHESIS	<i>T. brucei</i> (CF) Leishmania (various spp.)	95 89
FATTY ACID OXIDATION	Leishmania (various spp.)	89

Abbreviations are: GDH, glycerol-3-phosphate dehydrogenase; GK, glycerol kinase; BSF, bloodstream form; CF, culture form; PEPCK, phosphoenol pyruvate carboxykinase; MDH, malate dehydrogenase; OPRTase, orotate phosphoribosyl transferase; HGPRTase, hypoxanthine-guanine phosphoribosyl transferase.

longer the sole provider of energy in the form of ATP, being replaced by amino acids, especially proline, as the main energy sources (102).

Mammalian peroxisomes are known to be involved in a variety of metabolic functions including plasmalogen (ether-lipid) biosynthesis and beta-oxidation of fatty acids. The discovery of enzymes involved in these two metabolic pathways in the glycosomes suggests that peroxisomes and glycosomes may be more closely related than originally thought despite the apparent lack of characteristic peroxisomal enzymes such as catalase, peroxidase and urate oxidase in glycosomes. It is noteworthy that none of the enzymes of the glyoxylate cycle known to be associated with microbodies in plants could be detected in glycosomes of both bloodstream form (96) and culture form of *T. brucei* (93) (it is of note that malate dehydrogenase is involved in this cycle and is also found in glycosomes of culture form *T. brucei*). The integrated metabolic pathways that have been localized to date in glycosomes of bloodstream and culture forms of *T. brucei* are represented diagrammatically in Figures 7 and 8.

Glycosomes can be purified to almost total homogeneity by combined differential and isopycnic centrifugations (97). In sucrose density gradients, glycosomes of *T. brucei* equilibrate at a density of 1.23 g/cm^3 and their density in isotonic sucrose is 1.087 g/cm^3 . Electron microscopic examinations of bloodstream form *T. brucei* glycosomes both *in situ* and in isolated purified preparations reveal spherical or oval-shaped organelles with an average diameter of about 0.3 micron and bounded by a single membrane. Morphologically they are indistinguishable from microbodies of other eukaryotes. They display a remarkably homogeneous size distribution and probably represent a single class of microbodies in these organisms. The lumen or matrix is electron-dense and occasionally crystalloid cores of the

FIGURE 7
Schematic Representation of the Integrated Metabolic Pathways in
the Glycosomes of Bloodstream Form *Trypanosoma brucei*.

KEY

1= Hexokinase; 2= Phosphoglucose isomerase; 3= Phosphofructose kinase;
 4= Aldolase; 5= Triose phosphate isomerase; 6= Glyceraldehyde-3-
 phosphate dehydrogenase; 7= Phosphoglycerate kinase; 8= Glycerol-3-
 phosphate dehydrogenase; 9= Glycerol kinase; 10= Phosphoglycerate
 mutase; 11= Enolase; 12= Pyruvate kinase; 13= Glycerol-3-phosphate
 oxidase; 14= Adenylate kinase; 15= Orotate phosphoribosyl transferase; 16=
 Orotidine-5'-phosphate decarboxylase; 17= Hypoxanthine-guanine
 phosphoribosyl transferase

ABBREVIATIONS

FDP= Fructose-1,6-diphosphate; DHAP= Dihydroxy acetone phosphate;
 GAP= Glyceraldehyde-3-phosphate; G3P= Glycerol-3-phosphate; 1,3PGA=
 1,3-Phosphoglycerate; 3PGA= 3-Phosphoglycerate; 2PGA= 2-
 Phosphoglycerate; PEP= Phosphoenol pyruvate; OMP= Orotidine
 monophosphate; UMP= Uridine monophosphate; IMP= Inosine
 monophosphate; P_i= Inorganic phosphate

SUPERSCRIPTS

- a: The additional enzyme, phosphomannose isomerase, is required for mannose metabolism (Ref. 81).
- b: The reverse reaction of glycerol kinase proceeds in the direction of the broken arrow under anaerobic conditions resulting in ATP synthesis.
- c: At the time of writing, no reports have been published on whether or not the enzymes of alkoxyphospholipid biosynthesis are present in bloodstream glycosomes of *T. brucei*.

Substrates and products of glycolysis are enclosed in boxes.

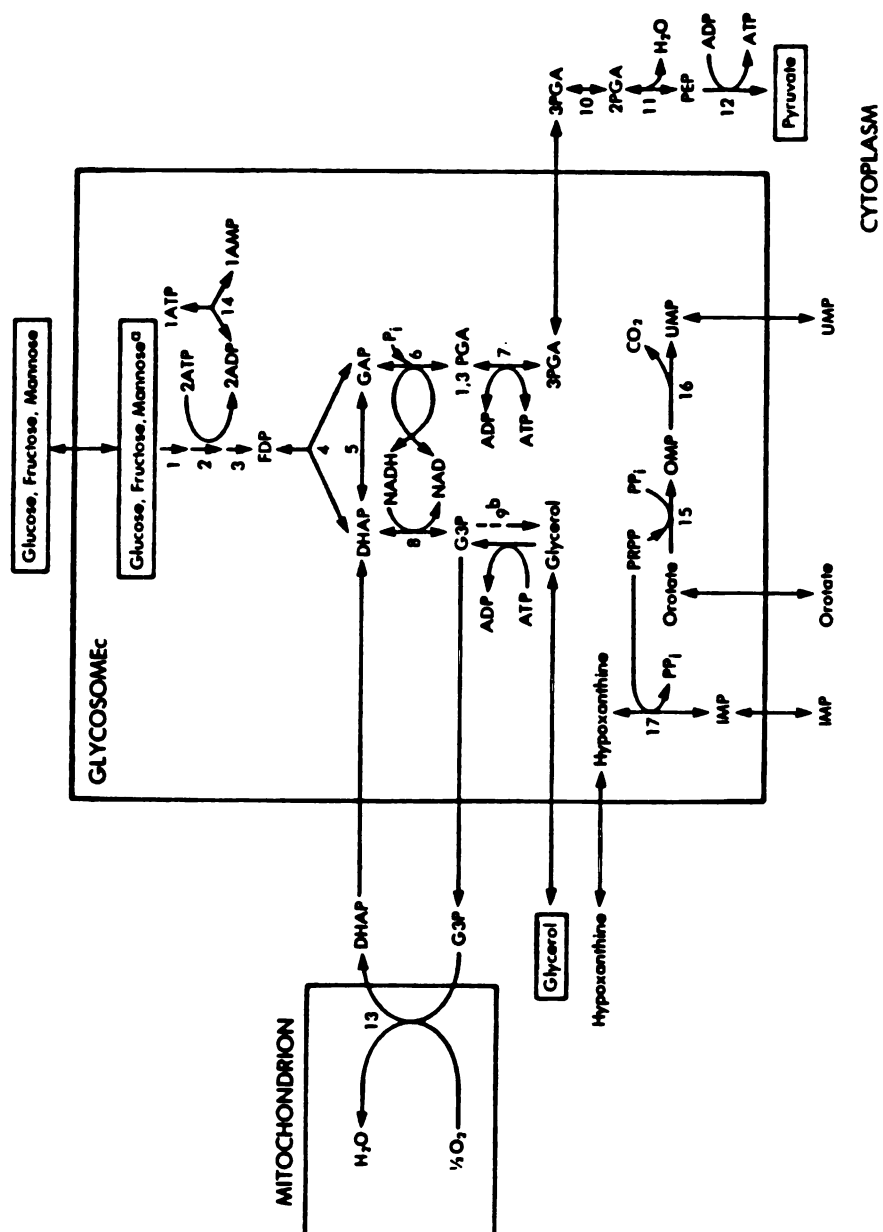


FIGURE 8**Schematic Representation of the Integrated Metabolic Pathways in the Glycosomes of Culture Form *Trypanosoma brucei*.****KEY**

1= Hexokinase; 2= Phosphoglucose isomerase; 3= Phosphofructose kinase; 4= Aldolase; 5= Triose phosphate isomerase; 6= Glyceraldehyde-3-phosphate dehydrogenase; 7= Glycerol-3-phosphate dehydrogenase; 8= Glycerol kinase; 9= Phosphoglycerate kinase; 10= Phosphoglycerate mutase; 11= Enolase; 12= Pyruvate kinase; 13= Phosphoenol pyruvate carboxykinase; 14= Malate dehydrogenase; 15= "Malic" enzyme; 16= Fumarate hydratase; 17= Fumarate reductase; 18= Aspartate aminotransferase; 19= Adenylate kinase; 20= Glycerol-3-phosphate oxidase; 21= Dihydroxyacetone phosphate acyl transferase; 22= 1-acyl glycerol-3-phosphate : NADP⁺ oxidoreductase; 23= Acyl CoA reductase; 24= 1-alkyl glycerol-3-phosphate : NADP⁺ oxidoreductase

ABBREVIATIONS

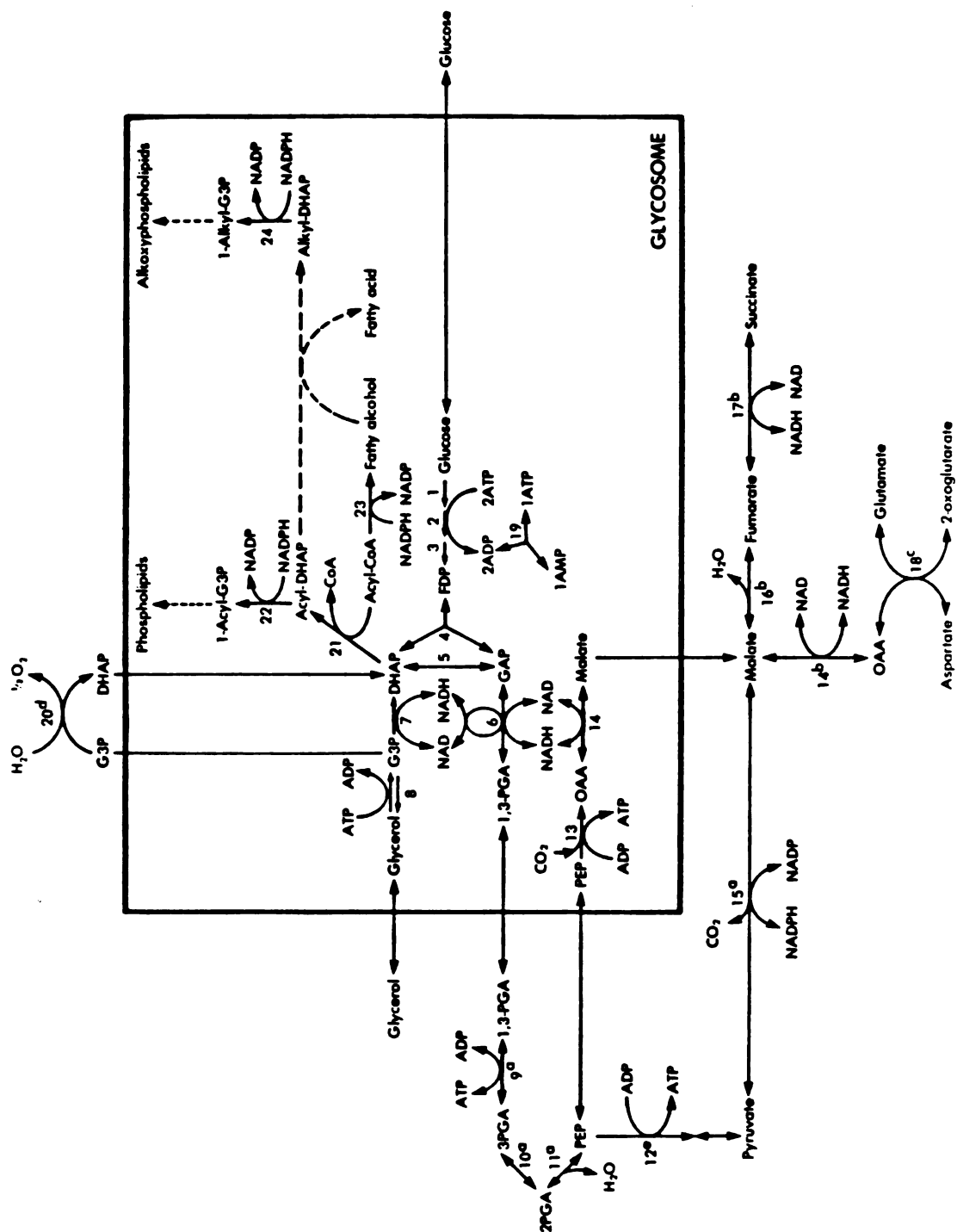
FDP= Fructose-1,6-diphosphate; DHAP= Dihydroxyacetone phosphate; GAP= Glyceraldehyde-3-phosphate; G3P= Glycerol-3-phosphate; 1,3PGA= 1,3-Phosphoglycerate; 3PGA= 3-Phosphoglycerate; 2PGA= 2-Phosphoglycerate; PEP= Phosphoenol pyruvate; OAA= Oxaloacetic acid

BROKEN ARROWS denote reactions either absent or not yet demonstrated in glycosomes.

SUPERSCRIPTS

- a: Cytosolic enzymes
- b: Precise subcellular location i.e. mitochondrial or cytosolic, not clearly demonstrated.
- c: Partly cytosolic and partly mitochondrial.
- d: Mitochondrial.
- e: Not detected and most likely absent.

N.B. Pyruvate kinase is absent in culture form *T. brucei* (Ref. 84)



kind described in mammalian peroxisomes may be seen. Morphometric analysis has shown that in bloodstream form *T. brucei*, glycosomes represent 4.3% of the cell volume (97). An average purification factor of 13 for the glycolytic enzymes indicates that glycosomes represent 8-9% of the total trypanosomal protein (97). The single membrane has a simple lipid composition with phosphatidyl choline and phosphatidyl ethanolamine as the only two major phospholipids. The high degree of latency exhibited by the glycolytic enzymes in isolated glycosomes is a result of the impermeability or limited permeability of phosphorylated glycolytic intermediates and cofactors through this membrane. As would be expected, latency is abolished by physical and chemical procedures that disrupt the integrity of the membrane, such as freezing and thawing, sonication, osmotic shock, treatment with phospholipases or detergent.

Apart from the fact of their unique location, the glycolytic enzymes in the glycosomes of *T. brucei* display properties that are variant from those of eukaryotic glycolytic enzymes. For instance, evidence suggestive of an association of these enzymes in the glycosome has been presented by several groups. Oduro et al (83) observed that the glycolytic enzymes in *T. brucei* behave as a multienzyme complex when subjected to gel-filtration chromatography in the absence of salt but elute off as separate peaks when 0.15 M NaCl is present. Salt dissociation of the complex appears to be reversible since removal of salt by dialysis results in reassociation of the enzymes. Glycolytic enzymes were found to band in two main clusters when detergent solubilized glycosomes were centrifuged in a sucrose gradient (98). Most of the glycolytic enzymes equilibrated at an intermediate density but hexokinase and phosphofructokinase both sedimented to the bottom of the gradient suggesting their association with a high density component most

likely the crystalloid core. Misset and Oppendoes (99) also noted that after removal of the glycosomal membrane, all glycolytic enzymes with the exception of phosphoglucose isomerase remained tightly associated with each other and behaved as a multienzyme complex in sucrose density centrifugation and gel-filtration experiments. Increasing the ionic strength promoted dissociation of the complex in a reversible fashion. The unusual properties of these enzymes is further demonstrated by the observation of Misset and Oppendoes (100) that ammonium sulphate precipitated all nine enzymes in the narrow range of 30 to 40% saturation. Furthermore, all nine enzymes strongly bound to an affinity medium of resin-linked NAD^+ in contrast to rabbit muscle enzymes in which the two dehydrogenases were separated from the other enzymes by this procedure. These rather strong interactions between these enzymes are probably largely hydrophobic in nature and in fact these investigators (100) have been successful in resolving these enzymes employing hydrophobic interaction chromatography. The kinetic properties of some of these enzymes may also be different from those of mammalian systems. Both hexokinase and phosphofructokinase appear not to conform to the classical modes of regulation of these enzymes in many systems (101). With the progress in purification of these individual enzymes, much should be learnt about their anomalous behaviour and their kinetic properties. The uniqueness of the glycosome, the peculiarities of its content enzymes and the importance of the organelle in survival of the bloodstream forms of African trypanosomes make the organelle an attractive target for rational chemotherapy.

OBJECTIVES

Typanosomiasis has long been a neglected disease despite the high morbidity and mortality rates in humans and animals afflicted with the disease. One overriding consideration in the economics of drug development by pharmaceutical industries has been largely responsible for this state of neglect. The disease is not a 'Western world' or 'developed world' disease and poses no threat of spreading to these regions because of the geographic constraint of the vector in tropical Africa. The stark reality is that it afflicts impoverished Africans and their animals, hardly a profitable market to warrant a program of drug development in the eyes of pharmaceutical industries. African nations themselves are unable to tackle the problem for the obvious reasons of lack of resources, technological know-how and expertise manpower; to mention but a few of the problems facing the emerging nations of Africa. The result is that no new trypanocidal agent has been introduced in the past three decades or so. The drugs in use today are not safe drugs to say the least and the occurrence of resistance to these agents is increasing.

Information on the fundamental biochemistry and cell biology of these organisms is scanty. It is ironic that after many years of neglect, there is suddenly a renewed interest in the biology of the African trypanosomes. This interest is fostered not so much so by a genuine desire to develop better drugs but more so by the recognition that the study of certain very unique attributes of this group of organisms may serve as good models to understand the phenomenon of gene expression in general. It is no surprise therefore that the antigenic variation in African trypanosomes has become a hot field of late. Whatever the motivation of studying these parasites, the

information that will be gathered is sorely wanting and the spin-offs of such studies could lead to development of better drugs to treat the disease.

Thus, the rational approach to the design of better trypanocidal agents calls for a thorough understanding of fundamental biochemical processes in the various forms of the organism. Naturally, the dividing bloodstream form (long slender trypomastigotes) should be the first focus of such studies, geared to recognize or unearth unique processes or biochemical pathways sufficiently different from those of the parasitized host to be exploited in drug design.

Grandiose as it may seem, this has been the guiding overall objective of this work. The glycosome was chosen as the target of this study for reasons that must be obvious by now. It is a unique organelle (at least to the family Trypanosomatidae) and it houses key glycolytic enzymes that are absolutely essential for the survival of the long slender bloodstream forms of the parasite. Any disruption of this vital process would be expected to lead to the death of the organism. The following specific objectives have been addressed in this study:

- i. To develop methods or improve existing methods of purifying glycosomes from long slender bloodstream form stages as well as the culture procyclic trypomastigotes of *T. brucei*.

- ii. To characterize these organelles especially in regard to their enzyme content and compare differences between the organelles from the two life stages of the parasite.

- iii. To investigate whether a specific organization of the glycolytic enzymes exists in the glycosome of long slender bloodstream forms of *T. brucei* that may account for the enormous rate of glycolysis in this stage of the parasite

iv. To determine the sub-organellar location of the enzymes in the glycosome of both bloodstream form and culture form of the parasite with particular emphasis on identifying the integral glycosomal membrane proteins

The work that follows describes the pursuit of the objectives stated above (not necessarily in the order stated). The existence of a multienzyme complex was investigated employing chemical crosslinking agents as trapping and stabilizing agents. The kinetic behaviour of the resulting complex was thoroughly analyzed to determine whether the system functioned as an efficient kinetic machinery (channeling). Glycosomal integral membranes have been described for the first time, opening up new avenues in understanding the functioning and biogenesis of the organelle.

CHAPTER II

CROSS-LINKING OF THE ENZYMES IN THE GLYCOSOME

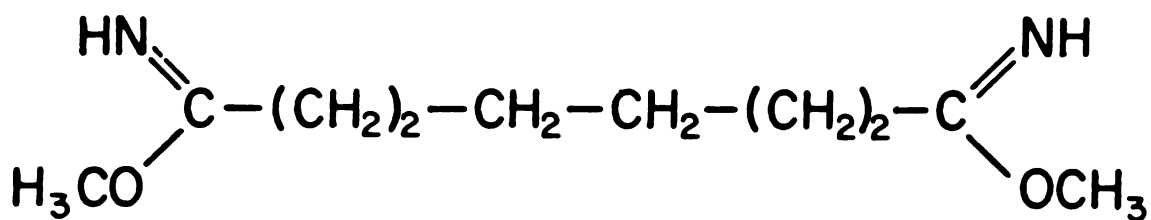
OF *TRYPANOSOMA BRUCEI*

Introduction

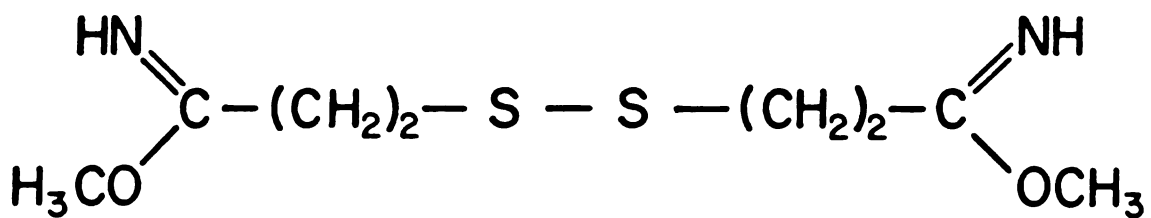
In the glucose-rich environment of the infected mammalian host blood, *T. brucei* long slender form thrives on glucose by converting it to pyruvate as the only end product with the net generation of two ATP molecules/glucose molecule consumed (104). NAD⁺ is regenerated by the concerted action of a glycosomal NAD⁺-linked glycerol-3-phosphate dehydrogenase and an oxygen-dependent mitochondrial glycerol-3-phosphate oxidase via a shuttle mechanism (64,104). To sustain its rapid growth in light of this poor energy yield from incomplete catabolism of glucose, the parasite consumes glucose at a rate of about 50 times that of mammalian cells (74). This high rate of glycolysis is apparently achieved by compartmentation of the first seven glycolytic enzymes within a microbody-like organelle termed the "glycosome" (64,104). Close physical association of the enzymes inside the glycosome into a multienzyme complex may be a factor contributing to the enhanced rate of glycolysis. Our current research interest has been to gain a better understanding of the detailed organization of such a multienzyme complex if it exists. If specific associations among the enzymes do exist within the glycosome, the question would arise as to whether this arrangement confers a kinetic advantage, such as an efficient channeling of substrates among the enzymes.

To address these questions, we set out first to isolate pure, intact glycosomes of the long slender bloodstream form of *T. brucei* and then treat them with bifunctional cross-linking reagents in the hope of trapping this complex in its native unperturbed form. Cross-linking reagents, particularly

the bisimido esters have been extensively used as probes of the spatial arrangement of polypeptides in extended biological systems such as cell membranes (105,106), ribosomes and nuclei (107,108), mitochondria (109) and multienzyme complexes (110,111). These compounds are readily soluble in water and react under mild conditions (pH range 7-10) with a high degree of specificity for primary amino groups. Of particular significance for our purpose is their ability to penetrate cell membranes and cross-link intracellular proteins (111). The chemistry and applications of chemical crosslinking reagents have been extensively reviewed (112,113,114). The chemical structures of the two compounds used in this study, dimethyl suberimidate (DMSI) and dimethyl-3,3'-dithiobispropionimidate (DTBP), are shown in Fig 9 . DTBP is an analog of DMSI in which two methylene groups have been replaced by a disulfide bridge, with the inherent advantage that proteins cross-linked with this compound can be cleaved at will by reduction of this disulfide bridge. It was anticipated that treating isolated intact glycosomes with these reagents under iso-osmotic conditions would yield a complex representing the natural state of the aggregated enzymes in vivo in the glycosome. The kinetic characterization of such a stabilized enzyme complex would hopefully provide insight into the functioning of the organelle and reveal potential sites of chemotherapeutic intervention.

Figure 9Chemical structures of two bisimidoester crosslinking reagents

Dimethyl suberimide (DMSI)



Dimethyl 3,3'-Dithiobispropionimide

Materials and Methods

Materials: Hydrochloride salts of the bisimido esters dimethylsuberimide (DMSI) and dimethyl-3,3'-dithiobispropionimide (DTBP) were obtained from the Pierce Chemical Company. Commercially available enzymes, substrates and cofactors were purchased from Calbiochem-Behring and Sigma Chemical Company. Concanavalin A-sepharose and Percoll were obtained from Pharmacia Fine Chemicals while Bio-Rad laboratories was the supplier of all electrophoretic reagents, SDS-PAGE molecular weight markers and RNase-free sucrose. A sample of purified variant surface glycoprotein (VSG) of *T. brucei* variant 118 was a kind gift from Dr. John Boothroyd of Stanford University. All other chemicals were of the highest purity commercially available. Two to three months old male Wistar rats were supplied by Simonsen Laboratories, Inc., Gilroy, CA.

Growth and isolation of the organism: Three hundred to 400 g male Wistar rats were anaesthetized with ether and inoculated intraperitoneally with 2×10^8 cells of long slender bloodstream forms of *T. brucei* monomorphic strain EATRO 110 maintained as a stablate. The animals, usually showing a peaking of parasitaemia at 10^9 cells/ml of blood after 2 days of infection, were anaesthetized again with ether and their blood collected by cardiac puncture. Two hundred to 220 ml of blood is usually obtained from 20 infected rats. The trypanosomes were separated from host erythrocytes and blood constituents by passage of the infected blood through a DEAE-cellulose column and elution with ice-cold phosphate-saline glucose buffer as described by Lanham (115). The parasites were pelleted by centrifugation at 3000 rpm in a SS-34 Sorvall rotor ($1000 \times g$) for 10 minutes. The cells were washed three times in this manner in the homogenization buffer composed of 25 mM Tris, 1 mM EDTA, 250 mM sucrose, pH 7.8 (TES buffer). The

purified parasites were either resuspended in TES buffer containing 15% (v/v) glycerol and stored frozen at -80°C or were used immediately for glycosome purification. All operations were conducted in the cold at $0-4^{\circ}\text{C}$ to lower the metabolism of the parasites.

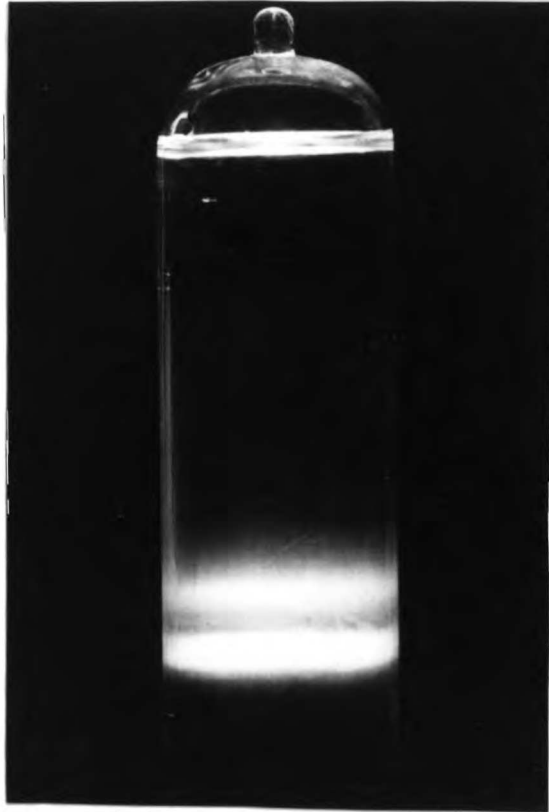
Purification of glycosomes: Glycosomes were prepared from trypanosomes that were freshly isolated (Method A) or trypanosomes that had been frozen at -80°C in 15% (v/v) glycerol (Method B). Frozen trypanosomes were supplemented with 50 mM glucose and thawed over a period of 20 minutes in a 37°C shaker water-bath. The parasites were pelleted by centrifugation at 3000 rpm in a Sorvall SS-34 rotor and the supernatant saved for later analysis. The pellet of *T. brucei* cells was weighed and resuspended in its own volume of TES buffer (1:1). The paste was transferred to a chilled mortar and homogenized by grinding with, one and a half times the wet weight of the pellet, of chilled silicon carbide (116). The degree of homogenization was monitored by microscopic examination of the homogenate and when few or none intact trypanosomes could be seen, the homogenate was centrifuged at $100 \times g$ for 5 minutes to sediment the abrasive. The supernatant was subjected to two successive spins of 10 minutes each at $1000 \times g$ and $5000 \times g$ to pellet the nuclear and large granule fractions, respectively (117). The post-large granule supernatant was subjected to Percoll gradient centrifugation as described by Oppenheimer (118). Briefly, it was mixed with an equal volume of 90% Percoll in a TES buffer pH 7.2, and centrifuged at $60,000 \times g$ for 30 min in a vertical VTi 50 rotor in a Beckman L8-80M ultracentrifuge. This procedure ensured a clear separation of two visible white-coloured bands. The lower glycosome-enriched band was drawn out of the tube by puncturing the side with a syringe and layered onto an 18% - 54% (w/w) linear sucrose gradient cast

in a buffer containing 25mM Tris and 1mM EDTA, pH 7.2 (TE buffer), and resting on a 66% (w/w) sucrose cushion pad in the same buffer. In later work, a 15 - 55% (w/w) gradient prepared by allowing a 5-step gradient to diffuse to linearity (see Appendix) was routinely used because of ease and speed of preparation. Both methods were shown to give identical results. After centrifugation at 170,000 x g for 30 min in the VTi 50 rotor, two closely associated bands were often seen in the lower half of the gradient (Figure 10). These bands, designated as top band and lower band, were present when freshly isolated trypanosomes were used for glycosome purification (Method A). However when trypanosomes that had been stored frozen at -80° C were thawed and used for glycosome purification (Method B), only one band was observed at this position of the gradient. The lower band consisting mainly of glycosomes was collected, diluted 3-fold in TES buffer pH 7.8 and centrifuged at 33,000 x g in a SW-28 rotor for 20 min both to reduce the final volume and to rid the organelles of Percoll particles. The glycosomal pellet was then resuspended in a minimal volume of TES buffer pH 7.8 (usually 2-3ml) and either stored frozen in small aliquots at -80° C or used immediately for other experiments.

Electron microscopy: Glycosomes and the top band from a sucrose gradient were prefixed with 2.5% glutaraldehyde in phosphate buffer pH 7.4 containing 0.25M sucrose according to the method of Opperdoes et al (63). The fixed samples were pelleted, post-fixed with osmium tetroxide, dehydrated, embedded in epon, sectioned, and stained with uranyl acetate. Intact live trypanosomes either freshly isolated or thawed were prepared for electron microscopy according to the procedure described by Cross (20).

Cross-linking and lysis of glycosome: A 12 mM solution of either DMSI or DTBP in TES buffer was prepared fresh each time and the pH adjusted to

Figure 10: Purification of glycosomes from *T. brucei* EATRO 110 bloodstream long slender forms. Two closely spaced bands after the final step of purification on 28 ml of a linear 18-54% (w/w) sucrose density gradient cushioned by a 2-ml 66% (w/w) sucrose pad. The gradient was centrifuged at 170,000 x g for 30 minutes in a VTi 50 rotor. The lower band contained the purified glycosomes.

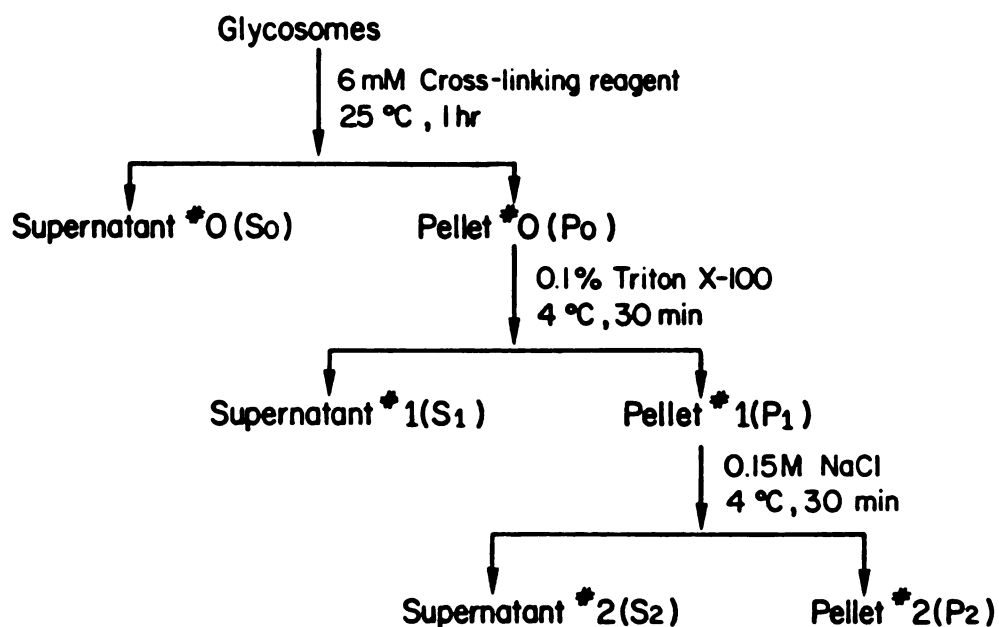


8.0 with NaOH solution. To a glycosome suspension in TES buffer (1-2ml) was added an equal volume of the cross-linking reagent solution and the reaction allowed to proceed at 25° C for one hour. The reaction was stopped by chilling the sample on ice and centrifuging at 33,000 x g for 20 min in a SW-41 rotor at 4° C. The supernatant fraction, designated S₀ as in Figure 11, was withdrawn and the pellet (P₀) subjected to the manipulations depicted in Fig. 11. All centrifugation steps were at 33,000 x g for 25 min in a SW-41 rotor (4° C). The pellet collected at each stage was resuspended in a buffer composed of 50 mM triethanolamine-HCl and 10 mM MgCl₂, pH 8.0 (TM buffer) containing either 0.1 % (v/v) Triton X-100 or 0.15 M NaCl. Resuspension was aided by a brief homogenization (10 strokes) in a hand-held Potter-Elvehjem homogenizer. The final pellet, P₂, was resuspended in the TM buffer containing 0.15 M NaCl. Native glycosomes (i.e. untreated) were also fractionated in an identical manner to give the corresponding control fractions of S₀, S₁, S₂, and P₂.

Sucrose density gradient centrifugation: Samples (0.5 ml) were overlayed on 12 ml of a 5-20% (w/v) linear sucrose density gradient resting on a 0.4ml 66% (w/w) sucrose cushion pad and centrifuged at 41,000 rpm in a Beckman SW-41 rotor for 13 hours at 4° C. Equal volume fractions of the gradient were collected by puncturing the bottom of the centrifuge tube. The fractions were assayed for enzyme activities as described below. The gradients as well as the samples were buffered by 25 mM Tris-HCl pH 7.8 containing 1 mM EDTA, 0.15 M NaCl, and 0.1% Triton X-100 (v/v).

SDS-PAGE: Samples were boiled for 5 min in a buffer containing 62.5 mM Tris-HCl, 2% SDS, 10% (v/v) glycerol, and 5% 2-mercaptoethanol, pH 6.8. Electrophoresis was carried out according to the method of Laemmli (119). Slab gels (1.5mm thick) composed of either 9% or 12% pH 8.8

Figure 11: Scheme outlining the crosslinking procedure and subsequent fractionation of the crosslinked glycosomes. At each centrifugation step (33,000 x g for 25 min) the protein pellet was resuspended in the TM buffer by a brief homogenization in a hand-held homogenizer and left on ice for 30 min before centrifuging in a SW 41 rotor. Native glycosomes, i. e. not treated with the crosslinking reagents, were also fractionated according to this scheme.



separating gel and 4% pH 6.8 stacking gel were used (see Appendix). The gels were stained for protein with Coomassie Blue R-250 dye or by the silver staining method. The Coomassie Blue-stained gels were scanned in an LKB 2202-001 Ultrosan laser densitometer linked to a Hewlett-Packard 3390-A integrator for quantitative estimation of individual protein bands.

Enzyme assays: Initial velocities were measured with a Beckman DU-7 spectrophotometer equipped with an automated six-cell sample selector with temperature control. Assays of the individual glycolytic enzymes were carried out according to the procedures described by Bergmeyer (120) and are detailed in the Appendix. All assays, conducted in a total volume of 1.0 ml, were validated for linearity with enzyme concentration. Phosphofructose kinase was assayed by a modification of the method of Maitra and Lobo (121), in which the assay mixture contained 70 mM triethanolamine-HCl, pH 8.0, 10 mM MgCl₂, 0.5 mM NADH, 1 mM ATP, 5 mM fructose-6-phosphate, and 0.5 Units/ml each of the coupling enzymes aldolase, glycerol-3-phosphate dehydrogenase, and triose phosphate isomerase. Glycerol-3-phosphate dehydrogenase was assayed as described by Oppendoes et al (96). All assays except those determining the latency of the enzymes were carried out in the presence of 0.1% Triton X-100. To determine latency of the enzymes, assays were conducted in the absence of Triton X-100 and in the presence of 0.25 M sucrose to ensure integrity of the organelles. Latency is defined as:

$$\% \text{ Latency} = \frac{[\text{Activity}]_{+\text{Triton X-100}} - [\text{Activity}]_{-\text{Triton X-100}}}{[\text{Activity}]_{+\text{Triton X-100}}} \times 100$$

Enzyme-linked oxidation or reduction of pyridine nucleotides was monitored at 340 nm and an extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for NADH

and NADPH was assumed in calculations of the specific activities. Specific activities are expressed as micromoles of substrate consumed per min per mg protein at 37° C.

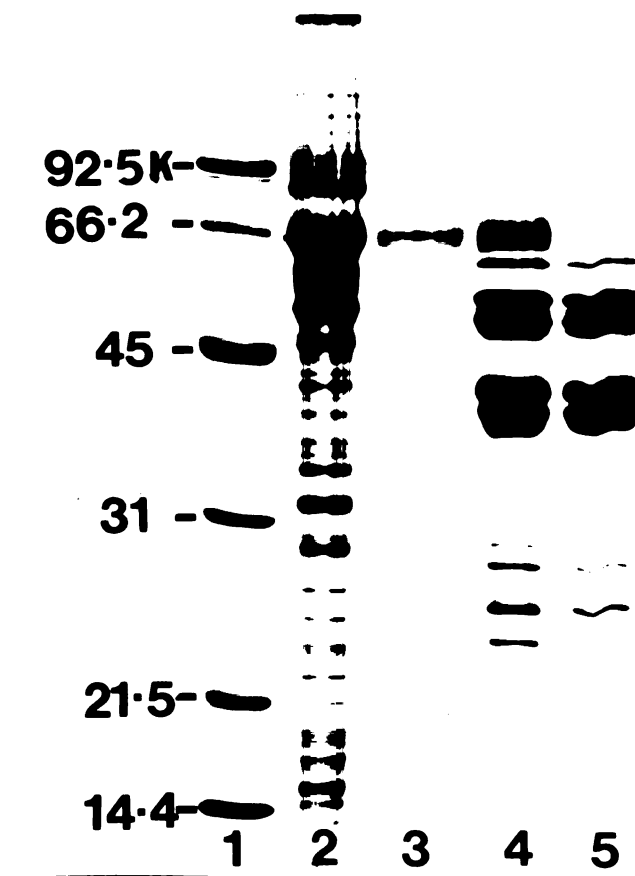
Protein determination: Protein concentrations were determined by the method of Lowry et al (122). Protein was first precipitated with trichloroacetic acid (final concentration 10%) and then dissolved by boiling for 2 minutes in 0.1 M NaOH. Bovine serum albumin Fraction V dissolved in 0.1 M NaOH was used as standard. This modification was devised to circumvent interference of the assay by reagents such as Triton X-100 and high salt concentrations.

Binding to concanavalin A-sepharose: Glycosomes, solubilized in TES buffer pH 7.8 containing 0.1% (v/v) Triton X-100 and 0.15 M NaCl, were incubated with shaking for 2 hours at 4° C with concanavalin-A sepharose beads that had been extensively washed and equilibrated in the same buffer. The beads were then sedimented by centrifugation, washed 3 times in the same buffer and bound material released from the matrix by incubating with shaking for 1 hour at 4° C with the initial buffer containing 0.75 M alpha-methylmannoside (123). The suspension was then loaded onto a small column and the effluent collected and analysed by SDS-PAGE.

Results

Purification of glycosomes: As noted earlier, when freshly isolated trypanosomes were used for glycosome purification, two very close bands almost always appeared after the final sucrose gradient centrifugation (Figure 10). Both bands were collected separately and examined by transmission electron microscopy. The lower band was found to be highly enriched in intact glycosomes and showed about 80% homogeneity (data not shown). However, there was still some degree of contamination with membrane fragments and broken flagellar structures which appear to be derived from the top band since this debris material seems to be abundant in this top band. Because the two bands are very close to each other in the gradient, it is difficult to collect the bands separately without the occurrence of some degree of cross-contamination. SDS-PAGE analysis of the purified glycosomal fraction (lower band) showed twelve major bands that can be loosely grouped into four clusters of a doublet (65K and 61K), two sets of triplets (51kD, 50kD and 48kD; 42kD, 40kD and 39kD) and a tetrad (29kD, 28kD, 26kD and 24kD) in their order of decreasing molecular weight (Fig.12, lane 4). The top band from the sucrose gradient contained all these polypeptides as well (not shown), but the relative contributions of the 11 bands from 61kD through 24kD were greatly reduced suggesting that their presence in this fraction is a result of cross-contamination with glycosomes. In contrast, the 65 kD polypeptide was consistently present as the major component in this top band fraction and its presence in the glycosomal fraction as a relatively minor component is probably due to the cross-contamination between the two bands. This assumption was tested by subjecting purified glycosomes to mild tryptic digestion (0.4 BAEE units/ml) in TES buffer at 37° C for 30 min followed by centrifugation and analysis by

Figure 12: SDS-PAGE analysis showing the absence of a 65,000 dalton protein band in glycosomes prepared after freezing and thawing the parasites in 15% (v/v) glycerol. Molecular weight standards used in all SDS-PAGE experiments are : phosphorylase B (92,000 kD), bovine serum albumin (66,200 kD), ovalbumin (45,000 kD), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). Lane 1, standards; Lane 2, supernatant fraction obtained after freezing and thawing parasites in 15% (v/v) glycerol; Lane 3, material eluted by alpha-methylmannoside from a concanavalin A-sepharose column after application of glycosomes contaminated with the 65,000 dalton protein; Lane 4, glycosomes prepared by the earlier procedure (method A); Lane 5, glycosomes prepared by the new procedure of freezing and thawing parasites in 15% (v/v) glycerol prior to homogenization (method B).



SDS-PAGE of the pelleted glycosomes. The result indicated an almost total elimination of the 65 kD protein band without affecting any of the other 11 bands seen in glycosome preparations suggesting that the 65 kD polypeptide is not a protein inside the glycosome. Several observations tended to suggest that this protein might be the variant surface glycoprotein arising by its release from plasma membrane fragments appearing in the top band from the sucrose gradient. First, it migrates on SDS-PAGE with a molecular weight similar to that of purified VSGs reported in the literature (20) although direct comparison of its mobility with a purified VSG 118 showed it to be slower migrating. It appears to be a major cell protein as assessed by its abundance in crude cell homogenates and is found largely in the soluble post-glycosomal supernatant fraction (33,000 x g) obtained by differential centrifugation of a trypanosomal homogenate. VSG, a major cell protein constituting ca. 10% of the cell protein, is known to be released into a soluble form on rupture of the organisms. Incidentally, this protein is not seen in homogenates or glycosomes of the culture form procyclics in which the surface coat glycoprotein is not present (155). The glycoprotein nature of this 65K protein was indeed established by its ability to bind to concanavalin-A sepharose beads and its specific elution from this lectin by alpha- methylmannoside as shown in lane 3 of Fig.12. This 65 kD protein which also lacks any of the enzyme activities known to be associated with the glycosome (see below) is thus most likely to be VSG.

A simple technique to eliminate this contaminant from purified glycosomes was devised. A suspension of freshly purified trypanosomes in TES buffer pH 7.8 containing 15% (v/v) glycerol as cryopreservant was frozen at -80° C and thawed at 37° C immediately prior to glycosome preparation. Light microscopic examination of the thawed suspension showed that over 90% of

the parasite population remained active and motile after this treatment. Centrifugation of the thawed suspension to pellet the organisms left a cloudy supernatant fraction, which when analysed by SDS-PAGE, was found to be composed largely of one major protein of molecular weight of 65,000 (Fig.12 lane 2). Electron microscopic examination of the frozen and thawed parasites revealed a reduced density or thickness of the surface coat glycoprotein when compared to that of freshly isolated parasites (Fig.13). The ultrastructure of frozen and thawed parasites was however, no different from that of fresh organisms.

When the frozen, thawed and washed *T. brucei* cells were used for purification of glycosomes by the procedure described, only a single band was observed in the final sucrose gradient. Electron microscopy of the purified glycosomal preparation showed a population of intact microbodies that were almost totally homogeneous (Fig.14). The morphological appearance and size of these isolated organelles is almost unaltered by the purification procedure as is evident by comparing the electron micrographs of these isolated organelles with those of the organelles in situ in intact parasites (Fig.15). Although occasionally the isolated organelles show a less dense granular matrix, the overall appearance of the organelles is quite well preserved. Both isolated and in situ organelles have an average diameter of 0.3 micron. Often, laminated crystalloid cores are seen in in situ as well as in isolated organelles. The protein profile of the purified glycosomes (method B) examined by SDS-PAGE (Fig.12 lane 5) now consisted of a typical band pattern of singlet-triplet-triplet-tetrad without the contaminating 65,000

Figure 13: Electron microscopic examination of bloodstream form *T. brucei* before (A) and after (B) freezing and thawing in 15% (v/v) glycerol. The scale bar corresponds to 0.16 micron.

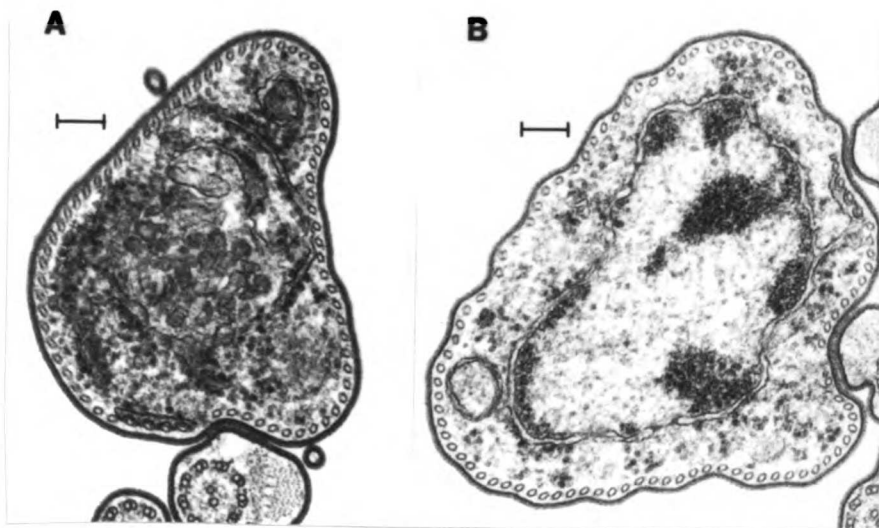


Figure 14: Electron microscopy of purified glycosomes.

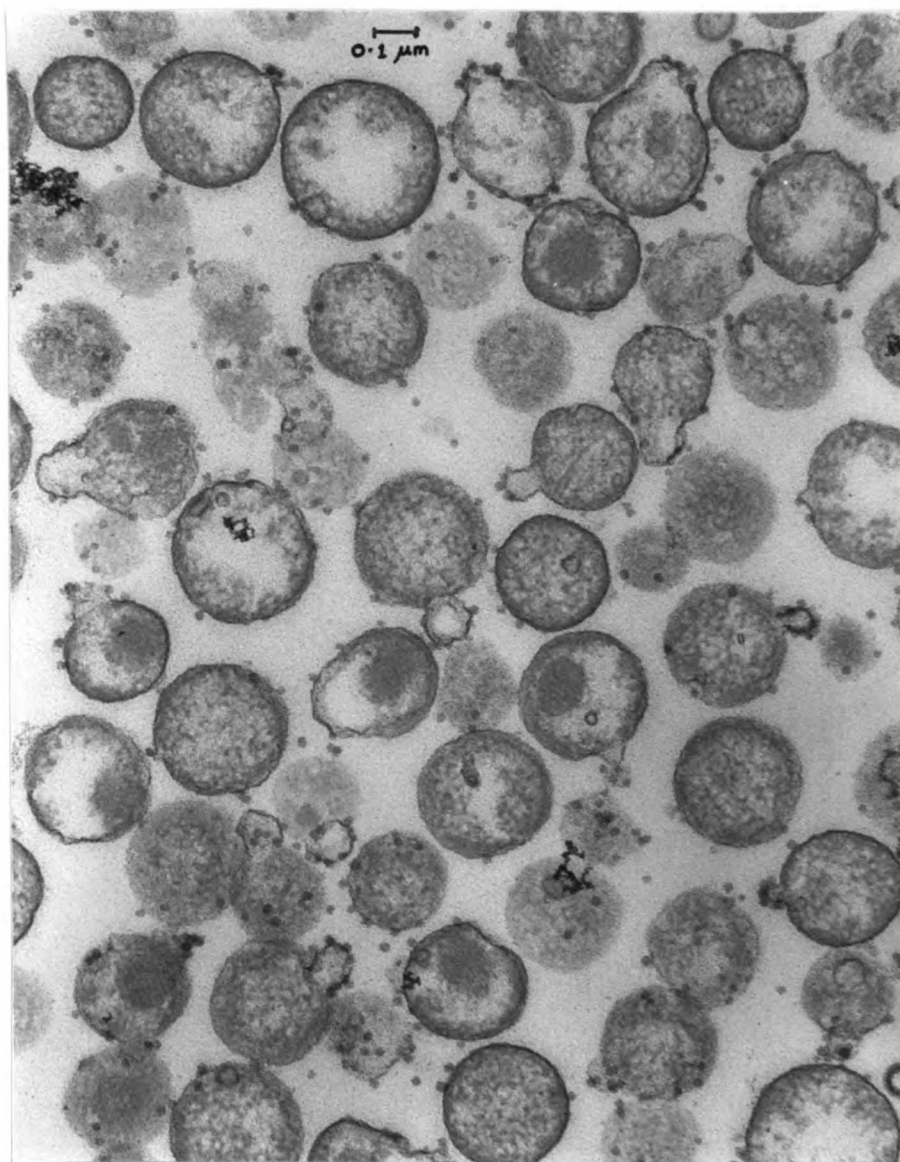
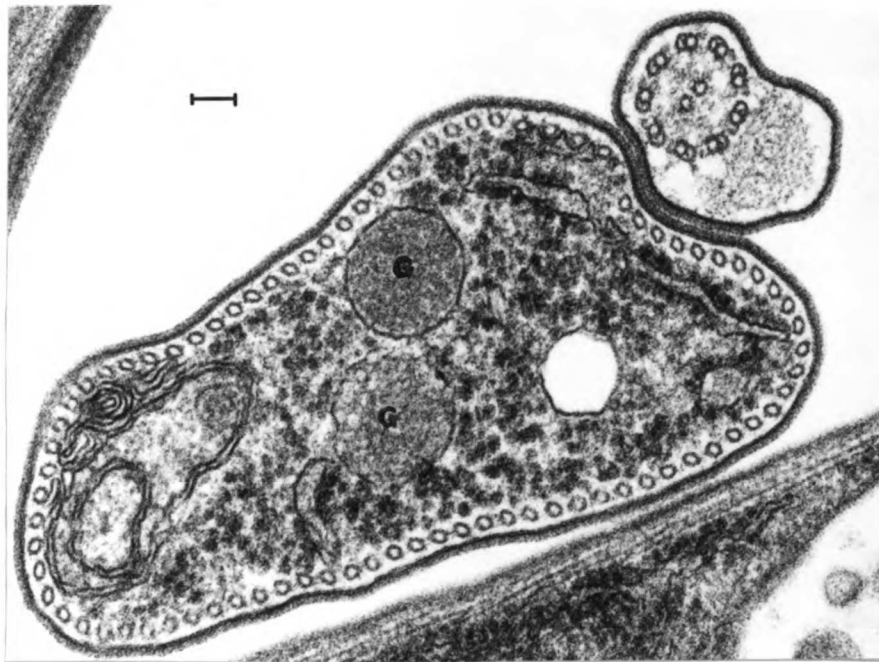


Figure 15: Electron microscopy of intact bloodstream form *T. brucei* showing the in situ appearance of glycosomes (G). The scale bar corresponds to 0.1 micron.



band (compare Lanes 9 and 5 of Fig. 12)[¶]. Apparently the procedure of freezing and thawing the parasites minimizes the extent of contamination of glycosomes with plasma membrane fragments (bearing VSG) that otherwise have a tendency to band very close to the glycosomal band in sucrose gradients.

Eleven major bands designated as bands 1 through 11 in their order of decreasing subunit molecular weight were consistently detected by SDS-PAGE analysis of the purified glycosomes (Fig.16). A quantitative estimation of the relative contribution of each of these bands to the total glycosomal protein was obtained by densitometric tracings of the gels and the data is presented in Table 6. According to the data presented by Hart et al (90) and our own results on the enzyme profiles both from sucrose gradients (Fig.20) and following fractionation of the glycosomes, a tentative identity was assigned to most of the bands as shown in Table 6. It is noteworthy that the two sets of triplets comprising bands 2 through 7 account for about 87% of the total glycosomal protein.

Cross-linking of the glycosomal proteins: The purified intact glycosomes shown in Fig. 14 were treated with either DMSI or DTBP at 6 mM and subsequently fractionated by solubilization in 0.1% Triton X-100 and 0.15 M NaCl by a stepwise procedure illustrated in Fig.11. The three supernatant fractions (S₀, S₁, S₂) and one pellet fraction (P₂) resulting from each experiment were analyzed for protein contents and the results are presented in Table 7. About 10% of the protein in each case seems to be released into the S₀ supernatant fraction by merely incubating glycosomes with buffer or

[¶] Although this procedure almost totally eliminates this contaminating protein from the purified glycosomes, the extent of its removal may vary from batch to batch and occasionally a faint broad band may be seen at this position of the gel.

Figure 16: SDS-PAGE analysis and densitometric tracing of purified glycosomes. The gel was scanned while wet in an LKB Ultrosan laser densitometer connected to a Hewlett-Packard 3390-A integrator.

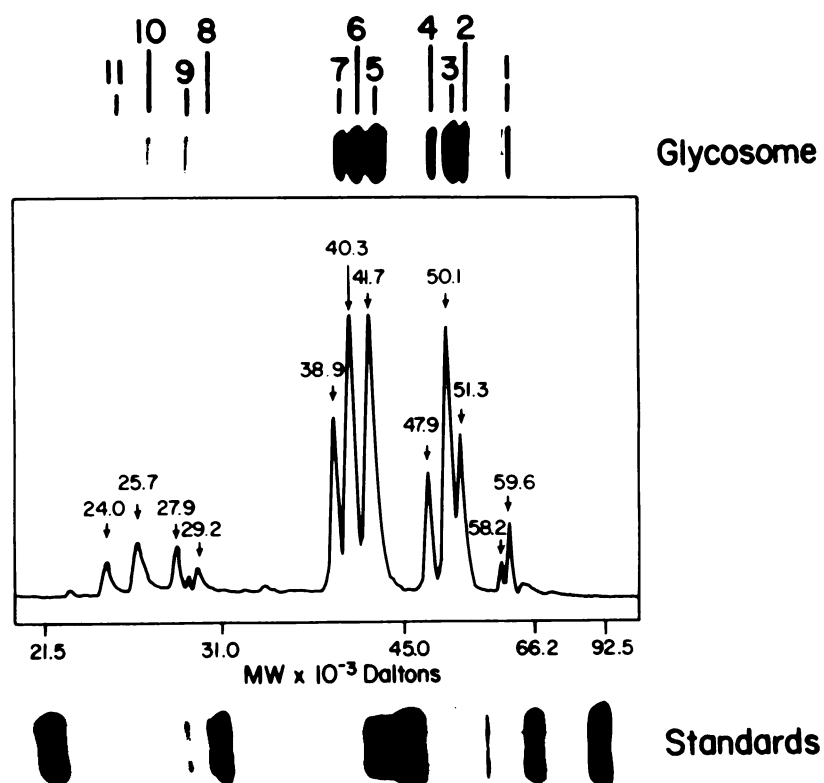


TABLE 6**SDS-PAGE analysis of the proteins from purified *T. brucei* glycosomes**

Protein band	Tentative ^a identification	Estimated ^b subunit M _r	Estimated % ^c of total glycosomal protein
1	Phosphoglucose isomerase	59,600	1.0
2	Phosphofructose kinase	51,300	8.4
3	Hexokinase	50,100	18.0
4	3-Phosphoglycerate kinase	47,900	5.0
5	Aldolase	41,700	28.3
6	Glyceraldehyde-3-phosphate dehydrogenase	40,300	17.9
7	Glycerol-3-phosphate dehydrogenase	38,900	9.4
8	Unidentified	29,200	1.8
9	Triose phosphate isomerase	27,900	3.0
10	Unidentified	25,700	1.7
11	Unidentified	24,000	2.7

^aAccording to Hart et al (90).^bSee Fig. 16.^cData from gel scanning in an LKB 2202-001 Ultrosan laser densitometer linked to a Hewlett-Packard 3390-A integrator.

cross-linking reagents under iso-osmotic conditions at room temperature for 1 hour. The proteins in this fraction could possibly arise from a few broken or leaky glycosomes but it seems more likely that it represents those proteins loosely associated with the outside of the glycosome, notably the 65,000 protein which is not totally eliminated even by the improved procedure. In fact when glycosomes prepared by the earlier procedure (Method A) were fractionated according to the scheme in Fig.11, the resulting S_0 fraction was found by SDS-PAGE analysis to contain exclusively the 65,000 protein contaminant.

The results presented in Table 7 indicate that for the untreated, native glycosomes, 60.7% of the total protein could be solubilized by 0.1% Triton X-100 and 0.15 M NaCl, leaving only 29.8% of the proteins associated with the P_2 fraction. In contrast, glycosomes treated with DMSI or DTBP released only 10.3% and 11.2% respectively of their protein content into the supernatant fraction on solubilization with detergent and salt. The greater portion of the protein, representing 81.2% and 78.1% respectively, remained insoluble and associated with the P_2 fraction. This shift of the proteins to a pelletable form following treatment with these reagents suggests successful cross-linkings by DMSI and DTBP of the glycosomal proteins.

SDS-PAGE and enzymatic analysis of fractions from native and cross-linked glycosomes: Samples of fractions S_0 , S_1 , S_2 , and P_2 derived from native (untreated) glycosomes and cross-linked glycosomes were analyzed by SDS-PAGE. Figure 17 indicates that most of the major protein bands from untreated, native glycosomes were solubilized by 0.1% Triton X-100 and ended up in the S_1 fraction except for protein bands 2 (phosphofructokinase), 10, and 11 which were relatively light in this fraction (Fig.17, lane 4). 0.15 M NaCl was able to extract into the S_2 fraction protein

TABLE 7
Alteration of protein distributions in crosslinked *T. brucei* glycosomes

Glycosome sample	Protein content (mg) ^a				
	S ₀	S ₁	S ₂	P ₂	Total
Untreated	0.44 (9.6) ^b	2.15 (47.0)	0.62 (13.6)	1.36 (29.8)	4.57
DMSI treated	0.40 (8.5)	0.26 (5.6)	0.22 (4.7)	3.80 (81.2)	4.68
DTBP treated	0.75 (10.7)	0.50 (7.2)	0.28 (4.0)	5.45 (78.1)	6.98

^aFor the fractionation procedure, see Fig. 10.

^bNumbers in parenthesis are the percentage of total protein in each fraction.

bands 10 and 11 together with substantial amounts of bands 3 (hexokinase), 4 (phosphoglycerate kinase) and 5 (aldolase) (Fig.17, lane 5). Appreciable levels of bands 5 (aldolase) and 6 (glyceraldehyde-3-phosphate) remained in the P₂ fraction (Fig.17, lane 6). These results are in good agreement with the glycosomal enzyme profiles in the fractionated native glycosomes (see Table 8) showing substantial percentages of phosphofructokinase and aldolase activities in the control P₂ fraction. Glycerol kinase activity appeared mainly in the S₀ fraction (74.4%) which is misleading because the enzyme is extremely unstable and is probably underestimated in the other fractions as indicated by the observation of low recoveries of total enzyme activity after fractionation of glycosomes. Although the protein band in SDS-PAGE gels corresponding to glycerol kinase has not been identified as yet, the absence of a prominent protein band in the S₀ fraction (Fig.17, lane 3) supports the view that the enzyme is probably present in an inactive state in other fractions as well and that it is not loosely associated with the outside of the glycosomal membrane as might be interpreted from the activity profile.

As mentioned earlier, the S₀ fraction from native glycosomes contaminated with the 65 kD protein (Method A) contained exclusively this protein (65 kD band) when analyzed by SDS-PAGE. All efforts to associate this 65 kD protein with any of the known glycosomal enzyme activities proved negative. The lack of any glycosomal enzyme activity associated with this protein further strengthened our suspicion that the protein represents a contaminant of the glycosomes.

Supernatant fractions S₀, S₁, and S₂ from either DMSI-treated or DTBP treated glycosomes did not show any appreciable bands when examined by SDS-PAGE (lanes 3, 4 and 5 of Figs. 18 and 19 respectively) in conformity with their low protein contents (Table 7). Enzyme activities also could not be

Figure 17: SDS-PAGE analysis of native glycosomes and their fractions obtained as shown in Fig. 10. Lanes 1 and 7, standards; Lane 2, native glycosomes; Lane 3, fraction S₀; Lane 4, fraction S₁; Lane 5, fraction S₂; Lane 6, fraction P₂.

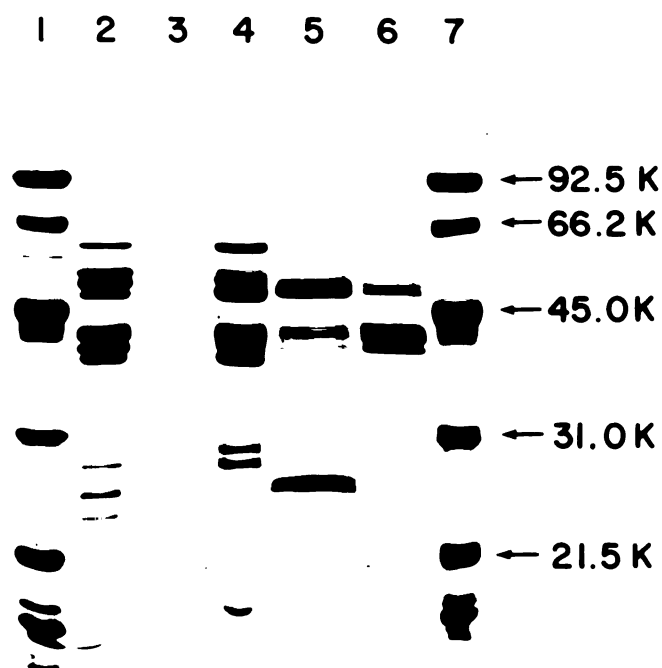


TABLE 8
The distribution of enzyme activities in different fractions of the
***T. brucei* glycosome**

Enzyme	Percentage of total activity ^a			
	S ₀	S ₁	S ₂	P ₂
Hexokinase	2.7	87.2	8.0	2.1
Phosphoglucose isomerase	12.3	78.3	6.9	
2.6				
Phosphofructose kinase	0.5	48.6	26.1	
24.8				
Aldolase	1.1	57.0	6.6	35.3
Glyceraldehyde-3-phosphate dehydrogenase	9.3	90.7	0	0
Triose phosphate isomerase	1.4	89.9	6.8	
1.9				
Glycerol-3-phosphate dehydrogenase	6.2	90.5	2.6	0
3-Phosphoglycerate kinase	4.5	88.5	7.0	0
Glycerol kinase	74.4	25.6	0	0

^aResults presented are the means from two independent experiments.

detected in these fractions. The recalcitrant P₂ fraction from DMSI-treated glycosomes did not penetrate the resolving gel resulting in the absence of protein bands in this track (Fig.18, lane 6). In the case of the P₂ fraction from DTBP-treated glycosomes, reduction of the disulfide bridges in the cross-linked complex by 2-mercaptoethanol present in the SDS-PAGE sample treatment buffer results in dissociation of the complex into its individual components as seen in lane 6 of Fig.19. This observation provides the strongest evidence that DTBP has indeed successfully cross-linked most of the eleven major glycosomal proteins under the present experimental conditions. The few high molecular weight protein bands and an intensely stained top band seen in the gel (Fig.16, lane 6) may represent products of incomplete dissociation of the DTBP-cross-linked protein complex by boiling with 2-mercaptoethanol.

Enzyme profiles of native and cross-linked glycosomal proteins: The P₂ fractions from DMSI-treated and DTBP-treated glycosomes were washed three times in TE buffer containing 0.1% Triton X-100 and 0.15 M NaCl to remove any free or non-complexed enzymes associated with the crosslinked complex. These samples along with similarly solubilized native glycosomes were then assayed for the nine enzymes hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), triose phosphate isomerase (TPI), glycerol-3-phosphate dehydrogenase (GDH) and glycerol kinase (GK). In native intact glycosomes, all the enzymes exhibited high latency ranging from 65% for aldolase to 94% for GDH as shown in Table 8. The results in Table 9 also indicate that all nine enzyme activities exist in the DMSI-P₂ and DTBP-P₂ fractions at reproducible but reduced levels when compared with the

Figure 18: SDS-PAGE analysis of fractions from glycosomes treated with DMSI. Lane 1, standards; Lane 2, native glycosomes; Lane 3, S₀ fraction; Lane 4, S₁ fraction; Lane 5, S₂ fraction; Lane 6, P₂ fraction

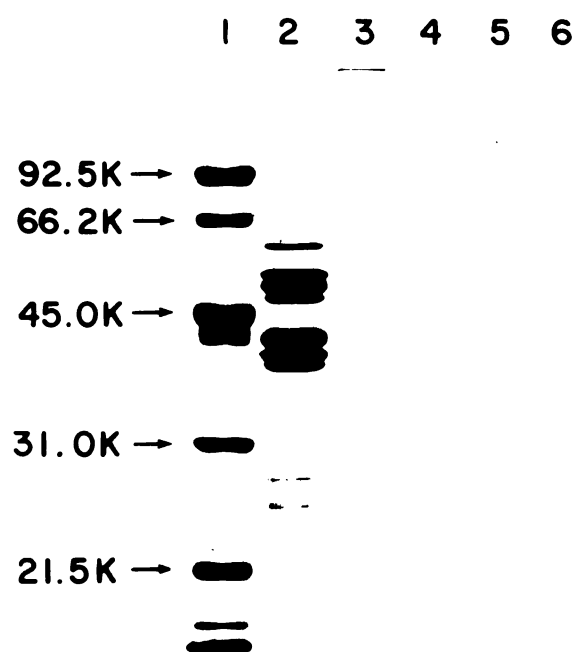
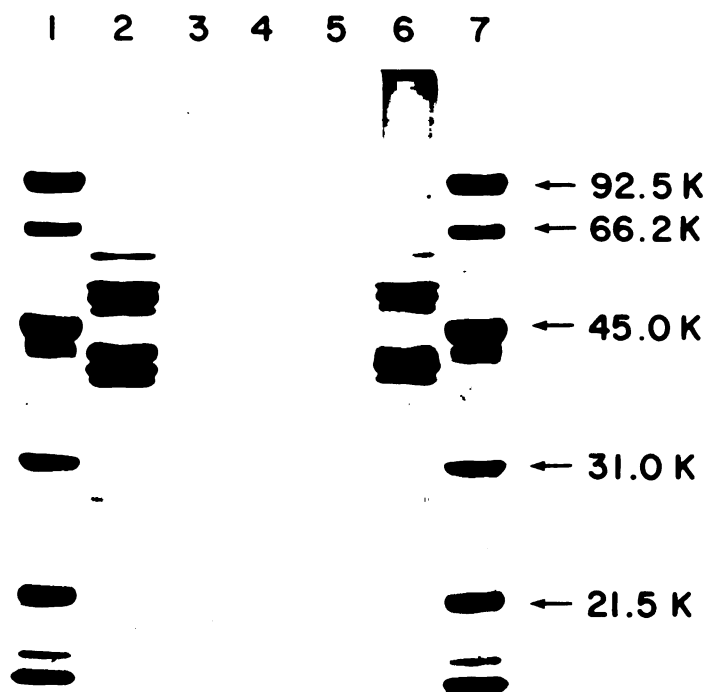


Figure 19: SDS-PAGE analysis of fractions from glycosomes treated with DTBP. Lanes 1 and 7, standards; Lane 2, native glycosomes; Lane 3, S₀ fraction; Lane 4, S₁ fraction; Lane 5, S₂ fraction; Lane 6, P₂ fraction



activities in a total lysate (by 0.1% (v/v) Triton X-100 and 0.15 M NaCl) of native glycosomes. All the enzymes appeared to be inactivated to the same degree by DMSI although phosphoglycerate kinase and glycerol kinase both seem to be more extensively inactivated by this reagent. DTBP caused a much higher level of inactivation of all the enzymes and activities were barely detectable in most cases. It is not clear why this reagent is more deleterious to the enzymes than DMSI but one possible explanation could be due to disulfide exchange between the reagent and critical thiol groups in the proteins. Even cleavage of DTBP-crosslinked P₂ fraction with dithiothreitol did not substantially restore enzyme activities. For this reason, further work with DTBP was not pursued.

Sucrose density centrifugation of fractionated and crosslinked glycosomes:

The possibility that the DMSI cross-linked P₂ fraction could comprise a heterogeneous mixture of many different cross-linked products was tested by subjecting this fraction to isopycnic sucrose gradient centrifugation. Samples of native glycosomal lysate, native glycosomal S₀+S₁+S₂ fraction and native glycosomal P₂ fraction were treated similarly by centrifugation in a 5-20% (w/v) linear sucrose gradient for 13 hours. Fractions of the gradient were collected and tested for each of seven enzyme activities. In the cross-linked sample (DMSI P₂), all of the enzyme activities were found to be exclusively associated with a pellet fraction sedimenting to the bottom of the tube (fractions 1 and 2)(Fig.20, panel D), thus ruling out the presence of multiple, intermediate-sized cross-linked complexes in the DMSI P₂ fraction.

The positions of enzymes in native glycosomal lysates in this gradient could be put into three categories (Fig.20, panel A). HK, PFK, and ALD sedimented toward the bottom of the tube while GDH, PGK and TPI banded in the middle of the gradient. The third category is PGI whose position in the

TABLE 9
Glycolytic enzyme activities in native glycosomes and DMSI and DTBP
crosslinked enzyme complexes from *T. brucei*

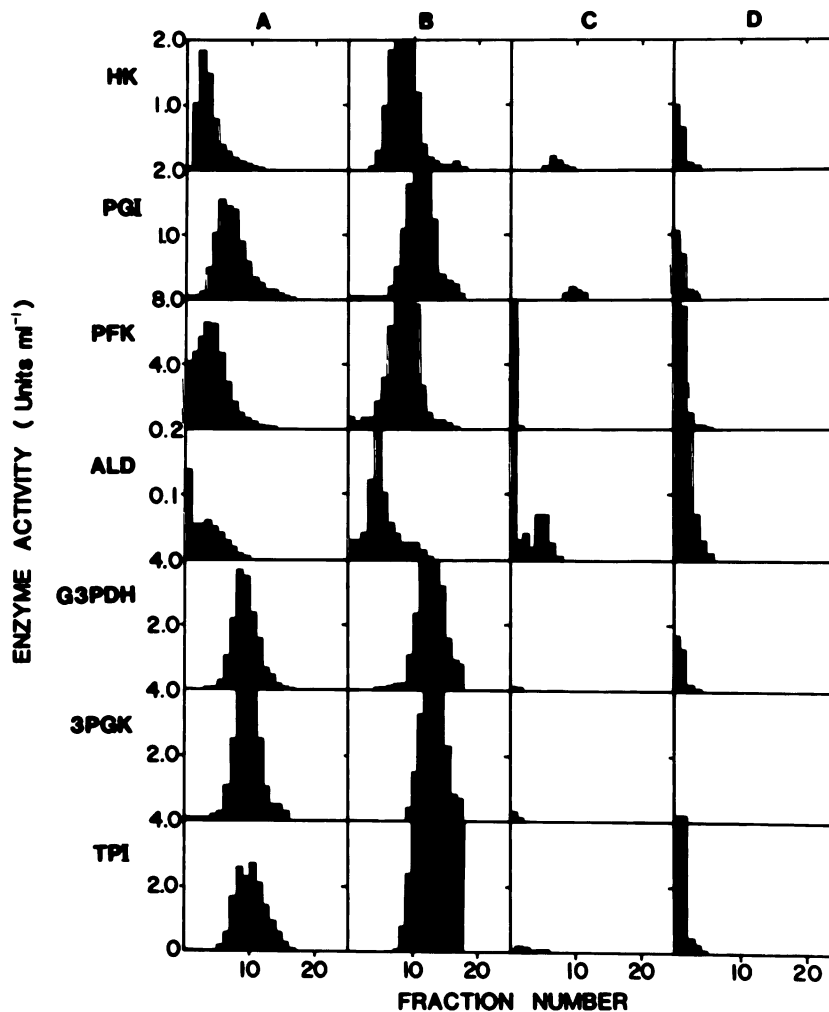
Enzyme	Specific activity (micromole substrate/min/mg protein)			
	Total glycosomal ^a lysate	c	DMSI P ₂ ^b	DTBP P ₂ ^b
Hexokinase	5.73 ± 1.26	(87.5) ^c	1.35	0.21
Phosphoglucose isomerase	4.98 ± 0.68	(80.1)	1.13	0.29
Phosphofructose kinase	22.09 ± 2.74	(75.7)	4.73	0.71
Aldolase	5.32 ± 0.87	(65.1)	1.45	0.35
Glyceraldehyde-3-phosphate dehydrogenase	2.38 ± 1.46	(78.4)	1.22	0.41
Triose phosphate isomerase	58.34 ± 11.71	(77.6)	12.25	5.07
Glycerol-3-phosphate dehydrogenase	7.98 ± 1.61	(94.0)	2.70	0.42
3-Phosphoglycerate kinase	10.35 ± 1.85	(93.8)	0.17	0.24
Glycerol kinase	3.00 ± 0.35	(82.1)	0.31	0

^aValues are mean (+ S.D.) of three independent experiments.

^bSpecific activity is the mean of two independent experiments.

^cValues in parenthesis are the percent latency of the native enzymes

Figure 20: Distribution of glycolytic enzyme activities in various fractions of glycosomes in a 5-20% (w/v) sucrose gradient centrifugation. Panel A, solubilized native glycosomes; Panel B, fractions $S_0 + S_1 + S_2$ from solubilized native glycosomes; Panel C, fraction P_2 from native glycosomes; Panel D, DMSI crosslinked P_2 fraction. Abbreviations are : HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructose kinase; ALD, aldolase; G3PDH, glycerol-3-phosphate dehydrogenase; 3PGK, 3-phosphoglycerate kinase; TPI, triose phosphate isomerase.



gradient was intermediate between the other two groups of enzymes. When glycosomal lysates are first centrifuged to remove the P₂ fraction, i.e. only the S₀+S₁+S₂ fraction is analyzed, the pattern of banding of various enzymes in the sucrose gradient does not change much except that HK, PFK and ALD do not band as low in the gradient as before although they still remain the most dense enzymes (Fig.20, panel B). The native glycosomal P₂ fraction contains largely PFK and ALD both of which sediment to the bottom of the gradient (Fig.20, panel C). This result is in good agreement with that of the SDS-PAGE in Fig.17 which indicates a substantial amount of PFK and ALD protein bands associated with the control P₂ fraction and with that in Table 8 which shows 24.8% PFK and 35.3% ALD in the control P₂ fraction. The possibility that the DMSI-cross-linked P₂ fraction might contain some free PFK and ALD that co-sediment with the complex has been ruled out by the absence of the corresponding subunit bands in the SDS-PAGE profile of the DMSI P₂ fraction (Fig.18, lane 6).

Discussion

The glycosomes of the long slender bloodstream forms of *T. brucei* are apparently capable of carrying out a self-driven and self-controlled process of glycolysis. The organelle in this life stage form of the parasite catalyses the net reaction of conversion of a glucose molecule to two molecules of 3-phosphoglycerate, a reaction in which the production and consumption of ATP and NAD⁺ are balanced(64). Net synthesis of ATP occurs in the cytosol where glycosome-generated 3-phosphoglycerate is converted to pyruvate. Not much is known about control of the seemingly high overall flux of glycolysis in the organism. Nwagwu and Opperdoes (101) have indicated that neither hexokinase nor phosphofructose kinase of *T. brucei* is subject to any feedback regulation. It is possible that the rate of glycolysis is controlled by the intraglycosomal ratios of ATP-ADP and NAD⁺-NADH since these cofactors are presumably confined within this compartment. On the other hand, the availability of glucose may be the rate-limiting step that regulates the glycolytic flux. *T. brucei* glycosomes have been estimated to occupy about 4.3% of the cellular volume which represents 7.8-8.7% of the total protein in the bloodstream form organism (97). With an estimated cellular protein concentration of 175 mg/ml, the glycosomes have been calculated to contain protein at a concentration of 320-360 mg/ml of glycosomal volume (97). Based on the information presented in Table 6, and the assumption that each identified band in Fig.16 represents a single protein, it is possible to calculate the approximate concentrations of individual glycolytic enzymes in the glycosome (Table 10). The specific enzyme activities presented in Table 9 have enabled us to predict the individual specific enzyme activities in their purified forms (Table 10). When these values are compared with those of the purified glycolytic enzymes from

yeast and mammals, most of them are closely comparable. One *T. brucei* enzyme, the hexokinase, appears, however, significantly different from those of yeast (124) and human (125). While the purified hexokinases from yeast and human have a specific activity of more than 1000 units, *T. brucei* hexokinase has a calculated specific activity of only 32 units in its pure form. This low value may suggest that the *T. brucei* glycosomal hexokinase serves more of a glucose-binding function than an enzymatic function. With an intraglycosomal concentration of 61.2 mg/ml (Table 10) and an estimated subunit molecular weight of 50,000 (Table 6), this enzyme is calculated to have an intraglycosomal subunit concentration of 1.2 mM. Assuming that each dimer binds one glucose molecule, the concentration of hexokinase-bound glucose in the glycosome will be 0.6 mM, not an unreasonable figure in view of the 5 mM glucose concentration in the host blood.

The specific activities of the glycolytic enzymes in pure glycosomes (Table 9) are in excess of those required to maintain the glycolytic flux at 85 nmole of glucose consumed per min/mg of total cellular protein, a value obtained from intact, viable *T. brucei* bloodstream form under optimal aerobic conditions by Fairlamb and Bowman (74). In a living *T. brucei*, the individual glycolytic intermediates have an intracellular concentration of 0.1-1.0 mM (76) of which 20-30% is inside the glycosome (77). Since the glycosome occupies 4.3% of the cellular volume, the intraglycosomal concentrations of glycolytic intermediates ought to be each in the 0.6-6 mM range, which is high enough to sustain a steady rate of glucose consumption at 85 nmol/min/mg of protein. Therefore, there is no real need for further shortening the diffusion time of the substrates by such mechanisms as substrate channeling. The glycosome could simply be a sac of enzyme soup and the rate of glycolysis could still reach the expected level. But some of

TABLE 10

Estimated concentrations and specific activities of the individual glycolytic enzymes in *T. brucei* glycosome

Glycolytic Enzymes	Calculated ^a concentrations in glycosome	Predicted specific ^b activities in pure forms
	mg/ml	micromole/min/mg
Hexokinase	61.2	32
Phosphoglucose isomerase	3.4	498
Phosphofructose kinase	28.6	263
Aldolase	96.2	19
Glyceraldehyde-3-phosphate dehydrogenase	60.9	13
Triose phosphate isomerase	10.2	1,945
Glycerol-3-phosphate dehydrogenase	32.0	85
3-Phosphoglycerate kinase	17.0	207

^aCalculations are based on the data in Table 6 and ref. 97.

^bCalculations from the data in Tables 6 and 9

our present evidence still argues for certain specific physical arrangement among the enzymes. For instance, the glycosomes are lysed by 0.1% Triton X-100 + 0.15 M NaCl and most of its proteins are released into the supernatant (see Fig. 17 and Table 8) except that some substantial amounts of phosphofructose kinase and aldolase are still associated with the pelletable fraction (see Table 8 and Fig. 20). Furthermore, all the glycosomal enzymes can be cross-linked together by either DMSI or DTBP. Since DMSI has an estimated maximal length of 11 Å and DTBP, 11.9 Å (126), the glycolytic enzymes must be closely aggregated with one another that many of their amino groups are within the rather short distance of 11-11.9 Å. On the other hand, however, the estimated intraglycosomal protein concentration of 320-360 mg/ml would represent a 10 mM solution of proteins with 32,000-36,000 molecular weights, which is higher than the 6 mM concentration of cross-linking reagents used in the present experiments. Assuming a 4 nm radius for a spherical 36,000 protein molecule (127), a 10 mM solution of such protein will leave an average intermolecular distance of about 20-25 Å from surface to surface (128), not much longer than the chain lengths of DMSI and DTBP.

Despite the successful cross-linkings, we are still left without a firm conclusion concerning whether or not the glycolytic enzymes are aggregated in a specific manner with specific substrate channelings. Fortunately, all the enzymes retained substantial levels of their original activities in the DMSI-cross-linked enzyme complex. The next chapter describes further investigations on this cross-linked complex. The kinetics of the chain reactions catalysed by the complex has been thoroughly studied in order to verify the possible presence of substrate channeling. It was also anticipated that glucose analogs, which may be recognized as substrates by the early

glycolytic enzymes in the glycosome, could either be converted to phosphorylated products trapped in the channel or accumulated to high concentrations in the glycosome. If the phosphorylated products are capable of inhibiting activities of the next glycolytic enzymes in line, they may exert a relatively powerful blocking effect on glycolysis in African trypanosomes due to the compartmentalization, and thus could be therapeutically useful.

In our effort to purify the glycosomes, we have also discovered a new method of releasing VSG from the bloodstream form of *T. brucei* apparently without damaging the integrity and motility of the parasites by simply freezing and thawing the organisms in the presence of glycerol. This finding indicates the ease in shedding the VSG from *T. brucei*. A similar observation by Reinwald et al (129) indicated that a simple gyratory shaking was enough to remove the VSG from *T. congolense*. Although it is not known whether the VSG shed by freezing and thawing is in its soluble form (20) or membrane form (130), it is likely the former since the membrane structure was not significantly perturbed by its release. We believe that this simple new method carries certain potential for studying the VSG's of African trypanosomes.

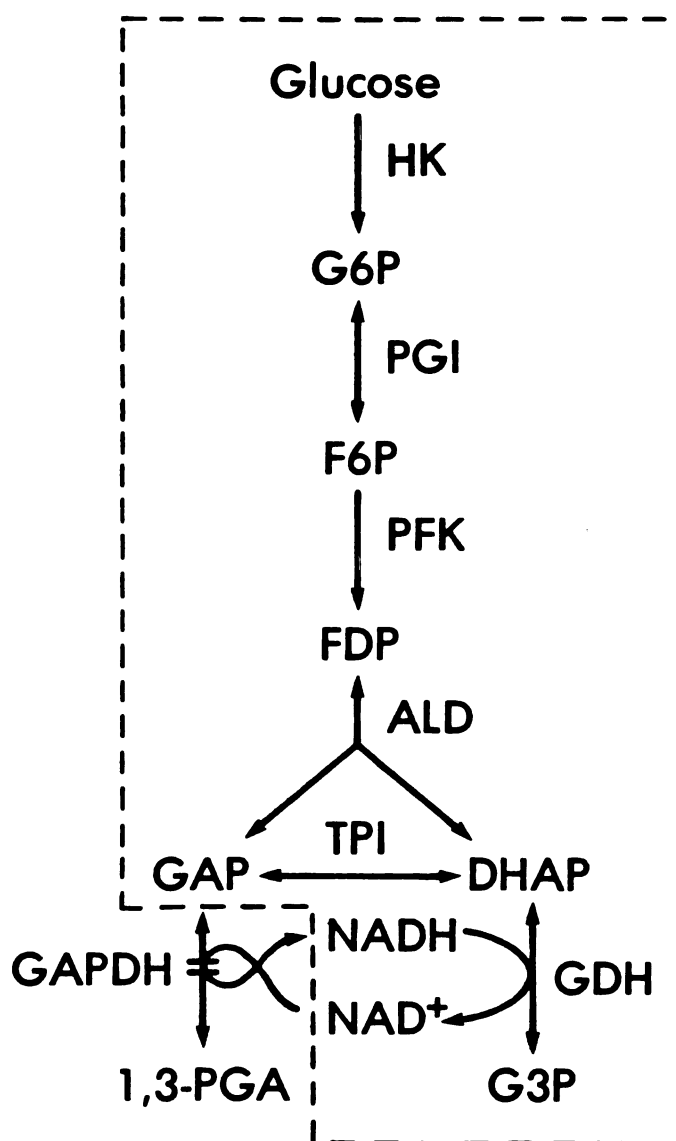
CHAPTER III
ABSENCE OF SUBSTRATE CHANNELING AMONG THE
GLYCOLYTIC ENZYMES IN THE GLYCOSOME OF
TRYPANOSOMA BRUCEI

Introduction

The unique location of the glycolytic enzymes in bloodstream form *Trypanosoma brucei* raises the important question as to what is the purpose of such a compartmentation. Does it play any role in ensuring the high rate of glycolysis in the organism? Membrane delineation of a glycosomal compartment from the rest of the cellular environment may be one way that the parasite can segregate the intermediates generated during glycolysis. Since these intermediates are charged with phosphate groups, impermeability or at the most a limited permeability of these sugar phosphates across the glycosomal membrane would be expected. The resulting high concentrations of these intermediates would be sufficient to drive glycolysis at the observed overall rate of 85 nmol/min/mg protein (74). Our work (133) and that of other investigators (83,98,100) indicates that a higher degree of organization may exist in the glycosome. The functional advantage of molecular compartmentation or enzyme aggregation within the glycosome is not clear but one possibility could be that it serves to promote the high glycolytic flux by 'channeling' of the glycolytic intermediates. In the previous chapter (133), we were able to show that the crosslinking reagent dimethyl suberimidate (DMSI), is capable of crosslinking the glycosomal enzymes in intact glycosomes into one large complex indicating a physical closeness of the enzymes. In this report we investigate the kinetic properties of this complex to address the question as to whether this complex represents an ordered aggregate that efficiently channels intermediates along the glycolytic

pathway. The kinetic profiles of the complex are compared to those of a parallel system composed of the corresponding unaggregated enzymes obtained by detergent and salt solubilization of native glycosomes. We have focussed on the multienzyme- catalyzed chain reaction in which the ATP and NADH dependent conversion of glucose to glycerol-3-phosphate (G3P) is catalyzed sequentially by the six enzymes hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructose kinase (PFK), aldolase (ALD), triosephosphate isomerase (TPI) and glycerol-3-phosphate dehydrogenase (GDH) as shown in Figure 21. For convenience of assay, 1 mM of iodoacetate is included in the assay mixture to inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and prevent a futile cycle of NADH oxidation and NAD reduction. Using this reaction sequence, we have examined spectrophotometrically the rate of NADH oxidation, the generation of radiolabeled intermediates from [^{14}C]-glucose and the competition by unlabelled intermediates in the two parallel systems of crosslinked complex , and solubilized native glycosomes. We further tested availability of in situ generated fructose-1,6-diphosphate (FDP) to exogenous fructose-1,6-diphosphatase (FDPase) and examined the inhibition of the systems by the glucose analog 2-deoxyglucose (2DG).

Figure 21: The multienzyme sequence of reactions catalyzing the conversion of glucose to glycerol-3-phosphate. The sequence of reactions studied is indicated by the boxed area. The cross-bar represents the point of inhibition by iodoacetate. Abbreviations are given in the abbreviations section on page .



Materials and Methods

Materials : Uniformly labeled [^{14}C]-glucose (specific activity of 295 mCi/mmole) and 2-deoxy- ^{14}C -glucose (specific activity of 11.3 mCi/mmole) were from ICN. The rest of the chemicals and enzymes were obtained as described in Chapter II.

Purification and DMSI treatment of glycosomes: The procedures were identical to those described in Chapter II.

Spectrophotometric assay of the multienzyme reaction : The enzymatic conversion of glucose to glycerol-3-phosphate by the six enzyme sequence (Figure 21) was assayed in a buffer containing 100 mM triethanolamine-HCl, 14 mM MgCl_2 , 1 mM iodoacetate, 0.5 mM NADH, 0.8 mM ATP and 0.1% Triton X-100 and maintained at 37° C unless otherwise stated. The reaction was started by adding the substrate and monitored spectrophotometrically in a Beckman DU 7 spectrophotometer by following the oxidation of NADH at 340 nm. In experiments investigating the effects of FDPase on the multienzyme reaction, the concentration of ATP in the reaction mixture was increased from 0.8 mM to 4 mM in order to minimize the effects from limited, nonspecific hydrolysis of ATP by FDPase. Controls in these experiments consisted of incubating FDPase at the highest concentration tested, in the reaction mixture with 4 mM ATP and in the absence of glucose and NADH for 30 minutes at 37° C. Following this period of preincubation, the activity of hexokinase in the reaction mixture was assayed by adding glucose, glucose-6-phosphate dehydrogenase and NADP. The rate of the reaction was not affected whether or not ATP and FDPase were preincubated before assaying for hexokinase. This concentration of ATP thus ensures that any observed effect of FDPase on the multienzyme reaction initiated by glucose is not due to degradation of ATP

by FDPase. In experiments testing effects of 2-deoxyglucose (2DG) on the multienzyme reaction with either glucose (G), glucose-6-phosphate (G6P) or fructose-6-phosphate (F6P) as substrate, 2DG was routinely preincubated in the assay mixture at 37° C for 10 minutes before adding substrate.

Radiometric assay of the multienzyme reaction : Radiometric assays of the conversion of [^{14}C]-glucose to sugar phosphates were carried out in the same reaction mixture as the spectrophotometric assays except where otherwise stated. [^{14}C]-glucose at a specific activity of 0.2 mCi/mmol and a concentration of 0.5 mM was used in a 1.0 ml incubation mixture containing from 10 to 20 micrograms of protein. In competition experiments, unlabeled sugar phosphate intermediates were included in the reaction mixture. After a period of incubation at 37° C, the reaction was stopped by the addition of 50 microlitre of 4 M ice-cold perchloric acid (final concentration of 0.2 M) and chilling the samples on ice for 30 minutes before a brief centrifugation to remove the precipitated proteins and nucleic acids. After adjusting the pH to ca. 5.5 by the addition of 40 microlitre of 4 M NaOH, the samples were again centrifuged to remove sodium perchlorate and then subjected to a routine procedure used in our laboratory (134) to trap the radiolabeled sugar phosphates on PEI-cellulose and the retained radioactivity counted in a Beckman LS-3133T liquid scintillation counter.

HPLC analysis of radiolabeled sugar phosphates: Perchloric acid treated samples (1 ml) as described above were neutralized by the addition of 0.4 ml of a 1 M solution of trioctylamine in Freon (ratio of 1:1.3) instead of NaOH. After 30 minutes on ice, the samples were centrifuged on a tabletop microfuge and the aqueous layer withdrawn. The aqueous layer was filtered by centrifugation through a nylon 66 Rainin filter and then either directly injected into the HPLC column or concentrated by lyophilization before

HPLC analysis. The sugar phosphates were separated on an Ultrasil AX anion exchange column using a phosphate buffer gradient as described by Wang et al (135). Radioactive peaks were detected and quantitated by a Flo-One radioactive detector as described previously (135). Radioactive peaks corresponding to G6P, F6P and FDP were identified by running samples obtained after incubation of [^{14}C]-glucose and cofactors with commercially pure HK; a mixture of HK and PGI; and a mixture of HK, PGI and PFK respectively. The peaks assigned to G6P and F6P were coincident and rarely ever separated. The triose phosphates were not resolved as distinct peaks and eluted off in a spread in the early phase of the run.

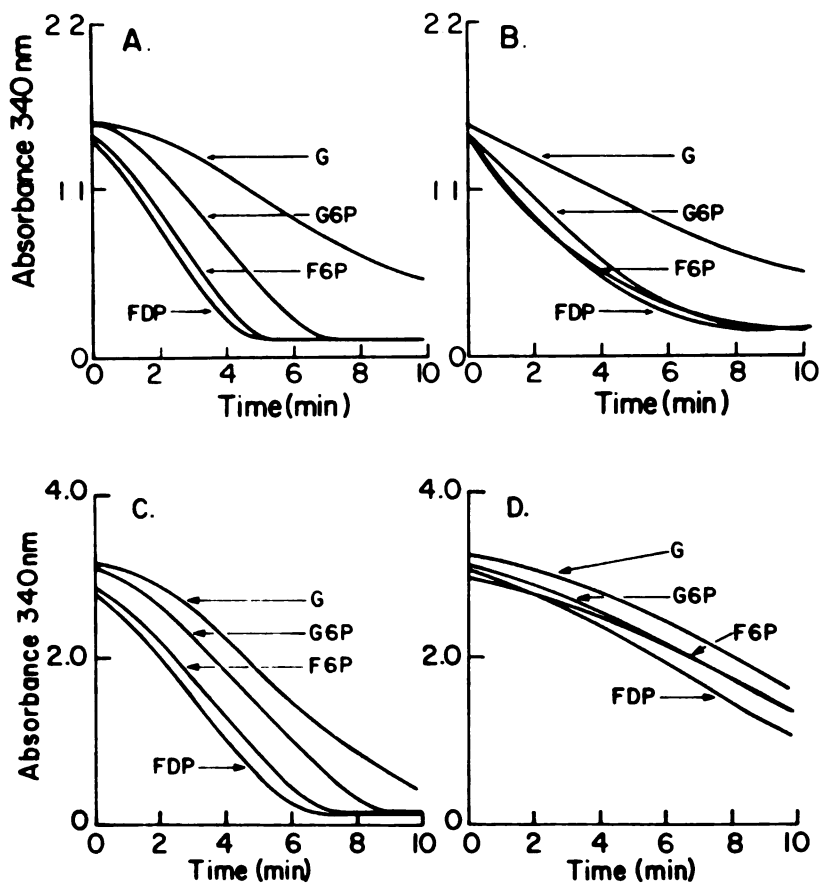
Enzyme assays : All assays of individual glycolytic enzymes were carried out as previously described (133).

Other procedures : Protein was determined by the method of Lowry et al (122). The samples were precipitated with TCA (10% w/v final concentration) and dissolved by boiling in 0.1 M NaOH. Bovine serum albumin fraction V dissolved in 0.1 M NaOH was used as standard.

Results

NADH oxidation by the crosslinked complex : In the previous chapter we have described that despite varying degrees of inactivation of the individual glycolytic enzymes in the DMSI P₂ fraction, the complex contained reasonable activities of most of the enzymes especially the six enzymes involved in the multistep conversion of glucose (G) to glycerol-3-phosphate (G3P). The DMSI P₂ fraction was tested for multienzyme activity by the spectrophotometric method and compared to the activity shown by native glycosomes solubilized by 0.1% Triton X-100 and 0.15 M NaCl, conditions that promote deaggregation of the glycosomal enzymes. Figure 22 shows that the crosslinked complex (Panel B) is capable of catalyzing the multienzyme conversion of glucose to G3P, demonstrating that the crosslinking procedure did not affect the functional relationship of the six enzymes of this reaction. At 20 mM concentration of either G, G6P, F6P or FDP as substrate, both the totally solubilized glycosomes (Panel A) and the crosslinked complex (Panel B) show overall catalytic rates of NADH oxidation that are not significantly different from each other. One major difference is, however, the lack of a lag period associated with the NADH oxidation catalyzed by the crosslinked complex (Panel B), while the same reactions catalyzed by the solubilized glycosomes (Panel A) show a distinct lag period before the maximal rates are attained. For glucose as substrate, the lag phase is of the order of 2 minutes in the deaggregated system and non-detectable for the crosslinked complex. When the substrate concentration is decreased from 20 mM to 0.5 mM (Panels C and D), however, both systems have a similar and distinct lag phase associated with NADH oxidation. Similar lag phases were also present with both enzyme preparations when glucose was added as substrate at 5 mM (data not shown). It appears

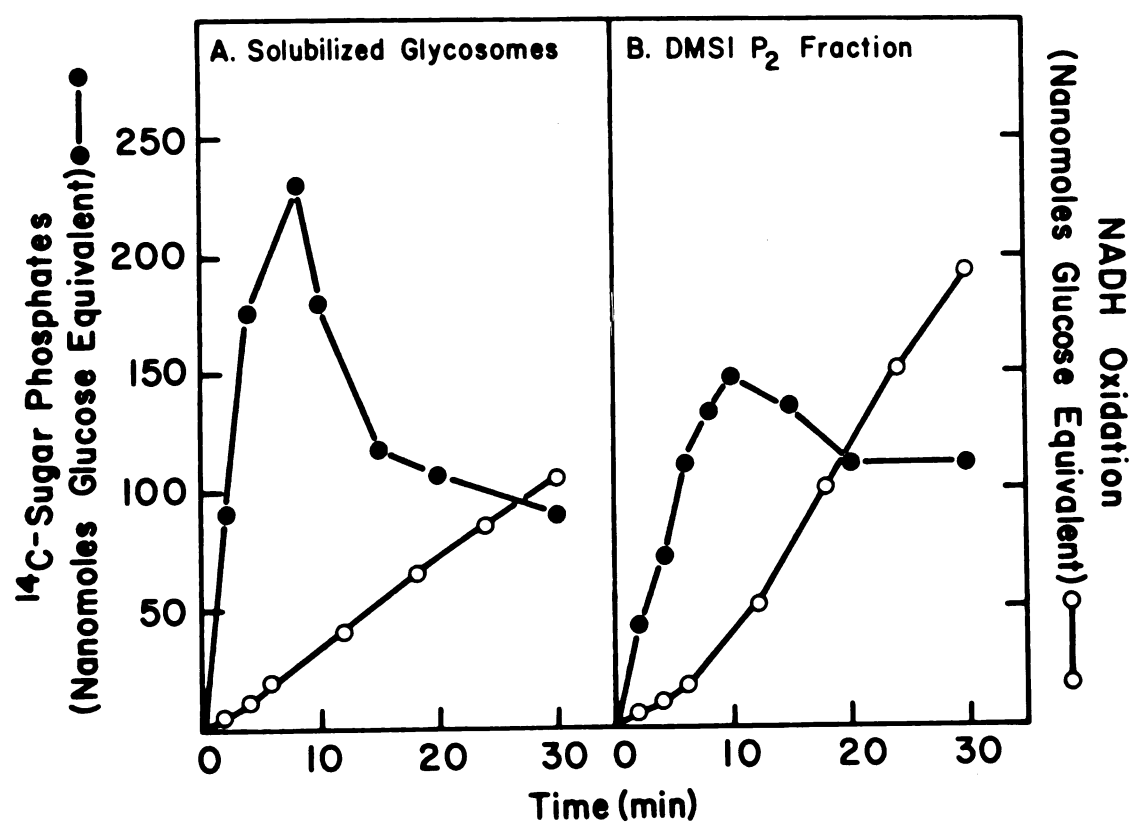
Figure 22: Production of G3P from glucose (G), G6P, F6P and FDP monitored spectrophotometrically by the oxidation of NADH at 340 nm. Panels A and C represent reactions catalyzed by the solubilized glycosome preparation while Panels B and D represent crosslinked complex-catalyzed reactions. 20 mM of each of the substrates and 10 microgram of protein per ml of reaction volume were present in (A) and (B) whereas in (C) and (D), 0.5 mM of each of the substrates and 23 microgram protein per ml of reaction mixture were present. 0.8 mM ATP and 0.5 mM NADH were present.



therefore that at physiologically relevant concentrations of glucose (ca. 5 mM in human blood), the crosslinked complex does not show a reduction of the lag phase over that of the nonaggregated glycosomal enzymes.

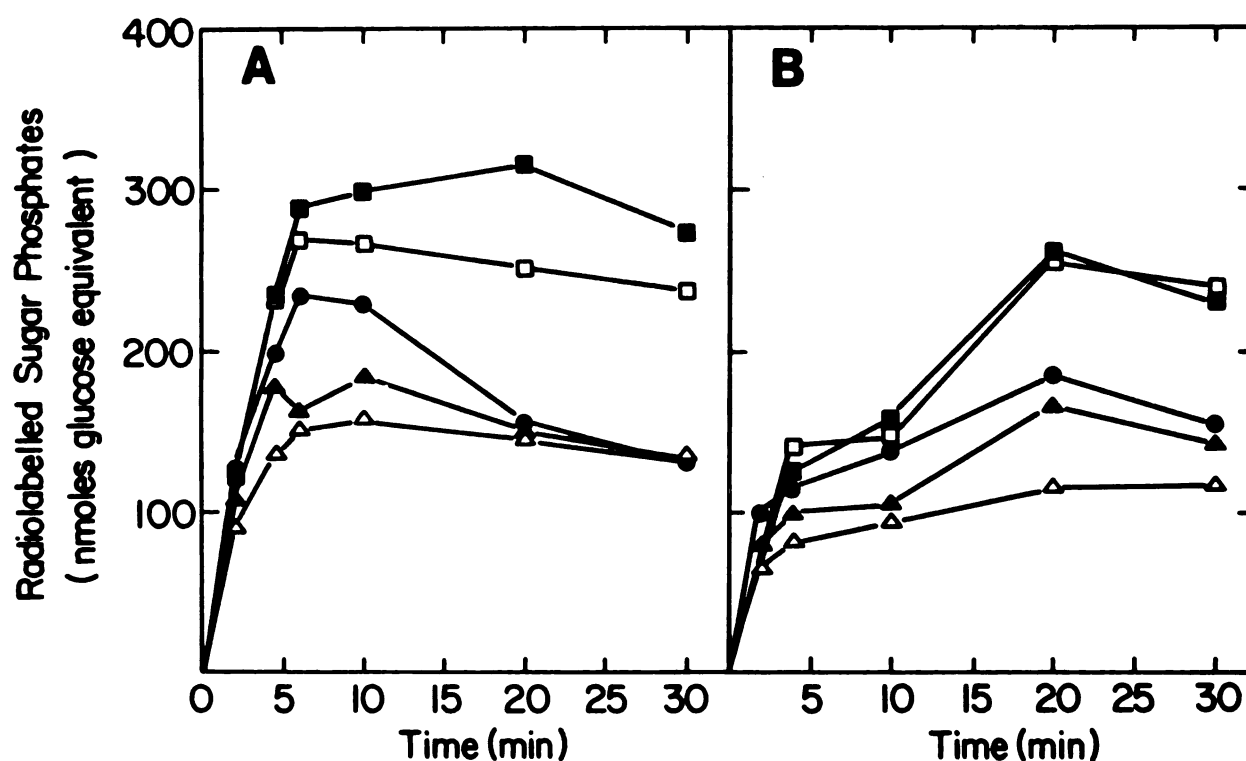
Sugar phosphate accumulation by the crosslinked complex : As a first step to determine the fate of sugar phosphate intermediates generated from [^{14}C]-glucose in the two systems, we carried out the radiometric assay described in 'Materials and Methods' to monitor the total sugar phosphate pool. In Figure 23, the course of the multienzyme reaction with 0.5 mM glucose as substrate was monitored both spectrophotometrically and radiometrically. The rate of NADH oxidation with time shows a profile of initial lag phase for both enzyme preparations as has been indicated before. In the radiometric assay which measures the total sugar phosphate pool derived from glucose (G6P, F6P, FDP, DHAP and G3P), however, a lag phase is not seen in either system (Figure 23). Sugar phosphates appear to accumulate rather rapidly reaching a peak in both systems within the initial 10 minutes before falling off to lower levels. The lack of a lag phase is probably due to the fact that aldolase is the rate-limiting enzyme in this chain reaction (133), and that a rapid accumulation of G6P, F6P and FDP in the initial phase is likely. The falling off of the levels of sugar phosphates coincides roughly with the ending of the lag phase of NADH oxidation and probably represents a dephosphorylation of perhaps some of the intermediates in the reaction. It is unlikely that loss of label is due to conversion of G3P into uncharged glycerol by the reverse reaction of glycerol kinase since the experimental conditions do not favour this reaction. However, it is possible that the action of a phosphatase(s) on the triose phosphates is responsible for the decline in radioactivity.

Figure 23: Dual monitoring of the time course of the multienzyme-catalyzed reaction by spectrophotometric and radiometric assays. 0.5 mM of [^{14}C]-glucose (specific activity of 0.2 mCi/mmol) was present as substrate. 20 microgram of solubilized glycosomes (Panel A) and crosslinked complex (Panel B) were present per ml of reaction mixture. ATP and NADH were present at concentrations of 0.8 mM and 0.5 mM respectively.



Dilution of the labeled sugar phosphate pool formed by the crosslinked complex by unlabeled sugar phosphates : In order to assess whether the sugar phosphate intermediates generated from [^{14}C]-glucose were channeled preferentially in the crosslinked complex, we investigated the effect of exogenously added unlabeled sugar phosphate intermediates on the total labeled sugar phosphate pool (see Figure 24). Solubilized glycosomes (Panel A) and DMSI P₂ fraction (Panel B) were incubated for various lengths of time with 0.5 mM [^{14}C]-glucose in the presence of equimolar concentrations of unlabeled G6P, F6P, FDP and DHAP. Figure 24 shows that the profiles of the two systems are very similar. In both cases, unlabeled G6P and F6P demonstrated suppressive effect on the maximal levels of the radiolabeled sugar phosphates generated. However, FDP and DHAP on the other hand appeared to increase the conversion of [^{14}C]-glucose label into sugar phosphates. This observation was not unique to the free or crosslinked glycosomal enzymes because the same pattern was observed with a mixture of the six commercially pure enzymes (HK, PGI, PFK, ALD, TPI and GDH) adjusted to activity levels similar to those of the corresponding enzymes in solubilized glycosomes or crosslinked complex. Since aldolase is the rate-limiting step, it is possible that added exogenous unlabeled FDP and DHAP will decrease the aldolase action on [^{14}C]-FDP and thus accumulate [^{14}C]-FDP, which in turn will cause increases in G6P and F6P. This possible explanation was tested by adding an excessive amount of pure rabbit muscle aldolase to the crosslinked complex and repeating the same experiment described in Figure 24. The results (not shown), indicated that when aldolase was no longer rate-limiting, the rate of accumulation of [^{14}C]-sugar phosphates was no longer affected by the addition of exogenous

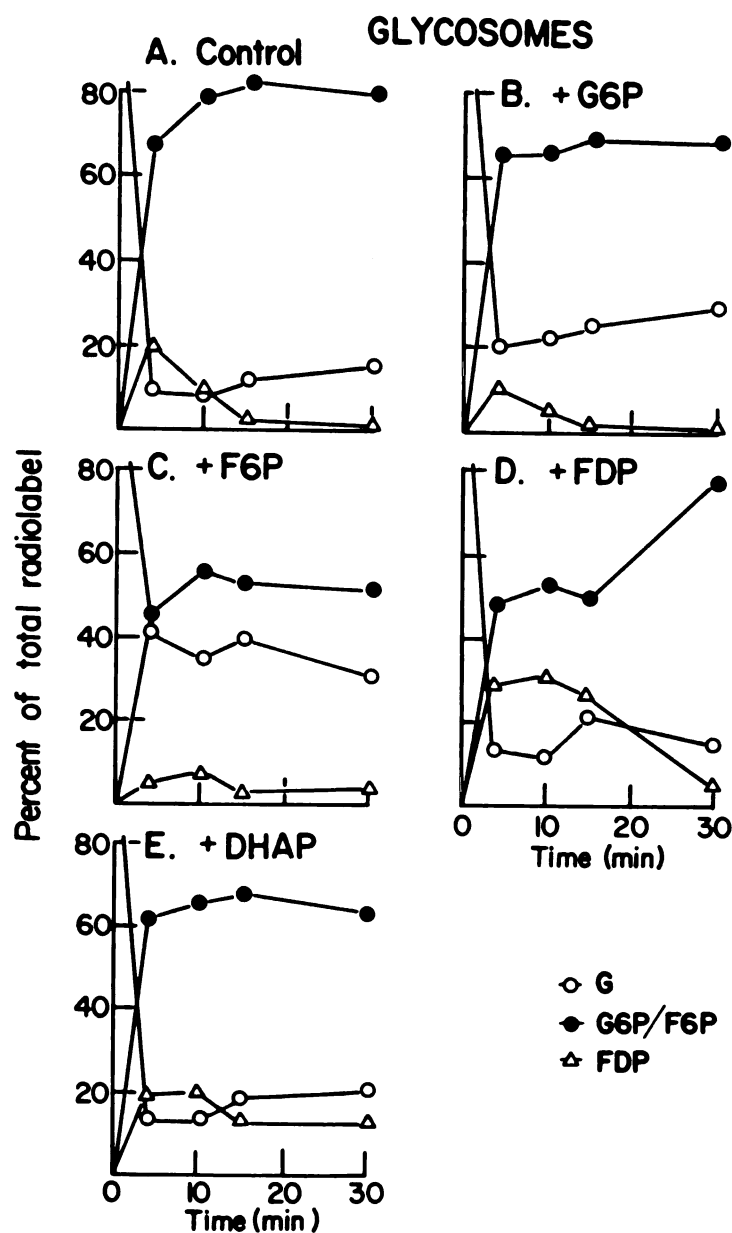
Figure 24: Radiometric assays of the time course of generation of labeled sugar phosphates from 0.5 mM of [^{14}C]-glucose under various conditions. ATP was present at a concentration of 0.8 mM. Reaction mixtures contained equimolar (0.5 mM) unlabeled G6P ($\blacktriangle$); F6P ($\triangle$); FDP ($\blacksquare$); or DHAP ($\square$). Control reactions ($\bullet$) contained only the labeled glucose. 20 microgram of solubilized glycosomes (Panel A) and DMSI-crosslinked complex (Panel B) were present per ml of reaction.



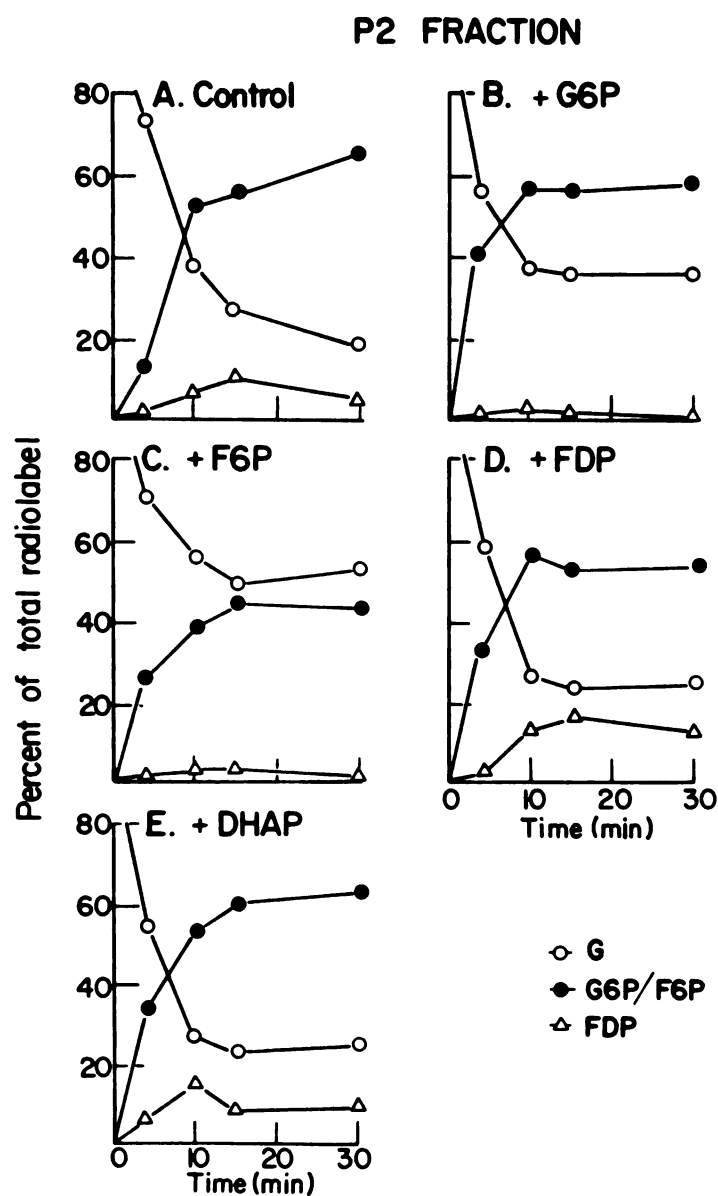
unlabeled G6P, F6P, FDP or DHAP to the crosslinked complex.

An important conclusion drawn from these studies is that under identical conditions, both the crosslinked complex and the solubilized glycosome preparations show similar kinetic profiles in these radioisotope dilution experiments. Levels of the individual radiolabeled sugar phosphates generated by these enzyme systems were then analyzed by a HPLC method that has been useful in our laboratory to separate nucleotides (135). This method gave a good separation of the hexose monophosphates (G6P and F6P) from the hexose diphosphate FDP, but we were unable to achieve resolution of the isomers G6P and F6P. The triose phosphates were very poorly resolved eluting off in a broad peak in the earlier phase of the run. Thus, the various time points in the experiment in Figure 24 were only analyzed for the relative contribution of labeled hexose mono-phosphates, FDP and the remainder of [^{14}C]-glucose as shown in Figure 25 (solubilized glycosomes) and Figure 26 (crosslinked complex). The figures reflect the levels of FDP and the sum levels of G6P and F6P generated from [^{14}C]-glucose in the absence (control) and in the presence of equimolar concentrations of competing unlabeled intermediates. As observed earlier, the suppressive effect of unlabeled G6P and F6P is reflected by the higher levels of unreacted [^{14}C]-glucose and lower levels of [^{14}C]-G6P and [^{14}C]-F6P present. In all cases, the major portion of the label (60-80%) resides in the hexose monophosphate peak which shows a cumulative rise to a plateau. Very low levels of [^{14}C]-FDP were generated from [^{14}C]-glucose in the presence of unlabeled G6P or F6P whereas it accounts for 20% of the total label when unlabeled FDP or DHAP is competing with radiolabeled intermediates. This is consistent with our earlier observation that under the condition of rate-limiting aldolase, addition of unlabeled FDP or DHAP

Figures 25: HPLC analysis of the time course of generation of hexose phosphates from 0.5 mM [^{14}C]-glucose in the absence and the presence of equimolar concentrations of unlabeled intermediates. The conditions of assay are exactly as described in Fig. 24. 20 microgram of solubilized glycosomes was added per ml of reaction volume. Results are expressed as percentages of the total radioactivity eluted off the column. Abbreviations are: G, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate.



Figures 26: HPLC analysis of the time course of generation of hexose phosphates from 0.5 mM [^{14}C]-glucose in the absence and the presence of equimolar concentrations of unlabeled intermediates. The conditions of assay are exactly as described in Fig. 24. 20 microgram of crosslinked complex was added per ml of reaction volume. Results are expressed as percentages of the total radioactivity eluted off the column. Abbreviations are as described in Fig. 25.

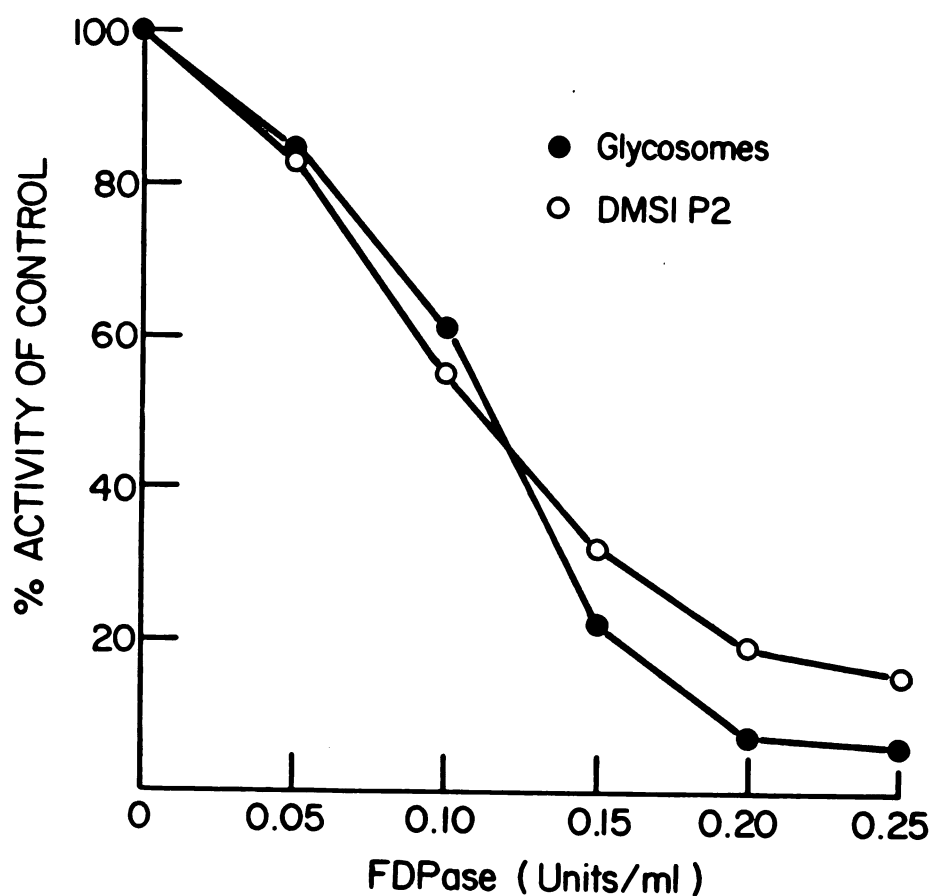


resulted in increased level of [^{14}C]-FDP. Again, these experiments show that there is virtually no difference between the two systems in the competition kinetics.

Effect of FDPase on the crosslinked complex-catalyzed chain reaction : If the multienzyme sequence did indeed exhibit channeling of intermediates, it would be expected that these intermediates would not be released into the general bulk solution but rather remain localized in the immediate vicinity or within the microenvironment of the enzymes. In such a situation, added exogenous enzymes utilizing these intermediates as substrates would not have access to them and would have no effect on the progression of the chain reaction. With this premise in mind, we investigated whether exogenously added FDPase had access to the FDP generated from glucose in the crosslinked complex. Figure 27 demonstrates that the rate of NADH oxidation initiated by glucose is inhibited by increasing concentrations of added FDPase in an identical manner in both the solubilized glycosomes and the crosslinked complex. These results clearly demonstrate that in situ generated FDP is not cryptic or protected from exogenous FDPase and is therefore not channeled.

Effect of 2-deoxyglucose on the crosslinked complex-catalyzed chain reaction: Of a dozen or so sugar and sugar phosphate analogs tested for their ability to inhibit the multienzyme-catalyzed chain reaction, only 2DG showed a significant effect albeit at concentrations several fold higher than that of glucose. In our efforts to verify whether substrate channeling is present in the crosslinked complex, we would like to know if 2DG can be converted to 2DG-6-phosphate (2DG6P) and becomes trapped in the 'channel' to become a more effective blocker of the glucose-initiated NADH oxidation in the complex compared to that in the solubilized glycosomes. Figure 28 shows

Figure 27: Effect of FDPase on the multienzyme-catalyzed conversion of glucose (0.5 mM) to G3P. The reaction in the presence of increasing levels of FDPase and 4 mM ATP was followed spectrophotometrically. Inhibition by FDPase is measured as the difference in the 30 minute levels of NADH between the test reaction and a control reaction not containing FDPase. Results are expressed as percentages of the control value. 20 microgram of protein was present per ml of reaction volume.



the per cent inhibition of the chain reaction leading to NADH oxidation by varying concentrations of 2DG. The reactions were initiated by 0.5 mM of either glucose (Panel A), G6P (Panel B) or F6P (Panel C) in the presence of 8 mM ATP. The percent of inhibition was measured by the difference in the final levels of NADH after a 30 minutes reaction in the absence and presence of 2DG. The results (Figure 28) indicate that with glucose as substrate, 4 mM 2DG caused a 50% inhibition of the reaction catalyzed by solubilized glycosomes and a 25% inhibition of the reaction in the crosslinked complex. With G6P as substrate, 2DG had a weaker inhibitory effect on solubilized glycosomes and even a lesser effect on the crosslinked complex (Panel B). No inhibitory effect by 2DG is observed when F6P (Panel C), FDP or DHAP (not shown) are substrates of the multienzyme reaction. This observed trend of inhibition suggests that the inhibitory effect of 2DG is exerted primarily on hexokinase and to a lesser extent on the phosphoglucose isomerase of the multienzyme sequence. The difference in sensitivity to 2DG between the two multienzyme systems, which is contrary to our original anticipation, could in part be due to a lower rate of production of 2DG6P by the crosslinked complex. This possibility was tested in another experiment and the result presented in Figure 29 shows the time course of production of phosphorylated [^{14}C]-2DG in the two multienzyme systems. Radiolabeled, phosphorylated product in the incubation mixture was analyzed by HPLC as described earlier. Only one radioactive peak other than [^{14}C]-2DG was observed (data not shown). This peak had the same retention time as the product seen when [^{14}C]-2DG was incubated with ATP and commercially available pure hexokinase and was most likely 2DG6P. The initial rate of production of this 2DG6P by solubilized glycosomes is almost twice as fast as that by the crosslinked complex which may thus account for the two-fold

Figure 28: Effect of 2DG on the multienzyme-catalyzed conversion of glucose to G3P. Conditions of assay are as described in 'Materials and Methods' section with ATP present at 8 mM. 2DG and all other assay components were preincubated for 10 minutes before start of the reaction by the addition of either 0.5 mM glucose (Panel A), 0.5 mM G6P (Panel B) or 0.5 mM F6P (Panel C). 20 microgram protein was present per ml of reaction volume.

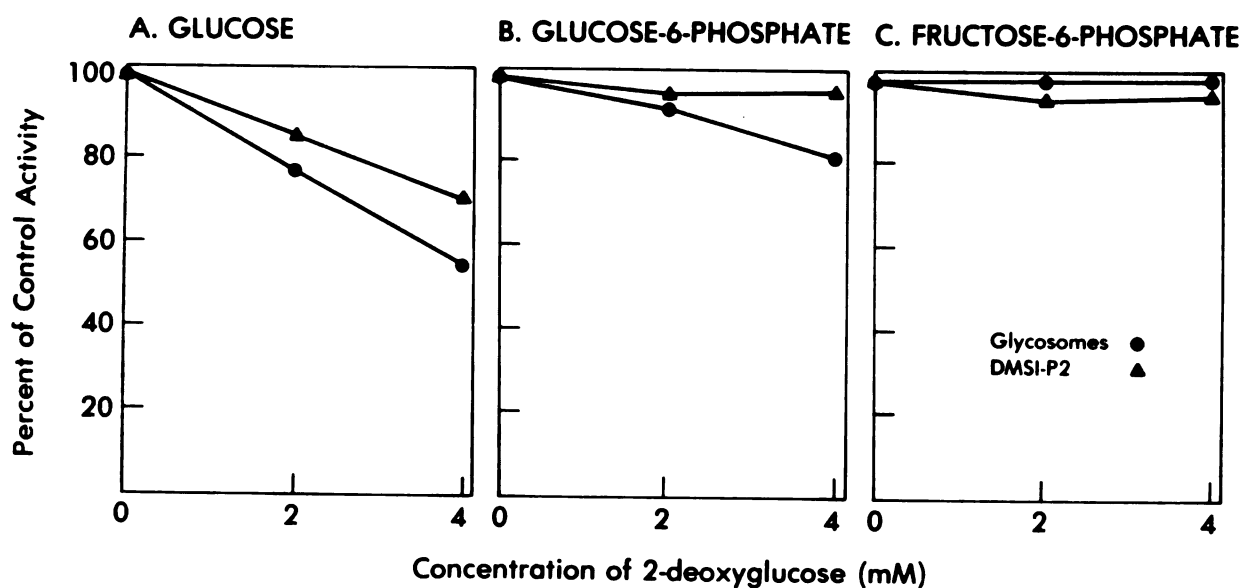
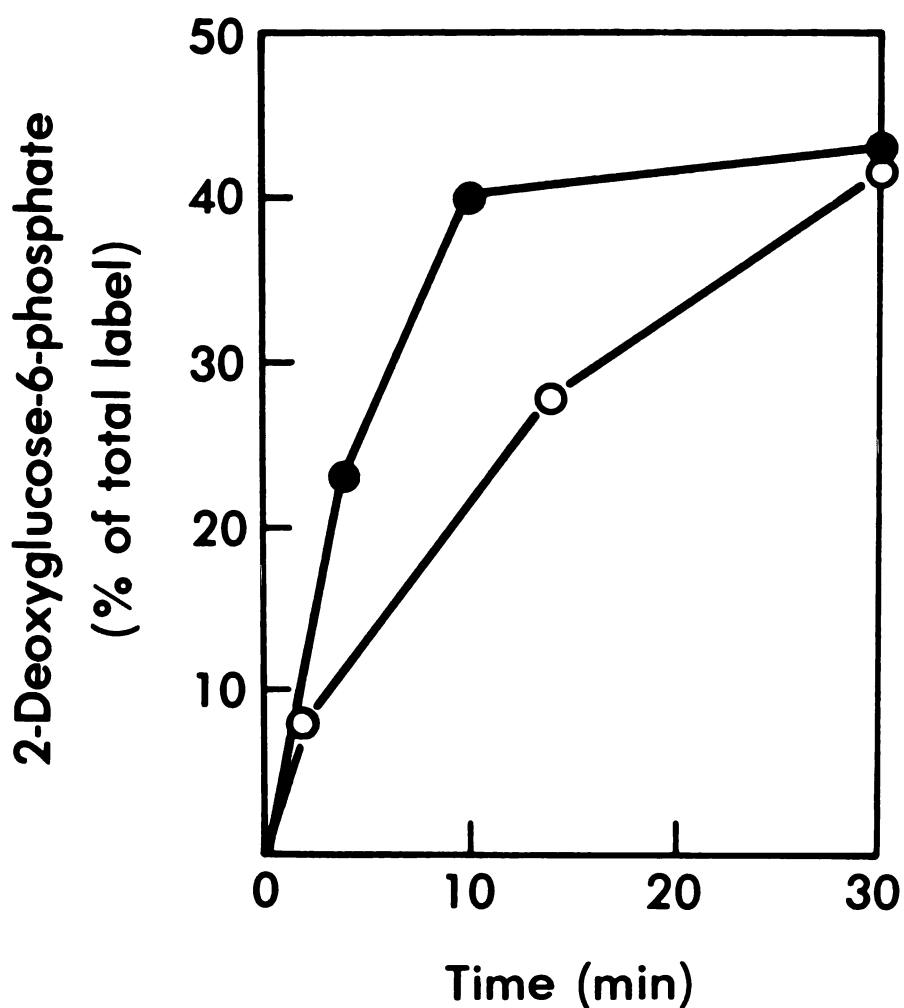


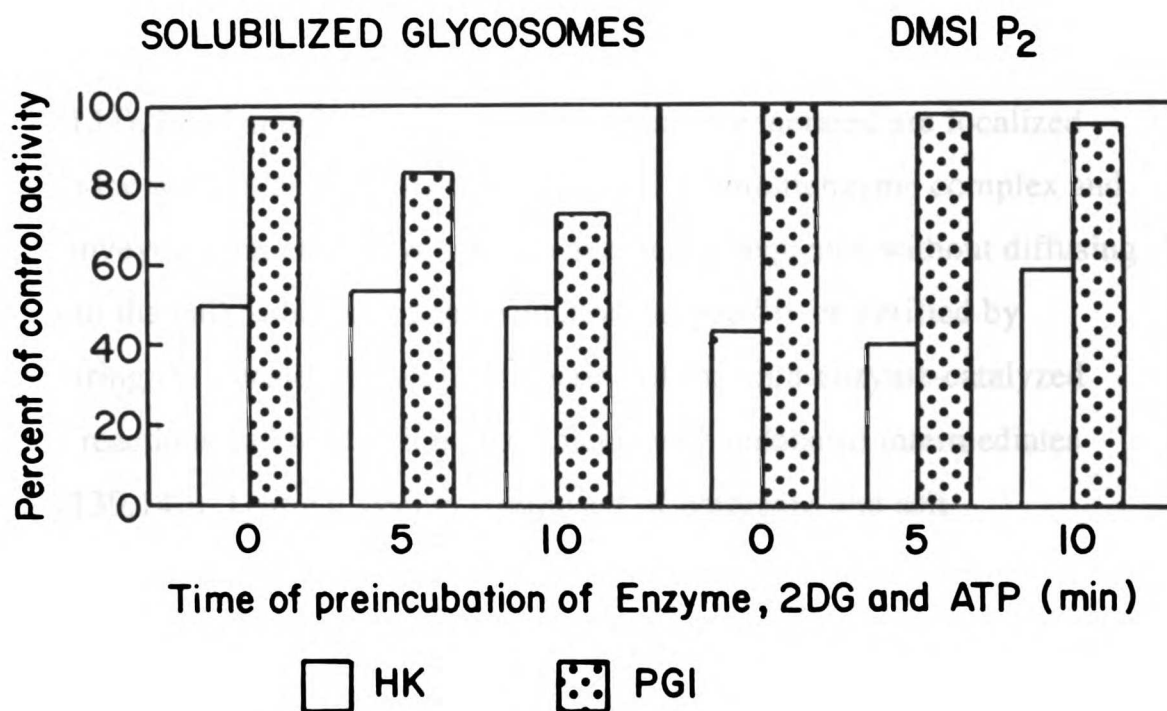
Figure 29: HPLC time course analysis of production of [^{14}C]-2DG6P from [^{14}C]-2DG by solubilized glycosomes (●) and crosslinked complex (○). 0.5 mM [^{14}C]-2DG (specific activity of 0.4 mCi/mmol) was incubated in an assay mixture containing all the components as described in Fig. 24. Results are presented as percentages of the total radioactivity recoverable from the HPLC column in a single run.



higher 2DG inhibition on solubilized glycosomes (Figure 28).

Since both the glucose- and G6P-initiated chain reactions were subject to 2DG inhibition, it became worthwhile to know whether 2DG6P acts as an inhibitor of PGI or both HK and PGI. If the *in situ* produced 2DG6P is contributing to the inhibitory effect observed with 2DG as seems likely from Figure 28, then the different rates of production of this compound may account for the higher sensitivity of the solubilized glycosomes to inhibition. In order to determine whether the inhibitory effect of 2DG observed with glucose as substrate was due to 2DG itself, its product 2DG6P or both, we carried out the following preincubation experiments. Solubilized glycosomes or crosslinked complex was incubated with 5 mM 2DG and 8 mM ATP for various periods of time before the activities of HK and PGI were assayed. It was anticipated that preincubation of the enzymes with 2DG and ATP would result in generation of 2DG6P which would be manifested as a time-dependent inhibition of HK or PGI or both. The results (Figure 30) show that inhibition of HK by 2DG occurs in both the solubilized glycosomes and crosslinked complex, and is independent of the time of preincubation. The inhibition of PGI is more pronounced in the solubilized glycosomes and is dependent on the time of preincubation among 2DG, ATP and the multienzyme systems. Apparently, 2DG inhibits HK whereas 2DG6P is an inhibitor of PGI. Neither 2DG nor 2DG6P has any effect on PFK activity (data not shown).

Figure 30: Effects of 2DG (5 mM) and *in situ* produced 2DG6P on HK and PGI activities in a solubilized glycosomal preparation (Panel A) and in the crosslinked complex (Panel B). 8 mM ATP and 20 micrograms of protein were present per ml of reaction volume. Results are expressed as a percentage of enzyme activities in the absence of 2DG.



Discussion

In the preceeding chapter (133), we demonstrated that treatment of purified intact glycosomes with the crosslinking reagent DMSI results in the formation of a large complex exhibiting all the original enzyme activities in the glycosome. This success was taken as evidence suggestive of a spatial proximity among all the enzymes to form a multienzyme complex.

Multienzyme systems or 'clusters' sometimes possess unique kinetic attributes that allow them to function as more efficient catalysts when compared to the analogous systems composed of free unaggregated enzymes or the theoretical models based on the kinetics of the free enzymes (136). Such comparisons have revealed that reduction of the transient phase often associated with multienzyme reactions is an important kinetic feature of those multienzyme complexes that demonstrate genuine substrate channeling (136,137). In 'channeled' systems, intermediates generated are localized within the molecular confines or 'channel' of the multienzyme complex and are conveyed from one enzyme to the next in the sequence without diffusing out into the bulk solution. Such a situation can readily be verified by measuring the transient phase or lag period of the multienzyme-catalyzed chain reactions and by dilution experiments with unlabeled intermediates (138,139,140). Using a system composed of detergent and salt solubilization and deaggregation of the glycosomal components to represent the mixture of the free enzymes, we have compared the kinetic features of such a system with those of the crosslinked complex. At the relatively high substrate concentrations of 20 mM, the lack of a lag period in the crosslinked complex-catalyzed chain reaction may be due to artificially high concentrations of intermediates trapped in the matrix created by crosslinking the proteins without the presence of true substrate channels. Srere et al (141)

have observed a rate increase in a three enzyme system known to have no substrate channeling by immobilizing the three enzymes on solid matrices which may bear some resemblance to our present observation. At the physiological concentration of glucose in mammalian blood (ca. 5 mM), no apparent reduction in the lag period of the crosslinked complex-catalyzed reaction over that of the free enzymes-catalyzed reaction was observed and thus suggests the absence of substrate channeling. Hammond et al (81) have carried out a more thorough analysis of the transient phase in the two multienzyme systems since then, and have further indicated the absence of a reduction of the lag phase in the crosslinked complex. The isotope dilution experiments described in Figures 24 through 26 show very similar kinetic behaviours of the free and crosslinked multienzyme systems. Channeling of hexose phosphate intermediates would have been detected in the HPLC analysis of the competition experiments as differences in the overall kinetics of intermediates accumulation and as low steady state levels of the intermediates in the crosslinked complex-catalyzed reaction, both of which were simply not observed. To make sure the control system of solubilized glycosomes represented a true mixture of free enzymes, we carried out similar experiments with a carefully constructed mixture of the six commercially available pure enzymes involved in the chain reaction and obtained essentially the same kinetic results as those from the solubilized glycosomes. The similar sensitivity of the two multienzyme system-catalyzed NADH oxidations to exogenous FDPase is another strong indication of the ready availability of the intermediate FDP to the general bulk solution. FDP must be freely released into the solution by the crosslinked complex.

2DG was found to inhibit the multienzyme-catalyzed reaction although rather high concentrations of the compound, five to ten fold higher than that

of the substrate, was required to cause any appreciable effect. Its inhibitory effect is exerted primarily on HK. It is phosphorylated by this enzyme to form a product which is most likely 2DG6P. This product is an inhibitor of the next enzyme PGI. Enzymes further down the sequence are not affected by 2DG or 2DG6P. The lack of a substantial inhibitory effect by in situ produced 2DG6P in the crosslinked complex, albeit present at a relatively low level, suggests that this dead end product is not trapped in a channel to have its inhibitory effect amplified.

Association of enzymes of a biochemical pathway into a multienzyme unit may serve to achieve one or more of several goals (136,137). Advantages of such molecular organizations in the kinetic sense are usually reflected in the form of rapid attainment of the overall rate, increased efficiency and enhanced catalytic rate or simply segregation of biochemical pathways or labile intermediates. In the case of *T. brucei* glycosome, the presence of the membrane barrier alone could serve to accomplish most of these conditions. For instance, impermeability of phosphorylated intermediates across the glycosomal membrane would result in high local concentrations of these intermediates in the glycosome. We have calculated (133) from data in the literature that the concentration of these intermediates in the glycosome may be as high as 6 mM. Given the rather high activities and concentrations of the glycolytic enzymes inside the glycosome (133), such concentrations of intermediates would be more than adequate to drive glycolysis at rates several fold higher than that required to ensure the observed glycolytic flux in live trypanosomes. Furthermore, calculations based on the estimated protein concentrations in the glycosome (97) indicate an average intermolecular distance of 20-25 Å between the surfaces of spherical protein subunits of an average size of 36,000 daltons (133). This

distance is not much larger than the 11 Å molecular stretch of DMSI such that the glycosomal proteins could be crosslinked with relative ease. Thus the observed crosslinking by DMSI may not necessarily be due to specific interactions among the glycosomal enzymes but rather a nonspecific effect arising from the volume constraint and hence crowding in the glycosome.

In summary then, several observations noted in this investigation are consistent with the absence of channeling of the glycolytic intermediates by the crosslinked complex. That any one of the intermediates can initiate the multienzyme-catalyzed chain reaction at rates higher than that with glucose as substrate (Figure 22) illustrates the easy accessibility of these intermediates to their respective enzymes. This fact is further demonstrated by the ability of unlabeled intermediates to compete with radiolabeled intermediates generated from [^{14}C]-glucose, and the equal inhibitory effect of FDPase on the chain reaction catalyzed by the two multienzyme systems. Should a channeled system exist, production of a dead end inhibitor such as 2DG6P within the channel would be expected to have a much enhanced inhibitory effect on the overall reaction rate due to its confinement within the 'channel' to a relatively high local concentration. The fact that such an enhanced inhibition by 2DG was not observed in the crosslinked complex has provided further evidence that substrate channels do not exist among the glycolytic enzymes involved in the conversion of glucose to G3P in the glycosome of *T. brucei*.

CHAPTER IV

SUBGLYCOSOMAL ORGANIZATION OF THE PROTEINS IN THE GLYCOSOMES OF BLOODSTREAM FORM AND CULTURE FORM *TRYPANOSOMA BRUCEI* WITH SPECIAL REFERENCE TO THE INTEGRAL GLYCOSOMAL MEMBRANE PROTEINS

Introduction

The glycosome is of vital importance in the survival of the bloodstream form stage of African trypanosomes (73). From the viewpoint of comparative biochemistry, this organelle is certainly an attractive target for rational chemotherapy and a fundamental understanding of its organization and functions is essential in the pursuit of this goal. Recent studies on the purification, ultrastructure, enzyme content and metabolic functions of the glycosome (90,95,97,133) have yielded valuable information but there is a paucity of information as to the sub-glycosomal location of the various proteins and the protein composition of the glycosomal membrane. Because the glycosomal membrane clearly plays a vital role in interfacing with the cytoplasm, an understanding of its components and properties is important for a variety of reasons:

- i. Studies on the glycosomal membrane might help in gaining a better understanding of the mode of functioning of the organelle and how it achieves selective permeability to some of the phosphorylated glycolytic intermediates. A good example of this would be the glycerol-3-phosphate : dihydroxyacetone shuttle operational during aerobic glycolysis of bloodstream form African trypanosomes and postulated to involve translocator systems on the glycosomal membrane (64).
- ii. From the cell biology point of view, the biogenesis of the organelle is of intrinsic importance and an understanding of the composition of the

glycosomal membrane is pertinent to the question of the intracellular assembly of the organelle and whether it exists as a separate intracellular compartment or arises by budding or vesicle formation from other intracellular membranes such as those of the endoplasmic reticulum. A comparative analysis of the protein composition of intracellular membranes (142) of the organism should provide clues to this question. Nothing is known about the biogenesis of the glycosome. It might follow the same biogenic pathway as other microbodies such as peroxisomes and glyoxysomes in which most, or all protein components are synthesized at free ribosomes and are imported post-translationally in the growing organelle (143). It would therefore be interesting to find out whether the glycosomal membrane contains proteins that may be involved in translocation of those glycosomal proteins that have to cross the membrane to reach their final destination in the organelle. Possible roles of such membrane proteins could be in the recognition and/or processing of precursor or mature glycosomal proteins synthesized at free ribosomes in the cytosol.

iii. During transformation of *T. brucei* from bloodstream-stage forms to culture or insect stage forms, several metabolic changes occur (84) including change of intracellular location of phosphoglycerate kinase from within the glycosome to the cytoplasm (85,90) and the appearance of phosphoenolpyruvate carboxykinase (93) and malate dehydrogenase (84) within the culture form glycosomes. It would be interesting to know at what level within the glycosome these changes take place and whether the reshuffling of the glycosomal contents during transformation involves changes in the glycosomal membrane composition.

iv. In addition to the preponderant glycolytic enzymes, the glycosomes also contain other enzymes which include adenylate kinase (84,94), orotate

phosphoribosyl transferase and orotidine-5-phosphate decarboxylase (91), hypoxanthine-guanine phosphoribosyl transferase (81), enzymes of alkoxypospholipid biosynthesis (95) and insect-stage specific phosphoenolpyruvate carboxykinase (93) and malate dehydrogenase (84). Although apparently distinct in their enzyme complement from peroxisomes and glyoxysomes of higher eukaryotes, it is probable that glycosomes may bear more than a morphological resemblance those organelles. A relatedness of these organelles is suggested by recent findings of the presence of enzymes of plasmalogen (ether-lipids) biosynthesis in *T. brucei* culture form glycosomes (95) and an enzyme involved in the beta-oxidation of fatty acids in the glycosomes of several species of *Leishmania* (89). Studies on the glycosomal membrane components along with the unravelling of the full metabolic potential of the glycosome would help to establish the evolutionary pathway of these organelles.

We have reported in an earlier chapter (Chapter II) on the major protein components of purified glycosomes from bloodstream form *T. brucei* (133). In this chapter we present investigations on the sub-glycosomal organization of these proteins in purified glycosomes from both bloodstream form and culture form of the organism. For this purpose, we developed a method for the purification of glycosomes from culture form procyclic trypomastigotes of *T. brucei* that has enabled us to make direct comparisons of the glycosomal components of culture form and bloodstream form glycosomes. Employing several criteria, we present evidence showing that the glycosomal membrane of organelles from the bloodstream form organism contains only two major integral membrane proteins that are also present in the membranes of glycosomes from the culture form organism.

Materials and Methods

Materials: DEAE-cellulose (DE-52) was from Whatman. Nycodenz was obtained from Accurate Chemical and Scientific Corporation. High quality sucrose, electrophoretic reagents and SDS-PAGE molecular weight markers were purchased from Bio-Rad Laboratories. Nitrocellulose paper was a product of Sartorius. Peroxidase-conjugated concanavalin A was obtained from Sigma Chemical Company. Enzymes, substrates, cofactors as well as all other chemicals and reagents were of the highest purity commercially available.

Isolation of long slender bloodstream forms of *T. brucei* and purification of glycosomes: All the procedures are the same as described in Chapter II, except that glycosomes were purified from the post-large granule supernatant by two slightly different procedures. One method employed the sequential Percoll and sucrose gradient centrifugation procedure described earlier (97,133). In a second procedure, the post-large granule supernatant was centrifuged at 33,000 x g (22,000 rpm in a Beckman SW-28 rotor) for 25 min at 4° C to obtain a glycosome-enriched pellet. This pellet was resuspended in TEDS buffer pH 7.8 and applied onto a linear 15-55% (w/w) sucrose gradient containing 25 mM Tris pH 7.2 and 1 mM EDTA. The gradients were centrifuged at 170,000 x g (49,000 rpm) in a Beckman VTi 50 rotor for 60 min. A slow acceleration and deceleration program was employed (the rotor was accelerated from 0 to 500 rpm over 6 min and the reverse for deceleration). The glycosomal band appearing about two-thirds of the way down the tube was collected into a syringe by puncturing the side of the tube, diluted threefold in TEDS buffer pH 7.8 and centrifuged at 33,000 x g for 25 min. The pellet was resuspended in a small volume of TEDS buffer pH 7.8 and layered onto a second sucrose gradient (15-55%

(w/w)) which was then centrifuged as before for 90 min. Again the glycosomal band was collected, diluted threefold in TEDS buffer pH 7.8 and the glycosomes pelleted by centrifugation at 33,000 x g for 25 min. The purified glycosomal pellet was resuspended in 1-2 ml of TEDS buffer pH 7.8 and either stored in aliquots at -80° C or used immediately for further experiments.

Cultivation of culture form parasites: Insect stage procyclic trypomastigotes of *T. brucei* stock EATRO 110 were grown in a semi-defined liquid medium (144) at 26° C with slight modifications. Eagle's MEM powder (Gibco) at 7 g/litre and MEM 199 (Gibco) powder (with Earle's salts, with glutamine and without sodium bicarbonate) at 2 g/litre were used as the equivalents of MEM F-14 powder (Gibco) and MEM 199 TC 45 powder (Wellcome) respectively (144). The concentration of sodium bicarbonate in the medium was reduced from 2 g/litre to 0.6 g/litre to achieve better growth. The medium was supplemented with 10% heat inactivated dialyzed fetal bovine serum. A total of 4 litres of culture was grown each time in several half-filled 150 cm² Falcon flasks in the absence of antibiotics or antimycotics. Cells were harvested during late logarithmic phase of growth, i.e. at cell densities of about 1.5×10^7 to 2×10^7 /ml by a 10 min centrifugation at 3,500 rpm in a GS-3 Sorvall rotor.

Purification of glycosomes from culture form parasites: The cells were washed twice in TEDS buffer (25 mM Tris pH 7.8, 1 mM EDTA, 1 mM DTT and 250 mM sucrose) by resuspension and centrifugation at 3000 rpm in a Sorvall SS-34 rotor at 4° C. The pellet was homogenized with silicon carbide and a post-large granule supernatant obtained as described previously (133). Glycosomes were purified from this post-large granule supernatant by three different density gradient centrifugation protocols. The first method

consisted of sequential Percoll and sucrose gradient centrifugations essentially as described for purifying the organelles from bloodstream form parasites (90,95). In the other methods described below, a glycosome-enriched pellet was first obtained by a further centrifugation of the post-large granule supernatant at 33,000 x g for 25 min at 4° C (22,000 rpm in a Beckman SW-28 rotor). The pellet was resuspended in an appropriate buffer and layered onto the gradients described below. In one method, the iodinated density medium Nycodenz was employed. A linear gradient of 10%-40% (w/v) Nycodenz in 5 mM Tris buffer pH 7.5 containing 0.3 mM EDTA (calcium disodium salt), 3 mM KCl and 220 mM sucrose (TEPS) was prepared by underlayering 7 ml each of a 10%, 20%, 30%, and 40% solution in that order in a Quick Seal tube and then allowing the step gradient to diffuse to linearity by laying the tube horizontally on its side for 1 hr at 4° C. The 33K x g pellet resuspended in TEPS was then layered on the top of the gradient and the tubes centrifuged in a Beckman VTi 50 rotor at 170,000 x g (49,000 rpm) for 90 min at 4° C. Two faint bands at lighter density and a dense band near the bottom of the tube were collected. The dense band, identified as the glycosomal band by its hexokinase activity, was diluted threefold in TEDS buffer pH 7.8 and centrifuged at 33,000 x g for 25 min. The pelleted glycosomes were resuspended in a small volume (1-2 ml) of TEDS and either used immediately or stored frozen in small aliquots at -80° C. In yet another method, a suspension of the 33K x g pellet in TEDS was layered onto a linear 15%-55% (w/w) sucrose gradient (in 25 mM Tris pH 7.2 containing 1 mM EDTA) and the gradient centrifuged at 170,000 x g for 60 min in the VTi 50 rotor. The glycosomal band equilibrating at a position about two-thirds of the distance from the top of the tube was collected, diluted threefold in TEDS and centrifuged at 33,000 x g for 25 min. The

pellet was resuspended in a minimum volume of TEDS, applied onto a second 15%-55% sucrose gradient and the gradient centrifuged as before for 90 min. The glycosomal band from this second sucrose gradient was washed by threefold dilution in TEDS and centrifugation as before. The purified glycosomes were resuspended in a small volume of TEDS and either used immediately for other experiments or aliquoted and stored frozen at -80° C until later use.

Electron microscopy: The organelles were fixed for 1 hr at 4° C in a 2.5% (v/v) solution of glutaraldehyde in 100 mM sodium cacodylate buffer pH 7.5 containing 0.25 M sucrose. The fixed material was pelleted in a microfuge, washed twice in cacodylate buffer and post-fixed with osmium tetroxide. After embedding in Epon 812, thin sections cut on a Sorval MT2B ultramicrotome were stained in uranyl acetate and lead citrate and examined in a JEOL-100S electron microscope at 80 KV. Membrane samples were prepared in the same manner except that sucrose was excluded from the initial fixing buffer. Live bloodstream form and procyclic organisms were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer pH 7.5 for 1 hr and processed for electron microscopy as described by Cross (20).

Triton X-114 phase separation: A 2% (v/v) solution of Triton X-114 in a Tris-glycine buffer (3mM Tris, 15 mM glycine) pH 8.6 was kept at 37° C overnight in a water bath. The resulting two phases were separated by centrifugation at 5000 rpm (SW-28 Beckman rotor) for 25 min at 37° C. The detergent-enriched lower layer was adjusted to the original volume with cold Tris-glycine buffer and the solution warmed for 30 min for clouding to occur. The detergent-rich lower layer was obtained by centrifugation as before and the procedure of resuspension, clouding and separation repeated two more times. The final Triton X-114 rich layer was used as the 11.4%

stock solution (145). For phase separation of glycosomal proteins, a 1.0 % Triton X-114 solution was prepared from the 11.4% stock by dilution in a 25 mM Tris, 1 mM EDTA, 1 mM DTT buffer (TED) of pH 7.2 containing 150 mM NaCl and 1.25 mg/ml of the protease inhibitor leupeptin. To a 100 microlitre sample of bloodstream or culture form glycosomes (ca. 5 mg/ml) was added 400 microlitre of the 1 % Triton X-114 solution and the suspension solubilized by homogenization at 4° C in a hand-held Potter-Elvehjem homogenizer (20 strokes). The sample was left at 4° C for 20 min and then subjected to one cycle of freezing (in a dry ice/ethanol bath) and thawing before centrifuging the sample at 4° C. The supernatant, containing the solubilized proteins was used for phase separation by warming in a 37° C heating block for 10 min for clouding to occur. The phases were separated by centrifugation at 37° C in a microfuge and the supernatant carefully drawn out. The supernatant was made to 1 % in Triton X-114 and the phase separation repeated once more. The combined Triton-rich sediments and the final aqueous supernatant were both examined by SDS-PAGE.

Salt treatment of intact bloodstream form glycosomes: To a 100 microlitre of bloodstream form glycosomes in TEDS buffer (ca. 5 mg/ml) was added an equal volume of either TEDS buffer (control), TEDS buffer containing 30 mM sodium pyrophosphate (PP_i) or TEDS buffer containing 400 mM MgCl_2 (all solutions added to the glycosomes had been adjusted to pH 7.8). The three suspensions were incubated on ice for 30 min following which the control reaction was diluted with 8 ml TEDS buffer and the other two test reactions with 8 ml of TEDS containing either 15 mM of sodium pyrophosphate or 200 mM MgCl_2 . The solutions were centrifuged at 33,000 x g (16,500 rpm in the SW-41 Beckman rotor) for 25 min at 4° C. Each

pellet was then resuspended to a volume of 200 microlitre and analyzed for enzyme activity and by SDS-PAGE.

Protease treatment of intact bloodstream form glycosomes: Glycosomes (in TEDS) were resuspended in three volumes of 25 mM Tris, 250 mM sucrose pH 7.8 (TS buffer) and centrifuged at 33,000 x g for 25 min to wash out most of the EDTA present in the sample. The glycosomal pellet was resuspended in fresh TS buffer to give a final protein concentration of ca. 0.5 mg/ml. To 1.5 ml of the glycosomal suspension was added either 5 microlitre of a 0.1 mg/ml pronase solution corresponding to 0.04 units of enzyme (pronase reaction) or 10 microlitre of a 5 microgram/ml solution of trypsin corresponding to 0.55 BAEE units of enzyme (trypsin reaction). A control 1.5 ml sample received no addition. All three samples were incubated in a 37° C water bath for 30 min following which 8 ml of ice-cold TEDS was added to each tube and centrifuged at 33,000 x g for 20 min at 4° C. The final pellets were resuspended in 200 microlitre of TEDS and assayed for enzyme activities as well as examined by SDS-PAGE.

Test for endogenous protease in bloodstream form glycosomes: A glycosomal sample was solubilized in 0.1% (v/v) Triton X-100 and 150 mM NaCl. The sample was split into two equal portions and to one was added one fifth its volume of a solution of antiproteases (0.2 mg/ml leupeptin, 0.5 mg/ml crude soybean trypsin inhibitor, 0.5 mg/ml aprotinin, 10 mM 1,10-phenanthroline, 10 mM benzamidine and 0.5 mM PMSF all at final concentration) while the other sample received an equivalent volume of buffer. The samples were incubated in a 37° C water bath for either 10 or 20 min after which an equal volume of SDS-PAGE sample preparation buffer (2x) was added to each sample. The samples were analyzed by SDS-PAGE.

Preparation of glycosomal membrane: Membranes from bloodstream form and culture form glycosomes were prepared by the sodium carbonate procedure described by Fujiku et al (146). Briefly, a 1 ml sample of glycosomes was added to 100 ml of ice-cold 100 mM Na_2CO_3 pH 11.5.

The diluted sample was left on ice for 30 min and then centrifuged at 42,000 rpm in a Beckman Type 60 rotor for one hour at 4° C. The membrane pellet was resuspended to the original sample volume (1 ml) in TEDS buffer. To the supernatant was added sufficient solid Tris base to give a final concentration of 50 mM and the pH adjusted to 7.8. The solution was then dialyzed and concentrated to the original sample volume (1 ml) in a Pro-Dicon apparatus against 5 litres of a 25 mM Tris, 1 mM EDTA, 1 mM DTT buffer pH 7.8. Both the membrane and matrix fractions were analyzed for enzyme activity and by SDS-PAGE. Glycosome samples were also subjected to hypotonic osmotic shock by diluting a sample 100-fold in a 5 mM Tris buffer pH 7.4 containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 0.05 mM leupeptin and leaving the sample on ice for 30 min. A portion of this osmotically-shocked solution was then further treated with 200 mM MgCl_2 and sonicated briefly (five 3-sec. pulses at 40% duty and output of 2). After another 30 min on ice, both samples were centrifuged at 42,000 rpm for 1 hr. Pelleted fractions were analyzed by SDS-PAGE.

Glycosomal membranes were also prepared by subjecting the glycosomes to six cycles of freezing and thawing. 100 microlitre of glycosome sample was diluted 10-fold in distilled water and frozen by immersion in a dry ice-ethanol mixture. The sample was thawed by placing the microfuge tube under a stream of hot tap-water and the cycle was repeated three more times. At this point, solid sodium chloride was dissolved in the thawed sample to give a final concentration of 0.15 M and the sample put through two more

cycles of freezing and thawing. The membrane fraction was pelleted by centrifugation and washed twice in 0.15 M NaCl. Both the supernatant and the pellet fractions were analyzed by SDS-PAGE.

Test for glycoproteins: Glycosylation of the glycosomal components was tested by two different methods. In one method, SDS-PAGE gels of glycosomal proteins were stained by the periodic-acid Schiff (PAS) reaction (see Appendix) with VSG 118 and ovalbumin as positive controls. In the second method, glycosomal proteins were separated by SDS-PAGE and transferred onto nitrocellulose paper according to the method of Towbin et al (147) (electroblotted at 60 V for 1 hr in a Bio-Rad Transblot apparatus). The transferred proteins were reacted with peroxidase-conjugated concanavalin-A and reactive bands visualized by the chromogenic oxidation of 1,4-chloronaphthol. Positive controls were VSG 118 and ovalbumin.

SDS-PAGE: Denaturing electrophoresis was according to the method of Laemmli (119) as described earlier (133). Nine or 12% gels were used and they were stained with Coomassie Blue R-250. Wet destained gels were scanned as described earlier (133).

Enzyme assays: Most enzyme assays were conducted spectrophotometrically as described earlier (133). Phosphoenol pyruvate carboxykinase (PEPCK) was assayed according to Oppenheimer and Cottem (93). Reactions were terminated by adding HCl (final conc. 1 M) and an aliquot of the reaction mixture were dried onto a circular (2.5 cm) Whatman filter paper in a scintillation vial with a blow drier in the fume-hood. Radioactivity retained on the filters was counted in a liquid scintillation counter. All enzyme assays were at 37° C unless otherwise stated.

Protein assay: Protein determination was by the method of Lowry et al (122) with bovine serum albumin as standard. Interfering substances were

eliminated by precipitating protein with trichloroacetic acid and dissolving the pellet by boiling in 0.1 M NaOH. Bovine serum albumin was also dissolved in 0.1 M NaOH.

Results

Purification of bloodstream form glycosomes: As reported earlier, bloodstream form glycosomes can be purified to almost total homogeneity employing sequential Percoll and sucrose gradient centrifugation steps (97,133). However the Percoll particles (colloidal silica coated with polyvinylpyrrolidone) are tenacious and persist in the glycosomal preparation even after extensive washing as is evident from electron microscopic examination of the final preparation (see Fig. 37 A). The presence of these particles was found to complicate the results obtained when glycosomes were fractionated to prepare the membrane fraction (see below). An alternative method that circumvented the use of Percoll for the purification of bloodstream form glycosomes was therefore developed. This procedure involved two identical sequential sucrose gradients and was adopted for all membrane preparation studies. Bloodstream form glycosomes prepared by this procedure when examined by electron microscopy (results not shown) were found to be morphologically identical to glycosomes prepared by the Percoll-sucrose procedure. Table 11 shows that the specific activities of the various glycosomal enzymes were very similar in both types of preparations indicating the same degree of purity of organelles by both procedures. The protein profile of glycosomes prepared by this modified method when analyzed by SDS-PAGE (Fig. 31) was also essentially identical to that of glycosomes prepared by the original method of sequential Percoll and sucrose gradient centrifugation with the exception that an additional protein band (marked by an arrowhead in Fig. 31) was present in glycosomes prepared by the new procedure.

Purification of culture form glycosomes: When the sequential Percoll and sucrose gradient centrifugation method was used to purify glycosomes from

culture form *T. brucei*, it became apparent that it was not the most suitable method. To begin with, the Percoll gradient step did not result in a clear separation of the mitochondrial (including other cellular components) and glycosomal fractions as two clearly separated bands as was observed during purification of glycosomes from bloodstream form parasites by the same method (118). Instead only one broad band was observed at the usual light density position of banding of mitochondria and other cellular components (unresolved lysosomes, flagellar pocket membranes and plasma membranes) of bloodstream form preparations in this gradient (118). The denser portion of this band was collected and identified as enriched in glycosomes by its phosphofructose kinase and glycerol-3-phosphate dehydrogenase activities. Subsequent sucrose gradient centrifugation of this fraction resulted in a broad band equilibrating at the normal position for glycosomes in this gradient. Fig. 32 A shows the fine structure of the glycosomal preparation obtained following this Percoll-sucrose procedure. It is evident that the preparation is not homogeneous and contains membranous and flagellar fragments in addition to the somewhat distorted and empty looking glycosomes. The poor degree of purification of glycosomes by this procedure is also reflected in the low relative specific activities of the various glycosomal enzymes (see Table 12). SDS-PAGE analysis of the preparation revealed a profile that contained several of the bloodstream form glycosomal proteins and a few rather major additional bands (see below) (Fig. 33).

In an effort to develop a better method of purifying culture form glycosomes, we used gradients made with either the iodinated density medium Nycodenz, or sucrose. Centrifugation of the post-large granule supernatant in a 10–40% (w/v) Nycodenz gradient resulted in separation of three distinct bands, the densest of which was the glycosomal fraction (see

'Materials and Methods' section). The appearance under the electron microscope of the purified glycosomes obtained from this gradient is shown in Fig 32 B. Although still somewhat distorted and empty in appearance, this procedure results in a glycosomal preparation with a higher degree of homogeneity than the Percoll-sucrose procedure. This improvement in purification is reflected both in the SDS-PAGE profile (Fig. 33) and in the higher values of the relative specific activities of the enzymes in the glycosomal preparation (Table 12). However, the use of two sequential and identical sucrose gradients was found to produce the best glycosome preparation under the circumstances as judged by the degree of homogeneity and the appearance of the organelles (see Fig. 32 C). While the structure and the shape of the organelles appears to have been retained by this procedure of purification, the preparation is still not totally homogeneous and contains few other larger undefined structures. The accumulation of electron-dense material towards one pole of the organelle appears to be an artefact caused by the purification procedure since in intact organisms (*in situ*), the entire lumen or matrix of the organelle is occupied by this electron opaque material (see Fig. 32 D). This displacement of the granular material is not very surprising in view of the fact that the purified organelles are found to have increased in diameter by twofold when compared to their original size in fixed intact cells (0.43 micron compared to 0.18 micron). It appears as though the organelles are at various stages of swelling and it may well be possible that the larger nondescript structures observed are overswollen organelles. Both the Nycodenz method and the double sucrose procedure result in a similar degree of purity of the organelles judging by the specific activities of the various glycosomal enzymes in the two preparations (Table 12) and their SDS-PAGE profiles (Fig. 33). The empty-looking appearance and distortion of

Nycodenz gradient-purified glycosomes (Fig. 32 B) does not indicate a relative loss of cisternal contents since the SDS-PAGE profile of this preparation is almost identical to that of the sucrose gradient-purified organelles (Fig. 33). In fact, the Nycodenz-prepared organelles appear to be more enriched in certain polypeptides namely band C (M_r 67.2 kD) and band D (M_r 64.2 kD). However since the double sucrose procedure results in organelles that retain much of their original shape and features, this method was routinely used for purifying glycosomes used in this study.

Comparison of glycosomes from bloodstream form and culture form parasites: The specific activities of a number of glycosomal enzymes in bloodstream and culture form glycosomes are compared in Table 13. The last column in this table gives the ratio of bloodstream form to culture form glycosomes, both prepared by the double sucrose procedure. It is evident that transformation from bloodstream form to culture form (equivalent to insect mid-gut form) is accompanied by a reduction in the activities of most of the glycolytic enzymes in accord with the changed ambient metabolic environment. Disregarding the high value for phosphoglycerate kinase arising due to the fact that this enzyme is absent from culture form glycosomes, hexokinase appears to be the enzyme most reduced in activity. The activities of the three enzymes glyceraldehyde-3-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase and triose phosphate isomerase are not very different between glycosomes of these two life stages. Hart et al (90) also noted little change with transformation in the activities of the last two of these enzymes as well as that of glycerol kinase. If we use the enzyme activity value for glycerol kinase from glycosomes purified by the Percoll-sucrose procedure as a basis of comparison, then our results are agreeable with those of these investigators. Since this enzyme is also very

labile, it is probable that the absence or low activity of the enzyme in the glycosomes obtained after the Nycodenz and double sucrose gradient procedures is due to inactivation of the enzyme. Both malate dehydrogenase and phosphoenolpyruvate carboxykinase are present only in the glycosomes of culture form parasites as has been reported by other investigators (84,90,93). We observe an extremely high activity of malate dehydrogenase which is about 75-fold that reported by Hart et al (90). Fig 34 shows the SDS-PAGE profile of glycosomes from the two different life stage forms of the parasite. Glycosomes from the culture form of the parasite contain several new proteins that are absent in the organelles of bloodstream form of the parasite. These additional bands have been designated with alphabetical letters to distinguish them from the originally described major proteins seen in bloodstream form glycosomes (133). Bands A (94.4 kD), B (80.4 kD), D (64.2 kD) and F (34.7 kD) are all unique to culture form glycosomes and comprise about 18 % of the total glycosomal protein (see Table 14). Bloodstream form glycosomes prepared by the Percoll-sucrose procedure do not contain protein band C (67.2 kD) but this band is present in these glycosomes when prepared by the sucrose-sucrose procedure (see also Fig. 31). Culture form glycosomes also contain this band. A protein band of M_r 56.2 kD and designated E is present in bloodstream glycosomes as a minor protein that migrates very close to band 1 (PGI). In culture form glycosomes, this protein (band E) is present as one of the major proteins (compare lanes 6 and 7 of Fig. 33 which shows better resolution in this region). This reciprocal switch in the intensities of these two polypeptides (band 1 and band E) with transformation is also evident from the SDS-PAGE data of Hart et al (90). The only bloodstream-stage glycosomal proteins apparently absent (or present to a minor extent) in culture form

glycosomes are bands 4 (PGK) and 8 (unidentified). Densitometric scans of the SDS-PAGE profiles have enabled us to obtain quantitative information on the various proteins as shown in Table 14 . Other than band 1 (PGI) and band 2 (PFK) whose relative contribution to the total glycosomal protein decreases by about twofold with transformation, the changes in the other proteins seem to parallel the changes observed in the corresponding enzyme activities. For instance, both bands 3 (HK) and 5 (ALD) show a severalfold decrease in culture form glycosomes whereas bands 6 (GAPDH), 7 (GDH) and 9 (TPI) hardly decrease in agreement with the unchanged enzymatic activities between the two forms of glycosomes. The decreased role of glycolysis in the overall metabolic function of the glycosomes of culture form organisms is reflected in the decreased contribution to the total glycosomal protein content of the enzymes phosphoglucose isomerase, hexokinase, phosphofructose kinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase and triose phosphate isomerase which make up about 35% of the glycosomal protein compared to an 87% contribution in bloodstream form glycosomes (including phosphoglycerate kinase). It is interesting to note that the relative contribution to the total glycosomal protein of band 10 increases almost twofold in culture form glycosomes. We shall come back to this particular protein in later discussion.

Salt treatment of bloodstream form glycosomes: The presence of peripheral membrane proteins associated with the cytoplasmic surface of the glycosomal membrane (external periheral membrane proteins) was investigated by subjecting intact glycosomes (prepared by the Percoll-sucrose procedure) to high salt concentrations. $MgCl_2$ has been shown to effectively dislodge peripheral membrane proteins from membrane preparations (148,149)

whereas sodium pyrrophosphate (15mM) has been found to be effective in stripping ribosome particles from microsomes of the rough endoplasmic reticulum (150). None of these procedures however was found to selectively remove any of the major glycosomal proteins as shown in Fig 35 A (70% of the initial protein was recovered in the control as well as in salt-treated samples). On a qualitative basis, glycosomes exposed to these salt solutions do not appear to lose any of the eleven major protein bands when compared to the control glycosomes. Protein content determinations as well as densitometric scans of the gel in Fig. 35 A show that there is also no quantitative change when compared to the control glycosome preparation. In line with this observation, no loss of activities of various glycosomal enzymes was seen after these treatments. Thus by this criterion, it appears that none of the major glycosomal proteins is a peripheral membrane protein associated with the cytoplasmic surface of the glycosomal membrane.

Protease treatment of intact bloodstream form glycosomes: Exposure of intact glycosomes (prepared by the Percoll-sucrose procedure) under iso-osmotic conditions to the proteases trypsin and pronase was used as another test for external peripheral membrane proteins and also as a test for integral membrane proteins with an exposed cytoplasmic or ecto-domain. Assuming that the integrity of the organelles has not been breached, only those proteins totally or partially exposed to the cytoplasm should be susceptible to digestion by these enzymes. In a control experiment whereby detergent solubilized glycosomes were exposed to these proteases, it was noted that all the major glycosomal proteins were either totally or partially digested by these enzymes and showed up as low molecular weight proteins in SDS-PAGE (results not shown). The results in Fig. 35 B indicate that trypsin tested on intact glycosomes did not affect most of the major glycosomal

bands except bands 10 and 11 (marked by arrowheads) which were reduced in intensity when compared to the control untreated glycosome preparation. The susceptibility of these two bands to trypsin has been demonstrated in several different preparations. Pronase on the other hand seemed to affect most of the glycosomal proteins as judged by their reduced intensities (25 to 50% reduction in most enzymes as assessed from densitometric scans) when compared to the bands in the untreated glycosomal sample. It is noteworthy that aldolase (band 5) has been cleaved by pronase into a slightly faster migrating species (both species marked by arrows in Fig. 35 B). We have found that this cleavage of aldolase occurs in glycosomes even under normal circumstances and especially in lysed organelles (see species marked by arrows in Fig. 31).. Misset and Oppendoorn (100) have also observed the same phenomenon and it may be responsible for the tendency of the enzyme to become inactivated as has been observed repeatedly by us. The decreased intensities of several of the enzymes in pronase-treated glycosomes does not appear to correlate well with the enzymatic activities in such preparations except for aldolase and phosphoglucose isomerase (see Table 15). It is not certain whether this means that cleaved products remain catalytically active or some inhibitory restraint in control glycosomes is removed by pronase treatment. It is interesting to note that both bands 10 and 11 are again totally eliminated in pronase treated glycosomes. Although it is difficult to draw conclusions as to the location of the various glycosomal proteins from the results of this experiment especially in view of the almost universal effect of pronase, both the salt and trypsin-treatment results are in support of the absence of external peripheral proteins. Thus the widespread effect of pronase most likely results from its entry into the glycosome.

The results from several trypsin treatment experiments consistently show that bands 10 and 11 are readily susceptible to this enzyme. When taken in conjunction with the pronase treatment results, the indications are that these two protein bands could very well be integral membrane proteins with a cytoplasmic domain cleavable by proteases. In view of this interpretation then, an explanation for the generalized effect of pronase could be that it is much more effective than trypsin (at the concentrations used here) in totally digesting these two membrane proteins resulting in the formation of pores that permit entry of the enzyme into the glycosomal lumen.

Presence of an endogenous protease in bloodstream form glycosomes:

During the course of our studies on glycosomes, we observed that when glycosomes were solubilized in 0.1% Triton X-100 and subsequently fractionated, there was a tendency for some of the glycosomal proteins, most notably bands 10 and 11, to disappear from the ensuing fractions as judged by SDS-PAGE analysis. In order to confirm our suspicion of the presence of an endogenous glycosomal protease that becomes activated on lysis of the organelle, we tested the effect of incubating detergent lysed glycosomes at 37° C for 20 min in the absence and presence of protease inhibitors.

Preparations so treated were then examined by SDS-PAGE to determine whether there was any change in the normal profile of glycosomal proteins. The results in Fig. 35 C show that in the absence of protease inhibitors, there is a complete loss of almost exclusively bands 10 and 11 (marked by arrowheads). The inclusion of a mixture of protease inhibitors prevents the loss of these two proteins. Intact glycosomes incubated under similar iso-osmotic conditions and in the absence of protease inhibitors show the complete profile of normal glycosomes. These results demonstrate that the glycosome contains a dormant protease which becomes activated when the

organelle is lysed and the action of this protease is directed almost specifically against bands 10 and 11 of the glycosome. The specificity of this protease is not known but it could well be a cysteine protease since it has recently been shown that proteases in long slender bloodstream forms of *T. brucei* almost exclusively belong to this class (151). In this context it is interesting to note that aldolase has been found to be inactivated by cleavage due to the action of a protease which can be inhibited by the serine protease inhibitor, leupeptin (100). Whether this aldolase-cleaving protease and the endogenous protease described here are one and the same remains to be established. An alternative possibility is that bands 10 and 11 are both proteases themselves and perform autodigestion upon lysis of the glycosome.

Integral membrane proteins of the glycosomal membrane: Glycosomal membranes were prepared from purified glycosomes by the sodium carbonate procedure of Fujiku et al (146). We began by using bloodstream form glycosomes prepared by the Percoll-sucrose procedure to obtain the purified membrane fraction. SDS-PAGE analysis of such a membrane fraction revealed that it contained mostly four protein bands corresponding to band 3 (HK), band 6 (GAPDH) and bands 10 and 11 (Fig. 36 A). Comparision with the SDS-PAGE profile of the corresponding matrix fraction showed that the two smaller protein bands (10 and 11) were almost exclusively present in the membrane fraction whereas the presence of band 3 in this fraction, although substantial, was not total. Only a small proportion of band 6 was associated with the membrane fraction. During several such experiments with bloodstream form glycosomes from different batches (prepared by the Percoll-sucrose procedure), we observed that the exclusive presence of bands 10 and 11 in the membrane fraction was highly

reproducible whereas the presence of the other two bands was variable and occasionally additional bands would also appear in the membrane fraction. Electron microscopic examination of this membrane fraction showed vesicular membrane structures entrapped in an amorphous mass of Percoll particles (Fig. 37 B). All attempts to separate the membranes from the Percoll particles including centrifugation in a 25-55 % (w/w) sucrose gradient met with no success. To overcome this problem, we circumvented the use of Percoll and developed a double sucrose gradient procedure to purify the organelles. Electron microscopic examination of the membranes prepared from this sample by sodium carbonate treatment (146), revealed membrane structures in the form of sealed vesicles (see Fig 37 C). The trilaminar appearance of the membranes is clearly evident (see inset). Fig 36 B shows the SDS-PAGE results of such a membrane preparation. As expected, the membrane contains only two proteins corresponding to bands 10 and 11. The fact that the other proteins observed in the membrane fraction of glycosomes prepared by the Percoll-sucrose procedure are absent from this preparation is suggestive of a strong interaction between these proteins and the Percoll particles. A quantitative comparison of the SDS-PAGE profiles of the membrane and matrix fractions shows that whereas band 10 is exclusively membrane-associated, a small proportion of band 11 is still present in the matrix fraction. The function of these two proteins remains unknown. Attempts to test, in the membrane fraction, for several glycosomal enzymes (adenylate kinase, orotate phosphoribosyl transferase and hypoxanthine-guanine phosphoribosyl transferase) found no activity presumably because of the destructive effect of sodium carbonate on protein structures. Even the exhaustively dialysed and concentrated matrix fraction, obtained after sodium carbonate treatment, failed to show most of the

expected glycolytic enzyme activities. Interestingly, the two isomerases (phosphoglucose isomerase and triose phosphate isomerase) were least affected by this procedure and showed 20% and 10% respectively, of the original activities.

To explore whether changes in the glycosomal membrane protein composition occur during transformation of the organism from the bloodstream form to the culture form, we carried out similar membrane fractionation studies on purified culture form glycosomes. Fig. 37 D shows the appearance under the electron microscope of this membrane fraction. As in bloodstream form glycosomal membrane preparations, sealed membrane vesicles with a well defined trilaminar appearance are seen. An important difference in this preparation however is the presence of patches of electron dense material similar to the granular material seen in the lumen of the intact organelles. Bloodstream form glycosomes have also been noted to contain electron opaque luminal or core material but these structures apparently do not withstand the sodium carbonate procedure since they are not present in the resulting membrane fraction. This is in agreement with the observation of Fujiku et al (142) who noted that the crystalloid core of rat liver peroxisomes which contains urate oxidase is dissolved when these organelles are subjected to the sodium carbonate procedure as evidenced by a quantitative release of this enzyme into the supernatant or matrix fraction. The persistence of the granular material in membrane fractions from culture form glycosomes can only mean that the structure of this material is different from that in bloodstream form glycosomes and it is not totally dissolved during the sodium carbonate treatment. The results in Fig. 36 B show that in addition to bands 10 and 11, two other protein bands (band B and band 6) are now associated with the procyclic glycosomal membrane. In light of the presence

of electron dense granules in the membrane preparation, it becomes difficult to draw conclusions as to whether the additional protein bands seen in the culture form glycosomal membrane fraction are indeed integral membrane proteins or are simply associated with the co-sedimented granular material. But the presence of the two protein bands similar to bands 10 and 11 of the bloodstream form glycosomal membrane strongly suggests that these two proteins are integral membrane proteins common to both bloodstream form and procyclic form glycosome.

Further evidence that bands 10 and 11 are the only integral membrane proteins of bloodstream form glycosomes comes from analysis of the matrix and supernatant fractions following exposure of these organelles to hypotonic shock. As shown in Fig. 38 A , osmotic shock of the glycosomes results in partial release of all the major glycosomal proteins into the supernatant fraction with the exception of bands 10 and 11 which remain in the pelletable fraction. Sonication of osmotically-shocked bloodstream form glycosomes in the presence of 200 mM $MgCl_2$ was found to yield a pellet fraction that contained mainly bands 10 and 11 and greatly reduced amounts of the other glycosomal proteins (Fig. 38 A, lane 3). The total absence of some of the glycosomal proteins (59.6 kD, 29.2 kD and 27.9 kD) in the pellet fraction indicates that this procedure breaks totally the glycosomes. Rupture of these organelles can also be achieved by several cycles of freezing and thawing. Fig 38 B shows the protein distribution in the supernatant and pellet fractions after subjecting glycosomes (both bloodstream and culture forms) to six cycles of freezing and thawing. Bands 10 and 11 were again found in the membrane (pellet) fraction. A few other proteins were also present in this fraction despite the inclusion of 0.15 M NaCl in the last two cycles of freezing and thawing. Nonetheless, the major

proportion of most of the glycosomal proteins are found in the supernatant fraction.

Triton X-114 phase separation of bloodstream form and culture form

glycosomal proteins: Triton X-114 phase distribution has been used to study integral membrane proteins in several systems (145,152). This detergent at low dilutions has a cloud point of ca 20° C. At or above this temperature, the micellar solution undergoes secondary association into larger micelle aggregates resulting in a clouding of the solution. The cloudy solution can be separated into a detergent-rich lower layer and a detergent-depleted upper phase by centrifugation at or above the cloud point. Integral membrane proteins of protein samples dissolved in Triton X-114 tend to associate with the detergent rich pellet (as a result of their hydrophobic nature) when subjected to phase separation. We examined the distribution of glycosomal proteins (bloodstream form and culture form) in the two Triton X-114 phases, and found both types of glycosomes poorly solubilized by this detergent at the concentration used (1% v/v at 4° C) after extensive homogenization on ice (about 30 strokes in a hand-held homogenizer) and a freeze-thaw cycle. The solubilized material was used for phase separation. Fig. 39 shows the results of such an experiment. The streaky migration of proteins in lanes 2 and 6 results from the presence of a high concentration of detergent in these samples which is difficult to get rid of (even after TCA precipitation of the diluted fractions). The appearance of band 10 in these fractions suggests a hydrophobic nature of the protein and supports the previous indication that this protein is an integral glycosomal membrane protein.

Test for the presence of glycoproteins in the glycosome: The PAS staining technique failed to stain any of the glycosomal proteins both in bloodstream

form and culture form preparations. Since the positive controls were stained by this procedure, this result suggests the absence of glycosylated proteins in the glycosome. The use of enzyme-coupled concanavalin A, for specific detection of glycoproteins containing D-glucose and D-mannose residues was also tried. The results proved negative in the case of the culture form glycosomes. In bloodstream form glycosomes however, the method resulted in the staining of two bands at M_r 64 kD and 58 kD both of which are not readily discernible in the Coomassie blue-stained gels (Fig. 40). Like the control glycoprotein, VSG 118, these two bands stain strongly and are not due to background nonspecific staining (Fig. 40). We have shown previously that the variant surface glycoprotein (VSG) of bloodstream form organisms (absent in culture form organisms), tends to contaminate glycosomes purified from these organisms. Although the method we reported earlier (133) greatly reduces the amount of VSG present in the glycosomal preparations, its removal may not be total. The positive bands seen with the concanavalin A-coupled reaction therefore could represent VSG and a breakdown product (the breakdown product comigrates with purified VSG 118). In view of this possible explanation, our results may indicate that none of the major glycosomal proteins are glycosylated.

Discussion

Glycosomes from cultured procyclic trypomastigotes of *T. brucei* have been isolated and studied by other workers (90,95). In isolating the organelles, these investigators employed a sequential Percoll and sucrose density centrifugation procedure that has been successfully used in purifying the organelles from the bloodstream form of the parasite. In our hands, we found this method to be unsuitable for purifying glycosomes from culture form parasites. Unlike the situation with bloodstream form preparations, the procedure does not yield a morphologically homogeneous preparation of organelles and there is a substantial contamination with other cellular components in the final preparation. This difficulty may account for the lack of any published accounts on the morphological appearance of isolated culture form glycosomes of *T. brucei*. We have investigated alternate procedures for purifying the organelles from these cultured organisms and found both the procedures of the use of a Nycodenz gradient and two identical and sequential sucrose gradient centrifugations to yield satisfactory preparations of these organelles. It is not clear why the sequential Percoll and sucrose gradient centrifugation procedure does not work well in the purification of procyclic form glycosomes. The different degree of mitochondrial development in the two different life stages of the parasite may be responsible for an altered buoyant density of the mitochondria such that the procyclic glycosomes and mitochondria are hardly separated from each other in the Percoll gradient. Although glycosomes from both forms of the parasite display the same buoyant density on sucrose gradients, they may differ in the nature and proportion of granular material as indicated by both electron microscopy of whole cells and the resistance of the granular material from procyclic glycosomes to dissolution by the sodium carbonate

procedure. The more abundant granular material in procyclic glycosomes may be the cause of the swelling of the organelles when isolated by the procedures described above.

In comparing the protein compositions of bloodstream form and culture form glycosomes, part of our results is in good agreement with those of Hart et al (90). Four major new protein bands (Bands A, B, D and F) are unique to culture form glycosomes and together contribute 18 % of the total glycosomal protein. Protein bands in the 94-110 kD range observed by Hart et al (90) in their preparations, however, are most likely contaminating proteins since we did not observe them in glycosomes better purified by the Nycodenz and sucrose-sucrose procedures but they were present in organelles prepared by the Percoll-sucrose procedure (see Fig. 34). The identities of the new proteins in culture form glycosomes is not yet known. It is tempting however to speculate that band F (M_r 34.7 kD) could represent malate dehydrogenase on the basis of the known subunit M_r of a number of malate dehydrogenases including that of cucumber cotyledon glyoxysomes (148). Band E represents yet another unidentified protein that probably assumes an increased metabolic role in culture form glycosomes since its protein contribution to the organelle increases greatly with transformation from bloodstream form to culture form. The absence in culture form glycosomes of a protein band corresponding to PGK as well as the enzyme activity is in agreement with the altered cellular location of this enzyme in culture form organisms (85,90). Band 8 is also absent from culture form glycosomes; its biological function remains to be identified.

It is important to note that in the normal life cycle of the parasite, transformation from the bloodstream long slender forms to the procyclic culture forms proceeds via a morphologically distinct stage termed the short

stumpy forms. These short stumpy organisms are believed to be forms preadapted to life in the insect midgut because of their increased mitochondrial metabolic capability (102). However, almost nothing is known about the glycolytic or glycosomal enzyme activities in these forms. The increased mitochondrial activity could suggest a decreased reliance on glycolysis and consequently reduced activities of the glycolytic enzymes. Carbon dioxide-fixing enzyme systems are known to be present in these short stumpy forms (51) and it is likely that the earliest appearance of one of these enzymes, phosphoenol pyruvate carboxykinase, in the glycosomes, may occur at this stage. In the absence of reliable methods for obtaining large numbers of short stumpy form organisms for glycosome purification and analysis, it is difficult to tell which of the changes finally observed in procyclic culture form glycosomes are actually initiated during the short stumpy stage.

Our results also strongly suggest that none of the eleven major proteins of bloodstream form glycosomes is associated with the cytoplasmic surface of the organelle. However, it is possible that external peripheral membrane proteins may be present in vivo but are stripped off during purification procedures.

In bloodstream form glycosomes, the two protein bands 10 and 11 behave as integral membrane proteins when assessed by several experimental criteria. In intact organelles, both proteins appear to be susceptible to digestion by exogenous proteases. They are associated with the membrane fractions following rupture of the organelle by osmotic shock, sonication or freeze-thaw cycles. In phase separation experiments, at least one of these bands (band 10) behaves as an integral membrane protein. The most convincing evidence that these two proteins are the only major integral

glycosomal membrane proteins in bloodstream form glycosomes is electron microscopic data of the membrane preparation which reveals clean membrane vesicles and the SDS-PAGE analysis which shows only bands 10 and 11 in these vesicles. The absence, from sodium carbonate-prepared membranes, of other glycosomal proteins that tend to be partly associated with the membrane fractions prepared by the other procedures (osmotic, sonication, freeze-thaw) corroborates the observations of Fujiku et al (146) that the method removes peripheral membrane proteins.

These two proteins are also present in the culture form glycosomal membranes. The sodium carbonate-prepared membrane fractions from culture form glycosomes contain electron dense granules, and additional protein bands in SDS-PAGE, which may be either integral membrane proteins or proteins associated with the granular material.

Most of the glycolytic proteins in both bloodstream form and culture form glycosomes are released as soluble proteins when the organelles are physically ruptured. Some band 2 (PFK) and band 6 (GAPDH) tend to remain in the pelletable fractions after freeze-thawing. It is difficult to ascertain whether these proteins are peripheral proteins associated with the internal surface of the glycosomal membrane (internal peripheral membrane proteins) or with dense granular material that is not disrupted by this procedure (as has been established in rat liver peroxisomes by Alexson et al [153]). We have previously observed that PFK (along with ALD) sediments even after detergent solubilization of the membrane of bloodstream form glycosomes (133). This may suggest a dense granule location for PFK (and ALD), and an internal peripheral membrane location for GAPDH.

To get a clue as to the intracellular pathway of synthesis and assembly of the glycosomal proteins (bloodstream form as well as culture form), we

tested for glycosylation of these proteins. None of the major glycosomal proteins appear to be glycosylated. This finding is not surprising since there is increasing consensus that proteins in microbody organelles such as peroxisomes are synthesized independent of the endoplasmic reticulum (143), at which site glycosylated proteins originate.

Opperdoes et al (97) have analyzed the phospholipid and protein content in membranes of bloodstream form glycosomes prepared by the sodium carbonate procedure. These investigators found that all the glycosomal phospholipid and 30% of the glycosomal protein was associated with the membrane fraction. Such membrane fractions had a composition of 32% phospholipid and 68% protein by weight. This phospholipid content of the glycosomal membrane may be an underestimate since our studies show that membranes prepared from glycosomes purified by the Percoll-sucrose procedure, as done by these investigators, contain extra proteins which are most likely associated with Percoll particles. The membrane fraction obtained from bloodstream form glycosomes purified by the sucrose-sucrose procedure contains only two integral membrane proteins. Since these two proteins together represent 7% of the total bloodstream form glycosomal protein, a recalculation of the data by Opperdoes et al gives the modified membrane composition of 67% phospholipid and 33% protein by weight.

The significance of the endogenous protease present in glycosomes of bloodstream form *T. brucei* is not known. However, the fact that it is apparently activated only in lysed organelles may suggest a role in the normal regulated protein turnover of the organelle. Its surprisingly specific digestion of the two integral membrane proteins band 10 and band 11 may suggest an involvement with the functions of glycosomal membrane. It could be involved in the import and assembly of the glycosomal proteins by

converting the precursor proteins to the mature proteins during their insertions into the glycosome as has been exemplified by mitochondrial biogenesis (156).

We conclude that glycosomes from bloodstream form *T. brucei* contain only two major integral membrane proteins which are also present in the membranes of glycosomes from the culture form of the parasite.

Figure 31: Comparison of the SDS-PAGE profiles of glycosomes from bloodstream form of *T. brucei* prepared by the Percoll-sucrose (lane 1) and the sucrose-sucrose (lane 2) procedures. Fifty microgram protein was loaded in each lane. Note the cleavage of band 5 (aldolase) into a slightly faster migrating species in these preparations (both parent and product bands marked by arrows). Molecular weight standards appearing in all SDS-PAGE figures are : Phosphorylase B (M_r 92.5 kD), bovine serum albumin (M_r 66.2 kD), ovalbumin (M_r 45 kD), carbonic anhydrase (M_r 31 kD), soybean trypsin inhibitor (M_r 21.5 kD) and lysozyme (M_r 14.4 kD).

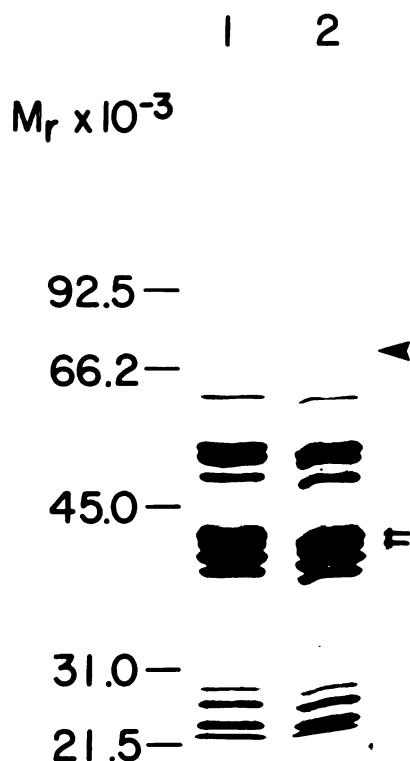


Figure 32: Electron microscopy of isolated and in situ glycosomes of culture form *T. brucei*.

A. Glycosomes purified by sequential Percoll and sucrose gradients. The scale bar corresponds to 0.48 micron.

B. Glycosomes purified by the use of a 10-40 % Nycodenz gradient. The scale bar corresponds to 0.23 micron.

C. Glycosomes purified by the use of two identical and sequential sucrose gradients. The scale bar corresponds to 0.48 micron. Magnification of inset is 42,000 X.

D. Glycosomes in situ in intact procyclic trypomastigotes. The scale bar corresponds to 0.22 micron.

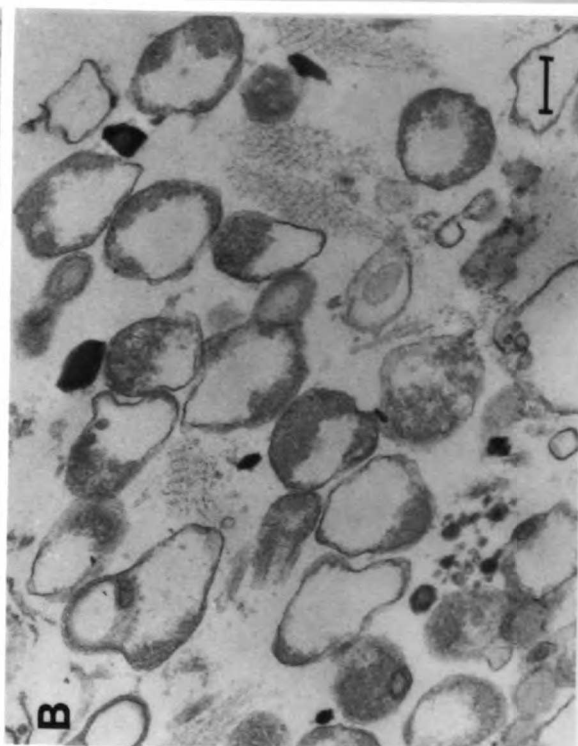
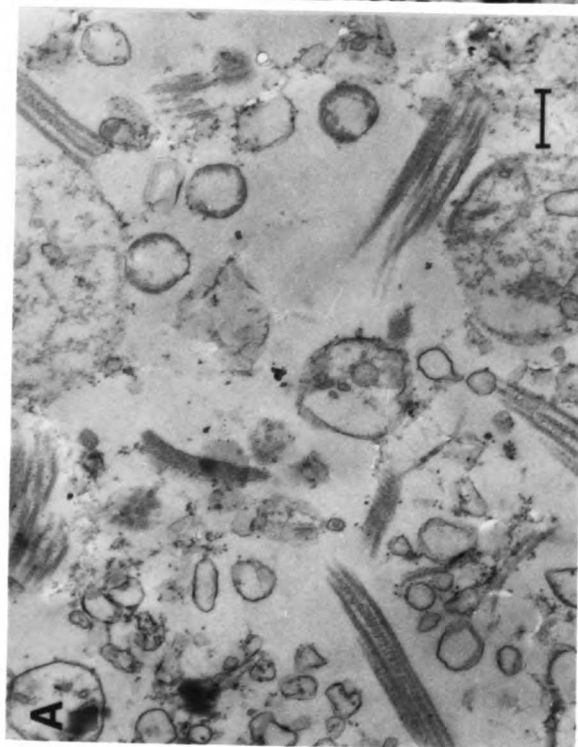
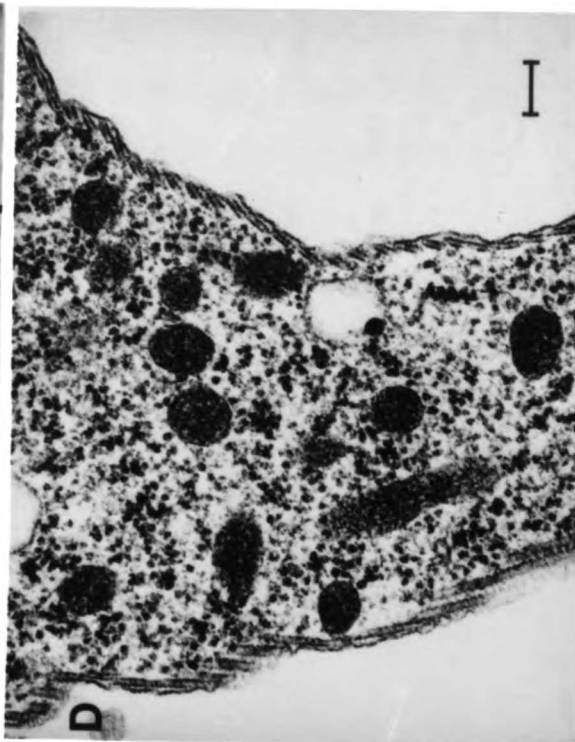
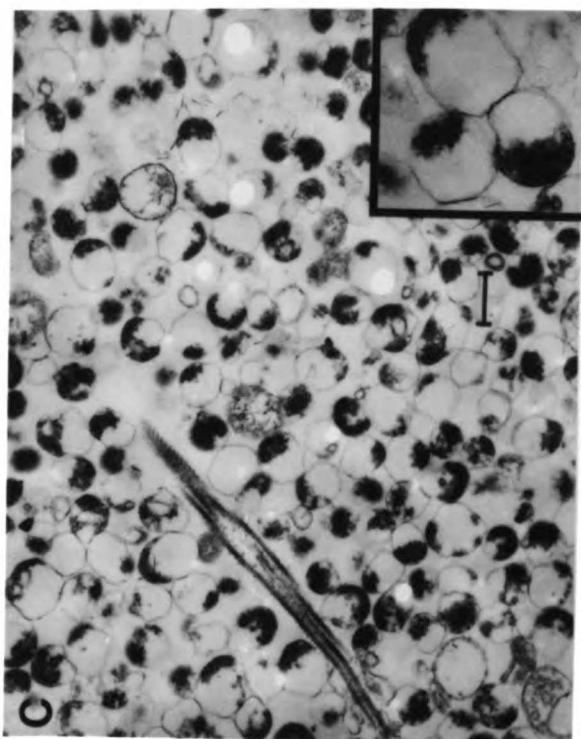


Figure 33: SDS-PAGE analysis of glycosomes isolated from culture form procyclic trypomastigotes of *T. brucei* by various procedures. Lane 1 : Glycosomes prepared by the Percoll-sucrose procedure. Lanes 2 and 3 : Glycosomes prepared by the Nycodenz procedure. Lanes 4 and 5 : Glycosomes prepared by the sucrose-sucrose procedure. Lane 6 : Bloodstream form glycosomes. Lane 7 : Molecular weight standards as in Fig. 31.

Figure 34: Comparison of the SDS-PAGE profiles of purified glycosomes from bloodstream form (Lane 1) and culture form (Lane 2) of *T. brucei* prepared by the sucrose-sucrose procedure. Fifty microgram protein was applied to each lane. The gels were scanned in an LKB Ultrosan laser densitometer linked to a Hewlett-Packard 3390-A integrator.

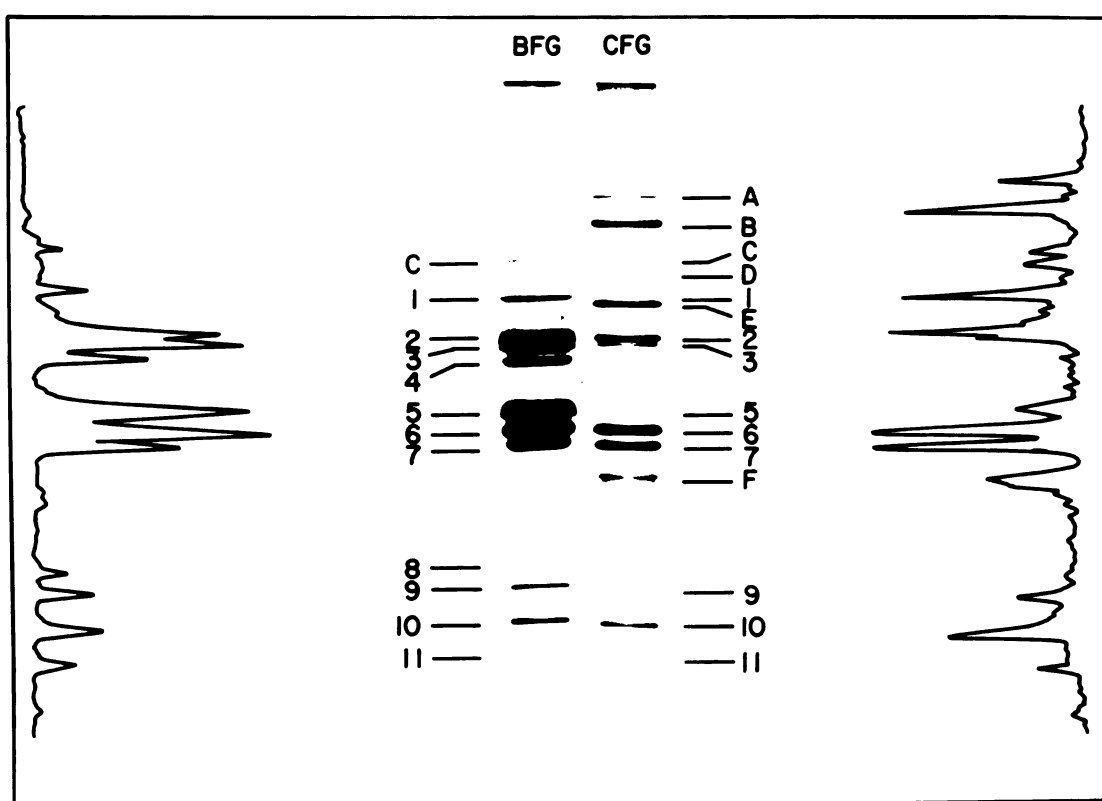


Figure 35:

A. Effect of salt-treatment on intact bloodstream form glycosomes. Lane 1 : Control. Lane 2 : Pyrophosphate-treated. Lane 3 : MgCl_2 - treated.

Forty microgram protein was applied per lane.

B. Effect of trypsin and pronase on intact bloodstream form glycosomes.

Lane 1 : Control. Lane 2 : Trypsin-treated. Lane 3 : Pronase-treated. Forty microgram protein was loaded in each lane.

C. Test for endogenous protease in bloodstream form glycosomes. Lane 1 :

Lysed glycosomes in the presence of protease inhibitors. Lane 2 : Lysed

glycosomes in the absence of protease inhibitors. Lane 3 : Intact

glycosomes. Sixty five microgram protein was loaded in each lane. The two intense bands of low mol.wt. in lane 1 represent the protease inhibitors soybean trypsin inhibitor (M_r 21.5 kD) and aprotinin.

Markers are positions of molecular weight standards (see legend to Fig. 31).

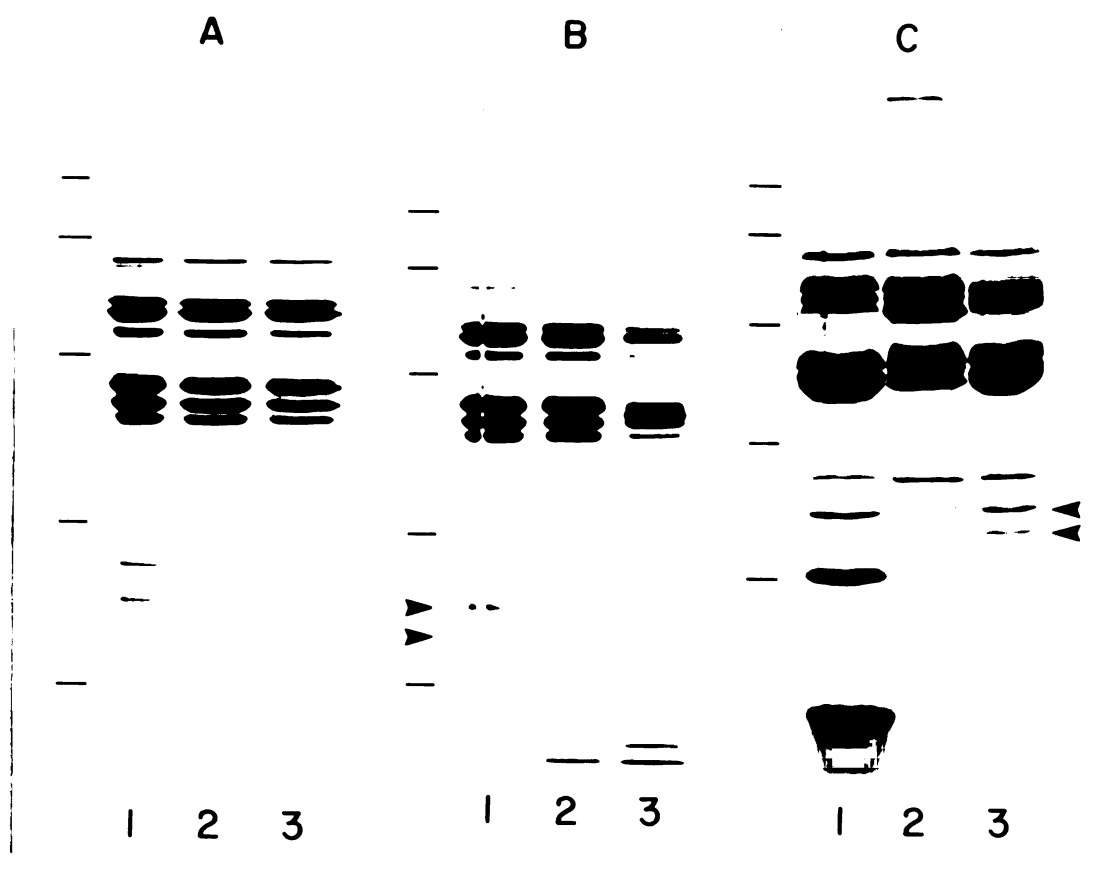


Figure 36: Fractionation of purified bloodstream and culture form glycosomes of *T. brucei*.

A. Membrane and matrix fractions generated by sodium carbonate treatment of bloodstream form glycosomes prepared by the Percoll-sucrose procedure.

Lane 1: Membrane fraction. Lane 2 : Matrix fraction. Lane 3 : Glycosomes.

Fifty microgram protein was loaded in each lane.

B. Membrane and matrix fractions generated by sodium carbonate treatment of bloodstream form and culture form glycosomes purified by the sucrose-sucrose procedure.

Lane 1 : Matrix fraction from culture form glycosomes.

Lane 2 : Membrane fraction from culture form glycosomes. Lane 3 : Culture form glycosomes. Lane 4 : Bloodstream form glycosomes. Lane 5 :

Membrane fraction from bloodstream form glycosomes. Lane 6 : Matrix

fraction from bloodstream form glycosomes. Fifty microgram protein was loaded in each lane except lanes 5 and 6 which received 25 microgram.

Markers are positions of molecular weight standards (see legend to Fig. 31).

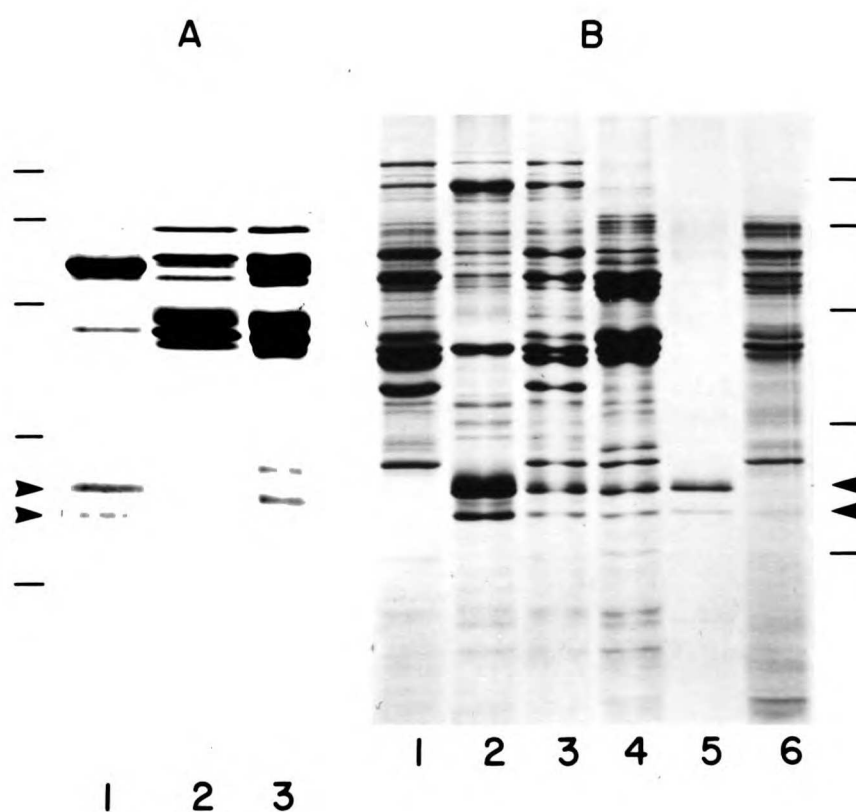


Figure 37: Electron microscopy of intact bloodstream form glycosomes and sodium carbonate-generated membrane fractions of bloodstream and culture form glycosomes.

A. Bloodstream form glycosomes purified by the original Percoll-sucrose procedure. The scale bar corresponds to 0.15 micron.

B. Membrane fraction generated from glycosomes in A. The scale bar corresponds to 0.39 micron.

C. Membrane fraction generated from bloodstream glycosomes purified by the sucrose-sucrose procedure. The scale bar corresponds to 0.26 micron. Magnification of inset is 100,000 X.

D. Membrane fraction generated from culture form glycosomes purified by the sucrose-sucrose procedure. The scale bar corresponds to 0.44 micron.

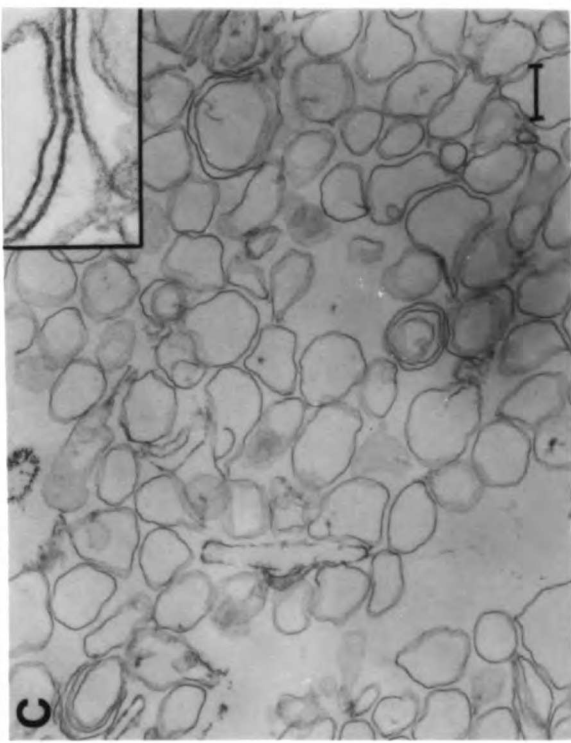
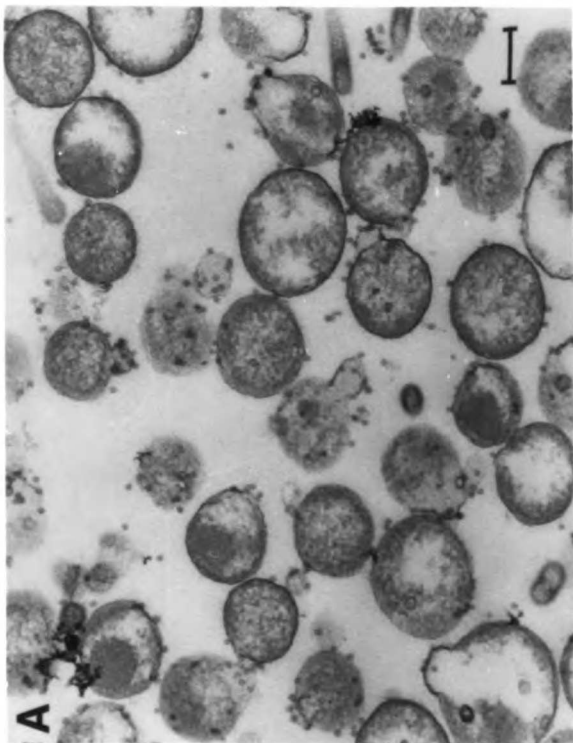
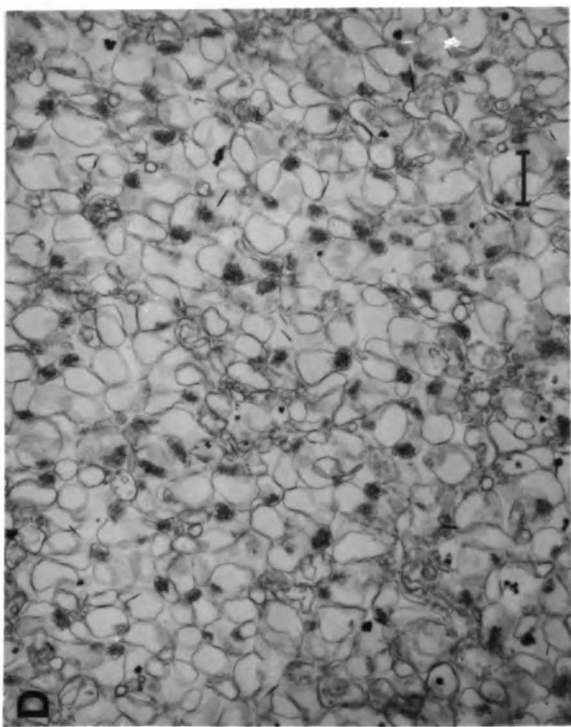
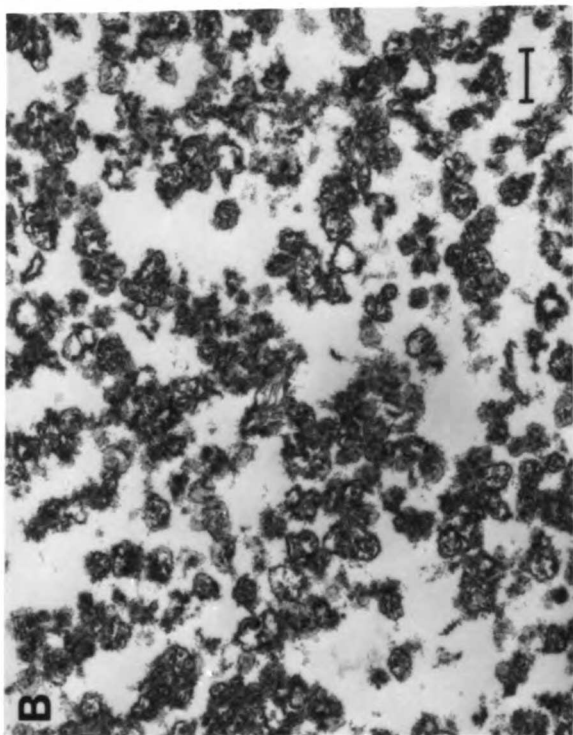


Figure 38: Rupture of bloodstream and culture form glycosomes by osmotic shock, sonication and freezing and thawing.

A. Effect of osmotic shock and sonication on bloodstream form glycosomes.

Lane 1 : Matrix fraction after osmotic shock. Lane 2 : Pellet fraction after osmotic shock. Lane 3 : Pellet fraction after osmotic shock plus sonication.

B. Effect of six cycles of freezing and thawing on bloodstream form and

culture form glycosomes. Lane 1 : Matrix fraction from bloodstream form glycosomes. Lane 2 : Pellet fraction from bloodstream form glycosomes.

Lane 3 : Bloodstream form glycosomes (prepared by the sucrose-sucrose procedure). Lane 4 : Bloodstream form glycosomes (prepared by the Percoll-sucrose procedure). Lane 5 : Culture form glycosomes. Lane 6 : Pellet fraction from culture form glycosomes. Lane 7: Matrix fraction from culture form glycosomes.

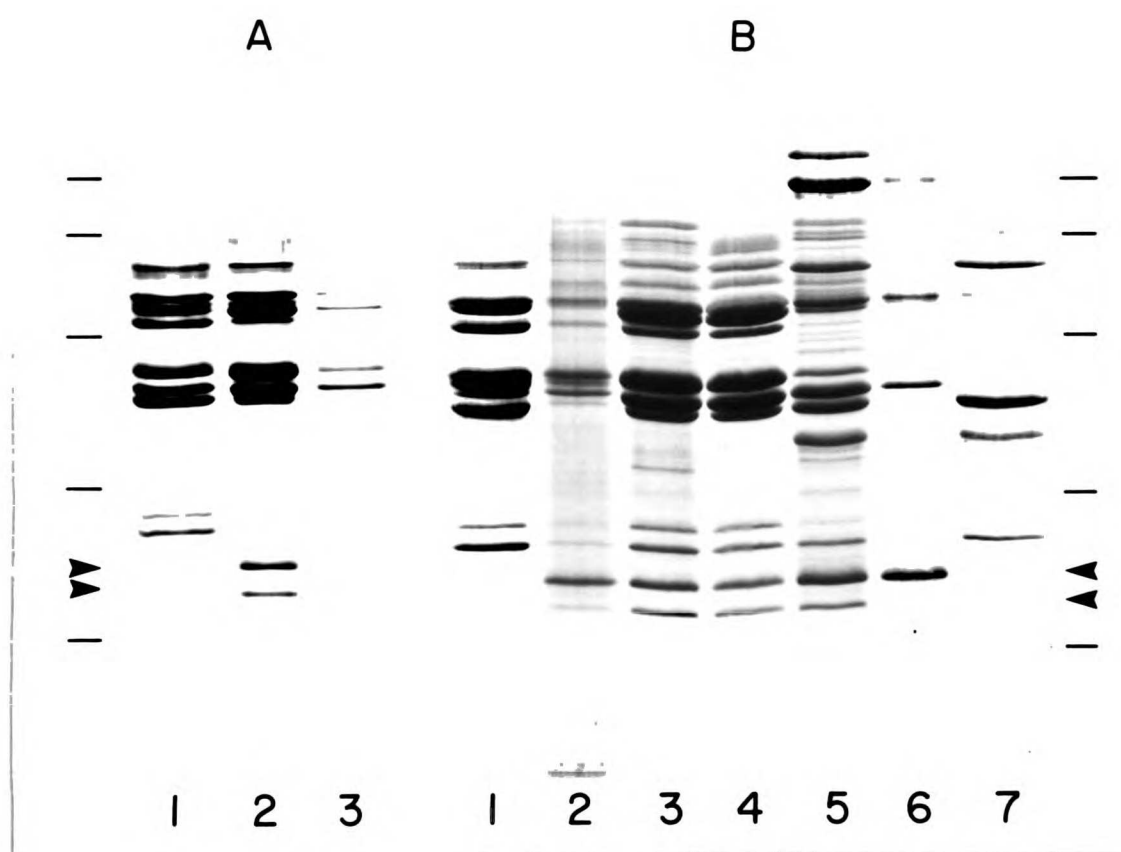


Figure 39: Triton X-114 phase separation of bloodstream form and culture form glycosomal proteins. Lane 1 : Supernatant aqueous fraction of culture form glycosomes. Lane 2 : Detergent-rich fraction of culture form glycosomes. Lane 3 : Culture form glycosomes. Lane 4 : Bloodstream form glycosomes. Lane 5 : Bloodstream form glycosomes. Lane 6 : Detergent-rich phase of bloodstream form glycosomes. Lane 7 : Supernatant aqueous fraction of bloodstream form glycosomes. All glycosomal samples were prepared by the sucrose-sucrose procedure except that in lane 4 which was made by the Percoll-sucrose procedure. Markers are positions of the molecular weight standards in the legend of Fig. 31.

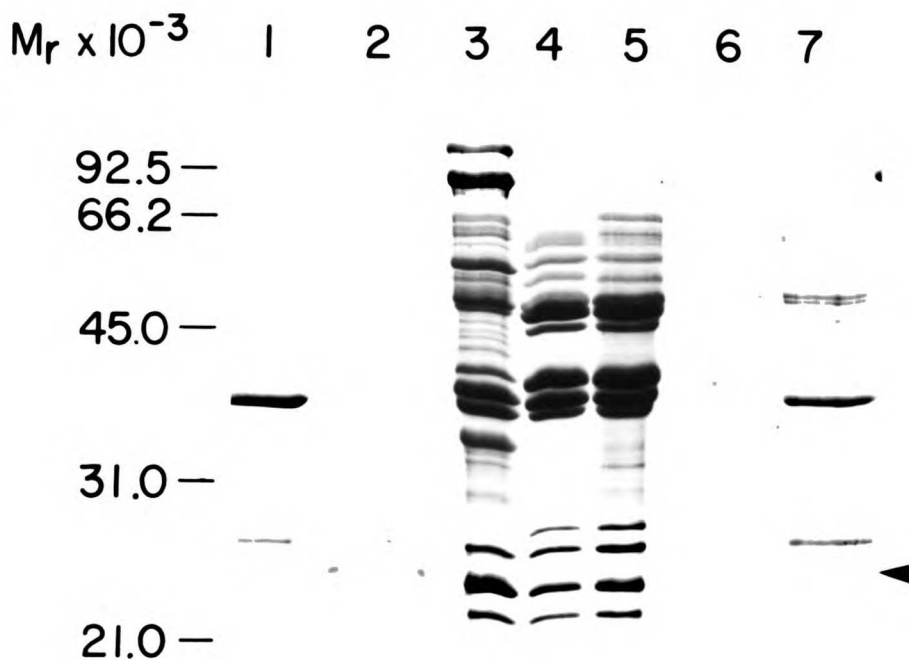


Figure 40: Test for glycoproteins in the glycosome. A Western blot of SDS-PAGE separated glycosomal proteins was tested for reactivity with peroxidase-conjugated concanavalin-A as described in 'Materials and Methods' section. Lane 1, ovalbumin; Lane 2, VSG 118; Lane 3, culture form glycosomal proteins; Lane 4, bloodstream form glycosomal proteins; Lane 5, molecular weight standards as in Fig. 31.

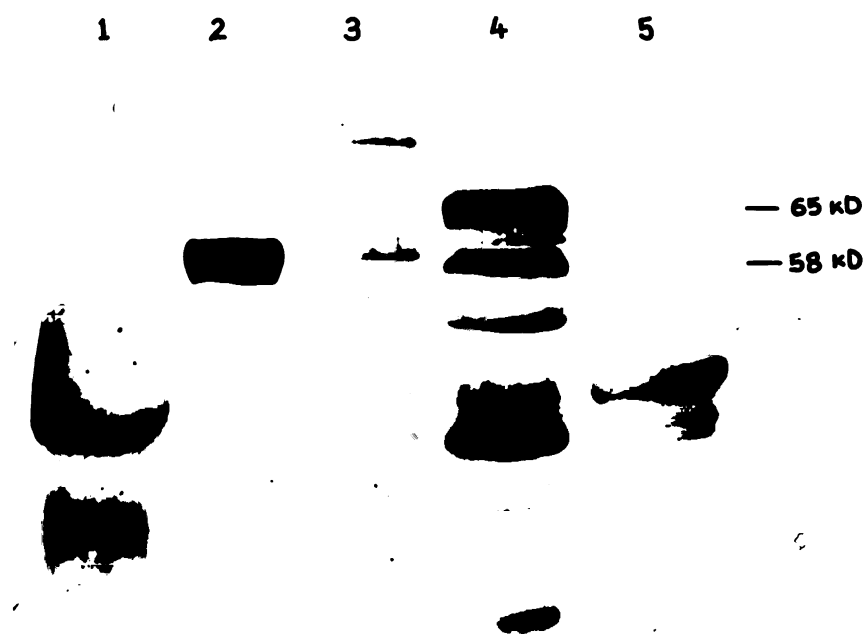


TABLE 11

Specific Activities of Enzymes in Glycosomes of Bloodstream Form
***T. brucei* Prepared by Two Different Procedures**

	Specific Activity ^a	
	Percoll-sucrose Procedure	Sucrose-sucrose Procedure
Hexokinase	8.52	7.12
Phosphoglucose isomerase	6.51	4.17
Phosphofructose kinase	57.45	49.36
Aldolase	7.73	5.96
Glyceraldehyde-3-phosphate dehydrogenase	0.38	0.32
Phosphoglycerate kinase	13.04	13.03
Triose phosphate isomerase	196.05	223.47
Glycerol-3-phosphate dehydrogenase	15.04	13.38
Glycerol kinase	3.59	3.08
Malate dehydrogenase	N.D	N.D
Phosphoenol pyruvate carboxykinase	N.D	N.D

^aSpecific activity in micromole substrate/min/mg protein is the mean of two independent experiments

TABLE 12

Enzyme Activities in Glycosomes of Culture Form *T. brucei* Prepared by
Three Different Procedures

	Specific Activity ^a (Relative Specific Activity ^b)		
	Percoll-sucrose Procedure	Nycodenz Procedure	Sucrose-sucrose Procedure
Hexokinase	0.30 (1.1)	1.21 (4.3)	0.31 (1.1)
Phosphoglucose isomerase	0.38 (1.5)	0.81 (3.2)	0.79 (3.2)
Phosphofructose kinase	1.71 (2.0)	2.60 (3.0)	3.91 (4.5)
Aldolase	0.24 (4.8)	0.82 (16.4)	1.24 (24.8)
Glyceraldehyde-3-phosphate dehydrogenase	0.17 (0.7)	0.23 (0.9)	0.18 (0.7)
Phosphoglycerate kinase	0.07 (0.7)	0.17 (1.7)	0.16 (1.6)
Triose phosphate isomerase	59.03 (2.2)	132.80 (5.0)	86.80 (3.8)
Glycerol-3-phosphate dehydrogenase	5.78 (2.3)	12.62 (5.1)	9.54 (3.8)
Glycerol kinase	1.24 (2.0)	n.d ^c	0.23 (0.4)
Malate dehydrogenase	81.20 (1.6)	224.30 (4.4)	235.80 (4.6)
Phosphoenol pyruvate carboxykinase ^a	1.0 (5.0)	n.d	2.0 (10)

^aSpecific activity is in micromoles substrate/min/mg protein except for phosphoenol pyruvate carboxykinase where the units are nanomoles substrate/min/mg protein

^bRelative specific activity (values in parenthesis) is expressed as a ratio of the specific activity in the post-large granule supernatant i.e the 6500 x g supernatant

^cNot detected

TABLE 13
Comparison of the Specific activities of Enzymes in Sucrose-Prepared
Glycosomes of Bloodstream Form and Culture Form *T. brucei*

	Specific Activity ^a		% Change	Ratio of S. A ^{b,c} (BFG/CFG)
	BFG	CFG		
Hexokinase	7.12	0.31	- 95.6	23
Phosphoglucose isomerase	4.17	0.79	- 81.1	5
Phosphofructose kinase	49.36	3.91	- 92.1	13
Aldolase	5.96	1.24	- 79.2	5
Glyceraldehyde-3-phosphate dehydrogenase	0.32	0.18	- 43.8	2
Phosphoglycerate kinase	13.03	0.16	- 98.8	82
Triose phosphate isomerase	223.47	86.80	- 61.2	3
Glycerol-3-phosphate dehydrogenase	13.38	9.54	- 28.7	1
Glycerol kinase	3.08	0.23	- 92.5	13
Malate dehydrogenase	n.d	235.80	-	-
Phosphoenol pyruvate carboxykinase	n.d	0.002	-	-

^aSpecific activity in micromole substrate/min/mg protein

^bAbbreviations are: S. A, specific activity; BFG, bloodstream form glycosomes; CFG, culture form glycosomes; n.d, not detected

^cRatio to the nearest whole number

TABLE 14
SDS-PAGE Analysis of the Proteins in Purified Glycosomes^a from
Bloodstream Form and Culture Form *T. brucei*

Band ^b	Tentative Identification ^c	Estimated Subunit M _r	Estimated % of total glycosomal protein ^d		Ratio of protein content (BFG/CFG)
			BFG ^e	CFG ^e	
A	Unidentified	94,400	-	3.2	-
B	Unidentified	80,400	-	7.5	-
C	Unidentified	67,200	1.8	1.0	1.80
D	Unidentified	64,200	-	1.7	-
1	Phosphoglucose isomerase	58,200	2.2	1.0	2.20
E	Unidentified	56,200	0.5	5.8	0.09
2	Phosphofructose kinase	50,700	13.4	6.3	2.13
3	Hexokinase	49,500	11.5	2.1	5.48
4	Phosphoglycerate kinase	47,300	5.9	-	-
5	Aldolase	41,200	22.3	2.3	9.70
6	Glyceraldehyde-3-phosphate dehydrogenase	38,700	19.0	11.0	1.73
7	Glycerol-3-phosphate dehydrogenase	37,600	8.2	9.2	0.89
F	Unidentified	34,700	-	6.8	-
8	Unidentified	28,200	2.1	-	-
9	Triose phosphate isomerase	26,800	2.9	2.8	1.00
10	Unidentified	24,800	4.5	7.8	0.58
11	Unidentified	23,200	2.5	1.2	2.00

^aGlycosomes from both forms of the organism were purified by the sucrose-sucrose procedure.

^bMajor proteins in glycosomes of bloodstream form organism are designated with numbers as in ref. 133. Major new proteins appearing in glycosomes of culture form organism are designated with alphabetical letters.

^cIdentifications are still tentative and according to references 90 and 133.

^dQuantitative data obtained from the densitometric scans shown in Fig. 34. The gel was scanned in a LKB 2202-001 Ultrosan laser densitometer linked to a Hewlett-Packard 3390-A integrator.

^eAbbreviations are: BFG, bloodstream form glycosomes; CFG, culture form glycosomes.

TABLE 15**Glycolytic Enzyme Activities in Protease-treated Glycosomes from
Bloodstream Form *T. brucei***

	Per Cent Activity of Control ^a	
	Trypsin-treated Glycosomes	Pronase-treated Glycosomes
Hexokinase	102	106
Phosphoglucose isomerase	103	67
Phosphofructose kinase	108	88
Aldolase	107	34
Glyceraldehyde-3-phosphate dehydrogenase	121	120
Triose phosphate isomerase	91	83
Glycerol-3-phosphate dehydrogenase	106	95
Phosphoglycerate kinase	88	83
Glycerol kinase	95	94

^asee 'Materials and Methods' section

CHAPTER V

CONCLUSION

Glycosomes purified from long slender bloodstream form *T. brucei* EATRO 110 monomorphic strain are invariably contaminated with the variant surface glycoprotein probably arising from plasma membrane fragments copurifying with the organelles. Such contamination can be greatly reduced if the isolated organisms are frozen in 15% glycerol and then thawed prior to breakage of the cells. Freezing and thawing the parasites in glycerol apparently promotes shedding of the VSG coat by intact organisms. Whatever the underlying mechanism of the VSG release, freezing and thawing the organisms in glycerol was adopted as a necessary procedure to ensure the isolation of highly purified and intact glycosomes, an important requirement for all the subsequent studies. The tentative identifications of 8 of the 11 major proteins present in these glycosomes are in good agreement with the work of Hart et al (90) on a different strain of *T. brucei* (stock 427). At least 90% of the total glycosomal protein in bloodstream form *T. brucei* is composed of glycolytic enzymes.

The bifunctional crosslinking reagents DMSI and DTBP both penetrate the membrane of intact glycosomes and cause extensive crosslinking of all the glycosomal proteins into a large complex. Since the distance between the two reactive functional groups in these compounds is rather short (span lengths of 11 Å for DMSI and 12 Å for DTBP), the formation of the complex implies that the glycosomal proteins are packed very close together in the intact glycosome. This close spatial relationship of the proteins in the glycosome does not seem to represent a specific organization to facilitate the glycolytic process since the complex was shown to be no different from the unaggregated enzymes in its kinetic behaviour. The demonstrated lack of

substrate channeling of glycolytic intermediates in the complex rules out any functional advantage gained by an association of the glycolytic enzymes in the glycosome. Fractionation of native glycosomes by detergent and salt reveals a differential release of proteins, implying some form of specific arrangements of the proteins inside the glycosome. Some of the proteins typified by phosphoglucose isomerase are readily released on rupture of the organelle whereas others notably phosphofructose kinase and aldolase are not readily released and remain in a pelletable fraction. What then is the purpose of this apparent association of the glycosomal enzymes? The answer to this question may lie in our simple perception of organelles and indeed cells as simple bags composed of an aqueous solution uniformly dispersed with enzymic proteins and freely diffusing metabolites. This long held notion is falling into disfavour and the modern concept of cellular metabolism maintains that enzymes operate within a framework of spatio-temporal structure probably involving structural cytoskeletal components of the cell. The crystalloid core seen in glycosomes may indeed provide a skeletal framework for the spatial organization of some of the glycosomal enzymes. Recent estimates of the protein concentration in bloodstream form glycosomes put it at 320-360 mg/ml whereas the glycolytic intermediates have been estimated to be present in the mM range. One can imagine that under such conditions, not only would there be crowding of protein molecules in the glycosome, but there would also be a serious solubility problem for the proteins because of the limited volume of aqueous solvent in the organelle. Association of the proteins with each other to form a crystalloid core could be seen as a reflection of the solubility constraint. In the glycosome, the association of the enzymes may be more for the fulfillment of the solubility requirement rather than achieving an enhanced or

more efficient glycolytic rate. Indeed the activities of the glycolytic enzymes in the glycosomes are far in excess of those required to drive glycolysis at the observed cellular rate and there should be no need for specific substrate channelings if availability of glucose is the rate limiting factor.

The lesser significance of glycolysis in the culture form of *T. brucei* is reflected in the much reduced activities of the various glycolytic enzymes in the glycosomes of this form of the parasite. The glycolytic enzymes also contribute much less to the total glycosomal protein. Several major new proteins appear reflecting the increased metabolic diversity of the glycosomes of the culture form. In the culture form glycosome, a specific organization of the glycosomal components is also apparent from the various fractionation studies. Many of the glycosomal proteins are matrix proteins either associated with the inside surface of the organelle or with the core. The glycosomal membrane contains two integral membrane proteins in both bloodstream and culture forms although there may be more in the glycosomes of culture form *T. brucei*. The function of these two integral membrane proteins has not yet been identified. They must serve some important function since they are present in organelles from both life forms of the parasite. The identification and characterization of these two integral membrane proteins will provide important ground work in understanding the biogenesis of the glycosome.

In closing then, it is hoped that the studies described have contributed to a better understanding of the biochemistry and cellular biology of the African trypanosome. Only through such fundamental approaches will the goal of rational chemotherapy be realized.

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APPENDIXES

A. Enzyme assays

All enzyme assays were conducted in a total volume of 1.0 ml in quartz cuvettes and at a controlled temperature of 37° C. Assays were validated for linearity with enzyme concentration and were investigated for interfering enzymatic reactions. The reaction mixture containing all components except substrate was allowed to equilibrate to a steady baseline before initiating the reaction by addition of prewarmed substrate solution. Control systems consisted of the complete assay mixture without the protein sample being assayed. All reactions except those determining the latency of the enzymes always contained 0.1% (v/v) Triton X-100. Enzyme activities were calculated from the initial rates assuming a molar absorptivity of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm for both NADH and NADPH.

Hexokinase [EC 2.7.1.1] : The reaction mixture contained 40 mM triethanolamine-HCl buffer (TEA) pH 7.6, 8 mM MgCl₂, 0.64 mM ATP, 0.91 mM NADP, 1.0 U/ml glucose-6-phosphate dehydrogenase (G6PDH), enzyme extract and 200 mM glucose.

Phosphoglucose Isomerase [EC 5.3.1.9] : The reaction mixture was composed of 85 mM TEA pH 7.6, 7 mM MgCl₂, 0.4 mM NADP, 1.0 U/ml G6PDH, enzyme extract and 1.4 mM fructose-6-phosphate (F6P).

Phosphofructokinase [EC 2.7.1.1] : The modified reaction mixture contained 70 mM TEA pH 7.6, 10 mM MgCl₂, 1.0 mM ATP, 0.45 mM NADH, 5 U/ml aldolase, 2 U/ml each of glycerophosphate dehydrogenase (GDH) and triose phosphate isomerase (TPI), enzyme sample and 5 mM F6P.

Aldolase [EC 4.1.2.13] : The reaction mixture contained 94 mM TEA pH 7.6, 0.2 mM NADH, 2 U/ml of GDH, 20 U/ml TPI, enzyme sample and 1.0 mM fructose-1,6-diphosphate (FDP).

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) [EC 1.2.1.12] : The reaction mixture contained 85 mM TEA pH 7.6, 2 mM MgSO₄, 1.0 mM EDTA, 10 mM dithiothreitol (DTT), 1.0 mM ATP, 0.2 mM NADH, 15 U/ml phosphoglycerate kinase (PGK), enzyme sample containing 10 mM DTT, and 6 mM glycerate-3-phosphate.

Phosphoglycerate Kinase [EC 2.7.2.3] : The reaction mixture contained 80 mM TEA pH 7.6, 2 mM MgSO₄, 1.0 mM EDTA, 1.0 mM ATP, 0.2 mM NADH, 2.5 U/ml of GAPDH, enzyme sample and 6.5 mM glycerate-3-phosphate.

Triose Phosphate Isomerase [5.3.1.1] : The reaction mixture contained 243 mM TEA pH 7.6, 0.2 mM NADH, 2 U/ml GDH, enzyme sample and 3.5 mM glyceraldehyde-3-phosphate (GAP).

Glycerol-3-phosphate Dehydrogenase [EC 1.1.1.8] : The reaction mixture was composed of 50 mM sodium phosphate buffer pH 7.2, 1.0 mM EDTA, 0.2 mM NADH, enzyme sample and 0.5 mM dihydroxyacetone phosphate (DHAP).

Glycerol Kinase [EC] : The reaction mixture contained 182 mM glycine buffer pH 9.8, 3.78 M hydrazine hydrate, 10 mM DTT, 2 mM MgCl₂, 1.4 mM ATP, 0.4 mM NAD, 4 U/ml GDH, enzyme sample containing 10 mM DTT, and 3 mM glycerol.

Malate Dehydrogenase [EC]: The reaction mixture contained 100 mM potassium phosphate buffer pH 7.6, 10 mM DTT, 0.2 mM NADH, enzyme sample and cis-oxaloacetate at a final concentration of 0.75 mM.

B. Hydrolysis of DL-Glyceraldehyde-3-Phosphate (GAP)

Diethylacetal, Monobarium salt.

Free GAP is a highly unstable compound and is available commercially as the more stable diethylacetal ester. Free GAP was liberated from this compound by the acid hydrolysis procedure described below.

12g Dowex 50 X4-200R (or 50-X8) was washed with boiling deionized water and added to 16 ml of hot water. 800 mg of the barium salt of the acetal was added to the resin and mixed thoroughly. The slurry was incubated in a boiling water bath for 5 minutes with intermittent stirring. The resin was then removed by filtration with suction and washed once with 4 ml deionized water. The filtrate, collected into a container in an ice bath, was adjusted to pH 4 with 0.1 M NaOH. The concentration of the solution was determined enzymatically. The solution was stored in 1 ml aliquots at - 20° C and is stable at this temperature for several months.

Reference: Methods in Enzymology Vol 89 1982

C. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Solutions

Monomer (30% T, 2.7% C_{Bis})

Acrylamide 146 g
 Bis 4 g
 Deionized water to 500 ml

The solution is filtered through a Whatman no. 1 filter and stored at 4° C in a light-protected container.

Separating Gel Buffer

Tris base 90.8 g (1.5 M)
 Deionized water to 500 ml

The pH is adjusted to 8.8 with HCl and the solution stored at 4° C after filtering.

Stacking Gel Buffer

Tris base 30.3 g (0.5 M)
 Deionized water to 500 ml

The pH is adjusted to 6.8 with HCl and the solution stored at 4° C after filtering.

Sample Buffer (2x)

Stacking gel buffer 2.5 ml (0.125 M in Tris)
 10% SDS solution 4.0 ml (4% SDS)
 Glycerol 2.0 ml (20% v/v)
 2-mercaptoethanol 1.0 ml (10% v/v)
 0.5% bromophenol blue 0.25 ml

1 ml aliquots of the solution are stored frozen at -20° C. Urea at concentration of 2 M may be included especially for insoluble protein samples.

Electrode Buffer

Tris base 24.0 g (0.05 M)
 Glycine 115.2 g (0.384 M)
 SDS 4.0 g (0.1%)
 Deionized water to 4 litres

The pH of this solution is ca. 8.3. It should not be adjusted by adding NaOH for this will result in precipitation of the SDS. Electrode buffer should not be reused.

1% Stain Stock

Coomassie Blue R-250 2.0 g

Deionized water to 200 ml

The solution is thoroughly stirred then filtered.

Staining Solution

1% stain stock 62.5 ml (0.125% in Coomassie Blue R-250)

Methanol 250 ml (50%)

Acetic acid 50 ml (10%)

Deionized water to 500 ml

Gels are stained in this solution for 8 to 12 hours.

Destaining Solution I

Methanol 500 ml (50%)

Acetic acid 100 ml (10%)

Deionized water to 1 litre

Stained gels are destained in several washes of this solution for 1 hour.

Destaining Solution II

Acetic acid 280 ml (7%)

Methanol 200 ml (5%)

Deionized water to 4 litres

After destaining gels in solution I, they are destained in this solution in the Bio-Rad destaining apparatus.

Preparation of Gels

The table below gives the recipe for preparing 9% or 12% separating gels and a 4% stacking gel. The volumes given are sufficient for casting two 1.5 mm thick slabs.

	Separating gel		Stacking gel
	9%	12%	4%
Monomer solution	18 ml	24 ml	2.66 ml
Separating gel buffer	15 ml	15 ml	-
Stacking gel buffer	-	-	5.0 ml
10% SDS	0.6 ml	0.6 ml	0.2 ml
Deionized water	26.1 ml	20.1 ml	12.2 ml
10% Ammonium persulfate (fresh)	0.3 ml	0.3 ml	0.1 ml

The solution is degassed for about 15 minutes and then TEMED is added and mixed with slow swirling (0.02 ml TEMED for separating gel mixture and 0.01 ml for the stacking gel mixture).

Reference: Hoefer Scientific Instruments catalog (1980) San Francisco.

D. Preparation of sucrose gradientsBuffers and solutions

TE Buffer
 25mM Tris
 1mM EDTA
 pH 7.2

66% (w/w) Sucrose Stock Solution
 855 grams sucrose
 450 mls. TE buffer
 Dissolve mechanically using a power stirrer.

Table of dilution of sucrose stock solution

Conc. of sucrose soln.		66% sucrose stock (ml)	TE buffer (ml)
% (w/w)	Molarity (M)		
15	0.464	17	83
18	0.564	21	79
25	0.806	32	68
35	1.177	47	53
45	1.581	63	37
54	1.975	78	22
55	2.020	80	20

Adapted from reference

Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation
 by Owen Mitch Griffith, Applications Research Department, Spinco Division, Beckman
 Instruments, Inc.

Preparation of gradients

Sucrose gradients can be prepared in one of two ways:

(a) Using Gradient Maker

1. Layer 2ml of a 66% (w/w) sucrose solution at the bottom of the Quick-Seal tube to form the cushion (density=1.34 g/c.c at 20° C)
2. Pour 14 ml of the heavy solution which is 54% (w/w) sucrose (density=1.2519 g/c.c at 20° C) into the mixing chamber of the gradient maker.
3. Pour 14ml of the light solution which is 18% (w/w) sucrose (density=1.0721 g/c.c. at 20° C) into the reservoir chamber.
4. Pump the gradient into the Quick-Seal tube always keeping the end of the delivery tube above the level of the meniscus (top loading)
Store the gradients at 4° C until use.

(b) Sucrose gradient prepared by allowing diffusion of a step gradient.

1. Place 6ml of the 15% (w/w) sucrose solution in the bottom of a Quick-Seal tube using a 10 ml syringe to which is attached a long 20 gauge needle.
2. Carefully underlay 6 ml of a 25% (w/w) sucrose solution below the 15% solution so that the later is lifted up without disturbing it or mixing the two solutions.
3. Continue underlaying successively 6 ml solutions of 35% (w/w), 45% (w/w) and finally 55% (w/w) sucrose solutions so as to form a five-step gradient.
4. Carefully place the tubes in the tube rack and place the rack on its side (horizontally) in a section of the refrigerator or cold box free from vibrations. Allow the step gradients to diffuse in this position for 45-60 minutes and then carefully turn the tube rack to the upright position.
Keep the gradients at 0-4° C until use.

E. Periodic acid Schiff (PAS) staining method for glycoproteins[¶]

The periodate Schiff reaction remains as the most sensitive staining method for glycoproteins. It is most important however that adequate periods be allowed for the several stages given below. If this is not done, poor contrast will result. The quality of the rosaniline is also very important.

For optimum contrast, the gels, including those containing SDS, should be treated as follows :

- (1) Soak for 30 minutes in a 12.5 % solution of trichloroacetic acid.
- (2) Rinse the gel several times in distilled water.
- (3) Soak the gel for one hour in a 1 % solution of periodic acid dissolved in 3 % acetic acid
- (4) Wash the gel repeatedly in distilled water until a test for IO_3^- with AgNO_3 is negative. Then wash in two further changes of water in the dark.
- (5) Immerse the gel in Schiff's reagent for 1 hour also in the dark. This reagent is prepared as follows : 2 % aqueous rosaniline is decolourized with SO_2 , treated with charcoal and then diluted 20 times with water.
The reagent is also available commercially (Sigma).
- (6) The gel is washed three times, each for 10 minutes in a 0.5 % sodium metabisulphite solution.
- (7) The gel is washed repeatedly in distilled water until the background is removed.
- (8) The stained gel is kept stored in a 7 % acetic acid solution

[¶]Reproduced from : Laboratory Techniques in Biochemistry and Molecular Biology. Electrophoresis of proteins in polyacrylamide and starch gels. Edited by Work, T. S. and Work, E.

F. Preparation of DEAE-cellulose

1. 500 g of DE-52 exchanger (Whatman, microgranular and preswollen) is added to 4 litres of 0.2 M NaH_2PO_4 solution.
2. The suspension is stirred and the pH adjusted to below 4 by adding concentrated HCl (ca. 20 ml). The pH is tested by placing a drop of the suspension on pH paper.
3. Adsorbed carbon dioxide is removed by degassing the suspension while stirring (vigorous stirring is avoided to minimize fines).
4. The pH is adjusted to ca. 7 by adding 50% NaOH solution (ca. 20 ml) (pH paper).
5. The suspension is allowed to settle down and the supernatant is aspirated out. Four litres of fresh phospho-saline buffer (PSG without glucose) is added, the slurry stirred briefly and the suspension allowed to settle y minutes where $y = 2.4 \times \text{column height in cm}$.
6. The supernatant containing fines is aspirated off and step 5 repeated two to three times and at the last time, about 50% of the supernatant volume is sucked out.
7. The slurry is transferred to a 4-litre screw-cap bottle and 0.02% sodium azide solution is added as a preservative. The exchanger is stored at 4° C.

The exchanger is equilibrated in PSG buffer prior to use in isolation of parasites from rat blood. Spent DEAE-cellulose from this procedure is regenerated by several washes (resuspension and decantation) in a 1.0-5.0 M sodium chloride solution interspersed with washes in distilled water and stored with preservative at 4° C. DEAE-cellulose regenerated in this manner can be used 5-6 times before its separating power is severely compromised.

G Phosphate saline glucose buffer pH 8 (PSG)

For large scale isolation of bloodstream forms of *T. brucei*, it has been found convenient to prepare a concentrated stock solution (16.7 X) of phosphate saline buffer (PS) as follows:

Phosphate Saline (PS) Stock Buffer (16.7 X)

Na ₂ HPO ₄ , anhydrous	67.4 g
NaH ₂ PO ₄ , anhydrous	3.0 g
NaCl	21.25 g
Distilled H ₂ O to 1 litre. pH adjusted to 8.0	

Working PSG buffer (30 mM sodium phosphate , 22 mM sodium chloride, 83 mM glucose, pH 8.0) is prepared from stock PS buffer as follows:

Phosphate Saline Glucose (PSG) Buffer pH 8.0

PS stock	60 ml
Glucose	15 g
Distilled H ₂ O to 1 litre. pH adjusted to 8.0 if necessary.	

For initial preparation of DEAE-cellulose, glucose is omitted from this buffer.

H. Preparation and maintainance of stabilate

Buffers

Alsevar's Buffer with 20% (v/v) Glycerol

Sodium Citrate	30 mM
Sodium Chloride	70 mM
Glucose	100 mM
Glycerol	20% (v/v)
BSA Fraction V	1 mg/ml

Blood collected from 3 or 4 heavily infected rats is placed in a beaker and an equal volume of Alsevar's buffer containing 20% (v/v) glycerol is added slowly with gentle stirring in the cold. The blood is aliquoted into 1 ml fractions and the samples cryopreserved at - 80° C. Stabilate prepared in such manner contains about 2×10^8 viable cells/ml and animals injected with 1 ml of this stabilate die of parasitaemia within 40-48 hours after innoculation. This time frame of killing is maintained even after several months of storage of the stabilate. Glycerol seems to be far more superior as a cryopreservant than dimethylsulfoxide.

I. Cardiac puncture procedure for collecting blood from infected rats

The infected animal is placed in a sealed dessicator jar saturated with ether and left there until unconsciousness sets in, usually within 3 to 4 min (the level of anaesthesia is gauged by pinching the animal and observing for a reflex). The unconscious animal is removed from the dessicator jar and quickly strapped onto a dissecting tray or table using surgical tape to hold down its outspread feet in the supine position. A mid-abdomen incision is made and the abdomen cut open along the bottom of the rib-cage on both sides so as to clearly expose the diaphragm. The diaphragm is cut away and the pumping heart unobstructively exposed by pressing up on the sternum with the thumb of the left hand (if right-handed). An 11½ gauge vacutainer needle is pierced into the left ventricle of the heart and the blood collected into a heparinized vacutainer tube. When the left ventricle is drained of blood, the needle is pushed through the septum of the heart (without withdrawing it from the left ventricle) into the right ventricle and whatever blood remains collected. This procedure usually yields 10-12 ml blood per 300-400 rat. If on exposing the heart, it is found to have stopped pumping, then blood is collected by direct entry into the right ventricle and auricle. At the end of the procedure, the heart is severed from the body before disposing the dead carcass. The blood, kept on ice throughout, is sieved through a kitchen strainer or cheese-cloth to remove any clots before it is further processed.

