TRYPANOSOMA BRUCEI: SUBCELLULAR DISTRIBUTION AND ORGANISATION OF THE ENZYMES OF GLYCOLYSIS

by

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I hereby declare that the work recorded here as well as the composition of this thesis was done by myself; and all sources of information have been specifically acknowledged by means of references.

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ABSTRACT OF THESIS

The cellular distribution of eleven Trypanosoma brucei enzymes involved in glucose breakdown has been studied, using the following six methods of cell disruption: saponin, Triton X-100, digitonin, freezing and thawing, and grinding with the abrasives alumina and silicon carbide.

By means of differential centrifugation of the homogenates of the bloodstream T. brucei obtained by these six different methods of cell lysis, it has been shown that the distribution pattern of the enzymes is greatly affected by the method of cell lysis as follows.

Only three of the eleven enzymes, namely, phosphoglycerate mutase, enolase, and pyruvate kinase were completely solubilised by at least five of the methods adopted for cell disruption. As well as these three enzymes, saponin lysis which appeared to be the most severe method of treatment, led also to the complete solubilisation of phosphoglucose isomerase and partial solubilisation of glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase and glycerokinase. At the other extreme, cell lysis by grinding T. brucei with alumina or silicon carbide completely solubilised phosphoglycerate mutase, enolase and pyruvate kinase, whereas hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, phosphoglycerate kinase and glycerolphosphate dehydrogenase were found to be concentrated in the post-nuclear fraction which sediments at 14,500 g (fraction 14.5KP). The patterns of distribution of the remaining two enzymes, glyceraldehyde-phosphate dehydrogenase and glycerokinase were found to be polydisperse.

The post-nuclear fraction was found to be capable of metabolising glucose to give glycerolphosphate without auxiliary enzyme supplementation, and this multienzyme activity proved to be very sensitive to inhibition by the trypanocidal compound, suramin. By means of Biogel column chromatography and acrylamide gel electrophoresis, it was shown that the multienzyme activity is concentrated in a particle probably bigger than a globular protein with a molecular weight of 5 million.

Kinetic studies of fraction 14.5KP, in the presence or absence of Triton X-100 indicated that the particles possibly possess a limiting membrane with an inner matrix to which the component enzymes are bound.

Isopycnic sucrose gradient centrifugation confirmed that the multienzyme complex is associated with large particles with a median equilibrium density of 1.22. Since the only subcellular organelles in T. brucei known to band at this density are the microbodies, it has been concluded that the probable intracellular location of the multienzyme complex is the microbodies of the bloodstream long slender form T. brucei.

ABBREVIATIONS

ALD	Aldolase [D-fructose-1,6-biphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13]
BSA	Bovine serum albumin
DAB	3,3'-diaminobenzidine
DHAP	Dihydroxyacetone-phosphate
DTT	Dithiothreitol
ENOL	Enolase [2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11]
GAP	Glyceraldehyde-phosphate
GAPDH	Glyceraldehyde-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12]
GK	Glycerokinase [ATP:glycerol 3-phosphotransferase, EC 2.7.1.30]
GP	L-glycerol-3-phosphate
GPDH	Glycerolphosphate dehydrogenase [sn-Glycerol-3-phosphate:NAD 2-oxidoreductase, EC 1.1.1.8]
GPO	L-glycerol-3-phosphate oxidase
HK	Hexokinase [ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1]
IAA	Iodoacetic acid
KPD	Solution of potassium phosphate containing dithiothreitol
PCV	Packed cell volume
PFK	Phosphofructose kinase [ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11]

ABBREVIATIONS (contd.)

PG	Phosphoglycerate
PGI	Phosphoglucose isomerase [D-Glucose-6-phosphate ketol-isomerase, EC 5.3.1.9]
PGK	Phosphoglycerate kinase [ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3]
PGlyM	Phosphoglycerate mutase [2,3-Biphospho-D-glycerate: 2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3]
PK	Pyruvate kinase [ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40]
TEA	Triethanolamine
TS	Tris-sucrose

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SECTION I

GENERAL INTRODUCTION AND SURVEY OF THE LITERATURE

I.1. GENERAL INTRODUCTION

The bloodstream forms of Trypanosoma brucei metabolise glucose at a rate which is about 50-fold higher than any of the mammalian host tissues which they infect (von Brand, 1951). These organisms are thought to rely completely on glycolysis for their energy requirements. The major end-product of this incomplete breakdown of glucose is pyruvate, and the organisms possess a unique metabolic system for reoxidizing the NADH that would otherwise accumulate during the conversion of glucose to pyruvate. It has been shown (Grant & Sargent, 1960; 1961; Grant et al., 1961) that the pathway for converting NADH back to NAD^+ involves a glycerolphosphate dehydrogenase and an L-glycerol-3-phosphate oxidase enzyme complex.

Thus in the area of energy metabolism, there appears to be at least two points at which these organisms differ from mammalian host tissues. These are (1) the process by which the long slender forms of T. brucei in the mammalian bloodstream deal with the pyruvate that is produced during glycolysis, and (2) the pathway involved in NADH oxidation back to NAD^+.

Since it is generally agreed that any rational approach to chemotherapy requires a judicious exploitation of differences existing betwen host and parasite metabolic apparatus as well as differences in enzyme structure and function, it would appear (at least in theory) that the possession of this rather limited means of energy production in T. brucei could be a target of drug action against the parasites; however, there is no evidence of a comprehensive study of this pathway in any Trypanozoon species. Hence, although there is evidence that specific enzymes associated with glucose catabolism in T. brucei are inhibited by trypanocidal compounds

(Flynn, 1971; Fairlamb & Bowman, 1975), it is still not known whether any of the trypanocidal compounds exert their chemotherapeutic effect by interfering with energy production in these organisms. This lack of detailed knowledge of the mode of action of the compounds already known to have trypanocidal action has, no doubt, been a contributing factor in the apparent reluctance of pharmaceutical companies in searching for new drugs against trypanosomiasis.

With regard to the enzymes involved in carbohydrate metabolism in the trypanosomatid flagellates, it is correct to say that the long standing interest of many workers has failed to produce a complete account on any given species. Yet, there is no doubt that partial study may lead to erroneous conclusions which might result in unproductive attempts to apply the knowledge so acquired. The dangers of a partial study are exemplified in the case of pleomorphic T. rhodesiense, where one morphological form contains the enzymes of the tricarboxylic acid cycle but closer study shows the cycle to be operating at an insignificant rate (if at all) in vivo (Flynn & Bowman, 1973).

The work to be described in this thesis was undertaken in order to study the isolation, the subcellular distribution and the activities of all the enzymes involved in glucose degradation in the bloodstream long slender forms of Trypanosoma brucei. It was expected that the establishment of these parameters would help lay the foundation for a more rational examination of the effect of trypanocidal compounds on these enzymes which are solely responsible for the total energy production in these parasites.

SURVEY OF THE LITERATURE

I.2. ENERGY PRODUCTION IN THE AFRICAN TRYPANOSOMES

In contrast to other eukaryotic cells, which can generate ATP by degradation of fatty acids, amino acids or carbohydrates, the bloodstream forms of the African trypanosomes probably depend entirely on a source of exogenous carbohydrate for their energy requirements (Ryley, 1956).

I.2.1. Oxidation of Amino Acids

Although some amino acids may be oxidised by the bloodstream trypanosomatids, the evidence available suggests that such oxidation of amino acids does not lead to the production of energy. Thurston (1958) found that with T. lewisi, glutamate, asparagine and aspartate supported oxygen uptake but at a much lower rate than with glucose and slight motility was maintained. The bloodstream form of T. equiperdum did not oxidize amino acids. Neither the long slender nor the short stumpy forms of T. rhodesiense are able to oxidize glutamate, alanine or proline (Flynn & Bowman, unpublished results). It appears that whereas the culture (insect mid-gut) form of some African trypanosomes such as T. rhodesiense and T. brucei can rely on proline and other amino acids as energy sources, (Srivastava & Bowman, 1971; Evans & Brown, 1972), the bloodstream forms make no use of amino acids for this purpose.

I.2.2. Oxidation of Fatty Acids

There is very little information concerning the oxidation of fatty acids in trypanosomes. Where such evidence is available, the rates

of utilization are found to be minimal. Dixon et al. (1971) estimated $^{14}CO_2$ formation from 1-^{14}C -palmitate by both bloodstream and culture forms of T. lewisi and T. rhodesiense, and found that the rates of evolution of CO_2 ranged from 0.008 nmoles/h/mg dry weight with bloodstream form T. rhodesiense to 0.375 nmoles/h/mg dry weight with culture form T. lewisi. These figures are extremely low as compared, for example, to the corresponding rate of glucose utilization by the former organism of 1500 nmoles/h/mg dry weight. Also, despite the rapid utilization of glycerol by many trypanosome species, the bloodstream forms of at least T. brucei and T. rhodesiense appear to be unable to cleave tristearylglycerol or tripalmitylglycerol to obtain this substrate for oxidation (Flynn, unpublished observations). It would appear, therefore, that lipids are not utilizable as energy sources by the Trypanosomatidae of the Brucei group.

I.2.3. Carbohydrate Catabolism

The major catabolic route of carbohydrate for energy production is via glycolysis and the tricarboxylic acid cycle. The tricarboxylic acid cycle functions only under aerobic conditions and leads to the breakdown of the pyruvate formed in glycolysis to CO_2 and reduced coenzymes. The reduced coenzymes are subsequently reoxidized by the electron transport chain. Under anaerobic conditions the pyruvate formed as the end-product of the glycolytic sequence of reactions is used to oxidize the NADH which would otherwise accumulate and bring glycolysis to a standstill. Thus, typical glycolysis leading from carbohydrate to lactic acid or some similar reduction product of pyruvate serves as the main energy-producing sequence of reactions under anaerobic conditions.

The classical tricarboxylic acid cycle and the electron transport chain do not appear to be universally established in parasitic organisms. On the other hand, the wide distribution of glycolytic enzymes and the demonstration of phosphorylated glycolytic intermediates within numerous parasites clearly indicates that their glycolytic sequences are in general quite similar to those found in other eukaryotic cells (see review by von Brand, 1973). There are instances, however, where some of the glycolytic enzymes are either lacking or so inactive that the classical glycolytic sequence has become modified.

I.2.4. Carbohydrate Metabolism in the Bloodstream Form of *Trypanosoma brucei*

In common with the other members of this group (*T. rhodesiense* and *T. gambiense*), the removal of exogenous carbohydrate results in a total loss of respiratory activity and cellular motility (Ryley, 1956). The incomplete oxidation of glucose is most marked in the slender forms of a natural pleomorphic infection, which appear to be identical to the monomorphic strains induced by syringe passage through rodent hosts (Flynn & Bowman, 1973). These organisms catabolise glucose aerobically to pyruvic acid (78%), the only other end-product being glycerol (von Brand, 1951; Ryley, 1956, 1962; Fulton & Spooner, 1957; Flynn & Bowman, 1973). No carbon dioxide is produced; the single mitochondrion is non-functional and the classical electron transport chain is absent. Since several enzymes of the tricarboxylic acid cycle are also either missing or present only to a quantitatively insignificant extent (Ryley, 1956; Fulton & Spooner, 1959; Bowman *et al.*, 1972), these

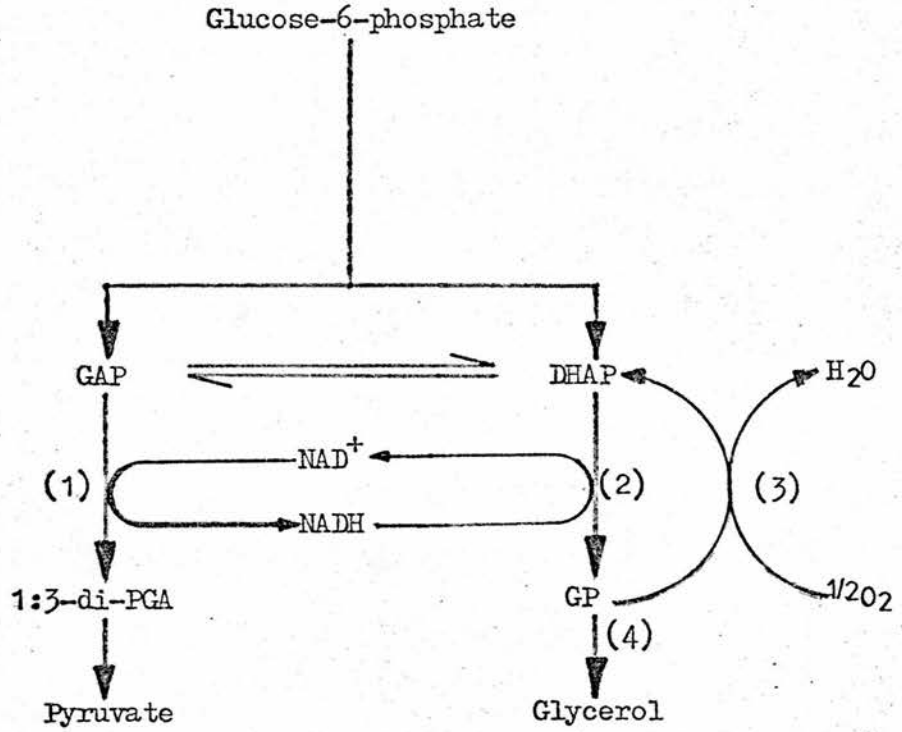


Fig. I. 1. The reaction sequence for the reoxidation of NADH generated in glycolysis in bloodstream long slender form T. brucei. GAP denotes glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 1:3 di-PGA, 1:3-diphosphoglycerate; GP, L-glycerol-3-phosphate. The figures in parentheses represent the following key enzymes: (1), glyceraldehyde-3-phosphate dehydrogenase; (2), NAD^+-dependent L-glycerol-3-phosphate dehydrogenase; (3), L-glycerol-3-phosphate oxidase enzyme system; (4), phosphatase.

organisms are totally dependent on glycolysis for their energy requirements.

The organisms differ further from other eukaryotic cells in that their glycolytic system does not utilize pyruvate to re-oxidize the NADH that is formed during glycolysis, since the enzyme, lactic dehydrogenase, appears to be absent (Dixon, 1966). Instead, these parasites utilize the dihydroxyacetone-phosphate formed in glycolysis to re-oxidize the NADH back to NAD^+. Grant and Sargent (1961) found the enzyme, an NAD^+-dependent glycerolphosphate dehydrogenase, which catalyses this reaction in T. rhodesiense.

The glycerolphosphate formed in the above reaction is re-oxidized back to dihydroxyacetone-phosphate in another reaction which requires molecular oxygen and is catalysed by an L-glycerol-3-phosphate oxidase enzyme system (Grant, et al., 1961). The relationship between glycolysis and the trypanosome glycerolphosphate oxidase system is shown in Figure I.1. It should be pointed out that only catalytic amounts of dihydroxyacetone-phosphate (DHAP) are required for the re-oxidation of NADH to NAD^+. By this coupling of glycolysis to glycerolphosphate oxidase by glycerolphosphate dehydrogenase, glucose is converted to pyruvate with trace amounts of glycerol and the redox balance is maintained.

Under anaerobic conditions, Grant and Fulton (1957) showed that one mol each of pyruvate and glycerol accumulate per mol of glucose metabolised by T. rhodesiense. In these circumstances, DHAP becomes the terminal acceptor and substrate (as opposed to catalytic) amounts of DHAP are reduced to glycerolphosphate and hence glycerol, presumably by the action of a phosphatase (Harvey, 1949).

Brucei group
The trypanosome glycerolphosphate oxidase, unlike that of insect flight muscle (Sacktor, 1961) and mammalian brain mitochondria (Sacktor, et al., 1959; Ringler & Singer, 1959), reacts with oxygen without the intervention of pyridine nucleotide coenzymes or of cytochromes. The L-glycerol-3-phosphate oxidase enzyme complex found in T. rhodesiense is insensitive to inhibitors of the mammalian cytochrome system such as cyanide, azide, amytal and antimycin A and is probably not coupled to the phosphorylation of ADP (Grant & Sargent, 1960). In the long slender form of the African trypanosomes there is then a net formation of only two molecules of ATP by substrate level phosphorylation occurring in the metabolism of glucose to pyruvate. These phosphorylations are the sole energy-trapping reactions so far observed for the bloodstream trypanosomes of the Trypanozoon subgenus; which is probably the reason why their rate of glucose consumption is so uncommonly high, corresponding to about 50-100% of their dry weight in 1 hr under in vitro conditions (von Brand, 1951).

SOME PROBLEMS ASSOCIATED WITH SUBCELLULAR FRACTIONATION BY DIFFERENTIAL CENTRIFUGATION

Introduction

The earlier methods employed to study subcellular organelles included histochemical techniques (Gomori, 1945) and the submicro-techniques of Linderstrom-Lang (1938) and Holter (1946). The former, aside from the possible artifacts introduced by the necessary procedures of freezing or fixation, are severely limited by their dependence on microscopic visualization for the localisation of an enzymic reaction or a chemical compound; as a result the method usually employs indirect procedures of analysis which cannot readily be quantitated and generally entails the possibility of still further artifacts. The latter technique, which most nearly approaches the ideal cytochemical tool in that it is aimed at the study of single cells and portions thereof, was not sufficiently sensitive to be applied to single mammalian cells.

The method of differential centrifugation of broken cell suspension was introduced by Bensley and Hoerr (1934), who described the isolation of mitochondria from guinea pig liver. Following their work, numerous publications appeared dealing with the isolation of individual cell components. Many dealt with the isolation of nuclei (Behrens, 1932; Dounce, 1943). Others described improvements in the procedures for the isolation of mitochondria (Claude, 1941, 1943, 1944, 1946, 1947-48; Hogeboom, et al., 1948), while still others dealt with the isolation of submicroscopic particles (Claude, 1946-48; Hogeboom, et al., 1948; Lazarow, 1943). Later papers described procedures for the complete fractionation of a tissue into

nuclei, mitochondria, submicroscopic particles, and a soluble material (Schneider, 1946, 1948; Schneider & Hogeboom, 1950). In spite of its obvious versatility, the cell fractionation technique had its limitations which even the early workers recognised. Indeed some investigators (Bradfield, 1950; Chambers, 1950; Danielli, 1946) took the view that the procedure of cell fractionation could be dismissed outright on the grounds that the very act of cell rupture necessarily produces artifacts such as redistribution, adsorption, and morphological alteration. There is no doubt that these objections were, and still are, valid; it is therefore important that such artifactual effects are recognised and, wherever possible, adequately accounted for.

Because biological systems differ very widely in their resistance to rupture, an array of diverse methods has been devised to disrupt cells. Thus, mechanical devices such as meat grinders, tissue mincers, the Waring Blendor and various grinding techniques have been used to shear through tough materials like plant cell walls; pressure devices like the filter press, or the method of nitrogen cavitation are widely used to explode cells in order to release their contents; chemical compounds like the surfactant agents, Triton X-100 and digitonin, are commonly employed to disrupt cell membranes by disorganising the delicate interaction between the polar and non-polar components of the membrane, while non-polar solvents such as acetone simply dissolve away the non-polar units in a membrane. The force of vibration has also been applied in the method of sonication, while autolytic enzymes have been employed to break down specific chemical groups in the cell wall of microorganisms. One obvious drawback of any scheme of cell

disruption is that since all biological membranes are thought to possess the same basic structure, it is virtually impossible to devise a method whose disruptive activity can be selectively directed towards the outer cell membrane thus leaving subcellular organelles completely intact.

Another problem associated with cell fractionation is that when cells are disrupted, their structural components are released into abnormal surroundings which are likely to affect the integrity of the subcellular components adversely. Hogeboom et al. (1948), for example, showed that the cytological and morphological properties of liver mitochondria are profoundly affected by the composition of the medium in which the liver cells are disintegrated. These authors observed that only when the cells were disrupted in hypertonic sucrose solutions was the morphology of the mitochondria maintained.

A second problem, namely, the possibility of adsorption of soluble material on cellular particles has been approached experimentally by washing the isolated particulate structures in various media. It is assumed that if the material is tightly bound to the structure and not adsorbed, repeated washing should fail to decrease either its total amount or its concentration (Schneider, 1948; Hogeboom, 1949). A consideration of the quantitative aspect of the intracellular distribution of the substances in question also has a decided bearing on the problem of adsorption. Thus, if a large percentage of the substance present in the whole tissue can be shown to be localised in a single fraction, then the probability of adsorption would appear to be minimal. On the other hand, the finding of a small percentage of a substance in a fraction must be seriously considered as an adsorption pheno-

menon. This need for establishing balance sheets in which the summation of the activities of the tissue fractions is compared with that of the whole tissue has been emphasized repeatedly (Hogeboom et al., 1946; Schneider, 1946, 1949; Holter, 1946).

A careful study of the early cell fractionation methods highlights yet another problem associated with this technique. Subcellular particles have been traditionally characterized by their morphology and/or behaviour in a centrifugal field. Until centrifuges were designed which could achieve high centrifugal force, it was the common practice to consider any cellular component which failed to sediment with the mitochondria and nuclei as 'soluble' (see for example, Sacktor, 1955).

This problem was fully appreciated by Chantrenne (1947) when he observed that "the cytoplasm of the liver cell was a heterogeneous collection of particles of varying size, enzymatic activity and chemical composition". Chantrenne concluded from his experiments that the separation of liver particles into large granules (mitochondria) and microsome fractions was a partly arbitrary procedure. Also, since the liver homogenates were prepared in electrolyte solutions, it seems probable that a large proportion of the large granules was lost during the initial centrifugation used to prepare the nuclei and cell-free extracts employed in the experiments. The fact that microsomes tend to be aggregated by electrolytes (Hogeboom & Schneider, 1950; Huseby & Barnum, 1950) would also have a bearing on such experiments. Chantrenne's views were later confirmed through the pioneering work of Hogeboom et al. (1948) who employed sucrose as a medium for differential centrifugation of liver homogenates, and succeeded in showing the distribution of various enzyme activities among the

fractions obtained, namely, the crude nuclear, mitochondrial (large granule), microsomal (small granule) and supernatant fractions (Hogeboom et al., 1953).

I.3. INTRACELLULAR LOCATION OF GLYCOLYTIC ENZYMES

Until recently, glycolytic enzymes were assumed to be a group of soluble and unassociated proteins found in the cytosol of all cells (Lehninger, 1951; Bucher & McGarraham, 1956). A close study of the early work on the subcellular localization of these enzymes however reveals that this view of glycolytic enzymes being universally soluble may reflect, not the true physiological situation but rather such factors as (a) the limitations of the fractionation techniques, or (b) interpretation based on insufficient knowledge.

For example, the limited power of the early centrifuges inevitably helped to create the situation where authors regarded the post-mitochondrial supernatant of cell homogenates as containing only soluble material (Abood, et al., 1952; Sacktor, 1955). To complicate the situation, the technique of subcellular fractionation was bedevilled by poor standardisation right from its inception (Roodyn, 1972). As a result, any finding which appeared to contradict the early concept of soluble glycolytic enzymes was understandably viewed with suspicion (see, for example, Brody & Bain, 1952; Aldridge, 1957; Tanaka et al., 1963; Mayer, et al., 1966).

Although it is still possible to encounter such objections, the various refinements which have been made in subcellular

fractionation techniques, have permitted the isolation of submicroscopic particles containing some of the enzymes previously thought to be soluble. Thus, the pioneering work of Hogeboom et al. (1948) in the use of sucrose as a medium for differential centrifugation of liver homogenates, led to the discovery in de Duve's laboratory by Hers et al. (1951) that the enzyme glucose-6-phosphatase is located in the microsomal fraction of liver homogenates. Since then, many workers have shown that various glycolytic enzymes are associated with subcellular entities in cells from a wide range of biological systems.

The Association of Glycolytic Enzymes with Cell Organelles or Membranes

I.3.1. Hexokinase

Hexokinase has been shown to be associated with subcellular particles from such tissues as the brain (Crane & Sols, 1953; Wilson, 1968), muscle (Hernandez & Crane, 1966; Sigel & Pette, 1969; Amberson & Bauer, 1971; Arnold et al., 1971; Mowbray & Ottaway, 1973), tumour cells (Rose & Warms, 1967), adipose tissue (Spydevold & Borreback, 1968) erythrocyte membranes (Green et al., 1965) and platelets (Doery et al., 1970). The subcellular organelles that have been implicated in this association include mitochondria (Johnson, 1960; Beattie et al., 1963; Bachelard, 1967) and microsomes (Spydevold & Borreback, 1968).

There is evidence to show that the degree of association of hexokinase with subcellular particles depends on a wide range of factors, some experimental and others probably genetic. Thus Wilson (1968), among others, has shown that the particulate hexokinase

activity of rat brain can be solubilised by changes in the levels of certain common metabolites or chemical substances in the incubation medium; these substances include glucose-6-phosphate, ATP, ADP, AMP and various chelating agents and high salt concentrations. Mayer et al. (1966) showed, on the other hand, that the localisation of myocardial hexokinase may reflect, not only the medium which is used for homogenization and fractionation, but also the species from which the heart was obtained.

I.3.2. Phosphofructose Kinase

This enzyme has been observed in the particulate form in cells of the sheep heart and rabbit muscle; in both tissues, the enzyme activity could be solubilized by means of $MgSO_4$ treatment. The solubilization is thought to result from dissociation of the particulate enzyme into subunits in an equilibrium (oligomer $\rightleftharpoons$ subunit) reaction, the position of which is dependent on pH, protein concentration or the presence of substrates and modifiers. Although there appears to be some disagreement on the size of the smallest fully active subunit (Uyeda & Racker, 1965; Parmeggini et al., 1966; Paetkau & Lardy, 1967) there is general agreement that disaggregation tends to reduce or abolish the enzyme activity.

I.3.3. Aldolase, Glyceraldehyde-Phosphate Dehydrogenase, Phosphoglycerate Kinase and Pyruvate Kinase

Tillmann et al. (1975) studied the organisation of glycolytic enzymes in isolated ghost membranes of human erythrocytes and

confirmed the earlier finding by other workers (Mitchell et al., 1965; Shrier, et al., 1966; Duchon & Collier, 1971) that some glycolytic enzymes are firmly bound to the membranes of the erythrocyte. Tillmann and his associates found four enzymes, aldolase, glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase to be so firmly attached to the erythrocyte membrane that treatment with toluene failed to desorb them completely.

It is interesting to note that even in the liver (in which the apparent soluble nature of all the glycolytic enzymes led to the classical concept of the universal solubility of glycolytic enzymes), Roodyn (1956a and 1956b) found aldolase to be associated with the nuclei. In another report, Roodyn (1957) observed that the particulate aldolase could be desorbed by such factors as long homogenization of the liver, delay in the isolation of the nuclei or high salt concentrations. It was concluded that salt linkages between the enzyme protein and some binding site (presumably nucleic acid), and not the integrity of the nuclear membrane, were responsible for the retention of aldolase within the nucleus.

I.3.4. Other Enzymes of Glycolysis

Of the remaining enzymes involved in the glycolytic sequence of reactions, the FAD-dependent glycerolphosphate dehydrogenase has been shown to be bound to the mitochondria (Green, 1936; Ringler & Singer, 1959; Ito & Horie, 1959), the sarcosomes of insect flight muscle (Estabrook & Sacktor, 1958) and the mitochondrion of the long slender bloodstream form of T. brucei (Opperdoes, 1977b); lactic

dehydrogenase is thought to be strongly bound to the actin-tropomyosin-troponin complex of skeletal muscle, along with phosphofructose kinase, aldolase, pyruvate kinase, glyceraldehyde-phosphate dehydrogenase and phosphoglucose isomerase while triosephosphate isomerase, phosphoglycerate mutase, enolase and hexokinase showed less adsorption to the muscle structural proteins (Clarke & Masters, 1975).

Thus, it is now generally recognised that like the enzymes of other major metabolic pathways, the glycolytic enzyme system is probably compartmentalized in the cell. This has recently been elegantly demonstrated by Kempner and Miller (1968) who used centrifugal stratification to demonstrate that all the enzymes of the major metabolic pathways of Euglena gracilis are associated with particulate fractions of the cell.

I.4. GLYCOLYTIC ENZYMES AS A MULTIENZYME SYSTEM

A common feature of metabolic pathways in intermediary metabolism is that the product of one enzyme in the sequence is the substrate for the next. One of the advantages of compartmentalized metabolic systems is the improved efficiency of the overall metabolic activity resulting from the relatively high concentration of the substrates maintained within the microenvironment of the enzymes. Free diffusion in the interior of the cell would also adversely affect the efficiency of a metabolic pathway through competition by enzymes operating in different pathways for the same substrate. Teleologically, therefore, compartmentalisation or localisation of all the enzymes involved in a particular metabolic sequence of reactions would be an advantage.

In spite of the obvious advantages inherent in such a system, however, the degree of intramolecular organisation within the glycolytic sequence is a topic which has generated a great deal of controversy. In a classic attempt at resolving this question, de Duve (1972) applied exacting sedimentation criteria to the behaviour of enzymes from rat liver and failed to uncover any evidence for the presence of a glycolytic particle, and concluded "that the enzymes of this sequence are molecularly dispersed in the cytosol of the cell."

On the other hand, Clarke & Masters (1974) applied de Duve's criteria to the myogen system and established the presence of an association of the glycolytic enzymes in the extracts of the rat and also in ovine skeletal muscle. The association was found to be sensitive to variations of pH and concentrations of proteins and specific metabolites such as ATP and fructose-1, 6-diphosphate. For example, the size of the complex of glycolytic enzymes was observed to increase with increasing protein concentration. Clarke and Masters therefore concluded from these experiments that the previous failure to substantiate the existence of glycolytic enzyme aggregates under isotonic conditions was probably due, at least in part, to the relatively low protein concentrations of the preparations studied.

Green et al. (1965) have also prepared membranes from beef erythrocytes and broken cells of Saccharamyces cerevisiae which catalysed the complete glycolytic sequence of reactions without supplementation with enzymes. These authors found that the conditions of isolation critically affected the binding of the individual glycolytic enzymes with the erythrocyte membrane. However, the glycolytic enzymes of the yeast exhibited no such

dependence of their binding to the membrane on the conditions of isolation.

The glycolytic complex of enzymes bound to the erythrocyte membrane was also resolved, and reassembled, by a single cycle of pH change. Green and his associates concluded from these experiments that in the intact cell the complete glycolytic complex of enzymes is associated with the plasma membrane and is not in free solution.

Balazs and Lagnado (1959) have shown that about 10% of the total glycolytic activity of the rat brain is associated with the mitochondrial fraction, and several washings of the mitochondria with 0.25 M sucrose solution failed to diminish the specific activity. When the mitochondria were extracted with Krebs-Ringer phosphate solution, the glycolytic activity was reduced by about 50%; this loss was found to be due mainly to the removal of triosephosphate dehydrogenase. Also, Mowbray and Moses have recently (1976) made a tentative report on the isolation from E. coli of a multienzyme complex capable of catalysing the whole sequence of glycolytic reactions.

It is therefore apparent that at least in some biological systems, the glycolytic enzymes probably exist as organised multienzyme systems formed either through association of the component enzymes among themselves, or with subcellular particles.

I.4.1. Efficiency of Complex Enzyme Systems in Catalysing Coupled Reactions

Following the various suggestions that the localisation of the enzymes involved in a specific metabolic sequence of reactions

may have metabolic advantages (see for example Green et al., 1965; Reed & Cox, 1966; Hubscher et al., 1971), there has been increasing effort to find out how the kinetics of enzymes that are immobilized on membranes or in subcellular particles differ from those of soluble enzymes; and such authors as McLaren & Packer (1970), Laidler & Sundaram (1971) have already demonstrated that the kinetic behaviour of enzymes in heterogeneous systems can be significantly different from that in dilute solution.

By immobilising enzymes on solid supports (Goldman et al., 1971) it has become possible to study enzyme reactions under conditions which are probably more representative of those prevailing in living systems. Thus, Mosbach & Mattiasson (1970) and Mattiasson & Mosbach (1971) have succeeded in preparing a two-enzyme system consisting of hexokinase + glucose-6-phosphate dehydrogenase and also a three-enzyme system made up of β-galactosidase + hexokinase + glucose-6-phosphate dehydrogenase. By comparing the kinetic behaviour of the matrix-bound enzyme system with that of the analogous system consisting of the enzymes unbound and in solution, these authors were able to demonstrate (1) that the overall rate of coupled reactions catalysed by the matrix-bound enzyme systems was higher prior to reaching steady state than the rate of the reactions catalysed by the corresponding soluble systems, and (2) that the observed increased reaction rate is enhanced by increasing the number of enzymes involved in the metabolic sequence.

Impressive though this method of binding enzymes to synthetic matrices may be, it is bound to meet the criticism that it does not represent in vivo conditions. In this regard, the glycolytic enzyme system isolated by Mowbray & Moses (1976) or the multienzyme

complex reported in this work may prove to be a useful alternative for such studies.

I.5. MICROBODIES AND PEROXISOME-LIKE ORGANELLES IN KINETOPLASTID FLAGELLATES

The occurrence of a special type of cytoplasmic particle containing hydrogen peroxide-producing oxidases and catalases was first observed in the liver by de Duve et al., (1960). These organelles were later found to be widely distributed in nature, being present in the cells of plants (glyoxysomes) and animals (peroxisomes) as well as in protozoa, where they have been referred to as microbodies. They contain one or more oxidative enzyme systems and appear, like mitochondria, to have a role in the oxidation of reduced pyridine nucleotides, though these oxidations are not coupled to ATP production (reviewed for Protozoa by Müller, 1975). According to Müller et al. (1968), peroxisomes may represent a repository particle with a very long evolutionary history, the importance of which decreased progressively as its functions were taken over by the more efficient mitochondria, except in certain types in which the requirements of special functions, such as gluconeogenesis or photosynthesis, have made its retention advantageous.

In possible support of the suggestion by Müller et al., was the finding by Levy (1970) that the level of peroxisomal enzymes (lactate oxidase and isocitrate lyase) of Tetrahymena depended upon the degree of anaerobiosis of the culture. The specific activity of both enzymes was high in cells from oxygen deficient cultures, and low in cells from aerated cultures.

The method commonly employed for the purification of peroxisomes is based on the equilibrium density of the particles in sucrose gradient, after the appropriate subcellular fraction has been prepared by differential centrifugation of the cell homogenate. The peroxisomes are heavier than mitochondria and they have a median equilibrium density of 1.20-1.25 g/ml sucrose (Beaufay, et al., 1964).

Microbodies of the Kinetoplastida present a unique problem. A crucial characteristic of the Kinetoplastida is the possession of the kinetoplast, to which a functional mitochondrial system is connected in most forms. In the most advanced kinetoplastid group (Trypanosoma brucei and related species), a functional mitochondrion is present only in certain stages of the life cycle and disappears from the long slender form living in the bloodstream of the host (Vickerman, 1971). This morphological change is accompanied by major changes in the metabolism. Microbodies are present whether or not the mitochondrial system is there.

Microbodies of T. brucei (Steiger, 1973) and of T. equiperdum (Warton et al., 1973) are of typical structure, often with a crystalline core. In culture forms both the size and relative volume increases (Müller, 1975), which probably suggests that deficiency in oxygen favours their development; a conclusion drawn by Levy (1970) from his studies of Tetrahymena microbodies.

Peroxidase activity was observed in T. brucei (Steiger, 1973) but not in T. equiperdum (Kallinikova & Warton, 1972). Although various workers have suggested the presence of L-glycerol-3-phosphate oxidase in T. brucei microbodies (Ryley, 1964; Vickerman, 1965; Bayne, et al., 1969; Muse, et al., 1970; Clarkson, 1975), Opperdoes, et al. (1977b) have used isopycnic sucrose gradient centrifugation to show that this enzyme is exclusively located

in the mitochondrion, and not the microbodies, of the parasite. They found, instead, that it is the NAD^+-linked glycerolphosphate dehydrogenase which is located inside the microbodies of the bloodstream long slender T. brucei.

The microbodies of the higher trypanosomatids are entities biochemically distinct from other known organelles. Their evolutionary relationship, if any, to microbodies of other aerobic protozoa is not yet known (Müller, 1975). Microbodies of typical structure and showing 3,3'-diaminobenzidine (DAB) positive reaction are also present in lower trypanosomatids (Cohen, 1972; Muse & Roberts, 1973). In Crithidia fasciculata grown on a defined medium, the microbodies occupy about 2.5% of the total cell volume (Cohen, 1972). The strong pellicle of the cells reinforced by microtubules makes the homogenisation of these organisms difficult which has made attempts to characterise their microbodies difficult (Cohen, 1972). Catalase has an unusually high activity in C. fasciculata (Wertlieb & Guttman, 1963) and C. luciliae (Eeckhout, 1972). Catalase in homogenates of these organisms is largely non-sedimentable. The particles may have some catalase that cannot be detected because of the very high activity in the non-sedimentable cytoplasm. The evidence available is therefore not sufficient to permit definition of the biochemical nature of the microbodies in lower trypanosomatids and their relations to other types of microbodies.

I.6. SURAMIN INHIBITION OF TRYPANOSOMAL ENZYMES INVOLVED IN GLUCOSE CATABOLISM

Issekutz (1933) and Glowazky (1937) reported the effect of suramin in depressing respiration and glycolysis in trypanosomes. Since then, the compound has been observed not to cross the red cell membrane (Wilson & Wormall, 1949). One would therefore have expected a great deal of effort to have been made to try and link the effect of suramin both as a prophylactic and curative agent against trypanosomal infections with its possible inhibition of the activity of the glycolytic enzymes of these organisms.

Yet, to date, the only enzyme linked to glycolysis in trypanosomes which has been shown to be inhibited by suramin at a curative dose is the glycerolphosphate oxidase complex of the bloodstream form of T. brucei (Fairlamb & Bowman, 1975, Bowman & Fairlamb, 1976). It will be shown, in this thesis, that suramin is very potent against the activity of a subcellular multienzyme complex of the bloodstream form of T. brucei; and this effect of suramin probably reflects its inhibition of two of the component enzymes of the complex, namely, glycerolphosphate dehydrogenase and phosphofructose kinase.

SECTION II

MATERIALS AND EXPERIMENTAL PROCEDURES

II.1. PRODUCTION OF TRYPANOSOMA BRUCEI

II.1.1. The Strain of T. brucei Studied

A monomorphic strain of Trypanosoma brucei TREU 55 (Trypanosomasis Research, Edinburgh University) was used throughout these studies. As Figure II.1. shows this strain had been derived from a wild type T. brucei by passage through laboratory mice to give strain TREU 1 which was subsequently passaged through rats to give strain TREU 55. This strain is completely monomorphic, producing a rapidly fulminating infection, which results in the death of its rodent hosts within 3-4 days after inoculation.

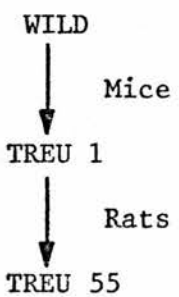


Fig. II.1

II.1.2. Host Animals Used

Throughout these studies, Wistar strain male white rats were used as the host animals for the T. brucei infection.

II.1.3. Stabilate Production and Establishment of Infection

Infection was established by intraperitoneal injection into rats of stabilates of T. brucei TREU 55 that had been stored in 7.5% glycerol (v/v) at -196^{o}C. Stocks of these stabilates were prepared

in the manner described below.

Infected rat blood was collected by cardiac puncture and taken into heparinised Krebs saline (5 I.U. heparin/ml). The pooled infected blood was made 7.5% (v/v) with glycerol, thoroughly mixed and dispensed into glass capillaries, approximately 10 cm in length and 1 mm internal diameter. After sealing the ends in a flame, the capillaries were left to cool to room temperature before being transferred to a bath of ice-cold methanol to ensure further cooling. Thereafter, they were packed in polystyrene to increase the time required for complete cooling and then slowly cooled to $-70^{\circ}C$ in solid carbon dioxide. After two days, the ampoules were removed from the solid carbon dioxide and stored in liquid nitrogen at $-196^{\circ}C$.

For infection of a batch of eight rats the contents of two such ampoules were usually diluted with 4 ml citrate-saline and 0.5 ml of the diluted stabilate was injected intraperitoneally into each animal.

II.1.4. Maintenance of Infection

Once the infection was established the strain was maintained by syringe passage every three days as follows. Infected blood from the tail of a parasitised animal was diluted with citrate saline so that between 5 and 10 parasites were visible under the high power (252×) of the light microscope. Approximately 0.5 ml of the diluted infected blood was routinely injected intraperitoneally into each rat. Under these conditions, the maximum parasitaemia occurred on the third day after injection.

II.1.5. Production of Rabbit Antibody to Rat Erythrocytes

Rabbit anti-rat erythrocyte serum was prepared by subjecting a rabbit to a course of six injections of 1 ml over a period of three weeks, each dose consisting of 50% (v/v) suspension of washed rat erythrocytes in Krebs saline. The injections were given via a marginal ear vein and when the rabbit serum titre against rat erythrocyte gave a titre of over 1/1,000, the rabbit was anaesthetised and bled via the femoral artery. The blood was allowed to clot at room temperature for 5 hours and the retracted clot was removed by centrifugation. The clear serum was then dispensed in 2 ml aliquots and stored at -20°C. The activity of this stabilate was not markedly decreased over the longest period of storage, of 1 year.

II.2. PREPARATION OF PARASITES FREE FROM BLOOD ELEMENTS

Parasites were harvested from three-day post infected rats. The technique used to prepare samples of trypanosomes which were practically free from contamination by platelets and other blood components was that of Flynn & Bowman (1972).

Infected blood was withdrawn from rats by cardiac puncture and diluted with citrate-saline solution containing about 200 mg % (w/v) of glucose at 1 ml per ml blood. Calcium chloride (1 ml of 0.2 M) was added to each 10 ml of citrated blood, with continuous shaking in the presence of glass beads, and the shaking was continued until the fibrin clot had separated. After filtration through glass wool the blood was centrifuged at 1,000 g for 10 min at 4°C

and the trypanosome layer removed and resuspended in Krebs saline-glucose buffer.

Rabbit anti-rat erythrocyte serum was then added (at the rate of one drop per ml) and the suspended cell preparation allowed to stand for 10 min at room temperature. The agglutinated red cells were packed by a 1-min spin at 1,000 g and the trypanosome-containing supernatant was then removed with a Pasteur pipette. The remaining clumped erythrocytes were eliminated along with the leucocyte and platelet contaminants by filtering the material through a No. 3 sintered glass funnel which had previously been roughened by treatment with a 1:1 (v/v) mixture of concentrated nitric acid and ethanol; this treatment increased the efficiency of the sintered discs in the removal of the blood contaminants. A further centrifugation at 1,000 g for 10 min at 4°C in a conical centrifuge tube usually led to only a few contaminating red cells in the preparation which appeared as a small stub of erythrocyte and trypanosome mass at the bottom of the centrifuge tube. By sacrificing this layer, completely pure preparations could be obtained as shown by the absence of lactate dehydrogenase (Dixon, 1966) or examination under the light microscope.

Resuspension was normally in Krebs saline-glucose solution; in the absence of glucose, the trypanosomes could remain viable in Tris-sucrose buffer (provided they were kept at 0°C) for no longer than 10 min before some began to disintegrate.

The list of chemicals and the composition of buffers employed throughout this work can be found on page 38.

II.3. CELL RUPTURE AND PREPARATION OF EXTRACTS FOR SUBCELLULAR FRACTIONATION

It has already been established (Simpson, 1972) that in isotonic media the Kinetoplastida are resistant to cell rupture by conventional techniques. Different methods were therefore used to help break the cell wall of the T. brucei and the effect that each method had on the activities of the glycolytic enzymes was studied. The methods selected were (i) the use of lytic agents, (ii) freezing and thawing and (iii) cell grinding with abrasives.

Unless otherwise stated all experiments on cellular treatment and subcellular fractionation were performed at 4°C or on ice.

II.3.1. Cell Disruption with Lytic Agents

The lytic agents used were saponin, digitonin and Triton X-100. A solution of the appropriate lytic agent in Tris-sucrose buffer (p.38) was added to a suspension of T. brucei cells in the same buffer. The mixture was then kept on ice for 20 min with periodic mixing by means of a Pasteur pipette. The final concentrations of the lytic agents in the incubation mixtures were:

Saponin, 0.5% (w/v)

Digitonin, 0.5 mg% (w/v)

Triton X-100, 0.5% (w/v)

II.3.2. Cell Disruption by Freezing and Thawing

A suspension of T. brucei cells in Tris-sucrose buffer was completely immersed in a bath of liquid nitrogen at -196°C until

the sample was completely frozen. It was then placed under cold running tap water until it was completely thawed. The process was repeated twice to kill the cells. On some rare occasions when this cycle of freezing and thawing failed to kill the cells, the process was repeated until virtually all the cells had become immotile.

II.3.3. Samples for Determining Enzyme and Protein Leakage

Samples for determining the extent of leakage of enzymes and other proteins were prepared as follows. Treated cell suspensions containing immotile whole trypanosomes were sedimented in an MSE Mistral 4L centrifuge in a swing out bucket rotor at 1,000 g for 20 min; the supernatant was carefully removed with a Pasteur pipette. The sedimented cells were washed and the procedure was repeated twice; the supernatants were pooled together and kept as sample 'L'.

II.3.4. Samples for Determining Total Enzyme Activity and Total Protein

Samples for the determination of total enzyme activity were obtained by homogenizing aliquots of treated cells which had not been previously subjected to sedimentation to remove the material which had leaked out of the cells. Such samples were referred to as 'T'.

II.3.5. Preparation of Cell Homogenates After Washing Treated T. brucei with Tris-Sucrose Buffer

The sediment of T. brucei cells obtained after the preparation of sample 'L' was resuspended in Tris-sucrose buffer (5 ml buffer per ml packed cell colume, PCV), and the suspension was subjected to homogenization by 40 strokes with a hand-operated Dounce homogenizer equipped with a tight fitting glass or perspex ball-type pestle (Wesley Coe, Cambridge, Ltd.). The homogenates were routinely examined by phase-contrast microscopy and if cell disruption appeared incomplete, homogenization was repeated by another 20-40 strokes of the pestle to ensure a more complete breakage of the cells.

II.3.6. Rupture of T. brucei Cells by the Use of Abrasives

Two abrasives, alumina and silicon carbide, were employed for grinding the cells.

Alumina (Koch-Light Labs., Ltd.: Alumina "Woelm" neutral TLC adsorbent) was first washed with distilled water and dried in an oven before use. Sufficient quantities of chilled alumina were stirred into a suspension of T. brucei in Tris-sucrose buffer to give a non-running paste of cells in alumina. The mixture was then subjected to grinding until examination by phase-contrast microscopy showed that practically all the cells had been disrupted.

Cell rupture with silicon carbide was similarly achieved by adding chilled silicon carbide abrasive grain (Crystalon: Norton Company: grit No. 37C 400) to a chilled mortar containing a suspension (2 ml buffer/ml PCV) of T. brucei cells in Tris-sucrose

buffer (approximately 5 g Crystalon/ml PCV); the cells were then ground with a chilled pestle until examination by phase-contrast microscopy again showed that practically all the cells had been disrupted.

II.3.7. Preparation of Extracts for Subcellular Fractionation

After the grinding, the method used for preparing material for subcellular fractionation was the same whether the grinding was performed with alumina or silicon carbide. To the paste of abrasive and disrupted cells was added just enough Tris-sucrose buffer to assist transfer of the contents of the mortar into a 50 ml centrifuge tube. Small quantities of the buffer were then employed to wash any remaining paste sticking to the mortar.

The abrasive was then sedimented by a 5 min centrifugation at 100 g in an MSE Mistral 4L centrifuge using the 8 x 50 ml swing out bucket rotor. The supernatant was carefully decanted; the sediment was then washed with the minimum volume of the Tris-sucrose buffer and the centrifugation repeated. This process was repeated until examination of the supernatant and the sediment showed that the bulk of the subcellular organelles had been removed from the sediment - this was usually achieved by three washings of the sediment. The pooled supernatants served as the stock material for subcellular fractionation studies.

II.4. SUBCELLULAR FRACTIONATION OF CELL HOMOGENATES

As used in this thesis the term 'homogenate' (which was the starting material for subcellular fractionation) refers either to

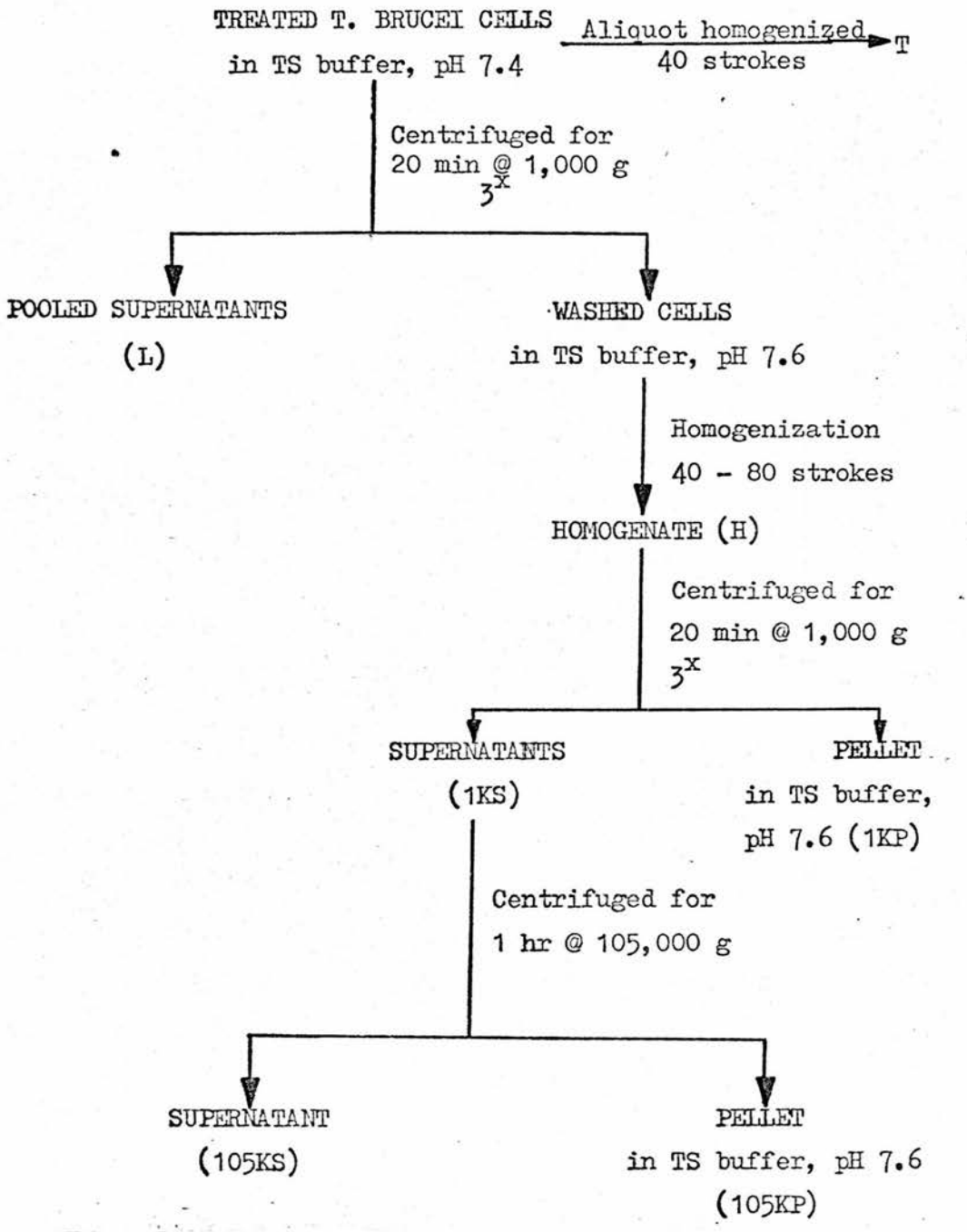


Fig. II. 2. Procedure for preparing samples from bloodstream long slender forms of T. brucei which had been lysed through incubation of the cells in 0.5% saponin, 0.5% Triton X-100, 0.5 mg% digitonin, or by three cycles of freezing and thawing. Experimental details have been given in the text.

(a) the homogenized suspension of the pellet obtained by 1,000 g centrifugation of cells which had either been treated with lytic agents or by freezing and thawing, or

(b) the material obtained after removal of the abrasive by centrifugation, in the case of cells disrupted by means of grinding.

For subcellular fractionation, a homogenate of T. brucei was centrifuged at 1,000 g max for 20 min in an 8 x 50 fixed angle rotor in an MSE 18 centrifuge at 4°C. The pellet was resuspended in the Tris-sucrose buffer and the centrifugation was repeated twice. The final pellet, which usually contained no whole cells but many nuclei and a mesh of flagella entrapping other subcellular particles, was resuspended in the Tris-sucrose buffer and referred to as sample IKP.

The supernatants were pooled together and subjected to centrifugation at 105,000 g_{max} for 1 hr in the Spinco Ultracentrifuge using the fixed angle rotor type 40. at 4°C. The pellet was resuspended in the Tris-sucrose buffer; this material and the supernatant were referred to respectively as samples 105KP and 105KS.

In some of the preparations, the supernatant obtained after the 1,000 g centrifugation was first subjected to centrifugation at 14,500 g max in the MSE 18 centrifuge in a fixed angle rotor for 20 min at 4°C. The resuspended pellet was referred to as sample 14.5KP. In such a preparation, it was the supernatant from the 14,500 g centrifugation which was fractionated in the Spinco to give samples 105KP and 105KS. The flow diagrams in Figure II.2 and Figure IV.1 summarise the fractionation procedures.

Table II. 1. Proportions of Solutions Used for Making Linear Sucrose Gradients. Experimental details for gradient preparation are given in the text.

		Volume (ml)	Sucrose molarity	Volume per tube (ml)
Gradient Formation	Mixing chamber	40	0.8	46
	Reservoir	40	2.5	
Cushion	Mixing chamber	20	2.5	5
Sample Layer	14,500 g pellet	-	0.25	2-4
Overlay		-	0	2-5 **

** Buffer solution consisting of 10mM potassium phosphate buffer, pH 7.6, containing 0.1mM dithiothreitol and 0.1% sodium azide.

II.5. ISOLATION OF THE MULTIENZYME COMPLEX OF BLOODSTREAM LONG SLENDER T. BRUCEI

The multienzyme complex activity was concentrated in the differential centrifugation subcellular fraction 14.5KP, isolated from homogenates obtained by lysing T. brucei cells either in 0.5 mg% digitonin, by three cycles of freezing and thawing, or by grinding the cells with alumina or silicon carbide. The details of the isolation technique are given in Section IV.

II.6. ISOPYCNIC SUCROSE GRADIENT CENTRIFUGATION

Linear sucrose density gradients from 0.8-2.2 M sucrose in 0.01 M potassium phosphate buffer, pH 7.6, containing 0.1 mM dithiothreitol and 0.01% sodium azide (KPDA buffer solution) were prepared at room temperature as follows. The reservoir of a conventional 2 x 60 ml gradient maker was filled with 40 ml of 2.5 M sucrose in the KPDA solution and the mixing chamber with 40 ml of the same buffer containing 0.8 M sucrose. The outflow of the mixing vessel was connected to a peristaltic pump. By connecting the pump outlet to a 60 ml cellulose nitrate centrifuge tube, it was possible to pump solution of increasing concentration of sucrose into the centrifuge tube; and in this way, reproducible linear gradients could be prepared. Table II.1. gives details of the volumes of solution used.

The gradients were cooled in an ice bath or in the cold room and were loaded with the samples to be centrifuged in 0.25 M sucrose. Each tube was then weighed and sufficient (2-5 ml)

KPDA buffer was carefully layered on top to serve both as an overlay and counter balance.

Centrifugation was performed with the Beckman 12-65B ultracentrifuge, using the SW 25.2 swing-out bucket rotor, at 23,000 rpm (i.e. 40,000 g min, 95,000 g max, or 65,000 g average) for 120 min. Thereafter, the gradients were fractionated in the cold room by means of the following bottom-sampling method. A fine bore metal tube was carefully inserted to the bottom of the centrifuge tube and the gradient was syphoned out by a peristaltic pump connected to a Uvicord analyser; the drop fractions were collected with an LKB Ultrorac fraction collector. The refractive index of the fractions was determined by an Abbe refractometer and converted to sucrose density (d_4^{20}) using data obtained from the International Critical Tables. The protein content of each fraction was determined either from the ratios of E_{260}/E_{280} by the method of Warburg and Christian (1942) or the protein-dye binding method of Bradford (1976).

II.7. BIOGEL COLUMN CHROMATOGRAPHY

Columns (30-60 cm in length, approx. 250 to 600 ml volume) of Biogel agarose (Calbiochem Ltd., Bio-Rad Laboratories, Richmond, California) were prepared according to the manufacturer's instructions and equilibrated with the appropriate buffer solution by washing with at least two column volumes of the buffer solution containing 0.01% sodium azide. Gel exclusion volumes were determined using Blue Dextran.

II.8. ACRYLAMIDE GEL ELECTROPHORESIS

The method used was essentially that of Davis (1964). The lower electrode vessel of the Shandon Gel Electrophoresis Apparatus was first filled to the mark with electrophoresis buffer, the gels were placed in position in the upper vessel and this was also filled with sufficient buffer to cover the gels. After ensuring that both the top and bottom ends of the gel tubes were completely filled with buffer, the samples for electrophoresis were made 0.5 M in sucrose; bromophenol blue was added as a marker and the samples were carefully layered on top of the large pore gels with a syringe.

Electrophoresis was run at a potential of 10 v/cm for 1-2 hr, or until the bromophenol blue marker just reached the bottom of the gel. The electrophoresis was stopped and the gels were extruded by squirting buffer solution between the gel and the inner surface of the glass tubes. The protein bands were either fixed overnight with a staining solution (0.1 % Coomassie Brilliant Blue or 1% Amido Schwartz in 7% acetic acid solution) or enzyme bands were detected by incubating the gels in the appropriate assay media into which had been incorporated tetranitro blue Tetrazolium-Cl. Fixed gels were destained in 7% acetic acid solution.

II.9. PROTEIN ESTIMATION

Estimation of protein was done either by the method of Lowry *et al.* (1951), Warburg & Christian (1942) or Bradford (1976).

When the chemical methods of Lowry et al. or Bradford were used, bovine serum albumin (BSA) served as the standard protein. The standard curves were linear from 0-250 µg (for the method of Lowry et al.) and 0-60 µg (when the Bradford method was used). Sucrose did not interfere with the latter method and therefore this method or that of Warburg & Christian was the method of choice for estimating protein in samples containing high concentrations of sucrose.

II.10. ENZYME ASSAYS

Unless otherwise stated, all enzyme assays were carried out at 25°C and pH 7.6, and the assay mixtures were incubated for at least 5 min before commencing the reaction by the addition of the appropriate sample.

The assay procedure involved oxidation or reduction of pyridine nucleotides. Changes in optical density at 340 nm were followed in a Unicam SP 8000 spectrophotometer fitted with a Unicam A 25 recorder and with silica cuvettes having a 1-cm light path. Apart from the method for the assay of the multi-enzyme complex which was developed by myself, the following enzyme assays were performed, sometimes with slight modifications which have been detailed out in the appendix: hexokinase [ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1] (Grignani & Lühr, 1960); phosphoglucose isomerase [D-Glucose-6-phosphate ketol-isomerase, EC 5.3.1.9] (Slein, 1955); phosphofructose kinase [ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11] (Ling et al., 1955); aldolase [D-fructose-1,6-biphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13] (Racker, 1947); glyceraldehyde-3-phosphate

dehydrogenase [D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12] (Delbrück et al., 1959); phosphoglycerate kinase [ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3] (Vogell et al., 1959); phosphoglycerate mutase [2,3-Biphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3] (Vogell et al., 1959); enolase [2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11] (Bock et al., 1958a); pyruvate kinase [ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40] (Bock et al., 1958b); glycerolphosphate dehydrogenase [sn-Glycerol-3-phosphate: NAD 2-oxidoreductase, EC 1.1.1.8] (see Bergmeyer et al., 1974).

With the exception of glycerolphosphate oxidase, all enzyme activities were calculated from the rate of production or utilization of reduced pyridine nucleotides which was obtained from the change in optical density at 340 nm using an extinction coefficient of 6.22×10^3/mole/cm. Assays were linked to the production or utilization of 1 mol of reduced nucleotide per mol of substrate.

The method used for assaying L-glycerol-3-phosphate oxidase activity was that of Srivastava & Bowman (1971). The enzyme activity was measured polarographically at 37°C with a Clark oxygen electrode (Yellow Spring Instrument Co.) polarised at -0.8 V, the electrode being adapted to fit a perspex reaction chamber (3 ml capacity) equipped with a magnetic stirrer and connected to a potentiometric recorder. A period of 1-2 min was required for temperature equilibration. Calculation of O_2 uptake was based on dissolved oxygen concentration of 217 µM in air-saturated medium at 37°C (Kielley, 1963). Assays were routinely performed in 0.02 M Tris buffer, pH 8.0, containing BSA, 3.3 mg/ml and 13.3 mM L-α-glycerolphosphate in 3 ml total volume.

II.11. BUFFERS

Tris-sucrose (TS) buffer, pH 7.6: 0.25 M sucrose, 10 mM Tris-HCl, buffer, pH 7.6, containing 0.1 mM dithiothreitol.

TEA buffer, pH 7.6: 0.3 M Triethanolamine hydrochloride-KOH, buffer, pH 7.6.

Potassium Phosphate buffer, pH 7.6: 10 mM K_2HPO_4-HCl buffer, pH 7.6, containing 0.1 mM dithiothreitol.

BSA/Tris buffer, pH 8.0: 10 mg serum albumin, per ml 60 mM Tris-HCl buffer, pH 8.0. 1 ml of this buffer was used in a 3 ml volume for the assay of L-glycerol-3-phosphate oxidase giving a final concentration of BSA (3.3 mg/ml) and Tris (20 mM).

II.12. CHEMICALS

All chemicals were of the highest purity available ('Analar' grade or its equivalent).

All enzymes, coenzymes and substrates (with the exception of D-glucose), were purchased from C.F. Boehringer and Sohne GmbH, Mannheim. Bovine serum albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, England. All other analytical reagents were purchased from BDH Biochemicals Ltd. or Sigma Chemicals Co. Ltd.

Silicon carbide was a generous gift from Norton Abrasives Ltd., Welwyn Garden City, Herts., England.

SECTIONS III - VI

EXPERIMENTAL RESULTS

SECTION III

RESULTS OF CELLULAR DISRUPTION WITH DETERGENTS OR BY FREEZING AND THAWING FOLLOWED BY EXTRACT FRACTIONATION BY MEANS OF DIFFERENTIAL CENTRIFUGATION

Introduction

It is generally agreed that any studies aimed at providing knowledge upon which a rational approach to chemotherapy can be based must emphasize subtle differences which may exist in the metabolic functions of host and parasite, and in the structure and kinetics of isofunctional host and parasite enzymes. However, there are many instances in the literature of disagreements on such pertinent questions as the activity of enzymes, their intracellular location and the effect that various factors have on these. Thus, for example, the respiration rate obtained for all developmental forms of T. rhodesiense by Srivastava & Bowman (1971, 1972) was ten times greater than the values found by Grant et al. (1961). Although the glycolytic enzymes have generally been referred to in the past as soluble enzymes, several findings which have been reported in the recent literature indicate that at least certain glycolytic enzymes are not present in soluble form within the cytoplasm. Biological systems which have been shown to contain glycolytic enzymes not freely dissolved within the cellular environment include muscle (Sigel & Pette, 1969; Clarke & Masters, 1972; Mowbray & Ottaway, 1973), beef erythrocyte and yeast (Green et al., 1965) and trypanosomatids (Risby & Seed, 1969a, 1969b). Such discrepancies may arise from differences in cell disruption techniques as well as the conditions under which the enzymes are assayed.

The results in this section were from experiments which were aimed at answering the following questions:

1) Are the activities of the enzymes responsible for glucose breakdown in T. brucei affected by some of the well-established methods of cell lysis?

2) Do such methods of cell disruption affect the distribution of the enzymes between the soluble and particulate fractions obtained from differential centrifugation of the extracts?

3) Since it has been shown that multienzyme complexes catalyse a chain of reactions more efficiently than the same enzymes in solution (Mattiasson & Mosbach, 1971), could it be that the unusually high rate of glucose catabolism by bloodstream forms of T. brucei may be due, in part at least, to the existence of such complexes?

Four methods of cellular disruption, by saponin, Triton X-100, digitonin or freezing and thawing, were studied. Cell lysis by sonication or nitrogen cavitation was not used because both methods are known to cause disruption of subcellular organelles (Fairlamb & Bowman, 1976); nor was lysis by cell incubation in solutions containing no sucrose since Agosin & von Brand (1955) have shown thathomogenization of T. cruzi in 0.15 M KCl or distilled water gave an extract with lower succinic dehydrogenase activity than the enzyme from cells homogenized in 0.25 M sucrose.

After showing that the four methods mentioned above produce artifacts in the distribution patterns of the enzymes the much milder method of cell disruption by grinding with abrasives was used in subsequent experiments (see section IV) to study enzyme latency, the probable intracellular location of the enzymes and for the isolation of a multienzyme complex of the glycolytic system of bloodstream T. brucei.

On exposure of bloodstream T. brucei to 0.5% saponin, 0.5% Triton X-100 or after freezing and thawing three times, the cells

Table III. 1. The Effect of Disruption Procedure on the Specific Activities of T. brucei Enzymes Involved in Glucose Catabolism.

The results have been given as units of enzyme specific activity (μ moles substrate converted/min/mg protein) in the total extract, T, and have been expressed as mean values ± the standard deviation (S.D). The figures in parentheses represent the number of experiments performed from which the means were calculated.

Enzyme	Enzyme Activities in Homogenates from Cells Lysed by			
	0.5% Saponin	0.5% Triton X-100	0.5 mg% Digitonin	Freezing & Thawing
HK	0.90±0.17 (4)	0.66±0.04 (3)	0.39±0.05 (3)	0.52±0.31 (3)
PGI	1.94±0.21 (2)	0.91±0.11 (2)	0.55±0.18 (2)	1.31±0.61 (2)
PFK	0.94±0.27 (4)	0.99±0.21 (2)	0.44±0.09 (3)	1.05±0.21 (4)
ALD	0.13±0.02 (3)	0.16±0.06 (2)	0.12±0.07 (2)	0.16±0.06 (2)
GAPDH	0.44±0.07 (4)	0.20±0.17 (2)	0.32±0.09 (3)	0.96±0.89 (2)
PGK	1.91±0.61 (3)	1.18±0.43 (2)	0.75±0.47 (3)	1.39±0.60 (3)
PGlyM	0.21±0.05 (2)	0.15±0.12 (2)	0.15±0.02 (2)	0.25±0.22 (3)
ENOL	1.75±0.93 (2)	0.30±0.04 (2)	0.53±0.18 (3)	0.76±0.34 (2)
PK	2.11±0.06 (2)	1.60±0.13 (2)	0.74±0.21 (3)	1.16±0.88 (2)
GPDH	1.18±0.48 (3)	1.16±0.38 (2)	0.67±0.24 (2)	0.67±0.13 (3)
GK	3.14±0.80 (3)	2.75±0.48 (3)	1.40±0.71 (2)	1.71±0.68 (3)

lose their motility within ten minutes and become swollen, but are easily sedimented by centrifugation at 1,000 g. Cells exposed to 0.5 mg% digitonin, on the other hand, looked slightly shrunk in appearance; such cells also become immotile within fifteen minutes, and are also easily sedimented by low speed centrifugation. After the exposure to the detergents or to freezing and thawing, the cells could be easily disrupted by 40-80 strokes of a hand-operated homogenizer.

III.1 EFFECT OF THE METHOD OF TREATMENT ON THE ACTIVITIES OF THE ENZYMES THAT CATALYSE THE BREAKDOWN OF GLUCOSE IN LONG SLENDER BLOODSTREAM FORMS OF T. BRUCEI

To find out how the different methods of cell disruption affect the gross activities of the enzymes, assays were performed on the total extract (T), and the results have been presented in Table III.1. as µmoles of substrate converted per min per mg protein in the extract. It is clear from these results that the enzyme activities vary considerably according to the disruption procedure adopted. Maximum activities were obtained using saponin lysis whereas treatment by digitonin gave the lowest activities.

This pattern becomes clear upon consideration of the relative activities of the enzymes as calculated in Table III.1.1. in which the activities of the enzymes in extracts from saponin treated cells have arbitrarily been assigned as unity. With the exception of phosphofructose kinase, aldolase and phosphoglycerate mutase, all the enzymes showed their highest activities in extracts prepared from cells which had been treated with saponin. On the other hand, seven of the eleven enzymes showed their lowest activities in extracts

Table III. 1. 1. Comparison of the Activities of the Enzymes in Saponin-lysed Cells with the Values from Cells Treated by Triton X-100, Digitonin or Freezing and Thawing.

The values are based on activities of 1 μ mole/min/mg protein in extracts from saponin-lysed cells.

Enzyme	in Saponin	in Triton X-100	in Digitonin	after Freezing & Thawing
HK	1	0.73	0.43	0.58
PGI	1	0.47	0.28	0.67
PFK	1	1.05	0.47	1.12
ALD	1	1.23	0.92	1.23
GAPDH	1	0.45	0.73	2.18
PGK	1	0.62	0.39	0.73
PGlyM	1	0.71	0.71	1.19
ENOL	1	0.17	0.30	0.43
PK	1	0.76	0.35	0.55
GPDH	1	0.49	0.28	0.28
GK	1	0.88	0.45	0.55

Table III. 1. 2. Hexokinase Activity Relative to the Activities of the Other Enzymes Listed in Table III. 1.

The values are based on hexokinase activity of 1 μ mole/min/mg protein in each extract.

Enzyme	in Saponin	in Triton X-100	in Digitonin	after Freezing & Thawing
HK	1.0	1.0	1.0	1.0
PGI	2.2	1.4	1.4	2.5
PFK	1.0	1.5	1.1	2.0
ALD	0.1	0.2	0.3	0.3
GAPDH	0.5	0.3	0.8	1.8
PGK	2.1	1.8	1.9	2.7
PGlyM	0.2	0.2	0.4	0.5
ENOL	1.9	0.5	1.4	1.5
PK	2.3	2.4	1.9	2.2
GPDH	2.6	1.8	1.7	1.3
GK	3.5	4.2	3.6	3.3

obtained from cells which had been lysed by treatment with digitonin. Enzyme activities in extracts obtained by treating T. brucei with Triton X-100 or by freezing and thawing appeared quite similar with the possible exception of glyceraldehyde-phosphate dehydrogenase. These results demonstrate quite clearly how discrepancies can arise when enzymes from the same source are studied under different conditions of cell extraction.

In Table III.1.2., the relative activities of the enzymes are once again presented, but this time the activity of hexokinase in each preparation has arbitrarily been assigned as unity. The results show that irrespective of the method of treatment the following enzymes show activities which are equal to or greater than the activity of hexokinase:- Phosphoglucose isomerase, phosphofructose kinase, phosphoglycerate kinase, pyruvate kinase, glycerolphosphate dehydrogenase and glycerokinase; in all cases, except Triton X-100 treated cells, enolase also shows a higher activity than hexokinase. The activities of the remaining three enzymes, aldolase, glyceraldehyde phosphate dehydrogenase and phosphoglycerate mutase are lower than the activity of hexokinase, except cells lysed by freezing and thawing where the activity of glyceraldehyde-phosphate dehydrogenase is almost double that of hexokinase. Hence although there is no doubt that these four different treatments of T. brucei affect the gross activities of the eleven enzymes, it appears nevertheless that there is a tendency for all the enzymes to be equally affected by a given method of cell disruption.

Table III. 2. The Effect of Disruption Procedure on the Leakage of Protein and Some Enzymes of Bloodstream Long Slender Form T. brucei.

The results, presented as percentages of the total extract, T, have been expressed as means ± the standard deviation (S.D.) and the figures in parentheses represent the number of experiments performed from which the means were calculated.

L denotes material that leaks out during lytic treatment of T. brucei cells; H, homogenate of treated cells after removal of L: T, total homogenate of treated cells (i.e. L + H).

	Cell Treatment					
	in 0.5% Saponin			in 0.5% Triton X-100		
	L	H	% Recovery	L	H	% Recovery
a) Protein	34 ± 9	66 ±15	100 (11)	54 ±16	64 ±13	118 (10)
b) Enzymes						
HK	6 ± 4	84 ± 9	90 (6)	14 ± 9	75 ±32	89 (3)
PGI	91 ± 8	13 ±11	104 (6)	88 ±30	5 ± 3	93 (4)
PFK	14 ± 8	75 ±20	88 (8)	7 ± 3	77 ±28	84 (2)
ALD	5 ± 3	95 ± 7	100 (7)	9 ± 3	106 ±15	115 (2)
GAPDH	38 ±12	52 ±18	90 (9)	16 ± 3	126 ±19	142 (2)
PGK	36 ± 3	66 ± 6	102 (5)	43 ±24	46 ±10	89 (2)
PGlyM	122 ±16	5 ± 4	127 (3)	101 ± 7	2 ± 2	103 (3)
ENOL	103 ±22	7 ± 4	109 (2)	75 ±12	12 ± 8	87 (2)
PK	80 ±34	19 ±18	99 (2)	81 ±13	3 ± 3	84 (3)
GPDH	13 ± 6	79 ±17	92 (3)	17 ±16	87 ±31	104 (3)
GK	39 ±14	61 ±13	100 (6)	26 ± 7	53 ± 4	79 (3)

	Cell Treatment					
	in 0.5 mg% Digitonin			by Freezing & Thawing		
	L	H	% Recovery	L	H	% Recovery
a) Protein	8 ± 5	82 ±12	90 (8)	66 ±16	54 ± 9	120 (9)
b) Enzymes						
HK	1 ± 1	88 ±12	89 (3)	6 ± 4	77 ±35	83 (3)
PGI	4 ± 4	88 ±12	92 (3)	20 ± 8	61 ±10	85 (3)
PFK	1 ± 1	87 ±18	88 (4)	9 ± 6	85 ±17	94 (4)
ALD	1 ± 1	95 ± 6	96 (3)	5 ± 2	93 ± 4	98 (2)
GAPDH	3 ± 0	59 ± 9	62 (3)	37 ± 4	58 ± 8	95 (2)
PGK	8 ± 8	71 ±37	78 (3)	15 ± 5	74 ±22	89 (3)
PGlyM	46 ±18	62 ± 9	108 (2)	237 ±99	8 ± 6	245 (3)
ENOL	14 ± 6	65 ±24	79 (2)	88 ±22	4 ± 0	92 (3)
PK	5 ± 5	90 ±14	95 (2)	91 ±10	9 ± 9	100 (2)
GPDH	5 ± 5	100 ±15	105 (3)	8 ± 0	71 ± 9	79 (3)
GK	2 ± 1	97 ± 3	99 (3)	13 ± 3	78 ±11	91 (2)

III.2. EFFECT OF LYTIC TREATMENT ON THE LEAKAGE OF PROTEIN AND ENZYMES ASSOCIATED WITH GLUCOSE CATABOLISM IN T. BRUCEI

Because the pellicular microtubules of the Kinetoplastida make these organisms resistant to cell rupture in isotonic media, various methods including detergents have been used to assist their disruption. When detergents are used in this way, it is important to appreciate that their action may not be limited to a weakening of the pellicular membrane; they are also likely to cause sufficient disruption of the cell membrane which would lead to leakage of macromolecules out of the cell. Table III.2. records the results of experiments designed to investigate whether cell treatment with the three detergents or by freezing and thawing causes such leakage of macromolecules out of bloodstream T. brucei.

These results show that, far from being innocuous, detergents or the process of freezing and thawing cause leakage of both protein and enzymes from T. brucei cells; and the extent of the leakage depends on the type of treatment employed. While lysis by saponin, Triton X-100 or freezing and thawing causes leakage of 34%, 46% and 55% respectively of the total protein in the cell, cells treated with digitonin lose less than 10% of their total protein to the suspending medium (Tables III.2. and III.2.1.).

It is also significant to note that the process of enzyme leakage appears to be quite specific, which is not what one would expect if all the enzymes under discussion existed in T. brucei as soluble proteins with unimpeded movement in the cytoplasm.

These observations become clear from Table III.2.1. where enzyme activities in fraction L have been expressed as percentages of the summed total activities in fraction L and the homogenate, H.

Table III. 2. 1. Enzyme Activities in Fraction L Expressed as Percentage of the Summed Activities in Fraction L and the Homogenate, H.

	Saponin	Triton X-100	Digitonin	Freezing & Thawing
a) Protein	34	46	9	55
b) Enzymes				
HK	7	16	1	7
PGI	87	95	4	25
PFK	16	8	1	10
ALD	5	8	1	5
GAPDH	42	11	5	39
PGK	35	48	10	17
PGlyM	96	98	43	97
ENOL	94	86	18	96
PK	81	96	5	91
GPDH	14	17	5	11
GK	39	33	2	14

Although digitonin treatment caused leakage of only 9% of the total protein, as much as 43% of the activity of phosphoglycerate mutase, 18% of enolase or 10% of phosphoglycerate kinase was found in fraction L; these values compare with leakages of 5% or less for the remaining enzymes.

The selective nature of the process of enzyme leakage is even more apparent with cells treated by the other three methods of disruption. Here, practically the total activities of phosphoglycerate mutase, enolase and pyruvate kinase were recovered in fraction L. This fraction also contained 42% (saponin), 11% (Triton X-100) and 39% (freezing and thawing) of the total activity of glyceraldehyde-phosphate dehydrogenase, 35% (saponin), 48% (Triton X-100) and 17% (freezing and thawing) of the total activity of phosphoglycerate kinase as well as 39% (saponin), 33% (Triton X-100) and 14% (freezing and thawing) of glycerokinase. Also as much as 87% of the total activity of phosphoglucose isomerase in cells treated with saponin, 95% in cells treated in Triton X-100 and 25% in cells treated by freezing and thawing was recovered in fraction L. The activities of the remaining enzymes that occurred in fraction L for cells treated by saponin, Triton X-100 or freezing and thawing ranged from as little as 5% for aldolase to 17% for glycerolphosphate dehydrogenase. It should be pointed out that the leakage does not involve loss of particulate material from the cell since centrifugation of fraction L at 105,000 g failed to produce a pellet.

In Table III.2.2., enzyme activities in fraction L and the homogenate, H, have been presented as μmoles/min/mg protein. A comparison of these results with those in Table III.1. shows how a distorted picture of enzyme activities may result, not only

Table III. 2. 2. Comparison Between the Specific Activities of Enzymes in Fraction L with the Specific Activities Found in the Homogenate, H.

	Saponin		Triton X-100		Digitonin		Freezing & Thawing	
	L	H	L	H	L	H	L	H
Enzymes	Specific Activity (μM substrate converted/min/mg protein)							
HK	0.26	1.15	0.17	0.85	0.05	0.61	0.17	0.73
PGI	3.67	0.14	1.68	0.05	0.60	0.70	0.31	0.89
PFK	0.31	1.12	0.13	1.30	0.07	0.67	0.09	0.73
ALD	0.02	0.20	0.02	0.22	0.01	0.13	0.08	0.25
GAPDH	0.40	0.40	0.11	0.32	0.15	0.34	0.34	0.58
PGK	1.29	1.49	0.97	0.73	0.69	0.65	0.30	0.87
PGlyM	0.69	0.04	0.69	0.00	1.37	0.11	0.82	0.06
ENOL	2.56	0.14	0.39	0.07	0.88	0.15	0.46	0.04
PK	2.28	0.50	1.83	0.08	0.23	0.83	2.23	0.21
GPDH	0.63	1.40	0.31	1.40	0.04	0.59	0.12	0.42
GK	3.07	3.29	2.77	2.26	0.37	0.94	0.34	1.78

by the method of cell disruption but also by the manner in which such results are presented. Thus, whereas the enzymes which undergo little or no leakage show similar specific activities in the homogenate, H, and the total extract, T, for enzymes like enolase and pyruvate kinase which are concentrated in fraction L, it is the specific activity in this fraction which compares with that in the total extract. In the case of phosphoglycerate mutase, the specific activity observed in fraction L was found to be much higher than the corresponding value found in the total extract, T, irrespective of the method of cell disruption.

The results therefore demonstrate quite clearly that not only do some of the enzymes leak out during treatment of blood-stream T. brucei but also such enzymes as phosphoglycerate mutase, enolase and pyruvate kinase tend to become selectively concentrated in the material that leaks out of treated cells. Phosphoglucose isomerase behaves similarly when T. brucei are treated with 0.5% saponin or 0.5% Triton X-100. A comparative examination of Tables III.1. and III.2.2. shows that once in solution some of the enzymes are open to inactivation probably by the action of proteases which themselves do not leak out of treated cells. This might explain why such enzymes as phospho-glycerate mutase are highly active in fraction L but tend to show very poor activities in the total extract, T.

III.3. THE EFFECT OF THE METHOD OF CELL DISRUPTION ON THE DISTRIBUTION OF PROTEIN AND ENZYMES IN SUBCELLULAR FRACTIONS OF T. BRUCEI

After treatment and homogenisation of the T. brucei cells,

Table III. 3. The Effect of Disruption Procedure on the Distribution of (i) Protein and (ii) Eleven Enzymes Involved in Glucose Metabolism in Subcellular Fractions of Bloodstream Long Slender Form T. brucei.

Absolute values are given as mg protein per ml packed cell volume (PCV), or units of enzyme activity (μmoles substrate converted/min/mg protein); the results for the subcellular fractions have been expressed as percentages of the summed values in fraction L and the homogenate, H of treated cells. All the results have been presented as means ± the standard deviation (S.D.), and the figures in parentheses represent the number of experiments performed from which the means were calculated.

L denotes material that leaks out during lysis of T. brucei cells; H, homogenate of treated cells which had been sedimented to remove L; 1KP, nuclear fraction and other cell debris which sediment at 1,000 g; 105KP, all the post-nuclear cell granules sedimenting at 105,000 g; 105KS, the final supernatant containing cell components which do not leak out during cell lysis but fail to sediment after 1 hr centrifugation at 105,000 g. Other details are given in Figure II. 2. and the text.

		Percentage Values				
	Absolute Values	L	1KP	105KP	105KS	Recovery
(a) SAPONIN						
i)Protein	78.0±7.5(8)	38.5±7.0	39.1±16.4	16.0±4.6	17.7±6.2	111.3±18
ii)Enzyme						
HK	0.90±0.17(5)	11.6±1.6	23.6±12.5	50.5±10.3	3.6±2.8	89.3±13
PGI	1.94±0.28(2)	90.5±2.0	0.9± 0.2	1.7± 0.3	4.3±0.9	97.4± 3
PFK	0.89±0.21(5)	14.9±7.3	26.4± 8.8	33.6±14.3	3.3±1.5	78.2± 8
ALD	0.10±0.02(5)	5.5±2.7	54.1±25.2	42.0±13.1	3.2±0.9	104.8±15
GAPDH	0.44±0.07(4)	44.0± 15	16.9± 8.6	12.7± 5.6	1.9±0.8	75.5± 1
PGK	1.91±0.61(5)	39.4±6.8	28.4± 4.0	17.4± 4.6	10.5±3.4	95.7±13
PGlyM	0.21±0.05(2)	91.7±3.5	0.8± 0.6	2.1± 0.8	3.1±1.1	97.7± 5
ENOL	1.75±0.93(2)	94.2±2.2	0.8± 0.8	0.2± 0.1	3.3±0.9	98.5± 2
PK	2.11±0.06(2)	79.9± 26	3.5± 3.4	1.2± 1.2	0.9±0.8	85.4±20
GPDH	2.36±0.48(2)	12.2±8.7	40.0±13.7	43.8± 9.5	3.6±0.8	99.6± 5
GK	2.81±0.84(4)	41.3±9.9	31.0±18.3	15.1± 6.4	7.8±3.6	95.2±26

Table III. 3. (continued)

	Absolute Values	Percentage Values				
		L	1KP	105KP	105KS	Recovery
(b) TRITON X-100						
i)Protein	87.9±20.2(8)	51.5±2.4	25.7±12.0	12.5± 5.5	14.4±13	104±19
ii)Enzyme						
HK	0.45±0.02(2)	26.0±4.1	26.0±14.2	32.7± 7.5	4.4±1.0	90±16
PGI	0.53±0.02(2)	97.1±0.7	0.8± 0.1	1.6± 0.6	0.6±0.2	100± 1
PFK	0.64±0.22(2)	11.1±3.9	30.1±14.4	13.3± 1.8	22.7±3.1	77±16
ALD	0.16±0.04(2)	8.7±2.0	52.9±16.5	23.7± 6.1	7.7±2.5	93± 2
GAPDH	0.20±0.17(2)	15.5±0.0	54.7±20.8	28.0±19.5	1.8±1.2	100±23
PGK	0.63±0.09(2)	48.8±4.9	37.0± 4.2	6.6± 1.0	9.9±1.5	102±11
PGlyM	0.08±0.04(3)	97.8±3.9	0.7± 0.7	0.2± 0.2	1.4±1.3	100± 0
ENOL	0.23±0.09(2)	98.0±0.5	0.3± 0.3	0.2± 0.1	1.6±0.8	100±13
PK	0.45±0.18(3)	95.5±5.5	1.6± 1.6	0.2± 0.0	0.3±0.2	98± 3
GPDH	0.70±0.14(2)	25.6±7.2	51.6± 6.3	20.5±10.8	4.9±1.0	103±22
GK	2.29±0.42(3)	33.4±7.9	37.9±13.2	13.7± 2.9	3.7±0.6	89± 8
(c) DIGITONIN						
i)Protein	79.7±22.6(10)	9.6±5.4	57.7±13.3	8.3± 6.8	15.0±8.7	91±20
ii)Enzyme						
HK	0.61±0.14(5)	1.6±1.2	62.2± 4.6	23.4± 6.0	8.7±2.2	96±13
PGI	0.76±0.37(2)	7.5±5.4	52.1± 1.4	10.0± 0.1	1.4±0.6	71± 7
PFK	0.77±0.16(4)	2.1±0.2	78.7±24.2	14.6± 3.2	7.7±2.2	103±15
ALD	0.12±0.02(3)	2.6±1.0	54.6± 6.4	21.4±11.0	15.9±3.5	95±18
GAPDH	0.55±0.01(2)	5.7±1.4	64.0±26.0	7.2± 5.2	15.2±1.5	92±46
PGK	1.03±0.01(2)	11.2±1.4	54.0± 4.1	24.1± 8.7	7.4±0.7	96± 4
PGlyM	0.09±0.03(3)	45.5±7.5	34.5±15.7	2.2± 2.0	68.0± 16	150±40
ENOL	0.41±0.14(2)	22.3±2.9	30.9± 8.8	7.6± 3.6	30.0±6.4	91± 9
PK	0.84±0.25(2)	0.8±0.3	83.1±20.0	0.3± 0.3	0.4±0.4	84±21
GPDH	1.13±0.71(2)	7.3±1.5	68.3±30.6	16.3± 2.6	0.2±0.1	91±18
GK	1.27±0.61(2)	2.0±0.5	79.0±23.1	10.0± 0.0	6.3±3.3	97±20
(d) FREEZING & THAWING						
i)Protein	83.4±14.7(10)	40.7±7.9	32.6±7.9	16.5± 3.8	10.3±2.2	100±16
ii)Enzyme						
HK	0.52±0.02(3)	8.4±7.4	33.7±16.6	50.5± 0.8	2.7±2.7	95± 7
PGI	1.31±0.65(2)	30.6±5.9	18.5± 2.4	48.8± 4.0	7.2±3.5	105± 8
PFK	1.15±0.21(3)	7.9±4.7	41.2±14.0	45.5± 6.9	1.3±0.6	96±20
ALD	0.16±0.06(2)	5.1±1.9	31.2±17.0	31.2± 7.0	5.9±5.8	73±30
GAPDH	0.76±0.50(3)	46.4±4.7	18.5± 7.2	10.0± 4.5	2.2±1.5	77± 2
PGK	1.39±0.60(3)	30.3± 19	20.6± 5.7	17.0±13.0	15.9± 14	84±25
PGlyM	0.27±0.02(2)	97.4±1.2	0.5± 0.1	0.1± 0.0	1.2±0.7	99± 1
ENOL	0.76±0.34(2)	91.0±6.8	0.6± 0.5	1.6± 1.2	6.1±4.4	99± 7
PK	1.16±0.88(2)	91.3±8.8	4.9± 4.8	4.0± 4.0	1.1±1.1	101± 2
GPDH	0.67±0.13(2)	10.6±0.9	18.1± 1.7	42.7± 5.7	2.3±0.3	74±19
GK	1.71±0.68(3)	12.9±2.4	37.9±17.3	56.6±15.0	8.7±5.1	100±44

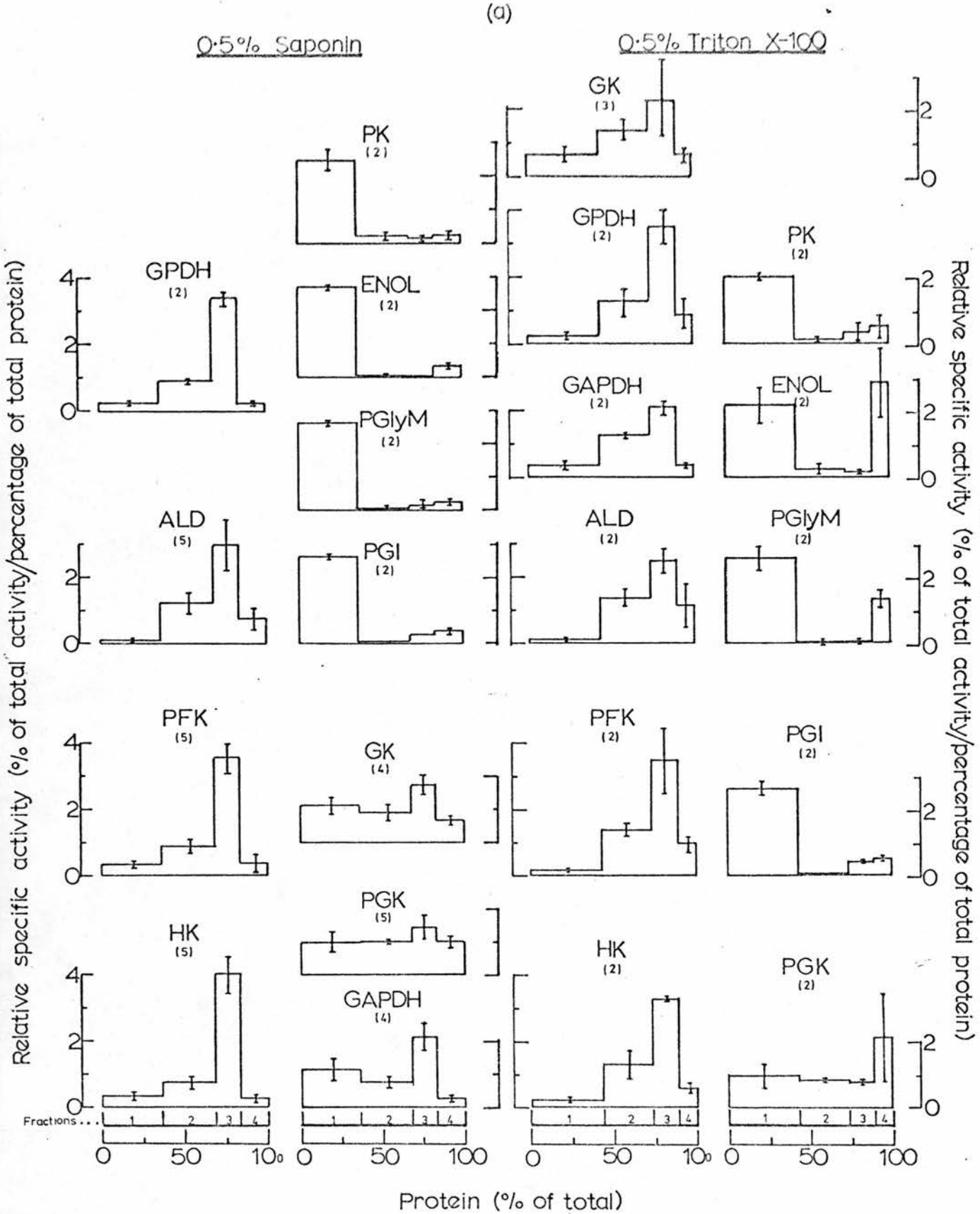


Fig III. 1. Distribution of Enzymes in Fractions Obtained by Differential Centrifugation of Homogenates of T. brucei Cells Lysed Either by 0.5% Saponin, 0.5% Triton X-100, 0.5mg % Digitonin, or by Three Cycles of Freezing and Thawing.

Ordinate: mean relative specific activity of enzymes. Abscissa: subcellular fractions represented by their relative protein content and in the order in which they were isolated, i.e. from left to right: 1, material which leaks out during cell lysis (fraction L); 2, nuclear and other cell debris which sediment at 1,000 g (fraction 1KP); 3, post-nuclear subcellular organelles sedimenting at 105,000 g (fraction 105KP); 4, the final supernatant containing cell components which fail to sediment after 1 hr centrifugation at 105,000 g (fraction 105KS).

Other experimental details are given in the text and in Table III. 3.

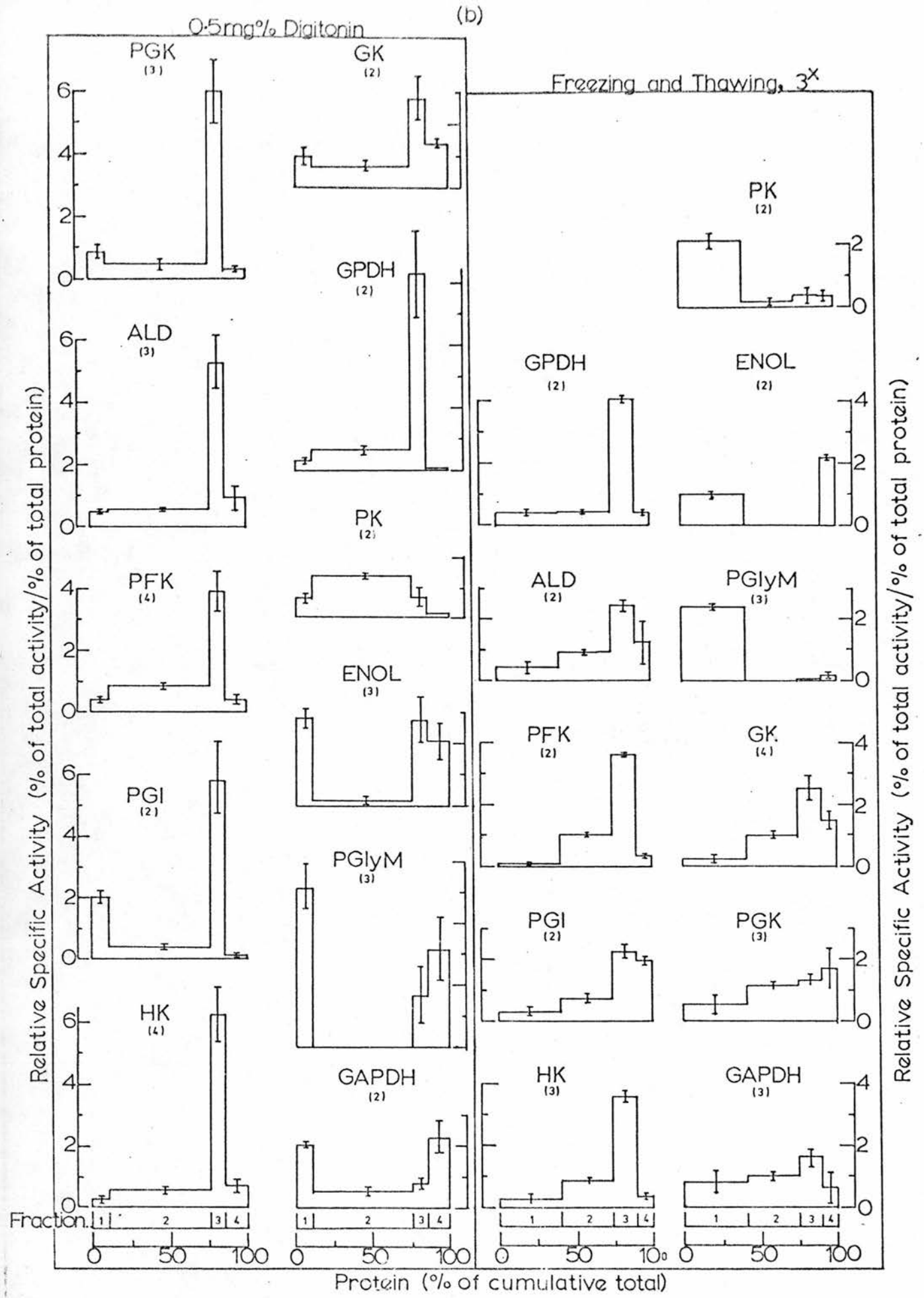


Fig. III. 1. (contd.)

the extracts were subjected to differential centrifugation as summarised in the fractionation scheme in Figure II.1. of the section on materials and experimental procedures (Section II).

The pattern of distribution of protein and T. brucei enzymes involved in the breakdown of glucose has been presented in Table III.3. Although these results show good recoveries of between 85% and 110% for protein and most of the enzymes, there were instances where recovery of enzyme activities were only a little over 70%. Conversely, there was one instance (phosphoglycerate mutase activity in cells treated by digitonin) where an excessively high recovery of 150% was recorded. In order to reduce any errors introduced by excessive or defective recoveries, the results were first recalculated in percentage of the sum of the recovered protein or enzyme activities; Figures III.1a and III.1b were then constructed by plotting the mean relative activities of the fractions against their mean relative protein content. The area of each block is thus proportional to the percentage of the activities recovered in the corresponding fraction, and its height to the degree of purification achieved over the combined activities of fraction L and the homogenate, H. Thus it is possible by studying Figures III.1a and III.1b, to determine which method of cellular disruption and which fraction from such material would serve as the best source for isolating the enzymes. Table III.3.1. summarises the data by listing those fractions which look most promising in this regard, the corresponding degree of purification for each enzyme in question as well as the recovery of activity found in the fraction. The last column in the table, which is the product of the degree of purification and the recovery, should therefore be an index of the

Table III. 3. 1. Subcellular Fractions Showing Highest Enzyme Activities.

Enzyme activities (μ moles substrate converted/min/mg protein) have been expressed as means ± the standard deviation, and the figures in parentheses represent the number of experiments from which the means were calculated. δ denotes the product of the specific activity and the percentage recovery.

	Cells Lysed								
	in 0.5% Saponin				in 0.5% Triton X-100			
Enzyme	Frn.	Specific Acty.	% Recovery	δ	Frn.	Specific Acty.	% Recovery	δ
HK	105KP	4.0 ± 0.6(5)	51	204	105KP	3.3 ± 0.1(2)	33	109
PGI	L	2.7 ± 0.1(2)	91	246	L	2.6 ± 0.4(2)	97	252
PFK	105KP	3.5 ± 1.7(5)	34	119	105KP	3.4 ± 1.9(2)	13	44
ALD	105KP	2.7 ± 0.9(5)	42	113	105KP	2.5 ± 0.8(2)	24	60
GAPDH	105KP	2.2 ± 0.8(4)	13	29	105KP	2.1 ± 0.4(2)	28	59
PGK	L	1.4 ± 0.5(5)	39	55	S	2.1 ± 1.3(2)	10	21
PGlyM	L	2.7 ± 0.1(2)	92	248	L	2.6 ± 0.7(3)	98	255
ENOL	L	2.7 ± 0.9(2)	94	254	L	2.2 ± 1.0(2)	98	216
PK	L	2.0 ± 0.4(2)	80	160	L	2.0 ± 0.2(3)	96	192
GPDH	105KP	3.4 ± 0.3(2)	44	150	105KP	3.5 ± 1.0(2)	21	74
GK	1KP	1.4 ± 0.7(4)	31	43	105KP	2.3 ± 2.2(3)	28	64
	in 0.5 mg% Digitonin				by Freezing & Thawing, 3^x			
HK	105KP	6.2 ± 1.8(5)	23	143	105KP	3.6 ± 0.4(3)	51	184
PGI	105KP	5.8 ± 2.2(2)	10	58	105KP	2.2 ± 0.4(2)	49	108
PFK	105KP	3.9 ± 1.3(4)	15	59	105KP	3.6 ± 0.0(3)	46	166
ALD	105KP	5.3 ± 1.8(3)	21	111	105KP	2.4 ± 0.4(3)	31	74
GAPDH	S	2.3 ± 0.3(2)	15	35	105KP	1.6 ± 0.5(3)	10	16
PGK	105KP	6.0 ± 2.1(2)	24	144	105KP	1.3 ± 0.4(3)	17	22
					S	1.7 ± 1.5(3)	16	27
PGlyM	L	5.2 ± 1.5(3)	31	161	L	2.4 ± 0.1(2)	97	233
	S	3.2 ± 2.1(3)	45	144				
ENOL	L	2.9 ± 0.6(2)	22	64	L	2.2 ± 0.0(2)	91	200
	S	2.1 ± 1.2(2)	30	63				
PK	1KP	1.3 ± 0.0(2)	83	108	L	2.1 ± 0.4(2)	91	191
GPDH	105KP	6.3 ± 2.8(2)	25	158	105KP	4.1 ± 0.2(2)	43	176
GK	105KP	2.8 ± 1.5(2)	15	42	105KP	2.5 ± 0.8(2)	56	140

quality of each fraction as a source material for isolating the enzymes.

The results in Table III.3. and Figures III.1(a and b) show quite clearly that the distribution of both protein and the enzymes among the fractions obtained by differential centrifugation of T. brucei extracts is affected by the method of cellular treatment employed. Assuming that fraction L and the final supernatant (fraction 105KS) represent soluble material, and fractions 1KP and 105KP material associated with subcellular particles or membranes, it is evident that whereas lysis by digitonin solubilises only about 25% of the total protein, the equivalent figures for cells treated with saponin, Triton X-100 or by freezing and thawing are respectively 56%, 66% and 51%. The only enzymes which undergo more than 50% solubilisation during digitonin treatment are phosphoglycerate mutase and enolase, whereas these two enzymes as well as pyruvate kinase are almost completely solubilised by the other three methods of cellular disruption. In addition, cell lysis with saponin or Triton X-100 almost completely solubilises phosphoglucose isomerase.

It should be emphasised here that if the assumption had been made that the glycolytic enzymes existed as soluble proteins in T. brucei and fractions 1KP and 105KP had not been assayed for enzyme activities, the activities of the following enzymes would have been grossly underestimated - hexokinase, phosphofructose kinase, aldolase, phosphoglycerate kinase, glycerolphosphate dehydrogenase and glycerokinase. The activities of phosphoglucose isomerase (in cells treated by digitonin or freezing and thawing), pyruvate kinase (in digitonin-lysed cells) and glyceraldehyde-phosphate dehydrogenase (in cells treated with Triton X-100, saponin

or by freezing and thawing) would have been similarly underestimated.

III.4. DETAILED ANALYSIS OF THE RESULTS ON CELLULAR TREATMENT AND FRACTIONATION BY DIFFERENTIAL CENTRIFUGATION

The four enzymes, hexokinase, phosphofructose kinase, aldolase and glycerolphosphate dehydrogenase show similar distribution patterns irrespective of any of the methods adopted here for disrupting T. brucei cells. This strongly indicates that all four enzymes have the same intracellular location; more than 50% of the recovered activity of each of the four enzymes occurs in the nuclear fraction (1KP) and fraction 105KP. The degree of purification shown in Figures III.1a and III.1b suggests that the post-nuclear fraction 105KP probably represents their intracellular location. This fraction should therefore serve as the best source material for isolating the four enzymes either separately or as a multienzyme complex. The pattern of distribution for glycerokinase (although not identical) is close enough to the ones described above to suggest that the intracellular location of this enzyme might be in fraction 105KP.

The distribution patterns of phosphoglucose isomerase, phosphoglycerate kinase and glyceraldehyde-phosphate dehydrogenase showed substantial variation according to the method of cellular treatment. Digitonin-lysed cells gave patterns for phosphoglucose isomerase and phosphoglycerate kinase which suggest that these two enzymes are probably also located in fraction 105KP. However, lysis with saponin or Triton X-100 completely solubilised phosphoglucose isomerase, whereas about 50% of phosphoglycerate kinase was solubilised by saponin, Triton X-100 or by freezing and thawing.

The distribution pattern of glyceraldehyde-phosphate dehydrogenase was the most complex; contrary to the general pattern established for the other enzymes, this enzyme appears to undergo the most extensive solubilisation in cells treated with digitonin. Thus, whereas the bulk of its activity in digitonin-lysed cells occurs in the soluble fractions, L and 105KS, it appears to be predominantly localised in the particulate fractions if the cells are disrupted with Triton X-100, saponin or by freezing and thawing. Although the soluble fractions from digitonin-lysed cells appear to be the best source material for preparing pure T. brucei glyceraldehyde-phosphate dehydrogenase, it should be possible to obtain the enzyme as part of a multi-enzyme complex by using fraction 105KP from cells lysed by freezing and thawing, saponin or Triton X-100.

Phosphoglycerate mutase and enolase occur as soluble enzymes irrespective of the method of treatment; the distribution pattern of pyruvate kinase from cells lysed by freezing and thawing, Triton X-100 or saponin is so similar to those of phosphoglycerate mutase and enolase that although it appears as a particle-bound enzyme from cells treated with digitonin, it seems reasonable to assume that it probably exists in the cell as a soluble protein. All three enzymes can be easily isolated from the soluble fractions of cells lysed by freezing and thawing, saponin or Triton X-100.

III.5. EFFECT OF SALT TREATMENT ON THE ASSOCIATION OF SOME ENZYMES WITH PARTICULATE COMPONENTS OF T. BRUCEI CELLS

To examine whether the isolation of fraction 105KP showing this multienzyme activity was an artifact resulting from the use of

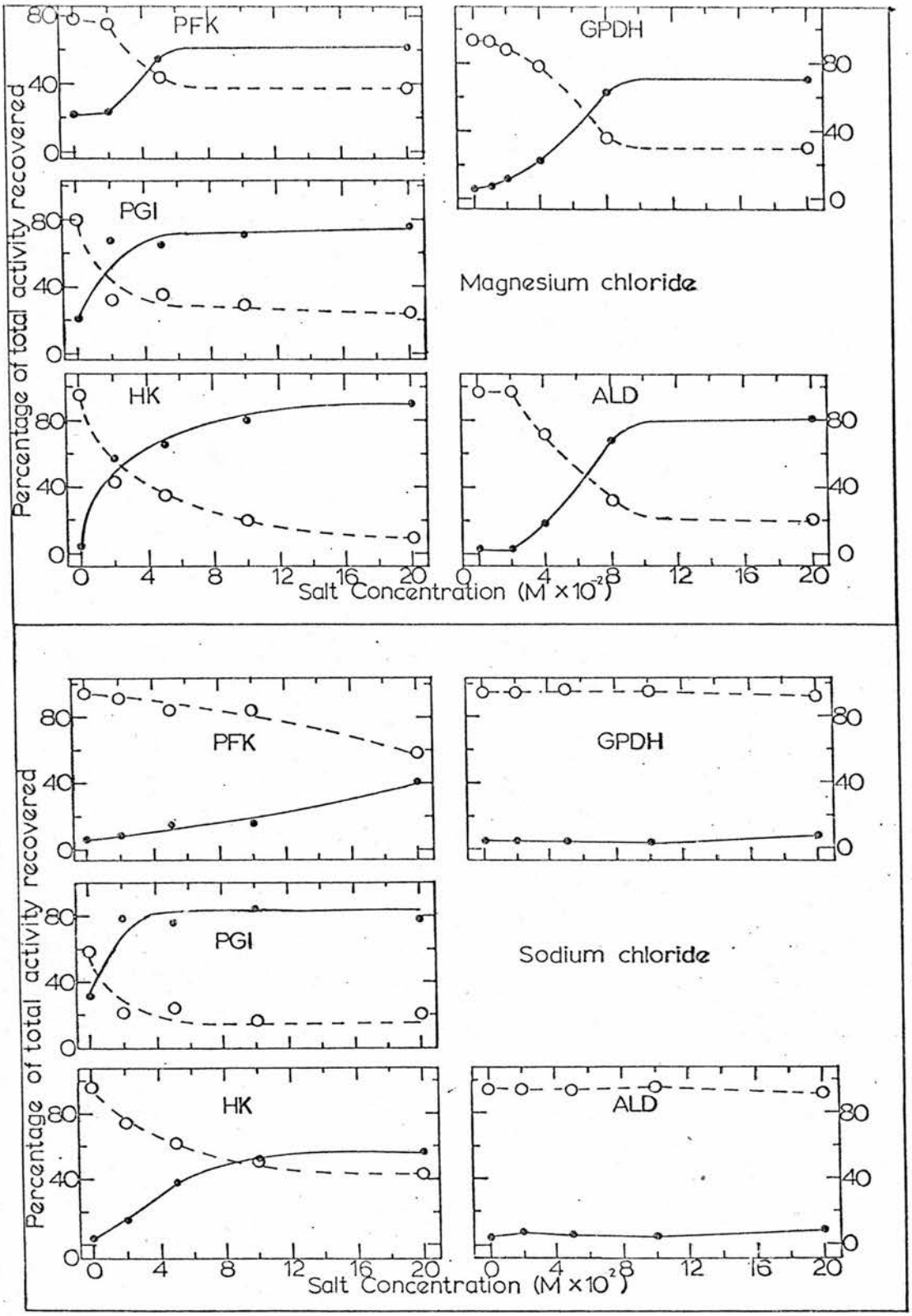


Fig. III. 2. Effect of Salt Concentration on the Solubilization of Five Particulate Enzymes of T. brucei.

T. brucei (3 ml PCV) in TS buffer was frozen overnight and homogenized by 40 strokes with the hand-operated Dounce homogenizer. Aliquots of the homogenate were diluted with solutions of NaCl or $MgCl_2$ to the desired salt concentration, and the samples were incubated on ice for 30 min. They were then centrifuged at 105,000 g for 1 hr; the supernatants were carefully removed and the pellets were resuspended in buffer solutions containing the appropriate concentrations of salt. The samples were assayed for enzyme activity, and the percentage of recovered activity in the supernatant (•) or the pellet (O) was plotted against salt concentration.

buffers of low salt concentration, homogenates of T. brucei were incubated at 25°C for 30 min in buffers containing increasing concentrations of NaCl or $MgCl_2$. At the end of the incubation period, the samples were centrifuged for 1 hr at 105,000 g. The pellets were resuspended in buffer and both the pellet suspension and the supernatants were assayed for enzyme activity; Figure III.2 presents typical results of such experiments. The highest concentration of NaCl employed (0.2 M) could not completely desorb any of the particulate enzymes, although those enzymes which were less firmly bound, such as phosphoglucose isomerase, could almost completely be brought into solution by $MgCl_2$ or NaCl concentrations of less than 0.04 M.

Summary of the Results on Cell Lysis by Detergents or Freezing and Thawing Followed by Fractionation by Differential Centrifugation

From the results on the differential centrifugation of hom-ogenates obtained by treating T. brucei cells with the detergents or by freezing and thawing, it became possible to make the following tentative propositions.

(1) That both the activities and the distribution patterns of T. brucei enzymes catalysing the breakdown of glucose may be affected by the procedure adopted for disrupting the cells.

(2) That four of the enzymes, hexokinase, phosphofructose kinase, aldolase and glycerolphosphate dehydrogenase are so firmly attached to membranes or subcellular organelles, or interact so strongly among themselves that irrespective of which method of treatment is used to lyse the cells, these enzymes appear in a particulate fraction.

(3) That a second group of enzymes, phosphoglucose isomerase, glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase and glycerokinase, also appear to be particle-bound although the proportions of these enzymes distributed between the soluble and particulate fractions are greatly affected by the method of cell lysis employed.

(4) The remaining three enzymes, phosphoglycerate mutase, enolase and pyruvate kinase probably exist in the cells as soluble proteins, since two of them, phosphoglycerate mutase and enolase were found in the soluble fraction irrespective of the method used for lysing the cells, and pyruvate kinase behaved similarly in all cases except where the cells were lysed by digitonin.

(5) Where cells were lysed by freezing and thawing or with digitonin, more than 50% of the total activity of each of the following five enzymes, hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase and glycerolphosphate dehydrogenase appeared in fraction 105KP. Hence this fraction which (according to the differential centrifugation scheme adopted here) should contain no cellular components larger than post-nuclear subcellular particles or membrane fragments should catalyse the conversion of glucose to glycerolphosphate without supplementation by auxiliary enzymes.

Preliminary experiments showed that fraction 105KP does indeed catalyse the conversion of glucose to glycerolphosphate. Evidence will be presented later (Section V) to show that the material containing this multienzyme complex activity is also eluted in the void volume during chromatography on columns of Biogel A-5m. Although acrylamide gel electrophoresis of fraction 105KP which had been treated with sodium dodecyl sulphate and urea leads to the separation of at

least six protein bands, untreated fraction 105KP fails to enter gels even when the acrylamide concentration is as low as 2%.

These preliminary experiments indicated quite strongly that fraction 105KP probably contains subcellular entities or protein aggregates of very high molecular weight capable of acting as a multienzyme complex for at least part of the glycolytic pathway of T. brucei. From now on, the fraction showing this complex activity shall be referred to as the "Multienzyme Complex" or simply as the "Complex", and section IV of this thesis describes the method adopted to isolate a complex which contained the greatest number of the enzymes under discussion; Section V deals with some of the properties of the complex and some of its component enzymes.

SECTION IV

ISOLATION OF THE MULTIENZYME COMPLEX

Introduction

As would be expected, the resistance to rupture caused by the pellicular microtubules presents problems to experiments aimed at the isolation of subcellular organelles of the kinetoplastida. Hill & White (1968) were the first to make a thorough study of a mitochondrial fraction from C. fasciculata. In their studies, these two authors ruptured the cells by grinding them with alumina in a mortar and pestle, using an isolation medium of 0.25 M sucrose-0.1 mM EDTA-0.1 M Tris-Cl, pH 7.6. The homogenate was centrifuged at 10,000 g x min to remove intact cells and alumina, and then the mitochondrial fraction was pelleted at 160,000 g x min.

Toner & Weber (1972) have used silicon carbide to grind C. fasciculata and obtained a homogenate from which they succeeded in isolating a mitochondrial fraction which was intact enough to show respiratory control. While this work was in progress, Opperdoes et al. (1977a) employed the silicon carbide grinding technique to prepare homogenates of T. brucei from which they isolated what was probably a microbody fraction. They proceeded to show by isopycnic sucrose density centrifugation and electron microscopy that the NAD^+-linked glycerolphosphate dehydrogenase of T. brucei is located in these microbodies. This technique of cell-grinding with abrasives was examined to see if a multienzyme complex could be isolated which contained more enzymes than was obtained by T. brucei lysis with digitonin or freezing and thawing, and the results of such experiments are reported in this section.

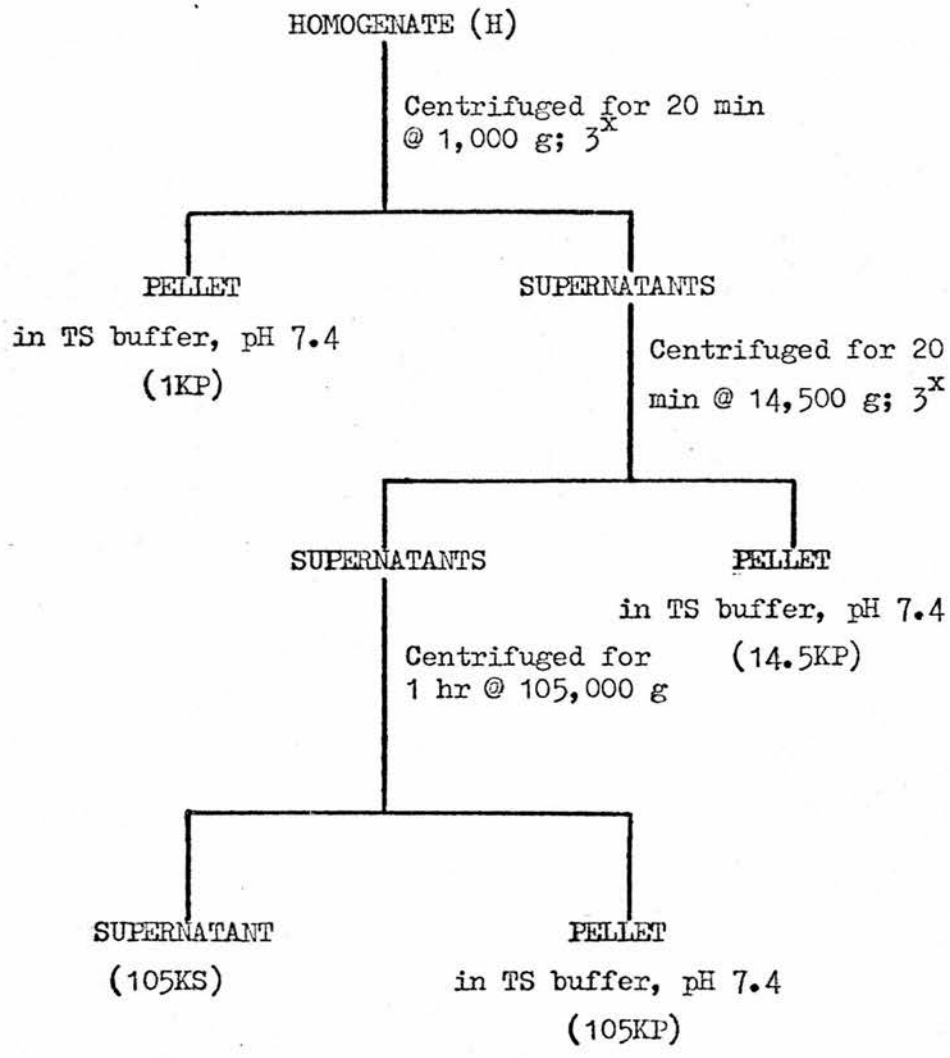


Fig IV. 1. Scheme for the Isolation of the Multienzyme Complex of Bloodstream Long Slender Form T. brucei.

The homogenate (H) obtained from T. brucei cells lysed either by digitonin treatment or three cycles of freezing and thawing, or by cell grinding with abrasives, was centrifuged to remove the nuclei and other cell debris. The supernatants were then centrifuged at 14,500 g max for 20 min in an MSE 18 centrifuge. The resuspended pellet served as the crude multienzyme complex and the other fractions were discarded, unless the subcellular distribution pattern of the enzymes was being studied.

IV.1. DIFFERENTIAL CENTRIFUGATION FRACTIONS OF HOMOGENATES OBTAINED BY GRINDING T. BRUCEI CELLS WITH SILICON CARBIDE OR ALUMINA

It would be recalled from Section III. that the fraction found to catalyse the conversion of glucose to glycerolphosphate consisted of the whole of the post-nuclear particles from T. brucei homogenates. In order to help identify the probable intracellular location of the complex more accurately, this fraction was subfractionated according to the scheme in Figure IV.1. as follows. The homogenate was centrifuged at 1,000 g max for 20 min, the supernatant was carefully removed and the pellet resuspended in the Tris-sucrose buffer. The centrifugation was repeated twice under the same conditions with the resuspended pellet. The washed pellet was finally suspended in the Tris-sucrose buffer and referred to as fraction 1KP. The supernatants were pooled together and subjected to centrifugation at 14,500 g for 20 min; the pellet was suspended in the Tris-sucrose buffer and the centrifugation repeated twice more. The pellet was finally resuspended in the Tris-sucrose buffer and identified as fraction 14.5 KP. The supernatant was centrifuged at 105,000 g for 1 hr; the pellet was resuspended in the Tris-sucrose buffer and referred to as fraction 105KP, and the final supernatant as fraction 105KS.

One problem found to be associated with cell grinding with abrasives is a tendency for pieces of broken flagella and possibly membranes to adhere strongly to the granules of the abrasives. Such material could only be released into solution after repeated washings of the slurry. The procedures detailed under Sections II.3.6. and II.3.7. in the Methods section were designed to

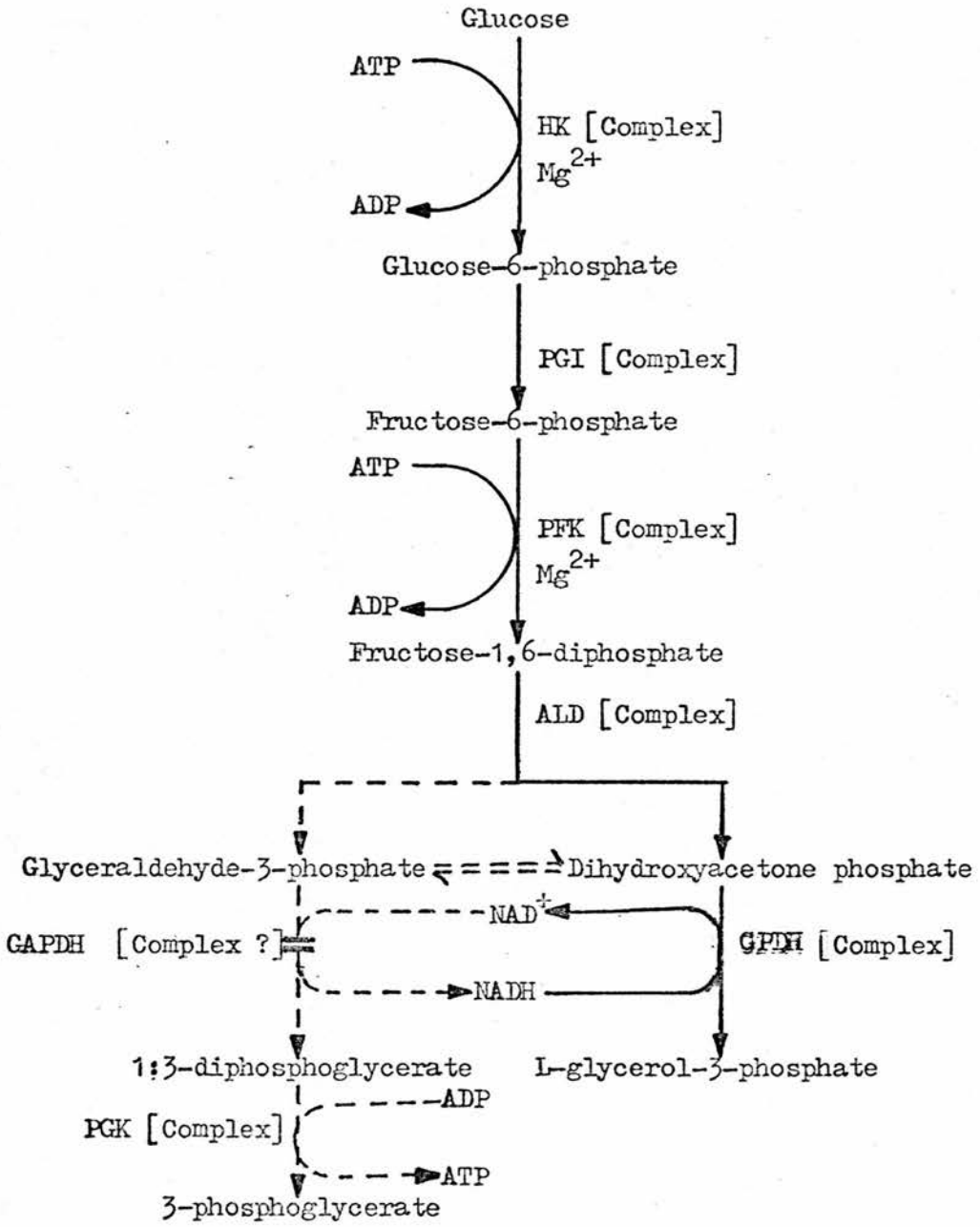


Fig. IV. 2. The Sequence of Reactions Potentially Catalysed by the T. brucei (Glycolytic) Multienzyme Complex.

The broken arrows indicate the reactions which were not included in the routine assay procedure for the complex activity; ═, reaction inhibited by iodoacetate.

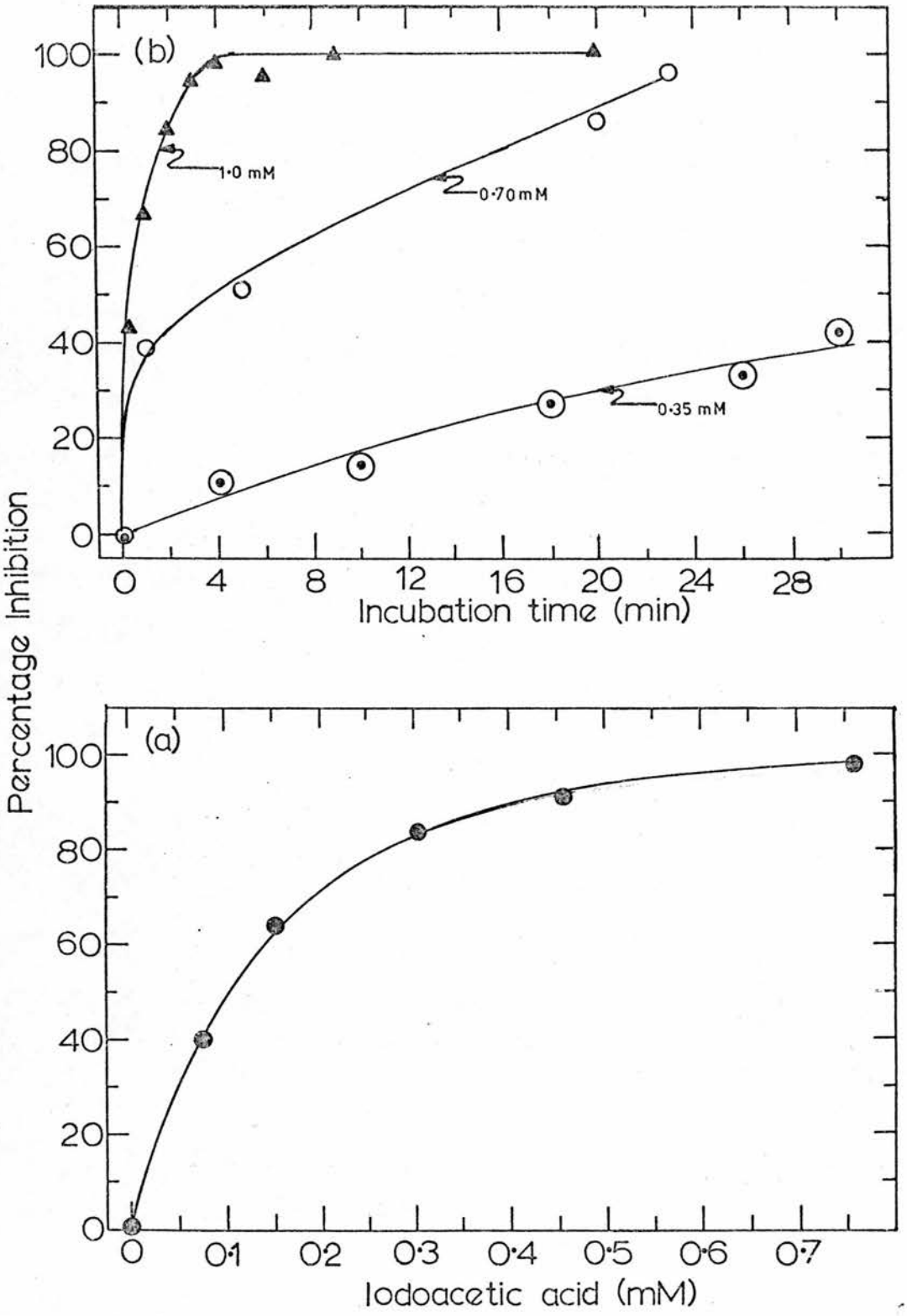


Fig. IV. 3. Inhibition of Glyceraldehyde-Phosphate Dehydrogenase by Iodoacetic Acid (IAA).

Aliquots of a post-nuclear fraction 14.5KP of T. brucei containing 1.9 enzyme units (μmoles substrate converted/min) per ml were incubated with (a) increasing concentrations of iodoacetic acid for 5 min or (b) 0.35 (◉), 0.70 (○) or 1 mM (▲) IAA, before being assayed for enzyme activity. The results have been presented as percentage enzyme inhibition against (a) inhibitor concentration or (b) incubation time.

achieve optimum recoveries of homogenates while at the same time minimising possible risks of disrupting subcellular organelles. While strict adherence to the details of these procedures helped to ensure reproducible results for other fractions, consistent recovery of material in the nuclear fraction, 1KP could not be assured. The total protein recovered also varied between 40 and 50 mg/ml packed cell volume as compared to the value of 80 mg/ml (approx.) per packed cell volume found in extracts from cells lysed with detergents or by freezing and thawing.

IV.2. ASSAY FOR MULTIENZYME COMPLEX ACTIVITY

Activity of the multienzyme complex was calculated from the gradient of the linear part of the curve (see Figure VI.3) obtained by measuring the decrease in optical density resulting from oxidation of NADH by the dihydroxyacetone-phosphate produced in the chain of reactions shown in Figure IV.2. In order to prevent the NAD^+ formed being reduced back to NADH by glyceraldehyde-phosphate dehydrogenase in the complex, iodoacetic acid was included in the assay medium to ensure a near-optimum inhibition of this enzyme. The results in Figure IV.3 show that with iodoacetic acid concentration of 1 mM in the incubation medium, only five minutes were required to achieve complete inhibition of glyceraldehyde-phosphate dehydrogenase. Similar experiments designed to study the effect of iodoacetic acid on the other component enzymes of the complex showed that none of them was inhibited by the acid.

The assay medium consisted of TEA-KOH, pH 7.6, 97 mM; iodo-acetic acid, 1 mM; ATP, 0.94 mM; $MgCl_2$, 13.9 mM; NADH, 0.18 mM; sucrose, 0.25 M; triosephosphate isomerase, 70 units; and glucose,

400 mM. The glucose was routinely added last to initiate the reaction after the rest of the assay mixture had been incubated for 5 min at 25°C to ensure inhibition of endogenous glyceraldehyde-phosphate dehydrogenase. In later experiments, when it was discovered that omission of triosephosphate isomerase had no effect on the complex activity, the auxiliary enzyme was omitted.

IV.3. THE EFFECT OF DETERGENT TREATMENT ON ENZYME ACTIVITIES IN HOMOGENATES OBTAINED BY GRINDING T. BRUCEI CELLS WITH ALUMINA OR SILICON CARBIDE

Since cell-lysis by the three chemical methods of detergent treatment as well as by freezing and thawing had been shown to affect enzyme activities, experiments were conducted to find out (1) what sort of activities would be recovered from homogenates of T. brucei obtained by grinding the cells with alumina or silicon carbide, and (2) to ascertain whether the presence of the detergents would have any effect on the values.

On the whole, the enzyme activities were similar whether grinding was done with alumina or silicon carbide. What appeared to be significant was that due to the difficulties associated with recoveries of homogenate from the slurries, and also the fact that some of the enzymes appeared to show latency, the individual enzyme activities could vary by as much as 50% even when experiments were repeated with the same abrasive. Preliminary experiments showed that the differences were probably due to differences in the degree of efficiency in the recovery of material for individual

Table IV. 1. Effect of Detergents on the Activities of Enzymes in Homogenates Obtained by Grinding T. brucei Cells with Alumina.

Enzyme activities have been expressed as μmoles substrate converted/min/mg protein. Other experimental details are given in the text.

Enzyme	Enzyme Activity in						
	Untreated Homogenate	Homogenate Pretreated in					
		0.5% Saponin	% Actn.	0.5% Triton X-100	% Actn.	0.5 mg% Digitonin	% Actn.
HK	0.28	0.38	36	0.42	48	0.28	0
PGI	0.50	0.57	14	0.57	14	0.50	0
PFK	0.63	0.66	5	0.71	14	0.50	-20
ALD	0.05	0.04	-20	0.04	-20	0.06	20
GAPDH	0.39	0.39	0	0.41	6	0.36	-6
PGK	1.14	1.81	58	2.24	97	0.99	-13
PGlyM	0.84	0.29	-66	0.60	-28	0.71	-16
ENOL	1.10	1.42	29	1.30	18	1.30	18
PK	1.19	0.97	-19	0.91	-25	1.16	-3
GPDH	0.30	0.34	14	0.34	14	0.33	11
GK	1.72	1.62	-6	1.45	-16	1.41	-18

experiments as well as the time lapse between preparation of the homogenates and assay for enzyme activities. For this reason, it was not considered meaningful to present the mean values from a number of experiments as was done in the case of homogenates obtained by treating the cells with detergents or by freezing and thawing. Instead, the results of a typical experiment have been presented in Table IV.1. Apart from the low specific activity of glyceraldehyde-phosphate dehydrogenase (0.39) the activities of the enzymes thought to be soluble, or not to be associated with the complex, were found to be higher than those of the enzymes considered to be components of the complex. For example, the specific activity of hexokinase in the untreated homogenate was 0.28 as compared with a value of 1.19 found for pyruvate kinase. It is interesting to note that the soluble phosphoglycerate mutase which consistently showed low activities in extracts from detergent lysis or cells treated by freezing and thawing now shows activity which is very close to the activities shown by the other soluble and more 'active' enzymes (cf. Table III.1). In this experiment, the effect of detergents on enzyme activities was also examined as follows. Samples of the homogenate obtained by grinding T. brucei with alumina were preincubated at $0^{\circ}C$ for 10 min with the appropriate detergent before being assayed for their enzyme activities.

With the exception of aldolase which is inhibited by saponin and Triton X-100, all the component enzymes of the complex are activated in the presence of these two detergents. Conversely, the soluble enzymes (with the exception of enolase) are inhibited by all three detergents. That phosphoglycerate mutase is inhibited by as much as 66% after a 10 min incubation

in 0.5% saponin may be a strong indication of how very labile this enzyme is, which could account for the low activities found previously in cells lysed with detergents or by freezing and thawing. While it is tempting to make similar statements about aldolase, a closer examination of the results indicates that the situation with this enzyme may be more complicated. Although aldolase is the only enzyme found to be associated with the complex which is inhibited by saponin or Triton X-100, it nevertheless appears unaffected or probably activated by digitonin. Since digitonin has already been shown to have the least solubilising effect on T. brucei proteins, this result could be interpreted as showing that aldolase is most stable when it is not in solution. If so, this might explain why it has consistently proved to be so inactive; for it would mean that those conditions which are most likely to favour the greatest access of substrates to the enzymes are precisely the conditions which are likely to expose aldolase to the inhibitory effects of the polar environment.

IV.4. DISTRIBUTION OF PROTEIN AND ENZYMES IN THE SUBCELLULAR FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES FROM GRINDING T. BRUCEI CELLS WITH THE ABRASIVES

The distribution of the enzymes as well as the multienzyme complex activity among the subcellular fractions is shown in Figure IV.4 (a and b). As in Figure III.1a and 1b, Figure IV.4 has been constructed by plotting the relative specific activities

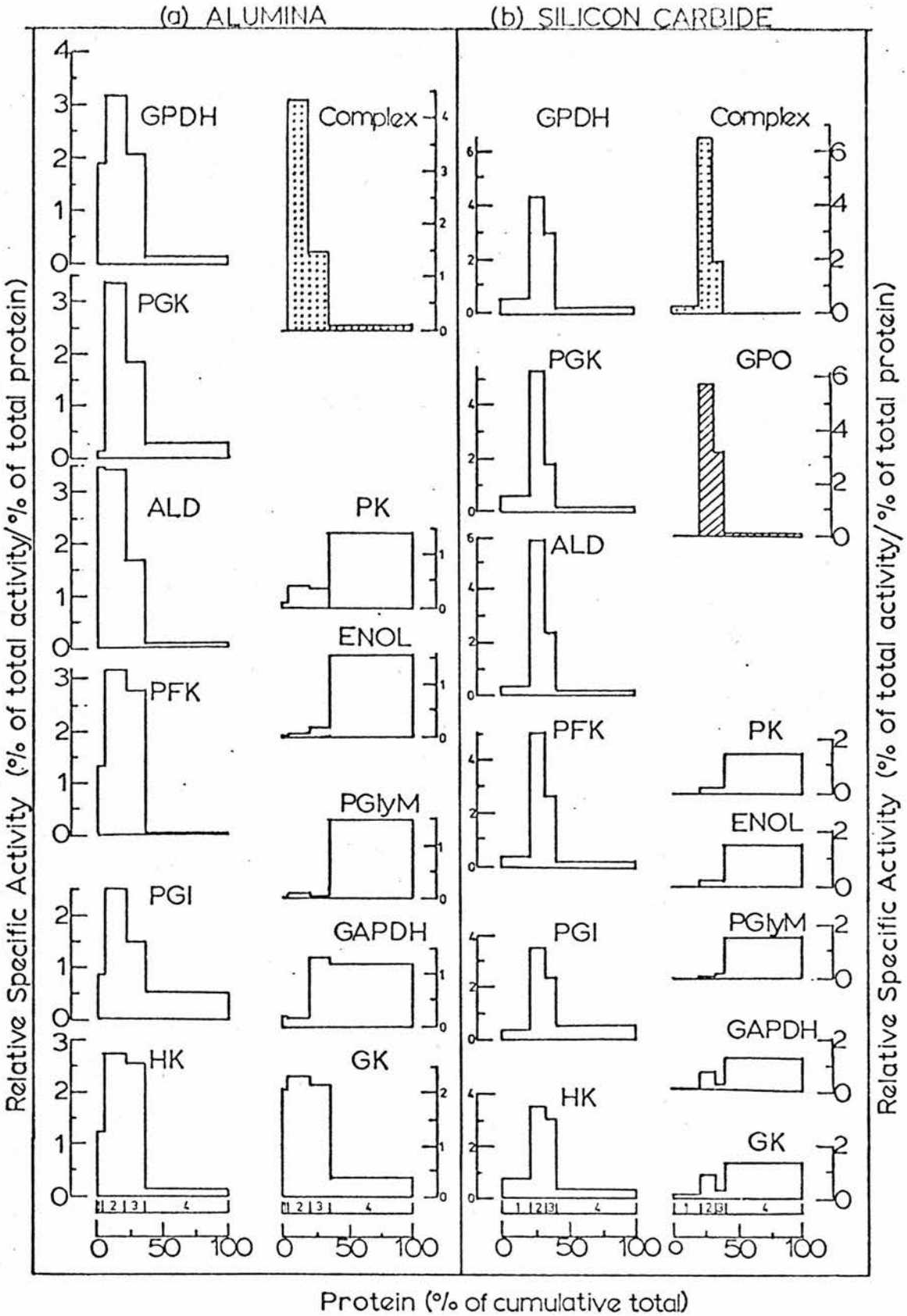


Fig. IV. 4. Distribution of Enzymes Among Subcellular Fractions from Homogenates Obtained by Grinding T. brucei Cells (a) with Alumina and (b) with Silicon Carbide.

Ordinate: relative specific activity of enzymes. Abscissa: subcellular fractions represented by their relative protein content and according to the order in which they were isolated; from left to right: 1, nuclear and other cell debris which sediment at 1,000 *g* (fraction 1KP); 2, post-nuclear subcellular organelles sedimenting at 14,500 *g* (fraction 14.5KP); 3, lighter subcellular granules which fail to sediment at 14,500 *g* but sediment during centrifugation for 1 hr at 105,000 *g* (fraction 105KP); 4, final supernatant containing only soluble cell components (fraction 105KS).

of the fractions against their relative protein content; thus the area in each block is proportional to the percentage of the activities recovered in the corresponding fraction, and the height to the degree of purification achieved. The pattern of distribution of glycerolphosphate oxidase has been included in Figure IV.4b to confirm the particulate nature of this enzyme (Fairlamb & Bowman, 1975).

The results show quite clearly that the distribution patterns of the following six enzymes, hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, phosphoglycerate kinase and glycerolphosphate dehydrogenase are similar to each other and to the distribution obtained when the fractions were assayed for complex enzyme activity. Up to 80% of the activities of five of these enzymes occurs in fractions 14.5KP and 105KP; the value for the sixth enzyme, phosphoglucose isomerase is 60%. It is therefore highly possible that all six enzymes are present in the same location in the cell. The degree of enzyme purification found for fraction 14.5KP from both alumina and silicon carbide extracts is approximately double the value for fraction 105KP. This, coupled with the fact that the complex activity is predominantly associated with fraction 14.5KP seems to suggest that the intracellular location of the activity of the complex is probably fraction 14.5KP and that fraction 105KP is either contaminated by this material or possibly it is largely made up of degraded material from fraction 14.5KP. Evidence is provided below (in Section IV.5) to show that the second interpretation is more likely to be correct.

The patterns of distribution for phosphoglycerate mutase, enolase and pyruvate kinase are almost identical; the bulk of the activities of all three enzymes is associated with the final

Table IV.2. Enzyme Activities in Subcellular Fractions of Homogenates of Alumina-Treated T. brucei Cells

Enzyme activities have been expressed as μmoles of substrate converted/min/mg protein.

		Subcellular Fractions			
Enzymes	Homogenate	1KP	14.5KP	105KP	105KS
HK	0.28	0.35	0.80	0.69	0.03
PGI	0.50	0.39	1.17	0.68	0.24
PFK	0.63	0.43	1.05	0.92	0.01
ALD	0.05	0.23	0.45	0.22	0.01
GAPDH	0.39	0.06	0.04	0.37	0.32
PGK	1.14	0.07	0.66	0.36	0.05
PGlyM	0.84	0	0.03	0.01	1.58
ENOL	1.10	0.03	0.09	0.16	1.66
PK	1.19	0.07	0.24	0.23	0.99
GPDH	0.30	0.20	0.32	0.21	0.02
GK	1.72	0.67	0.75	0.69	0.11

supernatant (fraction 105KS). These results strongly suggest that the three enzymes exist in T. brucei cells as soluble proteins in the cell sap.

The distributions of glyceraldehyde-phosphate dehydrogenase (approximately 80% of whose activity is found in fraction 105KS) and glycerokinase (for which silicon carbide solubilises approximately 60% but alumina solubilise less than 40% of the activity) are harder to interpret; it is suggested that either these two enzymes exist in the cell in association with sub-cellular organelles which break up easily to release the enzymes, or else that the enzymes actually exist in the cell as soluble proteins but have the tendency to adsorb to subcellular organelles or cell membranes during the fractionation procedures.

IV.5 EFFECT OF DETERGENTS ON ENZYME ACTIVITIES IN THE SUBCELLULAR FRACTIONS OF HOMOGENATES OBTAINED BY GRINDING T. BRUCEI CELLS

In Tables IV.2 and IV.2.1, enzyme activities of fractions treated with detergents have been compared with the activities in the respective untreated fractions as follows. Aliquots of untreated fractions and fractions treated in 0.5% saponin. 0.5% Triton X-100 or 0.5 mg% digitonin were incubated at 25°C for 10 min. Thereafter, the samples were assayed and the specific activities of the enzymes were calculated; the results in Table IV.2.1 compare the activities of detergent-treated fractions with the respective untreated materials.

In making these comparisons, where the specific activity of any enzyme in any of the fractions was found to be less than

Table IV. 2. 1. Effect of Detergent Treatment on the Activities of the Enzymes Listed in Table IV. 2.

The results were obtained by dividing the specific enzyme activities in detergent treated fractions by the values observed from the respective untreated fractions.

	a) Fraction 1KP			b) Fraction 105KS		
Enzyme	0.5% Saponin	0.5% Triton X-100	0.5 mg% Digitonin	0.5% Saponin	0.5% Triton X-100	0.5 mg% Digitonin
HK	2.1	2.1	1.2	*	*	*
PGI	1.7	1.7	1.3	1.0	1.0	1.0
PFK	1.6	1.7	1.0	*	*	*
ALD	0.8	0.7	0.9	*	*	*
GAPDH	*	*	*	1.1	1.1	0.6
PGK	*	*	*	*	*	*
PGlyM	*	*	*	0.5	0.6	0.7
ENOL	*	*	*	1.1	1.0	1.1
PK	*	*	*	1.4	1.1	1.2
GPDH	1.4	1.4	1.1	*	*	*
GK	2.1	1.9	1.7	*	*	*

	c) Fraction 14.5KP			d) Fraction 105KP		
Enzyme	0.5% Saponin	0.5% Triton X-100	0.5 mg% Digitonin	0.5% Saponin	0.5% Triton X-100	0.5 mg% Digitonin
HK	1.4	1.5	1.0	1.1	1.1	0.9
PGI	1.2	1.3	0.9	1.1	1.2	1.2
PFK	1.7	1.7	0.9	1.1	1.1	1.1
ALD	1.0	1.0	1.0	0.8	0.6	1.0
GAPDH	*	*	*	1.0	1.3	1.0
PGK	1.2	1.6	1.5	1.1	1.2	1.0
PGlyM	*	*	*	*	*	*
ENOL	*	*	*	1.2	0.9	0.7
PK	1.3	1.2	1.1	0.9	0.9	0.9
GPDH	1.3	1.3	0.7	0.8	0.9	0.8
GK	0.9	0.9	0.8	1.1	0.9	0.8

* Activities too low to be of significance.

20% of the specific activity recovered in the homogenate, the results were considered to be of little significance and were therefore not dwelt upon.

IV.5.1. Effect of Detergent Treatment on Enzyme Activities in Fraction 1KP

With the exception of aldolase which is inhibited by all three detergents, five enzymes show appreciable increases in their activities by both saponin and Triton X-100 treatment. Digitonin also activated four of these five enzymes, hexokinase, phosphoglucose isomerase, glycerolphosphate dehydrogenase and glycerokinase but it had no effect on the fifth enzyme, phosphofructose kinase. The activities of the remaining five enzymes in this fraction were too low to warrant meaningful discussion of their results.

That saponin and Triton X-100, both detergents known to have a strong solubilising effect on membrane proteins, should enhance enzyme activities in fraction 1KP, should not be surprising since this fraction contains cell debris and any cells not disrupted during the grinding process. It is therefore suggested that these results of detergent effect on enzyme activities in fraction 1KP may not have any bearing on the problem being investigated, which is to determine whether the detergents produce their effect by acting on the enzyme molecules themselves or on the membranes of subcellular organelles.

IV.5.2. Effect of the Detergents on Enzyme Activities in Fraction 105KS

As before, the results on hexokinase, phosphofructose kinase, aldolase, phosphoglycerate kinase, glycerolphosphate dehydrogenase

and glycerokinase are not considered significant since the activities of these enzymes in this fraction are very low. Of the remaining enzymes, the activity of phosphoglucose isomerase (the only particulate enzyme found in appreciable quantities in this fraction) is clearly not affected by any of the detergents. Glyceraldehyde-phosphate dehydrogenase activity in fraction 105KS is slightly enhanced by both saponin and Triton X-100, although it is inhibited by as much as 40% by digitonin. Of the three enzymes known to be preferentially located in the soluble fraction, both enolase and pyruvate kinase are activated by either saponin or digitonin although this effect on pyruvate kinase is higher than it is for enolase. Triton X-100 has no effect on enolase whereas it slightly activates pyruvate kinase.

Possibly the most significant effect of the detergents on enzyme activities in fraction 105KS is the strong inhibition of phosphoglycerate mutase activity caused by all three detergents. As pointed out earlier, the mean specific activity of this enzyme in cells lysed by detergent treatment or by freezing and thawing was found to be very low (never exceeding 0.3 µmole/min/mg protein). On the other hand, values of 0.8 µmole/min/mg protein or more were routinely recovered in homogenates prepared by grinding T. brucei cells with alumina or silicon carbide. In the experiment under discussion, the specific activity of 1.58 found in fraction 105KS was reduced to 0.81, 1.00 and 1.19 when this fraction was assayed after prior incubation in 0.5% saponin, 0.5% Triton X-100 and 0.5 mg% digitonin respectively. It is therefore concluded that phosphoglycerate mutase is strongly inhibited by all three detergents, and this must account for the low recoveries of this enzyme in homogenates obtained by treating

T. brucei cells with the detergents.

IV.5.3. Effects of Detergent Treatment on Enzyme Activities in Fractions 14.5KP and 105KP

Of the enzymes found to be associated with the multienzyme complex, aldolase in fraction 14.5KP appears not to be affected by any of the detergents, whereas aldolase in fraction 105KP is inhibited by both saponin and Triton X-100, but not by digitonin. Hexokinase, phosphoglucose isomerase, phosphofructose kinase, phosphoglycerate kinase and glycerolphosphate dehydrogenase in fraction 14.5KP are all activated by the two detergents, saponin and Triton X-100, but not by digitonin. Indeed, phosphoglucose isomerase, phosphofructose kinase and glycerolphosphate dehydrogenase are all inhibited by treatment of fraction 14.5KP with digitonin, the inhibition of glycerolphosphate dehydrogenase being as high as 30%.

Except phosphoglucose isomerase, whose activity in fraction 105KP is increased by about 20% in Triton X-100 or digitonin, and glycerolphosphate dehydrogenase which shows 20% inhibition by saponin or digitonin, the activities of these particulate enzymes are not affected by more than 10% after detergent treatment of fraction 105KP for 10 min at 25°C. It is therefore clear that where detergent treatment increases particulate enzyme activities, the effect is greater on fraction 14.5KP than it is for fraction 105KP. This point was confirmed in another experiment in which aliquots of fractions 14.5KP, 105KP and 105KS obtained by differential centrifugation of a homogenate of silicon carbide

Table IV.2.2. Effect of 0.1% Triton X-100 on Enzyme Activities in Fractions 14.5KP, 105KP and 105KS Obtained by Differential Centrifugation of a Homogenate of Silicon Carbide Treated T. brucei Cells

Enzyme activities have been expressed as μmoles/min/mg protein; the ratio, y/x compares the activities of Triton X-100 treated samples with untreated material.

	Fractions								
	14.5KP			105KP			105KS		
	(x) Untreated	(y) Treated	y/x	(x) Untreated	(y) Treated	y/x	(x) Untreated	(y) Treated	y/x
HK	1.037	3.449	3.33	0.797	0.774	0.98	0.093	0.076	0.82*
PGI	1.784	2.497	1.40	1.067	0.737	0.69	0.274	0.270	0.99
PFK	2.033	4.144	2.04	0.950	1.636	1.72	0.076	0.070	0.92*
ALD	0.503	0.500	0.99	0.178	0.175	0.98	0.009	0.006	0.67*
GAPDH	0.223	0.095	0.43	0.072	0.147	2.04*	0.404	0.189	0.47
PGK	2.731	6.710	2.46	0.584	0.757	1.30	0.039	0.035	0.90*
PGlyM	0.012	0.014	1.17	0.023	0.019	0.83*	1.960	1.830	0.93
ENOL	0.174	0.188	1.08	0.164	0.176	1.07	1.274	1.218	0.96
PK	0.140	0.014	0.10	0.026	0.016	0.62*	1.100	0.220	0.20
GPDH	0.928	2.667	2.87	0.560	0.871	1.56	0.042	0.039	0.93*
GK	1.175	0.195	0.17	0.345	0.210	0.61	1.770	1.315	0.74

* Results not significant

treated cells were incubated (in the presence or absence of 0.1% Triton X-100) for 10 min on ice before being assayed for enzyme activities (Table IV.2.2.).

To minimise the possibility of disrupting cell organelles, a much larger quantity of T. brucei cells was used in this experiment and the grinding process was stopped when light microscopic examination showed that about 50% of the cells had been disrupted. The slurry of silicon carbide was then centrifuged at 1,000 g for 5 min, the supernatant was carefully decanted and subjected to centrifugation at 1,000 g for 10 min to remove most of the contaminating silicon carbide granules; the clarified supernatant served as the homogenate. By studying the effect of only one detergent, it became possible to reduce considerably the time needed to complete the experiment, thus making it possible to use material from the same T. brucei preparation throughout; it was also intended to minimise errors caused by possible inactivation of enzymes during storage. Sample incubation with the detergent was also done on ice to reduce the possibility of enzyme degradation by proteases.

The results in Table IV.2.2. clearly support the conclusions previously arrived at. Apart from aldolase, all the particulate enzymes in fraction 14.5KP are activated by Triton X-100. Where similar activation occurs for the enzymes in fraction 105KP this never exceeds the values obtained for the enzyme in fraction 14.5KP. These results confirm the view that the intracellular location of the multienzyme complex is the subcellular fraction 14.5KP and that the activity found in fraction 105KP results from partial degradation of the material

in fraction 14.5KP. It should be noted that of the enzymes which are preferentially located in the final supernatant, glyceraldehyde-phosphate dehydrogenase, pyruvate kinase and glycerokinase are strongly inhibited by Triton X-100.

Summary of the Results on Isolation of the Multienzyme Complex from Homogenates Obtained by Grinding T. brucei Cells with Alumina or Silicon Carbide

A multienzyme complex of hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, phosphoglycerate kinase and glycerolphosphate dehydrogenase has been obtained from bloodstream long slender T. brucei after grinding the cells with either alumina or silicon carbide.

The complex activity is concentrated in the post-nuclear fraction which sediments at 14,500 g. With the possible exception of aldolase, the activities of all the enzymes associated with this fraction are greatly enhanced after pre-incubation of the fraction in either of the two detergents, saponin and Triton X-100. Similar activation may be observed for the activities of these enzymes in the material which sediments at 105,000 g after removal of the 14,500 g pellet. However, the activation observed in fraction 105KP is never greater, and may be considerably less than the increase in enzyme activities observed in the heavier fraction.

Since 0.1% Triton X-100 enhanced the activities of hexokinase phosphofructose kinase, phosphoglycerate.kinase, glycerolphosphate dehydrogenase and to a lesser degree phosphoglucose isomerase, it is suggested that these enzymes are all partly latent and become fully activated after treatment with Triton X-100.

It is therefore concluded that the complex enzymes are probably located in a subcellular organelle; that inside this organelle, hexokinase, phosphofructose kinase, phosphoglycerate kinase, glycerolphosphate dehydrogenase and, to a lesser extent, phosphoglucose isomerase are all partly latent and become fully activated by disrupting the organelle with saponin or Triton X-100. It is highly likely that aldolase is also strongly associated with this organelle, and it is possible that it fails to become activated in the presence of the detergents because it is buried more deeply in a non-polar environment within the organelle; or that although it is exposed by Triton X-100, it tends to be inactivated by the detergent. Three other enzymes, glyceraldehyde-phosphate dehydrogenase, glycerokinase and pyruvate kinase are also inhibited by 0.1% Triton X-100. Glyceraldehyde-phosphate dehydrogenase and glycerokinase are the two enzymes whose distribution among differential centrifugation fractions proved anomalous (see Section IV.4.).

Although substantial activities of glyceraldehyde-phosphate dehydrogenase (32%) and glycerokinase (34%) were found to be associated with fraction 14.5KP, these values could be reduced by as much as 57% (GADPH) or 83% (GK) after a 10 min incubation of fraction 14.5KP, on ice, in 0.1% Triton X-100. If the two enzymes are also buried in a subcellular organelle, it could mean that a higher percentage of their activities might be associated with the particle than the values quoted above. However, because detergent treatment inhibits the enzymes, their full potential had probably not been revealed by these experiments. It is therefore highly likely that these two enzymes also form an integral part of the multienzyme complex.

SECTION V

SOME PHYSICAL PROPERTIES OF THE MULTIENZYME COMPLEX

Introduction

Evidence was provided in Section IV. to show that the complex enzyme activity involved in the conversion of glucose to glycerol-phosphate is located in the post-nuclear subcellular fraction 14.5KP from homogenates obtained by grinding T. brucei cells with alumina or silicon carbide. In this section, data will be provided to show

(1) that the multienzyme complex is located in an organelle, probably the microbodies;

(2) that the membrane of the subcellular organelle probably forms a sufficient barrier to exogenous substrates and cofactors to ensure that enzymic reactions which depend on such exogenous compounds do not show maximum enzyme activity unless the limiting membrane is disrupted; and

(3) that the forces binding the individual enzymes of the complex to the subcellular organelle are sufficiently strong to withstand such methods of biochemical fractionation as chromatography in Biogel columns and electrophoresis in acrylamide gels.

V.1. DETERMINATION OF THE DENSITY OF THE MULTIENZYME COMPLEX BY ISOPYCNIC SUCROSE GRADIENT CENTRIFUGATION

In view of the fact that the distribution patterns of the oxidase activity, the NAD^+-dependent glycerolphosphate dehydrogenase

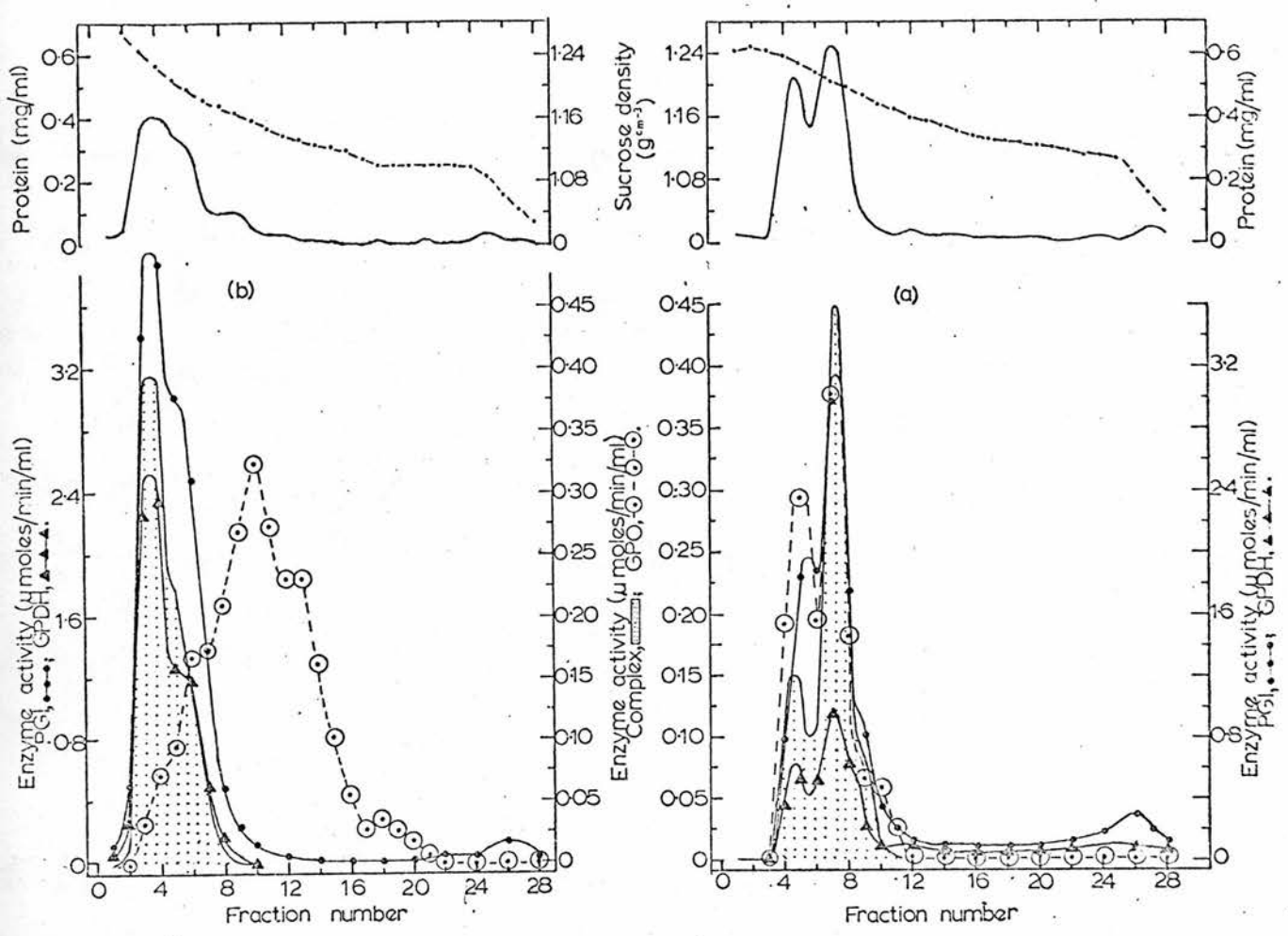


Fig. V. 1. Isopycnic Centrifugation in Sucrose Gradient of Post-Nuclear Fraction 14.5KP (12 mg protein) from Homogenates Obtained by (a) Three Cycles of Freezing and Thawing or (b) Silicon Carbide Grinding of Blood-stream Long Slender T. brucei.

Ordinate: (top) protein concentration (mg/ml) or density (g/ml sucrose); (bottom) enzyme activity (μmoles substrate converted/min/ml). Abscissa: fractions in the order of their position in the centrifuge tube, from bottom of tube (left) to meniscus (right). Enzyme activities are represented by ▨, multienzyme; •—•, PGI; ▲—▲, GPDH; ⊙- -⊙, GPO. The top graph represents protein (——) and concentration of sucrose (-·-·-).

Other experimental details are given in the text.

and the multienzyme complex overlap during differential centrifugation of T. brucei homogenates (Section IV), it was important to determine whether the complex would behave as an entity during isopycnic sucrose density centrifugation; and if so, whether it would band with the oxidase or the dehydrogenase.

Isopycnic sucrose density centrifugation of fraction 14.5KP from cells disrupted by freezing and thawing or with digitonin were found to be similar. In the same manner, the results with material from alumina or silicon carbide treated cells were almost identical. Since no further information could be provided by presenting results from cells lysed by all four methods, only representative results from cells disrupted by freezing and thawing (Figure V.1a) or by grinding with silicon carbide (Figure V.1b) have been presented.

The results in Figure V.1a and V.1b represent respectively, the typical patterns of isopycnic sucrose density centrifugation of the post-nuclear 14,500 g pellet from homogenates obtained by disrupting bloodstream T. brucei by means of freezing and thawing or by grinding with silicon carbide. Enzyme activities have been expressed as μmoles substrate converted/min/ml and protein concentration as mg/ml in each fraction.

Where cells had been disrupted by freezing and thawing (or by digitonin), sucrose gradient centrifugation resulted in two main protein peaks. As well as showing multienzyme activity, both peaks also showed activity for the NAD^+-dependent glycerol-phosphate dehydrogenase and the L-glycerol-3-phosphate oxidase. The median equilibrium density values for the two peaks are 1.232 and 1.203. In one such experiment, where samples from the two peaks were examined with the light microscope, the

peak with the higher median density was found to contain mainly pieces of flagella and nuclei, whereas the other peak contained particles with the same shape as, but much smaller than nuclei, contaminated by a few nuclei but no flagella. When the fractions were assayed for phosphoglucose isomerase, less than 5% of the activity was found in a small protein peak which bands near the meniscus in fraction 26; the remaining phosphoglucose isomerase activity was found in the two major protein bands with peaks at fractions 5 and 7 respectively.

Density gradient centrifugation of fraction 14.5KP from homogenates obtained by grinding bloodstream T. brucei with silicon carbide (or alumina) on the other hand, usually resulted in one main protein peak with a small shoulder towards the less dense part of the gradient. All the multienzyme activity is concentrated in this peak and is coincident with the activities of the only two component enzymes assayed for, the NAD^+-dependent glycerolphosphate dehydrogenase and phosphoglucose isomerase. The median equilibrium density of this peak is 1.226 and it is well separated from a peak of L-glycerol-3-phosphate oxidase activity which shows a median equilibrium density of 1.159.

Since microbodies isolated from protozoa are known to sediment in sucrose gradients with median equilibrium densities of 1.20-1.24 (Müller, 1975), these results appear to indicate that the multienzyme complex is located in bloodstream T. brucei microbodies. Whether these microbodies will sediment in sucrose gradients with the oxidase system appears to be dependent on the method chosen for cell disruption. Assuming that cell lysis by digitonin or freezing and thawing is a more severe method than disruption by grinding with silicon carbide or alumina, it could be argued that the association of the oxidase activity with the

Table V. 1. Relative Protein Content and Multienzyme Activity in Homogenates and Subcellular Fractions of T. brucei.

The results have been based on 1 ml packed cell volume (PCV) of bloodstream long slender form T. brucei.

(a) Cells Lysed by Three Cycles of Freezing and Thawing and Homogenized by 40 strokes with the Hand-operated Dounce Homogenizer.

	Protein (mg/ml PCV)	Activity (uM/min)	% of Activity in Homogenate	% Recovery	Sp. Acty. (μM/min/mg)	Purification
Homogenate	88.0	4.56	100.0	-	0.052	1.0
Differential Centrifugation Fraction						
1KP	22.3	1.33	29.2	82.7	0.059	1.14
14.5KP	4.4	2.28	50.0		0.518	9.96
14.5KS	19.4	0.16	3.5		0.008	0.15
Sucrose Gradient Fraction						
4	0.63	0.23	5.04	45.0	0.365	7.04
5	0.88	0.24	5.29		0.284	5.25
6	0.71	0.21	4.54		0.296	5.64
7	1.09	0.78	17.08		0.716	13.69
8	0.65	0.42	9.28		0.646	12.44
9	0.16	0.15	3.29		0.938	18.19
10	0.07	0.02	0.50		0.286	6.37

(b) Cells Homogenized by Grinding with Silicon Carbide.

Homogenate	54.0	1.35	100.0	-	0.025	1.0
Differential Centrifugation Fraction						
1KP	8.7	0.27	20.0	112.9	0.031	1.24
14.5KP	3.9	1.11	81.8		0.285	11.26
14.5KS	18.3	0.15	11.1		0.008	0.32
Sucrose Gradient Fraction						
2	0.06	0.02	1.35	112.4	0.333	12.88
3	0.44	0.47	34.93		1.068	42.56
4	0.51	0.48	35.08		0.941	37.08
5	0.45	0.28	20.68		0.622	24.96
6	0.39	0.18	13.37		0.462	18.72
7	0.17	0.07	5.32		0.412	16.92
8	0.11	0.02	1.70		0.182	4.76

NAD^+-dependent glycerolphosphate dehydrogenase (and also the complex activity) is an artefact of the method of T. brucei disruption with digitonin or by freezing and thawing. It is suggested that the oxidase system has a different location, probably in the mitochondria, in T. brucei cells (Opperdoes, et al., 1977b), and harsh methods of cellular treatment cause it to become adsorbed to the microbodies. For ease of detailed analysis, the results from the two experiments, on the multienzyme activity were normalised and presented in Table V.1. as follows. The complex activity in the homogenate was converted to enzyme units per ml packed cell volume (PCV). The results for the various fractions were then recalculated to correspond to the expected values for samples obtained by fractionating homogenates from 1 ml packed cell volume of bloodstream T. brucei.

The values of 88 mg and 54 mg protein per ml PCV respectively for cells lysed by freezing and thawing or by grinding with silicon carbide confirm the observation made earlier in Section IV. that cell grinding with abrasives leadsto loss of material as a result of adsorption of cell components to the granules. Comparison of the protein content in the fractions from differential centrifugation confirms that the bulk of the material lost during cell grinding with abrasives belongs to the nuclear fraction 1KP whose protein concentration of 23.3 mg per ml PCV in cells lysed by freezing and thawing falls to 8.7 mg when the cells are disrupted by grinding.

Other important features of the results detailed in Table V.1. may be summarised as follows. Where cells have been lysed by freezing and thawing, the multienzyme activity per ml PCV is 4.56 as against a value of 1.35 for cells treated by grinding; showing

clearly that freezing and thawing has caused over three-fold increase in the activity of the multienzyme complex. A similar pattern is followed by the differential centrifugation fractions, 1KP and 14.5KP but not the supernatant left after centrifugation at 14,500 g. These results may be interpreted in two ways; either cell grinding with abrasives, in some way, inactivates the multienzyme or else cell lysis by freezing and thawing enhances the enzyme activity. The evidence provided in Section IV., coupled with the fact that unlike fractions 1KP and 14.5KP the specific multienzyme activity in fraction 14.5KS is not affected by the method of cell disruption suggests that the multienzyme activity *per se* is not affected by either method of cell treatment. It would appear, instead, that the apparent increase in enzyme activity observed in homogenates and fractions 1KP and 14.5KP from cells lysed by freezing and thawing may be due to easier access of substrates and cofactors to the complex obtained by this method of treatment, but not by grinding. In other words greater latency is apparent in preparations made from grinding.

Approximately 61% of the total multienzyme activity in the homogenate of cells lysed by freezing and thawing is recovered in fraction 14.5KP as compared to a figure of 72% or more, for the corresponding fraction from cells disrupted by grinding with silicon carbide. Also, only 45% of the complex activity is recovered during isopycnic sucrose gradient centrifugation of fraction 14.5KP from cells lysed by freezing and thawing as opposed to the apparent total recovery of this activity when the experiment is performed with the complex from cells disrupted by grinding. Finally, approximately 70% of the total multienzyme

activity in the homogenate from cells disrupted by grinding is concentrated in only two fractions during sucrose gradient fractionation of the complex as opposed to about 26% when the experiment is conducted with samples from cells lysed by freezing and thawing.

Significantly, the specific multienzyme activity in isopycnic sucrose gradient fractions appears not to be affected by the method of cell lysis. This observation would appear to contradict some of the conclusions drawn from the results already discussed. However, there is evidence (Strauss, 1957) to show that after suspension in hypertonic sucrose, subcellular organelles may become completely ruptured by sudden osmotic changes when they are returned to a medium containing isotonic sucrose. Thus it is possible that under the assay conditions any membrane enclosing the complex becomes disrupted; a situation like that would reduce or completely abolish any differences in multienzyme activity that might originally exist for the sucrose gradient fractions. This probably explains the fact that while the post-nuclear fraction, 14.5KP showed similar degree of purification over the homogenate irrespective of which method of cell lysis was adopted, for the most active sucrose density fractions from cells treated by grinding, the degree of purification of multienzyme activity is over three-fold higher than the degree of purification found for cells lysed by freezing and thawing.

Summary of the Results on the Identification of the Probable Intracellular Location of the Multienzyme Complex by Isopycnic Sucrose Gradient Centrifugation

The results from the two experiments support the conclusion that the subcellular fraction 14.5KP is the intracellular location of the multienzyme complex, and that (1) the complex is a subcellular entity with the same median equilibrium density as established for protozoan microbodies; (2) while the integrity of this entity is preserved, at least partially, by grinding bloodstream T. brucei with silicon carbide or alumina, it may become substantially destroyed by such harsh methods of cell lysis as freezing and thawing or incubation in digitonin; (3) the subcellular particle containing the complex is not the same particle that contains the glycerolphosphate oxidase system of T. brucei, although experiments conducted with cells lysed by methods which degrade subcellular organelles may give the erroneous impression that the two particles are identical; and finally (4) silicon carbide or alumina grinding of bloodstream long slender forms of T. brucei gives a homogenate from which a fraction which shows a forty-fold increase in multienzyme activity can be isolated by differential centrifugation followed by isopycnic sucrose gradient centrifugation, although the apparently high purification achieved may have resulted from osmotic shock of the subcellular organelles during the assay for complex activity.

V.2. EFFECT OF SODIUM CHLORIDE ON THE BANDING OF THE MULTIENZYME COMPLEX DURING ISOPYCNIC SUCROSE DENSITY CENTRIFUGATION

Where protein-protein interactions or association of protein molecules with membranes occur, they do so mainly through ionic and/or hydrophobic interactions. In view of this, experiments were performed to study the possible effects that NaCl may have on the pattern of distribution of the multienzyme complex during isopycnic sucrose gradient centrifugation. The results of these experiments were expected to help provide answers to the question of whether the multienzyme activity results from a mere association of the component enzymes through ionic interactions, or whether, as suggested in Section IV., the enzymes are actually located in a subcellular organelle with a limiting membrane. Such experiments could also help to determine whether the individual enzymes (if located in an organelle), are associated with structural components of the organelle.

A post-nuclear fraction 14.5KP from silicon carbide treated cells was evenly suspended in a solution of 0.01 M TEA/KOH, pH 7.6, containing 0.25 M sucrose and 0.1 mM dithiothreitol (DTT). It was then divided into three 3 ml samples, A, B and C. Samples B and C were each added to 0.2 ml, 2.4 M NaCl so that the final concentration of NaCl in each sample was 0.15 M. They were then stood on ice, alongside the untreated sample, A, for 30 min. Thereafter, sample C was dialysed against the TEA-Sucrose-DTT solution to remove the NaCl; sample B was loaded onto a sucrose gradient which had been prepared in

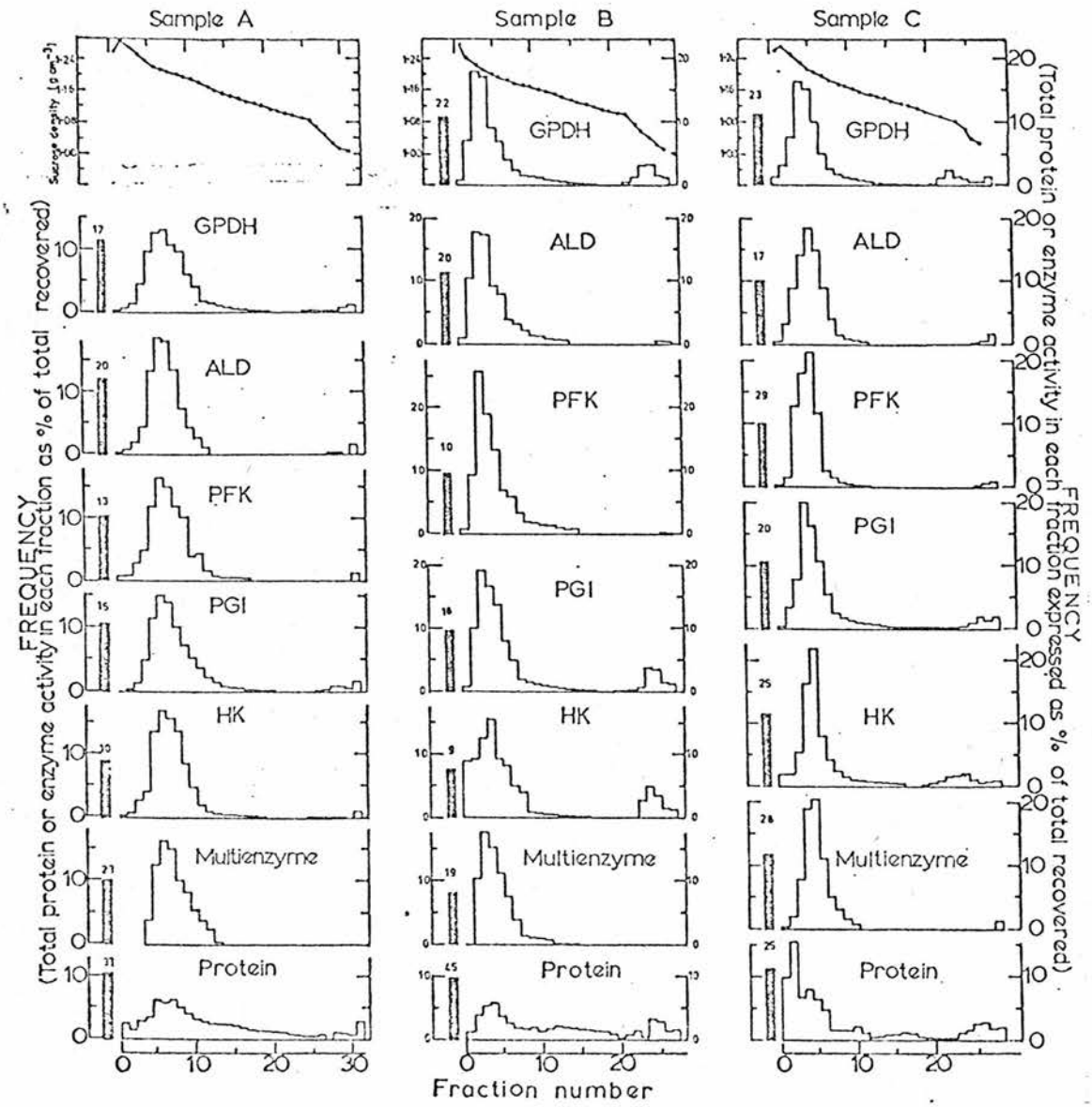


Fig. V. 1. 1. Isopycnic Centrifugation in Sucrose Gradient of Aliquots of Post-Nuclear Fraction 14.5KP (5 mg protein each) from Homogenates Obtained by Silicon Carbide Grinding of Bloodstream Long Slender Form T. brucei.

Sample A denotes untreated material; Sample B, material which had been incubated and centrifuged in 0.15M NaCl; Sample C, material which had been treated with 0.15M NaCl and dialysed to remove the salt before being subjected to centrifugation in sucrose gradient in the absence of the salt.

■, material not included in the density distribution.

Other experimental details are given in the text.

0.01 M potassium phosphate pH 7.6 containing 0.1 mM DTT and 0.15 M NaCl. Samples A and C were loaded separately onto sucrose gradients which had been prepared in the same buffer containing 0.1 mM DTT but no NaCl. The three samples were then subjected to isopycnic sucrose gradient centrifugation alongside each other; they were fractionated in the usual manner and the fractions were analysed for protein and enzyme activities. As shown in Figure V.1.1., the total activity (for enzymes) or total protein in each fraction has been expressed as a percentage of the total enzyme activity or protein recovered.

During experiments on sucrose gradient centrifugation of silicon carbide treated T. brucei preparations, it was discovered that a mixture of contaminating silicon carbide granules and sucrose frequently formed a ring near the bottom of the centrifuge tube. This material proved so sticky that it was impossible to recover it during the normal fractionation step and had to be removed by washing with buffer after all the fractions had been collected. Because the presence of silicon carbide granules made it impossible to determine the density with the refractometer, these materials were not included in the density distributions. To underline this fact, they have been represented by rectangles which have been drawn separately from the rest and been filled. Above each block of such shaded rectangle is a number which represents the percentage protein or enzyme activity associated with this material.

The results in Figure V.1.1. show that each of the three samples gave a peak of multienzyme activity which is coincident with the activities of the individual component enzymes of the

Table V. 2. Effect of NaCl Treatment on the Banding of the Multi-enzyme Complex and Its Component Enzymes During Isopycnic Sucrose Gradient Centrifugation.

Experimental details are given in Figure V. 1. and the text.

SAMPLE A		SAMPLE B		SAMPLE C	
Fraction Number	Density	Fraction Number	Density	Fraction Number	Density
6	1.2225	3	1.2219	4	1.2274
7	1.2176	4	1.2111	5	1.2127
Average	1.220	Average	1.217	Average	1.220

Table V. 2. 1. Specific Enzyme Activities in the Two Peak Fractions Obtained by Sucrose Gradient Centrifugation of Samples of the Post-nuclear Fraction 14.5KP.

Enzyme activities have been expressed as μmoles substrate converted/min/mg protein. A denotes untreated fraction 14.5KP; B, material which had been incubated and centrifuged in 0.15M NaCl; C, fraction 14.5KP which had been treated with 0.15M NaCl and dialysed to remove the salt before being subjected to sucrose gradient centrifugation. Other experimental details are given in Figure V. 1. and in the text.

(a) Assays Performed in the Absence of Triton X-100

		Enzymes					
Sample	Fr. No.	Complex	HK	PGI	PFK	ALD	GPDH
A	6	0.89	4.94	6.94	6.59	3.56	6.90
	7	0.67	4.22	5.78	6.03	3.21	5.37
	Average	0.78	4.58	6.36	6.31	3.39	6.14
B	3	1.02	6.64	7.70	4.53	3.91	6.90
	4	0.83	7.14	5.90	5.63	3.02	5.63
	Average	0.93	6.89	6.80	5.08	3.47	6.27
C	4	0.84	4.72	6.10	6.50	3.50	5.53
	5	0.90	5.92	5.87	6.00	2.97	5.91
	Average	0.87	5.32	5.99	6.25	3.24	5.72

(b) Assays Performed in the Presence of 0.02% Triton X-100

A	6	1.67	6.03	6.44	9.12	3.17	8.60
	7	1.44	5.15	5.65	7.64	2.75	8.27
	Average	1.56	5.59	6.05	8.38	3.01	8.44
B	3	2.58	6.46	9.63	10.07	4.19	18.76
	4	2.03	7.47	7.55	6.04	3.70	16.31
	Average	2.31	6.97	8.59	8.06	3.95	17.54
C	4	1.66	5.89	6.51	10.30	3.42	10.12
	5	2.29	10.13	5.26	8.65	2.88	11.88
	Average	1.98	8.01	5.89	9.48	3.15	11.00

Table V. 2. 2. Comparison of the Average Specific Enzyme Activities in the Two Peak Fractions from the Untreated Sample, A, with the Average Specific Activities in Similar Fractions from the Two Samples (B & C) which had Been Treated with Isotonic NaCl.

Enzyme	B/A	C/A
Multienzyme	1.19	1.11
HK	1.50	1.16
PGI	1.07	0.94
PFK	0.80	0.99
ALD	1.03	0.96
GPDH	1.02	0.93

Table V. 2. 3. Effect of 0.02% Triton X-100 on Enzyme Activities in the Peak Fractions from Isopycnic Sucrose Gradient Centrifugation.

The results represent the ratio (y/x) obtained by dividing the average specific enzyme activities (y) of the two peak fractions assayed in the presence of 0.02% Triton X-100 by the values (x) for the same fractions assayed in the absence of the detergent. Other experimental details are given in Fig V. 1. and in the text.

	Enzymes					
Sample	Complex	HK	PGI	PFK	ALD	GPDH
A	2.02	1.22	0.96	1.33	0.87	1.40
B	2.49	1.01	1.27	1.65	1.15	2.81
C	2.21	1.48	0.99	1.66	0.89	1.92

complex. It is also clear from these results that treatment of the post-nuclear fraction 14.5KP with 0.15 M NaCl causes solubilisation of no more than 10% of the total protein, and only three of the five component enzymes, hexokinase, phosphoglucose isomerase and glycerolphosphate dehydrogenase appear to have a small percentage (less than 20%) of their total activity transferred to low density fractions.

These results, which suggest that isotonic NaCl probably has very little effect on the integrity of the organelle containing the multienzyme complex, appear to be supported by the results in Table V.2 which give the average equilibrium density of the two peak fractions from the three samples, A, B and C as 1.220, 1.217 and 1.220 respectively. The results can only be interpreted as showing that in hypertonic sucrose solutions, isotonic NaCl fails to cause substantial leakage of macromolecules like proteins from the ruptured organelle.

In Table V.2.1. are recorded the specific enzyme activities of the two peak fractions from sucrose gradient centrifugation of the subcellular fraction 14.5KP. In order to highlight any effects that NaCl has on the specific enzyme activities, the results in Table V.2.1. have been compared by dividing the average specific activities of the two peak fractions from samples B and C by the values obtained for the corresponding fractions from sample A (Table V.2.2.). The results show that (with the exception of hexokinase), the specific enzyme activities are only moderately enhanced by incubation and gradient fractionation of the post-nuclear fraction 14.5KP in isotonic NaCl; phosphofructose kinase is inhibited by this treatment.

The effect of Triton X-100 on the enzyme activities is shown in Table V.2.3. where the average specific activities obtained by assaying the two peak fractions in the presence of 0.02% Triton X-100 have been divided by the results obtained when the assay was performed in the absence of the detergent. The results show that Triton X-100 also enhances activities of the enzymes. Thus, with the possible exception of phosphoglucose isomerase and aldolase in samples A and C which are not activated by this treatment, all the enzymes are activated by the detergent; on the whole this activation of the enzymes by 0.02% Triton X-100 is highest for 14.5KP preincubated and fractionated in isotonic NaCl (fraction B), and lowest where the subcellular fraction 14.5KP had not been subjected to NaCl treatment (fraction A).

Summary of the Results on the Effects of Isotonic Sodium Chloride on the Activities and Distribution of Enzymes During Isopycnic Sucrose Gradient Centrifugation of the Subcellular Fraction 14.5KP from Bloodstream T. brucei

In view of the fact that isotonic NaCl treatment of fraction 14.5KP does not affect the median equilibrium density of the complex; causes only small leakage (less than 20%) of either protein or any of the component enzymes; and does not abolish or effectively reduce the enzyme activation caused by treatment of the complex in 0.02% Triton X-100, it may be concluded that although isotonic NaCl does probably alter the integrity of the entity containing the complex, this effect is not sufficiently severe to cause substantial leakage of either the proteins or the component enzymes.

Table V. 3. Effect of Isotonic Sodium Chloride on Multienzyme Activity in Subcellular Fraction 14.5KP.

A sample of T brucei subcellular fraction 14.5KP was made 0.15M with respect to NaCl and incubated on ice for 30 min. It was then centrifuged at 105,000 g for 1 hr and the supernatant was carefully removed fromthe pellet. Aliquots of all three samples (untreated fraction 14.5KP, the pellet and the supernatant) were assayed for multienzyme activity and the results have been presented as the total enzyme activity (μmoles substrate converted /min) for each sample.

Sample	Total Enzyme Activity
14.5KP	4.34
Pellet	3.13
Supernatant	0
Pellet + Supernatant	2.88

Table V. 3. 1. Activity of Multienzyme Complex in NaCl-Treated Pellet or Supernatant Expressed as Percentage of the Activity in Untreated Fraction 14.5KP.

Sample	Activity Relative To Untreated Fraction 14.5KP
Pellet	72%
Supernatant	0
Pellet + Supernatant	66%

V.3. THE RELATIONSHIP BETWEEN THE MULTIENZYME ACTIVITY AND THE INTEGRITY OF THE COMPLEX

From the results of the experiments discussed in Sections V.1, and V.2., it was suggested that the multienzyme complex is located in a subcellular organelle, and that the component enzymes of the complex are probably linked to or even buried in the matrix of the organelle. Further evidence is provided in this section to confirm the view (1) that the individual enzymes are quite firmly bound to the matrix or inner membranes of the organelle and (2) that the membrane surrounding the organelle acts as a barrier towards exogenous substrates of the component enzymes.

V.3.1. Effect of Isotonic Sodium Chloride on the Multienzyme Activity

The effect of isotonic sodium chloride on the multienzyme activity was determined by incubating an aliquot of fraction 14.5KP in 0.15 M NaCl on ice for 30 min and subjecting the treated material to centrifugation at 105,000 g for 1 hr; thereafter, the supernatant was carefully separated from the pellet, the pellet was evenly resuspended in buffer and the supernatant, the untreated fraction 14.5KP and the pellet suspension were all assayed for multienzyme activity. The results have been presented in Table V.3. as the total activity (μmoles substrate converted/min); they show that whereas NaCl treatment results in loss of 28% multienzyme activity, all the activity recovered is in the pellet obtained by centrifuging the treated fraction 14.5KP at 105,000 g for 1 hr.

Table V. 3. 2. Solubilization of the Component Enzymes of the Multienzyme Complex by Incubation of Fraction 14.5KP with Triton X-100, Followed by Centrifugation at 105,000 g for 1 hr.

The results have been presented as total protein or total enzyme activity (μmoles substrate converted/min) in each sample.

	Assay in Absence of Triton X-100			Assay in Presence of 0.02% Triton X-100		
	14.5KP	Pellet	Supernatant	14.5KP	Pellet	Supernatant
Protein	7.40	3.80	0.10			
Complex	3.44	1.65	0	4.50	3.30	0
HK	22.45	17.08	0.25	32.25	22.36	0.10
PGI	71.34	50.29	9.32	95.40	48.79	10.03
PFK	29.00	13.32	0	28.90	14.40	0
ALD	21.62	10.18	2.46	19.63	11.70	2.93
GPDH	38.00	30.52	0.30	42.92	34.49	0.41

Table V. 3. 3. Comparison of Enzyme Activities in the Pellet with the Activities in the Untreated Fraction 14.5KP.

	(Enzyme Activity in Pellet)/(Activity in Fraction 14.5KP)	(Enzyme Activity in Supernatant)/(Activity in Fraction 14.5KP)
Complex	0.49	0
HK	0.76	0.11
PGI	0.71	0.13
PFK	0.45	0
ALD	0.47	0.11
GPDH	0.80	0.08

Indeed the multienzyme activity in the pellet could be reduced further by assaying a mixture containing the same volumes of the pellet suspension and the supernatant as employed in the individual assays.

A comparison of the results in Table V.3.1., where the recovered activities have been compared with the activity in the original untreated fraction 14.5KP, with the results in Table V.3.3. confirms the conclusion previously arrived at that Triton X-100 treatment of the post-nuclear fraction 14.5KP probably causes greater disruption of the subcellular organelle than treatment with isotonic NaCl.

V.3.2. Attempts at Solubilising the Component Enzymes of the Complex by Incubation of the Post-Nuclear Fraction 14.5KP with Triton X-100, Followed by Centrifugation at 105,000 g

In order to determine the efficacy of Triton X-100 in solubilising the enzymes of the multienzyme complex, a sample of the post-nuclear fraction 14.5KP obtained by differential centrifugation of a homogenate of bloodstream T. brucei was added to Triton X-100 so that the final concentration of the detergent was 0.02%. The mixture was incubated on ice for 30 min and then subjected to centrifugation at 105,000 g for 1 hr. The supernatant was carefully separated from the pellet and the latter was resuspended in the buffer; both were dialysed overnight at $4^{\circ}C$ to remove the detergent. The two samples, as well as samples of the untreated fraction 14.5KP were analysed for protein and enzyme activities. The results have been presented in Table V.3.2 as the total protein or total enzyme activity

Table V. 3. 4. Enzyme Activities Expressed as Percentages of the Total Recovered Activities in the Subcellular Fraction 14.5KP Treated with 0.02% Triton X-100.

	Percentage Activity in Pellet	Percentage Activity in Supernatant
Complex	100	0
HK	99	1
PGI	84	16
PFK	100	0
ALD	81	19
GPDH	99	1

(μmoles/min) for the untreated fraction 14.5KP, the pellet and supernatant obtained after centrifuging the Triton X-100 treated 14.5KP fraction at 105,000 g for 1 hr. It is clear from these results that there is substantial loss of both protein and enzyme activity through Triton X-100 treatment of fraction 14.5KP followed by dialysis.

To highlight these losses, the enzyme activities in the pellet and the supernatant have been compared with the activities in the untreated sample (Table V.3.3.). These results show that whereas the pellet still retains 49% of the multienzyme activity and component enzyme activities ranging from 45% for phosphofructose kinase to 80% for glycerolphosphate dehydrogenase, in no case does the activity in the supernatant reach 14%; indeed no multienzyme activity nor activity of phosphofructose kinase was detected in the supernatant. Thus, even assuming that the losses in enzyme activity result entirely from the effect of the detergent on solubilised enzymes (which become inactivated in the presence of the detergent) it is clear that such treatment still leaves as much as 49% of multienzyme activity associated with the structural components of the complex.

On the other hand, if the losses are attributed to unspecific adsorption of protein to the dialysis membrane, then it could be claimed as shown in Table V.3.4. that the treatment has so little solubilisng effect on the enzymes that activities ranging from 81% for aldolase to 100% for both phosphofructose kinase and the multienzyme are still left in the structural components of the complex. The fact that the specific multienzyme activity in the pellet (see Table V.3.2.)

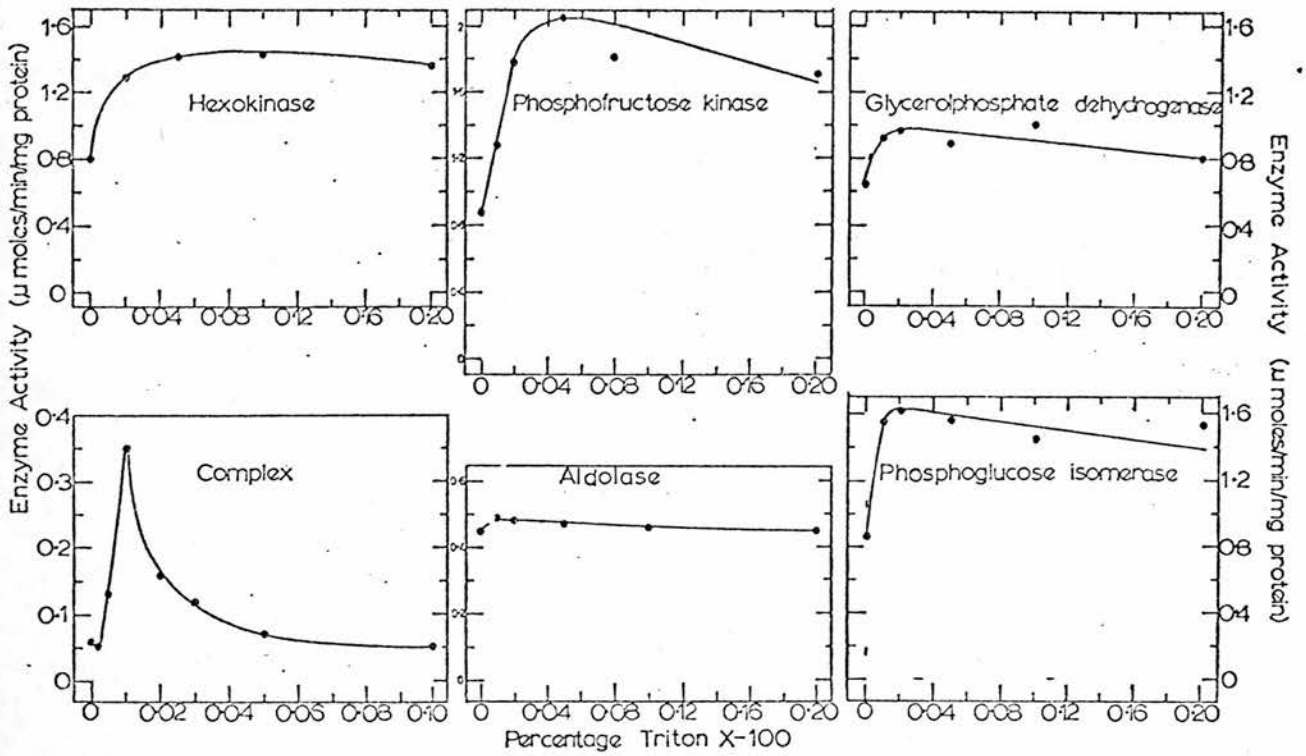


Fig. V. 2. Effect of Increasing Concentrations of Triton X-100 on the Multienzyme Activity and the Activities of Its Component Enzymes.

Aliquots of fraction 14.5KP of a homogenate of T. brucei were incubated on ice for 10 min in the presence of Triton X-100. Each sample was assayed for its multienzyme activity and the activities of the following enzymes: HK, PGI, PFK, ALD and GPDH. The enzyme activities (μmoles substrate converted/min/mg protein) were plotted against Triton X-100 concentration.

is doubled in the presence of Triton X-100 supports this view and suggests further that the treatment probably fails to expose the complex completely to substrates.

V.3.3. Effect of Increasing Concentrations of Triton X-100 on the Multienzyme Activity and the Activities of the Component Enzymes

On the assumption that solubilisation unmasks latent enzyme activity as well as disrupting any ordered arrangements between the individual component enzymes of the complex, it was expected that increasing concentrations of Triton X-100 might reduce multienzyme activity while at the same time increasing the activities of the component enzymes.

To verify this, aliquots of fraction 14.5KP from differential centrifugation of the homogenate obtained by grinding T. brucei were incubated on ice for 10 min with increasing concentrations of Triton X-100. They were then assayed for the activities of the complex as well as its component enzymes; and the results expressed as μmoles substrate converted/min/mg protein, against Trition X-100 concentration, are presented in Figure V.2.

The results show that the multienzyme activity is sharply increased by increasing concentrations of Triton X-100 of up to 0.01%; thereafter, the activity falls off, returning to the initial value (i.e. the activity prior to the Triton X-100 treatment) when the detergent concentration is about 0.1%. All the component enzymes are also activated by Trition X-100, the activities reaching the maximum values at detergent concentrations ranging from 0.01% for phosphoglucose isomerase to 0.04% for hexokinase and phosphofructose kinase. Further increases in

the concentration of Triton X-100 tend to inhibit phosphoglucose isomerase, phosphofructose kinase and glycerolphosphate dehydrogenase; however, detergent concentrations of up to 0.2% failed to cause any marked inhibitions of these enzymes. The activity of aldolase, which is only slightly enhanced in the presence of low concentrations of Triton X-100, does return to the initial value when the detergent concentration reaches 0.2%. Hence it could be argued that the seven-fold increase in the multienzyme activity when the complex is exposed to low concentrations of Triton X-100 is due entirely to the small increase (of up to 9%) in aldolase activity. However, such a view would appear unrealistic since at Triton X-100 concentration of 0.04%, when the aldolase activity has not returned to the initial value, the increased multienzyme activity is already practically abolished. It is therefore suggested that concentrations of Triton X-100 of up to 0.01% merely disrupt the limiting membrane and make the complex more accessible to glucose, and the cofactors required for the multienzyme activity without affecting the unique arrangement of the component enzymes, and that further increases in the detergent concentration tend to solubilise the component enzymes and disrupt this special arrangement with a consequent reduction in the efficiency of the complex as a multienzyme unit. If this is so it should be reflected in the kinetics of the component enzyme activities in untreated fraction 14.5KP and fraction 14.5KP that has been exposed to Triton X-100; this possibility is examined in Section V.4.

V.4. EFFECT OF DIFFUSION ON THE KINETIC PARAMETERS OF THE COMPONENT ENZYMES OF THE COMPLEX

An important feature of spherical particles and membranes containing immobilised enzymes is the relatively high enzyme concentrations in the solid phase. Since particles and membrane matrices tend to limit the transport of substrates and cofactors to the highly concentrated enzymic sites, it is expected that the kinetics of the enzymic reactions will differ from those in dilute solutions where diffusion effects may not be rate limiting.

This view is supported by a number of studies which have demonstrated that the behaviour of enzymes in heterogeneous systems can be significantly different from that in dilute solutions (McLaren & Packer, 1970; Laidler & Sundaram, 1971) and this effect has often been attributed to the microenvironment of the immoblised enzymes.

Engasser & Horvath (1973) did a theoretical investigation of the effect of internal diffusion on the overall rate in spherical particles and membranes containing immobilised enzymes. They found that since spherical particles and membranes containing immobilised enzymes represent open systems, the Michaelis-Menten kinetics is obeyed in the absence of diffusional effects at steady states even at high enzyme concentrations. When internal diffusion perturbs the reaction, the system can no longer be described by K_m and V_{max} alone, but is characterized by a factor which the authors referred to as the modulus. The modulus is directly related to three factors which are chatacteristic of immobilised enzymes, namely, the particle size, the facility for

Fig. V. 3.

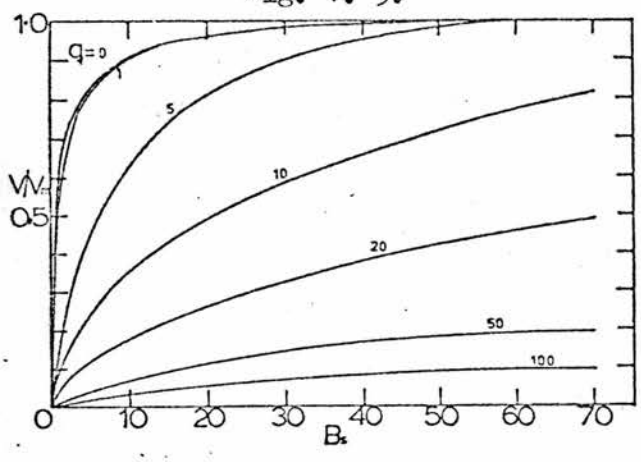


Fig. V. 3. 1.

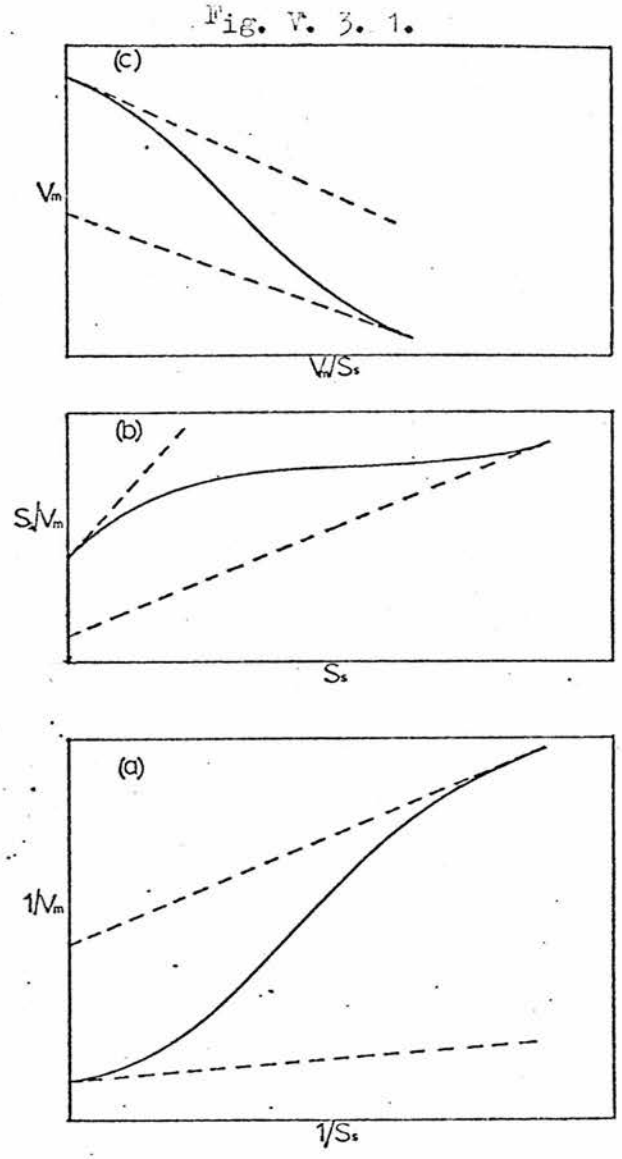


Fig V. 3. Theoretical Plots of the Overall Reaction Rate, Normalized to V/V_{max}, in Spherical Immobilized Enzyme Particles as a Function of the Dimensionless Surface Concentration (B_s) with the Modulus, q, as the parameter.

Fig. V. 3. 1. Illustration of the Effect of Internal Diffusion on the Overall Rate of Reaction in Spherical Particles Containing Immobilized Enzymes, Using Conventional Linearized Forms of the Michaelis-Menten Equation.

Both Figures were taken from Engasser, J-M and Horvath, C. (1973). Theor. Biol. 42, 137 - 155.

the substrates or cofactors to diffuse through the particle matrix, and the intrinsic activity of the immobilised enzyme.

The method used by the two authors was as follows. First, they derived a kinetic expression in the absence of diffusion limitations for reactions which take place in open systems at high enzyme concentrations. This expression was used to compute the overall reaction rate in spherical particles when diffusion is perturbing the reaction. They then demonstrated the effect of internal diffusion on the reaction rates obtained with heterogeneous enzyme systems by a series of curves on the three plots commonly used for graphical evaluation of the kinetic parameters of enzyme catalysed reactions. Figures V.3. and V.3.1. taken from Engasser & Horvath (1973) show respectively that the rate of reaction at a given surface substrate concentration decreases when the modulus (which represents the effect of diffusion on the reaction) increases, and that when diffusion perturbs the reaction rate, the classical linearised forms of the Michaelis-Menten equation do not yield straight lines since the external substrate concentration differs from the concentration at the site of the enzyme reaction.

On the assumption that the component enzymes of the T. brucei multienzyme complex are immobilised in their subcellular organelle, it was expected that plots of the overall reaction rate against substrate concentrations for untreated complex and complex treated with 0.02% Triton X-100 might reveal whether the structure of the complex is such as to cause progressive depletion of substrates towards the centre of the particle.

To examine this possibility, aliquots of the subcellular fraction 14.5KP of T. brucei were assayed for enzyme activity

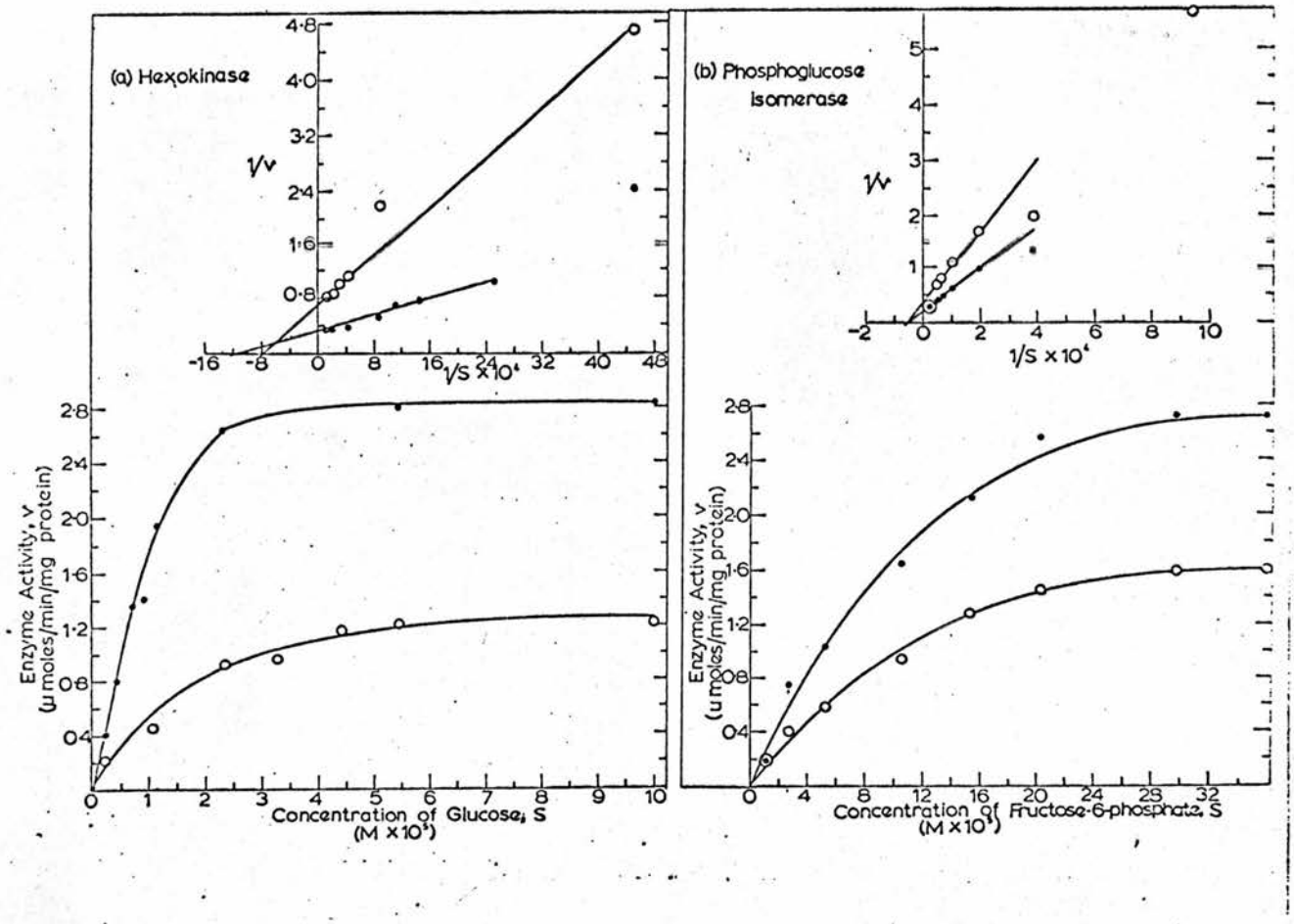


Fig. V.3.2. Effect of Diffusion on the Kinetic Parameters of the Component Enzymes of the Multienzyme Complex of T. brucei.

Aliquots of the post-nuclear fraction 14.5KP were assayed for enzyme activity against substrate concentration; O, denotes assay performed in the absence of Triton X-100; ●, assays performed in the presence of 0.02% Triton X-100. Enzymes studied were (a) hexokinase; (b) phosphoglucose isomerase; (c) aldolase; (d) phosphofructose kinase; (e) glycerolphosphate dehydrogenase.

The results have been plotted as the overall reaction rate, v, against substrate concentration, S, (lower plots) or 1/v versus 1/S (top plots). Other experimental details are given in the text.

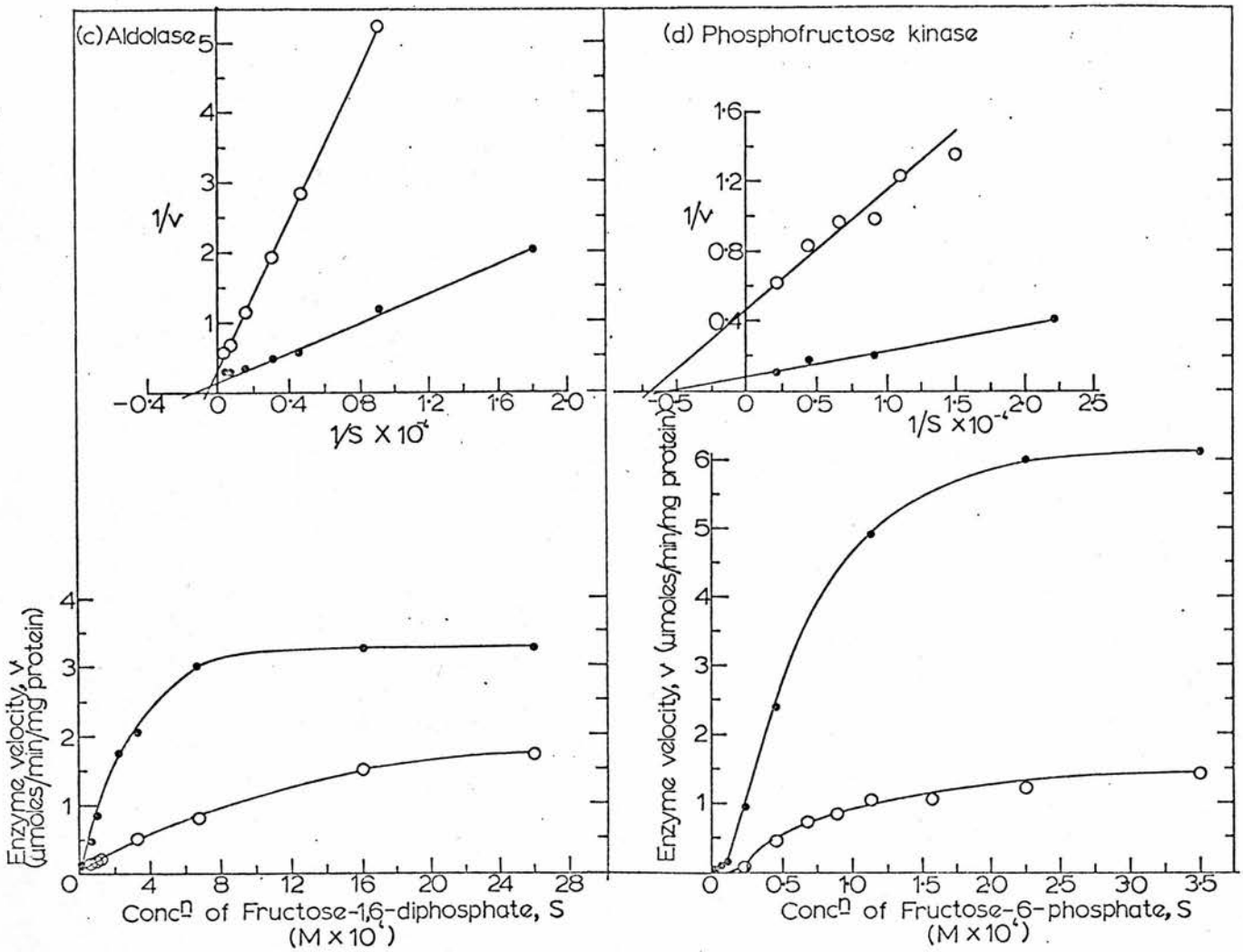


Fig. V. 3. 2. Effect of Diffusion on the Kinetic Parameters of the Component Enzymes of the Multienzyme Complex of T. brucei.

Aliquots of the post-nuclear fraction 14.5KP were assayed for enzyme activity against substrate concentration; O, denotes assays performed in the absence of Triton X-100; •, assays performed in the presence of 0.02% Triton X-100. Enzymes studied were (a) hexokinase; (b) phosphoglucose isomerase; (c) aldolase; (d) phosphofructose kinase; (e) glycerolphosphate dehydrogenase.

The results have been plotted as the overall reaction rate, v, against substrate concentration, S, (lower plots) or 1/v versus 1/S (top plots). Other experimental details are given in the text.

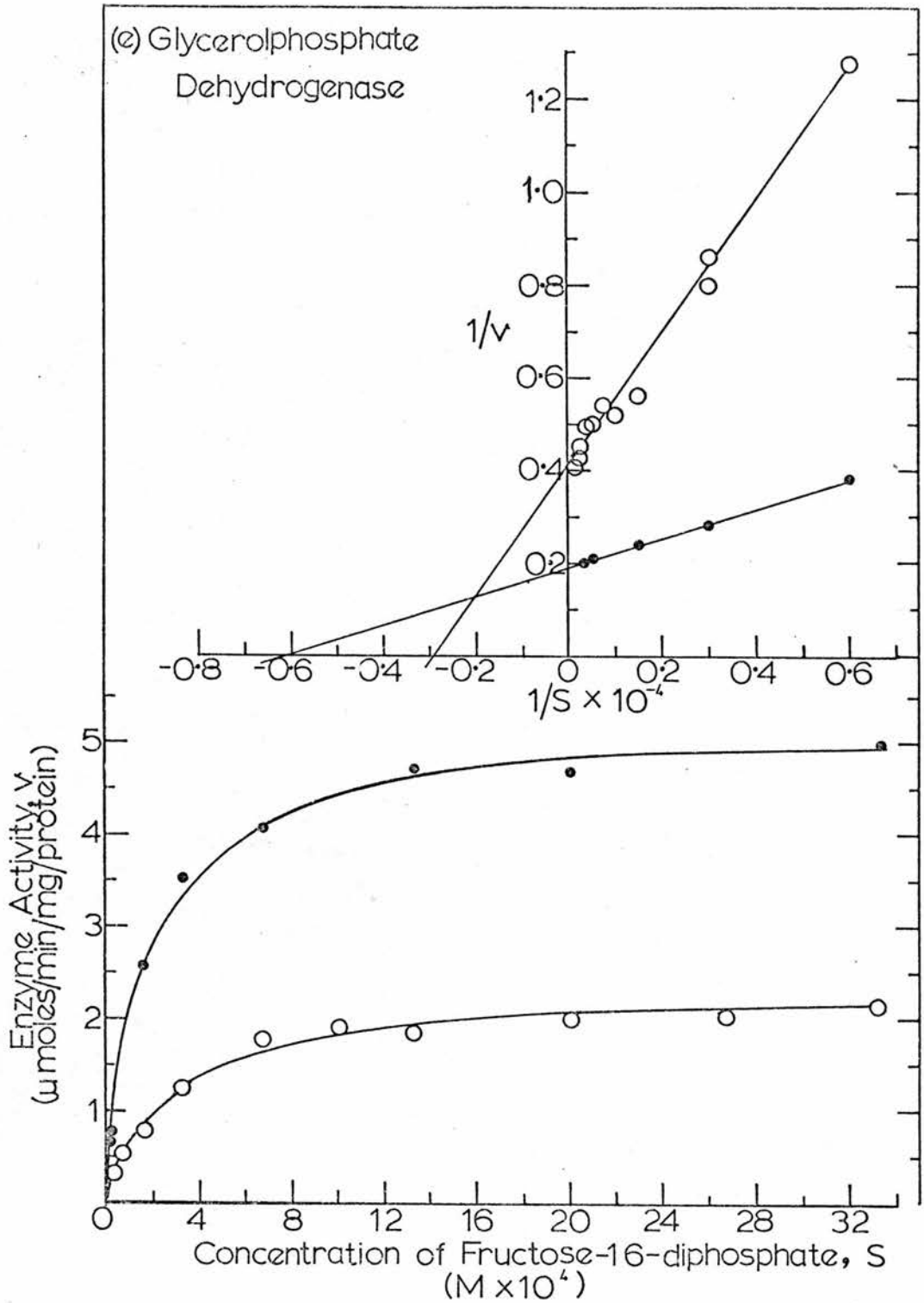


Fig. V. 3. 2. (contd.)

against substrate concentration. The experiment was repeated with fraction 14.5KP which had been incubated on ice for 10 min in the presence of 0.02% Triton X-100. The results of such experiments have been presented in Figure V.3.2.(a-e) as V (μmoles substrates converted/min/mg protein) against S (substrate concentration) or 1/V against 1/S. It should be mentioned here that no attempt was made to increase the rate of substrate diffusion by stirring the reaction mixture when the reaction was in progress.

These results show that the overall rate of reaction for each of the component enzymes of the complex at a given surface substrate concentration is greatly enhanced by Triton X-100 treatment of the complex. As Engasser & Horvath (1973) point out, when chemical reaction occurs simultaneously with mass transfer within porous structure, not all of the enzyme molecules inside of the particle are in contact with the substrate at the same concentration level that exists at the surface. Because of this lowering of the surface concentration, the overall reaction rate is lower than it would be in the absence of diffusional effects. This effect was quantitatively expressed by an effectiveness factor, β, defined as the ratio of the actual reaction rate V to the rate, V_{kin}, which would be obtained if all enzyme molecules inside of the particle were exposed to the same substrate concentration as that at the surface.

On the assumption that the T. brucei multienzyme complex is buried inside an organelle and the accessibility of substrates to the component enzymes increases after Triton X-100 treatment of the subcellular fraction 14.5KP, an apparent effectiveness, factor β′, has been calculated for each component enzyme (Table V.4.)

Table V. 4. The Apparent Effectiveness Factor (β') for the Component Enzymes of the Multienzyme Complex.

The values of β' were estimated by dividing the V_{max} (calculated from the Lineweaver-Burke reciprocal plots) found when the enzymes were assayed in the absence of Triton X-100 by the values obtained when the assays were conducted in the presence of 0.02% Triton X-100.

	V_{max} (μmoles substrate converted/min/mg protein)		
Enzyme	In Absence of Triton X-100	In Presence of 0.02% Triton X-100	Effectiveness Factor, β'
(a) HK	1.43	3.03	0.47
(b) PGI	2.86	5.00	0.57
(c) ALD	3.30	8.00	0.41
(d) PFK	2.20	12.50	0.18
(e) GPDH	2.44	5.30	0.45

as follows. The V_{max} calculated from the Lineweaver-Burke reciprocal plots for enzyme assays in the presence and absence of 0.02% Triton X-100 were recorded in Table V.4. From these results, the apparent effectiveness factor β', was determined by dividing the V_{max} for each enzyme in untreated fraction 14.5KP by the values obtained when the assays were repeated with material which had been incubated in 0.02% Triton X-100. The results were then recorded in Table V.4 as the apparent effectiveness factor, β'. Assuming 0.02% Triton X-100 treatment of fraction 14.5KP leads to the total exposure of the component enzymes to their substrates, these results show that prior to the detergent treatment, only 47% of the molecules of hexokinase, 18% phosphofructose kinase, 41% aldolase and 45% glycerolphosphate dehydrogenase are accessible to their respective substrates; these figures compare with 57% of the molecules of phosphoglucose isomerase which are already accessible to fructose-6-phosphate prior to treatment of the complex with the detergent. If, as it is assumed here, the effective factor truly reflects the accessibility or otherwise, of the enzymes to their substrates, then these results strongly support the earlier finding that phosphoglucose isomerase responds much more easily to attempts at solubilising the component enzymes of the complex.

The Lineweaver-Burke plots in Figure V.3.2.(a-e) indicate that the enzyme reactions follow the classical Michaelis-Menten kinetics irrespective of whether or not the enzyme assays are conducted with fraction 14.5KP which had been previously subjected to treatment with Triton X-100. However, as pointed out by Engasser & Horvath, the results of kinetic measurements with immobilised enzymes may give straight lines on reciprocal plots

if a relatively narrow concentration range is investigated. Therefore, the results of the reciprocal plots do not necessarily show that the component enzymes are accessible to their substrates prior to Triton X-100 treatment of the complex.

Summary of the Results on the Effect of Diffusion on the Kinetics of the Component Enzymes of the Multienzyme Complex

Other factors, apart from diffusion perturbations can affect the kinetics of membrane-bound or physically included enzymes (Laidler & Bunting, 1973) and discussion of the kinetics of the component enzymes should ideally take all such factors into consideration. However, because there is no detailed information on the multienzyme complex except that it is probably located in microbodies with a limiting membrane and an inner matrix, the present discussion has been limited only to diffusion effects on the kinetics of the component enzymes; and the main points that have been revealed by comparison of the enzyme reactions in the untreated complex with the complex disrupted with 0.02% Triton X-100 are as follows. (a) The rate of reaction for each of the enzymes at any given surface substrate concentration is lower for the untreated organelles than it is when the organelles are disrupted with the detergent. This means that detergent treatment improves diffusion of substrates and/or cofactors and auxiliary enzymes to the component enzymes either by reducing the particle size or disrupting the integrity of the organelles, or in some way as yet unknown, increases the intrinsic activities of the component enzymes. (b) The values of the effectiveness factor, β', and the V_{max} recorded

Table V. 5. Apparent K_m Values for the Component Enzymes of the Multienzyme Complex in the Subcellular Fraction 14.5KP of Blood-stream Long Slender Form T. brucei

	Apparent K_m ($M \times 10^4$)	
Enzyme	In Absence of Triton X-100	In Presence of 0.02% Triton X-100
(a) HK	0.130	0.087
(b) PGI	1.670	1.670
(c) ALD	14.300	6.670
(d) PFK	1.430	1.820
(e) GPDH	3.450	1.640

in Table V.4. confirm that even at saturating substrate concentrations the activities of the component enzymes in the treated complex are much higher than the activities found for the same enzymes in the complex which has not been exposed to the detergent. Assuming that the factor β' truly reflects the extent of exposure of the enzymes to the surrounding solution, it could be concluded from the results in Table V.4. that the component enzymes are probably arranged in the following order in the organelle, beginning from the most expossed to the most deeply buried enzyme: PGI>HK and GPDH>ALD PFK. The position of aldolase is complicated by its instability in the presence of Triton X-100. (c) With the exception of phosphoglucose isomerase, whose Michaelis-Menten constant is not affected by detergent treatment and phosphofructose kinase which showed slight increase in K_m in presence of the detergent, the K_m values of the remaining threeenzymes are all decreased by 0.02% Triton X-100 treatment of the complex (Figure V.5.). It is suggested that the lower K_m values obtained with the complex treated with Triton X-100 are closer to the true values, whereas the higher values observed in the absence of the detergent reflect the effect of diffusion limitations on the kinetics of the enzyme reactions.

V.5. EVALUATION OF THE STABILITY OF THE MULTIENZYME COMPLEX BY MEANS OF CHROMATOGRAPHY ON COLUMNS OF BIOGEL OR ELECTROPHORESIS IN ACRYLAMIDE GEL

Owing to the greater risk of artefacts, some of the most powerful biochemical tools for fractionation such as column chromatography and electrophoresis have not found wide usage in subcellular fractionation or identification. Yet, as pointed out by Beaufay et al. (1964) although identity of behaviour is never entirely conclusive, it becomes increasingly significant the more numerous and varied the conditions that are tried. This section presents the results obtained when the two powerful methods of separation of biological molecules, namely column chromatography and gel electrophoresis were employed to ascertain (a) the homogeneity and (b) the stability of the bloodstream T. brucei multienzyme complex.

V.5.1. Chromatography of the Multienzyme Complex on Columns of Biogel

In these experiments, the fraction containing the multienzyme complex was evenly suspended in 0.01 M potassium phosphate buffer, pH 7.6, containing 0.1 mM dithiothreitol (KPD buffer). It was then applied to a column of Biogel prepared and equilibrated in the KPD buffer and then eluted with the same buffer. The fractions collected were subjected to the appropriate analyses from the results of which the various elution patterns have been presented. The results obtained were essentially the same irrespective of whether the cells had been lysed by digitonin treatment, freezing and thawing or by grinding with the abrasives.

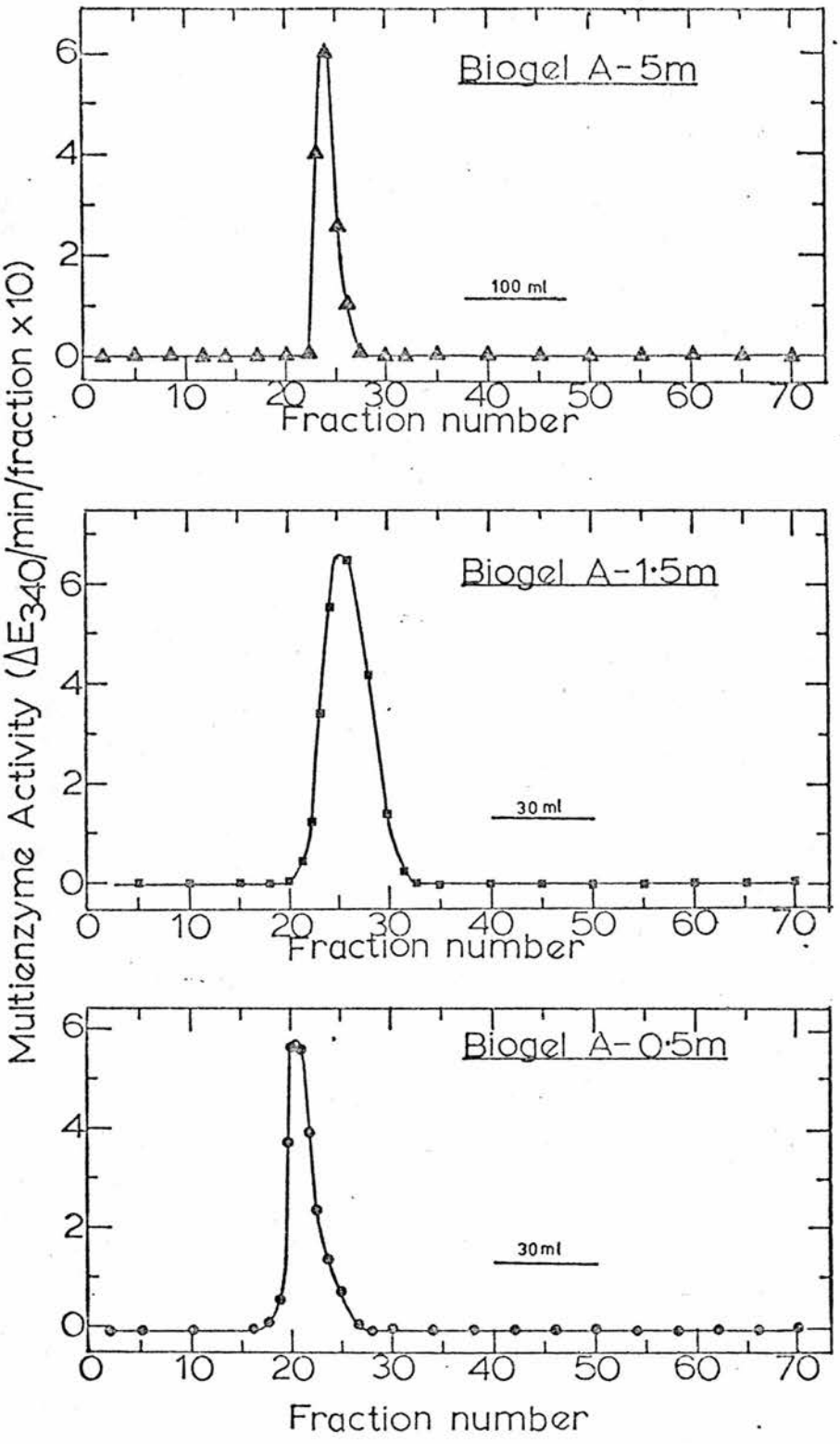


Fig. V. 4. Biogel Column Chromatography of the Multienzyme Complex of T. brucei.

Samples of the subcellular fraction containing the multienzyme activity were chromatographed on Biogel columns. The figures represent (from top to bottom) the elution pattern from Biogel A-5m column (67 x 3.2cm), A-1.5m column (47 x 1.8cm) and A-0.5m column (50 x 1.8cm) respectively. Fractions collected were 7ml (Biogel A-5m), 3ml (Biogel A-1.5m) and 3ml (Biogel A-0.5m).

In each case, the multienzyme complex activity was eluted in the void volume.

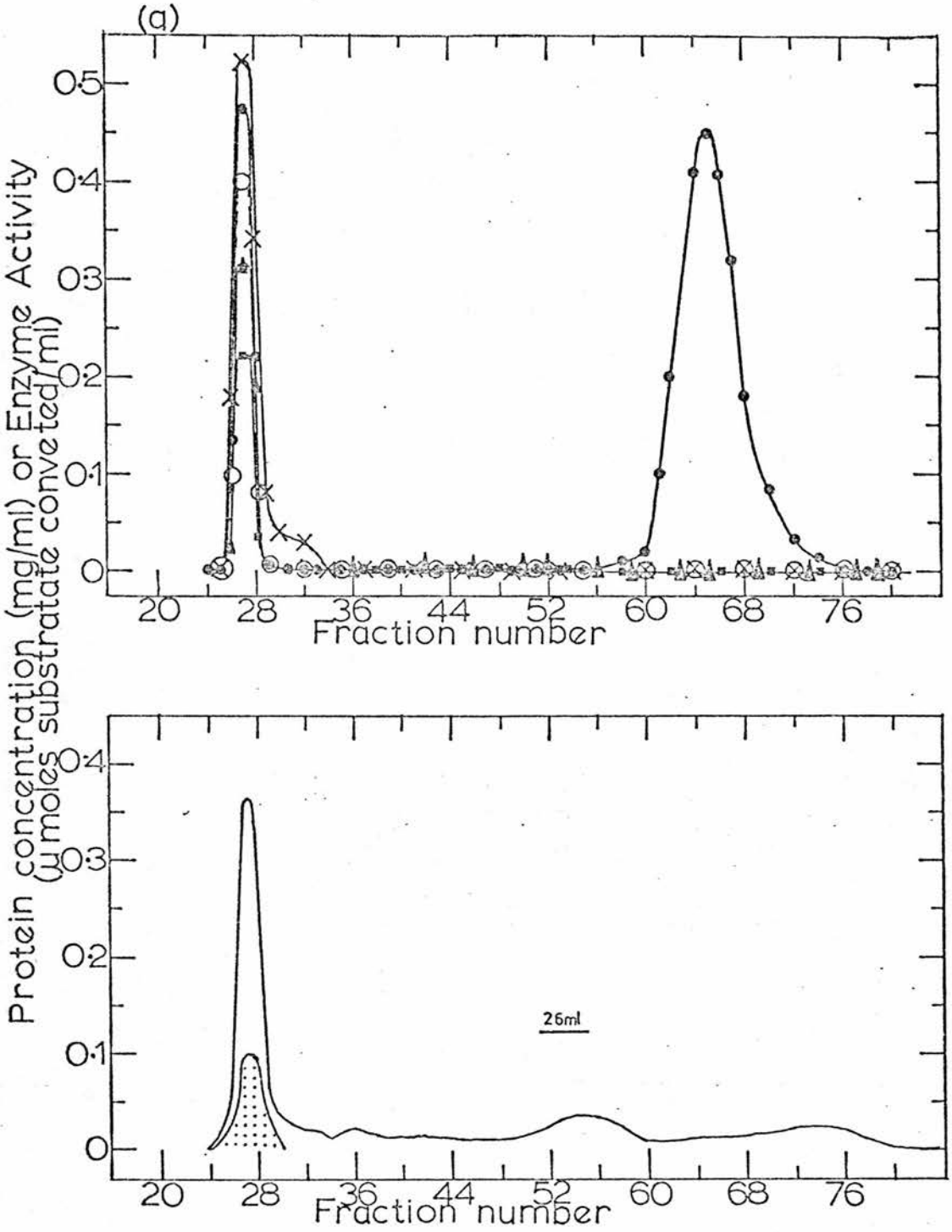


Fig. V. 4. 1. Effect of Isotonic Sodium Chloride Treatment of Fraction 14.5KP on the Elution Patterns of the Component Enzymes of the T. brucei Multienzyme Complex.

Samples of the post-nuclear fraction 14.5KP from homogenates obtained either by digitonin treatment, freezing and thawing, or by grinding T. brucei cells were subjected to the appropriate treatment before being chromatographed on a column (67 x 3.2cm) of Biogel A-5m. The figures represent the elution patterns of (a) fraction 14.5KP which had been incubated in the plain buffer prior to its chromatography in the same buffer solution; (c) sample pretreated in buffer containing 0.15M NaCl and dialysed against the plain buffer to remove the salt before being chromatographed in the plain buffer; (b) sample treated in 0.15M NaCl and chromatographed in buffer con- 0.15M NaCl. Eight ml fractions were collected in each case.

The enzyme activities shown are ⊡, multienzyme; O, HK; ●, PGI; x, PFK; ■, ALD; ▲, GPDH. Protein is represented by (——); E_{260}, (-.-.); and E_{280}, (- - -).

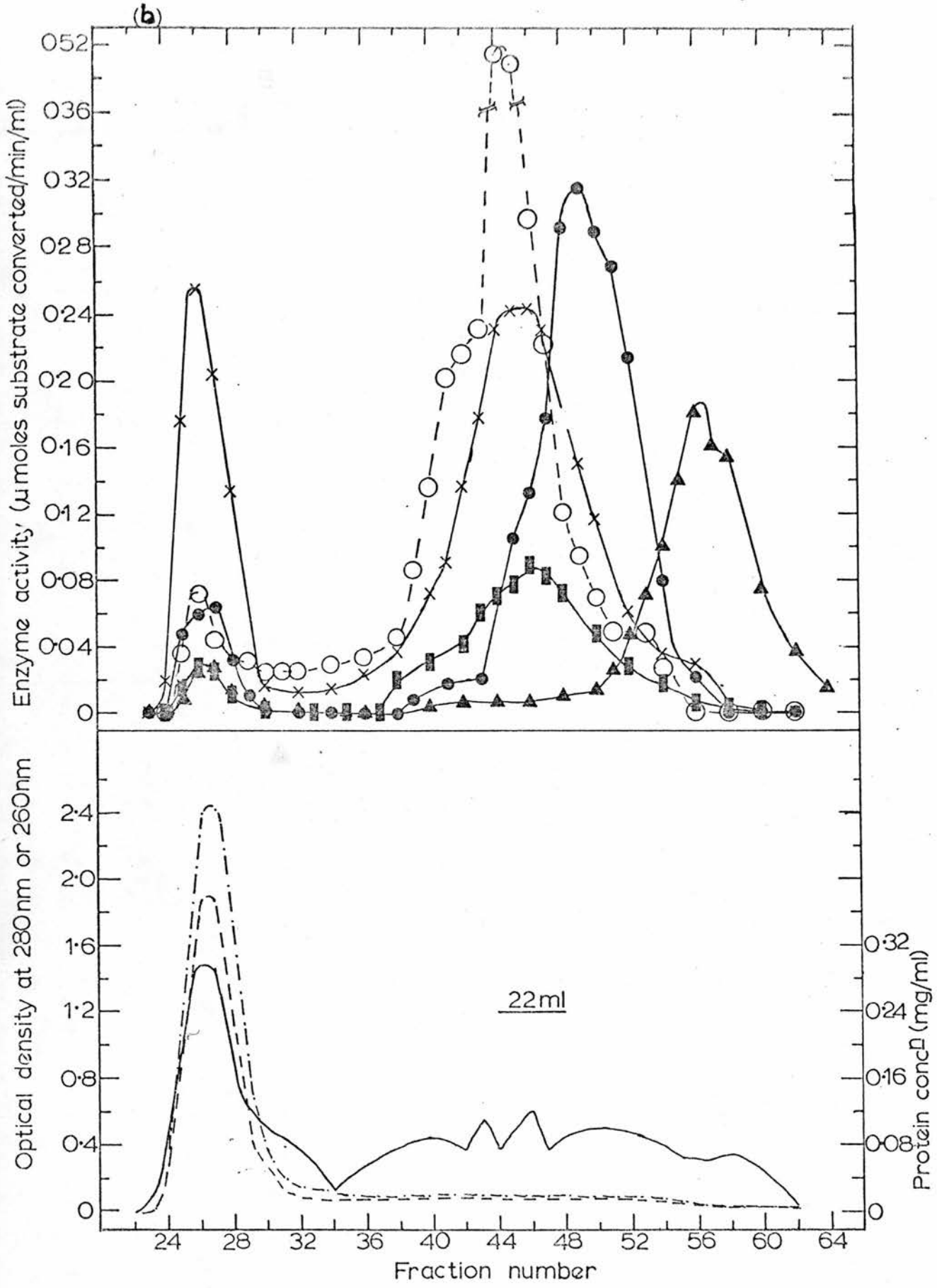


Fig. V. 4. 1. (contd.)

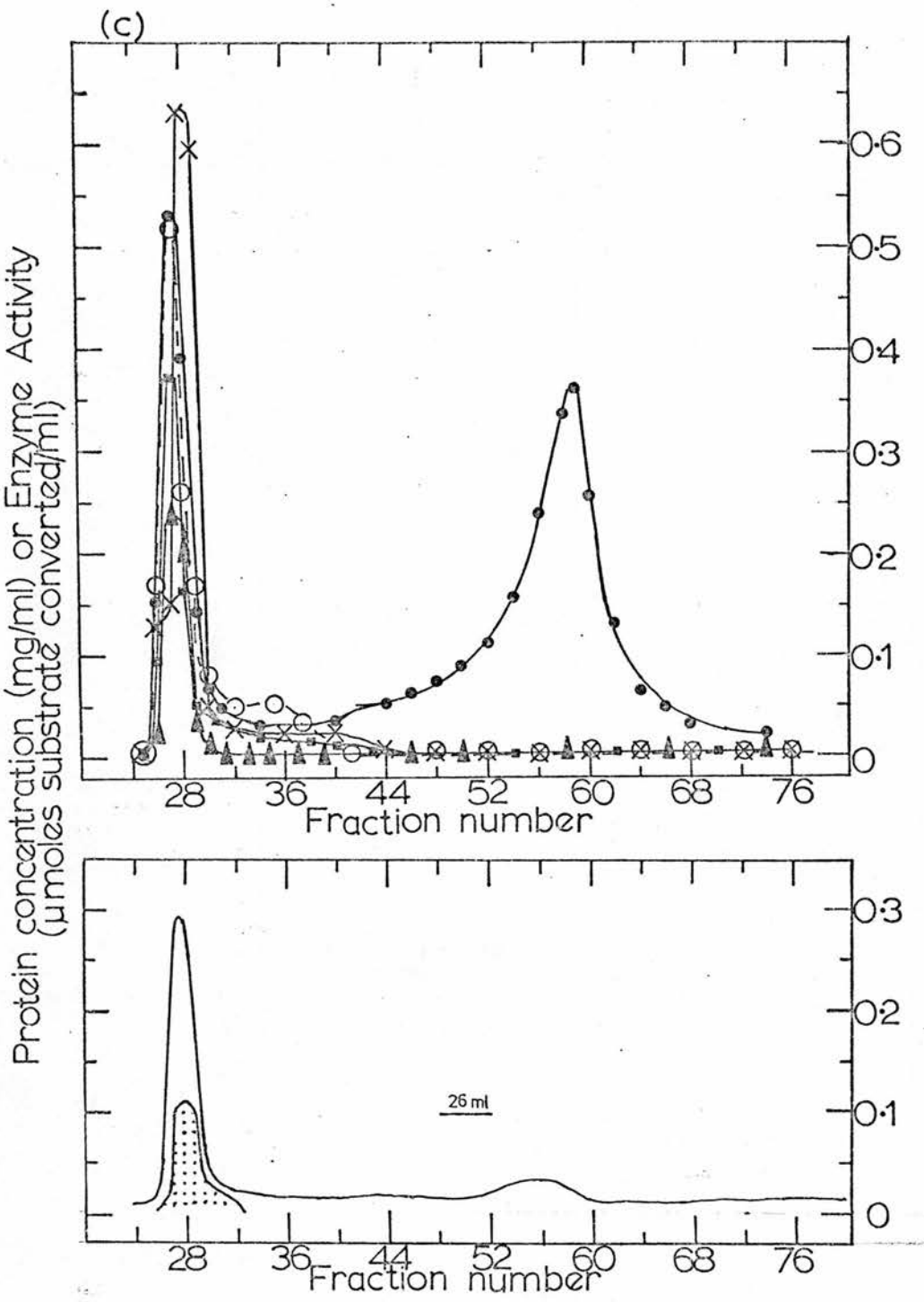


Fig. V. 4. 1. (contd.)

Similarly, whether the post-nuclear fraction 14.5KP or 105KP served as the source material of the multienzyme complex did not substantially affect the elution pattern, which comprised a leading sharp protein peak eluted in the void volume and well separated from minor protein peaks.

The results in Figure V.4. (presented as the total multienzyme activity in each fraction) shows that irrespective of which of the three grades of Biogel A-(0.5 m, 1.5 m or 5 m) was employed in the chromatography, the elution pattern of the complex activity is the same. In all three patterns, the leading peak contained over 80% of the protein and possessed all the multienzyme activity recovered.

Figure V.4.1. compares the elution pattern of untreated fraction 14.5KP chromatographed on Biogel A-5 m column in the plain KPD buffer with the pattern for fraction 14.5KP pretreated and chromatographed on a column equilibrated and eluted with KPD buffer containing 0.15 M NaCl. The pattern for the untreated complex (Figure V.4.1a.) shows that the leading protein peak contains all the multienzyme activity as well as the total activities of the component enzymes, hexokinase, phosphofructose kinase, aldolase and glycerol-phosphate dehydrogenase; the activity of phosphoglucose isomerase was divided approximately 1 to 3 between the leading peak and the slow-moving peak(s). The elution pattern for the complex treated and fractionated in KPD buffer containing 0.15 M NaCl (Figure V.4.1b.) on the other hand, shows a leading protein peak with very much reduced enzyme activities; these enzyme peaks are no longer coincident with one another, but they are all still well separated from the slower-moving peaks now greatly enriched in protein and enzyme activities; these

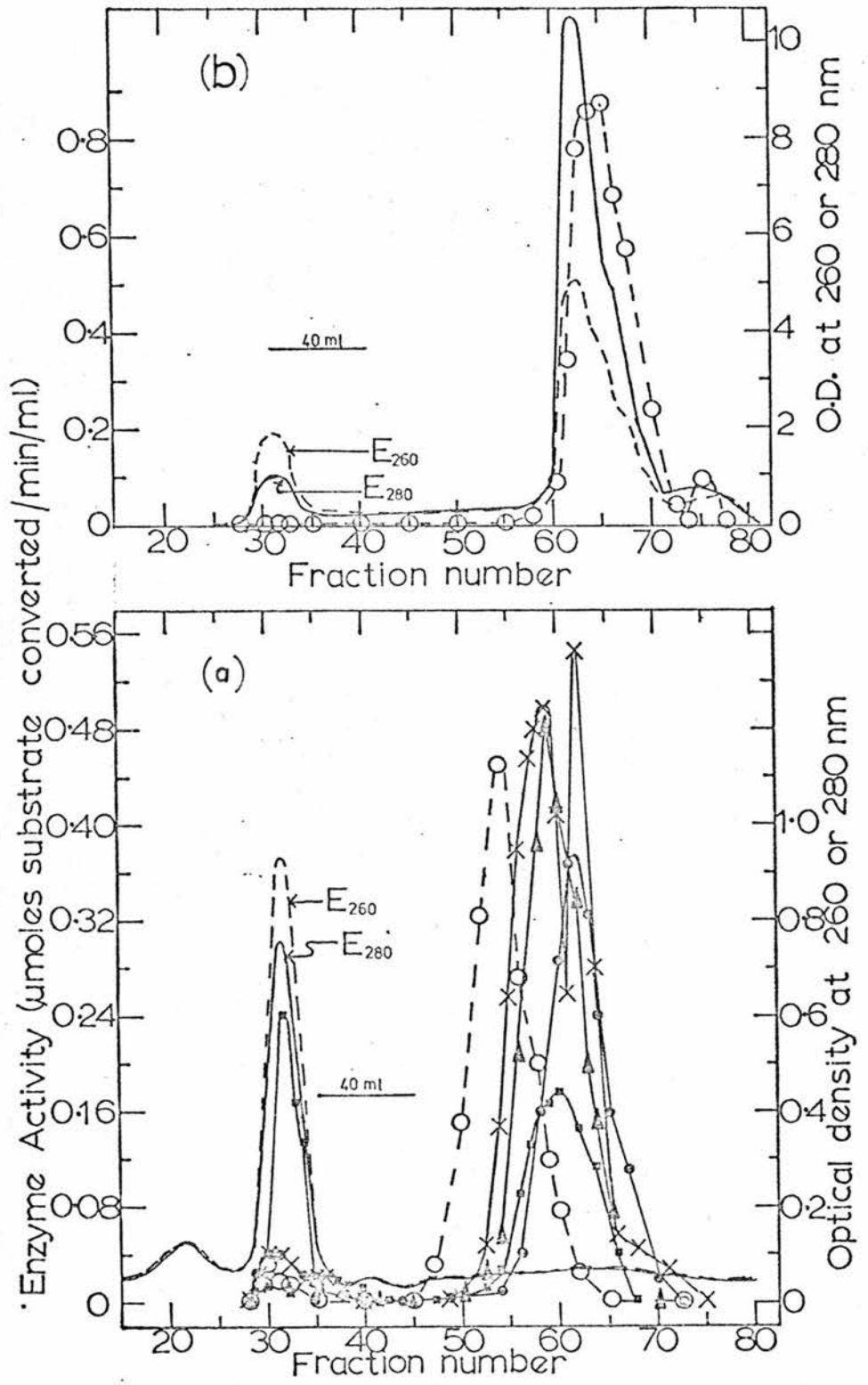


Fig. V. 4. 2. Effect of 0.2M $MgCl_2$ or a Mixture of 0.15M NaCl and 0.1% Triton X-100 Treatment of Fraction 14.5KP on the Elution Patterns of the Component Enzymes of the *T. brucei* Multienzyme Complex.

The figures represent the elution patterns from Biogel A-5m column (67 x 3.2cm) of (a) fraction 14.5KP treated and eluted with buffer containing 0.2M $MgCl_2$ and (b) fraction 14.5KP treated in buffer containing a mixture of 0.15M NaCl and 0.1% Triton X-100 and chromatographed with buffer containing 0.15M NaCl. Fractions, 6.5 ml, were collected in each case.

The enzyme activities shown are O, HK; ●, PGI; x, PFK; ■, ALD; ▲, GPDH. E_{280}, ____; E_{260}, - - -.

latter peaks are partially separated from one another.

When the experiment was repeated with fraction 14.5KP which had been treated with KPD buffer containing 0.15 M NaCl followed by dialysis to remove the salt and chromatography on a column of Biogel A-5 m equilibrated and eluted with the plain KPD buffer (Figure V.4.1c.), the elution pattern was similar to the pattern for the untreated complex except that the peaks of the component enzyme activities (still near the void volume) are no longer coincident with one another.

To determine whether desorption of the component enzymes of the complex could be further improved, a sample of fraction 14.5KP was treated with KPD buffer containing 0.2 M $MgCl_2$. It was loaded onto a column of Biogel A-5 m previously equilibrated with the buffer containing 0.2 M $MgCl_2$ and the enzymes were eluted with the same buffer solution. The elution pattern (Figure V.4.2a.) shows that treatment of fraction 14.5KP with 0.2 M $MgCl_2$ causes almost total desorption of each of the component enzymes of the complex.

Almost identical results could be obtained by incubating fraction 14.5KP in a buffer solution containing 0.15 M NaCl and 0.1% Triton X-100 followed by chromatography of the mixture on Biogel A-5 m column using KPD buffer containing 0.15 M NaCl, as the eluant. Figure V.4.2b. illustrates this point with the elution pattern for hexokinase; similar patterns were consistently obtained for the other component enzymes of the complex.

These experiments on the chromatographic behaviour of samples of fraction 14.5KP confirm that the multienzyme complex is a large particle of a size which is probably bigger than a globular protein of molecular weight of five million. They

also show (1) that the forces holding the component enzymes together are strong enough to withstand chromatography on Biogel columns; (2) that although long exposure of fraction 14.5KP to isotonic NaCl brings about more than 70% desorption of the activities of the component enzymes, removal of the salt through dialysis tends to cause reaggregation of the enzyme molecules; and (3) that magnesium chloride at a concentration of 0.2 M or a mixture of isotonic NaCl and 0.1% Triton X-100 causes nearly complete dissociation of the component enzymes from their matrix.

To find out whether the decrease in protein concentration that results from isolation of the subcellular fraction 14.5KP during differential centrifugation affects the chromatographic behaviour of the multienzyme complex, the experiments were repeated with homogenates obtained by grinding T. brucei cells with alumina. The homogenate was first divided into three equal volumes; the first sample, A, was not treated further; samples B and C were made 0.15 M with a strong solution of NaCl, and all three samples were incubated on ice for 30 min. Sample B was then dialysed against the KPD buffer to remove the salt and all three were subjected to chromatography on Biogel A-5 m columns as follows. A and B separately on columns which had been equilibrated with the plain KPD buffer and C on a column which had been equilibrated with KPD buffer containing 0.15 M NaCl; the samples were then eluted with the respective buffer solutions used to equilibrate the columns.

The results obtained (Figures V.4.3.(a-c), show elution patterns which are essentially the same as those found when

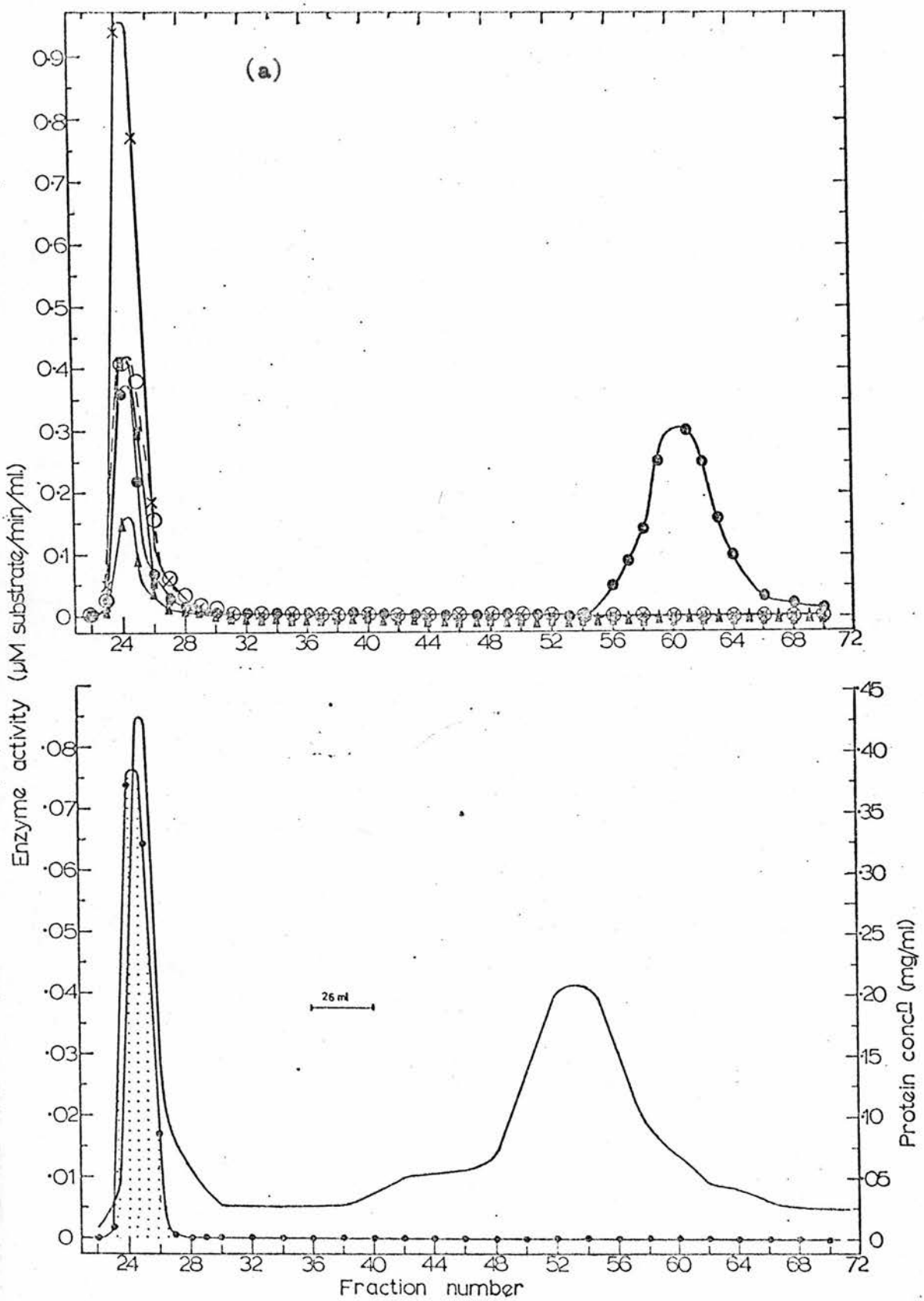


Fig. V. 4. 3. (contd.). Legend given in Fig. V. 4. 3b.

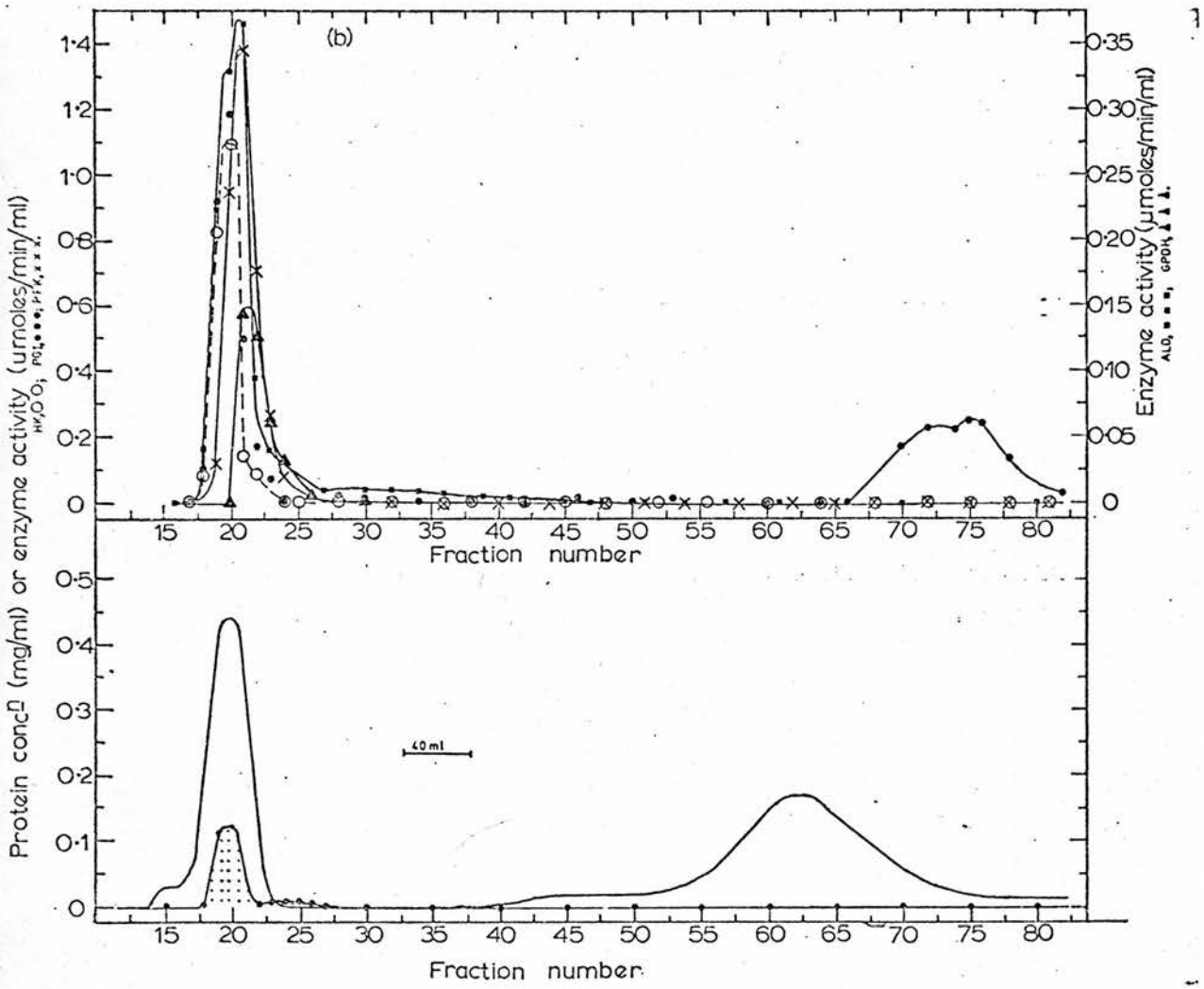


Fig. V. 4. 3. Elution Patterns of the Component Enzymes of the Multienzyme Complex During Biogel A-5m Column (67 x 3.2cm) Chromatography of T. brucei Homogenates.

Figures (a), (b) and (c) represent the patterns for homogenates pretreated and chromatographed as described for the equivalent patterns (a, c, and b) respectively in Fig. V. 4. 1. Other details are as described in Fig. V. 4. 1.

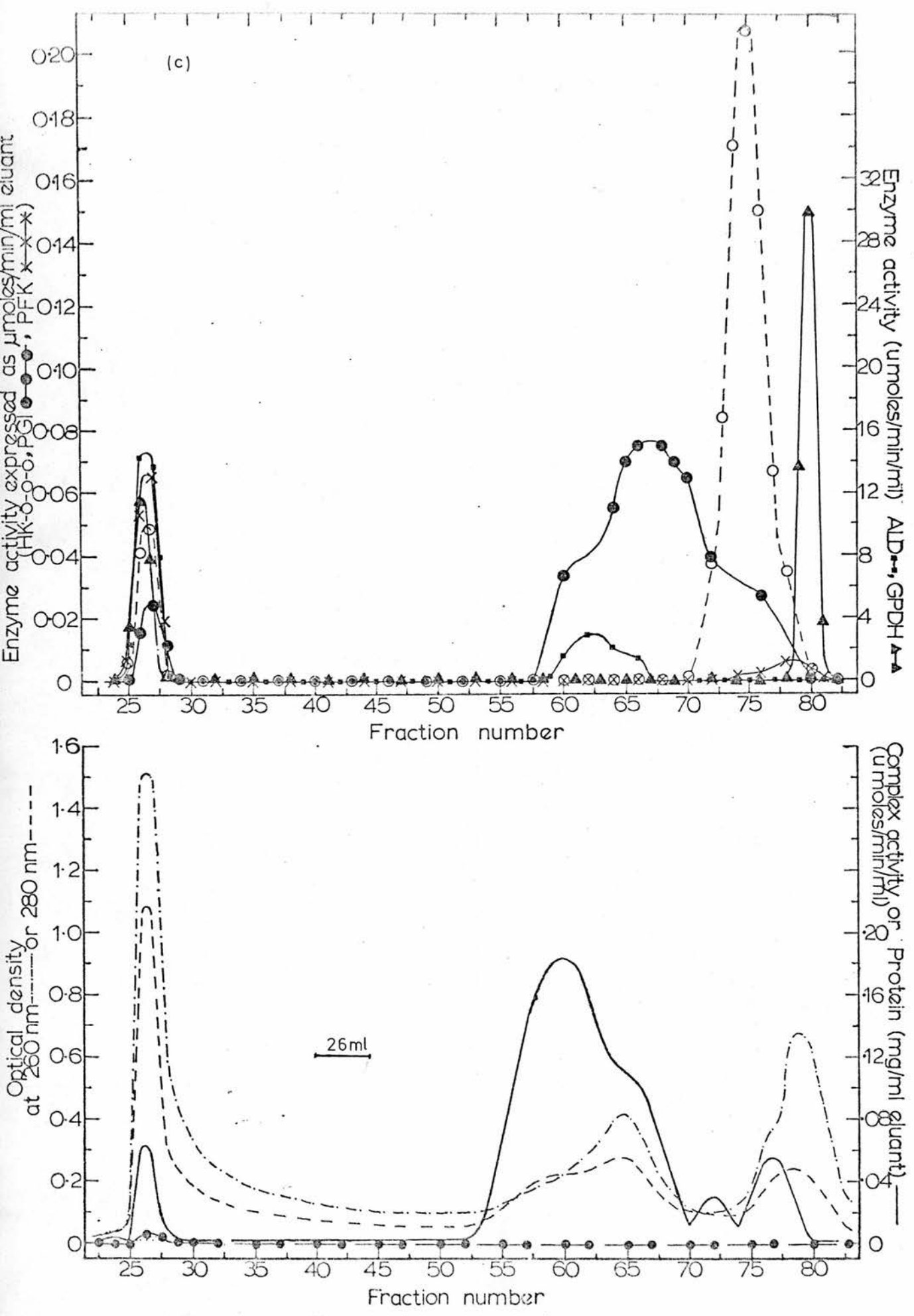


Fig. V. 4. 3. (contd.). Legend given in Fig. V. 4. 3b.

samples of the subcellular fraction 14.5KP were similarly treated ahd chromatographed (Figures V.4.1.(a-c) with only one major difference. Whereas more than 80% of the protein in the untreated fraction 14.5KP emerged in the void volume in association with the multienzyme activity, approximately 60% of the recovered protein penetrates the gel granules and therefore appears in the second peak during chromatography of the untreated homogenate. These results are consistent with the observation that during differential centrifugation of homogenates obtained by grinding T. brucei cells, over 50% of the protein is found in the final supernatant. It is interesting to note, however, that, apart from phosphoglucose isomerase, particulate enzyme activity was found to be associated with the second (soluble) protein peak only when the homogenate was incubated in and fractionated with buffer containing isotonic saline.

V.5.2. Acrylamide Gel Electrophoresis of the Multienzyme Complex

When samples of the subcellular fraction 14.5KP were subjected to electrophoresis, it was observed that no proteins penetrated the separating gel even when the acrylamide concentration in this gel was reduced to 2%; and the situation was not altered either by homogenization or incubation of the sample in 0.15 M NaCl. On the other hand, when fraction 14.5KP was incubated with a mixture of sodium dodecyl sulphate and urea, followed by boiling and acrylamide gel electrophoresis of the mixture, at least six fast-migrating protein bands could be observed.

SECTION VI

SOME BIOCHEMICAL PROPERTIES OF THE MULTIENZYME COMPLEX

Introduction

Determination of the multienzyme complex activity has been based on the following assumptions:

(1) That the complex is capable of converting glucose to metabolites which are sequentially acted upon to give dihydroxyacetone-phosphate;

(2) That in the presence of NADH, the complex reduces the dihydroxyacetone-phosphate so formed to give glycerol-phosphate and NAD^+; and

(3) That the optical density change observed at 340 nm accurately reflects the stoichiometric conversion of the dihydroxyacetone-phosphate to glycerolphosphate by the glycerolphosphate dehydrogenase in the multienzyme complex.

This section presents the results of experiments designed to study some of the biochemical properties of the multienzyme complex which were considered to verify adequately the assumptions listed above. The post-nuclear fraction 14.5KP from homogenates of bloodstream forms of T. brucei which had been disrupted by grinding with silicon carbide was used as the source of the multienzyme complex throughout these studies. Essentially identical results were obtained with fraction 14.5KP from T. brucei cells disrupted by freezing and thawing.

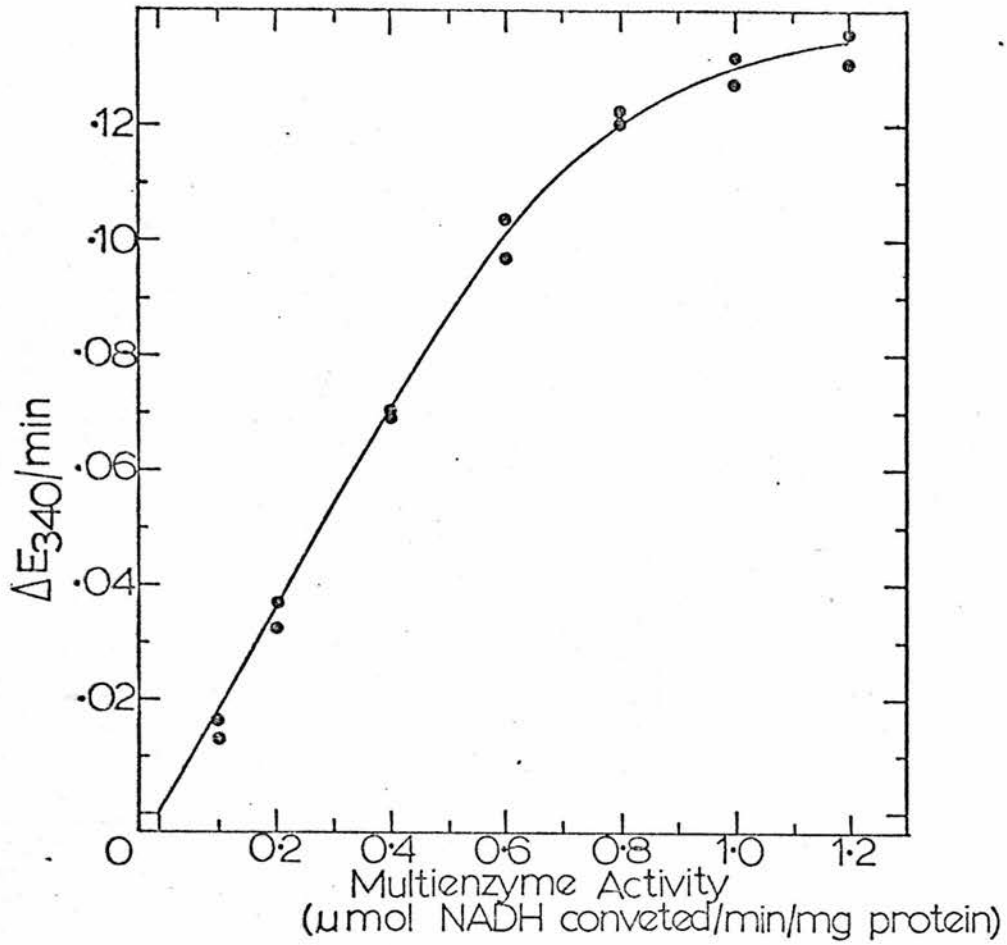


Fig. VI. 2. The Overall Rate of NADH oxidation Versus Multienzyme Activity in the Subcellular Fraction 14.5KP.

Experimental details are given in the text.

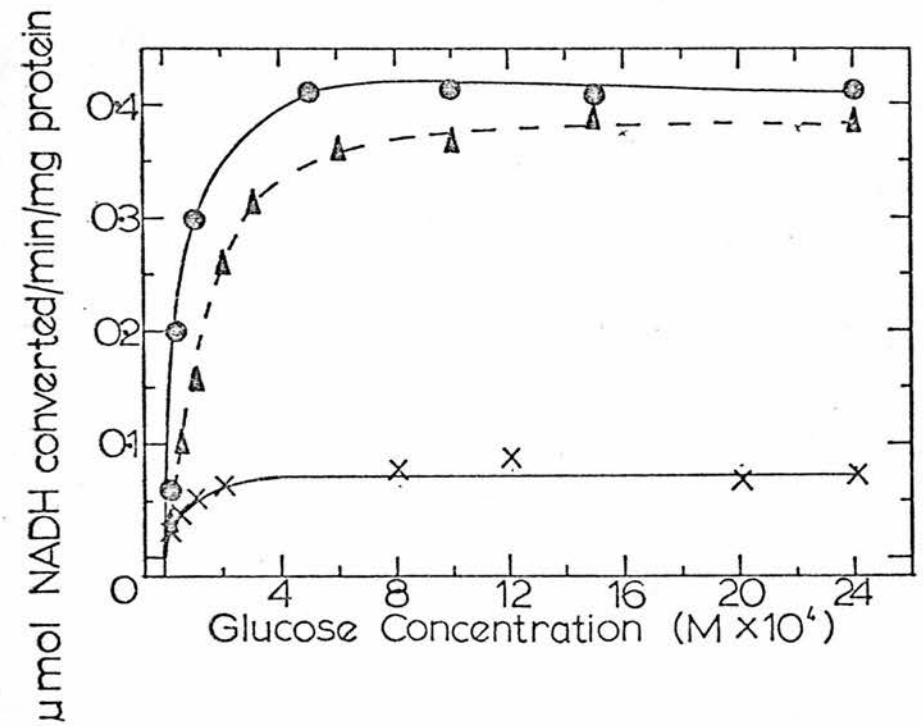


Fig. VI. 1. The Overall Rate of Multienzyme Activity Versus Glucose Concentration. x denotes untreated material; ▲, fraction 14.5KP which had been pretreated in 0.15M NaCl; ●, fraction 14.5KP which had been pretreated in 0.02% Triton X-100 prior to the assay.

VI.1. MULTIENZYME ACTIVITY VERSUS GLUCOSE CONCENTRATION

To confirm that the overall multienzyme activity follows Michaelis-Menten kinetics, aliquots of the subcellular fraction 14.5KP were assayed against glucose concentration. The results of such an experiment, as well as experiments in which samples of fraction 14.5KP were treated with 0.02% Triton X-100 or 0.15 M NaCl prior to the assay against glucose concentration have been presented as μmoles of NADH converted/min/mg protein in Figure VI.1. The results show the typical hyperbolic shapes of the velocity versus substrate concentration curves indicative of enzyme reactions represented by Michaelis-Menten kinetics.

VI.2. THE OVERALL RATE OF MULTIENZYME ACTIVITY VERSUS CONCENTRATION OF THE SUBCELLULAR FRACTION 14.5KP

Protein concentration in an assay mixture may decisively influence the stability of an enzyme during preincubation and the assay period, especially if long periods are required for the assay (Bergmeyer, 1974). Since the activity of the multienzyme complex had frequently been observed to show lag-phase of over 20 minutes it was important to find out how the overall multienzyme activity varies with concentration of fraction 14.5KP in the assay medium. The multienzyme activity was determined with varying concentrations of the subcellular fraction 14.5KP and the results have been presented in Figure IV.2. as μmoles NADH converted/min/mg protein in the complex versus $\Delta E_{340/min.}$ They show that up to multienzyme units of 0.5 μmoles NADH/min,

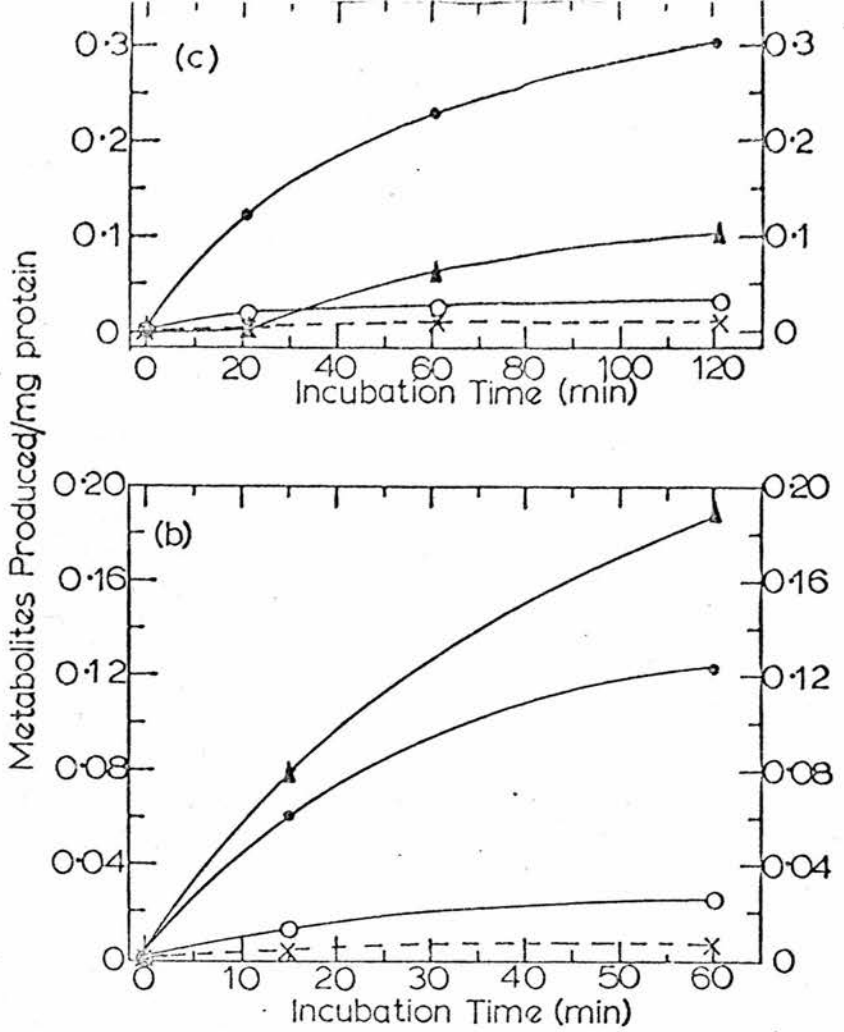


Fig. VI. 3(b&c). Product Formation During Activity of the Multienzyme Complex. (b) denotes results for untreated complex; (c), material preincubated in 0.02% Triton X-100 prior to its assay.

●, glucose-6-phosphate; 0, fructose-6-phosphate; ▲, fructose-1,6-diphosphate; x, dihydroxyacetonephosphate

Other experimental details are given in the text.

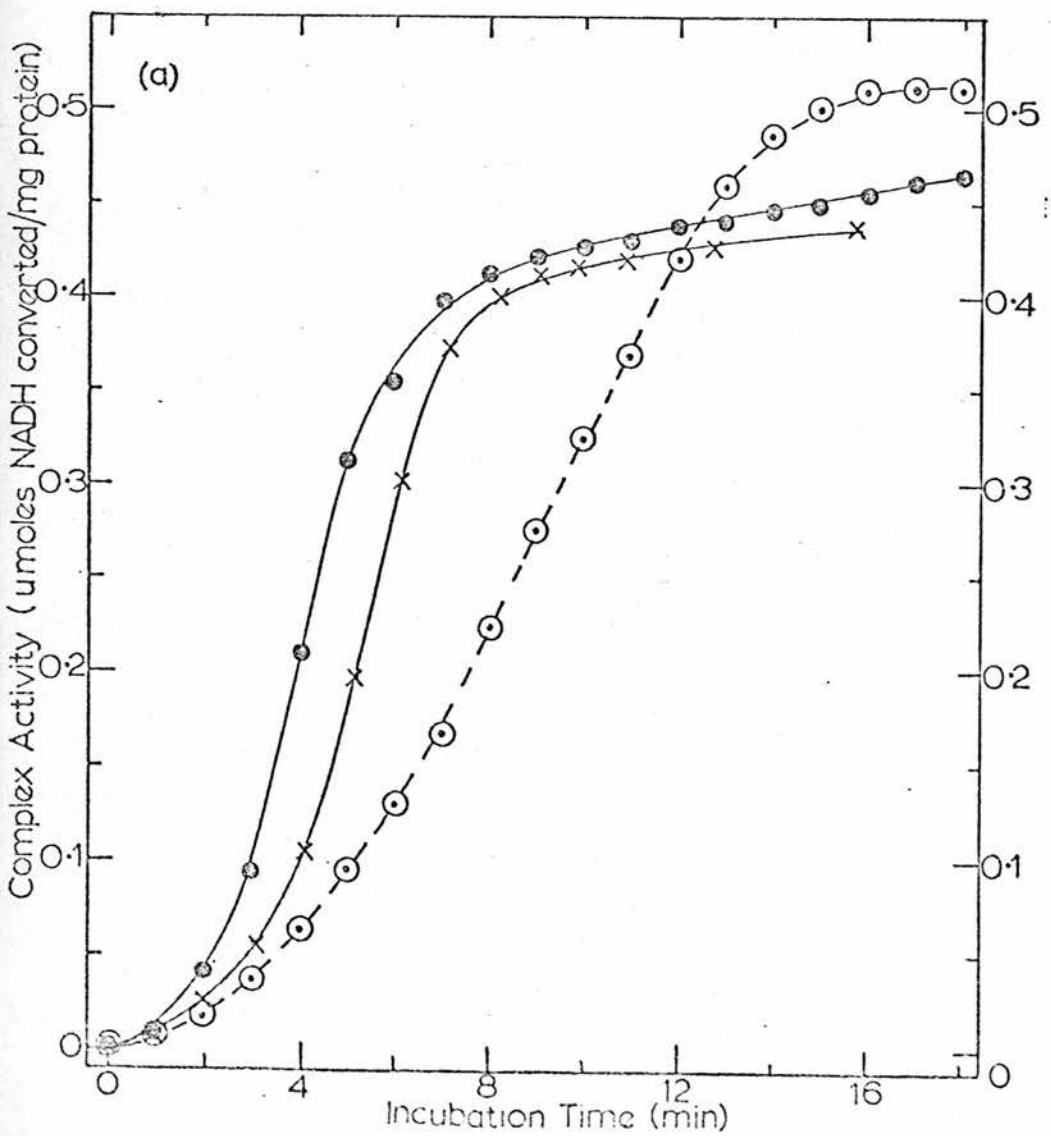


Fig. VI. 3a. Time Course for the Multienzyme Activity.

Other experimental details are given in the text.

0 denotes results from untreated complex; x, complex pretreated in 0.15M NaCl; ●, complex pretreated in 0.02% Triton X-100.

the reaction rate is proportional to the concentration of the multienzyme complex; thereafter, the velocity decreases and the reaction tends towards zero order.

To ensure reproducible results, all assays for multienzyme activity reported in this thesis were (wherever possible) performed with extract concentrations within the linear range of multienzyme activity.

VI.3. TIME COURSE FOR THE MULTIENZYME ACTIVITY

Long exposure of NADH to moisture, temperature and light (especially UV light) may result in the formation of inhibitors of dehydrogenases (Beaucamp, 1972). In view of the fact that the assay for the multienzyme activity shows a lag-phase the extent of which is inversely proportional to the multienzyme units in the assay medium it was important to find out how the multienzyme activity varies with time.

The results recorded in Figure VI.3a. were from experiments in which samples of fraction 14.5KP untreated, or treated either with 0.02% Triton X-100 or 0.15 M NaCl were assayed for their multienzyme activities against time. All three results show a lag-phase which could be considerably reduced or nearly abolished by increasing the concentration of fraction 14.5KP in the assay. Once the lag-phase ends, the velocity becomes linear; and the period during which this linearity lasts varies inversely with the units of multienzyme

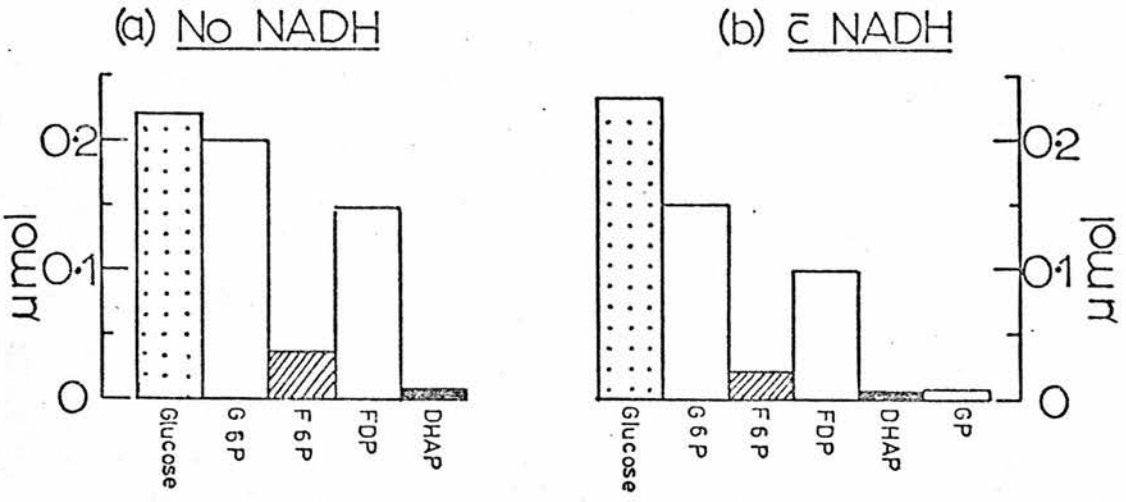


Fig. VI. 4. Relative Concentrations of the Products Formed After the Activity of the Multienzyme Complex Has Reached Steady State.

Aliquots of the subcellular fraction 14.5KP were assayed in the absence (a), or presence (b), of NADH. The reaction was allowed to proceed until steady state was reached; thereafter samples were treated and analysed for metabolites. The percentage of glucose conversion to metabolites varied from 73% to 116% during five experiments.

G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; DHAP, dihydroxyacetone-phosphate; GP, glycerolphosphate.

activity present in the subcellular fraction 14.5KP used in the assay.

When the experiments were repeated in the absence of glucose or fraction 14.5KP there was virtually no change in the optical density reading for up to 3 hr. It should be noted, however, that the maximum optical density changes observed for samples of fraction 14.5KP that had been pre-treated with 0.02% Triton X-100 or 0.15 M NaCl were always below the values obtained for the untreated samples. This result suggests that some component in the multienzyme complex is capable of decomposing NADH, and that this property is unmasked when the integrity of the complex is disrupted by detergent or isotonic NaCl treatment.

In Figure VI.3 (b and c) are the results of similar experiments in which samples of the incubation mixtures were withdrawn at various times, treated and analysed for metabolites. Only trace amounts of glycerolphosphate and practically no glycerol were detected in such experiments, whereas glucose-6-phosphate and fructose-1,6-diphosphate tended to accumulate. Although Triton X-100 treatment of fraction 14.5KP tended to change the relative amounts of glucose-6-phosphate and fructose-1,6-diphosphate, the extent of such variations were not consistently in favour of one compound or the other. Figure VI.4. shows that long (120 min) incubation does not alter very much the relative amounts of intermediates produced by the complex, except the increase in glycerolphosphate observed for the assay medium containing NADH. These results confirm (a) that the multienzyme activity follows the classical Michaelis-Menten kinetics for enzyme reactions, and (b) that the observed optical density changes

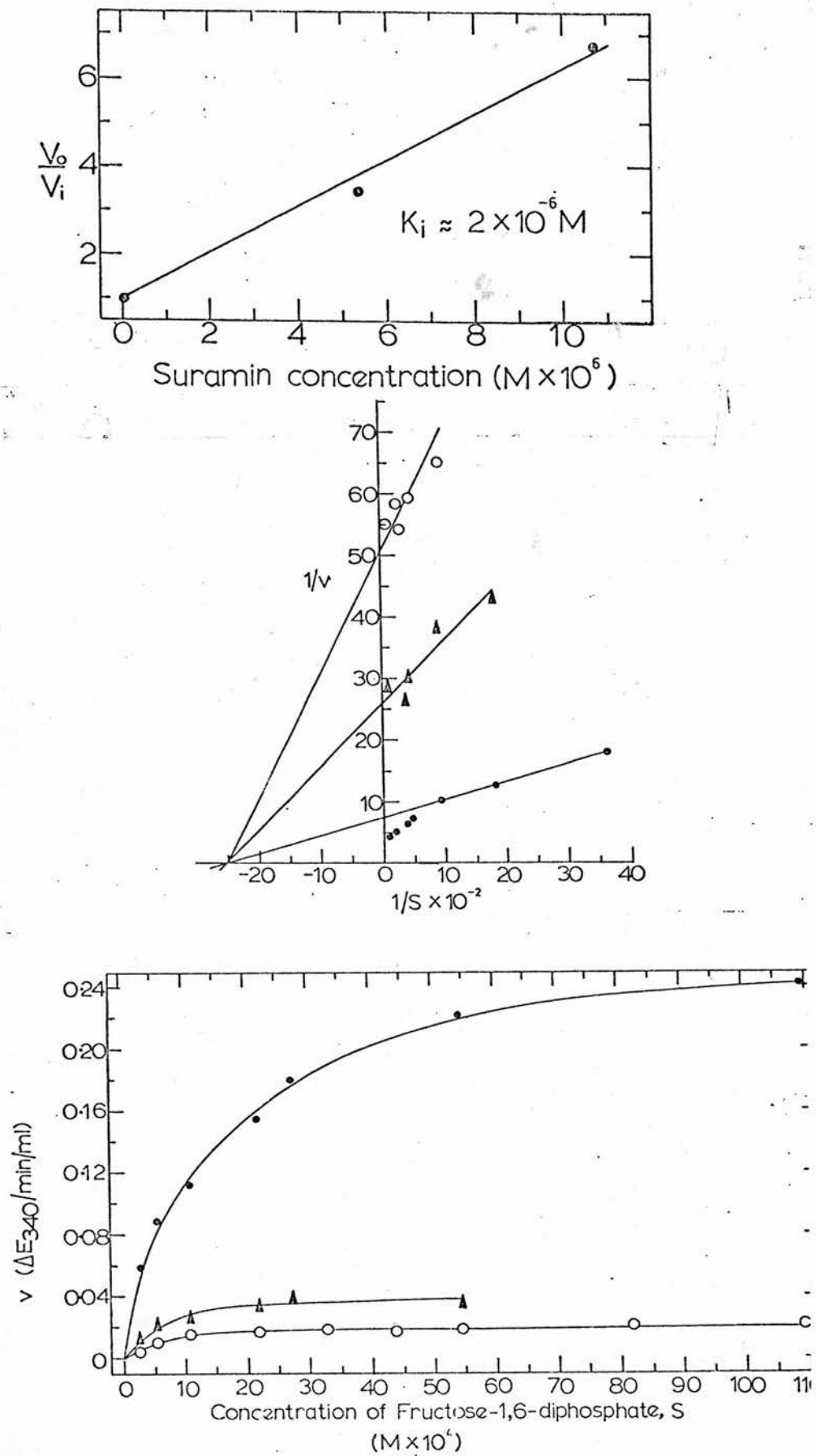


Fig. VI. 5. Suramin Inhibition of the NAD^+-dependent Glycerolphosphate Dehydrogenase. Samples of the subcellular fraction 14.5KP were assayed either in the absence (●), or presence of 6.7 μM (▲) or 13.4 μM (○) suramin.

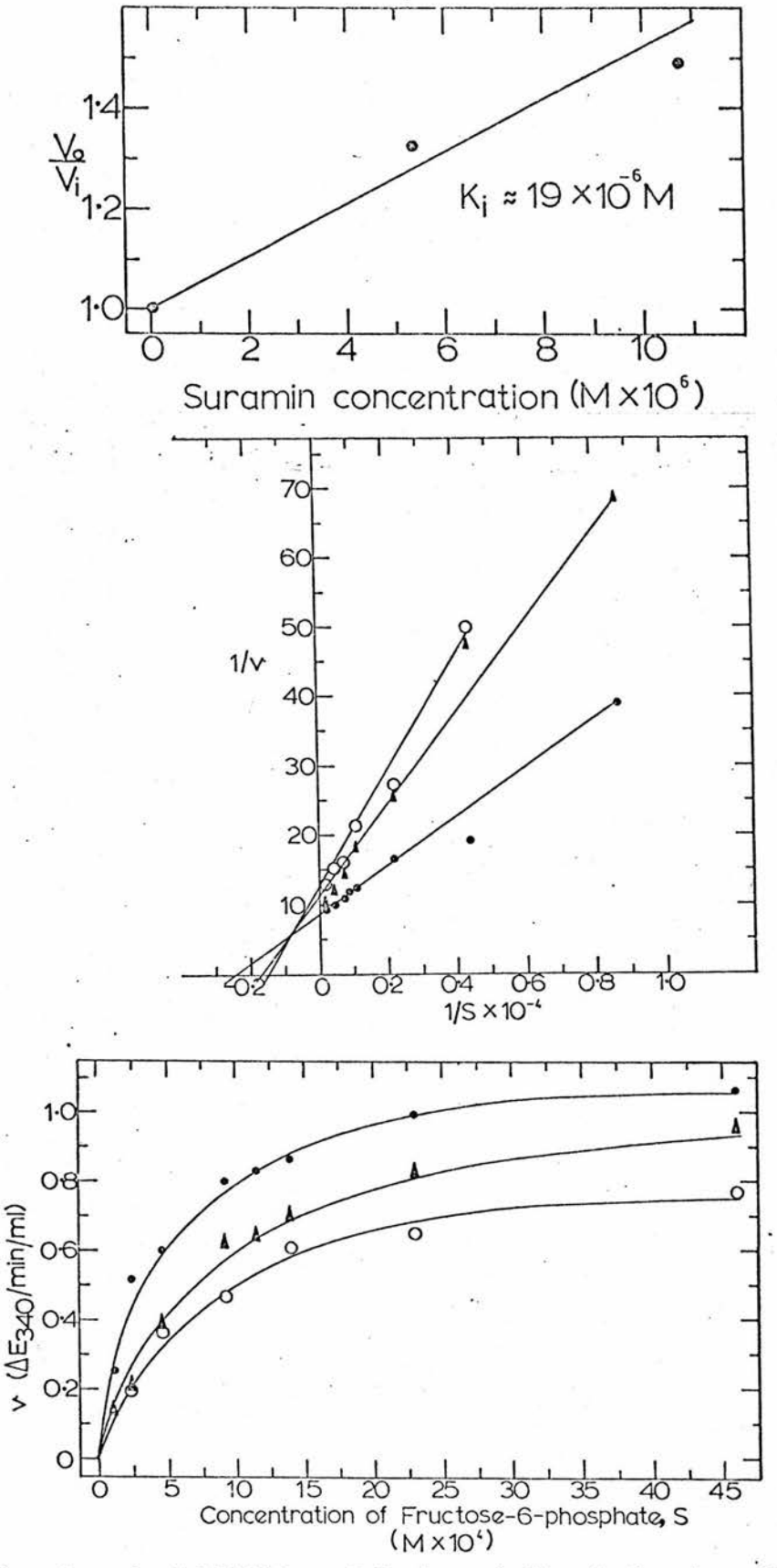


Fig. VI. 6. Suramin Inhibition of T. brucei Phosphofructose Kinase.

Samples of the subcellular fraction 14.5KP were assayed either in the absence (●), or presence of suramin at concentrations of 6.7 μM (▲) or 13.4 μM (○).

reflect the reduction of dihydroxyacetone phosphate formed from glucose by the concerted action of hexokinase, phospho-glucose isomerase, phosphofructose kinase and aldolase.

VI.4. INHIBITION OF MULTIENZYME ACTIVITY BY SURAMIN OR TRYPAN BLUE

Fairlamb & Bowman (1975) have shown suramin to be a potent competitive inhibitor of both the dehydrogenase and the oxidase components of the particulate glycerolphosphate oxidase system of long slender T. brucei, thus indicating, presumably, that the particulate nature of this enzyme complex does not greatly prevent the accessibility of suramin to these two enzymes. Since it has already been demonstrated (Section V.2.4.) that the particulate nature of the multienzyme complex limits diffusion of substrates to the component enzymes, it was interesting to find out whether any of the enzymes will be inhibited by suramin and if so, whether this inhibition will exhibit diffusion dependence.

First, the effect of suramin was tested on the activity of the NAD^+-linked glycerolphosphate dehydrogenase. As before, the substrate, dihydroxyacetone-phosphate, was first generated in the incubation mixture by the action of the auxiliary enzymes aldolase and triosephosphate isomerase on fructose-1,6-diphosphate. Under these conditions, suramin proved to be a potent, non-competitive inhibitor of the NAD^+-linked glycerolphosphate dehydrogenase, with a K_i value of 2 μM (Figure VI.5.), which is half the value reported for the glycerolphosphate oxidase by Fairlamb & Bowman (1975). The only other component enzyme of the complex found to be inhibited by suramin was phosphofructose

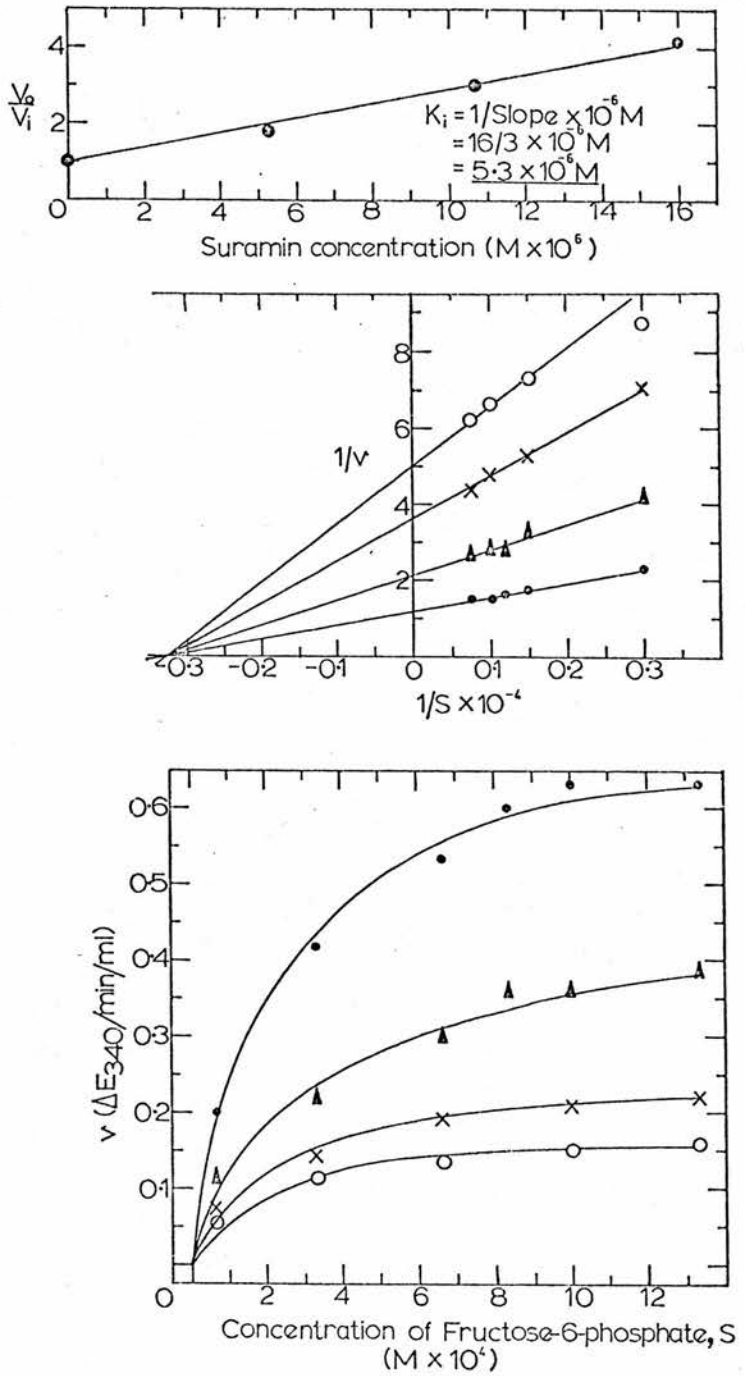
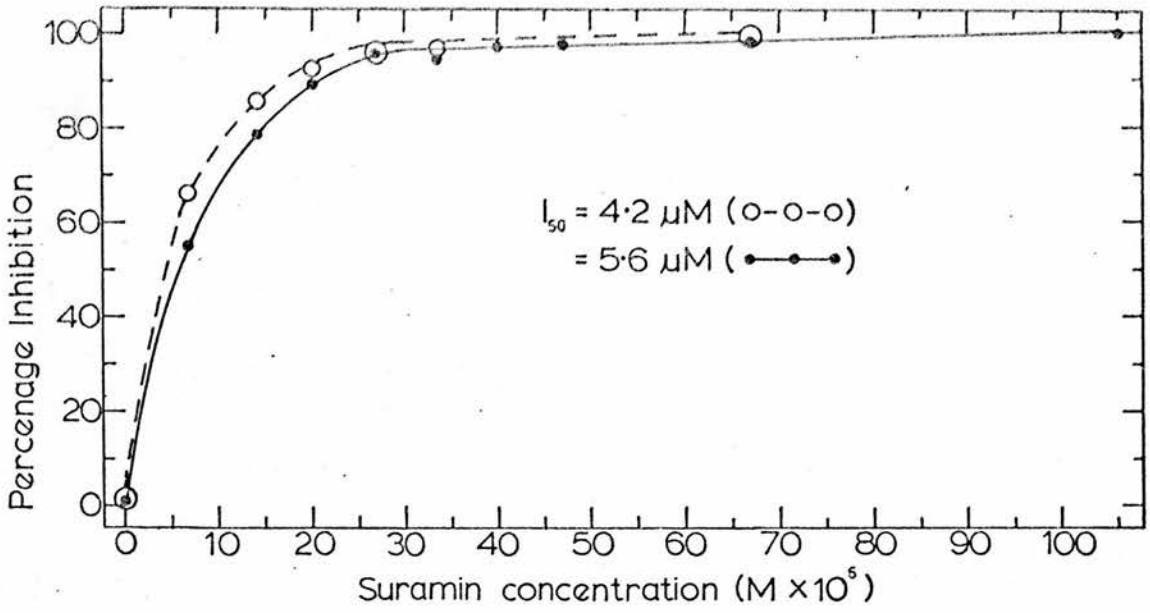


Fig. VI. 7. Effect of Suramin on the Combined Activities of Particulate Phosphofructose Kinase and Glycerolphosphate Dehydrogenase.

Samples of subcellular fraction 14.5KP of T. brucei were assayed either in the absence (•), or presence of 6.7 μM (▲), 13.4 μM (x), or 21.1 μM (0) suramin.



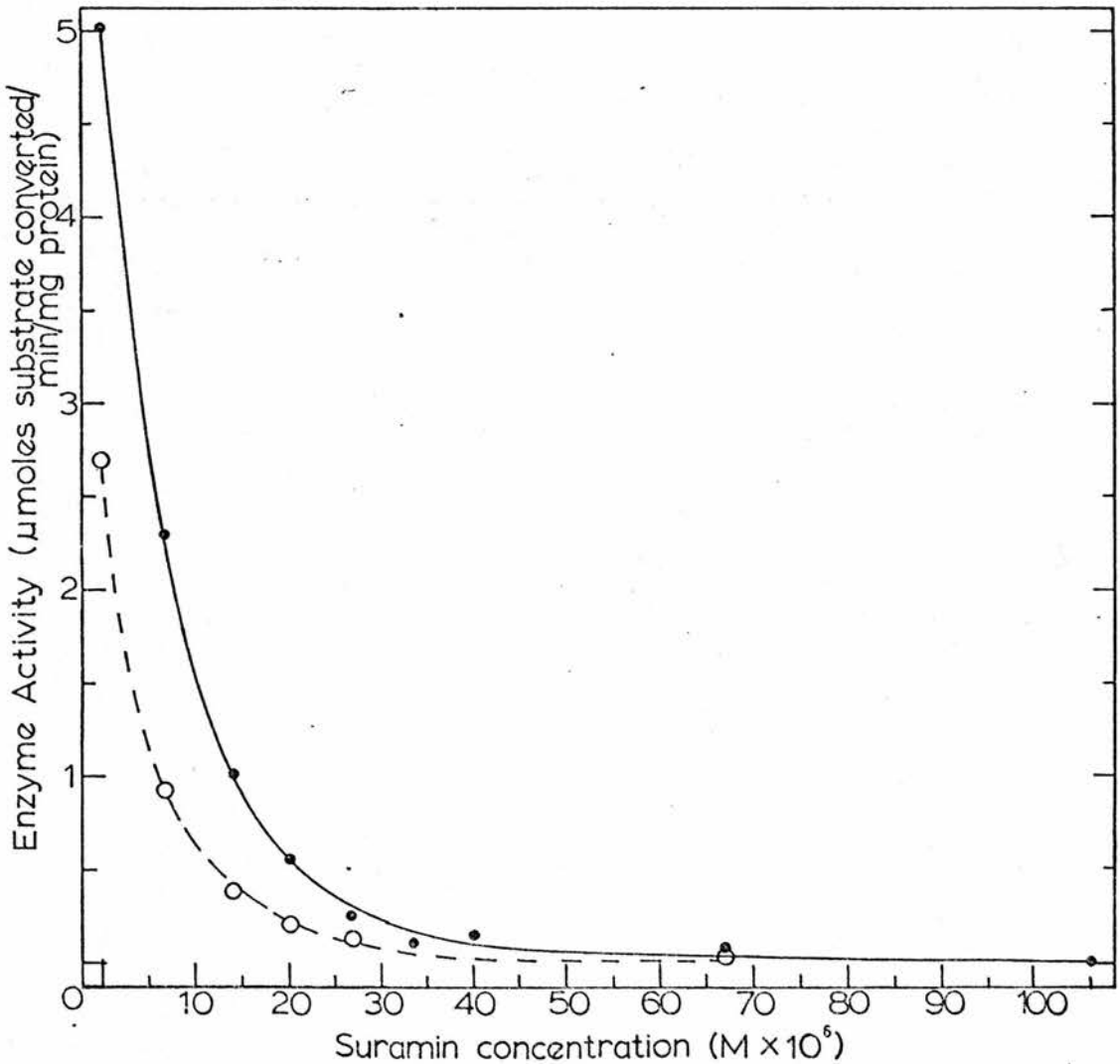


Fig. VI. 8. Effect of Diffusion on Suramin Inhibition of the NAD^+-dependent Glycerolphosphate Dehydrogenase. Incubation mixtures containing fraction 14.5KP in the presence of increasing concentrations of suramin were assayed in the absence (0) or presence (•) of 0.02% Triton X-100. Experimental details are given in the text.

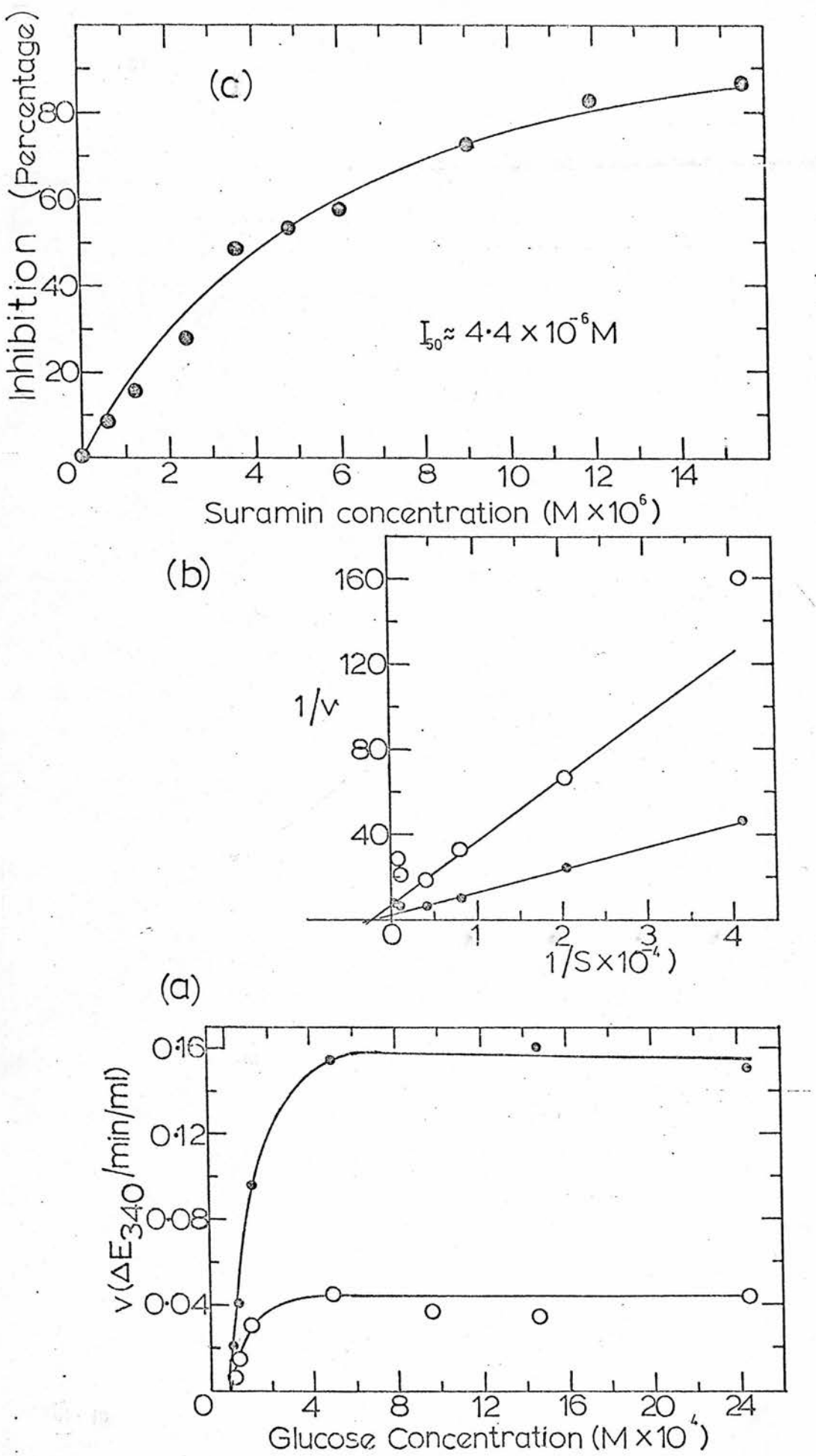


Fig. VI. 9. Suramin Inhibition of the Activity of the T. brucei Multienzyme Complex. (a & b) represent multienzyme activity of freaction 14.5KP determined in absence (•) or presence of suramin, 6.7μM (O); c, inhibition of multienzyme activity versus increasing concentrations of suramin.

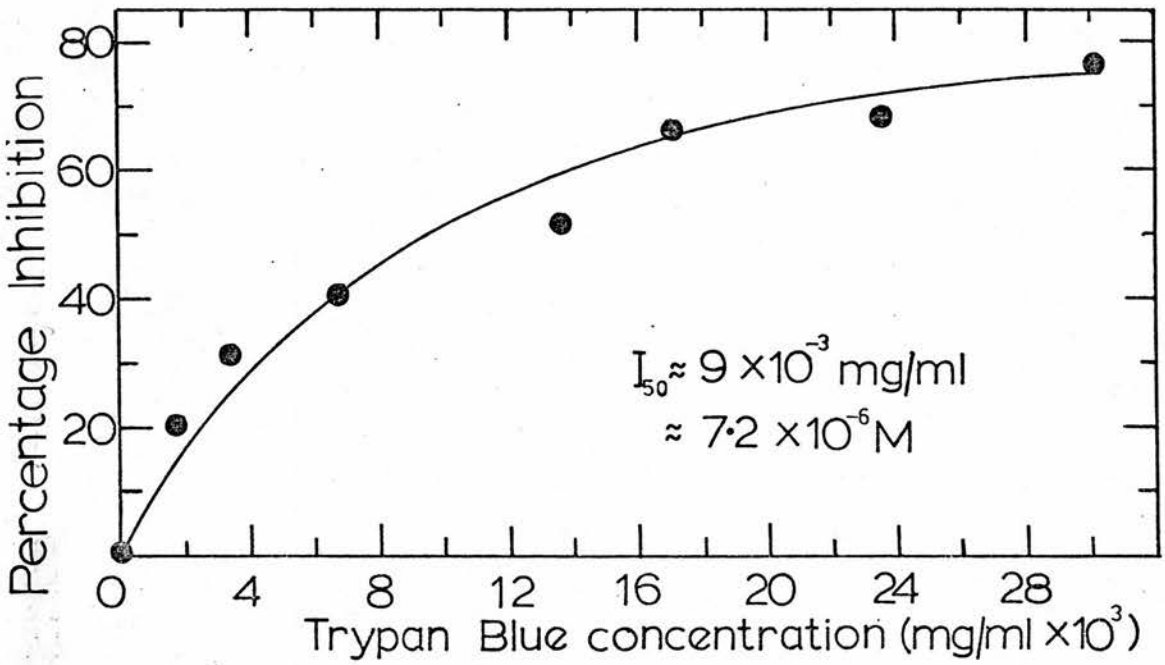


Fig. VI. 10. Trypan Blue Inhibition of the T. brucei Multienzyme Complex Activity. Samples of the subcellular fraction 14.5KP containing increasing concentration of trypan blue, were assayed for multienzyme activity, with glucose as the substrate.

kinase, although both the K_i value of 19 μM and the type of inhibition (probably uncompetitive) were significantly different (Figure VI.6.). When the effect of suramin was tested on the combined activities of phosphofructose kinase and the NAD^+-linked dehydrogenase using fructose-6-phosphate as the substrate and aldolase and triosephosphate isomerase as the auxiliary enzymes, the combined enzyme activity was non-competitively inhibited, with a K_i of 5.3 (Figure VI.7.), which is quite close to the value obtained for the dehydrogenase alone. The effect of diffusion on suramin inhibition of the NAD^+-dependent glycerolphosphate dehydrogenase was ascertained as follows. Untreated samples of fraction 14.5KP and samples of the material incubated in 0.02% Triton X-100 were assayed for enzyme activity in the presence or absence of suramin. The results (Figure VI.8.) show that, unlike the substrates (see Section V.2.4.), suramin interaction with the enzyme in the intact organelle is not diffusion dependent.

The multienzyme activity of the long slender T. brucei was also inhibited by suramin; with glucose as the substrate, the inhibition was observed to be non-competitive (Figure VI.9.) with an I_{50} value of 4.4 μM. Another surfactant trypanocide, trypan blue, was found to inhibit the multienzyme activity with an I_{50} value of 7.2 μM (Figure VI.10.).

SECTION VII

DISCUSSION

Introduction

In discussing the results recorded in this thesis, emphasis has been laid on the following:

(1) The methods of disruption of the T. brucei cells and how they affected the diffusion of proteins from the cells, as well as the activities and the subcellular distribution of the enzymes studied.

(2) The multienzyme system of T. brucei, its isolation and some of its physical and biochemical properties.

Suggestions have also been made indicating areas where further work may help to resolve some of the questions that could not be answered in the present studies.

VII.1. THE EFFECT OF CELL TREATMENT ON LIVE BLOODSTREAM FORMS OF T. BRUCEI

Although in isotonic media, the Kinetoplastida are known to be resistant to cell rupture (Simpson, 1972), Fairlamb (1975) has shown that exposure of bloodstream forms of T. brucei to isotonic sucrose solutions of 0.01 M Tris-HCl pH 7.4 containing 0.5% saponin, causes the cells to become immotile and such cells could easily be disrupted with a hand-operated Dounce homogenizer. The results reported in this thesis confirm Fairlamb's observations, in that T. brucei cells incubated on ice in the TS buffer containing 0.5% saponin became immotile within 10-20 min and could be easily disrupted with the Dounce homogenizer. Cell treatment with 0.5% Triton X-100, 0.5 mg% digitonin or by three cycles of freezing and thawing had similar

effects, except that the cells appeared to be more resistant to digitonin treatment and frequently required incubation periods of up to 30 min in solutions of digitonin before they became immotile; the digitonin-treated cells were also more resistant to homogenization and their complete disruption sometimes required up to 80 strokes of the pestle instead of the usual 40 strokes required to disrupt cells that had been lysed by the other three methods of treatment.

Since digitonin has a relatively low solubility in aqueous solutions the concentration of digitonin used in these studies was 0.5 mg% as compared with a value of 0.5% for saponin and Triton X-100. It could therefore be suggested that the relative resistance of the T. brucei cells to digitonin treatment was probably due to the low concentrations used. Such a view is supported by Segal & Milon-Goldweigi (1975) who found digitonin haemolysis of erythrocytes to increase with the concentration of the detergent. They observed that a 21% haemolysis by digitonin concentration of 2.7×10^{-6}M increased to 94% when the detergent concentration was increased to 5.9×10^{-6}M. Unfortunately it was not possible to resolve the question of whether the four different methods of cell treatment had the same effect on the T. brucei cell wall and ultrastructure by means of electron microscopy; however phase contrast microscopy showed that digitonin causes shrinkage of the cells whereas the other three methods cause cell swelling. It is therefore likely that digitonin affects the cells differently.

VII.2. THE EFFECT OF THE METHOD OF CELL LYSIS ON THE ACTIVITIES, THE SUBCELLULAR DISTRIBUTION AND THE LEAKAGE OF SOME ENZYMES OF THE BLOODSTREAM FORMS OF T. BRUCEI

Two reasons stand out (among others) why it is important to know what effect different methods of cell treatment have on the activities of the enzymes, their leakage from the cell and their subcellular distribution.

First, in chemotherapy, the importance of the ability to detect subtle differences that may exist in the metabolism as well as the activities of host and parasite enzymes has been regularly emphasised. Thus, although Ehrlich discovered the trypanocidal action of bis-azo dyes as far back as 1904, there is no doubt that the subsequent failure to determine the mode of action of these compounds in trypanosomes has contributed largely to the lack of success both in the discovery of new drugs based on the structure of these azo dyes as well as our inability to limit the undesirable side effects of a drug like suramin.

Secondly, comparative biochemistry has played a useful rôle in determining differences existing between organisms for which the conventional methods of morphological identification has proved insufficient or unreliable. The method has also been used sometimes to confirm the basis for a morphological identification. As pointed out in Section I in the literature review, the morphological differences that exist between the vector forms, the culture forms and the short stumpy blood-stream forms of the trypanosomes on one hand, and the long slender bloodstream forms of these organisms on the other,

have been shown to reflect the enzymic make-up of these different forms of the parasite (Flynn & Bowman, 1973).

The success of all such studies depends on the principle that the enzyme activities will not be affected by the method employed to treat the biological material; it sometimes works also on the assumption that the methods of cellular treatment either releases all the enzymes into solution, or does not affect their subcellular distribution. However, there are many examples in the literature to show that such assumptions may not be correct. The discussions which follow in Sections VII.2.1. to VII.2.3. compare the effects of the different methods of cell disruption on T. brucei enzymes in order to determine whether such assumptions might be justified in this case.

VII.2.1. The Effect of the Method of Cell Disruption on the Activities of the Enzymes Which Catalyze the Breakdown of Glucose in the Bloodstream Long Slender Form of T. brucei

When the activities of the eleven enzymes were determined, using the total homogenates (T) obtained by disrupting T. brucei cells in 0.5% saponin, 0.5% Triton X-100, 0.5 mg% digitonin or by three cycles of freezing and thawing, the results showed quite clearly that the specific activity observed for each of the enzymes was dependent on the method of cell lysis adopted. Seven enzymes, hexokinase, phosphoglucose isomerase, phosphoglycerate kinase, enolase, pyruvate kinase, glycerolphosphate dehydrogenase and glycerokinase, showed the

highest specific activities with cells lysed in saponin; and within experimental error, this treatment also gave specific activities for the remaining four enzymes which are comparable with those obtained from homogenates of cells disrupted by the other three methods of treatment. On the other hand, lysis by digitonin resulted in the lowest specific activity for each of the eleven enzymes.

When homogenates were prepared by grinding T. brucei cells with alumina, the following observations were made. With the exception of phosphoglycerate mutase, the specific activities of all the enzymes were below the values obtained from saponin-lysed cells. Preincubation of the homogenate from alumina-grinding with the detergents prior to assay, showed that saponin or Triton X-100 treatment activates hexokinase, phosphoglucose isomerase, phosphofructose kinase, phosphoglycerate kinase, enolase and glycerolphosphate dehydrogenase, whereas both detergents inhibit aldolase, phosphoglycerate mutase, pyruvate kinase and glycerokinase. Glyceraldehyde-phosphate dehydrogenase activity does not appear to be affected by saponin whereas Triton X-100 slightly activates this enzyme. On the other hand, all the enzymes are inhibited by digitonin except aldolase and glycerolphosphate dehydrogenase which are apparently activated by this detergent.

That the method of cell disruption may affect enzyme activities has been widely appreciated by many authors. Day et al. (1954), for example, suggested that varying homogenization techniques might explain some of the discrepancies between the results of various workers on liver catalase activity. Adams & Burgess (1957) confirmed that the disagreement in the literature

concerning liver catalase activity reflected the different homogenization techniques adopted by various authors. They showed that increasing the homogenizer-pestle strokes or incubation of the homogenate in Triton X-100 led to an increase in the enzyme activity. From such studies, these authors were able to show that a large amount of liver catalase activity is present in relatively stable particles associated with the large granule fraction, and that only about 10% of this activity is measurable so long as the particles remain unbroken.

The fact that the effect of a particular method of cell disruption may be more complicated than a simple activation of enzymes has been clearly demonstrated for the following enzymes in T. brucei, T. gambiense, T. rhodesiense and T. equiperdum: hexokinase (Risby & Seed, 1969a); phosphoglucose isomerase (Risby et al., 1969); and aldolase (Risby & Seed, 1969b). These authors showed that sonication of water-lysed homogenates of these trypanosomes led, not only to activation, but also to some denaturation of all three enzymes.

The results reported in this work support these authors in that detergent-treatment of homogenates obtained by grinding T. brucei cells with alumina causes activation of some enzymes and at the same time inhibits others. When some of the enzymes which are activated by detergent treatment were assayed in the presence of increasing concentrations of Triton X-100 (Figure V.2.), it became apparent that in every case, the process of enzyme activation had an optimum detergent concentration above which the enzyme became progressively inactivated.

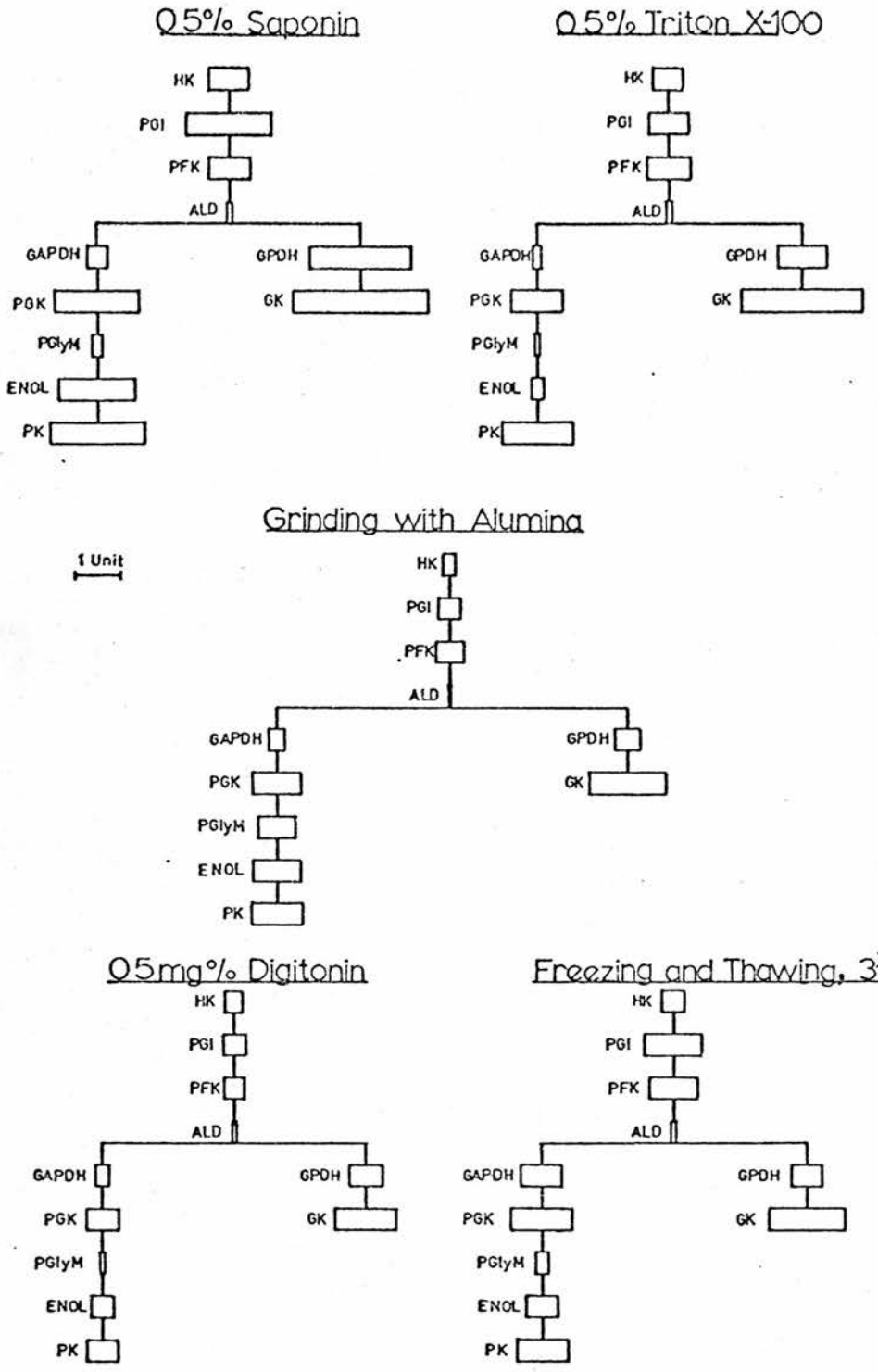


Fig. VII. 1. Schematic Representation of the Effect of the Method of Cell Disruption on the Specific Activities of the Enzymes which Catalyze Glucose Breakdown in Bloodstream Long Slender Form T. brucei.

The bar represents 1 unit of enzyme activity (μmol substrate converted/min/mg protein).

Thus it appears that, at least for T. brucei, not only do methods of cell disruption affect enzyme activities but also it is likely that for each method adopted there may be an optimum condition which ensures the most complete recovery of enzyme. This view is clearly illustrated by the schematic diagrams in Figure VII.1. which compare the specific activities of the enzymes observed in the total extract (T) obtained by the different method of disruption of T. brucei.

Although the corrected specific activity of 0.5 observed for aldolase by Risby & Seed (1969b) is much higher than the values recorded in this work, these authors performed their enzyme assays at 37°C as compared to the assay temperature of 25°C employed here. If it is assumed that every 10°C rise in temperature approximately doubles enzyme activity then the aldolase activity recorded for homogenates obtained by treating T. brucei cells with the detergents or by freezing and thawing, is in reasonable agreement with the value quoted by Risby & Seed; both results are however, still much higher than the value observed when homogenates obtained by grinding T. brucei cells with alumina were assayed for aldolase activity.

Notwithstanding any differences between the present results and the literature values, it is significant to note that aldolase showed the lowest activity of the eleven enzymes studied irrespective of the method of cell disruption adopted. If it is assumed that this reflects the in vivo physiological position, then it could be argued that this enzyme may be involved in the control of glycolysis in T. brucei. On this

principle, glycerokinase, which showed the highest activity irrespective of the method of cell lysis, would appear to be the least involved in any control mechanism for glucose catabolism in this organism.

VII.2.2. The Effect of Lytic Treatment on the Leakage of Protein and the Enzymes which Catalyze the Breakdown of Glucose in T. brucei

The investigations involving the effect of cell disruption techniques on the bloodstream form of T. brucei show quite explicitly that all three detergents as well as freezing and thawing cause protein and enzyme leakage, the nature and extent of which is dependent on the particular method adopted for lysing the cells.

On close examination of the results, it is possible to divide the eleven enzymes into four groups. The first group of enzymes comprises phosphoglycerate mutase, enolase and pyruvate kinase which completely leak out of T. brucei cells lysed with saponin, Triton X-100 or by freezing and thawing; then there is phosphoglucose isomerase which behaves similarly when the cells are lysed by saponin or Triton X-100 but not by digitonin or freezing and thawing; glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase and glycero-kinase whose leakage may be extensive or otherwise depending on the method of cell lysis; and finally the four enzymes, hexokinase, phosphofructose kinase, aldolase and glycerol-phosphate dehydrogenase which appear to be almost completely retained in the cell, irrespective of which method is adopted for cell lysis.

If it is assumed that the molecular weights of these enzymes are similar to the values recorded in the literature, then it becomes clear that the leakage of these enzymes, under the conditions described in this work, does not relate to their molecular weights. For example, between 80 and 91% of pyruvate kinase activity is recovered in the supernatant obtained by pelleting T. brucei cells which have been treated with saponin, Triton X-100 or by freezing and thawing, whereas only 8-17% of glycerolphosphate dehydrogenase activity is detected under similar conditions. Yet the molecular weights of the enzymes from T. brucei (Flynn, results unpublished) are 269,000 (PK) and 84,000 (GPDH). These results compare with the recorded values (see Bergmeyer et al., 1974) for the corresponding enzymes from rabbit muscle of 273,000 (PK) and 78,000 (GPDH). It therefore appears from these results that treatment of the bloodstream form of T. brucei with the three detergents or by freezing and thawing causes sufficient fragmentation of the plasma membrane to lead to substantial or complete leakage of those proteins which are not immobilised through linkage with membranes or subcellular organelles.

The ability of detergents to disrupt plasma membranes through extensive solubilization of proteins has been demonstrated by many authors. For example, Dulaney & Touster (1970) employed 1% solutions of sodium dodecyl sulphate, sodium deoxycholate or Triton X-100 to effect between 70 and 100% solubilization of rat liver plasma membrane proteins. They then proceeded to identify some of the plasma membrane enzymes by subjecting the solubilized proteins to electrophoresis in acrylamide gels which had been incorporated with the appropriate

detergent to a final detergent concentration of 0.1%. The same authors solubilized proteins from membranes of microbodies, lysosomes and mitochondria, and employed similar electrophoretic techniques to yield gels showing the presence of various membrane enzymes.

VII.2.3. The Effect of the Method of Cell Disruption on the Distribution of Protein and Enzymes in the Subcellular Fractions of T. brucei

Differential centrifugation of the cell homogenates confirmed the observations by Risby & Seed (1969a, 1969b) and Risby et al. (1969), that T. brucei hexokinase, phosphoglucose isomerase and aldolase exist in the soluble as well as particle-bound forms. Because the specific activities of these enzymes (as well as the other particulate enzymes) in the subcellular fraction 14.5KP consistently proved to be higher than the corresponding value, in the soluble fraction, it seems reasonable to conclude that the enzyme activities in the soluble fraction had resulted from desorption of particulate activity during cell lysis and homogenization. The results therefore show that probably in this organism only three of the glycolytic enzymes, namely, phosphoglycerate mutase, enolase and pyruvate kinase exist in the cell as soluble proteins, while the remaining eight enzymes are particle-bound. Since it is highly unlikely that the bonds involved in the association of the different members of a series of enzymes with a membrane structure will be of the same stability, it is not surprising that the proportions of some of the enzymes

distributed between the soluble and particulate fractions were found to vary with the different methods of cell disruption. This probably accounts for the failure of LePage & Schneider (1948) to demonstrate original (homogenate) glycolytic activity with any of their subcellular fractions, although this was achieved when all the fractions were recombined.

The method of grinding T. brucei cells with alumina or silicon carbide gave homogenates from which a particle could be isolated in which was concentrated at least six of the glycolytic enzymes as well as the glycerolphosphate oxidase. It should be emphasized however, that this should not be taken to mean that grinding is the best possible method for preparing T. brucei homogenates as other methods which were not examined might easily prove to be superior in this respect. In any case, the fact that some component(s) of T. brucei tend to become strongly adsorbed to the granules of the abrasives means that the specific enzyme activities obtained with this method may have been slightly exaggerated.

To summarise, the results of the experiments conducted to establish the effect of cell treatment with 0.5% saponin, 0.5% Triton X-100, 0.5 mg% digitonin or three cycles of freezing and thawing lead to the following conclusions:

(1) All four treatments render the cells immotile, although digitonin (at the low concentrations used) takes about double the time (20-40 min) required by the other three.

(2) Similarly, all four methods cause leakage of protein but again the 9% of the total protein which leaks out of cells incubated in digitonin is less than a quarter of the value observed for the other three methods. This, taken together

with the observation that digitonin, unlike the other three methods of cell disruption, causes shrinkage and not swelling of T. brucei cells suggests strongly that this detergent probably causes precipitation or adsorption of proteins to subcellular structures or membranes.

(3) Examination of the probable intracellular location of the enzymes by differential centrifugation confirmed that not only does digitonin solubilize fewer enzymes and to a lesser extent, but it also causes inhibition of most of them, probably through aggregation which would tend to curtail substrate accessibility to the enzymes. Four enzymes, hexokinase, phosphofructose kinase, aldolase and glycerol-phosphate dehydrogenase were found to be particulate irrespective of the method of cell lysis, whereas only two enzymes, phosphoglycerate mutase and enolase proved to be soluble regardless of which of the four methods was used to lyse the cells. The subcellular distribution of the remaining enzymes was completely dependent on the method adopted for lysing the cells.

(4) The pattern of enzyme distribution for homogenates obtained by grinding T. brucei cells with alumina or silicon carbide confirmed that hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, phosphoglycerate kinase glycerolphosphate dehydrogenase as well as the glycerolphosphate oxidase are all particle-bound, whereas phosphoglycerate mutase, enolase and pyruvate kinase are soluble enzymes; and that glyceraldehydephosphate dehydrogenase and glycero-kinase probably exist both in the soluble and particle-

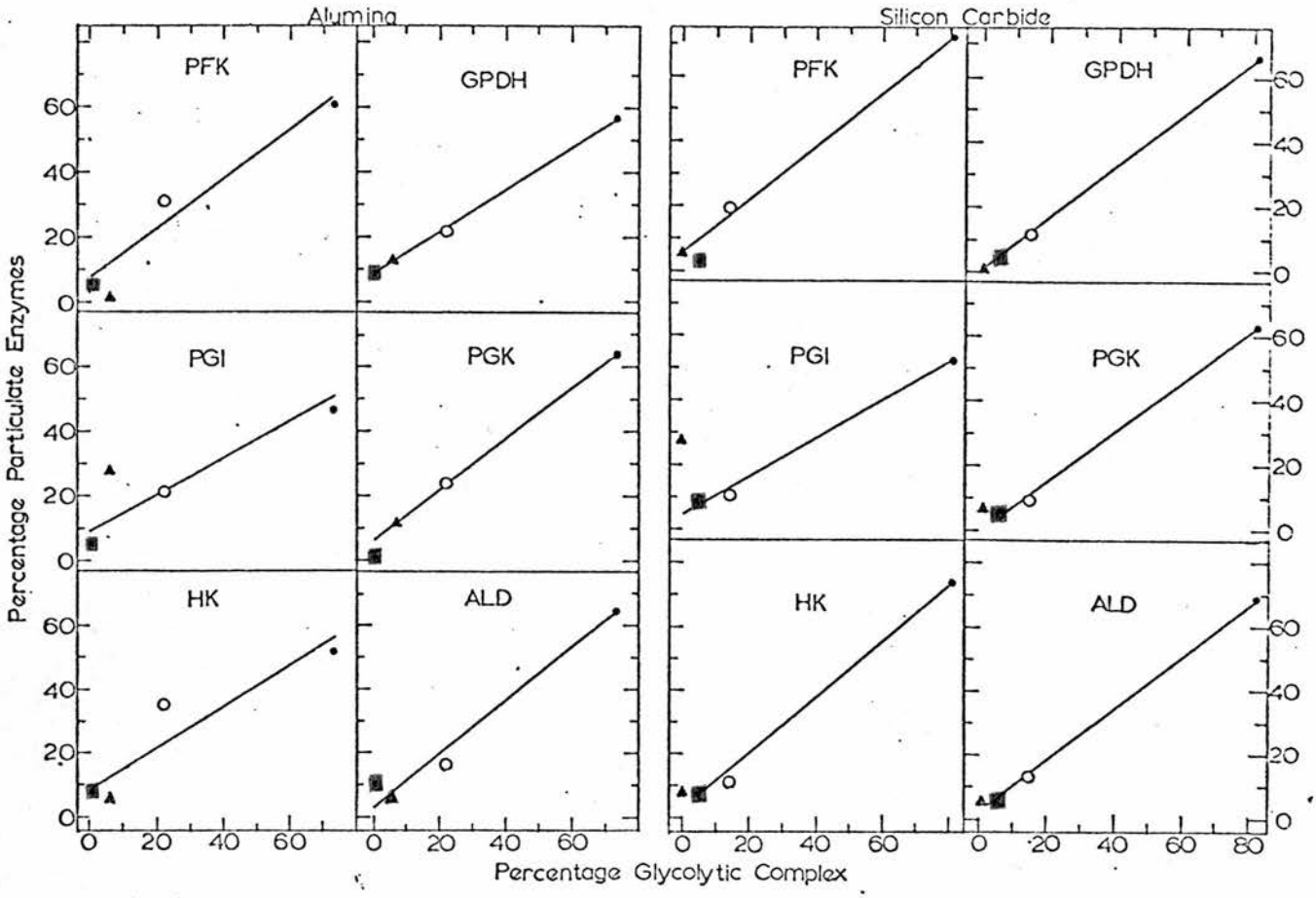


Fig. VII. 2. Correlation Between the Activity of the Multienzyme Complex and the Activities of Its Component Enzymes.

Subcellular fractions obtained by differential centrifugation of homogenates from alumina or silicon carbide treated T. brucei cells were assayed for enzyme activities. The recovered percentage activity of each component enzyme in the fractions was then plotted against the percentage recovered multienzyme activity in the same fractions.

■ denotes fraction 1KP; ●, fraction 14.5KP; 0, fraction 105KP; ▲, fraction 105KS.

bound forms, although no kinetic studies were conducted to verify this.

It should be emphasised that although grinding with the two abrasives appeared to be milder than the other methods, it has the disadvantage that there is a tendency for cell components to become strongly adsorbed to the granules of the abrasives. Thus this method fails to satisfy one of the important requirements of a good technique for cell disruption, which is that it does not permit examination of the cell in its entirety.

VII.3. THE INTRACELLULAR LOCATION OF THE MULTIENZYME COMPLEX

Reference to Table V.1b. and Figures IV.4a. and IV.4b. shows that grinding of T. brucei cells produces a homogenate whose post-nuclear fraction 14.5KP contains not less than 70% of the recovered multienzyme activity. The close relation between the multienzyme complex and its component enzymes is illustrated in Figure VII.2. which confirms that the relative content of each subcellular fraction in complex activity is equal (within experimental error) to its relative level in the activities of the following six enzymes: hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, phosphoglycerate kinase and glycerolphosphate dehydrogenase. Significant, but variable amounts of the activities of glyceraldehyde-phosphate dehydrogenase and glycerokinase were always found to be associated with fraction 14.5KP, thus suggesting that the two enzymes are probably bound to the complex through weak linkages the stability of

which depends on factors that are yet to be identified. The concentration of protein may be an important factor in this connection since glyceraldehyde-phosphate dehydrogenase in the subcellular fraction 14.5KP, but not the enzyme in the homogenate, was found to be inhibited by Triton X-100 treatment.

By inhibiting the residual glyceraldehyde-phosphate dehydrogenase activity in fraction 14.5KP, it became feasible to assay for multienzyme activity through the coupling of glucose breakdown to oxidation of NADH by the NAD^+-dependent glycerolphosphate dehydrogenase. Hence the method chosen to determine the complex activity did not take account of phosphoglycerate kinase, which was the sixth glycolytic enzyme concentrated in the subcellular fraction 14.5KP and also observed to be tightly bound to the complex.

VII.3.1. Identification of T. brucei Microbodies as the Subcellular Location of the Multienzyme Complex

Incubation of the post-nuclear fraction 14.5KP with saponin or Triton X-100 resulted in increased activities of the component enzymes, an observation consistent with the conclusion that the enzymes are located within closed membrane fragments. It is important to recall that although this unmasking of enzyme activities could always be demonstrated, the latent activity observed, even for the same enzyme, tended to vary from one preparation of fraction 14.5KP to another. It is impossible at the present moment to provide any concrete

explanation based on experimental data for this variability, except to say that there was enough tentative evidence to suggest that this may have been due partly to differences in the protein concentration of the different preparations. This, taken in conjunction with the observation that Triton X-100 could both activate as well as denature the enzymes, sufficiently emphasizes the need for further work to establish optimum conditions for the isolation of the complex.

Notwithstanding these difficulties, however, it is evident from Figure V.3.2.(a - e) which show the overall rate of reaction of each component enzyme in fraction 14.5KP in the presence and absence of 0.02% Triton X-100, that the detergent can cause increases of up to 132% (HK), 69% (PGI), 94% (ALD), 329% (PFK) and 127% (GPDH) in the activities of the enzymes. Figure V.2. also shows that the activity of the complex can be increased seven-fold by incubating fraction 14.5KP in 0.01% Triton X-100. Hence, there appeared to be no doubt that the multienzyme complex is located within a subcellular granule with a limiting membrane which has to be disrupted before the full potential of the complex can be revealed.

On subjecting fraction 14.5KP to isopycnic sucrose gradient centrifugation (Figures V.1. and V.1.1.), the complex and its component enzymes were observed to band at a density of 1.22-1.23, which is within the density range of 1.20-1.25 where protozoan microbodies are known to band (Müller, 1975).

Microbidies have been defined as cytoplasmic particles, with a limiting membrane and a dense granular matrix, which are widely distributed in nature. A characteristic feature of microbodies is their ability to remove excess reducing power generated during other metabolic activities of the cell; and depending on whether the reducing power is removed by an oxidase or by the action of glyoxylate enzymes, they have been referred to either as peroxisomes or glyoxysomes.

Protozoa in which microbodies have been observed include the Trichomonadida, Phytomastigophora, Chrysomodadida, Euglenida, Ciliata and the Trypanosomatida (Müller, 1975). There is evidence suggesting that in the bloodstream long slender forms of T. brucei and T. equiperdum the microbodies are the exclusive organelles of terminal respiration, and they contain a unique oxidase system that acts on glycerolphosphate (Bayne et al., 1969; Ryley, 1964). During isopycnic sucrose density centrifugation of the oxidase system from T. brucei, the oxidase activity separates from the activity of the NAD^+-dependent glycerolphosphate dehydrogenase, and the two enzymes show modal densities of 1.16 and 1.25 g/ml respectively (Fairlamb & Bowman, 1977). Since microbodies from other species are known to band in density zones extending from 1.20-1.25, Bayne et al. (1969) have suggested the name L-glycerol-3-phosphate oxidase body as the most appropriate designation for the biochemically defined entity in T. brucei and T. equiperdum which oxidizes α-glycerol-phosphate to dihydroxyacetone phosphate.

Müller et al. (1968) made the interesting observation that peroxisomes (or microbodies) may represent a repository

particle the importance of which decreased progressively as its functions were taken over by the more efficient mitochondria; except in certain types of organisms where the requirement of a special function such as gluconeogenesis or photosynthesis has made its retention advantageous. In view of this observation, it is interesting to find that in T. brucei, a multienzyme complex of glycolytic enzymes, including the NAD^+-dependent glycerolphosphate dehydrogenase which catalyses the first step in a chain of reactions responsible for the reoxidation of NADH, is located in the microbodies.

Opperdoes et al. (1977b) used differential centrifugation followed by isopycnic sucrose gradient centrifugation to isolate a particle which possessed the characteristics of a microbody from bloodstream long slender form of T. brucei; the particle contained the NAD^+-linked glycerolphosphate dehydrogenase and it was well separated from the material containing the L-glycerol-3-phosphate oxidase system of this organism. The authors put up a strong argument to support the view that the particle containing the NAD^+-linked glycerol-phosphate dehydrogenase was a microbody whereas the oxidase system was located in the mitochondrion. The gist of their argument included the following observations.

(1) That in Crithidia both the dehydrogenase and catalase (the peroxisome/microbody marker enzyme) band at identical positions in sucrose.

(2) That their particulate dehydrogenase in T. brucei has a density which is within the range found for microbodies or peroxisomes from other organisms.

(3) That this particle was observed to contain microbodies, often with the characteristic crystalloid inclusions (Opperdoes et al., 1977b). They also found the particle to be partially resistant against freezing and thawing, a phenomenon also observed for peroxisomes (Hayashi, et al., 1971).

(4) That an NAD^+-linked glycerolphosphate dehydrogenase has also been found in microbodies of animal tissues (Gee et al., 1974).

Although it was not possible to embark on electron microscopic examination of the multienzyme complex, there is sufficient evidence which strongly suggests that the peak of the isopycnic sucrose gradient fractionation which contained the multienzyme complex (see Figures V.1. and V.1.1.) was made up of microbodies.

First, the median equilibrium density (1.22-1.23) of this fraction is well within the range (1.20-1.25) found for protozoan microbodies (Müller, 1975), and specifically it is almost identical with the value found by Opperdoes et al. (1977) for their NAD^+-linked glycerolphosphate dehydrogenase particle.

Secondly, as found by Opperdoes et al. (1977b), the glycerolphosphate dehydrogenase peak is separated from a second peak of median equilibrium density of 1.159 in which the oxidase activity is located.

Thirdly, incubation of fraction 14.5KP in isotonic saline failed to cause substantial leakage of either the multienzyme activity or the activities of the component enzymes of the complex; indeed, the pellet obtained by incubating fraction 14.5KP in isotonic saline for 30 min on ice, followed by centrifugation at 105,000 g for 1 hr, could still be shown to

possess latent activity. In addition, isotonic NaCl treatment of fraction 14.5KP had only a slight effect (less than 10% solubilization of some of the component enzymes) on the banding of the multienzyme activity during isopycnic sucrose gradient centrifugation.

Fourthly, the homogenate obtained by subjecting T. brucei to three cycles of freezing and thawing also gives a post-nuclear fraction 14.5KP which shows multienzyme activity; true, such a preparation, in sucrose gradient centrifugation, gives two peaks of multienzyme activity with median equilibrium densities of 1.203 and 1.232 compared with the single peak with a value of 1.226 obtained with the preparation from cells that have been disrupted by grinding. These observations, however, merely emphasize the stability of the organelle containing the complex in partially resisting disruption by freezing and thawing.

Finally, the stability of the complex was further confirmed by means of Biogel A-5m column chromatography of fraction 14.5KP. The results of such experiments confirmed (a) that the complex is sufficiently stable to be eluted in a symmetrical peak; (b) that the elution pattern of the complex during Biogel A-5m chromatography is consistent with a particle whose molecular weight is above 5 million; (c) that although the component enzymes undergo partial desorption (presumably from the membrane-matrix of the complex-organelle) in isotonic saline, removal of the salt by dialysis tends to bring about enzyme reaggregation or possibly readsorption onto the organelle matrix; and (d) that the failure of the complex to penetrate the separating

gel during acrylamide gel electrophoresis supports the conclusion drawn from the experiments on the Biogel column chromatography of fraction 14.5KP.

Taken together, the evidence appears incontrovertible in proving that the multienzyme complex isolated from the long slender form T. brucei is located in an organelle with a matrix which is sufficiently firm to provide strong anchorage for strong binding and possibly trapping of the individual enzymes which make up the multienzyme complex. Since, among the organelles which have been identified with any certainty in these organisms, only the microbodies fit this description, it is concluded that the intracellular location of the multienzyme complex is probably in the microbodies. On the other hand, it appears from this work that the L-glycerol-3-phosphate oxidase is located in the pro-mitochondrion, and the earlier suggestions that this enzyme was located in the microbodies can probably be discounted.

The latency of the component enzymes of the complex suggests that, in common with similar organelles, the T. brucei microbody may be impermable to NAD^+ (Müller, 1975). Opperdoes et al. (1977b) have suggested that the T. brucei microbodies must contain an enzyme capable of reducing NAD^+, and they consider glyceraldehyde phosphate dehydrogenase as the possible candidate. The present work shows clearly that the T. brucei microbodies contain at least six enzymes, and the possibility that both glyceraldehyde-phosphate dehydrogenase and glycerokinase are also associated with this organelle has already been mentioned. Though the distribution of glyceraldehyde-phosphate dehydrogenase in subcellular fractions of homogenates obtained by grinding T. brucei cells suggests concentration of this enzyme in the soluble fraction, it should be pointed out that between 20 and 40% of the activity was routinely found to be associated with fractions 14.5KP and 105KP. Further, since particulate

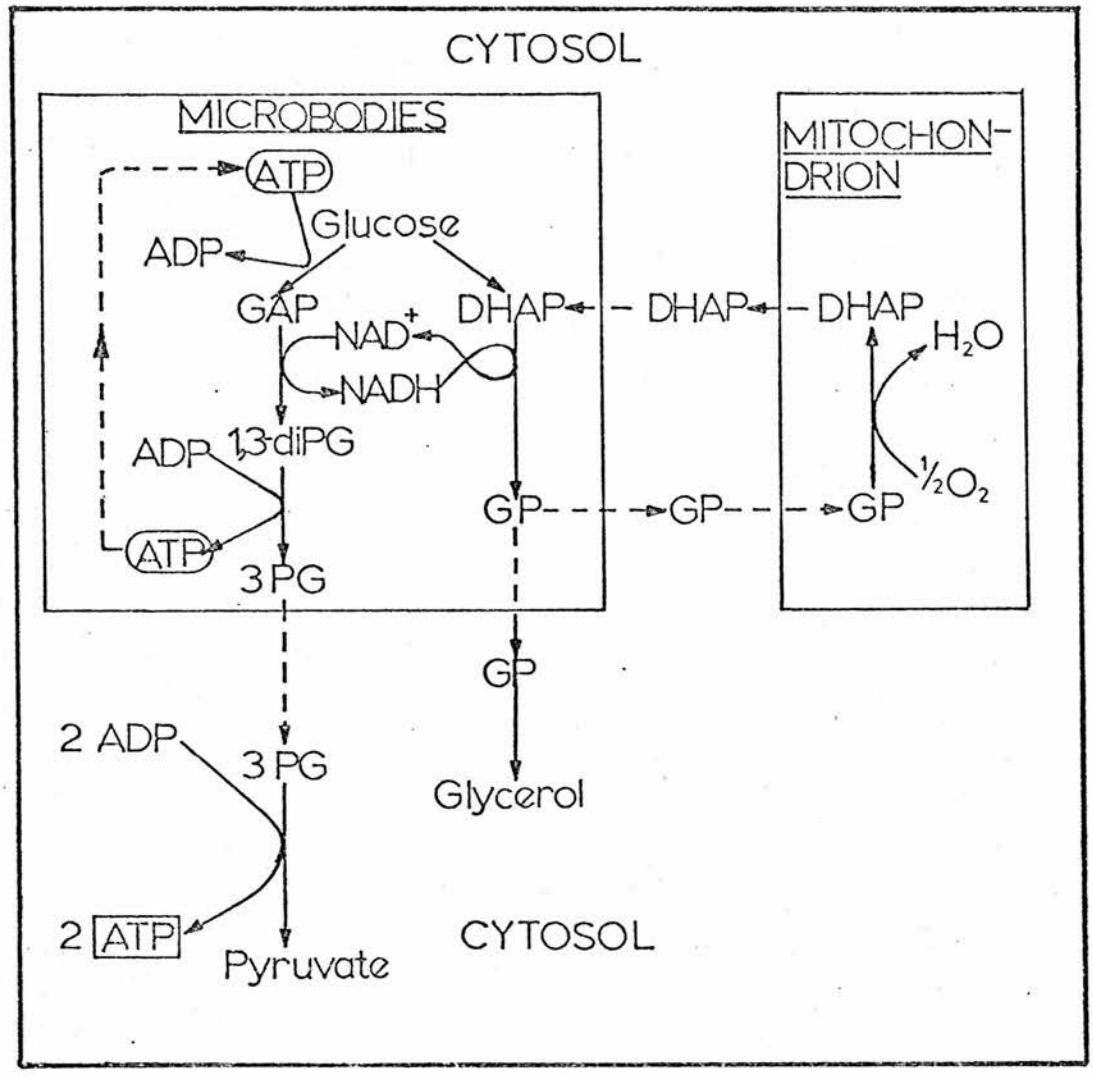


Fig. VII. 3. Diagramatic Representation of the Probable Intracellular Location of the Enzymes which Catalyze Glucose Catabolism in Blood-stream Long Slender Form T. brucei.

activity of up to 60% (saponin), 90% (Triton X-100), 50% (freezing and thawing) and 20% (digitonin) was obtained with the other methods of cell disruption, it is apparent that the distribution pattern of this enzyme is critically dependent on the isolation technique. Further work geared to the discovery of optimum conditions for isolating this enzyme is likely to confirm its particulate nature and possible association with the multienzyme complex.

The diagram presented in Figure VII.3. summarises the probable intracellular location of the enzymes involved in glucose catabolism in bloodstream long slender form T. brucei as follows.

1. The following enzymes, hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, phosphoglycerate kinase, glycerolphosphate dehydrogenase and most probably glyceraldehyde-phosphate dehydrogenase as well as glycerokinase, are located in the microbodies in the form of a tightly-linked multienzyme complex system. Consequently, the chain of reactions starting with glucose and ending in the production of 3-phosphoglycerate and glycerolphosphate takes place exclusively in these organelles.
2. That 3-phosphoglycerate and glycerolphosphate subsequently diffuse out of the organelles into the cytosol.
3. From the cytosol, glycerolphosphate may enter the pro-mitochondrion where it is oxidised to dihydroxyacetone phosphate by the action of the mitochondrial L-glycerol-3-phosphate oxidase. The dihydroxyacetone phosphate thus formed, then diffuses out of the mitochondrion and enters the microbodies to act as the catalyst for reoxidizing the NADH formed by the action of glyceraldehyde-phosphate dehydrogenase back to NAD^+.

Glycerolphosphate may also be converted to glycerol by the T. brucei phosphatase described by Harvey (1949).

If the above description is correct, it would mean that in the bloodstream long slender T. brucei, the following important steps in the glycolytic sequence of reactions take place exclusively in the microbodies:

(a) the two energy-consuming reactions of glucose to glucose-6-phosphate, and fructose-6-phosphate to fructose-1, 6-diphosphate;

(b) one of the energy-yielding steps, namely the conversion of 1,3-diphosphoglycerate and ADP to 3-phosphoglycerate and ATP; and

(c) the reoxidation of the NADH which is formed during the conversion of glyceraldehyde-3-phosphate to 1,3-diphospho-glycerate.

It would therefore appear that energetically, not only is the glycolytic system operating in the microbodies self-sustaining (given adequate supplies of glucose and catalytic amounts of ATP and NADH), but also it makes available to the rest of the cell 3-phosphoglycerate which can be converted to the 'high energy' compound, phosphoenolpyruvate; the latter compound may then be used directly to synthesize ATP which thus becomes available for other essential reactions in the cell such as protein synthesis, excretion, electrolyte balance and cell motility.

Because the system involves a closely-linked sequence of reactions, it offers the metabolic advantage of ensuring that the product of one enzyme in the sequence is directly available to the next enzyme for which it serves as the substrate. This

would ensure improved efficiency of the overall metabolic activity resulting from the maintenance of high concentration of substrates within the microenvironment of the enzymes. Also free diffusion of metabolites out of the organelle is reduced by the limiting membrane; this will reduce the risk of competition by enzymes operating in other pathways for the same substrates and, in this way, help to increase the efficiency of the system. This apparent efficiency of the system probably accounts for the unusually high rate of glucose oxidation observed in the bloodstream long slender form T. brucei.

Pertinent to this view of T. brucei microbodies being the centre for reactions leading to glucose breakdown and synthesis of ATP is de Duve's theory (1969) of a hypothetical ancestral peroxisome (or in this case, microbody) with the properties of "a functional unit of quite respectable importance and scope, involved in the metabolism of carbohydrates, fats, amino acids and purines; and using many of the (more) important coenzymes and prosthetic groups: NAD, NADP, coenzyme A, pyridoxal phosphate, flavins, haems, and others."
De Duve pointed out that although evidence that the peroxisomes could oxidise internally generated NADH or NADPH was lacking at the time, this could not rule out the possibility that other peroxisomes with the ability to oxidise reduced coenzymes might yet be uncovered. He went on to suggest the possibility that peroxisomes may participate in the oxidation of cytosol coenzymes through appropriate electron shuttles. For instance, in the liver or kidney, L-lactate formed in the cytosol by lactate

dehydrogenase could diffuse into the peroxisomes, there to be oxidised by the L-α-hydroxy acid oxidase. In turn, the pyruvate could diffuse out of the particles, and pick up another pair of electrons from the cytosol NADH.

The scheme in Figure VII.3. presents direct evidence for microbody oxidation of internally generated NADH which makes use of electron shuttles through glycerolphosphate and DHAP and which involves the microbodies, the cytosol and the pro-mitochondrion of T. brucei.

Another aspect of the possible importance of peroxisomes in the metabolism of the cell considered by de Duve is the possible threat that the progressive appearance of oxygen in the atmosphere must have posed to the early anaerobic organisms by causing the production of H_2O_2 which is highly toxic to some enzyme systems. It was, apparently because of the need to adapt to the rising concentration of oxygen in the atmosphere, that the cells developed the peroxidase and catalase reactions found in the peroxisomes. It is interesting, in this respect, to note that in T. brucei the oxidase responsible for the removal of the glycolytic electron equivalents is morphologically separated from the peroxisomes and delegated instead to the pro-mitochondrial oxidase complex.

The results recorded in this thesis partly confirm the forecast made by de Duve that a survey of microorganisms devoid of (functional) mitochondria may disclose the existence of a eukaryotic organism truly devoid of such organelles, but possessing peroxisomes showing evidence of the probable functions

of the ancestral peroxisomal particles.

The work also clearly confirms the frequent reports in the literature that methods adopted for cell disruption may affect gross enzyme activities. Differential centrifugation of the homogenates obtained by grinding bloodstream long slender form T. brucei results in glycolytic particles each with its advantages and disadvantages with respect to the degree of recovery of enzyme activities as well as the number of enzymic reactions that the isolated particles are capable of catalysing. It is suggested that if adequate assay methods can be devised, it should be possible to employ these particles as models for testing such reaction schemes as the glycerolphosphate/dihydroxyacetone-phosphate and ATP/ADP shuttles, as well as the screening of compounds for their possible inhibition of specific steps in the glycolytic sequence of reactions in T. brucei as described below for suramin in Section VII.3.2.

Since there appears to be no previous report of an association of a multienzyme complex of the glycolytic pathway with an organelle, the finding that intact microbodies obtained from T. brucei cells that have been disrupted by grinding with alumina or silicon carbide contain such an enzyme complex represents the first report of the location in a subcellular organelle of at least six (and most probably eight) enzymes of the glycolytic pathway[††]. Because it is capable of catalysing the major part of the glycolytic pathway, the particle may appropriately be called a "Glycolysome".

Finally, it should be stressed that although the scheme for relative enzyme activities presented in Figure VII.1. shows

variations according to the method of cell lysis, it nevertheless offers information (in diagramatic form) on the rate limiting steps which could also be exploited for chemotherapeutic studies on these organisms.

†† At the final proof stage of this thesis it was learnt (Fairlamb, personal communication) that Opperdoes et al. (unpublished) have observed essentially identical results in that they find T. brucei hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, glyceraldehyde-phosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, glycerolphosphate dehydrogenase and glycerokinase are all located in the microbodies.

VII.3.2. Suramin Inhibition of the Component Enzymes of the Multienzyme Complex

Only two enzymes of the multienzyme complex, phosphofructose kinase and glycerolphosphate dehydrogenase, are inhibited by suramin. The observation that suramin inhibition of the NAD^+-linked glycerolphosphate dehydrogenase is not affected by Triton X-100 treatment of fraction 14.5KP suggests that, unlike the substrates, suramin diffusion to the enzyme is not hindered by the limiting membrane of the microbodies. The inhibition of both the NAD^+-linked glycerolphosphate dehydrogenase and the glycerolphosphate oxidase complex is non-competitive, but the drug is two times more potent against the former enzyme. Suramin also inhibits phosphofructose kinase, but in this case, the inhibition is five times less potent than it is for the oxidase and it is also uncompetitive. It would be interesting to find out if any of the five enzymes, glyceraldehyde-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase or pyruvate kinase is inhibited by suramin. If none of them is, this would mean that the pathway from glucose to glycerolphosphate is at least ten times more sensitive to suramin inhibition than that from glucose to pyruvate. Since the pathway catalysed by the NAD^+-linked dehydrogenase and the oxidase is vital for the survival of the parasite but not the host erythrocyte or liver tissues, this would be a sufficient explanation for the observed chemotherapeutic effect of suramin in trypanosome infections.

It has been suggested that suramin uptake by the parasite is through pinocytotic ingestion of the drug bound to plasma proteins, followed by fusion of the phagosomes thus formed with lysosomes and release of the suramin through proteolytic breakdown of the carrier protein by lysosomal proteases. It would appear from the results reported here and those of Fairlamb & Bowman (1977) that once inside the parasite the drug diffuses freely into both the T. brucei pro-mitochondrion and the microbodies. It would be interesting, through both in vitro and in vivo studies, to find out whether once inside, the drug can easily diffuse out of these two organelles.

The advantages of employing a multienzyme complex, which catalyses a known sequence of reactions, as a system for screening compounds for their possible therapeutic value is illustrated by the observation that both suramin and trypan blue can inhibit the multienzyme activity (Section IV.4.). If such a test proves negative, it could be safely assumed that in vivo the compound would not inhibit any of the component enzymes of the complex. On the other hand, if the complex activity is inhibited, further tests could be performed to show the identity of the 'target' enzyme, and this could then be followed by in vivo studies to show whether the inhibition reflects potency of the compound against infection.

SUMMARY

The method adopted for cell lysis critically affects the differential centrifugation distribution pattern of the enzymes involved in glucose catabolism in bloodstream long slender form T. brucei.

By the use of mild methods of cell disruption, it is possible to isolate a multienzyme complex of T. brucei which catalyses the conversion of glucose to glycerolphosphate. The enzymic activity of this complex (which is probably located in the microbodies), is inhibited by the trypanocidal compound, suramin; so also are the activities of two of the six component enzymes of the multienzyme complex, namely phosphofructose kinase and the NAD^+-linked glycerolphosphate dehydrogenase.

The K_i value of 2 μM reported here for suramin inhibition of the dehydrogenase, makes this the most sensitive enzyme to suramin inhibition so far described for bloodstream long slender form T. brucei.

APPENDIX

The details and principles of the assay procedures were as follows:

1. Hexokinase (ATP:D-hexose-6-phosphate, EC 2.7.1.1)

Assay Principle:

(i) $\text{Glucose} + \text{ATP} \xrightleftharpoons[Mg^{2+}]{HK} \text{Glucose-6-phosphate} + \text{ADP}$

(ii) $\text{Glucose-6-phosphate} + NADP^{+} \xrightleftharpoons[Mg^{2+}]{(G6PDH)}$ 6-phospho-gluconate + NADPH + H^{+}

Reaction Mixture:	Final Concn.
TEA/KOH buffer pH 7.6	36.4 mM
ATP	1.0 mM
$MgCl_2$	7.3 mM
$NADP^{+}$	1.0 mM
Glucose-6-phosphate dehydrogenase	1.3 U
The reaction was started by the addition of glucose,	404.0 mM

2. Phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)

Assay Principle:

(i) $\text{Fructose-6-phosphate} \xrightleftharpoons{PGI} \text{Glucose-6-phosphate}$

(ii) $\text{Glucose-6-phosphate} + NADP^{+} \xrightleftharpoons[Mg^{2+}]{(G6PDH)}$ 6-phospho-gluconate + NADPH + H^{+}

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	96.2 mM
$MgCl_2$	3.8 mM
$NADP^{+}$	0.5 mM
Glucose-6-phosphate dehydrogenase	0.6 U
The reaction was started by the addition of fructose-6-phosphate,	3.5 mM

3. Phosphofructose kinase (ATP:D-Fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11)

Assay Principle:

(i) Fructose-6-phosphate + ATP $\underset{Mg^{2+}}{\overset{PFK}{\rightleftharpoons}}$ Fructose-1,6-diphosphate + ADP

(ii) Fructose-1,6-diphosphate $\overset{(ALD)}{\rightleftharpoons}$ Glyceraldehyde-3-phosphate + Dihydroxyacetone-phosphate

(iii) Glyceraldehyde-3-phosphate $\overset{(TIM)}{\rightleftharpoons}$ Dihydroxyacetone-phosphate

(iv) Dihydroxyacetone-phosphate + NADH + H^+ $\overset{(GPDH)}{\rightleftharpoons}$ Glycerol-phosphate + NAD^+

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	79.4 mM
Iodoacetic acid	0.1-0.3 mM
ATP	1.0 mM
$MgSO_4$	15.1 mM
KCl	60.5 mM
NADH	0.15 mM
Aldolase	0.2 U
Triosephosphate isomerase	19.0 U
Glycerolphosphate dehydrogenase	0.8 U
The reaction was started by the addition of fructose-6-phosphate,	3.2 mM

4. Aldolase (D-Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13)

Assay Principle:

(i) Fructose-1,6-diphosphate $\overset{ALD}{\rightleftharpoons}$ Glyceraldehyde-3-phosphate + Dihydroxyacetone-phosphate

(ii) Glyceraldehyde-3-phosphate $\overset{(TIM)}{\rightleftharpoons}$ Dihydroxyacetone-phosphate

(iii) Dihydroxyacetone-phosphate + NADH + $H^+ \overset{(GPDH)}{\rightleftharpoons}$ Glycerol-phosphate + NAD^+

Reaction Mixture	Final Concn.
TEA/KOH buffer, pH 7.6	83.2 mM
Iodoacetic acid	0.3 mM
NADH	0.16 mM
Triosephosphate isomerase	18.9 U
Glycerolphosphate dehydrogenase	0.8 U
The reaction was started by the addition of fructose-1,6-diphosphate	3.7 mM

5. Glyceraldehyde-3-phosphate dehydrogenase (D-Glyceraldehyde-3-phosphate:NAD oxidoreductase, EC 1.2.1.12)

Assay Principle:

(i) 3-Phosphoglycerate + ATP $\underset{Mg^{2+}}{\overset{(PGK)}{\rightleftharpoons}}$ 1,3-Diphosphoglycerate + ADP

(ii) 1,3-Diphosphoglycerate + NADH + $H^+ \overset{GAPDH}{\rightleftharpoons}$ 3-Phospho-glyceraldehyde + NAD^+ + P_i

Reaction Mixture:	Final Concn.
TEA/KOH	74.0 mM
3-Phosphoglycerate	4.2 mM
ATP	3.7 mM
EDTA	1.1 mM
$MgSO_4$	1.7 mM
NADH	0.2 mM
Phosphoglycerate kinase added to generate 1,3-diphosphoglycerate	21.6 U
The reaction was started by the addition of the extract	

6. 3-Phosphoglycerate kinase (ATP:3-Phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3)

Assay Principle:

(i) 3-Phosphoglycerate + ATP $\underset{Mg^{2+}}{\overset{PGK}{\rightleftharpoons}}$ 1,3-Diphosphoglycerate + ADP

(ii) 1,3-Diphosphoglycerate + NADH + H^+ $\overset{(GAPDH)}{\rightleftharpoons}$ Glyceraldehyde-3-phosphate + NAD + P_i

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	68.5 mM
3-phosphoglycerate	4.5 mM
EDTA	0.9 mM
$MgSO_4$	1.4 mM
NADH	0.2 mM
Glyceraldehyde-3-phosphate dehydrogenase	0.7 U
The reaction was started by the addition of ATP	3.0 mM

7. Phosphoglycerate mutase (2,3-Bisphospho-D-glycerate: 2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3)

Assay Principle:

(i) 3-Phosphoglycerate $\underset{2,3\text{-diphosphoglycerate}}{\overset{PGlyM}{\rightleftharpoons}}$ 2-Phosphoglycerate

(ii) 2-Phosphoglycerate $\overset{(ENOL)}{\rightleftharpoons}$ Phosphoenolpyruvate + H_2O

(iii) Phosphoenolpyruvate + ADP $\underset{Mg^{2+},\ K^+}{\overset{(PK)}{\rightleftharpoons}}$ Pyruvate + ATP

(iv) Pyruvate + NADH + H^+ $\overset{(LDH)}{\rightleftharpoons}$ Lactate + NAD^+

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	80 mM
2,3-Diphosphoglycerate (catalyst)	0.04 mM
ADP	2.9 mM
$MgSO_4$	1.0 mM

KCl	19.0 mM
NADH	0.14 mM
Enolase	4 U
Pyruvate kinase	4 U
Lactate dehydrogenase	20 U
The reaction was initiated by the addition of 3-phosphoglycerate	6 mM

8. Enolase (2-Phospho-D-glycerate hydro-lyase, EC 4.2.1.11)

Assay Principle:

(i) 2-Phosphoglycerate $\overset{ENOL}{\rightleftharpoons}$ Phosphoenolpyruvate + H_2O

(ii) Phosphoenolpyruvate + ADP $\underset{Mg^{2+},K^{+}}{\overset{(PK)}{\rightleftharpoons}}$ Pyruvate + ATP

(iii) Pyruvate + NADH + H^{+} $\overset{(LDH)}{\rightleftharpoons}$ Lactate + NAD^{+}

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	83.0 mM
2-Phosphoglycerate	0.94 mM
ADP	1.7 mM
$MgSO_4$	33.0 mM
KCl	133.0 mM
NADH	0.15 mM
Pyruvate kinase	3.2 U
Lactate dehydrogenase	15.0 U
The reaction was started by the addition of the extract	

9. Pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40)

Assay Principle:

(i) $\text{Phosphoenolpyruvate} + \text{ADP} \overset{\text{PK}}{\underset{Mg^{2+},K^{+}}{\rightleftharpoons}} \text{Pyruvate} + \text{ATP}$

(ii) $\text{Pyruvate} + \text{NADH} + H^{+} \overset{\text{(LDH)}}{\rightleftharpoons} \text{Lactate} + NAD^{+}$

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	76.1 mM
Phosphoenolpyruvate	0.84 mM
ADP	2.9 mM
$MgSO_4$	5.8 mM
KCl	23.1 mM
NADH	0.16 mM
Lactate dehydrogenase	18.2 U

The reaction was initiated by the addition of the extract

10. Glycerol-3-phosphate dehydrogenase (sn-Glycerol-3-phosphate dehydrogenase, EC 1.1.1.8)

Assay Principle:

(i) Fructose-1,6-diphosphate $\overset{\text{(ALD)}}{\rightleftharpoons}$ Glyceraldehyde-3-phosphate + Dihydroxyacetone-phosphate

(ii) Glyceraldehyde-3-phosphate $\overset{\text{(TIM)}}{\rightleftharpoons}$ Dihydroxyacetone-phosphate

(iii) Dihydroxyacetone-phosphate + NADH + $H^{+} \overset{\text{GPDH}}{\rightleftharpoons}$ Glycerol-3-phosphate + NAD^{+}

Reaction Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	83.2 mM
Fructose-1,6-diphosphate	3.7 mM
NADH	0.15 mM

Aldolase	0.2 U
Triosephosphate isomerase	18.7 U
Iodoacetic acid	0.1 mM

The above mixture was incubated for 5 min to allow the complete conversion of fructose-1,6-diphosphate to triosephosphates before initiating the reaction by the addition of the extract

11. Glycerokinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30)

Assay Principle:

(i) Glycerol + ATP $\overset{GK}{\rightleftharpoons}$ Glycerol-3-phosphate + ADP

(ii) Phosphoenolpyruvate + ADP $\overset{(PK)}{\underset{Mg^{2+},\ K^{+}}{\rightleftharpoons}}$ Pyruvate + ATP

(iii) Pyruvate + NADH + H^{+} $\overset{(LDH)}{\rightleftharpoons}$ Lactate + NAD^{+}

Assay Mixture:	Final Concn.
TEA/KOH buffer, pH 7.6	90 mM
Phosphoenolpyruvate	3.4 mM
ATP	1.3 mM
$MgSO_4$	3.1 mM
KCl	12.5 mM
NADH	0.26 mM
Pyruvate kinase	1.8 U
Lactate dehydrogenase	3.6 U

The reaction was initiated by the addition of glycerol 6.3 mM

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