ANAEROBIC CARBOHYDRATE METABOLISM BY TRYPANOSOMA BRUCEI BRUCEI

by

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I hereby declare that this Thesis has been composed by myself and has not been submitted in any previous application for a Degree. The work of which this is a record has been done by myself and all sources of information have been specifically acknowledged by means of references.

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ABSTRACT OF THESIS

The pathway of anaerobic glycolysis of Trypanosoma brucei has been studied by the following five approaches :

1. Enzyme activity required for the various different postulated schemes for anaerobic glycolysis (hexose monophosphate aldolase, glycerol dehydrogenase, glycerophosphate : glucose or glycerophosphate : fructose-6-phosphate or glycerophosphate : triose or glycerophosphate : ADP transphosphorylases) have been examined in a variety of assay conditions. Only significant activities of glycerophosphate : ADP transphosphorylase were detectable.
2. Broken cell incubation studies established that there were no significant differences in the sequence of the increase and decrease in glycolytic intermediates between aerobic and anaerobic pathways.
3. Whole cell adenylate charge and glycolytic intermediates were assayed in steady-state aerobic, anaerobic and in the transitions between aerobic to anaerobic, and anaerobic to aerobic showed glucose-6-phosphate production to be the rate limiting step in anaerobic glucose utilization. Salicylhydroxamic acid (0.5 mM) inhibited only glycerophosphate oxidase and so simulated anaerobiosis.
4. The inhibitory effect of glycerol on whole cells metabolising glucose anaerobically showed it to be dependent upon the intracellular concentration of glycerophosphate. Consequently its inhibitory effect is not caused through the inhibition of glucose transport.
5. The concentration of glycerophosphate in cells metabolising glucose under glycerophosphate oxidase inhibited conditions was found to increase rapidly to a concentration that was independent of time and extracellular glycerol concentration. Furthermore it was found to be an intermediate in anaerobic glucose utilization.

The results of this work were consistent with the pathway of carbohydrate metabolism under glycerophosphate oxidase inhibition which involved the production of glycerol plus ATP from ADP plus glycerophosphate catalysed by glycerokinase.

Glycerokinase is rapidly inactivated due to oxidation of a thiol group but may be stabilised by 2 mM-dithiothreitol, bovine serum albumin plus 0.2 mM-EDTA. It is a particulate enzyme associated in a multi-enzyme complex or microbody. A 17 fold purification of glycerokinase has been achieved through firstly preparing it in a post-nuclear fraction which sediments at 14000 g max. and then solubilising with 0.5% (w/v) triton X-100. The Michaelis constants for the solubilised, purified glycerokinase have been found to be Km^{ADP} = 0.12 ± 0.04 mM, Km^{GP} = 5.12 ± 1.47 mM, Km^{ATP} = 0.19 ± 0.04 mM, $Km^{glycerol}$ = 0.12 ± 0.05 mM, but the particulate enzyme shows a Km^{ADP} = 6.67 ± 3.27 mM. This difference probably reflects a conformational change of the enzyme during solubilisation.

ABBREVIATIONS

BSA	Bovine serum albumin
DHAP	Dihydroxyacetone phosphate
1,3-diPGA	1,3-Dihydroxyacetone phosphate
EDTA	Ethylenediaminetetra-acetic acid
F-D-P	D-Fructose-1,6-diphosphate
F-1-P	Fructose-1-phosphate
F-6-P	Fructose-6-phosphate
GA-3-P	D-glyceraldehyde-3-phosphate
G-1-P	D-glucose-1-phosphate
G-6-P	D-glucose-6-phosphate
GP	L-glycerol-3-phosphate
DL-GP	D,L-glycerol-3-phosphate
ISS	Intermediate short stumpy
LS	Long slender
PCV	Packed cell volume
PEP	Phosphoenol pyruvate
PGA	Phosphoglyceric acid
Pi	Phosphate
SHAM	Salicylhydroxamic acid
SS	Short stumpy

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SECTION 1

GENERAL INTRODUCTION

1.1 The Taxonomy of Trypanosomes

There are two divisions of parasitic flagellate protozoa within the family Trypanosomatidae, the monogenetic species that have one host, generally an arthropod in their life cycle and the digenetic species that have two, the second host being a vertebrate (Newton et al., 1973). The digenetic species may have evolved from monogenetic parasites resulting from the adoption of a vertebrate blood diet by some invertebrates (Baker, 1963; Hoare, 1972). Most digenetic species enter the vertebrate by contamination with or ingestion of infected faeces or intestinal contents of the invertebrate host, however the salivarian group of this genus, to which the Trypanozoon subgenus belongs, are injected into the vertebrate through the proboscis of the feeding insect vector (Hoare, 1966). The diversity of host produces very different environments not only between different species of digenetic Trypanosoma but also during the life cycle of a particular species. Thus this genus has undergone considerable biochemical adaptation.

1.2 Life Cycle and Biochemical Adaptation

Lines of the Trypanozoon subgenus that are infective to the insect vector show a wide and continuous variation in form, or pleomorphism, ranging from long slender (LS) flagellates with a free flagellum, through intermediate forms (ISS) to short stumpy forms (SS) with no free flagellum (Hoare, 1970; 1972). The transformation of the dividing LS to non-dividing SS forms is accompanied by the development of the single mitochondrion to contain an increased number of internal mitochondrial cristae and the acquisition of mitochondrial NADH tetrazolium reductase activity (Vickerman, 1965). Both LS and SS flagellates,

or trypomastigotes, possess cyanide insensitive respiration, mediated by a mitochondrial (Opperdoes et al., 1977) cytochrome independent L-glycerol-3-phosphate oxidase (Grant and Sargent, 1960). Monomorphic strains of the Trypanozoon subgenus are morphologically identical to LS trypomastigotes, but have lost the ability to develop ISS forms.

Upon ingestion of the bloodstream forms by the insect vector, the tsetse fly (Glossina spp) in the case of Trypanosoma Trypanozoon brucei brucei (hereafter T. brucei), the LS and ISS flagellates may form SS flagellates in the midgut (Wijers and Willett, 1960). SS trypomastigotes undergo further morphological and biochemical change to produce the insect midgut or procyclic trypomastigote form which is the form that develops in in vitro culture. This has frequently been called the culture form, but with the developments of Hirumi et al. (1977) in culturing LS trypomastigotes this term should be rejected to avoid ambiguity. The procyclic form is elongated with a free flagellum, but contrasting with the LS form the kinetoplast migrates to lie posterior to the nucleus and the mitochondrion becomes greatly branched with prominent cristae. Terminal oxidation is partially cyanide sensitive, cytochrome mediated.

The procyclic trypomastigote form migrates to the salivary gland of the tsetse fly where transformation via an epimastigote form to a metacyclic trypomastigote form occurs. Regression of the mitochondrion occurs at this stage and it is this metacyclic trypomastigote form that infects the vertebrate when the insect bites a susceptible host, thus completing the cycle.

Just as the SS trypomastigotes show a degree of adaptation to life in the invertebrate through mitochondrial development, mitochondrial regression at the

metacyclic trypomastigote form is a pre-adaptation to vertebrate bloodstream life. Trypanosomes cannot synthesise haem for cytochrome biosynthesis and free blood haem concentrations are very low (Putman, 1975) so consequently a cytochrome independent method of respiration will be advantagous in bloodstream trypomastigotes.

1.3 Carbohydrate Metabolism

This review of carbohydrate metabolism will be largely confined to Trypanosoma brucei brucei, the organism studied in this thesis and the closely related and morphologically identical Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense.

LS trypomastigotes of the African trypanosomes have an absolute dependence on a source of exogenous carbohydrate (Ryley, 1956). Glucose, mannose, fructose and also glycerol will support respiration and motility of monomorphic forms of T. rhodesiense (Ryley, 1962), while in addition α-oxoglutarate will support respiration (Flynn and Bowman, 1973) and motility of SS forms of T. rhodesiense (Vickerman, 1965) and T. brucei (this work).

In all bloodstream forms the oxidation of carbohydrate is incomplete; the LS trypomastigotes metabolise glucose aerobically to pyruvic acid (Ryley, 1956; Grant and Fulton, 1957), the only other end product reported being glycerol, but this appears only if oxygen becomes limiting (Clarkson and Brohn, 1976). No carbon dioxide is produced and even when all tricarboxylic acid cycle enzymes are present the cycle operates at insignificant rates, if at all in vivo (Flynn and Bowman, 1973).

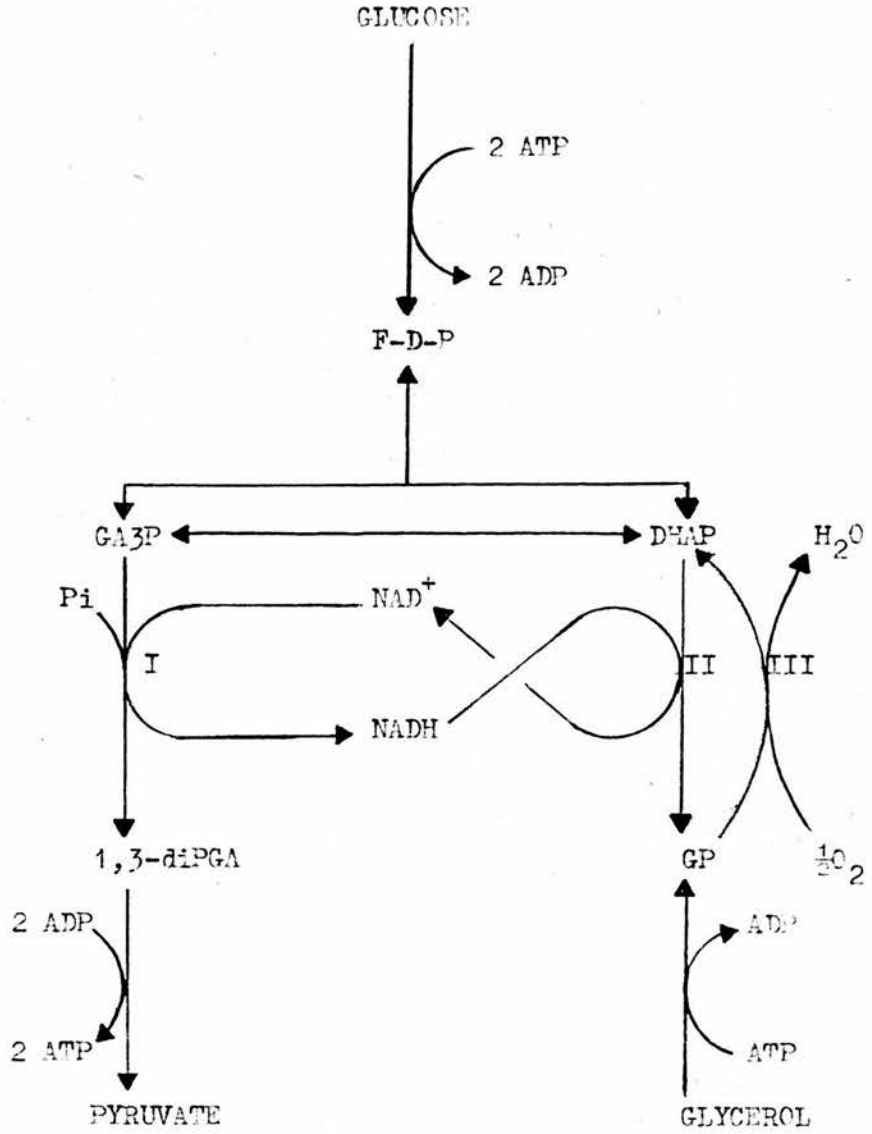


Fig. 1.1 : The glycolytic pathway in LS forms of T. brucei (showing the reoxidation of NADH generated at the glyceraldehyde-3-phosphate dehydrogenase step in glycolysis, I, by the glycerol-3-phosphate dehydrogenase, II, and GP oxidase, III, enzyme systems).

The NAD^+ reduced in the glyceraldehyde-3-phosphate dehydrogenase step of glycolysis is reoxidised aerobically by the L-glycerol-3-phosphate oxidase (GP oxidase) system, see Fig. 1.1. Dihydroxyacetone phosphate (DHAP) is reduced to L-glycerol-3-phosphate (GP) with concomitant oxidation of NADH. GP is then reoxidised to DHAP with reduction of $\frac{1}{2}O_2$ to H_2O. Under this scheme glucose is metabolised to pyruvate with a net yield of 2 moles of ATP per mole of glucose utilized, while glycerol is metabolised to pyruvate with a net yield of equimolar ATP to glycerol utilized.

Under anaerobic conditions or when GP oxidase is specifically inhibited, glucose forms equimolar pyruvate and glycerol (Ryley, 1956; Grant and Fulton, 1957). Glycerol cannot be utilized under these conditions but becomes an inhibitor of glucose metabolism (Ryley, 1962). When GP oxidase is made inoperative, DHAP is thought to be the terminal electron acceptor, being reduced to GP (Fairlamb, 1975). Thus for each mole of triosephosphate metabolised to pyruvate one mole of GP must be formed in the reoxidation of NADH. The production of glycerol from GP was originally thought to be through the action of a phosphatase. Harvey (1949) and Gerzeli (1955) have identified GP phosphatase activity in trypanosomes however this activity cannot account for a net yield of ATP from anaerobic glucose metabolism (Bowman, 1974; Bowman and Flynn, 1976) yet there is much evidence to indicate that ATP is synthesised anaerobically (Fulton and Spooner, 1959; Clarkson and Brohn, 1976; Opperdoes et al., 1976b).

Glucose utilization by LS forms of T. rhodesiense under anaerobic conditions proceeds at approximately the same rate obtained aerobically over an interval of 30 - 90 minutes (Ryley, 1956). The T. brucei anaerobic rate of glucose utilization has been found to be 40% of the aerobic rate (Ryley, 1956), although

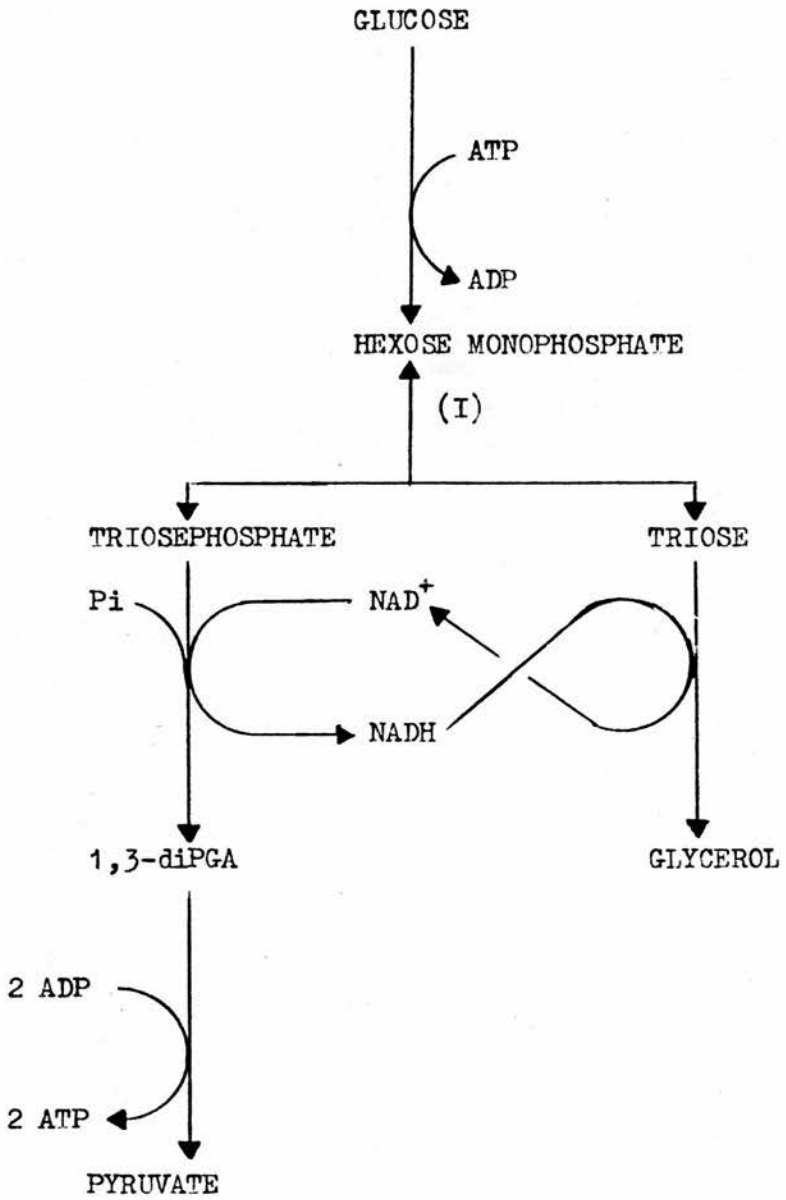


Fig. 1.2 : Postulated reaction sequence for anaerobic glycolysis in LS forms of T. brucei via hexose monophosphate aldolase, I, and glycerol dehydrogenase, II, catalysed reactions.

Brohn and Clarkson (1978) have shown this to be dependent upon the glycerol concentration which continually increases anaerobically being an end product of metabolism. Ryley (1962) found that the anaerobic glycolytic rate of LS forms of T. rhodesiense was markedly decreased when 5% carbon dioxide was absent from the nitrogen gas phase, yet carbon dioxide had no effect on the aerobic utilization of glucose or glycerol. At present these observations have not been satisfactorily explained, although several theoretical explanations have been proposed (Clarkson and Brohn, 1976; Opperdoes et al., 1976b; Opperdoes and Borst, 1977; Fairlamb et al., 1977).

1.4 Postulated Schemes of Anaerobic Glycolysis in LS forms of T. brucei

In order to account for the net synthesis of ATP by metabolism of glucose under anaerobic conditions Clarkson and Brohn (1976) and Opperdoes et al. (1976b) have both proposed that hexose monophosphate aldolase activity could decrease the ATP requirement to 1 mole per mole of glucose metabolised to yield equimolar triosephosphate and a triose. The triosephosphate could then yield two moles of ATP per mole metabolised to pyruvate with concomitant reduction of NAD^+. The NADH produced could then be reoxidised as the triose is reduced to glycerol, Fig. 1.2.

Some evidence exists for this proposed scheme of anaerobic glycolysis; Grant and Fulton (1957) have shown that C_1 of glucose is not found equally distributed between glycerol and pyruvate under anaerobic conditions but favours glycerol, the result expected if a hexose monophosphate is split rather than a hexose diphosphate, so that the 3-carbon products do not equilibrate. Furthermore Clarkson and Brohn (1976) have reported hexose monophosphate aldolase activity,

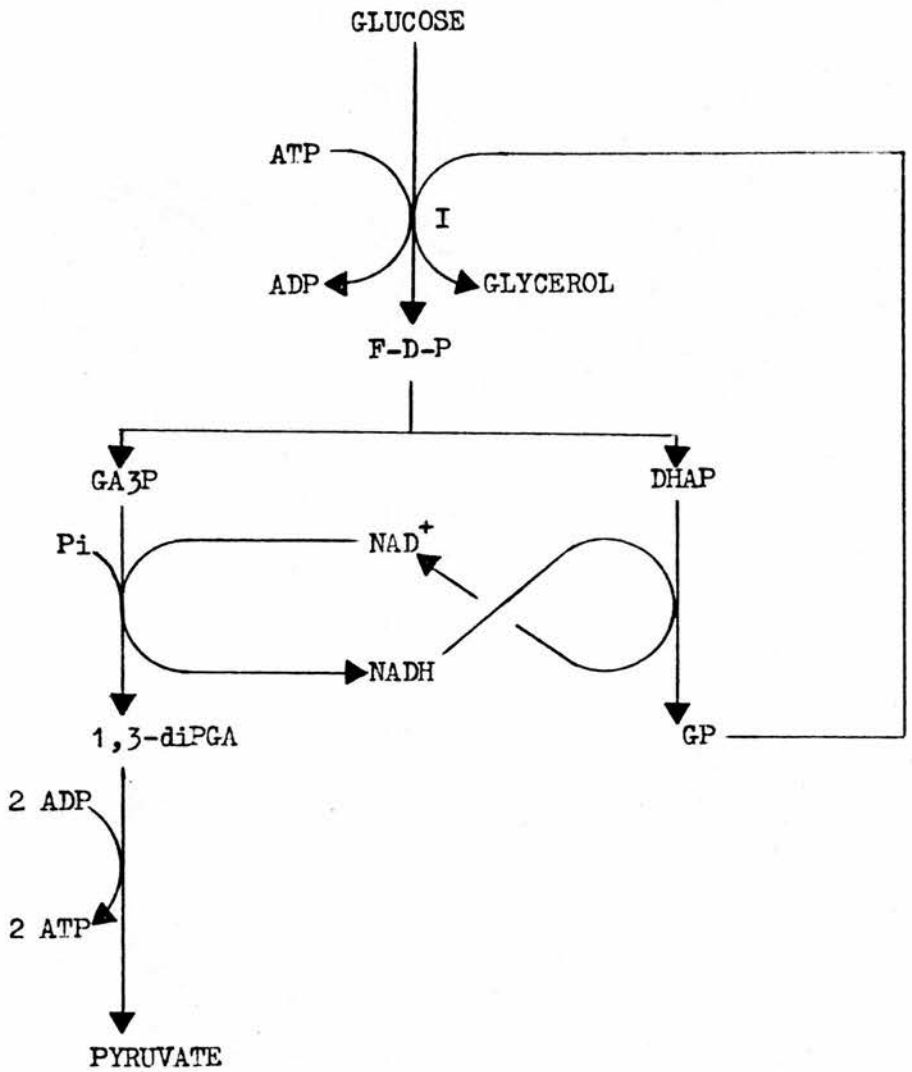


Fig. 1.3 : Postulated reaction sequence for anaerobic glycolysis in LS forms of T. brucei via a GP : hexose or hexose phosphate transphosphorylase, I, catalysed reaction.

although the specific activity or conditions have not been stated, but both Clarkson and Brohn (1976) and Opperdoes *et al.* (1976b) have failed to detect triose reductase activity which has previously been reported for *Trypanosoma hippicum* (Harvey, 1949).

It has also been postulated that GP could be used to transphosphorylate a hexose or hexosephosphate (Clarkson and Brohn, 1976; Opperdoes *et al.*, 1976b). This will decrease the requirement in forming 1 mole of fructose-1,6-diphosphate (F-D-P) from 1 mole of glucose to 1 mole of ATP plus 1 mole of GP. F-D-P would then be cleaved forming two moles of triosephosphate, one mole of which would form pyruvate with a gain of 2 moles of ATP while the other mole would maintain the redox balance and GP concentration via the glycerol-3-phosphate dehydrogenase catalysed reaction, Fig. 1.3. At present no transphosphorylase activity of the required specificity has been reported in the literature.

Another possibility proposed by Clarkson and Brohn (1976) is that a hexose monophosphate aldolase produces a triose and a triosephosphate. The triosephosphate could then be reduced to GP with concomitant oxidation of NADH, while the GP is used to transphosphorylate the triose. This would generate glycerol and one mole of the second triosephosphate would yield two moles of ATP and reduce NAD^+ in its metabolism to pyruvate, Fig. 1.4. Thus this postulate requires the presence of a hexose monophosphate aldolase and a GP : triose transphosphorylase; the latter at present is undetected.

A further possibility, proposed by Opperdoes and Borst (1977) suggests that if a locally high concentration of GP were found anaerobically this could lead

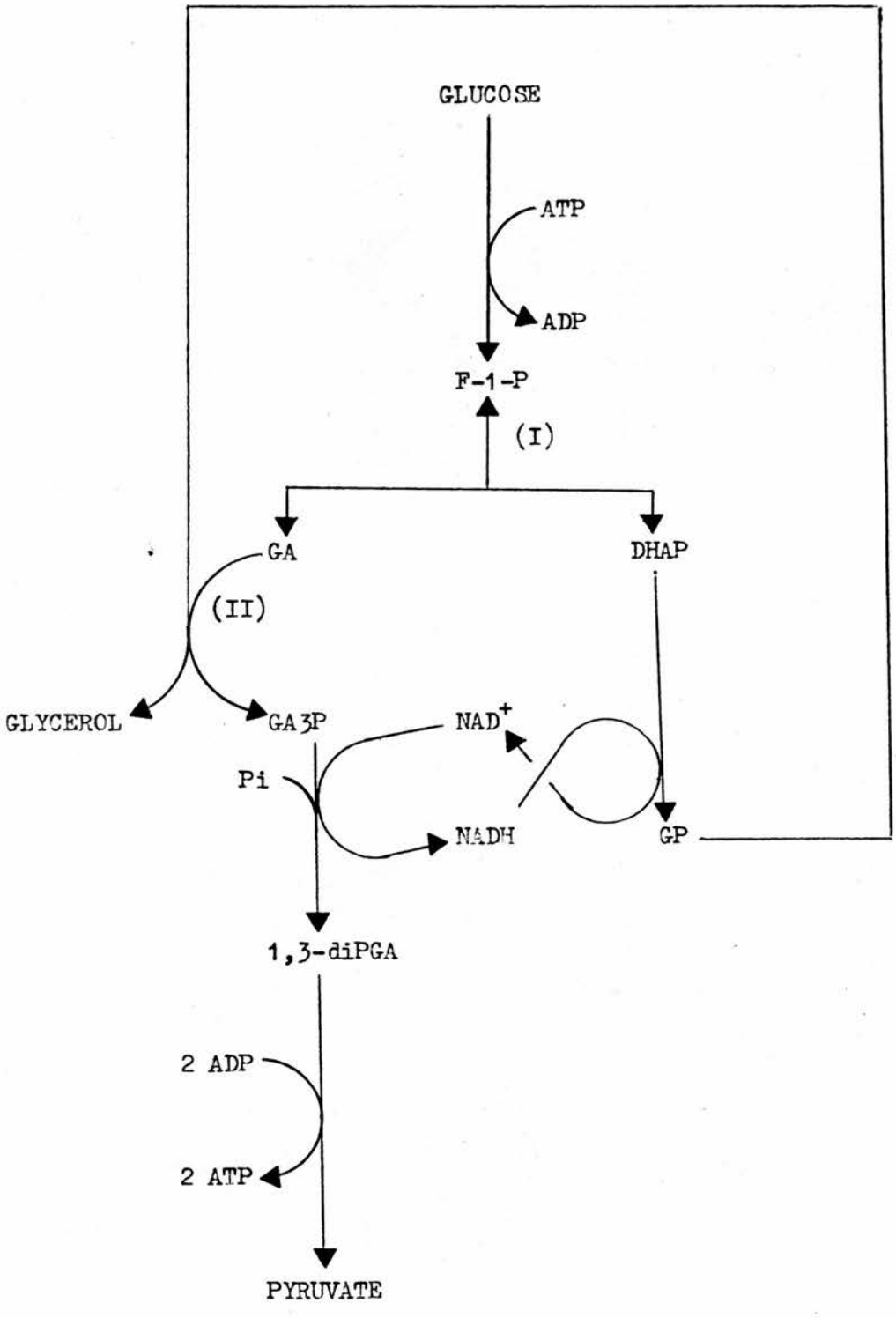


Fig. 1.4 : Postulated reaction sequence for anaerobic glycolysis in LS forms of T. brucei via hexose monophosphate aldolase, I, and GP : triose transphosphorylase, II, catalysed reactions.

to the formation of glycerol and ATP by the reversal of the glycerokinase catalysed reaction. This method would require no additional enzymes to those already found in T. brucei, but would require reversibility of the glycerokinase catalysed reaction to form ATP plus glycerol from GP plus ADP and compartmentation of enzymes and cofactors.

1.5 The Activities and Localization of Glycolytic Enzymes

Until relatively recently the enzymes involved in glycolysis were assumed to be soluble and unassociated proteins found in the cytosol of cells (Lehninger, 1951; Bucher and McGarraham, 1956). However this theory reflected limitations of the fractionation techniques and its standardization as well as incorrect interpretation of results (Roodyn, 1972) and many findings contradictory to the soluble glycolytic enzyme theory were viewed with suspicion (Brody and Bain, 1952; Aldridge, 1957; Tanaka and Abood, 1963; Mayer et al., 1966). However since the discovery by Hers et al. (1951) that the enzyme glucose-6-phosphatase is located in the microsomal fraction of liver homogenates, many workers have shown that various glycolytic enzymes are associated with subcellular entities in cells from a wide range of biological systems. Opperdoes et al. (1977), Oduro (1977) and Opperdoes and Borst (1977) have recently obtained much information on the localization of the glycolytic enzymes of T. brucei.

Hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, glycerol-3-phosphate dehydrogenase and phosphoglycerate kinase have been established to be localized within a particle (Oduro, 1977; Opperdoes and Borst, 1977). Opperdoes and Borst (1977) have found glyceraldehyde-3-phosphate dehydrogenase,

glycerokinase and triosephosphate isomerase to be localized in the same particle while Oduro (1977) has found glyceraldehyde-3-phosphate dehydrogenase and glycerokinase to be polydispersed. GP oxidase has until recently been thought to be contained within a microbody-like structure (Müller, 1975), but new evidence suggests a mitochondrial localization for this enzyme (Opperdoes et al., 1977). Enolase (Oduro, 1977; Opperdoes and Borst, 1977), phosphoglycerate mutase and pyruvate kinase (Oduro, 1977) have been found to be soluble enzymes.

The localization of the glycolytic enzymes indicates the particle should be able to catalyse the following reactions :

$$\text{glucose} + 2\ \text{DHAP} + 2\ \text{Pi} \rightarrow 2\ (\text{GP} + 3\text{-PGA})$$

$$\text{glycerol} + 2\ \text{DHAP} + \text{Pi} \rightarrow 2\ (\text{GP}) + 3\text{-PGA}$$

In both reactions ATP utilization balances ATP synthesis and NAD^+ reduction by NADH oxidation. However 3-phosphoglycerate (3-PGA) must leave the particle for further metabolism to pyruvate in the cytosol, where net ATP synthesis will occur :

$$3\ \text{PGA} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP} + H_2O$$

Similarly GP must leave the particle aerobically to be oxidised by the GP oxidase catalysed reaction in the mitochondrion :

$$\text{GP} + \tfrac{1}{2}O_2 \rightarrow \text{DHAP} + H_2O$$

Thus the particle must be at least permeable to inorganic phosphate, DHAP, GP, glucose and the other cabohydrates metabolised, and glycerol. The particulate enzymes show latency (Oduro, 1977) of the type shown in other microbody systems (Müller, 1975) suggesting a selective permeability or a diffusion barrier for glycolytic intermediates and cofactors. Opperdoes and Borst (1977) have hypothesised that the triosephosphate and phosphate should pass the membrane

by means of specific translocators e.g. one exchanging GP with DHAP, and another exchanging phosphate for hydroxyl ions. This hypothesis remains to be substantiated experimentally. Certainly this situation would result in locally high concentrations of GP which may trigger the switch to anaerobic glycolysis or alternatively it may lead to the formation of glycerol and ATP by the reversal of the glycerokinase catalysed reaction.

One of the advantages of having a compartmentalised system for glycolysis is that high concentrations of substrates can be maintained within the micro-environment of the enzymes, allowing the individual enzymes involved to be more highly saturated than if they were in cytoplasmic solution (Green et al., 1965; Reed and Cox, 1966; Hubscher et al., 1971). Diffusion controlled reactions will be similarly increased due to spatial confinement. Partitioning of reactions may also have a further importance in separating ATP requiring reactions from the cytoplasmic ATP pool, for example hexokinase is found in both mitochondrial and soluble fractions in the rat brain (Johnson, 1960; Crane and Sols, 1953; Craven and Basford, 1969). The distribution of this enzyme is important as the kinetic parameters of the bound and soluble forms of the same enzyme vary e.g. K_m^{ATP} for the soluble enzyme is 1.7 mM and 0.34 mM for the bound form. Under stress conditions the enzyme is found mainly in the mitochondrial form (Knull et al., 1974) which not only increases hexokinase activity but utilizes mitochondrial ATP which decreases the elevated cytoplasmic demand for ATP.

Since suggestions that compartmentalization of some enzymes catalysing a specific metabolic sequence of reactions may be advantagous, efforts have been made to find out how kinetic studies of enzymes that are immobilised on

membranes differ from those of soluble enzymes. McLaren and Baker (1970) and Laidler and Sundaram (1971) have already demonstrated that the kinetic behaviour of enzymes in heterogenous systems can be altered from those in dilute solutions through : pH optimum shifts, activation or inhibition of maximum activity, increasing or decreasing the apparent Michaelis constants and stabilization of activity.

Mosbach and Mattiasson (1970) and Mattiasson and Mosbach (1971) have succeeded in preparing a two enzyme system consisting of hexokinase and glucose-6-phosphate dehydrogenase, and also a three enzyme system of galactosidase, hexokinase and glucose-6-phosphate dehydrogenase immobilised through being covalently bound to a Sephadex gel matrix. By comparing the kinetic behaviour of the matrix-bound enzyme system with an analogous system of soluble enzymes they have demonstrated that the overall rate of coupled reactions by the matrix system was higher prior to reaching a steady-state than in the corresponding soluble system, and that the observed increase in reaction rate is enhanced by increasing the number of enzymes involved in the metabolic sequence.

Compartmentation of the glycolytic enzymes required to catabolise glucose to glyceraldehyde-3 phosphate (GA3P) and GP in T. brucei could explain the observations of Grant and Fulton (1957), in which the C_1 of glucose metabolised anaerobically was found predominantly in glycerol not pyruvate. In this case close enzyme juxtaposition could preferentially select DHAP to react with NADH via the glycerol-3-phosphate dehydrogenase catalysed reaction rather than forming GA3P catalysed by triosephosphate isomerase. The resulting distribution of C_1 from glucose favouring glycerol rather than pyruvate would be similar to that found with hexose monophosphate aldolase activity.

Table 1.1 : Relative enzyme activities in various tissues and cells (Glyceraldehyde phosphate dehydrogenase = 100)

ENZYME	RAT (1)						HUMAN (2)					(3)	ERYTHROCYTES (4) (5) (6)			T. brucei	
	LIVER	KIDNEY	BRAIN	HEART MUSCLE	SKELETAL MUSCLE	SPLEEN	LIVER	KIDNEY	HEART	LUNG	FAT	E coli	PERCH	CATTLE	SHEEP	(7)	(8)
HEXOKINASE	2	6	14	5	0.3	8	1	2	10	8	11	*	0.3	1	1	1580	205
PHOSPHOGLUCOSE ISOMERASE	178	138	99	72	60	280	242	167	281	500	*	58	104	39	38	1080	441
PHOSPHOFRUCTOSE KINASE	3	4	19	8	11	12	1	1	3	3	6	24	5	2	1	3260	214
ALDOLASE	9	9	10	13	17	9	4	3	7	5	11	48	19	4	4	720	30
TRIOSEPHOSPHATE ISOMERASE	756	838	512	644	901	1080	1240	820	1080	1580	*	116	6261	*	*	2360	*
GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
PHOSPHOGLYCERATE KINASE	119	122	90	82	57	184	86	108	82	141	148	168	289	64	49	860	434
PHOSPHOGLYCERATE MUTASE	57	20	33	30	34	56	42	40	48	62	*	173	*	67	17	*	48
ENOLASE	15	25	19	11	36	28	31	33	13	42	47	41	*	13	16	160	398
PYRUVATE KINASE	38	51	158	136	132	200	28	83	151	144	136	58	48	8	4	*	480
GLYCEROL-3-PHOSPHATE DEHYDROGENASE	103	36	9	12	167	6	23	27	6	7	224	*	*	*	*	1460	268
GLYCEROKINASE	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	5200	713

The following references were used : (1) Shonk and Boxer (1964); (2) Shonk et al. (1964); (3) Irani and Maitra (1977); (4) Bachand Leray (1975); (5) Agar et al. (1975a); (6) Agar et al. (1975b); (7) Opperdoes and Borst (1977); (8) Oduro (1977).

* not available from the selected reference.

The bloodstream forms of trypanosomes metabolise glucose at a rate which is about fifty fold higher than any of the mammalian host tissues which they infect (von Brand, 1951) this is expressed in high activities of glycolytic enzymes (Oduro, 1977; Opperdoes and Borst, 1977) and the rate limiting step in glycolysis has been recently established as glucose transport in T. brucei (Gruenberg et al., 1978).

Not only are the specific activities of the glycolytic enzymes high in T. brucei but they also show a very different ratio compared to other tissues or cells. Table 1.1 shows that for a wide range of tissues and cells phosphoglucose isomerase and triosephosphate isomerase have the highest activity, followed by glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase, then phosphoglycerate mutase and enolase, while the lowest activities are found for hexokinase, phosphofructose kinase and aldolase. In agreement with the observations of Pette et al. (1962) that glycolytic enzyme activities show constant proportions. Although Oduro (1977) and Opperdoes and Borst (1977) disagree in specific detail, phosphofructose kinase, glycerokinase and hexokinase are amongst the most active enzymes in T. brucei, aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase the least active. Glycerokinase is the most active of all the enzymes involved in either glucose or glycerol metabolism by T. brucei, its exceedingly high activity has been postulated by Opperdoes and Borst (1977) to be important in catalysing the production of glycerol plus ATP from GP plus ADP. The concentration of glycerol in the blood is low, about 0.1 mM (Lin, 1977) while glucose is high(5.5 mM) (Cahill et al., 1966), consequently it is thought that glucose would be the preferred substrate physiologically. The postulate of glycerokinase being involved in the anaerobic utilization of glucose by T. brucei would account precisely for its high activity (Opperdoes and Borst, 1977).

1.6 Glycerokinase

The properties of glycerokinase have been recently well reviewed by Lin (1977). However the published properties of this enzyme refer only to the catalysed production of GP plus ADP from glycerol plus ATP. The enzyme requires intact sulphydryl groups. Dihydroxyacetone and glyceraldehyde can serve in place of glycerol, while CTP and UTP can replace ATP. Physiologically ATP and glycerol are most likely to be the predominant reactants. Glycerol, GP, ATP, ADP and Mg^{2+} interact in a complex manner to determine the activity of this enzyme. The apparent Michaelis constant for glycerol ranges from 3 to 10 μM, for ATP (Mg^{2+}) from 60 to 170 μM, while GP and ADP act as inhibitors. The apparent Michaelis inhibition constant values of glycerokinase for GP and ADP are both about 0.5 mM. As revealed by double-reciprocal plots glycerol is inhibitory at high concentrations when ATP is low (0.02 mM), but not when high (1 mM). When glycerol concentrations are low (0.01 mM), ATP does not activate, but when glycerol is high (0.33 mM), ATP activates the enzyme. The collective kinetic features of this enzyme suggests that phosphorylation of glycerol occurs when the cell is not energy starved and that overphosphorylation in an affluent state is counteracted by the accumulation of GP (see Lin, 1977 for review). The kinetic features of the glycerokinase catalysed reaction GP plus ADP to glycerol plus ATP have not been reported in the literature.

1.7 The Possible Importance of Elucidating the Pathway of Anaerobic Glucose Metabolism by T. brucei.

At present there are four major drugs available for the prophylaxis and treatment of human sleeping sickness : suramin, tryparsamide, diamidines and

melarsoprol, while the treatment of animal trypanosomiasis is largely by phenanthridines and Berenil (Williamson, 1976). Occurrences of drug resistance in the wild are reported with distressing frequency; pentamidine resistance in T. gambiense (Kayembe and Wery, 1972), Ethidium resistant Trypanosoma congolense (Gadir et al., 1972; Scott and Pegram, 1974), Samorin (a phenanthridine) resistant T. congolense (Lewis and Thomson, 1974) and suramin-resistant Trypanosoma ninaekohlakimovae (= Trypanosoma evansi) (Petrarski and Khamiev, 1974). Indeed widespread resistance to Antrycide and Ethidium (phenanthridines) in large areas has left Berenil (a diamidine) as the only effective drug against cattle trypanosomiasis in these regions (Williamson, 1976). Resistance to one trypanocidal diamidine seems to confer resistance to all diamidines, a factor which is not encouraging when new derivatives of existing aromatic diamidines are synthesised as potential trypanocides.

The control of nagana (cattle trypanosomiasis), caused by T. brucei, has a restrictive requirement for a single dose treatment, so ideally a new non-toxic water soluble compound capable of absorption in active form with the blood and tissues after oral ingestion is required. It must also be capable of penetration into the cerebrospinal fluid and of forming an effective and harmless depot for prophylaxis. At our present stage of knowledge synthesis of such a drug is a remote possibility (Williamson, 1976).

A knowledge of the comparative biochemistry of the mammalian host and the bloodstream trypanosome could however prove useful in developing new drugs. GP oxidase peculiar to the parasite is a prime target, but inhibition of this enzyme alone will be expected to be of little chemotherapeutic value since Ryley (1956) and Grant and Fulton (1957) have shown that T. rhodesiense can

survive when
GP oxidase is inhibited by benzhydroxamates (Evans and Brown, 1973a; Evans and Brown, 1973b) and the trypanocidal drug suramin (Fairlamb and Bowman, 1977) although this drug must have another site of action, possibly glycerol-3-phosphate dehydrogenase (Oduro, 1977). Salicylhydroxamic acid (SHAM), a benzhydroxamate, is a potent inhibitor of GP oxidase and was found to have no therapeutic effect on a fulminatory T. brucei infection in rats (Opperdoes et al., 1976a). However it is possible that in a natural relapsing infection, where parasite attack and host defence are more evenly balanced a drug that decreases trypanosome energy production could still be of therapeutic use (Opperdoes et al., 1976a).

The existence of an alternative pathway for anaerobic glycolysis involving one enzyme not essential for host metabolism could allow an inhibitor of this enzyme and SHAM in combination to result in an effective trypanocidal agent, although either drug alone may have little or no effect. Glycerol has been found to inhibit the anaerobic utilization of glucose by T. rhodesiense (Ryley, 1962), and when injected with SHAM into Trypanozoon infected rodents, a rapid clearance of the parasites from the blood is seen (Clarkson and Brohn, 1976; Fairlamb et al., 1977; Evans et al., 1977). In mice showing LS and SS forms of T. rhodesiense, treatment with glycerol plus SHAM results in only SS forms remaining in the blood an hour after treatment (Clarkson and Brohn, 1976). Temporal separation of SHAM and glycerol by five minutes abolishes their therapeutic use suggesting that these compounds are rapidly cleared from the blood (Clarkson and Brohn, 1976). Glycerol in the serum rapidly equilibrates with tissue glycerol, but it enters the brain, cerebrospinal fluid and aqueous humour of the eyes at extremely low rates (Tourtellotte et al., 1972; Buckell and Walsh, 1964; Waterhouse and Coxon, 1970). T. brucei is not strictly a plasma parasite and the reason for the recurrence of infection after SHAM plus

glycerol treatment (Clarkson and Brohn, 1976; Fairlamb et al., 1977) could be due to insufficient glycerol and/or SHAM being present in certain interstitial fluids. Trypanosoma vivax, which is strictly a plasma parasite does not show recurrence of infection (Evans et al., 1977).

1.8 Selectivity of Introductory Review

This introduction has been specifically limited to reviewing the literature of carbohydrate metabolism by the bloodstream forms of T. brucei, and the closely related T. gambiense and T. rhodesiense. However the similarity of anaerobic glycolysis of other African trypanosomes : T. congolense, T. vivax, T. evansi, Trypanosoma equinum and Trypanosoma equiperdum (Ryley, 1956) as characterised by the anaerobic production of glycerol and pyruvate from glucose may indicate a more general occurrence of the T. brucei anaerobic glycolytic pathway.

The very different methods of energy production of the various stages in the life cycle of T. brucei and the very different method of energy production in Trypanosoma cruzi would exclude these from a general theory of anaerobic glycolysis in trypanosomes based on the method of LS forms of T. brucei.

SECTION 2

MATERIALS AND METHODS

2.1 The Line and Maintenance of T. brucei Studied

Unless otherwise stated in this thesis a monomorphic line of T. brucei, TREU 55 (Trypanosomiasis Research, Edinburgh University) was used. This line had been derived from a wild type T. brucei by passage through laboratory mice to give TREU 1, which was subsequently passaged through rats to give TREU 55. The line used was completely monomorphic and produced a rapidly fulminating infection resulting in the death of its rodent host within 4 days after innoculations. When a pleomorphic line was used, this was TREU 55 P, the parent line of TREU 55 that was stabilated from relapsing infections in rats before the technique of rapid syringe passage had eliminated pleomorphism.

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

Infection was established by intraperitoneal injection of stabilates of T. brucei, TREU 55 P, that had been stored in 7.5% (w/v) glycerol at -70°C. Syringe passages of a 48 hour old infection through six successive hosts ensured the monomorphic nature of the line. The stocks of stabilates were prepared in the following manner.

Blood from infected rats below the peak of parasitaemia was collected by exsanguination into heparinised (5.1 U/ml) Krebs' saline. Glycerol was then added to a final concentration of 7.5% (w/v), thoroughly mixed and the solution dispensed into glass capillaries, approximately 100 mm in length by 1 mm internal diameter. The capillaries were half filled, then sealed at both ends in a flame and were left to cool to room temperature before being transferred to a bath of

ice-cold ethanol. These capillaries were then packed in polystyrene to facilitate slow cooling to -70°C in solid CO_2. After a day the capillaries were removed and stored in a deep freeze at -70°C.

The established monomorphic line was maintained by syringe passage every 72 hours, as follows : infected blood from a parasitized rat was diluted with either citrate or phosphate saline until about 5 parasites were visible per high power field (252x) of a light microscope. A 0.3 ml aliquot of the diluted blood sample was routinely injected intraperitoneally into each rat.

2.2 Production of Rabbit Antibody to Rat Erythrocytes

Rabbit anti-rat erythrocyte serum was prepared by giving a rabbit a course of six injections of 1.0 ml of 50% (v/v) washed rat erythrocytes in heparinised Krebs' saline in 3 - 4 day intervals. The injections were given via a marginal ear vein, and when the rabbit serum titre against rat erythrocyte gave a titre of over 1/1000 the rabbit was anaesthetised and exsanguinated. The blood was allowed to clot at room temperature for 5 hours and the clot was removed by centrifugation. The supernatant was then dispensed in 2.0 ml aliquots and stored at -20°C.

2.3 Preparative Buffered Isotonic Solutions

Krebs' saline pH 7.0 : 129 mM-sodium chloride, 6 mM-potassium chloride, 17 mM-(K^+) phosphate, 1.2 mM-magnesium sulphate.

Citrate saline pH 7.0 : 157 mM-sodium chloride, 51 mM- Tri-sodium citrate.

Phosphate saline pH 8.0 : 53 mM-disodium hydrogen phosphate, 3 mM-sodium dihydrogen phosphate, 45 mM-sodium chloride.

Tris-sucrose pH 7.0 : 0.25 M-sucrose, 50 mM-Tris-HCl.

2.4 Preparation of Trypanosomes Free from Blood Elements

LS trypanosomes were harvested from rats 72 hours after infection when the parasitaemia was at a maximum. Pleomorphic infections were harvested when microscopic analysis of a tail blood smear revealed a high parasitaemia with a high proportion of SS parasites, usually about 120 hours after infection. One of two techniques was used to purify the trypanosomes free from blood elements.

One technique used was that of Flynn and Bowman (1973a), with the infected blood being withdrawn from the rats by cardiac puncture and diluted with citrate saline containing 20 mM-glucose. After defibrination the trypanosomes were prepared in Krebs' phosphate saline containing 11 mM-glucose.

The alternative method used was that of Lanham (1968). Infected rat blood obtained by cardiac puncture was transferred into 1.0 ml of phosphate saline containing heparin, (5 I.U./ml) and 55 mM-glucose. Centrifugation at 1000 *g* max. for 10 minutes at 4°C was followed by the removal of the white trypanosome layer, which was subsequently resuspended in phosphate saline containing 55 mM-glucose. The trypanosomes were then isolated from the remaining blood cells by filtration through a DEAE-52 cellulose column according to the method of Lanham (1968).

Both methods produced a trypanosome preparation of high purity as characterised by the lack of bloodcell contamination, judged by light microscopy and the absence of lactate dehydrogenase activity. The method of Flynn and Bowman (1973) proved much faster in preparing relatively large amounts (greater than 2 mls packed cell volume) of trypanosomes whereas the method of Lanham (1968) was quicker and more efficient in preparing smaller amounts of trypanosomes.

The prepared trypanosomes were routinely checked for motility and possible blood cell contamination by light microscopy. Only pure preparations of 100% intact and motile trypanosomes were used and all experiments were carried out on trypanosomes as soon as possible after preparation.

2.5 Preparation of Homogenates

Trypanosomes are resistant to cell lysis in isotonic media by conventional methods (Simpson, 1972), therefore different methods were used to break the pellicle of *T. brucei*. The methods selected were : (a) the use of hypotonic media, (b) cell grinding with abrasives and (c) the use of lytic agents.

(a) The Use of Hypotonic Media

1.0 ml packed cell volume (PCV) of trypanosomes were resuspended in 9.0 mls of 2x distilled water at about 2°C. The suspension was subjected to homogenization by 20 strokes of a hand-operated Dounce homogenizer, equipped with a tight fitting perspex ball-type pestle (Wesley Coe Ltd., Cambridge). The homogenates were routinely examined by phase contrast microscopy and if cell fragmentation was incomplete the homogenization procedure was repeated. The homogenates were left on ice before being studied, which commenced as soon as possible after preparation.

(b) The Use of Abrasives

The two abrasives used in the grinding of cells, were silicon carbide or alumina.

Silicon carbide was obtained from the Norton Company, grit No 37c 400, and before being used it was thoroughly washed with distilled water and dried. About 7 g of chilled silicon carbide were stirred into approximately 3 ml of 0.5 ml PCV of T. brucei in Tris-sucrose plus 5.5 mM-glucose in a chilled mortar to give a paste. This mixture was then ground until light microscopy revealed that virtually all the cells had been fragmented.

Cell rupture with alumina (Koch-Light Labs. Ltd. : Alumina "Woelm" neutral TLC absorbent) was carried out in a manner similar to that described for silicon carbide.

In both methods the ground material was resuspended in Tris-sucrose and centrifuged for 5 minutes at 100 g max. in a MSE Mistral 4L centrifuge using an 8 x 50 ml swing out rotor. The brake was not applied. The supernatant was carefully decanted while the pellet was resuspended in the original volume of buffer and the centrifugation was repeated; this washing process was repeated twice. The pooled supernatants were then centrifuged at 100 g max. for 5 minutes and decanted from the pelleted silicon carbide or alumina contamination. This supernatant was left on ice and used as soon as possible after preparation.

(c) Cell Fragmentation With Lytic Agents

The lytic agents were saponin and triton X-100. A solution of the appropriate agent was made up in tris-sucrose buffer and was added to a

suspension of T. brucei cells in the same buffer to give a ratio of 1 ml PCV to 10 ml of buffer. The final concentrations of the lytic agents in the suspension was 0.5% (w/v). The suspension was left on ice for 15 minutes and then lightly homogenized with 5 strokes of a hand operated Dounce homogenizer. The resulting suspension was examined by phase contrast microscopy to ensure cell lysis was complete and the resulting lysate was left on ice before being used for study.

2.6 Preparation of Subcellular Fractions

Broken cell preparations of T. brucei obtained by grinding with silicon carbide, as previously described, were initially centrifuged at 1000 g max. for 25 minutes in an 8 x 50 ml fixed angle rotor in a MSE 18 centrifuge maintained at 4°C. The supernatant was decanted and the pellet resuspended in tris-sucrose buffer, thoroughly mixed, then recentrifuged as before. This method of washing the pellet was repeated. The final pellet was resuspended in tris-sucrose buffer and was referred to as the 1 KP sample. It was seen under phase contrast microscopy to contain a mesh of flagella and subcellular particles including nuclei.

The decanted supernatants were pooled to form the 1 KS fraction. A portion of this fraction was centrifuged at 5000 g max. for 25 minutes in a MSE centrifuge with a fixed angle rotor at 4°C. The technique of pellet preparation, washing and supernatant pooling were as described for the 1 KP and 1 KS fractions except that centrifugation was at 5000 g max. throughout the period of centrifugation. The resulting pellet fraction was referred to as the 5 KP, the supernatant - the 5 KS.

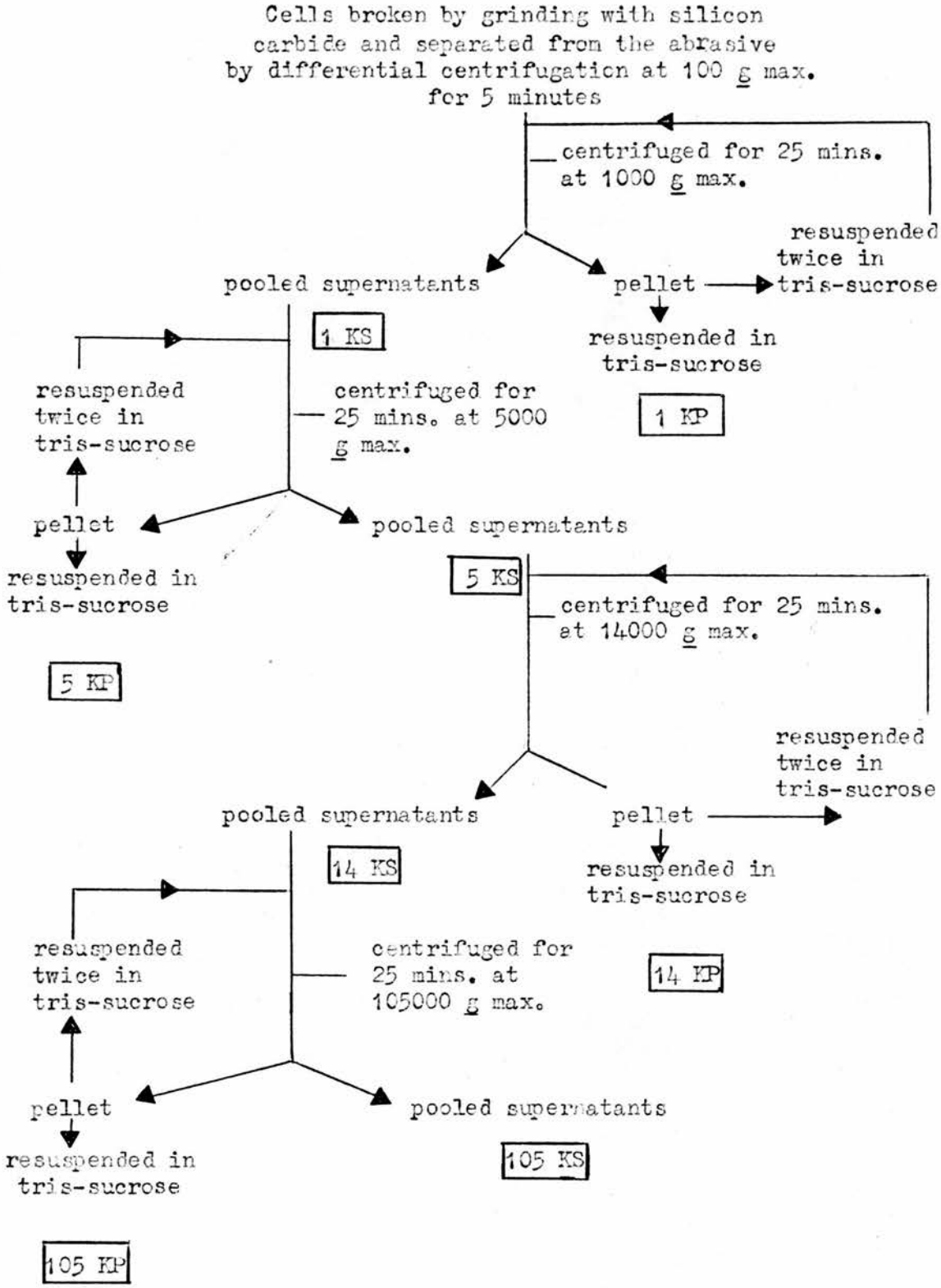


Fig. 2.1 : Flow diagram of subcellular fractionation technique.

The technique for preparing the 14 KP and 14 KS fractions was identical to that described for the 5 KP and 5 KS except that a portion of the 5 KS fraction was used and centrifugation was at 14000 *g* max. throughout the period of centrifugation.

For preparing 105 KP and 105 KS fractions, a portion of the 14 KS fraction was centrifuged at 105000 *g* max. for 25 minutes at 4° C in a Spinco Ultracentrifuge with a fixed angle rotor, type 40. The supernatant was decanted and the pellet was resuspended in tris-sucrose, thoroughly mixed and recentrifuged at 105000 *g* max. for 25 minutes. The supernatant was decanted and the pellet again resuspended in tris-sucrose and thoroughly mixed. The resulting pellet suspension was referred to as the 105 KP, the pooled supernatants - the 105 KS.

In some experiments only the 14 KP fraction was wanted and for these the procedures for obtaining the 1 KP, 1 KS, 5 KP, 105 KP and 105 KS fractions were eliminated. Instead, a 5 KS fraction was prepared by the standard method except from an unfractionated broken cell preparation not a 1 KS fraction. The 14 KP and 14 KS fractions were prepared from the 5 KS fraction as previously described. No further fractionation of the 14 KS took place. A flow diagram summarizing the technique employed for sub-cellular fractionation is given in Fig. 2.1.

2.7 Analysis of Whole Cell Glycolytic Intermediate and Adenine Nucleotide Concentrations of *T. brucei*

There are several problems inherent in the evaluation of whole cell steady-state metabolite concentrations of *T. brucei* under aerobic and GP oxidase inhibited

conditions of incubation. The organisms cannot be sufficiently concentrated to allow metabolite analysis by spectrophotometric determinations, without the organisms rapidly altering their environment during incubation. In concentrated trypanosome suspensions under aerobic conditions glycerol is formed as an end product, probably due to oxygen becoming limiting (Clarkson and Brohn, 1976), while in GP oxidase inhibited suspensions glycerol is being rapidly and continuously produced. The increase in glycerol concentration will progressively inhibit glucose utilization, thus creating a transient condition. Furthermore the concentration of dilute trypanosome extracts by freeze drying produces high salt concentrations from the concentrated incubation medium rendering many substrate assays unreliable. The use of hypotonic media was considered undesirable for trypanosome swelling caused by this condition may well produce artefacts in the results.

The method adopted here was to incubate trypanosomes under various conditions at a final concentration of 1 ml PCV to 10 ml of media. Anaerobiosis was achieved by passing oxygen free nitrogen over the incubation media for 30 minutes prior to the addition of an anerobic trypanosome suspension and throughout the subsequent incubation. The trypanosome suspension was deoxygenated by sucking it into a chilled syringe, filling with oxygen free nitrogen, and leaving a moment for gaseous equilibration between liquid and gas phases before inverting and excluding the gas. This process was repeated four times.

18° C was selected to be the incubation temperature as the rate of oxygen consumption and glycerol production by the trypanosomes was very much reduced compared to the standard 37° C or 25° C, thus allowing a more concentrated trypanosome suspension to be used without incurring an oxygen deficiency

aerobically or too rapid an increase in glycerol concentration anaerobically. At the start of the incubations the media were well mixed and throughout the period of incubation they were continually agitated by 100 strokes per minute through 6 cms. At predetermined times incubations were terminated by aliquots of suspension being injected into 4 x their volume of identical medium at about 0°C. The anaerobic suspension was injected through a paraffin film about 5 mm thick into previously deoxygenated medium. The diluted suspensions were quickly centrifuged for 3 minutes at 1000 g max. at 0°C in a MSE Mistral 4L centrifuge, using an 8 x 50 ml swing and rotor, with the brake applied. The supernatants were then rapidly decanted and the pellets quickly deproteinised with excess formic acid. The formic acid extract was left on ice for 10 minutes before being neutralized to pH 7 with 18 M-ammonia. After a further hour on ice the extract was centrifuged at 1000 g max. for 20 minutes at 4°C. The resulting supernatant was carefully decanted and freeze dried, the residue being resuspended in double distilled water added to a volume that resulted in the ratio of the extract of 1 ml PCV to 3 mls of water.

2.8 Broken Cell Incubation and Extract Preparation Techniques

Round bottom conical flasks containing the incubation media were left on ice prior to the addition of the broken cell preparation. Anaerobiosis was achieved and maintained throughout the incubation by the method outlined in the previous sub-section (2.7). Upon the addition of the broken cell preparation the suspensions were well mixed and placed in a thermostatically controlled water bath and continually agitated with 100 strokes per minute through 6 cms. 1.0 ml aliquots were taken at specified times and rapidly deproteinised in 0.5 ml of 1.5 M-perchloric acid. These extracts were left on ice for 10 minutes before

being neutralised with 0.5 ml of 2.5 M-dipotassium hydrogen phosphate. After being left on ice for a further hour, the extracts were centrifuged at 1000 g max. for 10 minutes at 4° C in a MSE Mistral 4L centrifuge using an 8 x 50 ml swing out rotor. The supernatants were then decanted and analysed.

2.9 The Preparation of Extracts for Measuring the Incorporation of (1-^{14}C)-Glycerol into T. brucei

Trypanosomes prepared by the method of Lanham (1968) at 22° C were twice washed in phosphate saline buffer at 25° C containing 11 mM-glucose plus 1.0 mM-SHAM. The trypanosomes suspension was divided into five equal portions and each diluted with an equal volume of phosphate saline buffer with varying glycerol concentrations. These suspensions were incubated in a thermostatically controlled water bath at 18° C continually agitated by 100 strokes per minute through 6 cms. After 2 minutes radioactive glycerol of specific activity 56 mCi/m mole, final activity 0.25 μ Ci/ml was added. 1.0 ml aliquots were taken at specified times, deproteinised with 0.5 ml of 1.5 M-perchloric acid, then left on ice for 10 minutes before being neutralised with 0.5 ml of 2.5 M-dipotassium hydrogen phosphate. After a further hour on ice the neutralised samples were centrifuged at 1000 g max. for 10 minutes at 4° C in a MSE Mistral 4L centrifuge using an 8 x 50 ml swing-out rotor.

The trypanosome concentration had to be high, about 4 mg of protein/ml of incubation media, to allow for accurate estimations of purified GP to be made. The method of concentrating the trypanosomes by centrifugation prior to deproteinisation could not be used as this would inevitably result in a locally high concentration of non-radioactive glycerol produced by the organism from

glucose in the environment of the pelleted trypanosomes, thus decreasing the radioactivity present in the intracellular GP.

2.10 Thin Layer Chromatography

Whatman CM-cellulose (CC41) was twice washed with distilled water and dried before being made into an easy spreading liquid paste with distilled water. This paste was evenly spread over glass plates (200 mm by 200 mm) to a thickness of 1 - 2 mm. The paste was allowed to dry to room temperature before being spotted 20 mm from the "bottom" edge and at intervals of 25 mm with the appropriate solutions. The plates were then warmed in an oven to 60°C for 10 minutes before cooling to the temperature of the chromatography tanks, this process evaporated water present in the spotted solutions.

The solvents present in each tank were given 24 hours to equilibrate with the vapour phase before being used. The plate was developed until the solvent front was about 5 mm from the top edge. The plates were then removed and dried in a current of warm air. Any remaining traces of solvent were removed by heating in an oven at 75°C for 10 minutes.

For sugar phosphate analysis each plate was held within a fume chamber and sprayed with the following solution : 5 mls of 60% (w/v) perchloric acid, 10 mls of 0.1 M-hydrochloric acid, 25 mls of 4% (w/v) ammonium molybdate and water to 100 mls. The plates were dried in a current of warm air then heated for 20 minutes at 70°C in an oven equipped with an extractor fan. This method causes sufficient hydrolysis of even fairly resistant esters e.g. PGA and G-6-P (Hanes and Isherwood, 1949). Although many of the esters are visible at this stage

owing to decomposition products reducing phospho-molybdate, the plates were allowed to regain moisture from the atmosphere and were then held in a tank containing dilute H_2S for 10 minutes. The position of the esters appeared as intensely blue spots against a buff background.

When ammonia is present in the solvent system the background often became coloured blue but this could be removed by placing the developed plate in a tank of dilute hydrogen chloride vapour.

2.11 Partial Separation of Glycerol-3-Phosphate and ADP from Glycerol

In several experiments it was found necessary to estimate the concentration of glycerol when comparatively high concentrations of GP and ADP were present. This rendered the conventional methods impractical unless either the GP or ADP concentrations were decreased. This was achieved by the following method. An anion exchange resin, DEAE-cellulose (Whatman DE23) was stirred into 0.1 M-triethanolamine and adjusted to and then maintained at pH 7.4 for 15 minutes with potassium hydroxide and hydrochloric acid. The equilibrated cellulose was then centrifuged at 1000 g max. for 5 minutes at 4°C in a MSE Mistral 4L centrifuge. The pelleted resin was then resuspended in the extract to be analysed (1 vol resin : 10 vol of extract), thoroughly mixed and allowed to stand for 15 minutes. The resin with bound anions was repelleted and the anion depleted supernatant was decanted and analysed for glycerol by conventional methods.

The recovery of glycerol was usually less than 90%, consequently standards had to be run for each set of glycerol concentrations determined by this method.

2.12 Purification of Glycerol-3-Phosphate from Glycerol in Whole Cell Extracts

This technique was based on the method of Bartlett (1959). GP and glycerol were separated by ion exchange using 30 mm by 3 mm internal diameter columns of Dowex 1-8 x and Whatman DE23 resins in the ratio 4 : 1 (v/v) as this was found to provide optimal flow rates. The resins were treated for 12 hours with 0.1 M-hydrochloric acid and thoroughly washed with distilled water. 1.0 ml aliquots of trypanosome extracts were slowly added to the column, left for 5 minutes to allow anion exchnage before glycerol plus a portion of the GP was eluted off with 100 mls of distilled water. A glycerol free, GP fraction could then be eluted off in 3 mls of 0.2 M-hydrochloric acid. Control experiments adding radioactive glycerol to non-radioactive extracts established that no significant glycerol contamination was present in the GP fraction.

2.13 Radioactivity Counting

(1-14C)-glycerol was the only radioactive compound used for this thesis, and all radioactivity counting was performed in a Packard Model 3330 Tri-carb scintillation counter. Aqueous samples were counted in the following scintillation fluid : 0.02% (w/v) 1, 4-Bis-(5-pheny loxazol-2yl) benzene, 0.4% (w/v) 2, 5-Diphenyloxazole, 33% (v/v) triton X-100 and 67% (v/v) toluene, 10 ml of scintillation fluid being added to 1.0 ml of sample. When 1.0 ml of sample was not available or the activity too high, then the sample was made up to 1 ml with distilled water. The efficiency of this counting system was 56% and did not vary significantly with the different constituents in the samples.

The radioactivity of the thin layer chromatograms was counted by taking 5 mm widths from the spot base line to the solvent front and adding 10 mls of

scintillation fluid. The radioactivity in each width was counted and together they provided an R_F value for the radioactive compound(s).

2.14 Trypanosome Motility

In some experiments trypanosome motility was qualitatively evaluated according to the following scheme :

Description	Rating
Most trypanosomes show maximum activity	+++++
Most trypanosomes show sub-maximal activity but flagella and cell body movements are too rapid to be followed microscopically	++++
Flagella and cell body movements can be followed microscopically in most of the organisms	+++
The trypanosomes have frequent and regular cell body and flagella movements which can be easily followed microscopically	++
The trypanosomes are moving with Brownian motion except for infrequent flagella and cell body movements	+
About half the cells are lysed, the rest immotile	$\frac{+}{-}$
Extensive cellular disintegration is seen microscopically	-

In experiments where motility was estimated a sample of results were verified by an independent observer.

2.15 Protein Estimation

Protein estimation was carried out either by the method of Lowry et al. (1951) or Bradford (1976). Bovine serum albumin (BSA) standards were included in each assay. Calibration curves were linear from 0 - 200 µg of BSA per assay by the method of Lowry et al. (1951) and from 0 - 100 µg per assay by the method of Bradford (1976). Sucrose interferes with protein estimation by the method of Lowry et al. (1951) (Hinton et al., 1969) but not with the method of Bradford (1976). The latter method was therefore always used for samples containing high concentrations of sucrose.

Triton X-100 was however found to affect protein estimation by the method of Bradford (1976), and when present in the sample analysed an identical concentration of triton X-100 was added to the BSA standards.

Trypanosome protein concentrations determined by the method of Bradford (1976) repeatedly gave values of about 55% of that obtained using the method of Lowry et al. (1951), and all results obtained based on the method of Bradford (1976) were corrected to the equivalent value that would be obtained by the method of Lowry et al. (1951).

2.16 Statistical Methods

The mean ($\bar{x}$), standard deviation and standard error of the mean were calculated using either a Hewlett-Packard HP45 calculator or a Texas SR-51-11 calculator, employing the following formulae :

$$\bar{x} = \frac{\sum x}{n}$$

$$\text{standard deviation} = \sqrt{\frac{\sum x^2 - n\bar{x}^2}{n - 1}}$$

$$\text{standard error of the mean} = \sqrt{\frac{\sum x^2 - n\bar{x}^2}{n(n - 1)}}$$

Unweighted linear regression analyses of data were carried out on a Texas SR-51-11 calculator and kinetic data was analysed using Wilkinson's method of regression using an Emas computer.

2.17 Enzyme and Substrate Assays

Unless otherwise stated all enzyme assays were performed at pH 7.0, 25°C and the assay mixtures were incubated for at least 5 minutes before initiating the reaction by the addition of the appropriate substrate. The assay procedures that involved oxidation or reduction of NAD or NADH were measured by change of absorbance at 340 nm unless high concentrations (above 1.0 mM) of SHAM were present in which case 366 nm was chosen to minimize interference due to absorbance of SHAM at 340 nm. Reactions were monitored in an Unicam SP 8000 or SP 800A spectrophotometer fitted with a slave recorder. Silica cuvettes with a light path of 1 cm were routinely used.

Most assays performed were based on standard procedures with slight modifications as detailed in the appendix : hexokinase [ATP : D-hexose-6-phosphotransferase, EC 2.7.1.1] (Grignani and Lohr, 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-dipohosphate 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] (Delbruck 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); glycerol-3-phosphate dehydrogenase (NAD^+), [sn-glycerol-3-phosphate : NAD^+ 2-oxidoreductase, EC 1.1.1.8] (Bergmeyer, 1974a); glycerol-3-phosphate oxidase [sn-glycerol-3-phosphate : oxygen oxidoreductase, EC 11.2.99] (Srivastava and Bowman, 1971); glycerokinase [ATP : sn glycerol 1-3-phosphotransferase, EC 2.7.1.30] (Kreutz, 1962); adenylate kinase [ATP : AMP phospho-transferase, EC 2.7.4.3] (Bergmeyer, 1974b); glycerol dehydrogenase [glycerol NAD^+ 2 oxidoreductase EC, 1.1.1.6] (Toews, 1967); glycerol dehydrogenase ($NADP^+$) [glycerol : $NADP^+$ 2 oxidoreductase, EC 1.1.1.99] (Toews, 1967). Hexose monophosphate aldolase activity was assayed by a method similar to that of aldolase; GP : glucose transphosphorylase, that of hexokinase; GP : fructose-6-phosphate transphosphorylase; that of phosphofructose kinase. The assays for GP : triose transphosphorylase and glycerokinase in the direction of GP plus ADP forming glycerol plus ADP were developed by myself and are described in the appendix.

With the exception of GP oxidase all enzyme activities were calculated from the rate of production or utilization of NADH or NADPH, obtained from the change in absorbance at 340 nm using an extinction coefficient of 6.22×10^3 cm^{-1} mol^{-1}

and of 3.11×10^3 cm^{-1} mol^{-1} at 366 nm. GP oxidase activity was assayed polargraphically in a Clark oxygen electrode. This was polarised at -0.8 volts with the electrode adapted to fit a perspex reaction chamber (3 ml capacity) equipped with a magnetic stirrer and was connected to a potentiometric recorder. Calculation of oxygen utilization was based on the dissolved oxygen concentration in air saturated medium being 217 μM at 37° C or 258 μM at 25° C (Kielley, 1963).

The majority of substrate assays involved oxidation or reduction of NAD or NADP. Changes in absorbance were read at 340 nm unless SHAM was present at concentrations greater than 0.1 mM in which case 366 nm was selected. Substrate concentrations were calculated similarly to enzyme analysis but the results were expressed in μ moles/ml. The details of these assay methods are given in the appendix.

It was found more convenient to assay glucose by the method of Werner et al. (1970) than through measuring $NADP^+$ reduction as described in the appendix when many samples required glucose determination. This method oxidases glucose by the glucose oxidase catalysed reaction producing gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts with di-ammonium 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonate) forming a coloured complex and water. The absorbance of the complex is measured at 615 nm and compared with a standard calibration curve ranging from 0 - 0.1 ml of 0.55 mM-glucose per 5 ml of assay reagent.

2.18 Chemicals

All chemicals were of the highest purity available ('Analar' grade or its equivalent) purchased from BDH Chemicals Ltd. or Sigma Chemical Co. Ltd. The

concentration of sn glycerol-3-phosphate in the hydrated DL-GP was determined enzymatically and all references to GP concentration in this thesis, unless otherwise stated refer specifically to the concentration of sn glycerol-3-phosphate in a mixture of DL-GP.

Most enzymes, cofactors and substrates were purchased from C. F. Boehringer and Sohne Gmbh, Mannheim except glucose, fructose, mannose and α-oxoglutarate, glyceraldehyde and dihydroxyacetone which were purchased from BDH Chemicals Ltd. SHAM was purchased from Aldrich Chemical Co. Ltd., Wembley, Middlesex. Radioactive glycerol was purchased from the Radiochemical Centre, Amersham, England and melarsen oxide and glycerol from May and Baker Ltd., Dagenham.

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

SECTION 3

RESULTS (PART 1)

Table 3.1 : The molar ratio of glycerol and pyruvate produced to glucose utilized in aerobic and anaerobic conditions

Aerobic		Anaerobic	
$\frac{\text{Moles pyruvate produced}}{\text{Moles glucose utilized}}$	$\frac{\text{Moles glycerol produced}}{\text{Moles glucose utilized}}$	$\frac{\text{Moles pyruvate produced}}{\text{Moles glucose utilized}}$	$\frac{\text{Moles glycerol produced}}{\text{Moles glucose utilized}}$
1.95 ± 0.04 (10)	0.10 ± 0.02 (10)	1.05 ± 0.07 (10)	0.94 ± 0.06 (10)

The results were taken from several different experiments with the incubation medium being Krebs' saline containing 5.5 mM-glucose at 25° C. They have been expressed as means $\pm$ the standard error of the mean and the figures in parentheses represent the number of results obtained from which the means were calculated.

3.1 Products of Glucose, Mannose, Fructose and Glycerol Catabolism by T. brucei under Aerobic and Anaerobic Conditions

The products of glucose catabolism under aerobic and anaerobic conditions were examined to establish if T. brucei, line TREU 55, behaved similarly to other lines of the subgenus Trypanozoon as reported by other authors (Ryley, 1956 and 1962; Grant and Fulton, 1957). The catabolism of fructose and mannose was examined to determine whether these substrates could support anaerobic as well as aerobic survival (Ryley, 1962). The observations of Ryley (1956) that glycerol can only be utilized aerobically was also tested for T. brucei, TREU 55.

Highly dilute trypanosome suspensions (less than 0.5 mg of protein/ml) were used in these analyses to avoid oxygen becoming limiting aerobically. Therefore long incubation times were necessary to obtain sufficient substrate concentration changes to allow for accurate determination.

Table 3.1 gives the molar ratio of glycerol and pyruvate produced per glucose utilized in aerobic and anaerobic conditions. Aerobically glucose is quantitatively metabolised to pyruvate with minimal glycerol production while anaerobically glucose quantitatively forms equimolar glycerol and pyruvate. In both cases the carbon recovery was about 100% establishing that there are no other accumulations of end products of glucose metabolism. These results are in agreement with the observations of Ryley (1956) and Grant and Fulton (1957).

Mannose and fructose are metabolised to pyruvate aerobically and glycerol and pyruvate anaerobically, Table 3.2. This demonstrates that the method of glycerol

Table 3.2 : The rates of pyruvate and glycerol production from glucose, fructose, mannose and glycerol by T. brucei

Substrate	Aerobic		Anaerobic	
	pyruvate production	glycerol production	pyruvate production	glycerol production
Glucose	130	1	66	58
Fructose	115	1	41	43
Mannose	76	0	38	36
Glycerol	56	-	0	-

The results are the average of duplicate experiments performed on one trypanosome preparation incubated in Krebs' saline containing 10 mM-substrate at 25° C, and are represented in n moles/minute/mg of protein.

production anaerobically must be a common factor in fructose, glucose and mannose metabolism. The differing rates of end product formation from glucose, mannose and fructose metabolism probably reflects a difference in the rate of carbohydrate transport into the organism, which Gruenberg et al. (1978) have found to be the rate determining step in the aerobic utilization of glucose.

Aerobically glycerol is metabolised to pyruvate but not anaerobically. This finding is in agreement with the observations of Ryley (1962) for T. rhodesiense, although Ryley found glycerol utilization to be 26% faster than glucose utilization the results of Table 3.2 show it to be 16% slower for T. brucei, TREU 55.

3.2 Inhibition of T. brucei Motility by Glycerol

In the previous subsection it was established that glycerol cannot be metabolised to pyruvate anaerobically and is an end product of anaerobic glucose metabolism. Ryley (1962) found that glycerol was an inhibitor of anaerobic glucose utilization and these observations are verified for T. brucei in Table 3.3, where motility is examined, under various conditions. Table 3.4 shows the results of a similarly designed experiment performed using a pleomorphic trypanosome preparation. The pleomorphic character of the trypanosomes was established by their survival with α-oxoglutarate as the sole substrate present. LS forms quickly became immotile and lyse under these conditions.

LS and SS trypanosomes can metabolise glucose both aerobically and anaerobically, but only aerobically when 20 mM-glycerol is present. These observations have been repeated using SHAM to simulate anaerobiosis. Glycerol plus SHAM is a lethal

Table 3.3 : The effect of glycerol on the motility of a monomorphic T. brucei preparation under aerobic and glycerol-3-phosphate oxidase inhibited conditions

Time mins	Additions to phosphate saline buffer							
	Glucose	Glucose SHAM	Glucose Glycerol	Glucose SHAM Glycerol	Glycerol	SHAM Glycerol	α-Oxoglutarate	
1	++++	+++	+++	–	++	–	++	+++
7	+++	+++	++	–	++	–	±	±
14	++	+++	++	–	+++	–	–	–
25	+++	++	+++	–	++	–	–	–
35	+++	+++	+++	–	++	–	–	–

LS trypomastigotes were incubated in phosphate saline buffer at 22° C with glucose, glycerol and α-oxoglutarate when present at 20 mM and SHAM, 1.0 mM. Several motility results were verified by an independent observer.

Table 3.4 : The effect of glycerol on the motility of a pleomorphic T. brucei preparation under aerobic and glycerol-3-phosphate oxidase inhibited conditions.

Time mins	Additions to phosphate saline buffer								
	Glucose	Glucose SHAM	Glucose Glycerol	Glucose SHAM Glycerol	Glycerol	SHAM Glycerol	α-Oxoglutarate	SHAM α-Oxoglutarate	
0	++++	+++	++++	–	++	–	++	+	+++
7	+++	+++	++	–	++	–	+	–	–
12	++	++	+	–	++	–	+	–	–
21	+++	+++	+++	–	+	–	+	–	–
35	+++	+++	+++	–	+	–	+	–	–

The incubation media were as described in Table 3.3 and several motility results were verified by an independent observer.

combination to bloodstream trypanosomes *in vitro* with or without glucose present, while 1.0 mM-SHAM alone can inhibit α-oxoglutarate utilization by SS trypomastigotes. This shows that anaerobic energy production of the type found in glucose metabolism is not seen in α-oxoglutarate metabolism.

The lethal effect of glycerol plus SHAM on pleomorphic preparations was re-established in other experiments but this finding contrasts with the observations of Clarkson and Brohn (1976) who found that when SHAM and glycerol are injected into a rat with a pleomorphic *T. rhodesiense* infection SS forms are found in the blood an hour after treatment whereas LS forms are cleared.

3.3 Inhibition of Pyruvate Production from Carbohydrates in *T. brucei* under Anaerobic Conditions

Glucose is metabolised to equimolar pyruvate and glycerol anaerobically while 20 mM-glycerol was found to lyse trypanosomes utilizing glucose anaerobically. In this subsection the effect of glycerol on anaerobic glucose utilization was examined. Pyruvate production was measured rather than glucose utilization because this was more accurate and as a result less glucose needed to be metabolised which in turn meant less glycerol was produced, thereby lessening the variation in concentration of glycerol added exogenously as the variable inhibitor.

Fig. 3.1 gives the results of two separate experiments with varying initial glycerol concentrations and measuring the rate of pyruvate production from anaerobic glucose metabolism. An average I_{50} (concentration of inhibitor required for half maximal inhibition) value of 2.5 mM-glycerol was obtained.

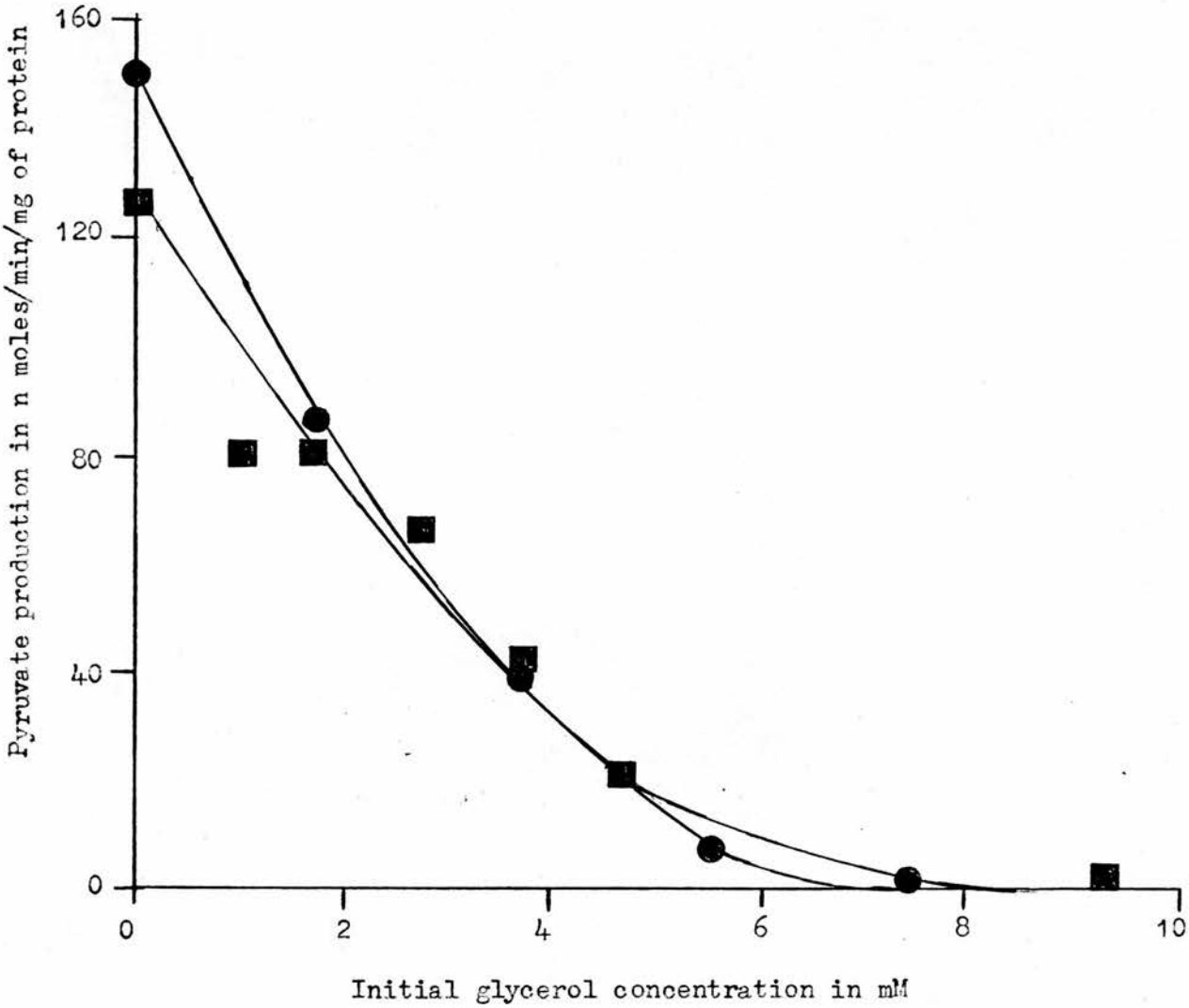


Fig. 3.1 : Glycerol inhibition of the anaerobic production of pyruvate from glucose by T. brucei. The incubation medium used was Krebs' saline at 37° C with 11 mM-glucose and varied glycerol concentrations. ●-● and ■-■ refer to two separate experiments identically performed. Other experimental details are given in the text.

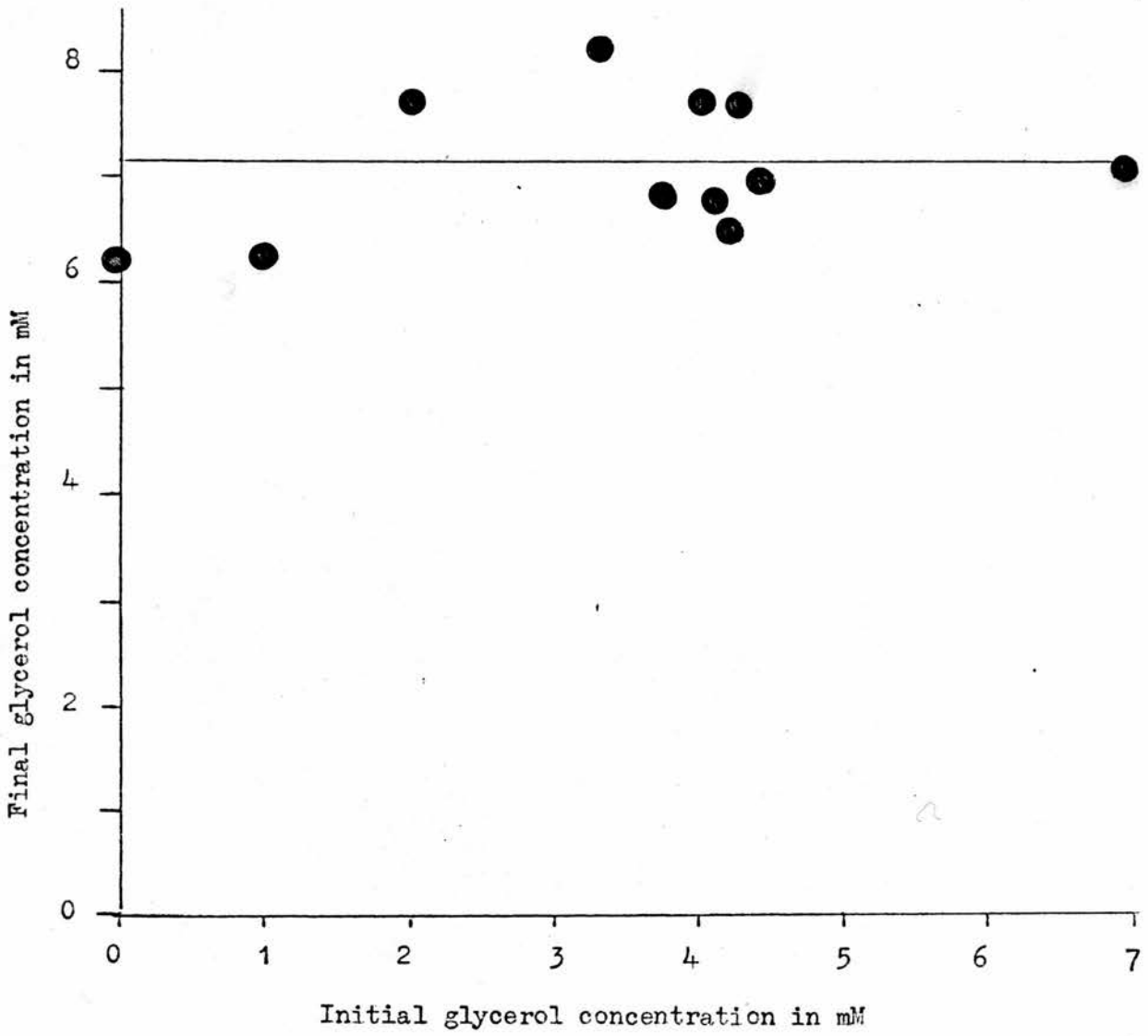


Fig. 3.2 : Final glycerol concentration produced from an initial glycerol concentration by the anaerobic metabolism of glucose by T. brucei. The incubation medium used was Krebs' saline at 37° C containing 11 mM-glucose and varied glycerol concentrations. Glycerol estimations were carried out at time 0 and when microscopic analysis revealed extensive cell lysis.

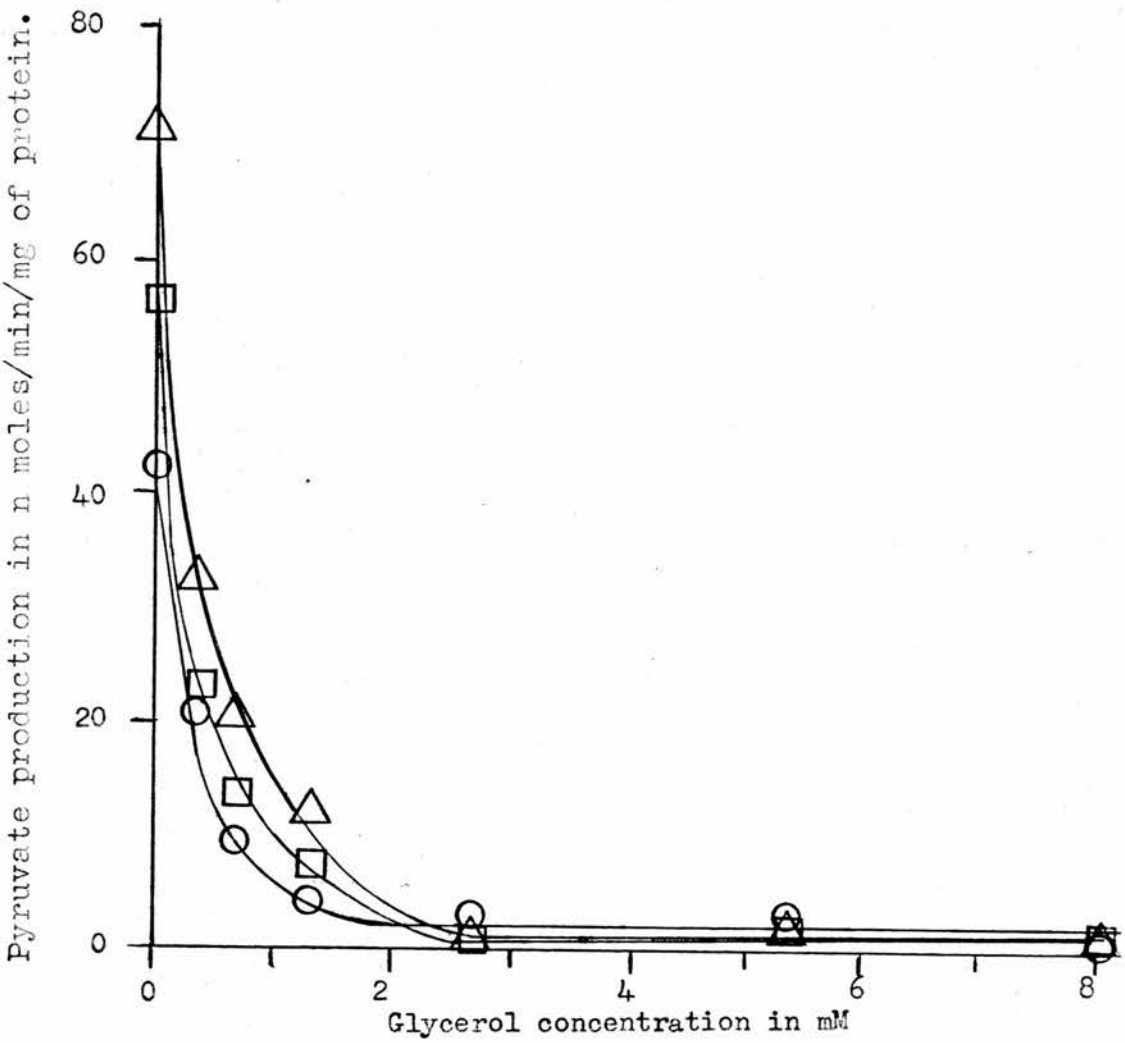


Fig. 3.3 : Anaerobic production of pyruvate from glucose, fructose and mannose against varying glycerol concentrations for T. brucei. Trypanosomes were prepared by the standard method except they were washed glucose-free before being incubated in Krebs' saline at 37° C with 11 mM-substrate and varied glycerol present. All experiments were carried out on the same preparation, with Δ-Δ representing the glucose incubation; □-□ fructose and 0-0 mannose.

This value is higher than the average value of 0.8 mM reported by Fairlamb et al. (1977), while Brohn and Clarkson (1978) obtained an intermediate value. The I_{100} value obtained from Fig. 3.1 lies between 6 and 8 mM and was more accurately assessed by the experiment summarized in Fig. 3.2. This method relied on establishing the final glycerol concentration at which trypanosome motility ceased and cellular fragmentation began. A value of 7.05 ± 0.7 mM was obtained with the final glycerol concentration independent of the initial glycerol concentration providing the initial glycerol concentration did not exceed the I_{100} value. This value is in general agreement with that found by Fairlamb et al. (1977) of 5 mM and Brohn and Clarkson (1978) of 4.5 mM, but like the I_{50} value it is higher.

As a corollary to these experiments the I_{50} values of glycerol inhibition of anaerobic mannose and fructose utilization were compared to those for glucose. Fig. 3.3 shows the results of one experiment, in which the trypanosomes were washed free from glucose present in the preparative salines before being added to the various incubation media. Glycerol inhibited the anaerobic utilization of mannose, fructose and glucose with similar I_{50} values (of below 0.5 mM) and I_{100} values (2 mM) but these were very much lower compared to the previous I_{50} and I_{100} result for inhibition of glucose utilization. Subsequent experiments showed that the I_{50} was decreased in suspensions that had previously been prepared in substrate free salines. However glycerol inhibition of anaerobic glucose utilization was found to be independent of the glucose incubation concentration between 2 - 200 mM.

The I_{50} and I_{100} values for glycerol inhibition of anaerobic glucose utilization are about 2.5 mM and 7 mM respectively for trypanosomes maintained in a

Table 3.5 : Adenylate charge, motility and adenine nucleotide concentrations of T. brucei under aerobic, anaerobic and SHAM treated incubations

Substrate	Gas phase	SHAM concn in mM	Motility	Concns in n moles/mg of protein			Adenylate charge	Glycerol concn in mM
				ATP	ADP	AMP		
	aerobic		±	0	8	9	0.23	0.02
Glucose	aerobic		++++	18	3	2	0.88	0.16
Glucose	aerobic	1.0	++++	9	10	8	0.52	0.94
Glucose	anaerobic		++++	10	8	8	0.53	0.92

This experiment was performed in Krebs' saline at 18° C. The concentration of glucose when present was 11 mM and the glycerol concentrations refers to the medium glycerol concentration at the end of the experiment. The motility tests were substantiated by an independent observer.

substrate rich medium before analysis. The lack of quantitative agreement with Fairlamb et al. (1977) and Brohn and Clarkson (1978) may well reflect a difference in preparation technique.

Glycerol cannot inhibit the uptake of sugars as the I_{50} and I_{100} values would be independent of the preparation method. The similar I_{50} and I_{100} values obtained for glycerol inhibition of the anaerobic utilization of fructose, mannose and glucose suggests glycerol must inhibit a reaction common to the metabolism of all three sugars. From substrate depleted media at 0^{o} C to glucose rich media at 25^{o} C trypanosomes require 4 - 5 minutes to attain maximum oxygen utilization whereas with trypanosomes permanently maintained with saturating glucose at 0^{o} C less than a minute is required. It is assumed that this reflects a difference in glycolytic intermediate concentrations with the substrate depleted condition requiring a longer incubation time before steady-state concentrations are achieved. Therefore it is proposed that the I_{50} and I_{100} values are variable and will depend upon intracellular glycolytic intermediate concentrations.

3.4 The Effect of Anaerobiosis on the Motility and Adenylate Charge of T. brucei

Previously it has been shown that glucose can support trypanosome motility under anaerobic conditions as effectively as it can aerobically (Tables 3.3 and 3.4). This subsection examines the effects of anaerobiosis on the adenylate charge of T. brucei.

The results of Table 3.5 refer to one experiment although similar results were obtained in subsequent studies. The incubation time for these experiments was

8 minutes, and a low temperature was employed to allow high trypanosome concentrations to be used without causing too rapid an increase in glycerol concentrations in the anaerobic or SHAM inhibited media. If the glycerol concentration were allowed to approximate the I_{100} value for inhibition of anaerobic glucose utilization, cell lysis may occur. The decreased adenine nucleotide concentration in the substrate depleted condition may well be due to cell lysis. A further advantage of using 18° C in preference to 37° C is that the oxygen concentrations of the buffer are similar but oxygen consumption aerobically is very much decreased at the lower temperature, thus helping to maintain sufficient oxygen in the aerobic condition.

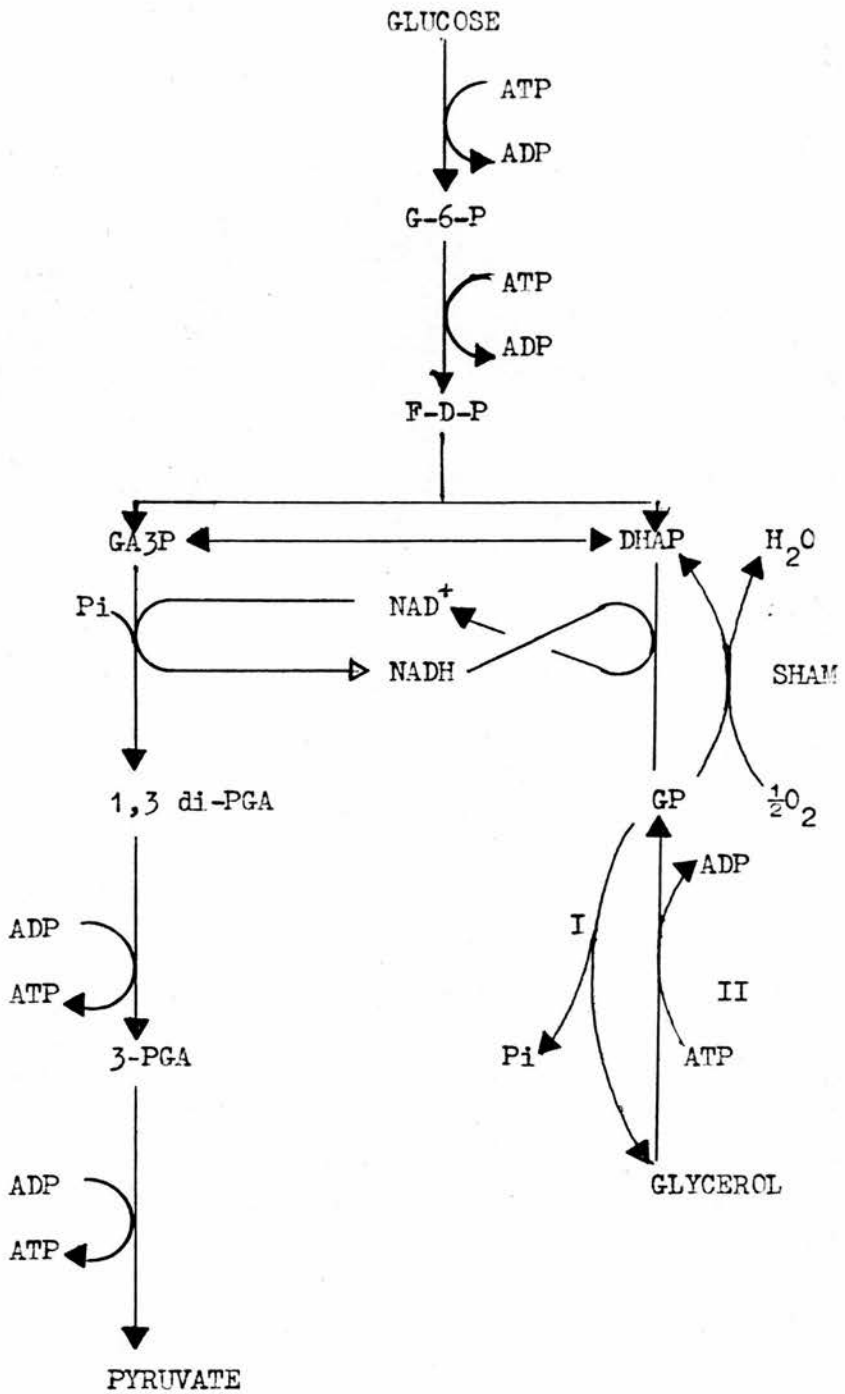
The lack of trypanosome survival in the substrate depleted media establishes the absence of a significant endogenous energy source in LS trypomastigotes. Net ATP synthesis must therefore occur anaerobically and in the SHAM treated condition, although at a much decreased rate compared to the aerobic condition. Two factors contribute to the decrease in ATP synthesis from the anaerobic metabolism of glucose, firstly the glycolytic flux is decreased anaerobically and secondly on thermodynamic efficiency grounds only 1 mole of ATP per mole of glucose metabolised anaerobically can be synthesised compared with 2 moles aerobically. In subsequent experiments the adenylate charge was found to decrease anaerobically with increasing extracellular glycerol concentration.

These findings are qualitatively in good agreement with those of Opperdoes et al. (1976a). They do differ quantitatively in ATP concentrations. Using a luciferin/luciferase bioluminescence assay A. Smith (unpublished results) showed ATP concentrations of between 20 - 30 n moles/mg of protein, comparable to those of Table 3.5 but a factor of 10 greater than Opperdoes et al. (1976b).

Table 3.6 : Glycerol-3-phosphate phosphatase activity in *T. brucei* lysates

Method of preparation	Gas phase	Limits of detection (+) or activity (*) in n moles/min/mg of protein
Saponin lysate	nitrogen	0.6+
Saponin lysate	air	4*
Water lysate	nitrogen	0.8+
Water lysate	air	4*

The incubation medium used was : 50 mM-GP, 16 mM-$MgSO_4$, 6 mM-KCl, 1 mM-NaCl, 200 mM-TEA, and 3 mg/ml BSA at pH 7.0 with 0.5 mg of protein/ml of trypanosome lysate added. Glycerol production was allowed to proceed for 60 minutes at 37° C before samples were taken and assayed for glycerol content.



I catalysed by glycerol-3-phosphate phosphatase

II catalysed by glycerokinase

Fig. 3.4 : Glucose utilization in LS forms of T. brucei under SHAM inhibition with postulated glycerol-3-phosphate phosphatase activity.

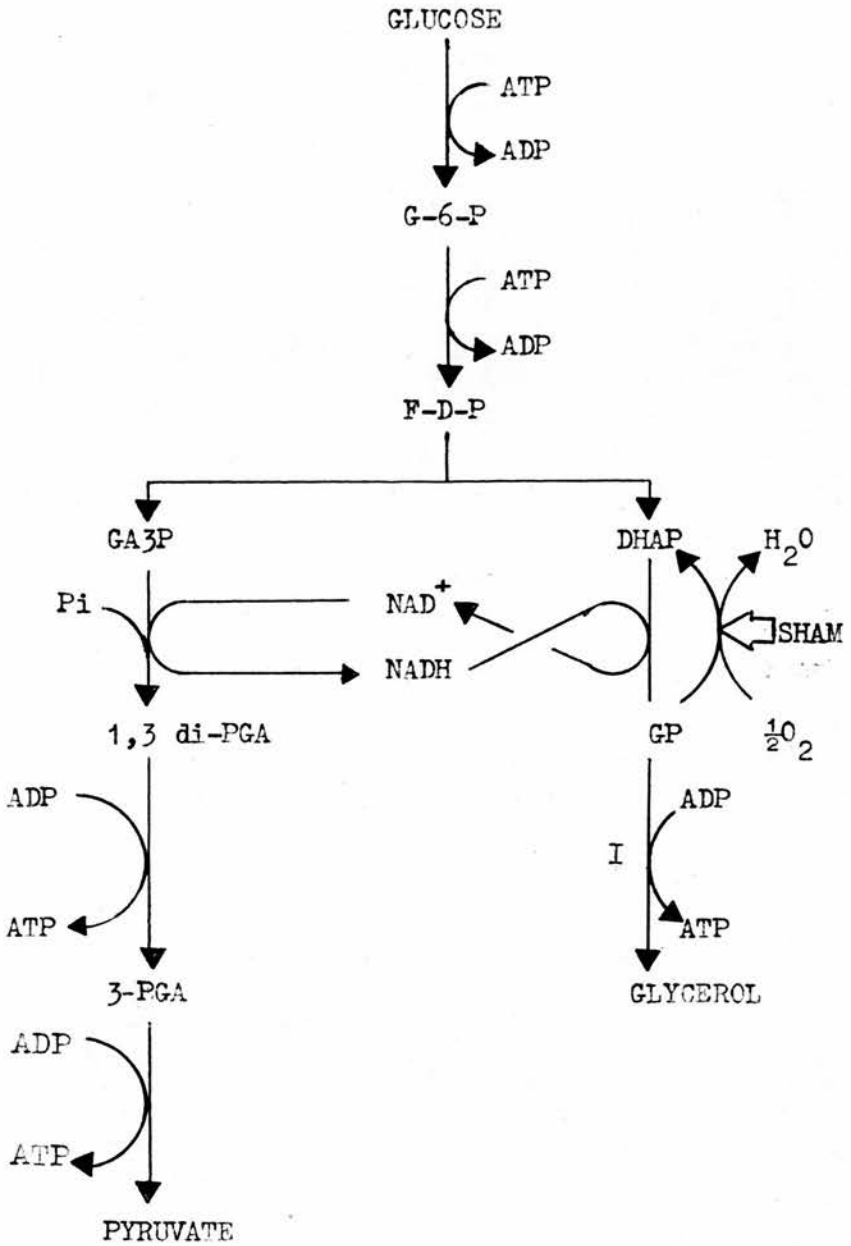
The present results confirms Smith's values and Opperdoes has retracted his original estimates (Opperdoes, personal communication). The adenylate charge found in T. brucei under a high rate of ATP synthesis, aerobically and a decreased rate, anaerobically, compares favourably with that found by Chapman et al. (1971) in Escherichia coli during growth and starvation respectively.

3.5 The Various Postulated Schemes of Anaerobic Glucose Metabolism Yielding Equimolar Pyruvate and Glycerol

It has been previously demonstrated that despite inhibition of the GP oxidase, trypanosomes can metabolise glucose, mannose and fructose with net ATP synthesis. The end product, glycerol, has been found to be an inhibitor of this pathway, but does not act on carbohydrate uptake. Its mode of action therefore is thought to be through mass action effect of the enzyme required for glycerol formation. To account for these observed facts various schemes have been postulated for anaerobic glycolysis and are tested experimentally in this subsection.

(i) Glycerol-3-phosphatase activity

Although the scheme represented in Fig. 3.4 will not account for net ATP synthesis from the anaerobic metabolism of glucose it has been tested due to its publication in the literature (Honigberg, 1967). Several experiments were carried out on lysates prepared by detergent lysis, water lysis and abrasive grinding from cells previously maintained under anaerobic and aerobic conditions. GP phosphatase activity was tested for at pH values 5, 6, 7.2 and 9.5 and at GP concentrations up to 200 mM but under none of these conditions was glycerol produced from GP at a rate comparable to that found in whole cell glycerol production of 60 n moles/min/mg of protein. The results of Table 3.6 are from a typical experiment.



I catalysed by glycerokinase

Fig. 3.5 : Glucose utilization in LS forms of T. brucei under SHAM inhibition with postulated glycerol-3-phosphate dependent ADP phosphorylation.

Table 3.7 : The activities of the enzymes required for anaerobic glycolysis by the scheme represented in Fig. 3.5

Enzyme	Method of preparation	Activity
Hexokinase	water lysate	0.48
Phosphoglucose isomerase	water lysate	0.50
Phosphofructose kinase	water lysate	0.81
Aldolase	saponin lysate	0.33
Glycerol-3-phosphate dehydrogenase	water lysate	0.48
Glycerol-3-phosphate oxidase	water lysate	0.14
Glyceraldehyde-3-phosphate dehydrogenase	water lysate	0.22
Phosphoglycerate kinase	water lysate	0.41
Phosphoglycerate mutase	water lysate	0.14
Enolase	water lysate	0.24
Pyruvate kinase	water lysate	0.20
Glycerokinase (f)	water lysate	2.9
Glycerokinase (r)	water lysate	0.017

All assays were carried out at 25° C and the activities are expressed in μ moles/minute/mg of protein. All were carried out in the direction shown in Fig. 3.5 except glycerokinase (f), phosphoglucose isomerase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase.

(ii) GP dependent phosphorylation of ADP catalysed by glycerokinase

The scheme represented in Fig. 3.5 will account for net ATP synthesis from anaerobic glucose metabolism and could account for glycerol inhibition by mass action effect of ATP and glycerol production from GP and ADP catalysed by glycerokinase. All enzymes required for this scheme are present in the trypanosome and Table 3.7 is a summary of the specific activities of these enzymes found in various buffered system from largely water lysate preparations. The assay systems for phosphoglucose isomerase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase measured the reverse reaction catalysed by the respective enzymes to that found in glycolysis. All other enzymes necessary to metabolise glucose to pyruvate are found at sufficiently high activities to account for the whole cell aerobic production of pyruvate of about 100 n moles/minute/mg of protein at 25° C, even although ion concentration, pH and the method of preparation were not necessarily optimal. Substrate concentrations were however saturating.

The specific activity of glycerokinase in catalysing the production of GP and ADP from glycerol and ATP is very high compared to the other glycolytic enzymes. This is in agreement with the findings of Opperdoes and Borst (1977) and Oduro (1977). The very high activities are surprising since glycerol is not likely to be a significant source of energy for T. brucei in the vertebrate bloodstream and a physiological role of this enzyme in catalysing the reverse reaction will explain this observation (Opperdoes and Borst, 1977).

The rates of glycerol plus ATP production from GP plus ADP given in Table 3.7 have been subsequently found to be sub-optimal but none the less are of the correct order of magnitude to account for the whole cell anaerobic rate of glycerol production at 25° C of about 50 n moles/min/mg of protein. However

Table 3.8 : Glycerol-3-phosphate : glucose transphosphorylase activity in T. brucei water lysate

Additions to media	Concentration of G-6-P plus F-6-P combined in μ moles/ml		
	0 mins	10 mins	80 mins
GP, glucose	0.0	0.0	0.0
GP	0.0	0.0	0.0
Glucose	0.0	0.0	0.0
GP, glucose, ATP	0.0	0.9	1.0
Glucose, ATP	0.0	0.9	1.0
ATP	0.0	0.0	0.0

The incubation medium contained : 10 mM-$MgSO_4$, 50 mM-Na_2HPO_4, 100 mM-TEA, 3 mg/ml BSA, and when appropriate 100 mM-GP, 26 mM-glucose and 2 mM-ATP at pH 7.0 with 0.78 mg of protein/ml of trypanosome water lysate added. Incubation was carried out at 37° C and samples were taken and analysed for G-6-P plus F-6-P and glycerol concentrations. Glycerol concentrations were all below 0.024 μ moles/ml, the limit of detection with high GP concentrations present.

for this scheme to be viable a degree of compartmentation of glycolytic enzymes and adenine nucleotides must exist to provide a high GP times ADP to glycerol times ATP ratio before net glycerol and ATP will be formed from GP and glycerol catalysed by glycerokinase.

(iii) Transphosphorylase activity

a) GP : glucose transphosphorylase

The presence of a GP : glucose transphosphorylase will decrease the ATP requirement in forming F-D-P from glucose to 1 ATP resulting in a net ATP production per glucose metabolised to glycerol and pyruvate, Fig. 1.3. Glycerol could inhibit the anaerobic metabolism of glucose by inhibiting the production of G-6-P through mass action effect of this transphosphorylase catalysed reaction.

Enzyme activity of the correct specificity has not been detected in lysates prepared from detergent lysis (triton X-100 and saponin), water lysis or silicon carbide grinding of cells preincubated in both aerobic and anaerobic conditions. Five different pH conditions, range 5 - 9.5, and from 5 - 500 mM-glucose and 1 - 670 mM-GP were tried for each lysate but under no conditions was G-6-P formed. G-6-P was however produced when ATP was added (via the hexokinase catalysed reaction) showing the assay system to be operational.

Lower transphosphorylase activity could be detected through incubating high concentrations of trypanosome lysates in various media and measuring G-6-P plus F-6-P combined and glycerol production from glucose and GP with time. The method of lysate preparation, pH range, glucose and GP concentrations were as described in the continual assay system and the results of a typical experiment are shown in Table 3.8. In these experiments the assay systems were found to

Table 3.9 : Glycerol-3-phosphate : fructose-6-phosphate transphosphorylase activity in T. brucei water lysates

Addition to media	Concentration of F-D-P plus ½(DHAP + GA3P) combined in μ moles/ml	
	0 mins	20 mins
GP, fructose-6-phosphate	0.0	0.1
GP	0.0	0.1
Fructose-6-phosphate	0.0	0.0
GP, fructose-6-phosphate, ATP	0.0	9
Fructose-6-phosphate, ATP	0.0	9
ATP	0.0	0.0

The incubation media contained : 10 mM-$MgSO_4$, 50 mM-KCl, 20 mM-$NaHPO_4$, 100 mM-TEA, 3 mg/ml BSA, 0.5 mM-SHAM and when appropriate 16 mM-F-6-P, 12 mM-ATP and 15 mM-GP at pH 7.4 with 1.5 mg of protein/ml of trypanosome water lysate added. Incubations were carried out at 37° C and samples were analysed for F-D-P plus ½(DHAP plus GA3P) combined and glycerol concentrations. Glycerol concentrations were below 0.024 μ moles/ml, the limit of detection with high GP concentrations present.

produce G-6-P plus F-6-P upon the addition of ATP to control incubations. The addition of glucose to GP did not enhance glycerol production indicating that other possible products of GP : glucose transphosphorylase are absent in this system.

b) GP : F-6-P transphosphorylase

The presence of a GP : F-6-P transphosphorylase will decrease the ATP requirement in forming F-D-P from glucose to 1 ATP, resulting in a net synthesis of 1 ATP per glucose metabolised to pyruvate and glycerol, Fig. 1.3. Glycerol could inhibit anaerobic glycolysis by inhibiting the production of F-D-P by mass action effect of this transphosphorylase catalysed reaction.

Enzyme activity of this specificity was examined in lysates prepared from detergent lysis (triton X-100 and saponin), water lysis and silicon carbide grinding of both aerobically and anaerobically preincubated cells. The standard assay system was employed (see appendix). Five different pH values ranging between 5 and 9.5, and from 1 - 100 mM-F-6-P and 1 - 200 mM-GP were tested for each lysate. Under none of the experimental conditions was F-D-P formed from F-6-P and GP although the system rapidly responded to F-D-P production from F-6-P upon the addition of ATP (via the phosphofructose kinase catalysed reaction). High endogenous 'NADH oxidase' activity considerably decreased the sensitivity of this system.

A lower limit of possible GP : F-6-P transphosphorylase activity could be detected through incubating high trypanosome lysate concentrations in various media and measuring the production of F-D-P and DHAP and GA-3-P combined from F-6-P and GP with time. The method of lysate preparations and pH and F-6-P and

Table 3.10 : Hexose monophosphate aldolase activity in T. brucei triton X-100 lysates

Additions to media	Activity
Fructose-1-phosphate	0
Fructose-1-phosphate, control enzyme	26
Fructose-6-phosphate	0
Fructose-6-phosphate, fructose-1-phosphate, control enzyme	30
Glucose-1-phosphate	0
Glucose-1-phosphate, fructose-1-phosphate, control enzyme	26
Glucose-6-phosphate	0
Glucose-6-phosphate, fructose-1-phosphate, control enzyme	30

The assay procedure is given in the appendix. The final concentrations of sugar phosphates was 10 mM and the final lysate protein concentration 0.1 mg/ml. These assays were carried out at pH 7 at 25° C. The enzyme activities are represented in n moles/minute and the control enzyme (at liver fructose-1-phosphate aldolase) specific activity was uninhibited by the trypanosome lysate.

GP concentration ranges were as described in the above, continual assay method and the results of a typical experiment are given in Table 3.9. The maximum rate of F-D-P plus DHAP plus GA3P combined production from F-6-P and GP was found to be similar to that obtained for GP alone, suggesting incomplete inhibition of GP oxidase is responsible for this production. Under none of the experimental conditions was significant glycerol formed from GP nor was F-D-P plus $\frac{1}{2}$(DHAP plus GA3P) production from GP increased by the addition of F-6-P.

(iv) Hexose monophosphate aldolase and glycerol dehydrogenase activity

The general scheme outlined in Fig. 1.2 requires the presence of at least two as yet undetected enzymes, a hexose monophosphate aldolase and a glycerol dehydrogenase. This scheme will produce net ATP synthesis from glucose metabolism to pyruvate and glycerol. Glycerol could inhibit anaerobic glucose utilization by inhibiting the reoxidation of NADH at the glycerol dehydrogenase catalysed step by mass action reversal.

Table 3.10 shows the results of assays for hexose monophosphate aldolase activity in water lysates by the assay technique detailed in the appendix. Similar results were however obtained for triton X-100 lysates and for homogenates prepared by silicon carbide grinding and assayed at pH 5, 7 and 9. The effect of adding 100 mM-GP to the assay media and preparing lysates from anaerobically preincubated cells were also tested. The control enzyme for these experiments was fructose-1-phosphate aldolase prepared from rat liver by the method of Leulhardt and Wolf (1955). Under none of the experimental conditions tested were fructose-1-phosphate, F-6-P, glucose-1-phosphate or G-6-P directly or indirectly metabolised to DHAP or GA-3-P plus a triose. The limit of detection of this method was about 5 n moles/minute/mg of protein.

Table 3.11 : Glycerol dehydrogenase activity in T. brucei water lysates

Pyridine nucleotide	Additions to media	Activity
NADH	Glyceraldehyde	0
	Glyceraldehyde, control enzyme	50
	Dihydroxyacetone	0
	Dihydroxyacetone, glyceraldehyde, control enzyme	45
NADPH	Glyceraldehyde	0
	Glyceraldehyde, control enzyme	20
	Dihydroxyacetone	0
	Dihydroxyacetone, glyceraldehyde, control enzyme	19

The assay procedures are given in the appendix. The final concentrations of trioses was 5 mM and the final lysate protein concentration 0.1 mg/ml. The assays were carried out at pH 7.0 at 25° C. The enzyme activities are represented in n moles/minute with both control enzyme's (NAD^+ and $NADP^+$ linked glycerol dehydrogenases from rat liver and femoral muscle respectively) specific activities being uninhibited by the trypanosome lysate.

Clarkson and Brohn (1976) have reported hexose monophosphate aldolase activity from T. brucei. The specific activity was not reported and may be too low to be accurately detected by the methods used in this thesis.

The same homogenate preparations were assayed for glycerol dehydrogenase activity (see appendix for method) at pH 5, 7 and 9. The effect of adding 100 mM-GP was also examined, but like the other assays gave negative results, see Table 3.11 for the results of a representative experiment. Both $NADP^+$ and NAD^+ linked glycerol dehydrogenase assays were tested with control enzymes NAD^+ linked glycerol dehydrogenase prepared with fructose-1-phosphate aldolase from rat liver by the method of Leulhardt and Wolf (1955) and $NADP^+$ linked glycerol dehydrogenase prepared from rat femoral muscle prepared by the method of Toews (1967). The limit of detectability using this glycerol dehydrogenase assay method was 3 n moles/minute/mg of protein.

These findings are in agreement with the observations of Clarkson and Brohn (1976) and Opperdoes et al. (1976b) but contrast with the situation found in T. hippicum by Harvey (1949).

(v) GP : triose transphosphorylase

The scheme outlined in Fig. 1.4 has been postulated by Clarkson and Brohn (1976) as a possible pathway for glucose metabolism to pyruvate and glycerol with net ATP synthesis. It could also account for glycerol's inhibiting anaerobic glucose metabolism at the GP : triose transphosphorylase catalysed step by mass action effect. However this scheme requires the existence of at least three enzymes as yet not detected with the necessary activity in T. brucei : a xylose isomerase, a monophosphate aldolase and a GP : triose transphosphorylase.

Evidence for the existence of a hexose phosphate aldolase has already been discussed. A direct assay for detecting GP : glyceraldehyde or dihydroxyacetone transphosphorylase has been developed (see appendix for details). No significant activity was found in homogenates prepared by water lysis, detergent lysis or silicon carbide grinding, at pH 5, 7 or 9 with different triose concentrations and GP concentrations up to 100 mM being tested. The assay system responded to the production of DHAP and GA3P formed from F-D-P via the aldolase catalysed reaction.

In subsequent experiments (Fig. 3.6) the incubation of trypanosome lysates in reconstituted systems containing glucose plus ATP but minus NAD quantitatively formed G-6-P, F-6-P, F-D-P and DHAP suggesting that a pathway for glucose isomerisation to fructose and then further metabolism to fructose-1-phosphate is operating at a low rate, if indeed at all under these conditions.

Conclusion

In this subsection various postulated schemes to account for anaerobic glucose metabolism to pyruvate and glycerol with net ATP synthesis have been examined. Homogenates prepared by a variety of methods have been used to test for specific enzyme activities sufficient to account for whole cell anaerobic metabolism. The effect of adding high concentrations of GP to the assays that do not directly implicate GP was examined to find if GP were required for the triggering on of the enzyme activity required for the alternative pathway (Opperdoes and Borst, 1977). Lysates were also prepared from whole cells metabolising glucose anaerobically to find if enzyme activation through intracellular modification occurred upon anaerobiosis, that could not be reproduced in a broken cell preparation.

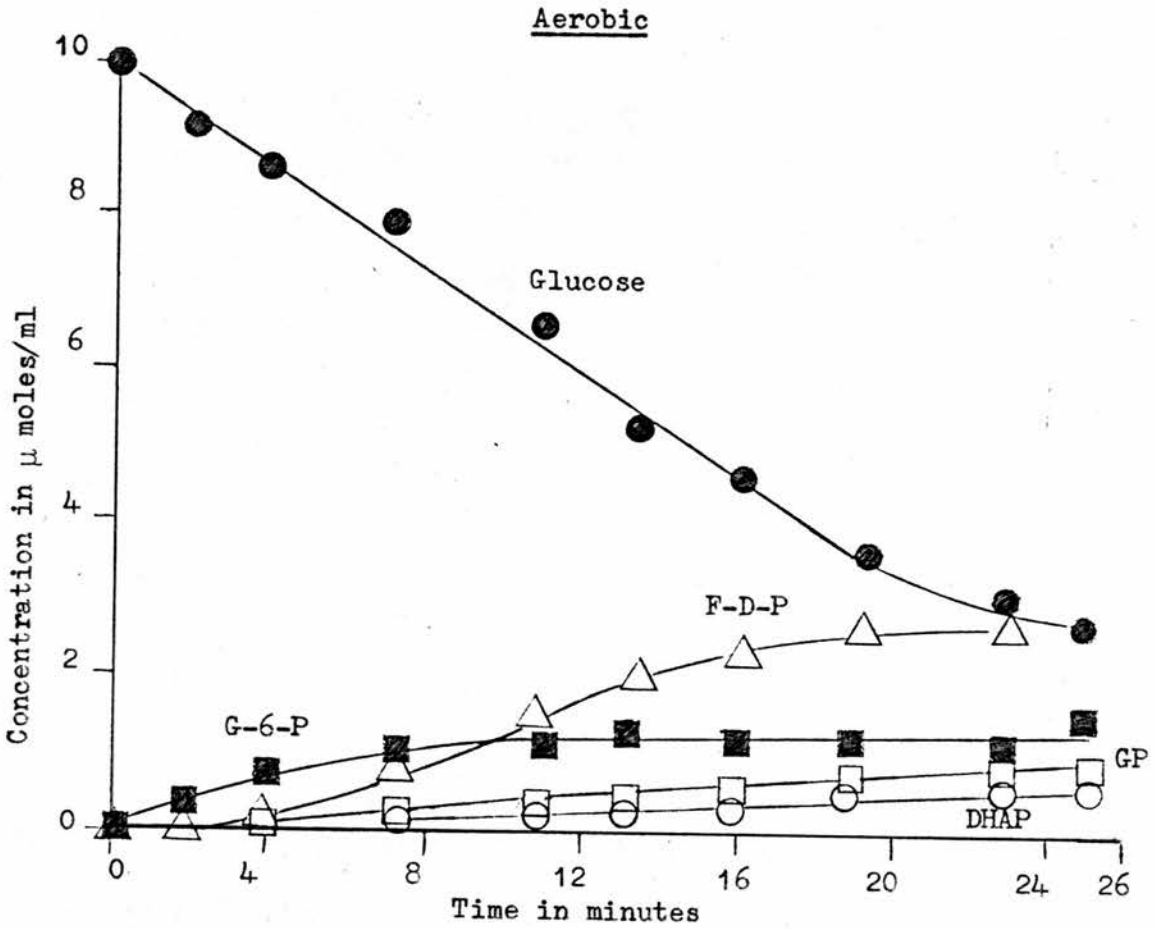


Fig. 3.6 : Concentrations of glucose and the products of its metabolism by T. brucei lysates under aerobic and anaerobic conditions.

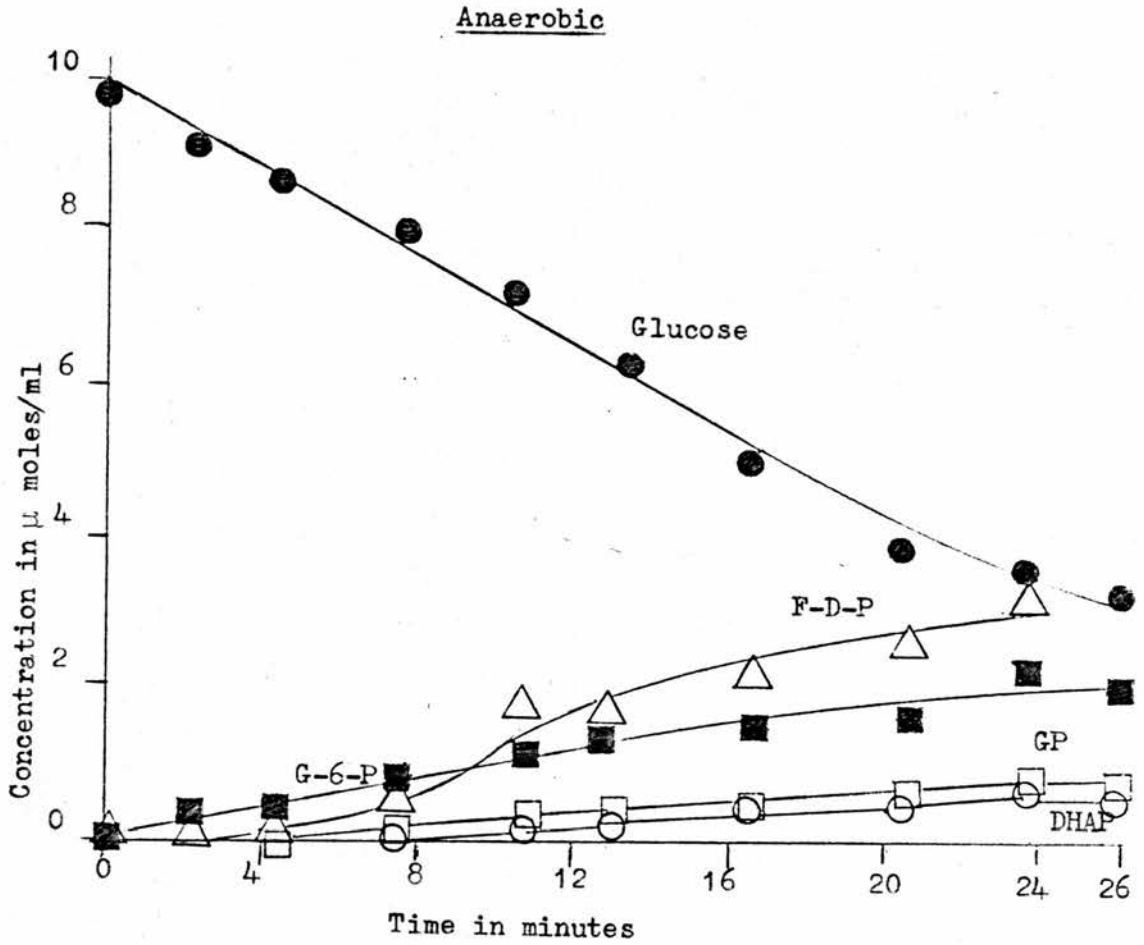
●—● Glucose

■—■ G-6-P

△—△ F-D-P

○—○ DHAP

□—□ GP



The incubation of the trypanosome water lysate of protein concentration 0.31 mg/ml was performed at pH 7.0 and at 37° C in the following medium : 50 mM-$MgSO_4$, 20 mM-$NaHPO_4$, 50 mM-KCl, 3 mg/ml BSA, 10 mM-glucose, 7.5 mM-ATP, 0.75 mM-NAD^+, 0.75 mM-NADH and 0.2 mM-nicotinamide. Also estimated but not included on the graphs were 3-PGA (max. 0.9 mM) and F-6-P (max. 0.6 mM). PEP, 2-PGA, glycerol, pyruvate and GA3P were too low for accurate determination.

Only one postulated scheme provided activities of all the enzymes of the correct order of magnitude required for glycerol's production from glucose. This scheme involved the production of glycerol plus ATP from GP plus ADP and will be further examined in this thesis. Enzyme activities to account for the other postulated schemes were found to be undetectable, which does not prove their absence but other methods of analysis must also be used to eliminate the method of anaerobic glycolysis in T. brucei proceeding by these postulated pathways.

3.6 Glucose Utilization by Lysates of T. brucei under Aerobic and Anaerobic Conditions

Specific enzyme analysis of some of the possible alternative methods of glucose utilization under anaerobic conditions or those in which GP oxidase is specifically inhibited have been tested in the previous subsections. Only the production of glycerol and ATP from GP and ADP according to the scheme outlined in Fig. 3.5 showed significant activity. This subsection deals with attempts to elucidate possible differences between aerobic and anaerobic glycolysis through estimating intermediate substrate concentrations with time and looking for points of difference between the two conditions. In this way indications of pathway differences may become evident.

Fig. 3.6 shows results from an incubation of glucose, ATP and a T. brucei water lysate in a reconstituted system under aerobic and anaerobic conditions. In another experiment F-D-P and ADP replaced glucose and ATP (Fig. 3.7) to allow the rise and fall of intermediate concentrations between F-D-P and pyruvate to be more clearly differentiated. Similar results were obtained for detergent lysates and from lysates prepared from cells preincubated in SHAM prior to lysis.

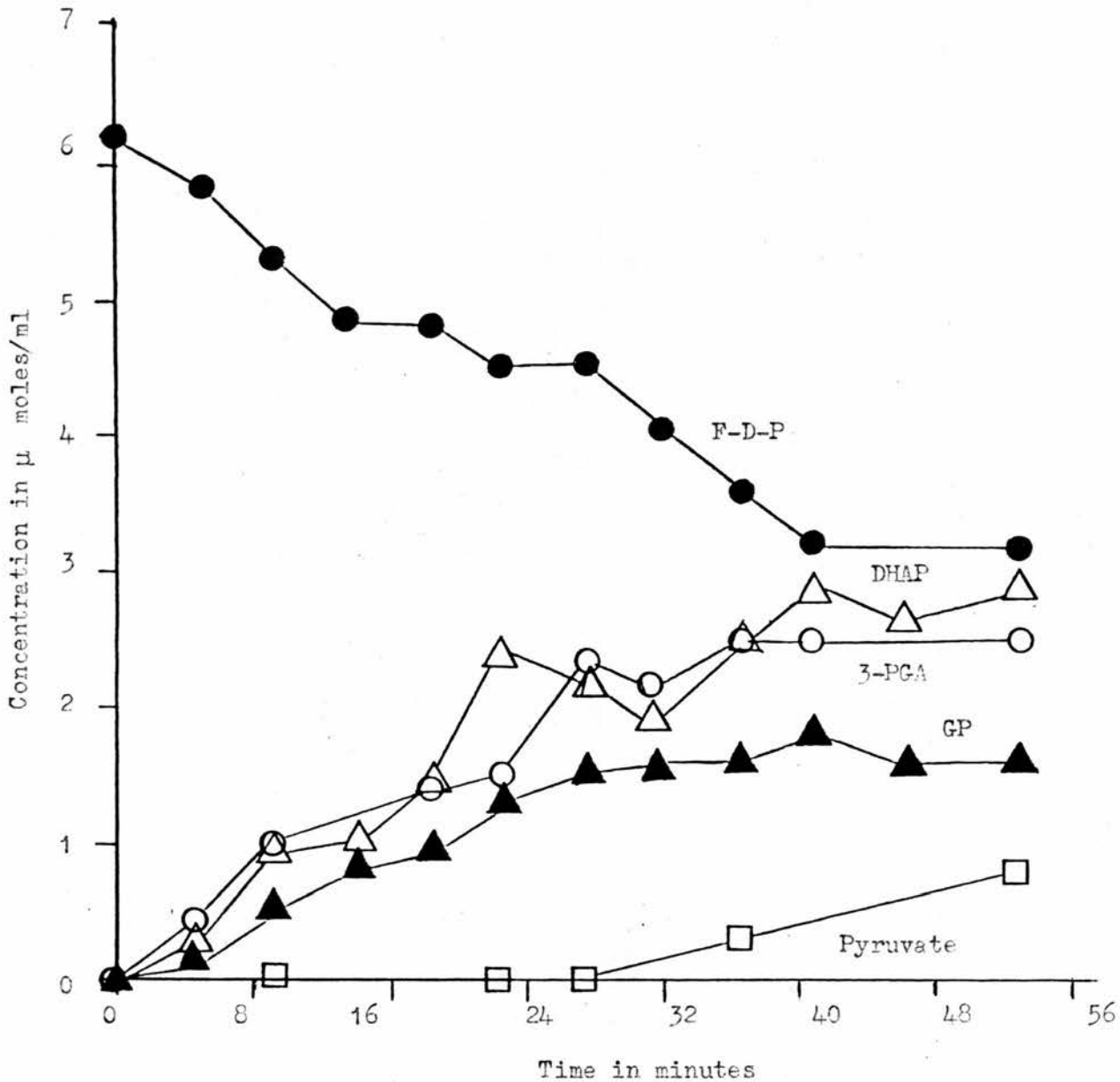
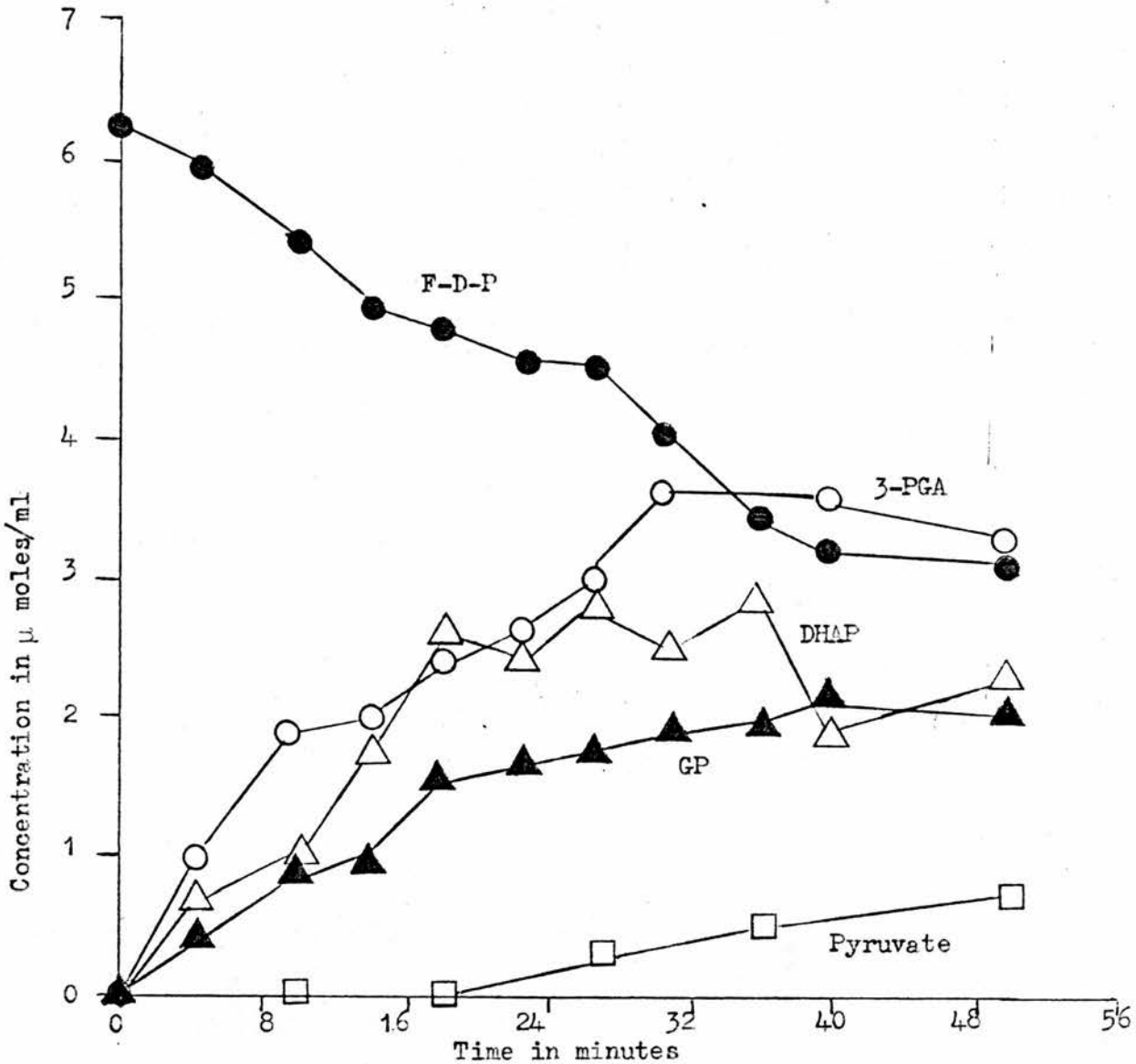
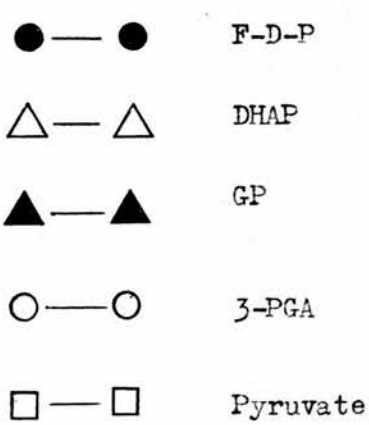


Fig. 3.7 : Concentrations of fructose-1,6-diphosphate and the products of its metabolism by T. brucei lysates under aerobic and anaerobic conditions. The lysate protein concentration was 0.56 mg/ml and the incubation was performed at pH 7.0 and 37° C in the following medium : 50 mM-$MgSO_4$, 20 mM-$NaHPO_4$, 50 mM-KCl, 3 mg/ml BSA, 6.5 mM-F-D-P, 6 mM-ADP, 0.75 mM-NAD^+, 0.75 mM-NADH and 0.2 mM-nicotinamide. GA3P, PEP, glycerol and 2-PGA were all too low to be accurately determined.

Anaerobic


Concentration in μ moles/ml
Time in minutes
F-D-P
3-PGA
DHAP
GP
Pyruvate
0
1
2
3
4
5
6
7
0
8
16
24
32
40
48
56


F-D-P
DHAP
GP
3-PGA
Pyruvate

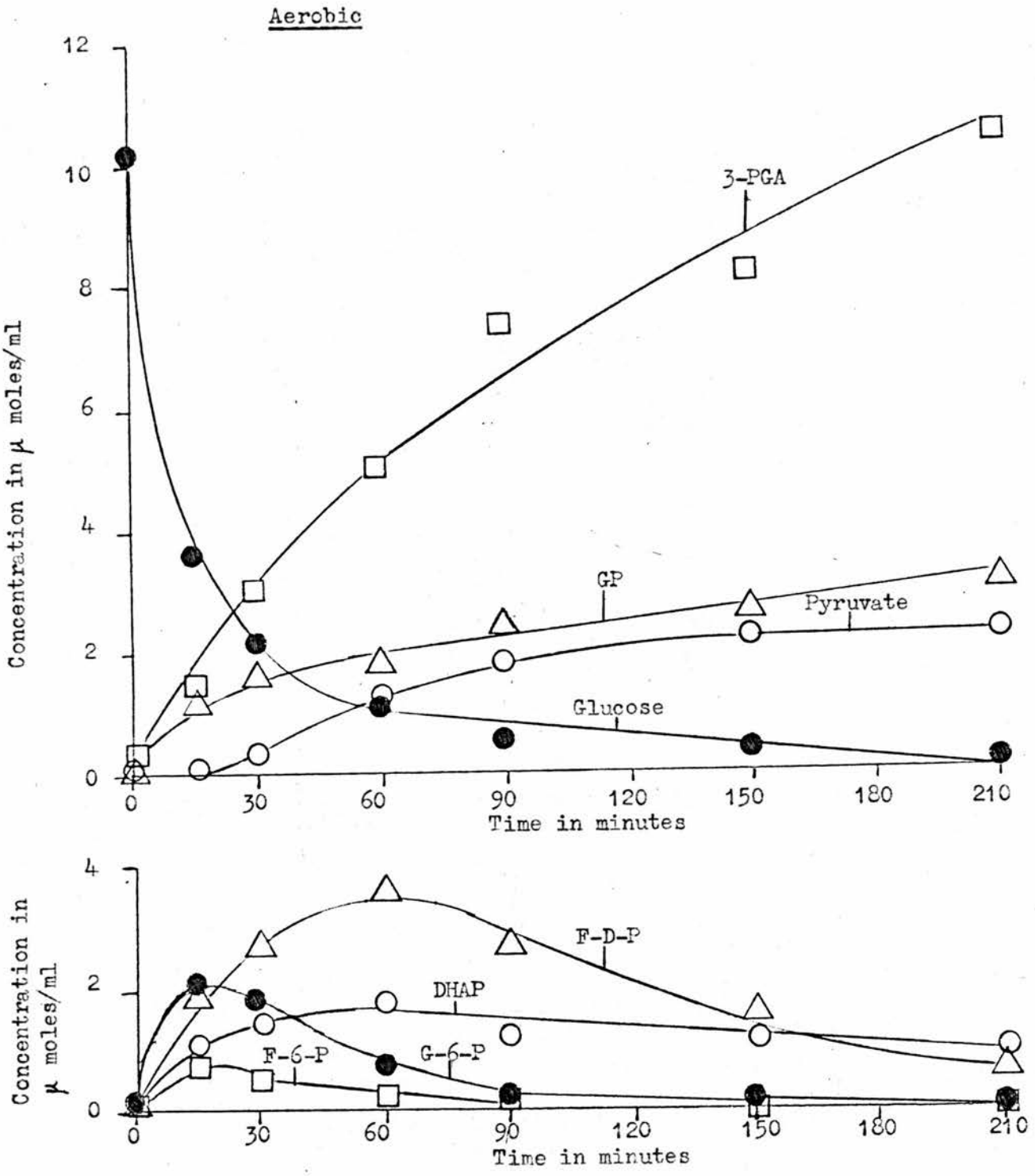
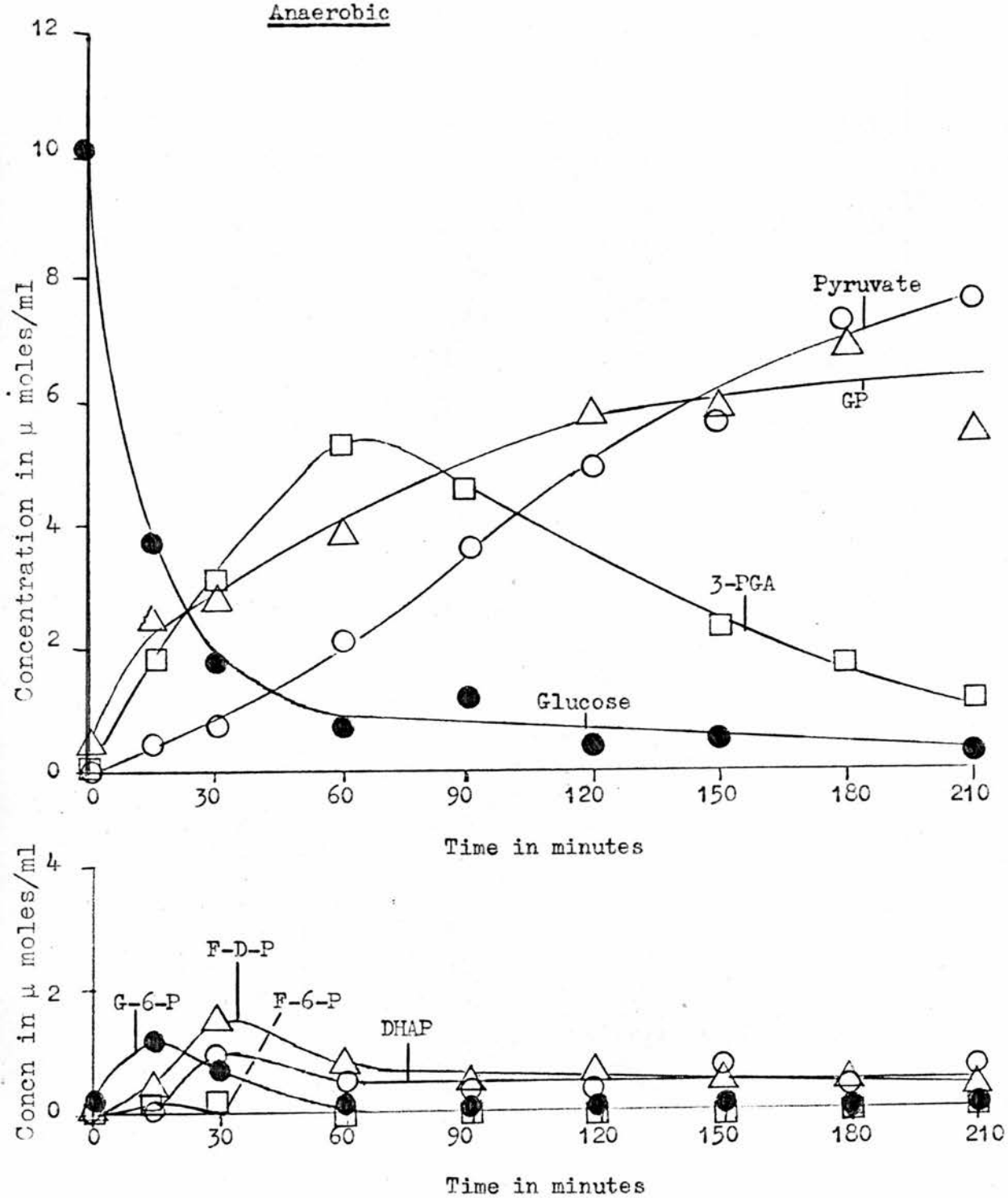


Fig. 3.8 : Concentrations of glucose and the products of its metabolism by T. brucei lysates under aerobic and anaerobic conditions for long duration incubations.



The incubation of the trypanosome lysate of protein concentration 0.94 mg/ml was performed at 37° C in the following medium : 250 mM-sucrose, 50 mM-tris, 50 mM-$MgSO_4$, 20 mM-$NaHPO_4$, 50 mM-KCl, 3 mg/ml BSA, 11 mM-glucose, 10 mM-ATP, 0.75 mM-NAD^+, 0.75 mM-NADH and 0.2 mM-nicotinanide. GA3P, PEP, 2PGA and glycerol were all too low to be accurately determined.

Glucose and ATP incubations of longer duration were performed to allow a greater time for glycerol production. Two experiments were performed with similar results, one using a water lysate, the other described in Fig. 3.8 with saponin lysed cells prepared from aerobically and anaerobically preincubated cells.

Under all conditions of glucose or F-D-P metabolism by broken cell preparations of T. brucei approximately 100% of the carbon utilized can be accounted for indicating that all the major products of metabolism have been assayed. The time dependence of the increase in the concentration of glycolytic intermediates from Fig. 3.6 demonstrates that G-6-P and F-6-P are formed first from glucose, followed by the production of F-D-P, DHAP, GP and 3PGA. Fig. 3.7 however demonstrates that DHAP and 3PGA are formed from F-D-P before GP and finally pyruvate.

The results in Fig. 3.8 verifies these observations and also shows a carbon pulsing effect through the various intermediates. This is due to glucose becoming limiting during the course of the incubation, although similar effects are observed when ATP becomes limiting. For instance the time course of the concentration changes of the reactants in this process.

$$\text{glucose} \xrightarrow{k_1} \text{G-6-P} \xrightarrow{k_2} \text{products}$$

(with glucose the rate determining factor for G-6-P production and k_1 and k_2 irreversible)

is described by the following equations :

$$-\frac{d\left[\text{glucose}\right]}{dt} = k_1\left[\text{glucose}\right]$$

and where $\left[\text{glucose}\right]_i$ = initial concentration

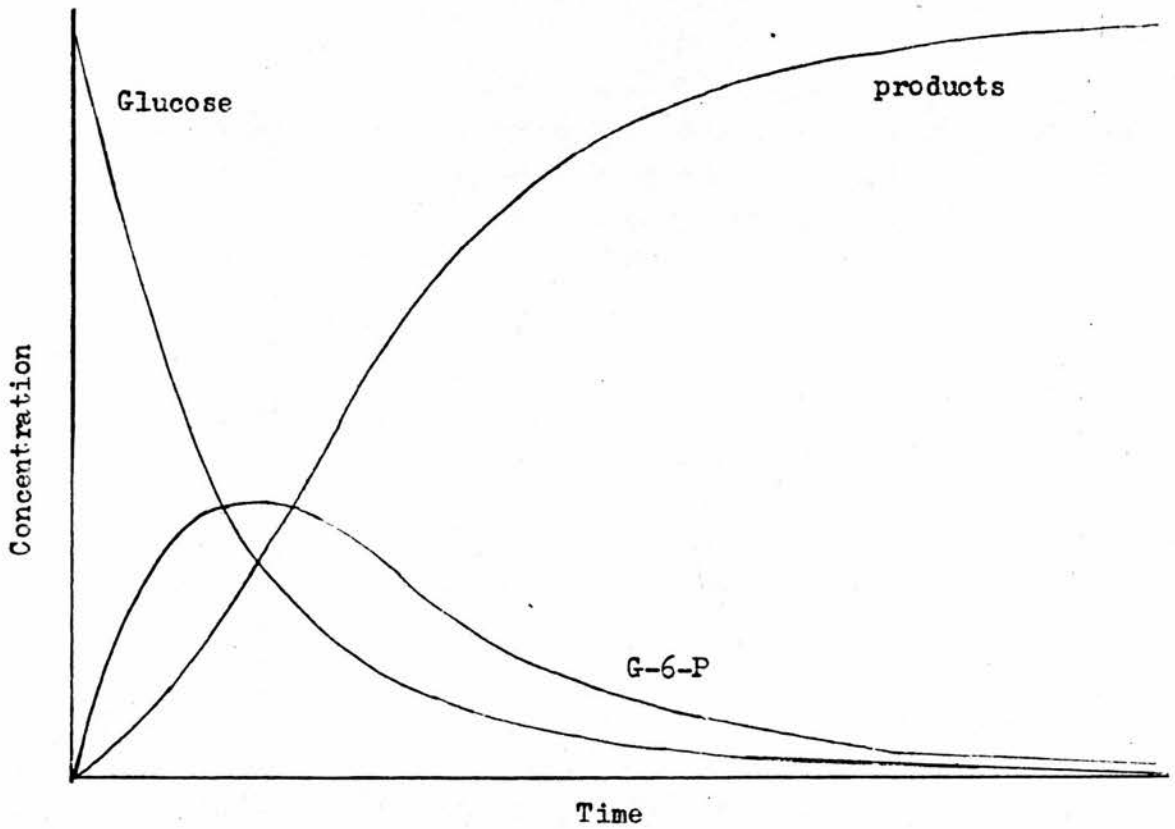


Fig. 3.9 : Analog Computer simulation of the concentration changes during the reaction glucose to glucose-6-phosphate to products, adapted from Gutfreund, 1972. Both steps of the reaction are taken as irreversible and have the same rate constant.

$$\left[\text{glucose}\right] = \left[\text{glucose}\right]_i e^{-k_1 t} \quad *$$

$$\frac{d\left[\text{G-6-P}\right]}{dt} = k_1 \left[\text{glucose}\right] - k_2 \left[\text{G-6-P}\right] = k_1 \left[\text{glucose}\right]_i e^{-k_1 t} - k_2 \left[\text{G-6-P}\right] \quad *$$

$$\left[\text{G-6-P}\right] = k_1 \left[\text{glucose}\right]_i \frac{e^{-k_1 t} - e^{-k_2 t}}{k_2 - k_1}$$

and for the condition $\left[\text{G-6-P}\right]_i = 0$

$$\left[\text{products}\right] = \left[\text{glucose}\right]_i - \left[\text{glucose}\right] - \left[\text{G-6-P}\right]$$
$$= \left[\text{glucose}\right]_i \left[1 + \frac{1}{k_1 - k_2}\left(k_2 e^{-k_1 t} - k_1 e^{-k_2 t}\right)\right]$$

A computer representation of the reaction discussed illustrates the formation of a pulse through G-6-P, Fig. 3.9. Similar pulses will be seen through the other intermediates although the concentration of the substrate will itself be a transient with its rate of formation dependent upon $\frac{d\left[\text{products}\right]}{dt}$ of the discussed reaction.

The sequence of the increase and decrease in glycolytic intermediate concentrations are in agreement with the schemes outlined in Figs. 3.4 and 3.5. However if a different scheme were operating such as that described in Fig. 1.4 a different profile of intermediate concentration increase would be expected. There are several quantitative differences in intermediate and end product concentrations between the aerobic and anaerobic incubations.

*

The rate of change of the concentrations of glucose and the products of its metabolism will be described approximately by these equations only when the following two conditions are met : firstly the concentration of ATP is saturating for hexokinase and secondly the concentration of glucose is below its Michaelis constant value for this enzyme.

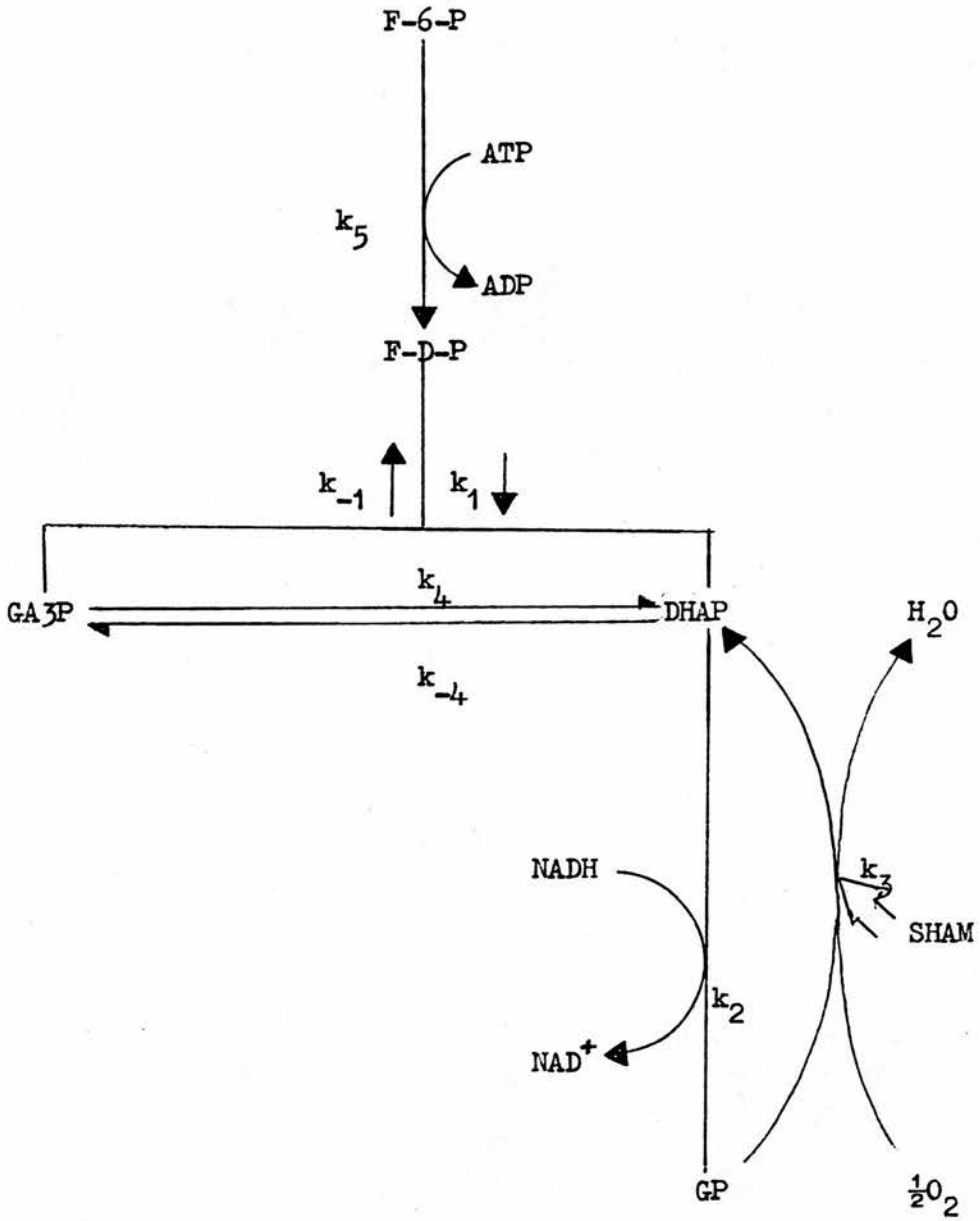


Fig. 3.10 : Reactions involving glycerol-3-phosphate, fructose-1,6-diphosphate and dihydroxyacetone phosphate in aerobic glycolysis of T. brucei.

The ratio of the concentration of DHAP to that of GP is much higher aerobically than that found anaerobically. This situation would be a consequence of the scheme outlined in Fig. 3.10. Aerobically the rate of DHAP formation minus its utilization is given by

$$\frac{d[DHAP]}{dt} = [FDP]\,k_1 + [GA3P]\,k_4 + [GP][\tfrac{1}{2}O_2]\,k_3 - k_2[NADH][DHAP] - k_{-4}[DHAP] - k_{-1}[DHAP][GA3P] \quad *$$

when GP oxidase is inhibited the rate of DHAP formation is decreased by the term $[GP][\frac{1}{2}O_2]\,k_3$. Thus the rate of DHAP formation will be lower under anaerobiosis; furthermore this will also decrease the GP concentration in aerobically compared to the anaerobic condition for the rate of GP formation minus its utilization aerobically is given by :

$$\frac{d[GP]}{dt} = k_2[DHAP]\frac{[NADH]}{K} - k_3[\tfrac{1}{2}O_2][GP] \quad *$$

and anaerobically by :

$$\frac{d[GP]}{dt} = k_2[DHAP][NADH] \quad *$$

The increased DHAP concentration aerobically will also account for the aerobic increase in F-D-P concentration using the scheme outlined in Fig. 3.10.

The rate of F-D-P formation minus its utilization will be given by :

$$\frac{d[FDP]}{dt} = k_5[F\text{-}6\text{-}P][ATP] + k_{-1}[DHAP][GA3P] - k_1[FDP] \quad *$$

Since DHAP is greater aerobically than anaerobically the rate of formation of F-D-P will be greater aerobically due to the increase in the $k_{-1}[DHAP][GA3P]$ term.

* see page 123

G-6-P and F-6-P increase to a higher concentration aerobically than anaerobically in long duration incubations but this observation is not seen in the short incubations. These facts cannot be accounted for by the available data but phosphofructose kinase is known to be effected by many intermediates and cofactors, and it is possible that different intermediate concentrations in the aerobic and anaerobic conditions may alter the activity of this enzyme.

The production of pyruvate from 3-PGA is found to be inhibited aerobically, but the addition of dithiothreitol to the incubation medium was found to greatly decrease this inhibition. Subsequent studies have shown that pyruvate kinase is rapidly inactivated in an oxidising environment, probably due to the oxidation of an essential thiol group. Dithiothreitol protects against thiol oxidation.

Only in long duration incubation studies was glycerol production measurable, and even in these conditions producation was low and varied with time. This may be due to (a) the structural integrity of the cell being required for glycerol production, (b) excess exogenous ATP being used to rephosphorylate any glycerol formed, (c) the methods of experimentation were at fault.

These incubation studies showed that all the enzymes required for pyruvate production from glucose were operational in broken cell preparations, and that the sequence of glycolytic intermediate build up was in agreement with that described in Fig. 3.4. However little glycerol was produced. No anomalies in the glycolytic flux through the various intermediates in the aerobic and anaerobic conditions were seen. Thus no indication of the existence of alternative pathways operating was obtained from these studies.

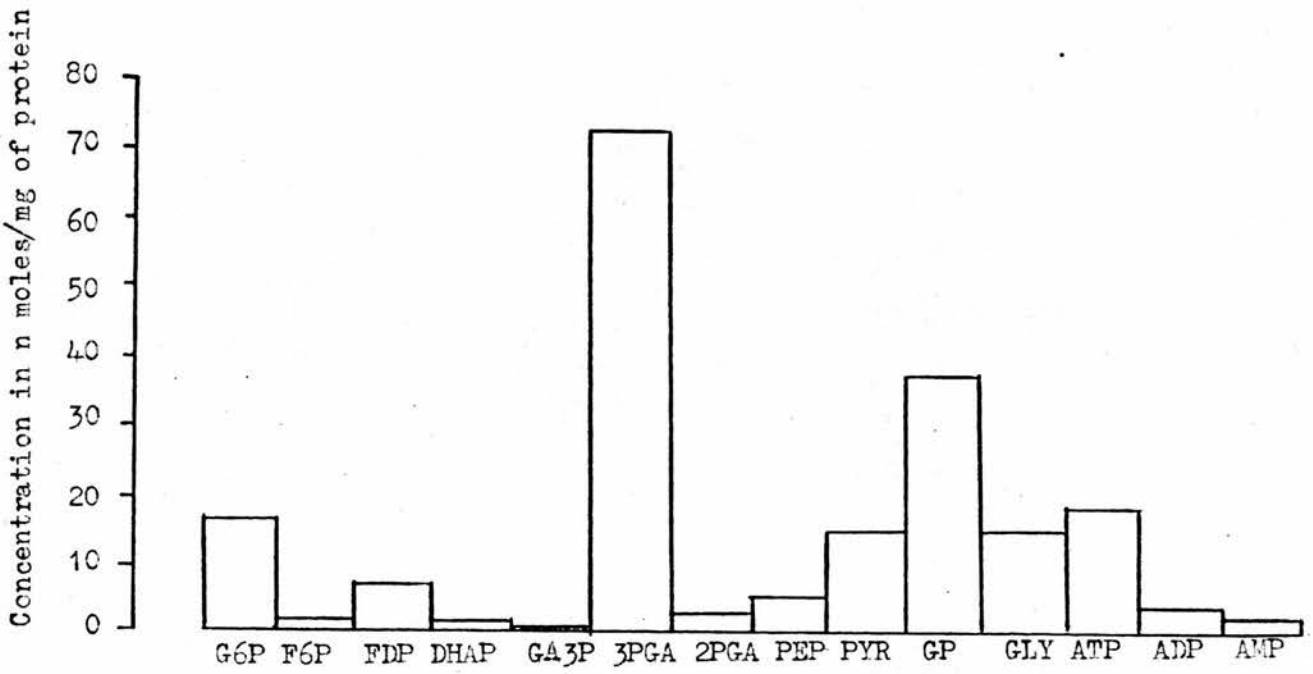
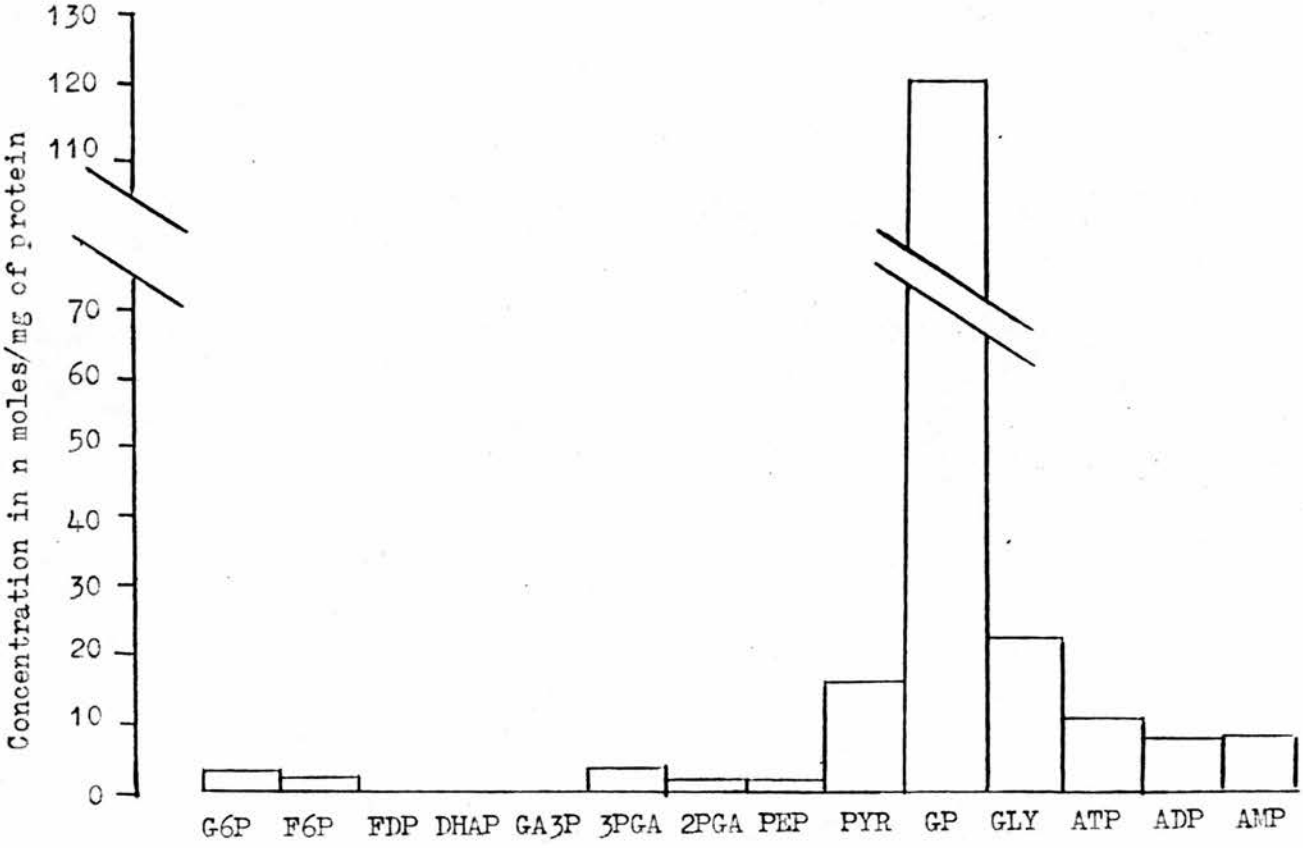
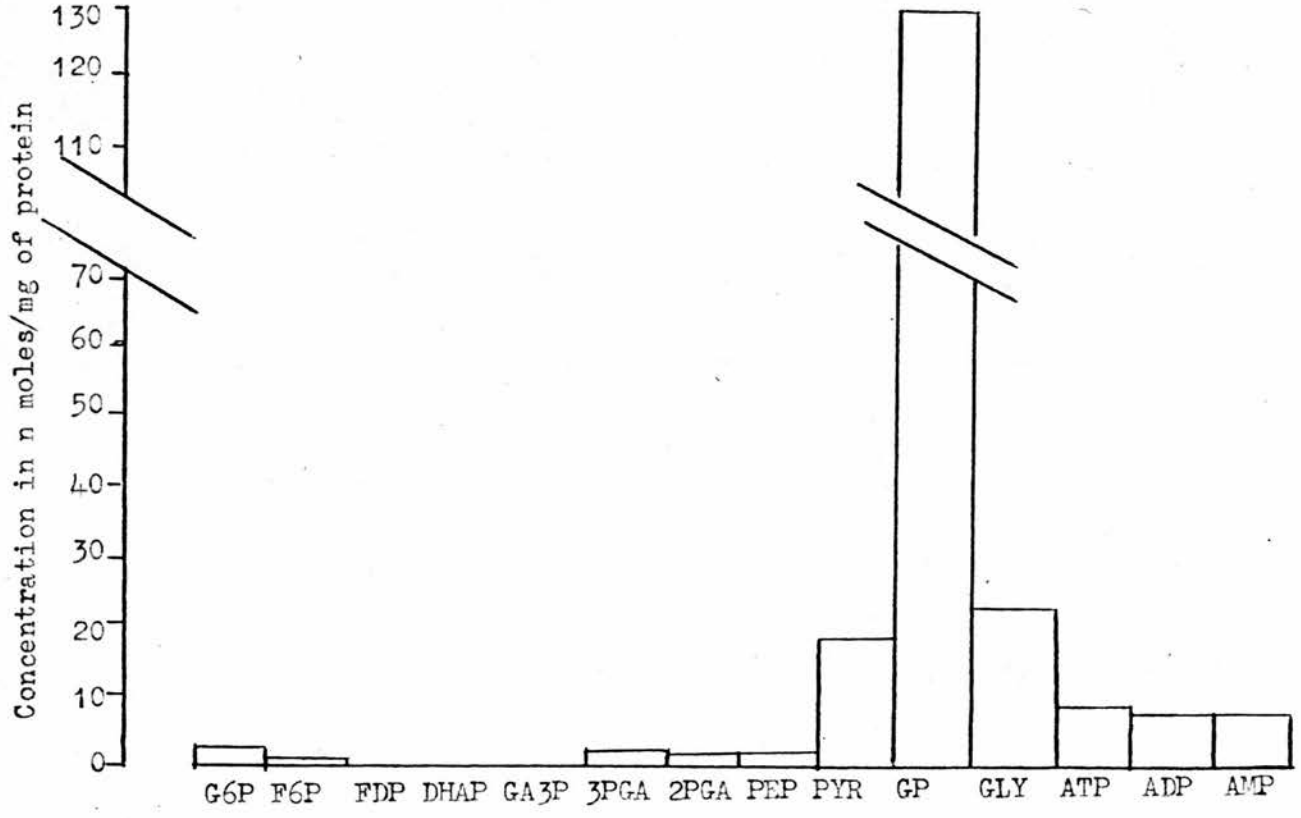


Fig. 3.11 : Glycolytic intermediates products and adenine nucleotide concentrations of T. brucei under aerobic and glycerol-3-phosphate oxidase inhibition by SHAM and through anaerobiosis. The incubations were carried out in phosphate saline at pH 8.0 and 18° C. The trypanosomes were incubated for 7 minutes before samples were taken and analysed.

Anaerobic



Aerobic plus 0.5 mM-SHAM



3.7 Determination of the Steady-state Intracellular Glycolytic Intermediate Concentrations of T. brucei under Aerobic, Anaerobic and SHAM Inhibited Conditions

Broken cell preparations have been found to possess all the enzymes required to produce pyruvate from glucose, but have so far failed to produce significant concentrations of glycerol anaerobically corresponding to those found *in vivo*. The steady-state concentrations of the glycolytic intermediates of whole cells were therefore examined to establish if differences in glycolytic intermediate concentrations could provide an indication of the alternative method for metabolising glucose anaerobically.

The glycolytic intermediate concentrations for SHAM inhibited, aerobic and anaerobic conditions are given in Fig. 3.11. These results are taken from one set of experiments, although two other identically conducted experiments provided qualitatively similar results. The SHAM inhibited and anaerobic conditions produced extremely similar glycolytic intermediate, adenine nucleotide and pyruvate and glycerol concentrations, showing SHAM inhibition causes a condition identical to anaerobiosis.

The concentrations of pyruvate and glycerol each consists of an intracellular and an extracellular component. Pyruvate is produced from glucose at least twice as fast aerobically as anaerobically resulting in a much higher extracellular pyruvate component aerobically than anaerobically. Consequently the higher overall pyruvate concentrations found anaerobically, see Fig. 3.12, indicates that a higher intracellular pyruvate concentration is found anaerobically suggesting a possible inhibition of pyruvate excretion under this condition.

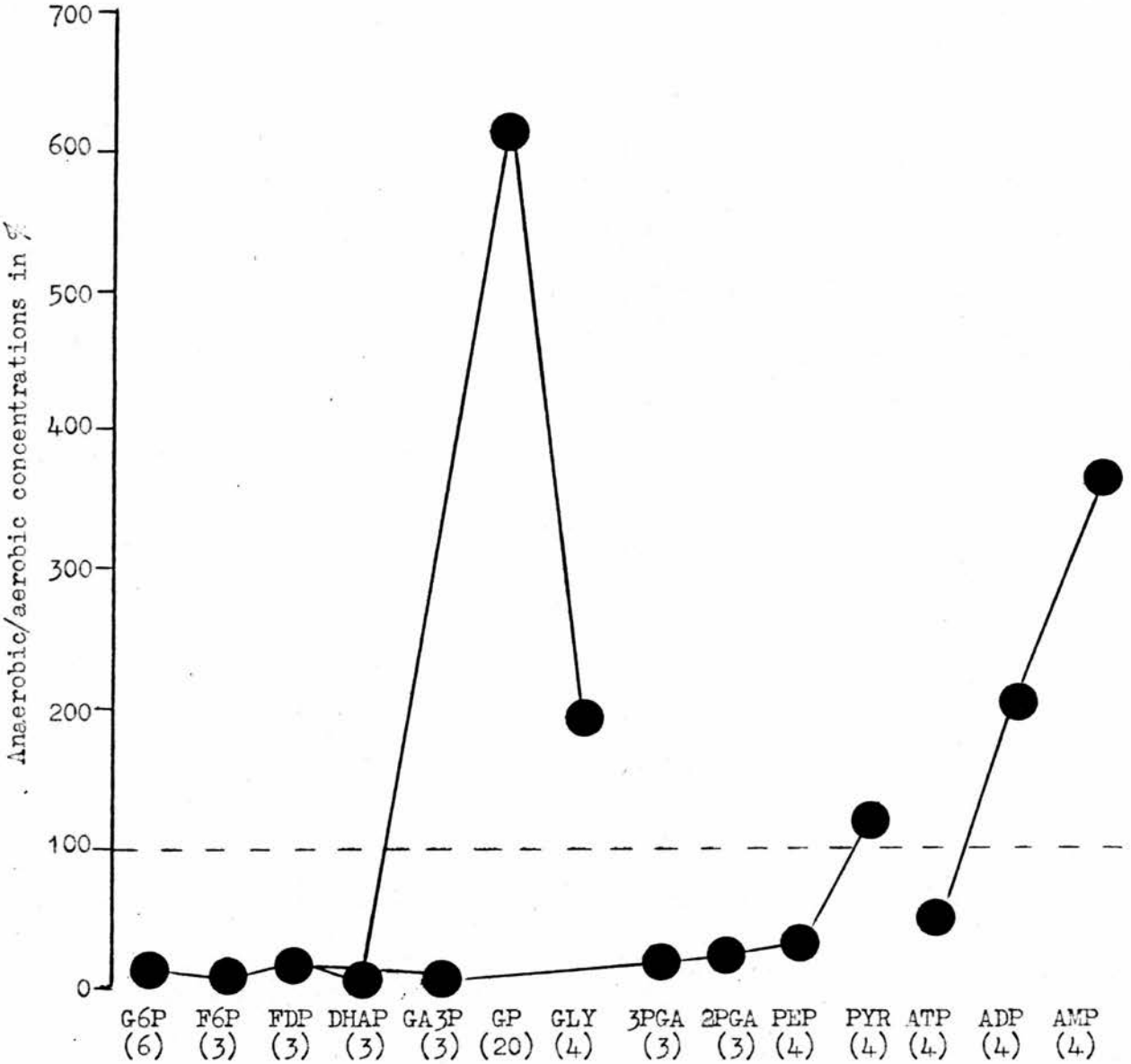


Fig. 3.12 : Anaerobic metabolite concentrations divided by the corresponding aerobic concentrations. These results were compiled from different experiments conducted as described in Fig. 3.11. The number of separate results for each compound are given in parentheses and the results averaged.

Low intracellular glycerol concentrations have always been found aerobically even if the method of extract preparation is altered to avoid concentrating the trypanosomes before deproteinisation. This would suggest that even in aerobic incubations the alternative pathway of glucose metabolism is proceeding to some extent but glycerol is re-phosphorylated by the cell with little being lost to the external medium. The concentration of trypanosomes used in subsection 3.1 would make this glycerol concentration undetectable by the standard assay procedure.

The carbon concentration of glycolytic intermediates is similar under aerobic and anaerobic conditions (Fig. 3.11), but the ratios of their concentrations varies considerably (Fig. 3.12). All glycolytic intermediates are decreased anaerobically except GP which is considerably higher than in the aerobic condition. The decrease in G-6-P, F-6-P and F-D-P concentrations can be explained by the decrease in glycolytic flux upon anaerobiosis (Table 3.2), while the concentrations of DHAP, GA3P, 3-PGA 2-PGA and PEP will be further decreased by the flux through these intermediates per glucose utilized being halved upon anaerobiosis. It is for this reason that Fig. 3.12 is branched at the triosephosphate stage of glycolysis with DHAP being metabolised to glycerol anaerobically and GA3P forming pyruvate.

The observed increase in GP concentration anaerobically is in agreement with the observations of Opperdoes and Borst (1977). This increase is expected when GP utilization via the GP oxidase catalysed reaction is inhibited and compares favourably with the broken cell situation.

These differences in glycolytic intermediate concentrations show that G-6-P production is greatly decreased anaerobically compared to the aerobic condition.

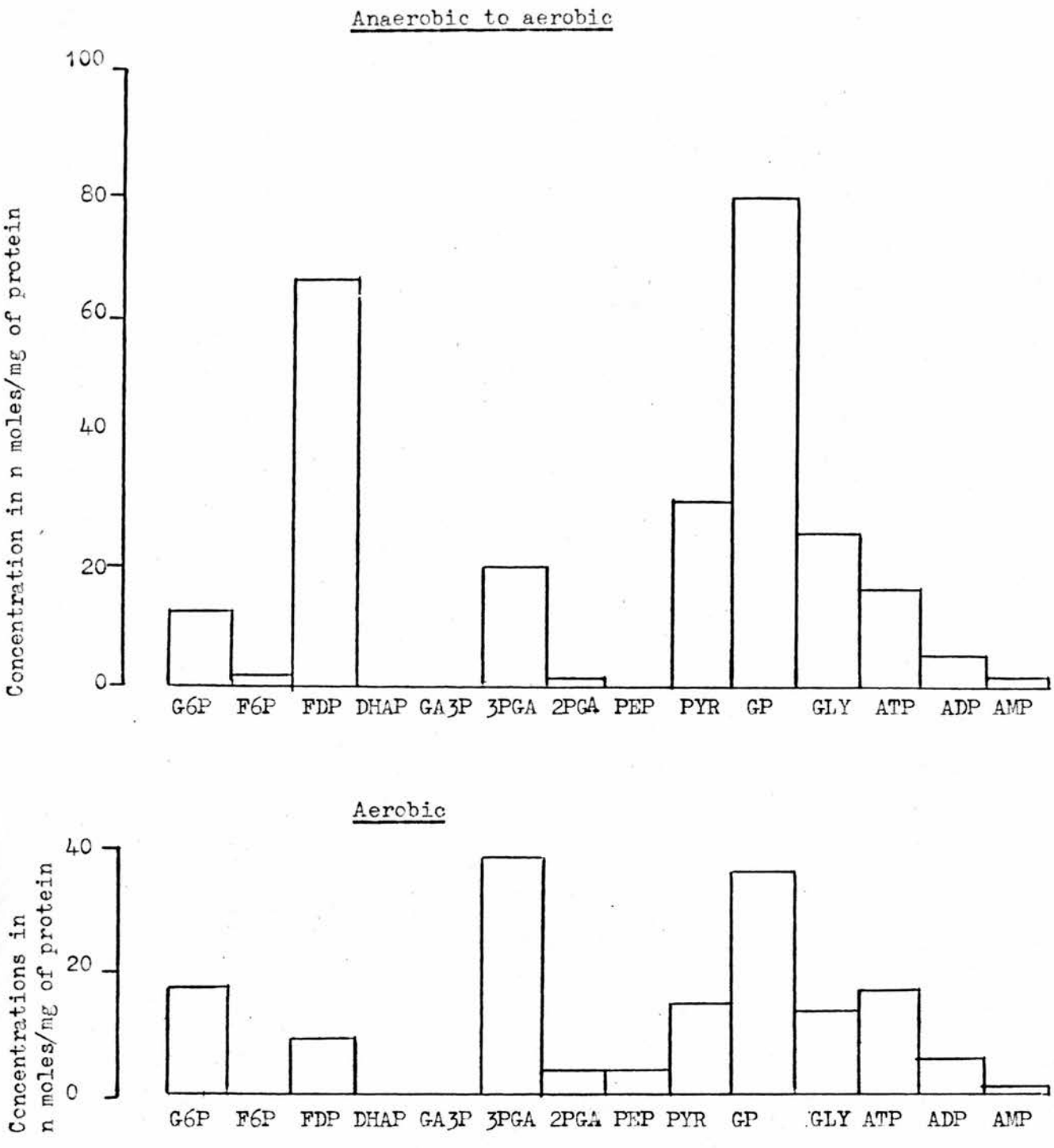
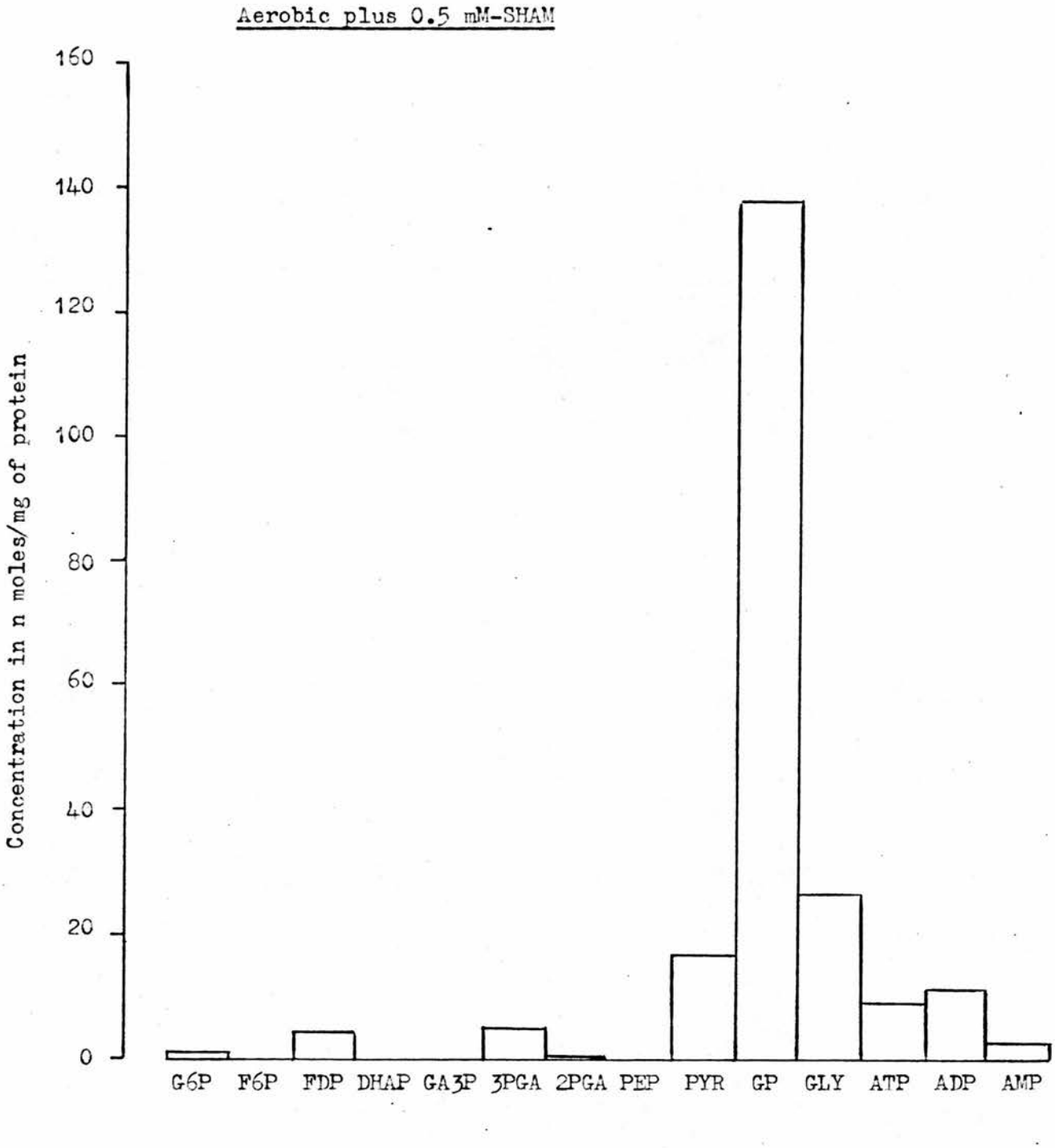


Fig. 3.13 : Glycolytic intermediates, products and adenine nucleotide concentration in T. brucei under aerobic, glycerol-3-phosphate oxidase and anaerobic to aerobic transitional conditions.



The incubations were performed in phosphate saline buffer containing 5.5 mM-glucose at pH 8.0 at 18° C. The anaerobic to aerobic transitional state was achieved by resuspending anaerobically metabolising cells into an air-saturated buffer before being concentrated and deproteinised.

Since anaerobic glucose utilization varies inversely with glycerol concentration and that this is not due to transport inhibition the decrease in G-6-P production could be due to one of three possibilities :

1. Anaerobically glucose is phosphorylated by GP catalysed by a postulated GP : glucose transphosphorylase. Glycerol could therefore inhibit glucose utilization by mass action effect of this catalysed reaction.
2. Anaerobically ATP becomes the limiting factor in the hexokinase catalysed reaction. Glycerol could inhibit ATP production by mass action effect on the glycerokinase catalysed reaction.
3. The high concentrations of GP could inhibit hexokinase and glucose is then utilized by an alternative pathway forming glycerol by means of a hexose monophosphate aldolase and glycerol dehydrogenase catalysed reactions. Glycerol could inhibit glucose utilization by mass action effect of the glycerol dehydrogenase catalysed reaction.

3.8 Intracellular Glycolytic Intermediate Concentrations of T. brucei During the Transition from Anaerobic to Aerobic Glycolysis

The previous subsection examined the steady-state glycolytic intermediate concentrations under aerobic and anaerobic conditions, while this subsection is concerned with the anaerobic to aerobic transitional state. It was hoped that the rise and fall of certain intermediates in this transitional state could lead to evidence suggesting the existence of a particular alternative pathway.

Fig. 3.13 shows the results of an experiment in which a trypanosome suspension that had been metabolising glucose anaerobically was made aerobic 3 minutes before deproteinisation. This condition is compared to the aerobic and GP

oxidase inhibition conditions. The transitional state G-6-P, GP, 3PGA and adenine nucleotide concentrations show values between those of the aerobic and anaerobic conditions. The major points of departure are the very high concentrations of F-D-P and high pyruvate concentrations found only in the transitional state. A plausible explanation for this observation is that the high GP concentrations found anaerobically are a source of stored energy, for when released under aerobic conditions 2 moles of ATP can be synthesised as GP is metabolised to pyruvate. This high ATP synthesising potential could be used in the phosphorylation of glucose, which is the rate limiting factor in glucose utilization anaerobically and subsequently F-6-P resulting in the accumulation of F-D-P. This explanation would also account for the increased pyruvate concentrations. It is possible that a small percentage of the F-D-P is formed from GP by the reversal of the aldolase catalysed reaction from the triosephosphate intermediates of the aerobic metabolism of GP.

The intracellular concentration of phosphate found covalently bound as glycolytic intermediates or adenine nucleotides is greatly increased in the transitional state, 67% higher than the aerobic and 53% higher than the anaerobic state showing phosphate availability is not limiting either aerobically or anaerobically. The concentration of carbon found as glycolytic intermediates increases by 80% in the transitional state compared to the anaerobic state and with the increase in pyruvate concentration being indicative of an increased glycolytic flux glucose and glycerol metabolism must be dramatically increased in this transition. This is in agreement with the observation that ATP availability is limiting anaerobically.

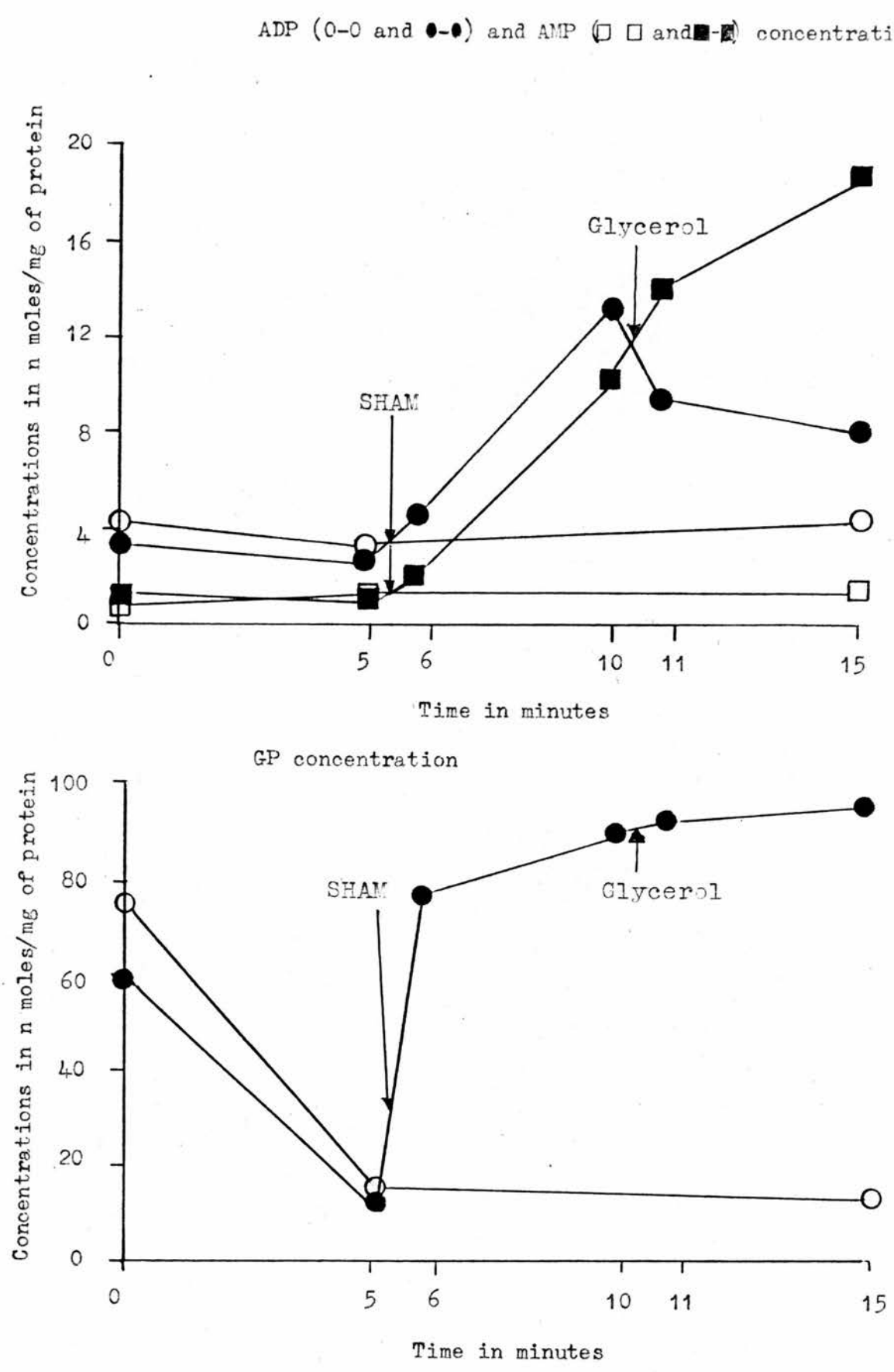
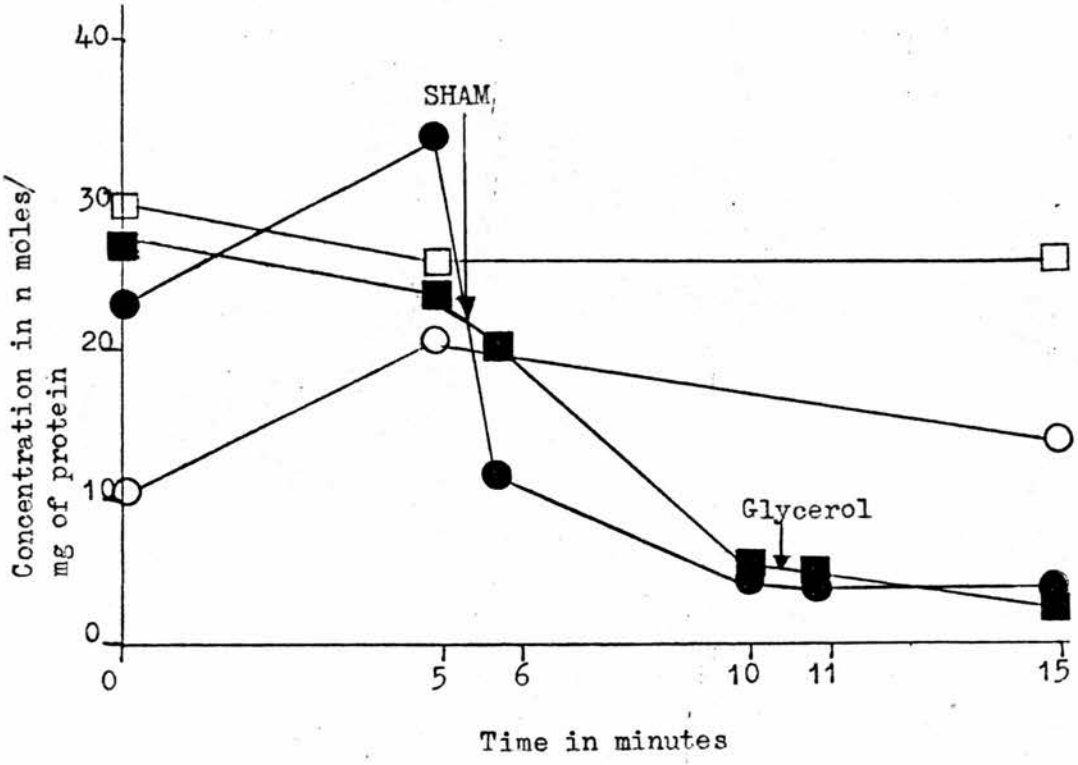
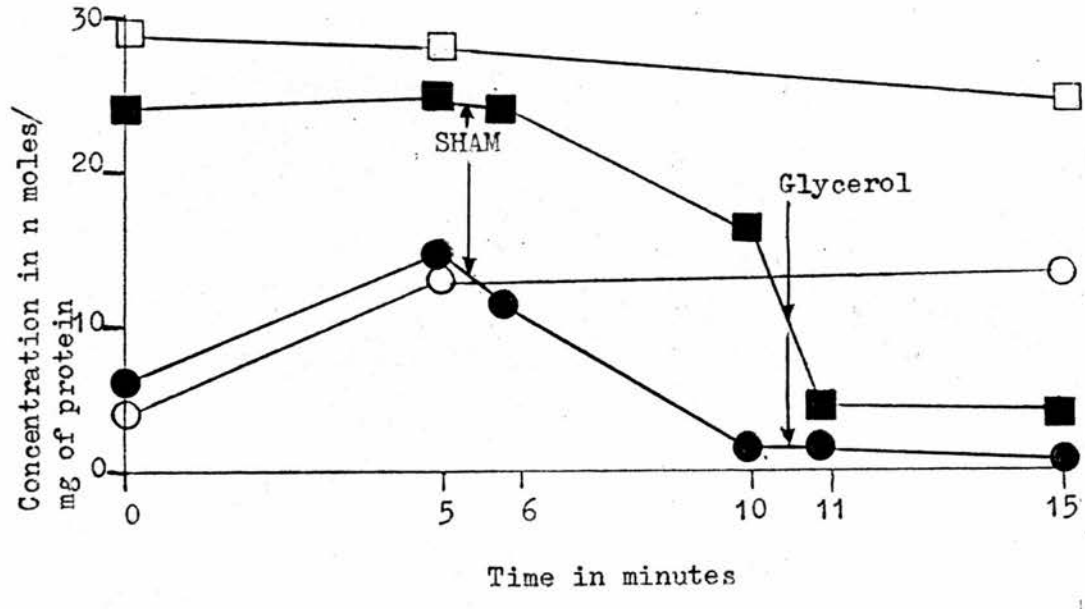


Fig. 3.14 : Glycolytic intermediate and adenine nucleotide concentrations in aerobic to glycerol-3-phosphate oxidase inhibited conditions.

F-D-P (O-O and ●-●) and 3-PGA (□-□ and ■-■) concentrations



ATP (□-□ and ■-■) and G-6-P (O-O and ●-●) concentrations



The incubation media employed was phosphate saline buffer containing 5.5 mM-glucose at pH 8.0 and 18° C. To one condition (●-● and ■-■) 0.5 mM-SHAM was added after $5\frac{1}{2}$ minutes and 5 mM-glycerol after $10\frac{1}{2}$ minutes. The other condition (□-□ and O-O) was maintained aerobically throughout.

3.9 Intracellular Glycolytic Intermediate Concentrations of T. brucei During the Transition from Aerobic to Anaerobic Glycolysis

This experiment was conducted to elucidate what happens when an aerobically respiring cell is made anaerobic and to find out what effect glycerol has on the glycolytic intermediates and adenine nucleotide concentrations of cells metabolising glucose anaerobically. It was hoped that this approach will help in the elucidation of the alternative pathway and will allow the intermediate concentrations under high glucose utilization inhibition by glycerol to be studied. An increase in the concentration of a glycolytic intermediate upon the addition of glycerol would indicate that its further metabolism is linked to glycerol formation. Glycerol could inhibit, through mass action effect, the reaction leading to its formation, causing a concomitant increase in reactant concentration e.g. F-6-P in the postulated GP : F-6-P transphosphorylase scheme.

The results in Fig. 3.14 show that at time 0 the trypanosome suspensions did not possess steady-state aerobic intracellular glycolytic intermediate concentrations but this was achieved within the first 5 minutes of incubation at 18° C. Upon the addition of SHAM, after $5\frac{1}{2}$ minutes of incubation, there is a rapid increase in the GP concentration (120 n moles/minute/mg of protein), which is much faster than the glycolytic rate at 18° C where glucose utilization is 50 n moles/minute/mg of protein, Fig. 3.16. The increased GP concentration must be at least partly derived from glycolytic intermediates present at the onset of GP oxidase inhibition most likely mainly F-D-P. The rapid response of the GP concentration shows SHAM to be a fast acting inhibitor. Compared to the F-D-P concentration, the 3-PGA and G-6-P concentrations do not decrease very rapidly during the first $\frac{1}{2}$ minute of GP oxidase inhibition, but fall dramatically over the next four minutes of inhibition. In contrast the adenine nucleotide concentrations are not rapidly

affected by the addition of SHAM, the adenylate charge only falls from 0.91 to 0.86 in the first $\frac{1}{2}$ minute and to 0.57 after $4\frac{1}{2}$ minutes, although the turnover time of ATP is about once every $\frac{1}{2}$ minute at 18° C.

An increase in the concentration of a glycolytic intermediate with increased glycerol would be expected if glycerol inhibited the anaerobic pathway of glucose metabolism by mass action effect on a GP dependent transphosphorylation. However no glycolytic intermediate concentration increases significantly upon the addition of glycerol. The rapid increase in ADP concentration is consistent with the postulated reversible glycerokinase scheme but this could be satisfactorily explained by the inhibition of ATP production from ADP by inhibition of anaerobic glucose metabolism at a different site in the pathway.

The concentration of GP rises rapidly upon GP oxidase inhibition but appears to form a plateau that is independent of time or glycerol concentration. This effect will be examined in the next subsection. The intracellular concentrations of adenine nucleotides were found to remain relatively constant between different experiments, however the concentration of GP was found to vary considerably. Higher concentrations were routinely established for trypanosomes prepared in substrate rich media. This observation agrees well with the fact that GP must be partly rapidly formed from glycolytic intermediates present at the onset of GP oxidase inhibition and that substrate depleted cells have decreased glycolytic intermediate concentrations (see 3.3).

3.10 The Effect of Glycerol on the Concentration of Glycerol-3-Phosphate of T. brucei under Aerobic and SHAM Inhibited Conditions

The results of the previous subsection showed that glycerol had minimal effect upon the intracellular GP concentrations of anaerobically metabolising cells.

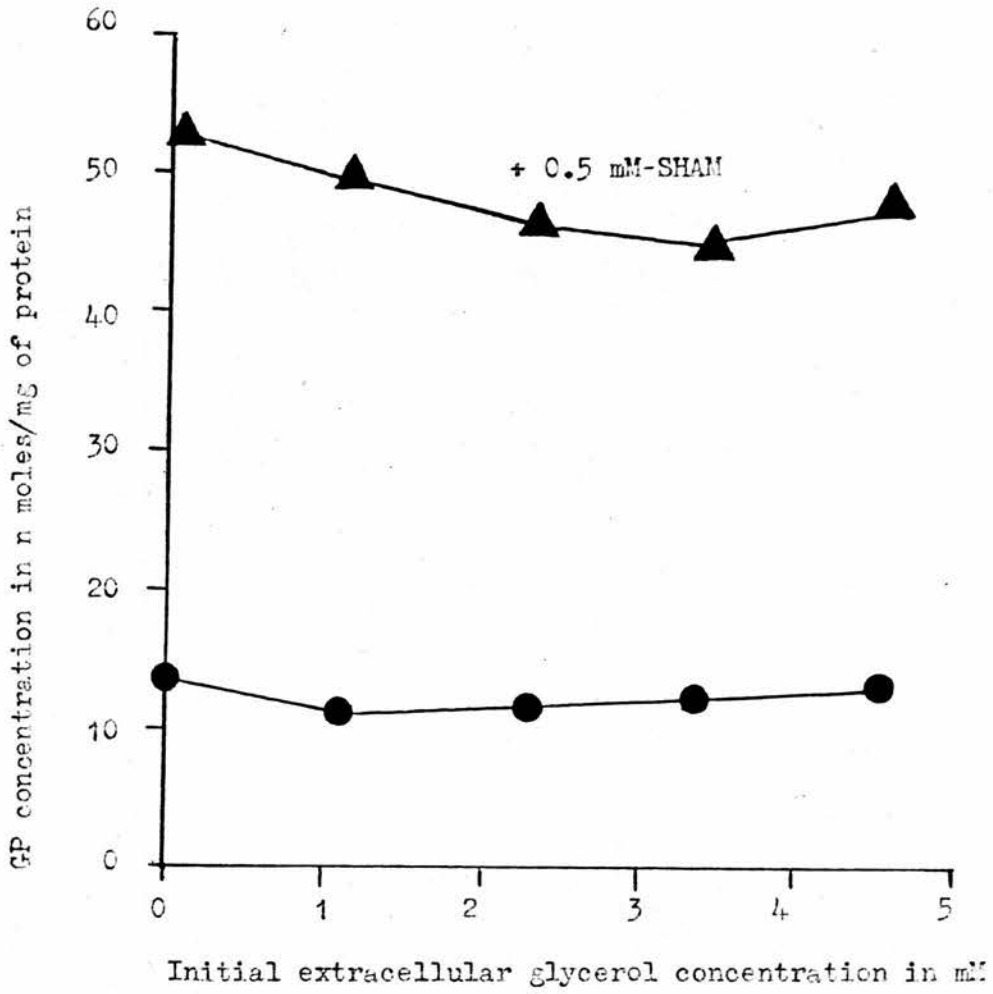


Fig. 3.15 : Glycerol-3-phosphate concentrations of T. brucei under aerobic and glycerol-3-phosphate oxidase inhibited conditions with varying extracellular glycerol concentrations. The trypanosomes were incubated for 6 minutes in phosphate saline buffer at 18° C and at pH 8.0 containing 5.5 mM-glucose and varying glycerol concentrations. ●-● refers to the aerobic and ▲-▲ to the SHAM added media.

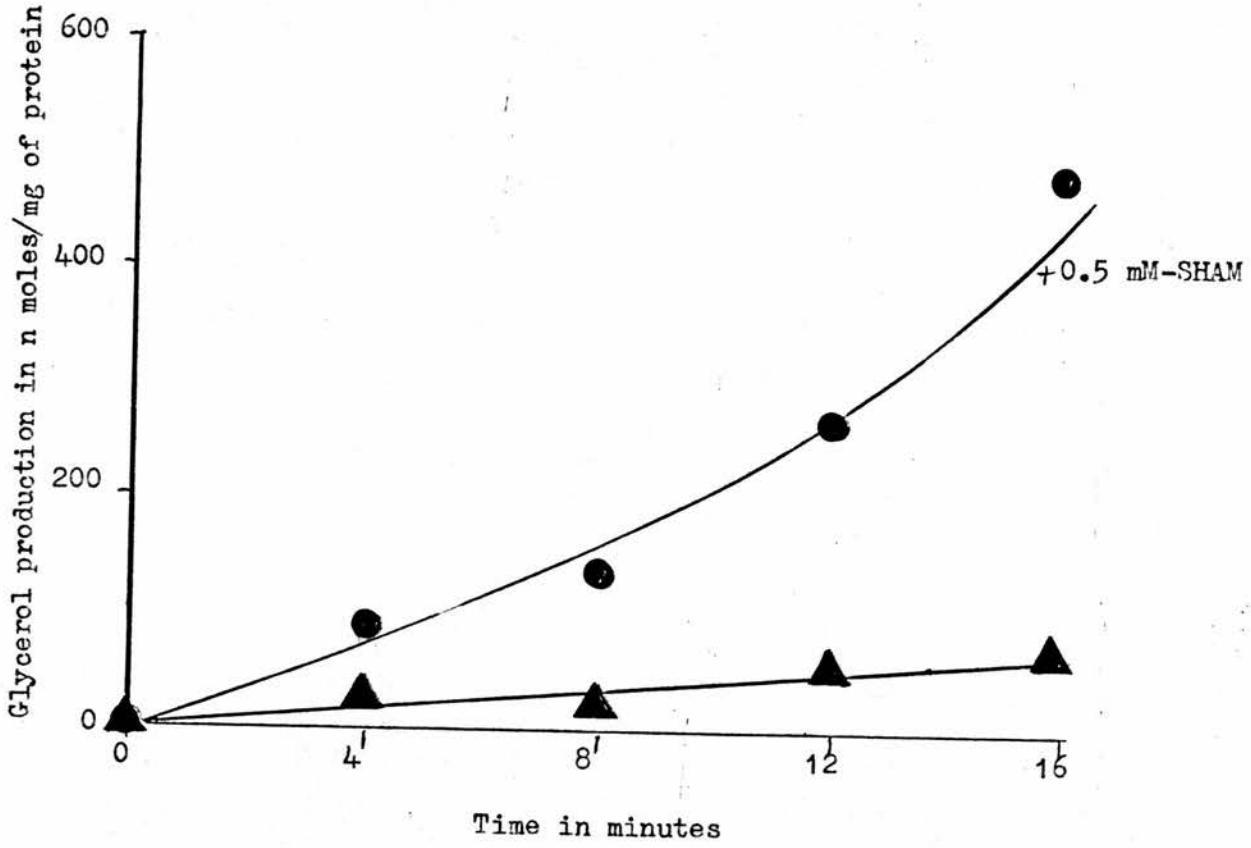


Fig. 3.16 : Glycerol production from glucose by T. brucei under aerobic and glycerol-3-phosphate oxidase inhibited conditions.

▲—▲ Aerobic

●—● Aerobic + 0.5 mM-SHAM

In this subsection this observation was analysed in greater detail through selecting a range of glycerol concentrations and measuring the intracellular GP concentration.

The results given in Fig. 3.15 refer to one such experiment in which trypanosomes were incubated at 18° C for 6 minutes both aerobically and under SHAM inhibition. A low temperature and short incubation time were selected as steady-state concentrations would be established but glycerol production from the anaerobic metabolism of glucose will be minimal, leaving the final extracellular glycerol concentration approximately that of the initial concentration.

Increasing the extracellular glycerol concentration was seen to have no significant effect upon the GP concentration of either the aerobic or SHAM inhibited conditions. The ratios of the GP concentrations in the two conditions were in keeping with previous findings. The conditions of this experiment will not show a GP dependence with increasing glycerol through possible glycerokinase activity as the K_m glycerol for glycerokinase is about 0.1 mM consequently glycerol must be very nearly saturating for glycerokinase within the experimental range.

The difference in the rate of anaerobic glucose utilization under 0.1 mM and 4.5 mM extracellular glycerol concentration is high (Fig. 3.1), but throughout this range GP production from glucose must equal its utilization. This can be achieved by either of the following :

1. The formation of 1 mole of F-D-P from glucose requires 1 mole of GP donating a phosphate group through either a GP hexose or hexosephosphate transphosphorylase. The mole of F-D-P is then cleaved forming GA3P and DHAP, the latter then forming 1 mole GP via the glycerophosphate

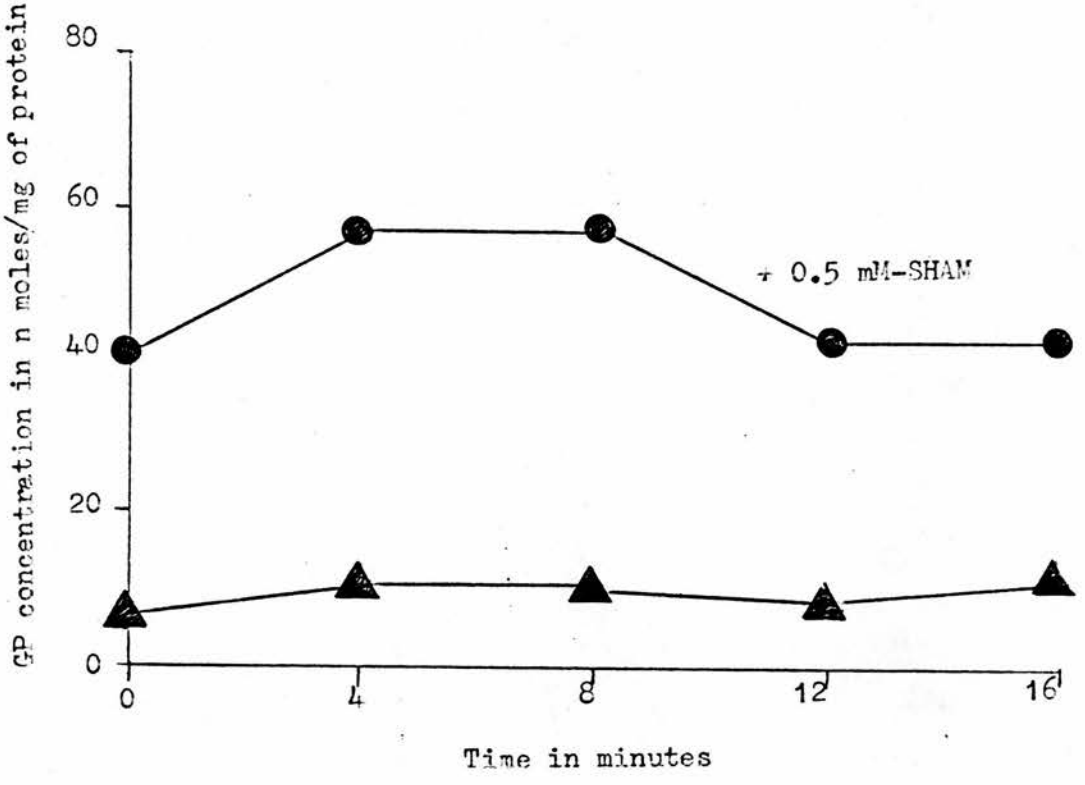


Fig. 3.17 : The intracellular concentration of glycerol-3-phosphate under aerobic and glycerol-3-phosphate oxidase inhibited conditions.

▲—▲ Aerobic

●—● Aerobic + 0.5 mM-SHAM

dehydrogenase catalysed reaction. Thus 1 mole of GP is formed to every mole of GP utilized.

2. The rate of glucose phosphorylation being limited by ATP availability and when ATP is phosphorylated by GP via the glycerokinase catalysed reaction the ATP produced can either be used in phosphorylating glucose or glycerol but through either method GP will be produced at a rate equal to its utilization.
3. When GP concentration increases to a certain value it inhibits its own production by activating a shunt pathway that bypasses F-D-P and hence GP production. Such a pathway could be the postulated hexose monophosphate aldolase and glycerol dehydrogenase scheme as represented in Fig. 1.4.

3.11 To Determine if the Glycerol-3-Phosphate Concentration of T. brucei Varies with Time

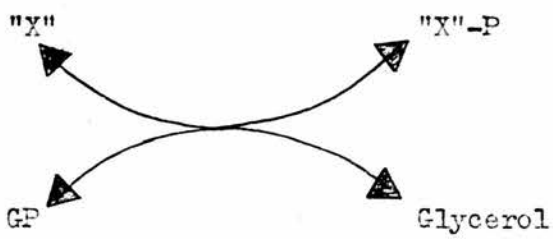
This experiment was designed to elucidate whether the GP concentration increased significantly with time. Such would be the case if an active glycerokinase were operating in conjunction with an GP : hexose or hexosephosphate transphosphorylase or with a hexose monophosphate aldolase glycerol dehydrogenase system, but not if a freely reversible glycerokinase were obligatory for anaerobic glycolysis. The production of glycerol was measured to determine the turnover rate of GP if it were involved as an intermediate in anaerobic glycolysis.

The results of Fig. 3.17 show that over the 16 minute duration of the experiment GP concentrations have fluctuated but have not significantly altered. During the 16 minute incubation the GP pool would have turned over 10 times based on the glycerol production of Fig. 3.16 (if it were an anaerobic glycolytic

intermediate). Thus the rate of GP production from glucose and the rate of its utilization to glycerol must be constant with time.

3.12 The Incorporation of ^{14}C-glycerol into Glycerol-3-Phosphate in T. brucei Under Conditions of Glycerophosphate Oxidase Inhibition

It has been previously demonstrated that under conditions of GP oxidase inhibition the concentration of GP remains constant with time, Fig. 3.17, and with varying extracellular glycerol concentrations, Fig. 3.15. From these observations it was concluded that the production of GP and its utilization to form glycerol must be equal with time and glycerol variation, or alternatively GP upon reaching a certain concentration must switch off its further production by activating an alternative pathway for glucose utilization which bypasses GP production and utilization. To elucidate which of these possibilities applies to T. brucei, the rate of (1-^{14}C)-glycerol incorporation into GP under conditions of GP oxidase inhibition was examined. If glycerol were rapidly incorporated as GP then the following general scheme must be operating :



"X" could be glucose in the postulated GP : glucose transphosphorylase scheme, F-6-P in the postulated GP : F-6-P transphosphorylase scheme or ADP in the reversible glycerokinase postulated scheme. However a rapid incorporation of glycerol as GP could not be explained by a scheme not involving a GP : "X" transphosphorylation, because in this scheme glycerol must be phosphorylated to GP requiring ATP while the phosphate group must be subsequently lost through

Table 3.12 : Pyruvate and glycerol production from glucose in T. brucei inhibited by SHAM under varying extracellular glycerol concentrations

	Initial glycerol concentrations				
	0.19 mM	0.63 mM	1.32 mM	1.93 mM	2.45 mM
Glucose utilization	200	155	140	125	105
Pyruvate production	185	160	140	120	105
Glycerol production	190	155	130	120	115

Glucose utilization and pyruvate and glycerol production were obtained through assaying a sample taken immediately after addition of the radioactive glycerol to the incubation media and another sample taken 16 minutes later. Further experimental details are given in the text. The results are expressed in n moles/ml.

Table 3.13 : Glycerol and glycerol-3-phosphate concentrations with time for T. brucei metabolising glucose under glycerol-3-phosphate oxidase inhibition with SHAM

Time mins	Initial glycerol concentration									
	0.19 mM		0.63 mM		1.32 mM		1.93 mM		2.45 mM	
	GP	Glycerol	GP	Glycerol	GP	Glycerol	GP	Glycerol	GP	Glycerol
0	1.3	0.2	1.1	0.6	1.2	1.3	1.2	1.9	1.2	2.4
1	1.2	0.3	1.2	0.7	1.2	1.4	1.2	2.0	1.2	2.5
2	1.1	0.4	1.4	0.8	1.2	1.6	1.0	2.1	1.2	2.6
4	1.2	0.6	1.2	1.0	1.2	1.7	1.1	2.2	1.1	2.6
8	1.3	1.0	1.4	1.3	1.2	2.0	1.2	2.5	1.2	2.9
16	1.2	1.7	1.2	2.0	1.2	2.6	1.1	3.0	1.2	3.4

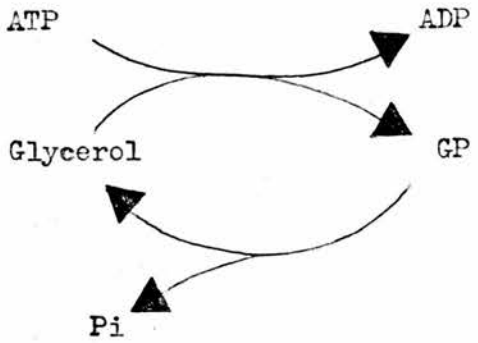
All concentrations are given in mM. The relevant experimental data is given in the text and in section 2. The protein concentration of each incubation was 4.1 mg/ml.

Table 3.14 : Radioactivities of various incubation conditions

	Initial glycerol concentration				
	0.19 mM	0.63 mM	1.32 mM	1.93 mM	2.45 mM
Radioactivity	0.234	0.239	0.252	0.259	0.254

These results represent the average of three taken at 1, 4 and 8 minutes after the addition of radioactive glycerol to the incubation media. The results are expressed in μ Ci/ml of medium.

dephosphorylation to maintain a constant GP concentration. Thus the following futile cycle will exist :



The rate of ATP expenditure by this pathway cannot be greater than the rate of ATP production (the rate of glucose utilization) as the cell will rapidly become depleted of ATP.

The trypanosome suspension used for this experiment was prepared in saline containing 11 mM-glucose and was incubated at 25° C for 5 minutes before SHAM was added to a final concentration of 1,0 mM and the incubation temperature was lowered to 18° C. This ensured high glycolytic intermediate concentrations resulting in a high GP concentration upon inhibition by SHAM. This made the relevant assays and purification procedures more convenient. Further details of the experimental procedure are given in sections 2.9 and 2.10.

The results of Table 3.12 reaffirm the observations made in section 3.1 that glucose is metabolised under SHAM inhibition to equimolar pyruvate and glycerol, but also shows that the I_{50} for glycerol inhibition of glucose utilization is greater than 2 mM. This supports the observation that high intracellular glycolytic intermediate concentrations produce higher I_{50} values for the inhibition of anaerobic glucose utilization by glycerol.

The results of Table 3.13 gives the concentrations of GP and glycerol in the various incubation media. The trypanosomes were not concentrated by

Table 3.15 : Specific radioactivity of glycerol-3-phosphate in various media at varying times

Time mins	Specific radioactivities of GP in the incubation media whose initial glycerol concentrations were				
	0.19 mM	0.63 mM	1.32 mM	1.93 mM	2.45 mM
0	0.0037	0.0018	0.0004	0.0018	0.0012
1	0.0675	0.0153	0.0088	0.0044	0.0053
2	0.0881	0.0440	0.0394	0.0063	0.0042
4	0.121	0.0617	0.0497	0.0203	0.0200
8	0.0995	0.0420	0.0349	0.0251	0.0193
16	0.0520	0.0554	0.0292	0.0252	0.0153

The radioactivity is expressed in Ci/ mole of GP. Further experimental details are given in the text.

Table 3.16 : Specific radioactivities of glycerol in various incubation media at varying times

Time mins	Specific radioactivity of glycerol in the incubation media whose initial glycerol concentrations were :				
	0.19 mM	0.63 mM	1.32 mM	1.93 mM	2.45 mM
0	1.149	0.3951	0.1938	0.1330	0.1054
1	0.5117	0.3152	0.1726	0.1268	0.0992
2	0.3441	0.2219	0.1281	0.1203	0.0921
4	0.1485	0.1651	0.1133	0.1075	0.0894
8	0.1051	0.1387	0.1052	0.0915	0.0797
16	0.1013	0.0887	0.0835	0.0770	0.0694

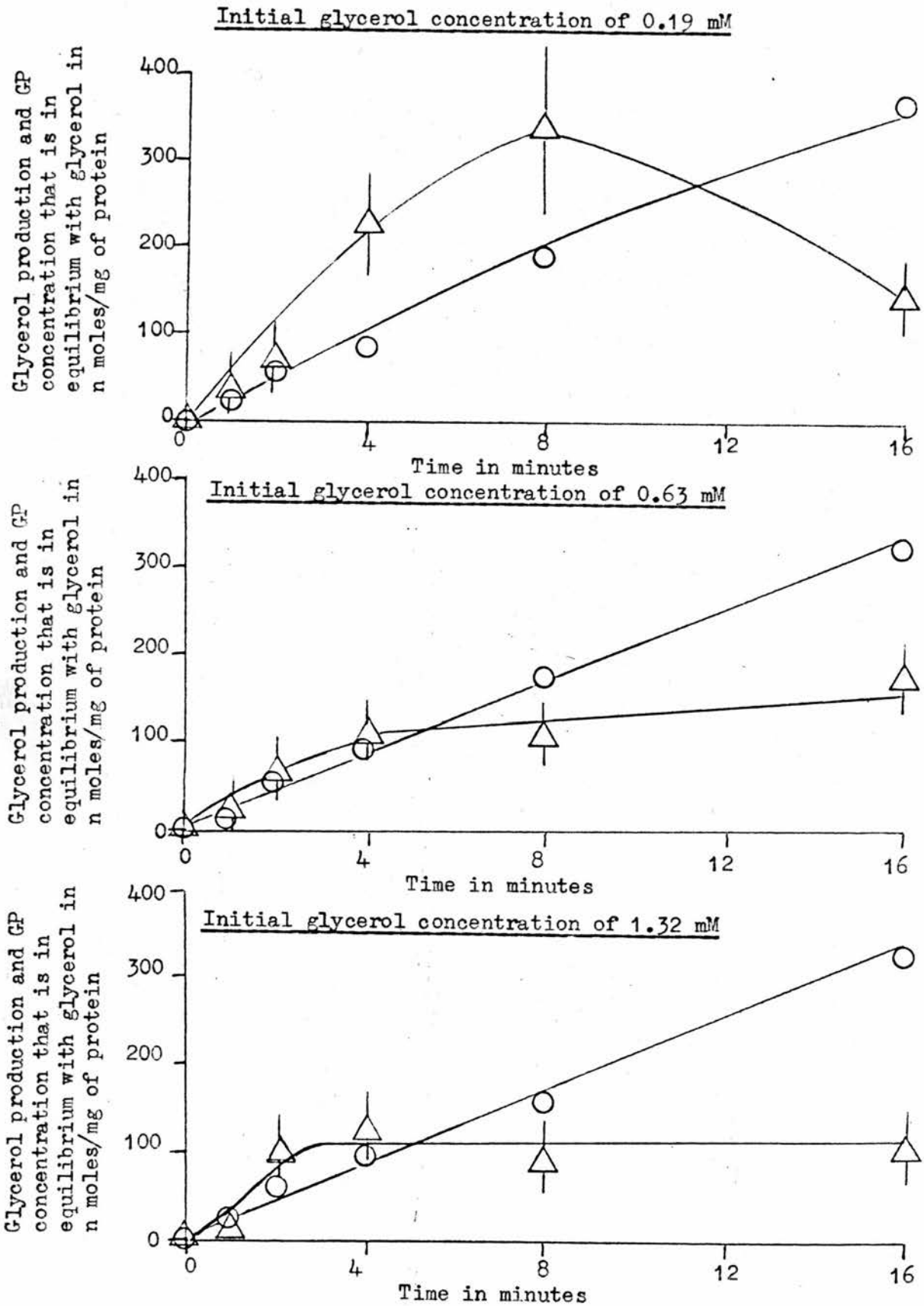
The radioactivity is expressed in Ci/ mole of glycerol. Further details are given in the text.

centrifugation before deproteinisation so these figures represent the incubation media concentrations which contain both intracellular and extracellular components. The average GP concentration was 290 n moles/mg of protein. This concentration does not vary with time or with increasing extracellular glycerol concentration, in keeping with previous findings.

An aliquot of each sample taken was used to prepare a GP fraction free from glycerol by the method described in section 2.12. The specific radioactivity of these fractions were obtained and are given in Table 3.15. By knowing the specific radioactivity of the GP, the specific radioactivity of the glycerol present at each sampling time for each incubation can be obtained by :

1. multiplying the specific radioactivity of GP by the concentration of GP in μ moles/ml in the incubation media (Table 3.13). This gives the total radioactivity of GP/ml of incubation media.
2. The total radioactivity of GP/ml of incubation media is subtracted from the total radioactivity/ml of incubation media (Table 3.14). This gives the total radioactivity of glycerol/ml of incubation media.
3. The specific radioactivity of glycerol is then obtained by dividing the total radioactivity of glycerol/ml of incubation media by the respective glycerol concentration in μ mole/ml. These results are given in Table 3.16 and show a progressive decrease in specific radioactivity with time. This is due to radioactive glycerol being incorporated into GP and also due to dilution with glycerol derived from glucose.

The percentage of the GP pool in equilibrium with glycerol can then be obtained by dividing the specific radioactivity of GP by that of glycerol and multiplying by 100. The amount of GP in equilibrium with glycerol at each sampling time can then be evaluated by multiplying the concentration of GP in each sample by the percentage in equilibrium with glycerol. These results are shown in graphical form in Fig. 3.18.


Initial glycerol concentration of 0.19 mM
Glycerol production and GP concentration that is in equilibrium with glycerol in n moles/mg of protein
400
300
200
100
0
0
4
8
12
16
Time in minutes
Initial glycerol concentration of 0.63 mM
Glycerol production and GP concentration that is in equilibrium with glycerol in n moles/mg of protein
400
300
200
100
0
0
4
8
12
16
Time in minutes
Initial glycerol concentration of 1.32 mM
Glycerol production and GP concentration that is in equilibrium with glycerol in n moles/mg of protein
400
300
200
100
0
0
4
8
12
16
Time in minutes

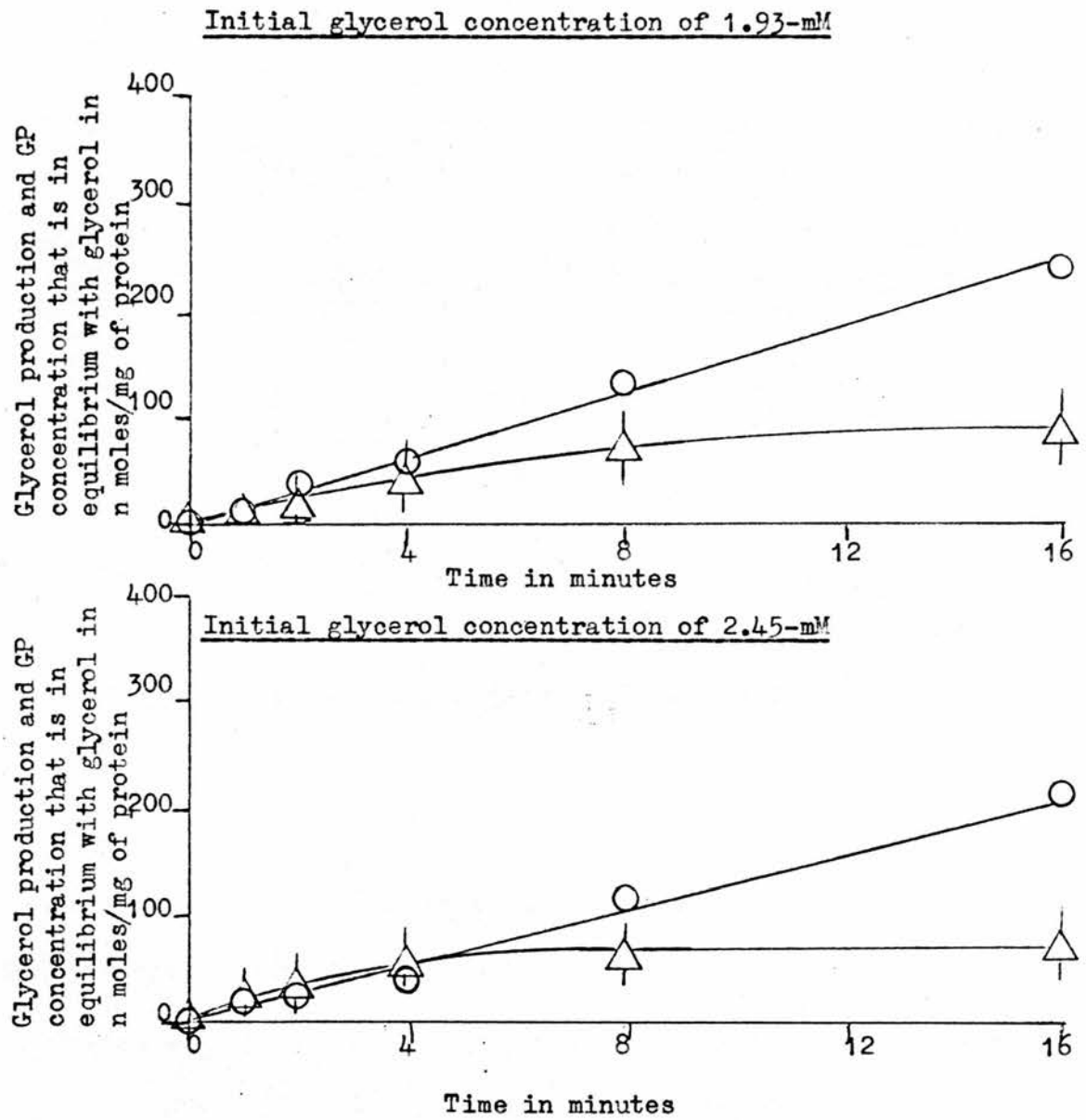


Fig. 3.18 : Glycerol production from glucose and the concentration of glycerol-3-phosphate in equilibrium with glycerol at varying times for T. brucei under glycerol-3-phosphate oxidase inhibited conditions (see text for further details). O-O refers to glycerol production and △-△ to the GP concentration that is in equilibrium with glycerol.

Evaluation of the rate of glycerol incorporation into GP for an initial low glycerol concentration has been repeated in a similarly designed experiment, which produced a maximum rate of glycerol incorporation into GP of greater than twice that of glucose utilization. The identity of GP as being the major radioactive product from glycerol under conditions of GP oxidase inhibition has been determined in five separate thin-layer chromatography systems which had been developed to clearly separate all the glycolytic intermediates. No peaks of radioactivity other than those migrating with synthesised radioactive GP could be detected (Table 3.17).

Fig. 3.18 shows that glycerol is being incorporated into GP under conditions of GP oxidase inhibition at a fast rate compared to glycerol production. At low glycerol concentrations this rate is significantly faster than glucose utilization demonstrating that the processes of glycerol phosphorylation and GP dephosphorylation must be coupled to the phosphorylation and dephosphorylation of a moiety, "X". Glycerol cannot be phosphorylated to GP by ATP via the glycerokinase catalysed reaction to be dephosphorylated by the action of a phosphatase as this cycle would require ATP at a rate faster than its possible production from glucose. GP must therefore be an intermediate in glycolysis.

The rate of GP production from glycerol can be quantitatively estimated from Fig. 3.18. Over the glycerol concentration range 0.2 - 0.6 mM, GP is produced from glycerol at a rate of 59 n moles/minute/mg of protein at 18° C, while this is decreased to about 15 n moles/minute/mg of protein over the range 2.4 to 2.6 mM. Over the same glycerol concentration ranges, glucose utilization (as measured by glycerol production) is decreased from 23 to 17 n moles/minute/mg of protein. These observations are contrary to that expected if glycerol were to inhibit the anaerobic metabolism of glucose by a mass action effect upon GP

Table 3.17 : Chromatography of a glycerol-3-phosphate fraction against a radioactive glycerol-3-phosphate control

	1	2	3	4	5
R_F of radioactivity in experimental spot	0.60	0.53	0.04	0.23	0.17
R_F of radioactivity in control spot	0.60	0.57	0.04	0.19	0.18

The chromatography systems used are described in section 2.10 and the solvents systems employed were :

1. 16 M-ammonia/10% (w/v) trichloroacetic acid/water/ethanol, (3 : 1 : 3 : 10 by volume).
2. Propionic acid/water/n-butanol (7 : 10 : 15 by volume).
3. 16 M-ammonia/formic acid/water/methanol/ethanol (3 : 1 : 3 : 6 : 4 by volume).
4. 16 M-ammonia/10% (w/v) trichloroacetic acid/water/methanol/ethanol (3 : 1 : 3 : 6 : 4 by volume).
5. Formic acid/isopropyl ether (2 : 3 by volume).

200 μl of trypanosome extract plus 10 μl of deproteinised GP synthesis medium, experimental spot was run opposite 200 μl of the same extract plus 10 μl of deproteinised GP synthesis medium containing radioactive GP, control spot.

dependent transphosphorylation as glycerol incorporation as GP would increase with increasing glycerol. However these results are consistent with the postulated pathway of anaerobic glucose metabolism involving a reversible glycerokinase, since increasing the glycerol concentration must decrease the ATP concentration thus limiting its availability for further glycerol and glucose phosphorylation.

SECTION 4

RESULTS PART 2

Table 4.1 : Production of glycerol and ATP from glycerol-3-phosphate, ADP and glycerol-3-phosphate plus ADP combined in T. brucei lysates

Lysate preparation	GP concentration	ADP concentration	ATP production	Glycerol production
Saponin lysate	6.3 mM	3.0 mM	60	20
	-	3.0 mM	41	0
	6.3 mM	-	0	1
Water lysate	6.3 mM	3.0 mM	43	19
	-	3.0 mM	30	0
	6.3 mM	-	0	2

The incubation medium used was : 200 mM-TEA, 6 mM-$MgSO_4$, 0.2 mM-EDTA and 1 mM-SHAM at pH 7.2 at 37° C and with 50 mM-glucose and 0.3U/ml hexokinase added to trap ATP as G-6-P and F-6-P. Samples were taken upon the addition of the lysate and again 30 minutes later. These samples were assayed for glycerol and ATP production as measured by the formation of G-6-P plus F-6-P combined. The results are expressed in n moles/minute/mg of protein.

4.1 Introduction to Glycerol-3-Phosphate Dependent ADP Phosphorylation

The previous section demonstrated that the phosphorylation of glycerol and dephosphorylation of GP is proceeding at a fast rate compared to the other net reactions in anaerobic glycolysis, and that the free reversibility of the glycerokinase catalysed reaction has previously been implicated as a possible enzyme catalysing this conversion. This section demonstrates that in broken cell preparations GP dephosphorylation and glycerol phosphorylation are catalysed by the same enzyme, glycerokinase and some of the properties of this enzyme, with particular relevance to the production of glycerol plus ATP from GP plus ADP are examined.

4.2 Glycerol-3-Phosphate Enhanced ADP Phosphorylation

Preliminary tests showed that the rate of GP dephosphorylation in homogenates is very low (Table 3.6), however ADP was found to enhance significantly the rate of dephosphorylation. Subsequent experiments showed that dephosphorylation of GP was linked to phosphorylation of ADP to ATP provided a mechanism for trapping the ATP was present.

Table 4.1 shows the results of a typical experiment producing a rate of GP : ADP transphosphorylation of 13 and 19 n moles/minute/mg of protein, measured as ATP trapped by glucose and exogenous hexokinase as G-6-P and F-6-P. Glycerol production from GP is greatly enhanced by the addition of ADP, the net rates of glycerol production being 19 and 17 n moles/minute/mg of protein.

These results suggest that the increase in ATP production is not due to incomplete inhibition of GP oxidase, with subsequent ATP synthesis by the

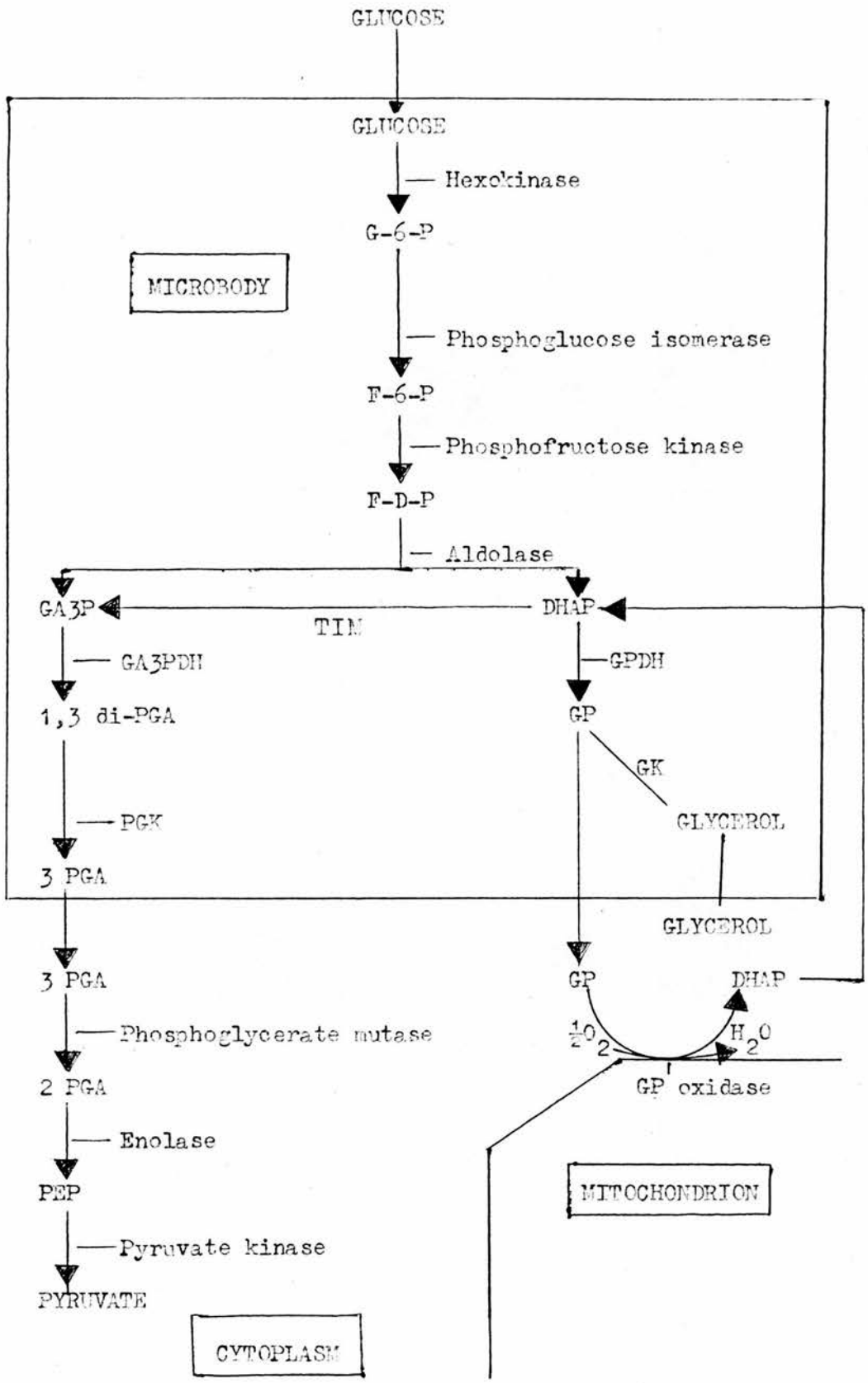


Fig. 4.1 : Compartmentation of the enzymes and reactions involved in glucose and glycerol metabolism by T. brucei. TIM = triosephosphate isomerase, GPDH = glycerol-3-phosphate dehydrogenase, GK = glycerokinase, GA3PDH = glyceraldehyde-3-phosphate dehydrogenase and PGK = phosphoglycerate kinase. Constructed from the work of Oduro (1977), Opperdoes et al. (1977) and Opperdoes and Borst (1977).

Table 4.2 : Specific activities of some enzymes in a fractionated *T. brucei* lysate

Enzyme	Fraction				
	1 KP	5 KP	14 KP	105 KP	105 KS
GP oxidase	0.16	0.23	0.06	0.01	0.02
Adenylate kinase	0.20	0.14	0.29	0.13	0.10
Glycerokinase	1.8	1.9	3.5	1.0	0.66
Hexokinase	0.51	0.54	1.1	0.28	0.08
Phosphoglucose isomerase	0.15	0.23	0.54	0.14	0.03
Pyruvate kinase	0.06	0.07	0.02	0.10	0.28
GP : ADP transphosphorylase	0.11	0.15	0.21	0.06	0.04
Protein	7.3	8.5	1.0	8.4	33

The enzyme activities are expressed in μmole/minute/mg of protein determined at 25° C. Adenylate kinase and GP : ADP transphosphorylase were assayed through incubation similar to that described in Table 4.1. The GP : ADP transphosphorylase activity being the difference in ATP production as measured by G-6-P plus F-6-P formation from ADP plus GP combined minus the ATP formation from ADP (adenylate kinase) and from GP. The method of fractionation is outlined in Fig. 2.1.

phosphoglycerate kinase catalysed reaction, but rather GP : ADP transphosphorylase activity is present. The low specific activity of the GP : ADP transphosphorylase in the unfractionated lysate makes purification a necessity before detailed study of this transphosphorylase can be undertaken.

4.3 Initial Purification of Glycerol-3-Phosphate : ADP Transphosphorylase Activity

Oduro (1977) and Opperdoes and Borst (1977) have shown that the enzymes involved in glucose metabolism are compartmented according to the scheme outlined in Fig. 4.1. The first stage of GP : ADP transphosphorylase purification was to establish if it co-sedimented with marker enzymes from the various sub-cellular compartments.

The results of Table 4.2 show that GP oxidase sediments at 5000 g whereas glycerokinase, hexokinase and phosphoglucose isomerase co-sediment at 14000 g and pyruvate kinase is essentially soluble. These results are in good agreement with the findings of Oduro (1977) and Opperdoes and Borst (1977). The GP : ADP transphosphorylase activity shows a similar sedimentation profile to the microbody enzymes, but the sedimentation characteristics of adenylate kinase are not so well defined. Oduro (1977) found that homogenates prepared by silicon carbide grinding results in a soluble glycerokinase but this conclusion was not confirmed by these or subsequent findings.

4.4 Inhibition of Glycerol-3-Phosphate : ADP Transphosphorylase and Glycerokinase by Melarsen Oxide

The co-sedimentation of GP : ADP transphosphorylase and glycerokinase suggested that these activities could be of the same enzyme. In water lysates melarsen

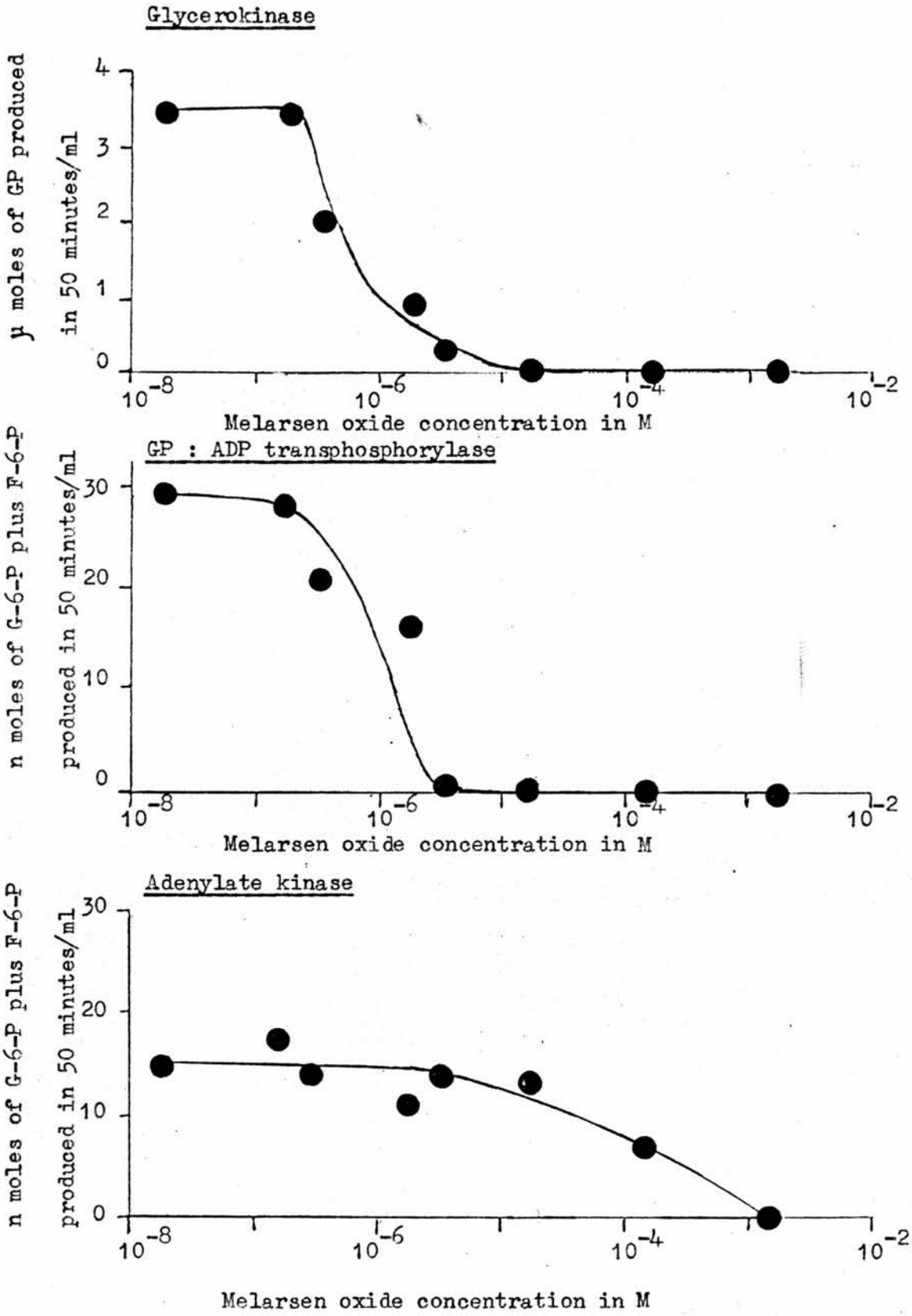

Glycerokinase
μ moles of GP produced in 50 minutes/ml
0
1
2
3
4
10^{-8}
10^{-6}
10^{-4}
10^{-2}
Melarsen oxide concentration in M
GP : ADP transphosphorylase
n moles of G-6-P plus F-6-P produced in 50 minutes/ml
0
10
20
30
10^{-8}
10^{-6}
10^{-4}
10^{-2}
Melarsen oxide concentration in M
Adenylate kinase
n moles of G-6-P plus F-6-P produced in 50 minutes/ml
0
10
20
30
10^{-8}
10^{-6}
10^{-4}
10^{-2}
Melarsen oxide concentration in M

Fig. 4.2 : Inhibition of glycerokinase, glycerol-3-phosphate : ADP transphosphorylase and adenylate kinase by melarsen oxide.

A 14 KP fraction was prepared by the standard method, but after washing in tris-sucrose it was resuspended in distilled water, and homogenized with a hand operated Dounce homogenizer. Aliquots of this preparation were preincubated for 10 minutes at 25° C in substrate deficient assay media with varying concentrations of melarsen oxide present. The assay media consisted of : 50 mM-tris, 125 mM-sucrose, 55 mM-$MgSO_4$, 46 mM-KCl, 8 mM-$NaHPO_4$, 0.2 mM-EDTA and 1.0 mM-SHAM. 60 mM-glucose and 0.4 U/ml hexokinase were present except in the glycerokinase assay medium. When added the final concentration of ATP was 20 mM; glycerol, 10 mM; ADP, 2.3 mM and GP 5.5 mM. All solutions were adjusted to pH 7.0 at 25° C.

Glycerol and ATP were added to the glycerokinase assay. GP plus ADP to the GP : ADP transphosphorylase assay, ADP to the adenylate kinase assay and GP to a transphosphorylase control. GP : ADP transphosphorylase activity was measured by the GP : ADP transphosphorylase minus the adenylate kinase and minus the control assay results.

The results are the average of duplicate incubations expressed in n or μ moles of GP or G-6-P plus F-6-P combined produced in 50 minutes.

oxide has been found to be a potent inhibitor of glycerokinase with an I_{50} value of between 3 - 4 μM, Fig. 4.2. Inhibition of glycerokinase and GP : ADP transphosphorylase by melarsen oxide were compared to elucidate if glycerol plus ADP production from GP plus ADP could be catalysed by glycerokinase.

A 14 KP fraction was used to increase the ratio of activity of the GP : ADP transphosphorylase to adenylate kinase activity. The 14 KP fraction was then water lysed before assay analysis because when prepared by silicon carbide grinding in isotonic media glycerokinase is insensitive to melarsen oxide inhibition. This is presumably due to melarsen oxides being unable to penetrate the microbody membrane, a factor that becomes important in the interpretation of section 4.14.

The results in Fig. 4.2 show very similar melarsen oxide inhibition profiles with an I_{50} of about 4 μM for glycerokinase and GP : ADP transphosphorylase suggesting these to be the same enzyme.

4.5 Comparing Glycerol-3-Phosphate : ADP Transphosphorylase and Glycerokinase Inactivation with Time

Glycerokinase was found to be a labile enzyme with maximum stability below 4° C. This property was exploited to correlate GP : ADP transphosphorylase activity with the enzyme glycerokinase.

Fig. 4.3 shows the inactivation of glycerokinase and GP : ADP transphosphorylation with time, with and without 2 mM-dithiothreitol. Very similar rates of inactivation are found for the two activities, strongly suggesting that only one enzyme is being studied, and that GP : ADP transphosphorylation is catalysed

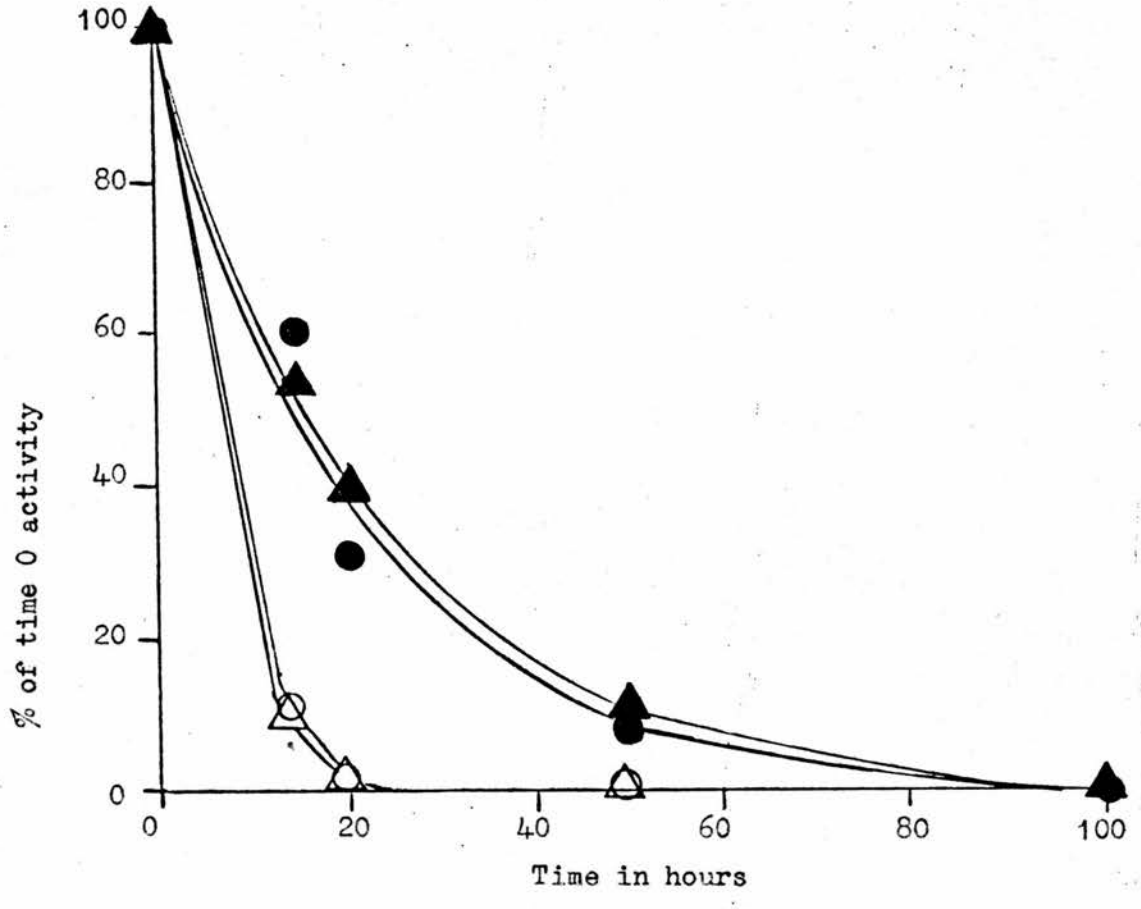


Fig. 4.3 : Inactivation of glycerokinase and glycerol-3-phosphate : ADP transphosphorylase with time. A 14 KP fraction was solubilised in 0.5% (w/v) triton X-100 and divided into two portions. Dithiothreitol to a final concentration of 2 mM was added to one portion. Both contained a crystal of thymol to act as an anti-bacterial agent and were kept at 4° C and at pH 7.0. Samples were taken at various times and assayed for glycerokinase and GP : ADP transphosphorylase activity. O-O refers to GP : ADP transphosphorylase activity and△-△to glycerokinase; ●-● and▲-▲ are the respective activities with 2 mM-dithiothreitol present.

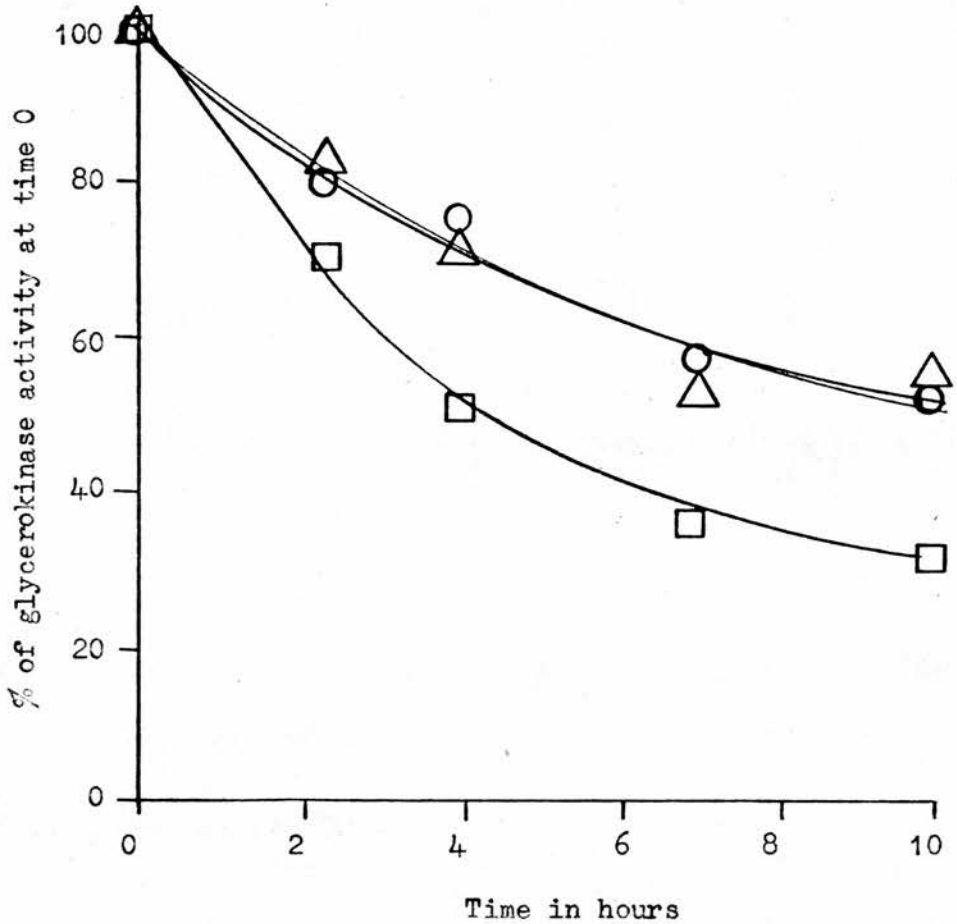


Fig. 4.4 : Inhibition of glycerokinase activity with time, with 0, 0.2 and 2 mM-EDTA present. A 14 KP fraction was solubilised in 0.5% (w/v) triton X-100 and divided into three portions : □- □ containing no EDTA, 0-0, 0.2 mM-EDTA and Δ - Δ 2 mM-EDTA. The portions were left at 4° C at pH 7.0 and each contained a crystal of thymol. Samples were taken at various times and glycerokinase was assayed.

by glycerokinase. The protection afforded by dithiothreitol varied with its concentration but showed a maximum of 2 mM and greater. Dithiothreitol also protected against melarsen oxide inhibition of the solubilised enzyme, indicating that the major site of inactivation of glycerokinase is oxidation of a thiol group present at the active site of this enzyme.

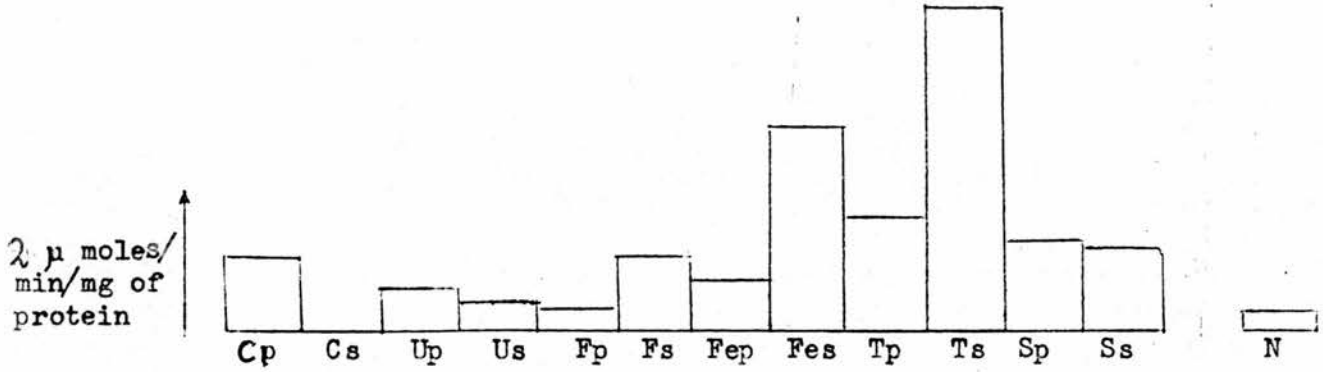
BSA was found to have an inconsistently beneficial effect upon glycerokinase stability in the solubilised preparations of glycerokinase. BSA can give only limited conformational stability due to the high detergent concentrations present, but may possibly protect the enzyme from proteolytic factors contaminating the preparation.

EDTA was also found to have an inconsistent but beneficial effect upon the stability of glycerokinase. The results of one experiment in which EDTA proved significantly beneficial in stabilising glycerokinase is given in Fig. 4.4. The protection of glycerokinase by EDTA was not increased from 0.2 - 2 mM consequently 0.2 mM-EDTA was routinely added to glycerokinase preparations.

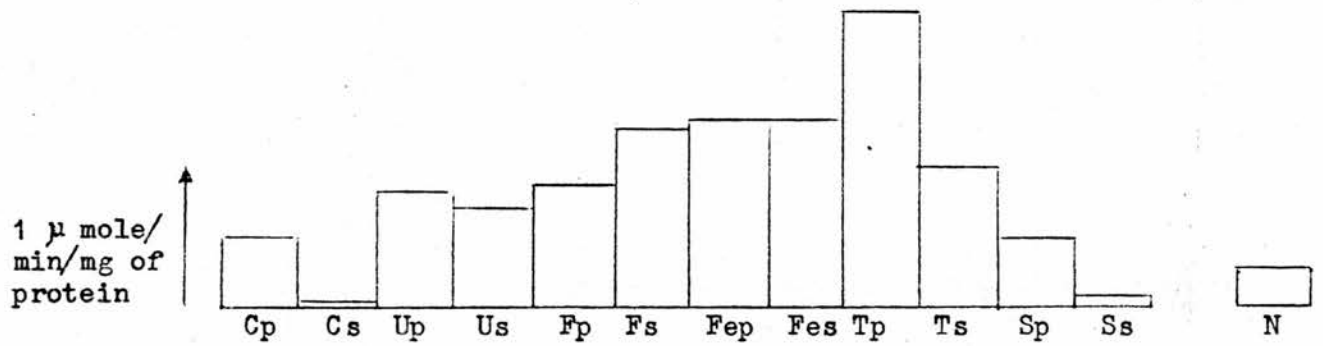
4.6 Further Purification of Glycerokinase

It may be recalled (Table 4.2) that the highest specific activity of glycerokinase is found in a fraction sedimenting at 14000 g (14 KP), when prepared from silicon carbide grinding. The preparation of a 14 KP fraction provided an initial 3-4 fold purification and was therefore used as the first stage in glycerokinase purification. The second stage of purification involved solubilization of glycerokinase from the 14 KP fraction. A variety of different methods for solubilization were tried, and the specific activities of the glycerokinase remaining in the 14 KP sedimentary fraction and solubilised fraction are given in Fig. 4.5 for the different treatments.

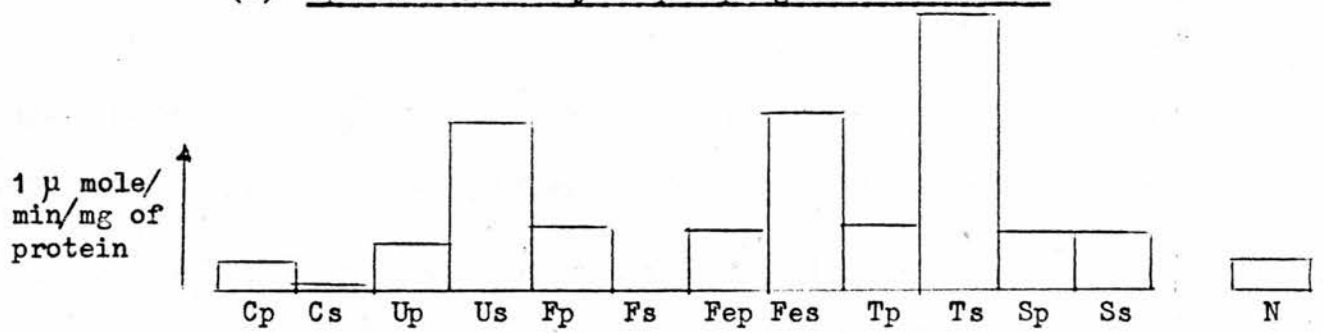
(a) Specific activity of glycerokinase



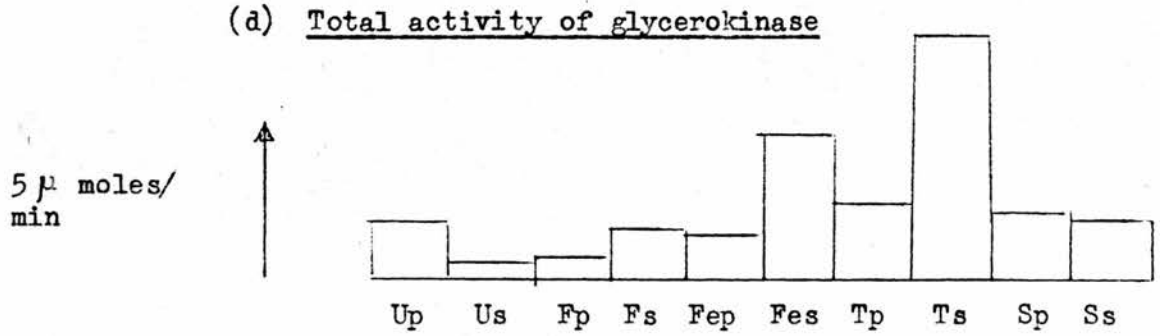
(b) Specific activity of hexokinase



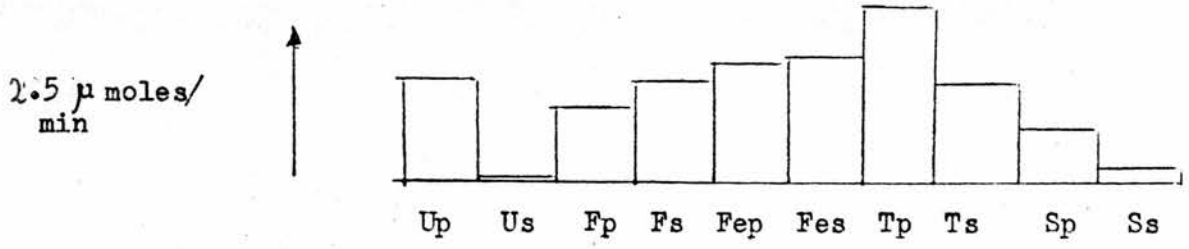
(c) Specific activity of phosphoglucose isomerase



(d) Total activity of glycerokinase



(e) Total activity of hexokinase



(f) Total activity of phosphoglucose isomerase

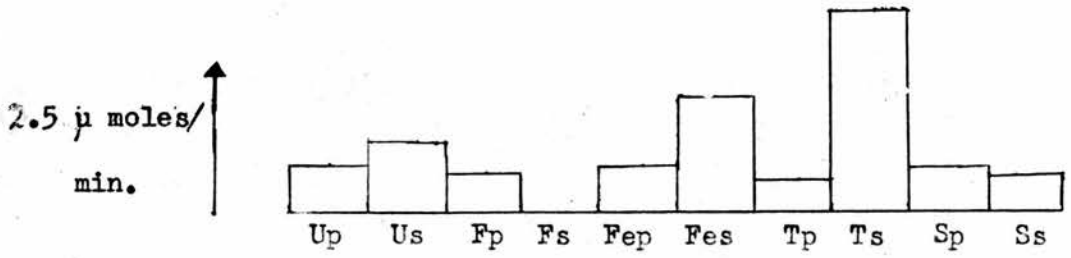


Fig. 4.5 : Specific activities of glycerokinase, hexokinase and phosphoglucose isomerase prepared from various treatments. A portion of a trypanosome lysate prepared by silicon carbide grinding in tris-sucrose was kept on ice for 3 hours before being assayed for enzyme activities (donated N). 14 KP and 14 KS fractions were prepared from the remaining lysate and a portion of each was assayed for enzyme activities (donated Cp and Cs respectively). The remaining 14 Kp was variously treated.

U = An aliquot was diluted in an equal volume of tris-sucrose and left on ice for 3 hours before being centrifuged at 14000 g max. for 25 minutes. Subscript S refers to the decanted supernatant while p refers to the resuspended pellet.

F = Treated as in U except the diluted 14 KP fraction was freeze dried for 3 hours and resuspended in an equal volume of 2x distilled water to that evaporated before being centrifuged.

Fe = Treated as in F but before the residue was resuspended it was suspended in ether. The ether was allowed to evaporate before being resuspended in distilled water and centrifuged as in F.

T = Treated as in U except triton X-100 (0.5% w/v) was present.

S = Treated as in U except saponin (0.5% w/v) was present.

All fractions were assayed for glycerokinase, hexokinase and phosphoglucose isomerase activities and protein concentration. Each result represents the average of two assays.

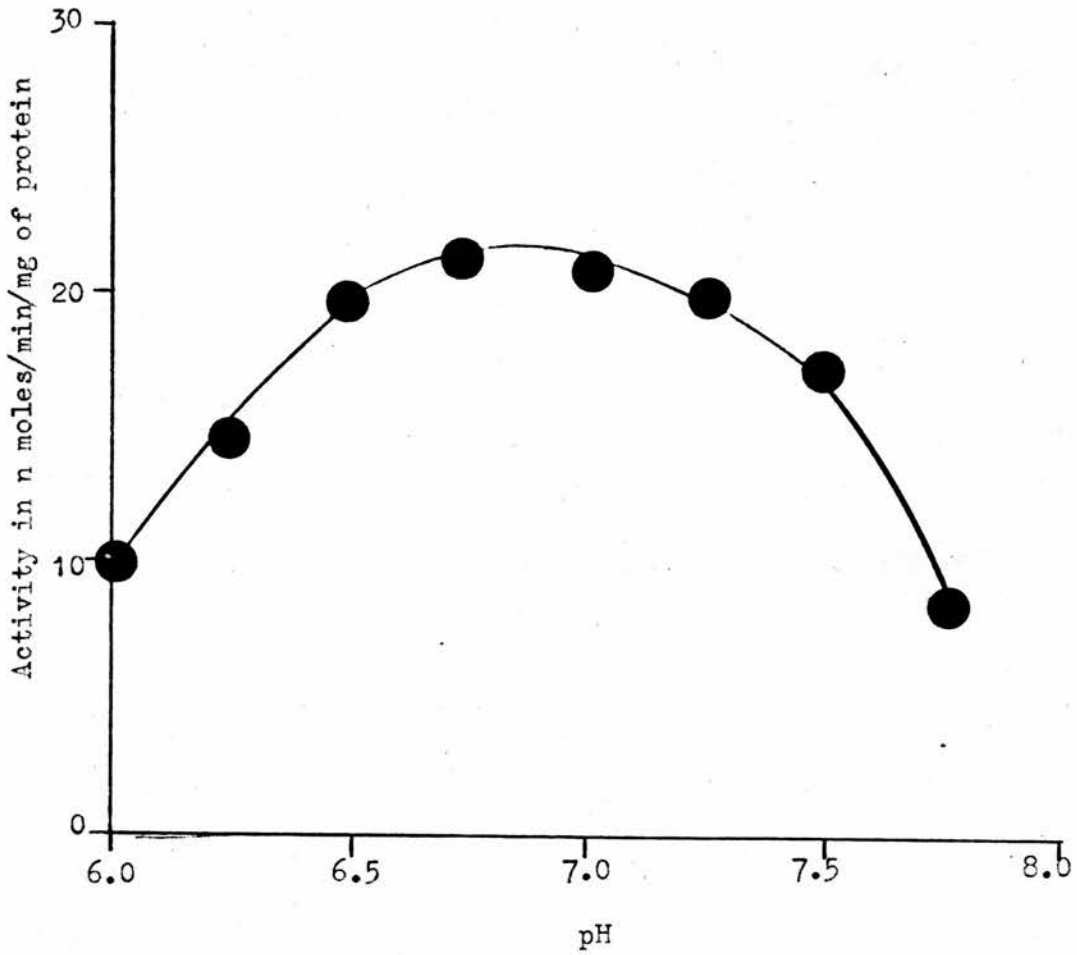


Fig. 4.6 : The effect of pH on glycerokinase activity. The activity of glycerokinase was measured by the production of glycerol plus ATP from GP plus ADP. An unfractionated homogenate prepared by triton X-100 lysis was used and assayed by the method described in the appendix.

The total activity of glycerokinase was increased about 4 times upon triton X-100 solubilisation and about 3 times upon the freeze-drying and ether extracting technique for solubilisation. These observed latency effects of the 14 KP fraction agree with the findings of Oduro (1977). The maximum purification of glycerokinase was found to be about 17 fold, although this figure is artificially high due to only a fraction of the glycerokinase activity being measured in the 14 KP fraction probably due to latency effects with intact microbodies. The length of the purification procedure for glycerokinase made the addition of dithiothreitol to the tris-sucrose buffer medium essential to prevent oxidation inhibition decreasing too greatly the specific activity.

4.7 The Effect of pH on the Rate of Glycerol-3-Phosphate : ADP Transphosphorylase Activity

The pH optimum for GP dependent ADP phosphorylation catalysed by glycerokinase was examined only for the triton X-100 solubilised enzyme. The standard buffer system need not be changed to operate in the pH range 6 - 8 because the phosphate groups of ADP and GP were themselves buffering the assay system in this range.

The results of Fig. 4.6 demonstrate a pH optimum between 6.5 and 7.25; pH 7.0 was selected for assaying glycerokinase for standardization with the assay systems of other glycolytic enzymes.

4.8 Determination of the Michaelis Constants of Glycerokinase

These parameters have been calculated for the glycerokinase of *T. brucei* prepared by differential centrifugation of a silicon carbide ground preparation

Table 4.3 : The Michaelis parameters of solubilised glycerokinase of *T. brucei* and *Candida mycoderma*

(a) *T. brucei*

Substrate	Saturating substrate and concentration	Michaelis constant in mM	V max. ratio
GP ADP	1 mM-ADP 28 mM-GP	5.12 ± 1.47 mM (18) 0.12 ± 0.04 mM (16)	1
Glycerol ATP	4 mM-ATP 15 mM-glycerol	0.12 ± 0.05 mM (17) 0.19 ± 0.04 mM (16)	234

(b) *C. mycoderma*

Substrate	Saturating substrate and concentration	Michaelis constant in mM	V max. ratio
GP ADP	1 mM-ADP 28 mM-GP	0.11 ± 0.02 mM (12) 0.57 ± 0.14 mM (14)	1
Glycerol ATP	4 mM-ATP 15 mM-glycerol	0.09 ± 0.02 mM (12) 0.06 ± 0.02 mM (15)	531

The *T. brucei* glycerokinase was prepared by the method described in section 4.6, while *C. mycoderma* glycerokinase was a commercial preparation (Boehinger, Mannheim). The assay was conducted by the method described in the appendix. The Michaelis constants are given in mM with the standard error. The numbers in parentheses refer to the number of results used for the determination. The V max. ratios are taken from Table 4.4 and are the ratio of the maximum velocity of GP plus ADP formation from ATP plus glycerol to the maximum velocity of glycerol plus ATP from GP plus ADP.

in tris-sucrose that was subsequently solubilised with triton X-100 according to the procedure outlined in section 4.6, the T_s fraction being used.

The method for assaying ATP production from GP and ADP as measured by the formation of G-6-P was found to be inhibited by ADP. Subsequently it was shown that ADP was inhibiting the marker enzyme glucose-6-phosphate dehydrogenase, in agreement with the observations of Domagk *et al.* (1969). This inhibition is not significant in the determination of either Km^{GP} or Km^{ADP} for the solubilised glycerokinase of *T. brucei*, but is important in applying this assay system to other glycerokinases e.g. of *Candida mycoderma*, which has a Km^{ADP} of 0.6 mM and for the particulate glycerokinase of *T. brucei* Km^{ADP} of about 6 mM.

The ratio of the maximum velocities of glycerokinase in the forward direction, glycerol plus ATP forming GP plus ADP and the reverse direction, GP plus ADP forming glycerol plus ATP were obtained from Table 4.4, and compared to the theoretical value obtained from the Haldane relationship using the Michaelis constants of Table 4.3. When the glycerokinase catalysed reaction is at equilibrium :

$$\Delta G'_o = - RT \ln K$$

Since $\Delta G'_o = 22.2$ kJ/mole

$R = 8.32$ J/degree absolute/mole

$T = 298^o$

$$K = 7740 = \frac{[\text{glycerol}]\,[\text{ATP}]}{[\text{GP}]\,[\text{ADP}]}$$

If the affinity of one substrate to glycerokinase is uninfluenced by the binding of another then the following relationship holds :

Table 4.4 : The relative rates of the forward and reverse reactions catalysed by glycerokinase

Method of Preparation	Maximum velocity in n moles/minute/mg of protein		Forward / Reverse
	Forward	Reverse	
Water lysate	3.1	0.014	221
Triton X-100	3.0	0.016	188
Silicon carbide	0.80	0.036	22
Silicon carbide plus triton X-100	3.0	0.018	167
Alumina	0.78	0.038	21
Alumina plus triton X-100	2.9	0.017	170

All lysate preparations were unfractionated and were obtained from the same trypanosome preparation. The forward reaction refers to the production of GP plus ADP from glycerol plus ATP, and the reverse, the production of glycerol plus ATP from GP plus ADP. All assays were performed at 25° C.

$$\text{The theoretical Keq} = \frac{Vf}{Vr} \; \frac{Km^{ADP}\; Km^{GP}}{Km^{ATP}\; Km^{glycerol}} \qquad \text{(Alberty, 1953a)}$$

where Vf = maximum velocity of the reaction : glycerol + ATP → GP + ADP

and Vr = maximum velocity of the reaction : GP + ADP → glycerol + ATP

The theoretical value for Vf/Vr is 282, while the observed value compares favourably at 234. A similar calculation was performed using the Km values of glycerokinase from C. mycoderma where a theoretical Vf/Vr value of 720 is obtained comparing with an observed value of 531. The Km results obtained for C. mycoderma and T. brucei contrast greatly especially in Km^{GP} values. The differences in Vf/Vr values of the two glycerokinases reflect the different Michaelis constants, with the T. brucei glycerokinase being more favourable for the production of ATP and glycerol from GP and ADP than that of C. mycoderma.

4.9 The Relative Rates of the Forward and Reverse Reactions Catalysed by Glycerokinase

The relative rate of ATP and glycerol production from GP and ADP compared to GP and ADP production from ATP and glycerol were found to agree with the theoretical value based on the Michaelis constants for glycerokinase and the standard free energy change for the reaction. However the relative rates of these reactions varied according to the method of preparation and Table 4.4 summarizes assay results obtained for lysates prepared by water lysis, triton X-100 treatment and alumina or silicon carbide grindings. The effect of solubilising the glycerokinase of lysates prepared by grinding with abrasives was also studied.

Table 4.5 : Michaelis constant of ADP for particulate glycerokinase of *T. brucei*

Michaelis constant	Standard error of mean	No. of results
6.67 mM	± 3.27 mM	17

The experiment was conducted in the assay medium described in the appendix but without glucose-6-phosphate dehydrogenase and $NADP^+$ incubation was at 25° C with (a) 7 mM-GP plus varied ADP, (b) varied ADP alone and (c) 7 mM-GP. Samples were taken at 5 and 30 minutes deproteinised by PCA and neutralised with K_2HPO_4 as described in section 2.8, then ATP production was measured by the formation of G-6-P plus F-6-P combined. The rate of ATP formation from GP plus ADP, via the glycerokinase catalysed reaction was obtained by substrating the results of (b) plus (c) from those of (a).

It can be seen from Table 4.4 that lysates prepared from water and triton X-100 treatment produced a glycerokinase with the same activities in the forward and reverse directions. However in the silicon carbide and alumina ground cell preparations the specific activity of glycerokinase in catalysing GP plus ADP formation from glycerol plus ATP is greatly decreased (3 - 4 times) but glycerol plus ATP formation from GP plus ADP is enhanced by about 2 times compared to the solubilised enzyme. In these experiments 1.0 mM was the maximum ADP concentration used as the method of assay employed is inhibited by higher concentrations of ADP. However there is much evidence from discontinuous incubation studies that this value may be well below saturating and consequently far from optimal.

Table 4.5 gives a Km^{ADP} value of 6 mM but with a high degree of error. This error may be due to many contributing factors, primarily some of the particles may be partially lysed and also steady-state production of ATP may not have been achieved and maintained throughout the incubation time which was necessarily long to allow for accurately measurable concentrations of G-6-P and F-6-P to accumulate.

The Km^{ADP} for particulate glycerokinase is significantly higher than that found for the solubilised enzyme comparable to the situation found for hexokinase in the rat brain. This may reflect a permeability barrier to ADP or a structural change in glycerokinase upon solubilisation, or both. The increased production of ATP from GP plus ADP in the particulate enzyme suggests glycerokinase undergoes a structural change upon solubilisation, since an ADP permeability barrier could not explain the increased reaction rate found under particulate conditions. Such a permeability barrier may still exist for ADP if an increase in reaction rate derived from a more efficient method of ATP trapping due to the close juxtaposition of the endogenous particulate hexokinase is greater than the decrease in reaction rate due to ADP impermeability.

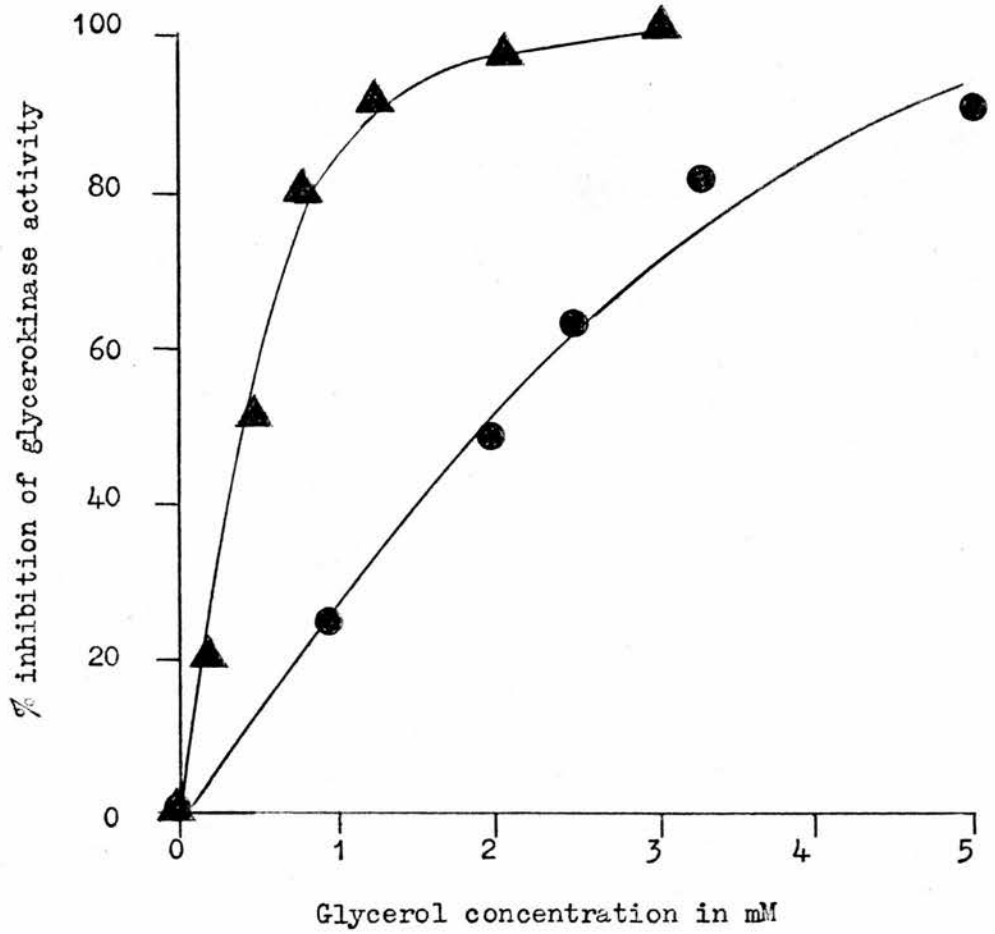


Fig. 4.7 : Inhibition of ATP production from glycerophosphate plus ADP via the glycerokinase catalysed reaction against glycerol concentration. Glycerokinase was prepared by the method described in section 4.6 but with 28 mM-GP and 1 mM-ADP present. ▲—▲ refers to one experiment carried out with 0.1 U/ml hexokinase present as opposed to the standard assay activity of 1.0 U/ml, ●—●

The decreased production of GP plus ADP from glycerol plus ATP can be explained along with the decreased activities of hexokinase and phosphoglucose isomerase by diffusion of substrates to the active site of these enzymes being retarded by a permeability barrier or active site masking by components of the particulate system.

4.10 Inhibition by Glycerol of ATP Production from Glycerol-3-Phosphate Plus ADP Catalysed by Glycerokinase

Possible inhibition of the glycerokinase catalysed production of ATP from GP plus ADP by glycerol was examined to elucidate whether this could be the site of glycerol's trypanocidal action in GP oxidase inhibited cells.

Fig. 4.7 demonstrates that glycerol exerts an I_{50} of about 2 mM at saturating GP in the solubilised glycerokinase, but this value is dependent upon the activity of the ATP trapping enzyme, hexokinase. With the hexokinase activity reduced to 1/10th of the standard assay activity (0.1 U/ml) greater than 90% inhibition was seen with 2 mM-glycerol.

These findings suggest that in the broken cell assay medium, glycerol is affecting apparent ATP production as measured by trapping with glucose and hexokinase, by decreasing the steady-state concentration of ATP.

At equilibrium the ratio of $[GP][ADP]$: $[glycerol][ATP]$ is given by the formula :

$$\Delta G'_o = - RT \ln K = - RT \ln \frac{[GP][ADP]}{[glycerol][ATP]}$$

where $\Delta G'_o$ = standard free energy change at pH 7.0

= 22.2 kJ/mole

R = gas constant = 8.32 J/degree absolute/mole

T = degree absolute = 298^{o}

K equals 7740

Thus using 28 mM-GP and 1 mM-ADP, for net glycerol and ATP production, the product of glycerol and ATP concentrations must be less than 0.002 mM^2. The steady-state ATP concentration of the assay system employed :

$$GP + ADP \underset{k-1}{\overset{k1}{\rightleftharpoons}} \text{glycerol} + ATP$$

$$\text{glucose} + ATP \xrightarrow{k3} \text{G-6-P} + ADP$$

is given by the formula :

$$\frac{d[ATP]}{dt} = 0, \text{ at a steady-state} = k1\,[GP]\,[ADP] - k-1\,[\text{glycerol}]\,[ATP] - k3\,[\text{glucose}]\,[ATP]$$ *

Rearranging :

$$[ATP] = \frac{k1\,[GP][ADP]}{k-1\,[\text{glycerol}] + k3\,[\text{glucose}]}$$

and with k1 [GP] [ADP] fixed, and saturating glucose the equation may be simplified to :

$$[ATP] = \frac{A}{k-1\,[\text{glycerol}] + k3\,[B]}$$

where A and B are constant in the assay conditions.

Thus the steady-state ATP concentration is inversely dependent upon the glycerol and k3 (the hexokinase activity) terms. With a fixed glycerol concentration, the steady-state ATP concentration will be given by the formula :

$$[ATP] = \frac{A}{C + k3.B}$$

where C is a constant.

When k3 is increased by increasing hexokinase the steady-state ATP concentration

* See page 123

must decrease. Glycerol inhibition of ATP production depends upon the rate of $[GP][ADP]\ k1 - k{-}1\ [glycerol][ATP]$. When GP, ADP and glycerol concentrations are kept constant glycerol inhibition at any fixed concentration is dependent upon the steady-state ATP concentration, i.e. is dependent upon the hexokinase activity. The competition between glucose, F-6-P and glycerol for ATP may explain the high specific activities of hexokinase and phosphofructose kinase in T. brucei compared with other cells and tissues (Table 1.1).

As a corollary to this experiment the effect of GP and ADP upon ATP phosphorylation of glycerol was examined. Concentrations of up to 100 mM-GP and 7 mM-ADP were found to be without effect. These findings conflict with the properties of glycerokinase from other sources (Lin, 1977) e.g. rat liver in which GP and ADP inhibition of the glycerokinase catalysed reaction of glycerol plus ATP were found to have a Ki value of 0.5 mM for each substrate. However Grunnet (1970) found the lack of GP inhibition of glycerokinase from C. mycoderma when inorganic phosphate or sulphate were present at concentrations below 0.1 mM was apparently due to competition between GP and the anion. It is therefore possible that since both phosphate and sulphate were present in the assay medium that the glycerokinase from T. brucei could be inhibited by GP. Whether the lack of inhibition of glycerokinase by GP when phosphate or sulphate is present is as Grunnet (1970) believes it to be an artifact created during the preparation procedure or if this reflects the whole cell state in which phosphate has been found not to be a limiting factor is not known at present.

4.11 The Determination of the Michaelis Constant of Hexokinase and Phosphofructose Kinase of T. brucei for ATP

The importance of hexokinase activity on the steady-state concentration of ATP

Table 4.6 : The Michaelis constant for ATP of hexokinase and phosphofructose kinase of T. brucei

Enzyme	Michaelis constant	Standard error of mean	No. of results
Hexokinase	0.060	± 0.012	12
Phosphofructose kinase	0.082	± 0.013	12

The assays were performed by the methods described in the appendix at pH 7.0 with the hexokinase assay medium containing 60 mM-glucose and the phosphofructose kinase assay medium containing 10 mM-F-6-P. Unfractionated trypanosome water lysates were used.

when produced by transphosphorylation of GP and ADP and its subsequent trapping by phosphorylation of glucose has been described in the previous section. It has been noted that the maximum activities of the ATP utilizing enzymes, hexokinase and phosphofructose kinase are high in comparison to the other glycolytic enzymes in T. brucei contrasting with other cells and tissues, Table 1.1. However the ability to trap low concentrations of ATP also depends upon the Km^{ATP} value. In this section the Km^{ATP} values of hexokinase and phosphofructose kinase are determined and compared to that obtained for glycerokinase.

Table 4.6 shows the Km^{ATP} value for hexokinase to be 0.060 $\pm$ 0.012 mM and 0.082 $\pm$ 0.013 mM for phosphofructose kinase. Both values were obtained from unpurified water lysates according to the methods described in the appendix. Contamination by the many ATP binding enzymes in an unpurified lysate will decrease the free ATP concentration available for binding to hexokinase and phosphofructokinase in the assay medium, consequently these Km^{ATP} will both be expected to decrease upon purification. None the less both hexokinase and phosphofructose kinase have Km^{ATP} values significantly lower than that of purified glycerokinase (Km^{ATP} = 0.19 $\pm$ 0.04, Table 4.3), indicating that they could compete favourably with glycerokinase for ATP at low ATP concentrations.

Phosphofructose kinase is known to be affected positively and negatively by many metabolites including PEP, ATP, ADP and F-D-P present in the assay system employed. Consequently the Km^{ATP} for phosphofructose kinase may be greatly influenced by the assay conditions employed.

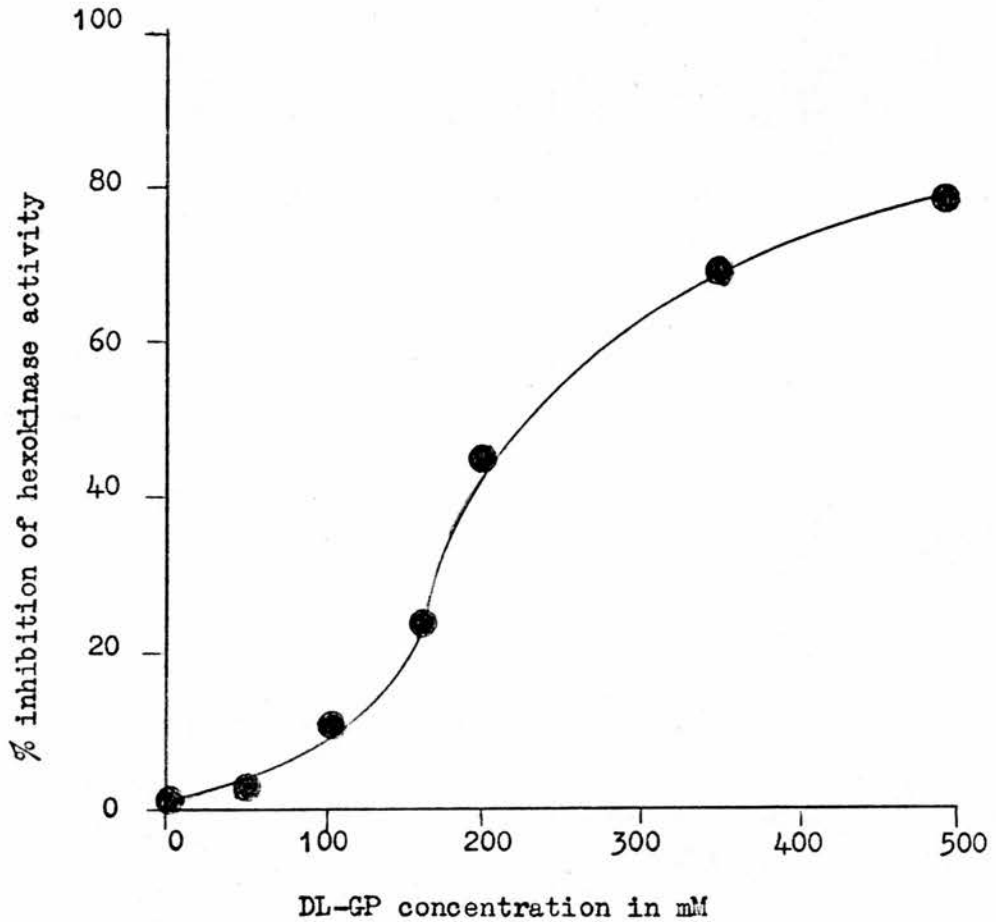


Fig. 4.8 : Inhibition of hexokinase activity by DL glycerol-3-phosphate. The hexokinase was prepared with glycerokinase by the method described in section 4.3, the 14 KP fraction being used. The assays were performed using 5.5 mM-glucose and 0.5 mM-ATP as substrate concentrations.

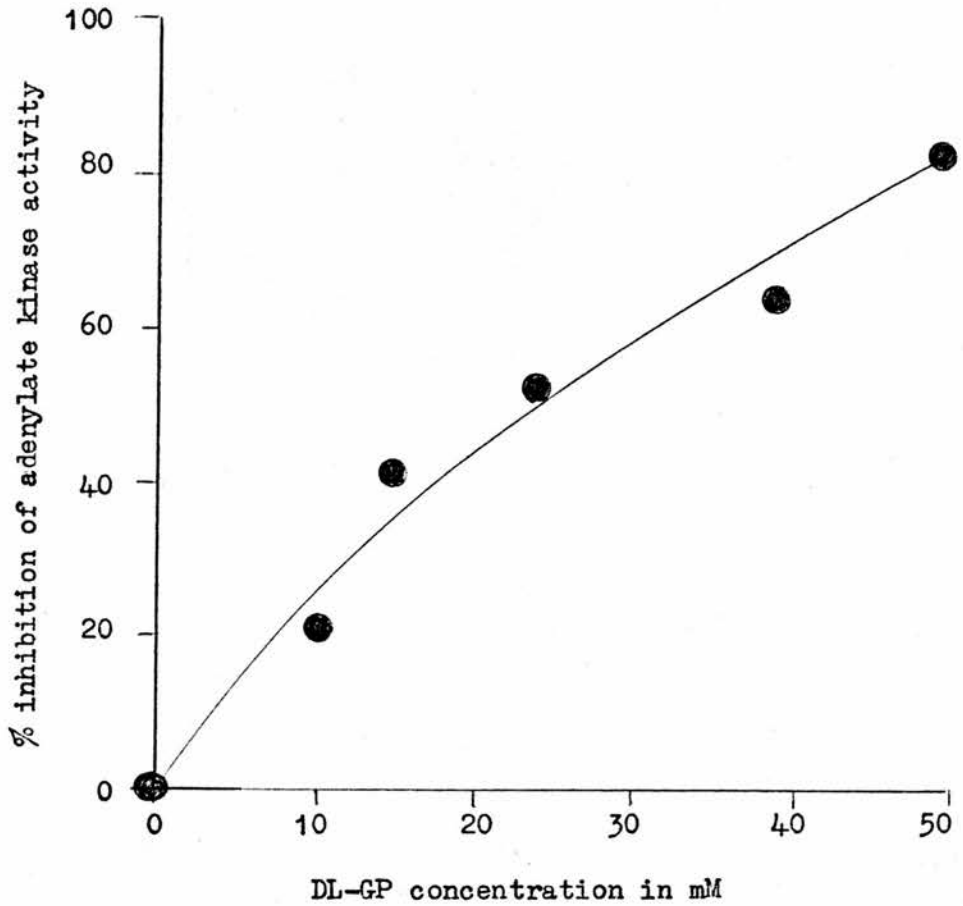


Fig. 4.9 : Inhibition of adenylate kinase activity by DL-glycerol-3-phosphate. The extract was prepared by the method described for the purification of glycerokinase. Adenylate kinase was assayed by measuring the production of ATP from 1 mM-ADP with varying DL-GP concentrations. Glycerokinase activity was inhibited by 0.05 mM-melarsen oxide present in the media.

4.12 Effect of Glycerol-3-Phosphate on Hexokinase, Phosphofructose Kinase and Adenylate Kinase

A fundamental requirement of all the postulated pathways except the free reversibility of glycerokinase in producing net ATP synthesis from the anaerobic metabolism of glucose is that the activity of either hexokinase or phosphofructose kinase must be low compared to the activity of the transphosphorylase or hexose monophosphate aldolase. However the activities of hexokinase and phosphofructose kinase are high in broken cell preparations, but a method of decreasing their activity could be through GP inhibition. Under GP oxidase inhibited conditions, trypanosomes rapidly increase their GP concentration and this has been postulated by Opperdoes and Borst (1977) to be a possible triggering mechanism for an alternative pathway. The effect of GP on the activities of hexokinase and phosphofructose kinase was therefore examined to establish if either of these enzymes were inactivated by GP. The effect of GP upon adenylate kinase was also examined due to its relevance in the method of assaying GP : ADP transphosphorylation by the production of ATP from GP plus ADP combined minus the production of ATP from ADP and minus ATP production from GP.

Fig. 4.8 shows that hexokinase is inhibited by a mixture of DL-GP, but only at concentrations above 0.1 M. Phosphofructose kinase was not found to be significantly inhibited by concentrations up to 100 mM-GP. The adenylate kinase used in the experiment whose results are given in Fig. 4.9 was prepared with solubilised glycerokinase from a 14 KP fraction. This solubilised adenylate kinase proved sensitive to DL-GP inhibition in the range of GP concentrations significant to GP : ADP transphosphorylation assay procedures.

The concentration of GP in the whole cell, assuming no compartmentation will be approximately 25 mM based on the following assumptions :

a) The concentration of adenine nucleotides does not vary significantly between cells and is equal to about 5 mM (Dyson, 1975).

b) The concentration of GP anaerobically equals 100 n moles/mg of protein, five times the adenine nucleotide concentration of 20 n moles/mg of protein.

Thus the effect of GP upon hexokinase and phosphofructose kinase when extrapolated to the whole cell situation would be negligible even if GP were concentrated at the site of hexokinase through compartmentation in a microbody by a factor of five times.

4.13 Some Properties of the 14 KP Fraction

The glycolytic enzymes present in a microbody fraction have been well characterised by Oduro (1977) and Opperdoes and Borst (1977). However some points of conflict between published work and results recorded in this thesis have emerged. Hexokinase and phosphoglucose isomerase have been recorded by Oduro (1977) and Opperdoes and Borst (1977) as being enzymes contained within a microbody. Glycerokinase has previously been found (Table 4.3) to co-sediment with these microbody enzymes from lysates prepared by silicon grinding, in contrast to the observations of Oduro (1977). The latency effects of hexokinase, glycerokinase and phosphoglucose isomerase have been found to be greater (Fig. 4.5) than those obtained by Oduro (1977). This is probably due to the 14 KP fraction being less permeable than those obtained by Oduro or the longer treatment with triton X-100 used by this author produced a greater degree of solubilisation than the methods of Oduro (1977). The observations of Oduro (1977) and Opperdoes and Borst (1977) that the microbody fraction contains all the enzymes required to convert glucose to 3-PGA and GP was repeated in this section. Since this metabolism requires net ATP the concentration of intermediates was examined for indications of the possible mechanism by which GP can decrease the ATP requirement.

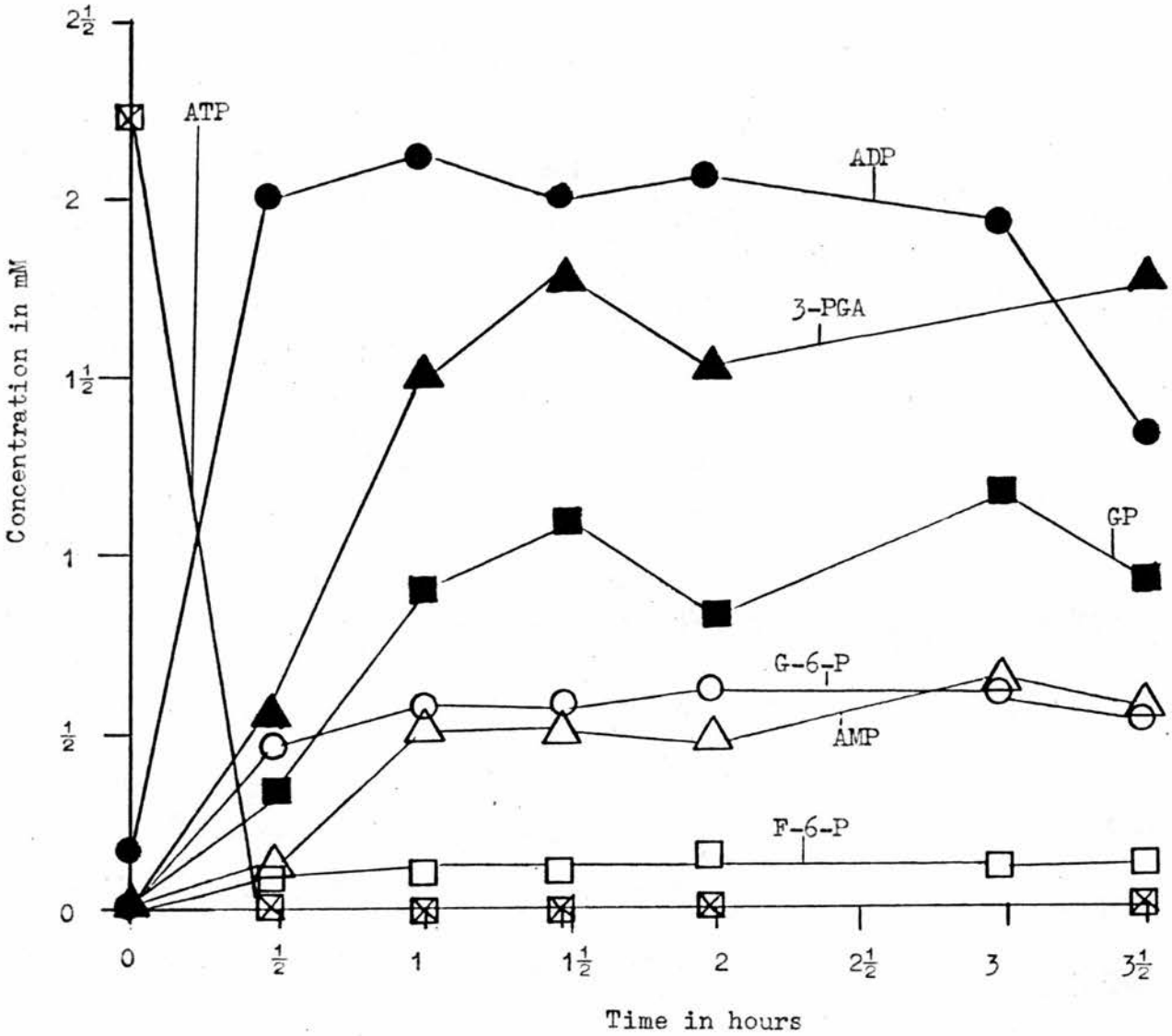


Fig. 4.10 : Concentrations of the products of glucose plus ATP metabolism of a 14 KP fraction under anaerobic conditions. The 14 KP fraction was incubated in the following medium : 0.25 mM-sucrose, 50 mM-tris, 60 mM-glucose, 2.25 mM-ATP, 1.0 mM-$MgSO_4$, 10 mM-KCl, 10 mM-Na_2HPO_4, 0.2 mM-EDTA and 3 mg/ml BSA at pH 7.0 and 25° C. Samples were taken at various times and assayed for adenine nucleotides and glycolytic intermediates. The concentrations of GA3P, DHAP, PEP and pyruvate were below detection. The maximum concentration of F-D-P was 0.07 mM and glycerol, 0.3 mM.

Incubation of a 14 KP fraction in a reconstituted system plus glucose and ATP (Fig. 4.10) showed that a complex of enzymes were present to convert 3-PGA and GP. However GA-3-P, 2-PGA, PEP and pyruvate were not detectable suggesting that the production of pyruvate from GP cannot take place in the 14 KP fraction due to the absence of one or more enzymes. Only low concentrations of F-D-P DHAP, maximum 0.07 μ mole/ml were found and glycerol showed variable but low concentrations (maximum 0.3 μ mole/ml).

In an aerobic incubation lower GP and higher DHAP concentrations were found suggesting contamination by GP oxidase. GP oxidase has been established by Opperdoes et al. (1977a) to be present in the promitochondrion and Table 4.7 demonstrates it to sediment at lower centrifugal forces than the 14 KP fraction enzymes. Pure preparations of 14 KP fractions would be expected to show the same glycolytic intermediate concentrations in both aerobic and anaerobic conditions.

No pulsing effect of carbon through the glycolytic intermediates was seen in contrast to the unfractionated lysate incubation (Fig. 3.8). ATP is limiting at $\frac{1}{2}$ hour and consequently inhibits further G-6-P production from glucose by the hexokinase catalysed reaction and F-D-P production from F-6-P by the phosphofructose kinase catalysed reaction. Thus the concentration of G-6-P and F-6-P remains constant. F-D-P once formed can be metabolised through to 3-PGA and GP without an ATP requirement.

4.14 Melarsen Oxide Inhibition of Glucose and Glycerol Utilization by T. brucei

Melarsen oxide has been established as a potent inhibitor of glycerokinase, but is believed to act on whole cell respiration at the site of pyruvate kinase,

Table 4.7 : Toxicity of melarsen oxide to T. brucei metabolising glycerol aerobically and glucose aerobically and under GP oxidase inhibition

Substrate	SHAM	Melarsen Oxide	Time in minutes				
			1	6	10	20	30
Glucose	0.0 mM	0 μ M	++++	++++	++++	+++	+++
Glucose	0.5 mM	0 μ M	++++	+++	+++	+++	+++
Glycerol	0.0 mM	0 μ M	++++	++++	+++	+++	+++
Glucose	0.0 mM	0.17 μ M	+++	++++	+++	++	+
Glucose	0.5 mM	0.17 μ M	+++	++++	+++	+++	+
Glycerol	0.0 mM	0.17 μ M	+++	+++	+++	++	+
Glucose	0.0 mM	1.7 μ M	++	++	+	±	-
Glucose	0.5 mM	1.7 μ M	++	++	+	+	-
Glycerol	0.0 mM	1.7 μ M	+++	++	++	+	-
Glucose	0.0 mM	17.0 μ M	+	-	-	-	-
Glucose	0.5 mM	17.0 μ M	+	-	-	-	-
Glycerol	0.0 mM	17.0 μ M	-	-	-	-	-

The trypanosomes were incubated in phosphate saline buffer at pH 8.0 at 20° C. The buffer contained either 10 mM-glucose or 10 mM-glycerol and when present SHAM at 0.5 mM. All samples were preincubated for 5 minutes before varying concentrations of melarsen oxide were added. Motility tests were carried out at various times after the addition of melarsen oxide.

Flynn and Bowman (1974). If glycerokinase were implicated as a necessary enzyme in anaerobic glucose metabolism a higher sensitivity towards melarsen oxide inhibition should be seen in cells metabolising glucose anaerobically compared to those metabolising aerobically.

The results of Table 4.7 show that even when glycerokinase is a mandatory enzyme in substrate utilization, as in the case of aerobic glycerol utilization, the same inhibition by melarsen oxide is found compared to aerobic and anaerobic glucose utilization.

From these results it is evident that an enzyme common to glucose and glycerol metabolic pathways must be inhibited, in agreement with the observations of Flynn and Bowman (1974) that pyruvate kinase is the major site of action metabolically. That melarsen oxide does not inhibit glycerol utilization at the reaction stage catalysed by glycerokinase despite having a very low I_{50} for glycerokinase could be due to the inaccessability of melarsen oxide to this enzyme. This most likely would be due to glycerokinase's compartmented nature affording protection against melarsen oxide inhibition, in comparison to the situation in the particulate glycerokinase, section 4.4. Pyruvate kinase has a higher I_{50} value for melarsen oxide inhibition but will be a more rapidly attacked target enzyme due to its cytoplasmic location.

SECTION 5

DISCUSSION

5.1 Evidence for the Existence of an Alternative Pathway of Glucose Metabolism by T. brucei under Glycerol-3-Phosphate Oxidase Inhibited Conditions

The work of Ryley (1956) and Grant and Fulton (1957) demonstrated that some trypanosomes of the subgenus Trypanozoon including T. brucei metabolised 1 mole of glucose to 2 moles of pyruvate under aerobic conditions. However anaerobically a different pathway was operating metabolising glucose to equimolar pyruvate and glycerol, showing that glycerol has acted as a trap for electrons from NADH generated in the glycolytic conversion of glucose to a mole of pyruvate. These observations have been reproduced for T. brucei, TREU 55 (Table 3.1) and have been extended to show the aerobic and anaerobic metabolism of fructose and mannose produces the same end products as glucose metabolism (Table 3.2). The anaerobic production of pyruvate and glycerol from glucose must be accompanied by net ATP synthesis (Fulton and Spooner, 1959; Clarkson and Brohn, 1976; Opperdoes et al., 1976b) since glucose can support trypanosome motility anaerobically, but without a substrate source the organisms rapidly lyse. These observations were repeated in Table 3.3 and Table 3.4 demonstrated that a similar situation existed for SS trypomastigotes.

The possible evolutionary importance of T. brucei possessing a bioenergetic pathway for the anaerobic metabolism of glucose is considered in the following subsection.

5.2 Possible Evolutionary Importance of an Anaerobic Method of Glycolysis in T. brucei

The parasitaemias of T. brucei found in its natural hosts are low compared to the fulminating infections of syringe passaged monomorphic forms parasitising

laboratory animals. Even in the latter anaerobic survival will not be of importance as the following calculations show for a 230 g rat at the height of a T. brucei parasitaemia :

a) Calculating the oxygen demand of the trypanosome infection :

   i) The optimum yield of trypanosomes from the rat is about 150 mg of protein (personal observation).

   ii) The rate of oxygen consumption of T. brucei at 37° C = 200 n moles/mg of protein (personal observation).

   Therefore the maximum oxygen demand of the trypanosomes in the rat is 200 x 150 = 30μ moles/minute.

b) The minimum oxygen demand of a 230 g rat is 133μ moles/minute (Denckla, 1970).

c) The arterial blood of the rat is about 98% saturated with oxygen, while the venous value is 64% (Dyson, 1975) consequently the rat consumes about 34% of the available oxygen and the trypanosome infection at a maximum can only decrease this value by $\frac{30}{133}$ x 34 = 8%. An increased oxygen demand of this order can easily be met with an increased oxygen supply by the host thus maintaining a relatively constant venous oxygen concentration (Denckla, 1970).

T. brucei are able to utilize oxygen from blood at a steady-rate to below an O_2 concentration of 10 μ Molar, about 4% saturation, a value far lower than it will experience in laboratory animals. Consequently oxygen deficiency is extremely unlikely when T. brucei is parasiting its natural host where trypanosome concentrations are much lower than in laboratory animals.

Neither is oxygen diffusion into the cell likely to be a rate limiting factor in its utilization, for oxygen is a lipophilic substance (Battino et al., 1968) which will diffuse through membranes at much faster rates than those expected from the Fick-Einstein theory of diffusion. Oxygen is expected to experience

gaps between the molecules of the membrane and consequently its movement is relatively unimpeded (Fischkoff et al., 1975) and much faster than glucose transport across the membrane even with facilitated diffusion.

It is therefore unlikely that trypomastigotes have developed, or retained an alternative pathway as a selective advantage in the vertebrate host, but it may well be vital in the transition from trypomastigote to procyclic trypomastigote forms found in the fly. Trypomastigotes give rise to procyclic trypomastigotes after they have been taken up by the tsetse fly with a meal of infected blood. It is probably in the low oxygen tension environment of the fly's midgut that the trypomastigotes will encounter an oxygen deficiency, and with the transformation of SS trypomastigotes to procyclic forms taking at least twelve hours, based on morphological studies (Wijers and Willet, 1960), this anaerobic environment is far from temporary. SS trypomastigotes would therefore be expected to possess a pathway for anaerobic survival. Forms morphologically identical to SS trypomastigotes that can utilize α-oxoglutarate aerobically and glucose anaerobically are not resistant to a 10 mM-glycerol plus 0.5 mM-SHAM challenge, Table 3.4. This shows a similarity of anaerobic glucose metabolism between LS and SS trypomastigotes, in contrast with the reported findings of Clarkson and Brohn (1976) who found SS forms were not cleared from the blood after a SHAM plus glycerol challenge. The method of anaerobic ATP production must be directly involved in anaerobic glycolysis since α-oxoglutarate will not support SS trypomastigote motility anaerobically whereas glucose will.

5.3 The Thermodynamic Efficiencies of ATP Production from Glucose and Glycerol Metabolism by T. brucei

The standard free energy change ($\Delta G'_o$) in metabolising glucose to pyruvate and glycerol with ATP synthesis from ADP plus inorganic phosphate as an overall

Table 5.1 : The standard free energy changes of glycolytic, cofactor, redox reactions and ATP synthesis

No	Reaction	$\Delta G'_o$ in kJ/mole	Reference
1	Glucose + ATP $\rightarrow$ G-6-P + ADP + H^+	-14.2	1
2	G-6-P $\rightarrow$ F-6-P	+ 2.1	1
3	F-6-P + ATP $\rightarrow$ F-D-P + ADP + H^+	-14.2	1
4	F-D-P $\rightarrow$ GA3P + DHAP	+23.9	1
5	DHAP $\rightarrow$ GA3P	+ 7.6	1
6	GA3P + Pi + NAD^+ $\rightarrow$ 1,3-diPGA + NADH + H^+	+ 6.2	1
7	1,3-diPGA + ADP + H^+ $\rightarrow$ 3-PGA + ATP	-28.3	1
8	3-PGA $\rightarrow$ 2-PGA	+ 4.4	1
9	2-PGA $\rightarrow$ PEP	- 4.4	1
10	PEP + ADP + H^+ $\rightarrow$ pyruvate + ATP	-23.8	1
11	Pyruvate + NADH + H^+ $\rightarrow$ lactate + NAD^+	-25.0	1
12	ATP $\rightarrow$ ADP + Pi	-32.1	2
13	H^+ + NADH $\rightarrow$ NAD^+ + H_2	+61.6	1
14	H_2 + $\frac{1}{2}O_2$ $\rightarrow$ H_2O	-157.4	1
15	DHAP + NADH + H^+ $\rightarrow$ GP + NAD^+	-28.3	3
16	GP + $\frac{1}{2}O_2$ $\rightarrow$ DHAP + H_2O	-67.5	4
17	$Glycogen_n$ + Pi $\rightarrow$ G-1-P + $glycogen_{(n-1)}$	+ 3.0	5
18	G-1-P $\rightarrow$ G-6-P	- 7.2	5
19	GP + ADP + H^+ $\rightarrow$ glycerol + ATP	+22.1	2

References : (1) Dyson (197), (2) Gutfreund (1972), (3) Bergmeyer (1974a), (4) obtained by considering the GP cycle, the net change of reactions 15 and 16 combined is $\frac{1}{2}O_2$ + NADH + H^+ $\rightarrow$ H_2O + NAD^+, the $\Delta G'_o$ for these combined reactions must be equal to that of reactions 13 and 14 combined, -95.8 kJ/mole. Since reaction 15 has a $\Delta G'_o$ value of -28.3 kJ/mole, the $\Delta G'_o$ value for reaction 16 must be -95.8 + 28.3 = -67.5 kJ/mole, (5) Mahler and Cordes (1971).

Table 5.2 : Standard free energy changes and ATP synthesising efficiencies of various bioenergetic pathways

No	Overall reaction change	Reactions involved	$\Delta G'_o$ in kJ/mole	Energy involved in ATP synthesis	Efficiency
1	Glucose + O_2 + 2ADP + 2Pi → 2pyruvate + $2H_2O$ + 2ATP	1 → 5,2x(6 → 10), 2x(15 + 16)	-278	64.2	19%
2	Glucose + ADP + Pi → pyruvate + glycerol + ATP	1 → 4,6 → 10,15,19	-54	32.1	37%
3	Glucose + 2ADP + 2Pi → 2lactate + 2ATP	1 → 5,2x(6 → 11)	-137	64.2	32%
4	Glycogen + 3ADP + 3Pi → 2lactate + 3ATP + $glycogen_{(n-1)}$	2 → 5,2x(6 → 11), 17,18	-126	96.3	43%
5	Glycerol + ADP +Pi + O_2 → pyruvate + ATP + $2H_2O$	5 → 10,15,2x(16), 19	-223	32.1	14%

reaction sequence is independent of the pathway postulated. The value for this $\Delta G'_o$ and the efficiency of this pathway have been evaluated (Table 5.2) based on the production of glycerol plus ATP from GP plus ADP catalysed by glycerokinase, Table 5.1, reaction 19. This pathway was selected as the relevant thermodynamic values are available from the literature.

Table 5.2 shows that the efficiencies of ATP production, calculated by the $\Delta G'_o$ of net ATP synthesis divided by the sum of the overall $\Delta G'_o$ of the reaction sequence plus the $\Delta G'_o$ of net ATP synthesis for various systems. The anaerobic metabolism of glucose by T. brucei shows an efficiency of 37% comparable to that obtained for other bioenergetic pathways such as Table 5.2, no. 4. Table 5.2 also demonstrates the relative inefficiency of the aerobic metabolism of glucose of 19% and of glycerol (15%). Aerobic ATP production is inefficient due largely to the high $\Delta G'_o$ value (-67.5 kJ/mole) of the GP oxidase catalysed reaction.

The thermodynamic basis for an anaerobic pathway producing 1 ATP per glucose metabolised to pyruvate plus glycerol agrees well with the experimental findings that T. brucei can utilize glucose anaerobically. However the less efficient aerobic pathway has a rate of ATP production of at least twice that obtained anaerobically because 1 mole of glucose will synthesise 2 moles of ATP aerobically compared with 1 mole anaerobically and can therefore maintain a much higher adenylate charge (Table 3.5).

5.4 Testing the Various Postulated Schemes of Anaerobic Glucose Metabolism Yielding Equimolar Pyruvate and Glycerol by Specific Enzyme Analysis

Honigberg (1967) hypothesised that the GP formed from glucose by the pathway described in section 3.5.1 is dephosphorylated to glycerol plus inorganic phosphate under GP oxidase inhibited conditions. However this scheme will not

account for the net ATP synthesis found when glucose is metabolised anaerobically (Fulton and Spooner, 1959; Clarkson and Brohn, 1976; Opperdoes et al., 1976b). The results of section 3.5(1) demonstrate that in a variety of assay conditions and with different broken cell preparations only very low activities of GP phosphatase was present compared to the whole cell rate of anaerobic glycerol production (Table 3.6).

To account for glycerol plus ATP production from glucose in GP oxidase inhibited cells a number of different metabolic pathways have been postulated (Opperdoes et al., 1976b; Clarkson and Brohn, 1976; Opperdoes and Borst, 1977; Fairlamb et al., 1977). These schemes have been tested by looking for specific enzyme activities required in each different scheme.

1) GP : glucose or F-6-P transphosphorylase

There are two ATP utilizing steps in glucose metabolism, the hexokinase and phosphofructose kinase catalysed reactions. If one of these ATP utilizing reactions were to be substituted by transphosphorylation with GP as the phosphate donor the metabolism of 1 mole of glucose to 1 mole of F-D-P would require 1 mole of ATP and 1 mole of GP. The F-D-P could then be metabolised to GA-3-P plus DHAP. The further metabolism of GA-3-P to pyruvate will produce 2 moles of ATP while the DHAP will maintain the redox balance and GP concentration via the glycerol-3-phosphate dehydrogenase catalysed reaction. Thus a net production of 1 mole of ATP will be formed per mole of glucose metabolised to pyruvate plus glycerol (see Fig. 1.3) (Clarkson and Brohn, 1976; Opperdoes et al., 1976b).

A GP : glucose tranphosphorylase has only been tested with glucose as the phosphate acceptor, but this postulated enzyme would also have to phosphorylate mannose and fructose as these carbohydrates are metabolised by a pathway similar to glucose (Table 3.8) since glycerol inhibits a reaction involved in the

anaerobic metabolism of all three carbohydrates or alternatively the trypanosome must be able to interconvert these sugars. At present, GP : glucose transphosphorylase activity remains undetected (section 3.5.1) although a wide range of lysate preparations and assay conditions were tested. A further requirement for this anaerobic glycolytic scheme is the inactivation of hexokinase and glycerokinase. A GP : glucose transphosphorylase would have to compete with the very active hexokinase in phosphorylating glucose. The effect of GP on hexokinase activity was therefore examined to find if the increased GP concentrations found anaerobically (Fig. 3.12) inhibits hexokinase and hence activates the anaerobic pathway. The results of section 4.12 show that GP at physiological concentrations has no significant effect upon hexokinase. Glycerokinase must also be inhibited in this scheme for extracellular glycerol concentrations of 1 mM and above will be almost saturating for glycerokinase, which if active anaerobically would rapidly utilize ATP creating an increasing GP concentration at the expense of ATP. GP plus ADP formation from glycerol plus ATP is not inhibited by 100 mM-GP (section 4.12). Consequently none of the three conditions necessary for this postulated method of anaerobic glycolysis : GP : glucose transphosphorylase activity, hexokinase inactivation and glycerokinase inactivation have been established in broken cell preparations.

The other possible transphosphorylase, the GP ; F-6-P transphosphorylase has also been tested for in a whole range of lysate preparations and assay conditions, but activity of the correct specificity remains undetected (section 3.5.1). A further necessity for this scheme of anaerobic glycolysis is the inactivation of phosphofructose kinase and glycerokinase. Phosphofructose kinase is a very active enzyme in T. brucei (Table 3.7) and would compete for F-6-P with the proposed GP : F-6-P transphosphorylase. The effect of GP on phosphofructose kinase was therefore examined to find if the increased GP concentrations found

anaerobically could inhibit phosphofructose kinase and hence activate the anaerobic pathway. However the results of section 4.12 showed that up to 100 mM-GP had no effect upon phosphofructose kinase activity. The rational behind the requirement for glycerokinase inhibition and the lack of inhibition with GP are summarized in the preceeding paragraph. None of the three criteria for the postulated GP : F-6-P transphosphorylase scheme (GP : F-6-P transphosphorylase activity, phosphofructose kinase inactivation and glycerokinase inactivation) have been established in broken cell preparations.

11) Hexose monophosphate aldolase and glycerol dehydrogenase scheme

This scheme has been postulated by Clarkson and Brohn (1976) and Opperdoes et al. (1976b) and is outlined in Fig. 1.2. A hexose monophosphate aldolase will decrease the ATP requirement of 1 mole of glucose to 1 mole by forming a hexose phosphate which is cleaved to equimolar triosephosphate and triose. The triosephosphate could then yield 2 moles of ATP per mole metabolised to pyruvate with concomitant reduction of NAD^+. The NADH produced could then be reoxidised as the triose is reduced to glycerol via a glycerol dehydrogenase catalysed reaction. This will produce a net ATP synthesis from glucose metabolism to pyruvate plus glycerol.

Hexose monophosphate aldolase has been detected by Clarkson and Brohn (1976) but these findings have not been confirmed by experiments described in this thesis (Table 3.10) with F-1-P, F-6-P, G-1-P or G-6-P as substrate metabolised either directly or indirectly. Glycerol dehydrogenase activity has also remained undetected in a variety of enzyme preparations and assay conditions (section 3.5.3).

iii) Hexose monophsophate aldolase and GP triose transphosphorylase scheme
This scheme has been postulated by Clarkson and Brohn (1976) and is outlined in Fig. 1.4. 1 mole of glucose forms F-1-P with utilization of 1 mole of ATP, F-1-P is then cleaved to glyceraldehyde and DHAP. The DHAP can then be reduced to GP with concomitant oxidation of NADH. The GP is then used to transphosphorylate the triose generating 1 mole of glycerol and 1 mole of a second triosephosphate, which can then yield 2 moles of ATP and reduce NAD^+ in its metabolism to pyruvate. Thus this scheme provides net ATP synthesis but requires a hexose monophosphate aldolase and a GP : triose transphosphorylase.

The evidence for a hexose monophosphate aldolase has been discussed in the previous subsection. The presence of a GP : glyceraldehyde or dihydroxyacetone transphosphorylase has also been tested (section 3.5.4) for a variety of different broken cell preparations of T. brucei and for a wide range of assay conditions but GP triose transphosphorylase activity remained undetected.

iv) GP : ADP transphosphorylation catalysed by glycerokinase
This possibility, proposed by Opperdoes and Borst (1977) suggests that GP and pyruvate can be formed by the scheme represented in Fig. 3.5 but that GP is used to phsophorylate ADP directly, catalysed by glycerokinase. Consequently 2 moles of ATP will be utilized in forming F-D-P which is then cleaved to GA-3-P and DHAP. GA-3-P can then synthesise 2 moles of ATP in its metabolism to form 1 mole of pyruvate. Therefore net ATP synthesis is achieved by the direct phosphorylation of ADP by GP in forming ATP plus glycerol.

Table 4.1 demonstrated that the rate of GP dephosphorylation was greatly enhanced by ADP and that this was linked to an increased production of ATP from ADP provided that a mechanism for trapping ATP was present. Subsequent experiments showed glycerokinase was the enzyme catalysing this reaction (sections 4.4 and 4.5) and a 17 fold purification of glycerokinase has been achieved (section 4.6).

Maximum activities of the purified, solubilised glycerokinase have been established (Table 4.4) and show the enzyme capable of producing glycerol plus ATP from GP plus ADP. The maximum rate of this activity was low, about 1/200 th the rate of GP plus ADP formation from glycerol plus ATP. This could account for a glycerol production of about 15 n moles/minute/mg of protein at 25° C for a lysed preparation contrasting with the observed rate of glycerol production of about 58 n moles/minute/mg of protein for whole cells metabolising glucose anaerobically (Table 3.2). The ratio of the forward and reverse maximum activities was however found to depend upon the method of preparation, for when lysates were prepared in isotonic media by grinding compared to detergent lysates the specific activity of GP plus ADP production from glycerol plus ATP decreased but the reverse reaction was found to increase (Table 4.4). The decrease in the rate of formation of GP plus ADP from glycerol plus ATP from 3 to 0.8 μ mole/minute/mg of protein can be explained by a permeability barrier of the unlysed organelle inhibiting the transport of substrates to the particulate glycerokinase and the transport of products to the marker enzymes. A similar phenomenon has been observed by Oduro (1977) for other particulate enzymes. The production of glycerol plus ATP from GP plus ADP shows an increase in activity (0.015→0.034 μ moles/minute/mg of protein) the opposite of that expected if a permeability barrier existed. Furthermore the method of assay employed relied upon measuring ATP production by trapping through glucose phosphorylation and linking this to $NADP^+$ reduction via the glucose-6-phosphate dehydrogenase catalysed reaction. Since permeability barriers exist for some glycolytic intermediates and cofactors (Oduro, 1977) and melarsen oxide (section 4.4) the marker enzymes and $NADP^+$ are not likely to be freely accessible to the site of ATP production. Thus the value of 34 n moles/minute/mg of protein at 25° C may well be only a fraction of the true intra-organelle activity.

The increased activity of ATP production via the glycerokinase catalysed reaction found in the particulate preparation could reflect a conformational change in the glycerokinase occurring upon solubilisation. McLaren and Baker (1970) and Laidler and Sundaram (1971) have demonstrated that the kinetic behaviour of enzymes in heterogenous systems can be altered from those in dilute solutions. The kinetic properties of glycerokinase may be therefore altered upon solubilisation resulting in the inhibition of glycerol plus ATP formation from GP plus ADP. Alternatively a close juxtaposition of the endogenous hexokinase may be more efficient in trapping ATP as it is produced from GP and ADP than the hexokinase in the solubilised system. Mosbach and Mattiasson (1970) and Mattiasson and Mosbach (1971) have demonstrated that two enzyme systems immobilised on a matrix show a faster overall rate of the coupled reactions prior to reaching a steady-state than a corresponding soluble system. Therefore if the rate of ATP formation depends upon how rapidly it can be removed at low concentrations a faster maximum velocity will be found in the matrix bound (particulate) systems. Certainly a specific spatial organisation of the glycolytic enzymes in the organelle would account for the observations of Grant and Fulton (1957) that the C1 of glucose is preferentially found in glycerol rather than pyruvate for T. rhodesiense under anaerobic conditions. DHAP and GA3P formed from the aldolase catalysed reaction could be preferentially oxidised or reduced by their respective dehydrogenases due to these enzymes having a close proximity to aldolase rather than being interconverted by the triosephosphate isomerase catalysed reaction.

If anaerobic glucose metabolism to pyruvate plus glycerol with net ATP synthesis were dependent upon glycerol plus ATP formation from GP plus ADP the following conditions must be met :

1) The rate of ATP production must be able to account for the observed rate of whole cell anaerobic glycerol production. This has not yet been achieved although values of about 50% of the expected value have been obtained and there is evidence to suggest the assay conditions employed were sub-optimal (section 4.9).
2) The ATP trapping enzyme, either hexokinase or phosphofructose kinase must not be inhibited by GP. This is found (section 4.12), although hexokinase is inhibited by GP at concentrations well above the physiological range (up to 100 mM).
3) Hexokinase or phosphofructose kinase and glycerokinase must be present within the same organelle with a limiting membrane. This is again found. All three enzymes are found with high activities especially glycerokinase, a condition necessary for the postulated scheme of anaerobic glycolysis.

Specific enzyme analysis has revealed that only the scheme postulated by Opperdoes and Borst (1977) has shown detectable enzyme activities of the correct specificity and in the order of magnitude to account for glycerol production from anaerobic glucose metabolism. The lack of detectability of enzymes vital for the other postulated scheme does not prove their absence for inoperative assay systems or faulty preparation methods may be responsible for the lack of detection. These factors were minimized by selecting three different preparation techniques and employing assay systems with varied pH and substrate concentrations. Control enzymes of known specificity were used whenever possible to show the system sensitive to measuring the formation of the desired product. Lysates were prepared from anaerobically preincubated cells in case enzyme modification upon anaerobiosis necessarily preceded glycerol formation. The addition of GP to assays in which it was not a substrate was tested to elucidate if GP acted as a triggering device for switching on the anaerobic pathway by activation or inhibition of various enzymes. A metabolic control

for the anaerobic glycolytic pathway seems most likely because the time required for glycerol production after anaerobiosis is too fast for protein synthesis (section 3.9), and only GP of the glycolytic intermediates was found to increase upon GP oxidase inhibition (Fig. 3.14).

5.5 Testing the Various Postulated Schemes of Anaerobic Glucose Metabolism Yielding Equimolar Pyruvate and Glycerol by Incubation Studies of Cell Free Preparations

These studies attempted to elucidate possible differences between aerobic and anaerobic pathways through estimating intermediate substrate concentrations with time and looking for points of difference in the two conditions. In this way it was hoped indications of pathway differences may become evident, perhaps suggesting a pathway not previously postulated.

All enzymes required for pyruvate production from glucose were present but minimal glycerol was produced in cell free studies, suggesting that whole cell integrity could be vital for glycerol production. The rise and fall of the glycolytic intermediates showed that glucose was first metabolised to G-6-P and F-6-P, then F-D-P next DHAP and 3PGA followed by GP and finally pyruvate. The recovery of the glucose carbon in the various intermediates and end products was about 100% indicating that all the major accumulations of carbon from glucose has been measured (section 3.6).

These results are in agreement with the schemes outlined in Fig. 3.4 and 3.5, however if a different scheme were operating such as a hexose phosphate aldolase, glycerol dehydrogenase scheme a different profile of intermediate concentration rise and fall would be expected, e.g. the triosephosphates would accumulate before F-D-P.

5.6 Intracellular Glycolytic Intermediate Concentrations

Broken cell preparations were found to possess all the enzymes required to produce pyruvate from glucose but failed to produce significant concentrations of glycerol anaerobically. Therefore the steady-state and transient glycolytic intermediate concentrations of whole cells metabolising glucose under both aerobic and GP oxidase inhibited cells were examined in the hope that indications of pathway differences may become evident.

The major conclusions from these experiments were that the phosphorylation of glucose was inhibited under GP oxidase inhibited conditions (Fig. 3.13). Three possible explanations were attributed to this observation :

a) Glucose transport could be inhibited by glycerol.

b) ATP availability for the hexokinase reaction could be the rate limiting factor anaerobically.

c) Hexokinase may be inhibited anaerobically and glucose is phosphorylated by a less active GP : glucose transphosphorylase.

All glycolytic intermediate concentrations except GP decreased upon GP oxidase inhibition. GP increased to a maximum concentration which was dependent upon the method of trypanosome preparation but was independent of extracellular glycerol concentration, Fig. 3.16 and independent of time, Fig. 3.17 thus GP production from glucose must equal its utilization. This can be achieved by one of the following :

d) The formation of 1 mole of F-D-P from glucose which requires 1 mole of GP donating a phosphate group through transphosphorylation of glucose or a hexosephosphate. F-D-P is then cleaved to DHAP plus GA3P by the aldolase catalysed reaction and 1 mole of GP is reformed by the glycerol-3-phosphate dehydrogenase catalysed reaction (Fig. 1.3).

e) 1 mole of glucose forming 1 mole of GP and 1 mole of a triose by the method postulated in Fig. 1.4. The 1 mole of GP is then used to phosphorylate the 1 mole of triose to a second triosephosphate.

f) The rate of glucose phosphorylation is limited by ATP availability and when ADP is phosphorylated by GP catalysed by glycerokinase, the ATP produced can either be used in the phosphorylation of glucose or glycerol but either will produce 1 mole of GP per mole of ATP utilized (F-6-P could theoretically substitute for glucose).

g) When the concentration of GP increases beyond a certain value it inhibits its own production from glucose through activating an alternative pathway that bypasses F-D-P and hence GP production. Such a pathway could be the postulated hexose monophosphate aldolase and glycerol dehydrogenase scheme as represented in Fig. 1.2.

5.7 Glycerol Inhibition of Anaerobic Glucose Utilization

Glycerol was found to inhibit anaerobic glucose utilization with I_{50} and I_{100} values dependent upon the method of trypanosome preparation (section 3.3), with higher I_{50} values being obtained from trypanosomes prepared in substrate rich media. Glycerol cannot inhibit the transport of sugars in agreement with the conclusions of Gruenberg *et al.* (1978) as the I_{50} and I_{100} values would be independent of the preparation method. Since glycerol does not act upon glucose transport it could inhibit glucose phosphorylation by either decreasing the ATP available for the hexokinase catalysed reaction or by the mass action effect on a GP : glucose transphosphorylase.

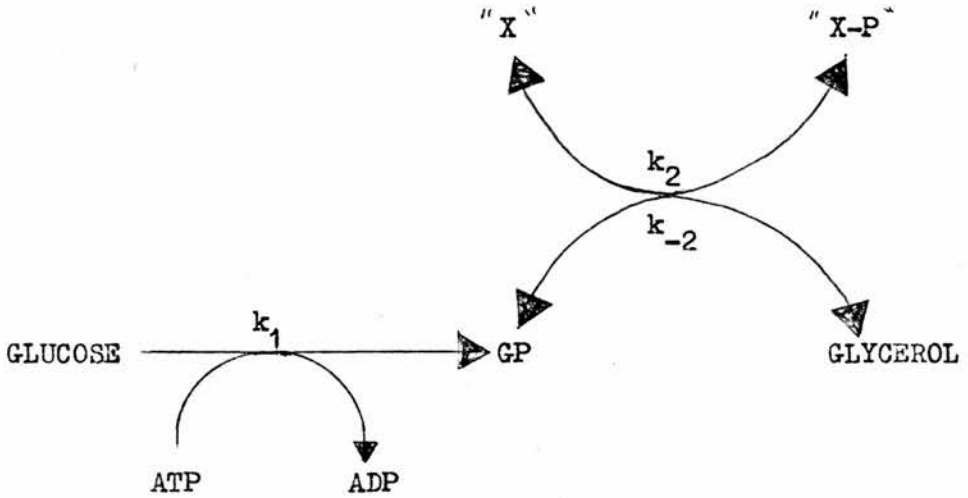
Considering the latter option first. Neither glucose transport (section 3.3) nor GP concentration (section 3.10) are altered with increasing glycerol

concentrations, consequently the decreased rate of G-6-P formation with increasing glycerol must be due to the reverse reaction, G-6-P : glycerol transphosphorylation increasing with increasing glycerol. However glycerol phosphorylation to GP is greatly decreased with increasing glycerol (Fig. 3.18). Consequently by deduction ATP must be the rate limiting factor in anaerobic G-6-P formation (alternative 'b' in section 5.6).

Glycerol could inhibit ATP production by inhibiting glycolysis through the mass action reversal of the glycerol producing reaction and hence inhibiting ATP synthesis from the pyruvate kinase and perhaps also the phosphoglycerate kinase catalysed reactions depending upon the position of this reaction in the anaerobic glycolytic sequence. The glycerol producing enzyme could be a GP : F-6-P transphosphorylase (alternative 'd'), glycerol dehydrogenase (alternative 'g'), GP : triose transphosphorylase (alternative 'e') or GP : ADP transphosphorylation (glycerokinase catalysed) (alternative 'f') according to the postulated schemes.

The hexose monophosphate aldolase and glycerol dehydrogenase scheme ('g') can be invalidated by the results of Fig. 3.18 showing that glycerol is being incorporated into GP at a fast rate compared to glycerol and hence ATP production. At low glycerol concentrations this rate is significantly faster than glucose utilization demonstrating that glycerol cannot be phosphorylated by ATP to form GP, then dephosphorylated by the action of a phosphatase to glycerol. Instead phosphorylation of glycerol must be reversibly coupled to the transphosphorylation of a metabolisable compound such as ATP or a glycolytic intermediate.

If this compound were a glycolytic intermediate increasing the glycerol concentration would be expected to increase the rate of its incorporation to GP compared to the rate of glycerol formation from GP. Consider the anaerobic system :



$$\frac{d[GP]}{dt} = 0 \text{ (experimentally proven Fig. 3.19, 3.11)}$$

$$= [\text{glucose}]\,[ATP]\,k_1 + k_{-2}\,[X\text{-}P]\,[\text{glycerol}] - [X][GP]\,k_2 \quad *$$

$$[\text{glucose}]\,[ATP]\,k_1 + k_{-2}\,[X\text{-}P]\,[\text{glycerol}] = [X][GP]\,k_2$$

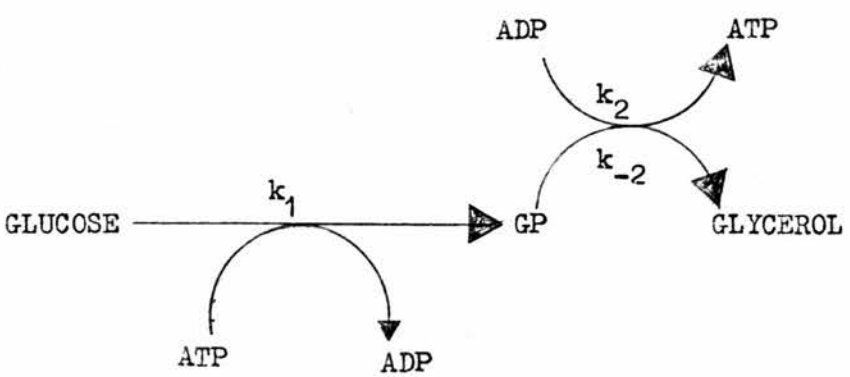
Since the rate of glucose phosphorylation is glucose independent the term $[\text{glucose}]\,[ATP]\,k_1$ can be simplified to $[ATP]\,kg_1$

rearranging and simplifying

$$[ATP]\,kg_1 = [X][GP]\,k_2 - k_{-2}\,[X\text{-}P]\,[\text{glycerol}]$$

The term $[ATP]\,kg_1$ decreases with increasing glycerol (Fig. 3.1). An increase in glycerol would be expected to decrease the glycolytic flux ($[ATP]\,kg_1$) by increasing the value $k_{-2}\,[X\text{-}P]\,[\text{glycerol}]$. However the opposite is found Fig. 3.18. Indicating that if 'X-P' were a glycolytic intermediate glycerol must have a site of inhibition other than the mass action effect on the glycerol forming enzyme.

Considering the alternative scheme with 'X' the cofactor, ADP i.e. scheme f



* See page 123

Again $\frac{d[GP]}{dt} = 0 = [ATP][glucose]\,k_1 + k_{-2}[ATP][glycerol] - k_2[ADP][GP]$ *

$$[ATP]\,kg_1 + [ATP][glycerol]\,k_{-2} = k_2[ADP][GP]$$

$$[ATP](kg_1 + [glycerol]\,k_{-2}) = k_2[ADP][GP]$$

Increasing glycerol would be expected to decrease the ATP concentration according to the following relationship :

$$[ATP] = \frac{k_2[GP][ADP]}{k_{-2}[glycerol] + kg_1}$$

Thus the steady-state ATP concentration is inversely proportional to the glycerol concentration and since the hexokinase catalysed reaction is dependent upon the ATP concentration, the glycolytic rate will be inversely proportional to the glycerol concentration. On thermodynamic grounds ATP plus glycerol may only be formed from GP plus ADP when the free energy change (ΔG) of the reaction : GP + ADP $\longrightarrow$ glycerol + ATP is less than zero kJ/mole. ΔG is given by the equation :

$$\Delta G = \Delta G'_o + RT \ln \frac{[glycerol][ATP]}{[GP][ADP]} \qquad \text{Equation 1}$$

where $\Delta G'_o$ = 22.1 kJ/mole (Table 5.1)

R = 8.32 J/degree absolute/mole

T = 298°

ΔG is found to be negative when the ratio of $[GP][ADP]$: $[glycerol][ATP]$ is greater than 7740 : 1

Consequently a higher GP concentration will allow the glycerol concentration to increase without the ratio of ADP : ATP being affected. This could explain the observation that the I_{50} for the inhibition of anaerobic glucose utilization by glycerol varies with the cellular GP concentration according to the method of

* See page 123

trypanosome preparation. However if ATP and ADP concentrations were solely determined by this ratio then increasing glycerol should lead to an increase in ADP. With a constant GP concentration and an increasing ADP concentration with glycerol, the rate of GP : ADP transphosphorylation should increase. This is not found (section 3.12). Fig. 3.14 shows the concentration of ADP in the whole cell metabolising glucose anaerobically decreases as glycerol is increased. This decrease is met with a concomitant increase in the AMP concentration, and it is therefore probable that the ATP : ADP ratio is also dependent upon adenylate kinase activity.

5.8 The Thermodynamic Feasibility of GP : ADP Transphosphorylation

The thermodynamic feasibility of a self contained multi-enzyme complex as described by Oduro (1977) and Opperdoes and Borst (1977) being capable of catalysing the overall reaction sequence glucose plus inorganic phosphate to 3-PGA plus glycerol is favourable, as a $\Delta G'_o$ value of - 30.7 kJ/mole is obtained (Table 6.1). The value is independent of the pathway postulated. However for net glycerol plus ATP formation from GP plus ADP via the glycerokinase catalysed reaction a $\frac{[GP]\,[ADP]}{[GLYCEROL]\,[ATP]}$ ratio of greater than 7740 must be obtained (section 4.10).

Since glycerol phosphorylation is very rapid at low extracellular glycerol concentrations, glycerol must be freely permeable and show similar intracellular and extracellular concentrations. The intracellular GP concentration when based on the assumptions of section 4.12 equals about 25 mM, whereas the I_{50} value of glucose utilization by glycerol is about 2.5 mM at this GP concentration. Assuming the glycerol to GP ratio at the site of glycerokinase is equal to the whole cell ratio, the ADP : ATP ratio at a 2.5 mM glycerol concentration will be given by this formula :

$$\frac{[GP]\,[ADP]}{[glycerol]\,[ATP]} = 7740 \qquad \text{(from p. 98 equation 1)}$$

when GP = 25 mM and glycerol 2.5 mM

$$\frac{[ADP]}{[ATP]} = 774$$

A figure can be obtained for the whole cell $[ADP]:[ATP]$ ratio, Fig. 3.10, but from the evidence of a limiting membrane separating the ATP requiring enzyme in glycolysis (hexokinase and phosphofructose kinase in the glycosome) from the net ATP generating enzyme pyruvate kinase in the cytoplasm it is highly probable that the adenylate charge in the glycosome will be significantly different to that of the whole cell. The following calculations have been made to consider how a concentration ratio of ADP to ATP of 774 : 1 could influence glucose phosphorylation via the hexokinase catalysed reaction.

Initially we must assume a figure for the adenylate nucleotide concentration at the site of glycerokinase and that this concentration is made up mainly of ADP. Based on the assumptions of section 4.12 this figure is taken as 5 mM. An ADP : ATP concentration ratio of 774 : 1 would give a maximum ATP concentration of 7μM. At this ATP concentration the maximum velocity of the hexokinase catalysed reaction may be obtained from the Michaelis-Menton equation :

$$v = \frac{V_{max\,g}\,[ATP]}{K_m^{ATP} + [ATP]}$$

where v = velocity at saturating glucose and 7μM-ATP

V_{max_g} = maximum velocity (taken from Table 3.7)

K_m^{ATP} = Michaelis constant for ATP (taken from 4.6)

ATP = 7μM-ATP

$$\text{Consequently } v = \frac{0.48 \times 0.007}{0.06 + 0.007} n \text{ moles/minute/mg of protein at } 25^{\circ} C$$

$$\text{evaluating } \quad v = 50\ n \text{ moles/minute/mg of protein at } 25^{\circ} C$$

Consequently even at this low ATP concentration the rate of glucose phosphorylation could be sufficient to account for the whole cell rate of glucose phosphorylation. The estimated ATP concentration may be significantly enhanced by ATP's binding to at least five enzymes present in the glycosome : phosphofructose kinase, hexokinase, glycerokinase, phosphoglycerate kinase and adenylate kinase (Alberty, 1953b). Furthermore Mattiasson and Mosbach (1971) have shown that the kinetic behaviour of matrix bound enzyme systems compared to an analogous system of soluble enzymes show higher overall rates of coupled reactions prior to reaching a steady-state maximum. A comparable situation may well exist in the multi-enzyme complex compared to the solubilised assay systems (section 4.9) in respect to this coupled system :

$$GP + ADP \xrightarrow{(i)} \text{glycerol} + ATP$$

$$\text{glucose} + ATP \xrightarrow{(ii)} G\text{-}6\text{-}P + ADP$$

Catalysed by (i) glycerokinase and (ii) hexokinase

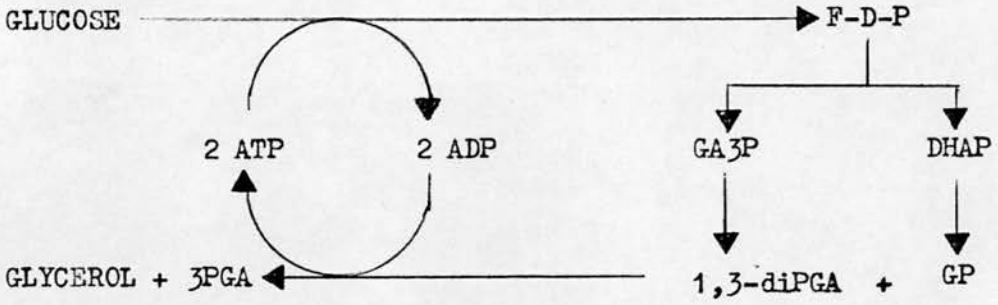
Such a situation would increase the ability of hexokinase to phosphorylate glucose by ATP produced by GP : ADP transphosphorylation when ATP concentrations are low.

5.9 The Properties of the Glycosome

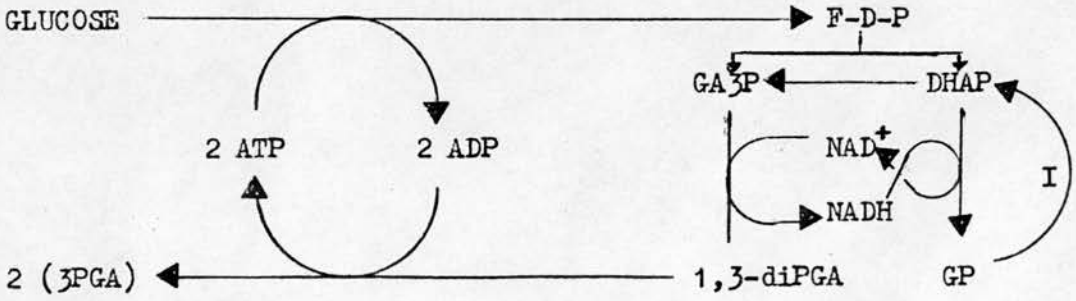
An organelle, the glycosome (Opperdoes and Borst, 1977) has been found in T. brucei to contain these enzymes : hexokinase, phosphoglucose isomerase, phosphofructose kinase, aldolase, glycerol-3-phosphate dehydrogenase phosphoglycerate kinase (Oduro, 1977; Opperdoes and Borst, 1977) glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase and glycerokinase (Opperdoes

and Borst, 1977) although Oduro (1977) believes these enzymes to be polydisperse. Glycerokinase and a DL-GP sensitive (Fig. 4.9) adenylate kinase have been found to co-sediment (Table 4.2) and with these enzymes in the 14 KP fraction from this author's researches. The permeability of substrates of some of these enzymes have been analysed by Oduro (1977) and indicate the presence of a limiting membrane. Similar findings are presented in this thesis for glycerokinase (section 4.9) further indicating the presence of a limiting membrane.

The enzymes present in the 14 KP fraction are sufficient to metabolise glucose to 3-PGA and GP. When GP is converted to glycerol by transphosphorylation the particle is self sufficient in ATP generation and in the reoxidation of NADH. In theory if this system were a completely independent unit in glycolysis the glycolytic intermediate concentrations within this particle should show the following relationship anaerobically :



and aerobically :



I - catalysed out of the glycosome by GP oxidase.

Thus the glycosomal glycolytic intermediate plus adenosine covalently bound phosphate concentration should remain constant. Figure 3.13 shows a glycosomal glycolytic intermediate phosphate concentration of 160 n moles/mg of protein greater in the anaerobic to aerobic transitional state compared to the aerobic state, although there is little difference in ATP, ADP or AMP concentrations.

Most conveniently this situation can be accounted for by F-6-P being phosphorylated (catalysed by phosphofructose kinase) by extra organelle ATP as well as an organelle component. Certainly this situation would account for the regulatory properties of phosphofructose kinase as described by Bowman et al. (1977). However this postulate has yet to be tested.

5.10 The Effect of Melarsen Oxide on T. brucei

Melarsen oxide has been found to be a potent inhibitor of solubilised glycerokinase (Fig. 4.2), but is far less active on particulate glycerokinase. It is also equally effective in killing trypanosomes respiring on glucose or glycerol, showing that its major metabolic site of action is an enzyme common to both pathways (section 4.14). Flynn and Bowman (1974) have established that pyruvate kinase is a major site of inhibition aerobically. The lack of increased melarsen oxide toxicity to trypanosomes under anaerobic conditions may be due to glycerokinase being protected by the organelle membrane.

5.11 Summary

It was originally hoped that by establishing the method by which trypanosomes could metabolise glucose anaerobically a pathway may be discovered which requires enzyme activity not found in the host. Specific inhibition of this pathway in conjunction with GP oxidase inhibition was hoped to provide a new and powerful chemotherapeutic combination of high selective toxicity to the trypanosome.

The results in this thesis are consistent with the postulated theory of Opperdoes and Borst (1977) that the alternative pathway of carbohydrate metabolism under conditions of GP oxidase inhibition involves the production of glycerol plus ATP from GP plus ADP catalysed by glycerokinase. If this postulate is confirmed it presents a difficulty for the drug combination approach to

chemotherapy of African trypanosomiasis for glycerokinase is a vital enzyme in vertebrate metabolism and specificity for the trypanosome enzyme may well be very difficult to achieve. Furthermore glycerokinase of T. brucei is found in an organelle and therefore a specific inhibitor of the trypanosome enzyme must be capable of being taken up by the parasite but must also be permeable to the organelle membrane.

SECTION 6

APPENDIX

6.1a List of Enzyme Assays

b List of Substrate Assays

6.2 Enzyme Assay Procedures

a) Hexokinase (HK)

Assay principle :

(i) $\text{glucose} + \text{ATP} \xrightarrow[Mg^{2+}]{HK} \text{G-6-P} + \text{ADP}$

(ii) $\text{G-6-P} + \text{NADP}^+ \xrightarrow[Mg^{2+}]{(G6PDH)} \text{6-phosphogluconate} + \text{NADPH} + H^+$

Reaction mixture at pH 7.0 :	Final Concentration or activity
TEA	100 mM
ATP	1 mM
$MgCl_2$	1 mM
$NADP^+$	1 mM
Glucose-6-phosphate dehydrogenase (G6PDH)	0.13 U
The reaction was started by the addition of glucose	60 mM

When ATP concentration was varied, pyruvate kinase, 1.3 U, plus 2 mM-PEP was added to maintain a constant ATP concentration through rephosphorylation of ADP formed from ATP by the hexokinase catalysed reaction and endogenous ATPases.

b) Phosphoglucose isomerase (PGI)

Assay principle :

(i) $\text{F-6-P} \xrightarrow{PGI} \text{G-6-P}$

(ii) $\text{G-6-P} + \text{NADP}^+ \xrightarrow[Mg^{2+}]{(G6PDH)} \text{6-phosphogluconate} + \text{NADPH} + H^+$

Reaction mixture at pH 7.0 :	Final Concentration or Activity
TEA	100 mM
$MgCl_2$	1 mM
$NADP^+$	1 mM
Glucose-6-phosphate dehydrogenase (G6PDH)	0.13 U
The reaction was started by the addition of F-6-P	10 mM

All assays performed on phosphoglucose isomerase measured G-6-P production from F-6-P and not the direction in glycolysis F-6-P production from G-6-P.

c) Phosphofructose kinase (PFK)

Assay principle :

(i) $\text{F-6-P} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{PFK}} \text{F-D-P} + \text{ADP}$

(ii) $\text{F-D-P} \xrightarrow{(\text{ALD})} \text{GA-3-P} + \text{DHAP}$

(iii) $\text{GA-3-P} \xrightarrow{(\text{TIM})} \text{DHAP}$

(iv) $\text{DHAP} + \text{NADH} + H^+ \xrightarrow{(\text{G-3-PDH})} \text{GP} + NAD^+$

Reaction mixture at pH 7.0 :	Final Concentration or Activity
TEA	200 mM
Iodoacetic acid	0.1 mM
ATP	1 mM
$MgCl_2$	1 mM
NADH	0.16 mM
SHAM	0.5 mM
Aldolase (ALD)	0.7 U
Triosephosphate isomerase (TIM)	20 U
Glycerol-3-phosphate dehydrogenase (G-3-PDH)	0.13 U
The reaction was started by the addition of F-6-P	10 mM

When the ATP concentration was varied 1.3 U pyruvate kinase plus 2 mM-PEP were added to maintain a constant ATP concentration through rephosphorylation of ADP formed from ATP by the phosphofructose kinase catalysed reaction and endogenous ATPases.

d) Aldolase (ALD)

Assay principle :

i) $\text{F-D-P} \xrightarrow{\text{ALD}} \text{GA3P} + \text{DHAP}$

ii) $\text{GA3P} \xrightarrow{(\text{TIM})} \text{DHAP}$

iii) $\text{DHAP} + \text{NADH} + \text{H}^+ \xrightarrow{(\text{G3PDH})} \text{GP} + \text{NAD}^+$

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
Iodoacetic acid	0.1 mM
$MgCl_2$	1 mM
NADH	0.16 mM
SHAM	0.5 mM
Triosephosphate isomerase (TIM)	20 U
Glycerol-3-phosphate dehydrogenase	1.3 U
The reaction was started by the addition of F-D-P	10 mM

e) Glyceraldehyde-3-phosphate dehydrogenase (GA-3-PDH)

Assay principle :

i) $\text{3-PGA} + \text{ATP} \xrightarrow{(\text{PGK})} \text{1,3-diPGA} + \text{ADP}$

ii) 1,3-diPGA + NADH + H^+ $\xrightarrow{\text{GA-3-PDH}}$ GA3P + NAD^+ + Pi

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
3-PGA	10 mM
ATP	4 mM
$MgCl_2$	1 mM
NADH	0.16 mM
Suramin	0.01 mM
Phosphoglycerate kinase (PGK)	42 U

The reaction was initiated by the addition of the extract, the control was minus 3-PGA.

All assays performed on glyceraldehyde-3-phosphate dehydrogenase measured NADH utilization, not NADH production, the direction found in glycolysis.

f) Phosphoglycerate kinase (PGK)

Assay principle :

i) 3-PGA + ATP $\xrightarrow{\text{PGK}}$ 1,3-diPGA + ADP

ii) 1,3-diPGA + NADH + H^+ $\xrightarrow{\text{(GA-3-PDH)}}$ GA3P + NAD^+ + Pi

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
ATP	4 mM
$MgCl_2$	1 mM
NADH	0.16 mM
Suramin	0.01 mM
Glyceraldehyde-3-phosphate dehydrogenase (GA-3-PDH)	1.4 U
The reaction was started by the addition of 3PGA	10 mM

All assays performed on phosphoglycerate kinase measured 1,3-diPGA production, not 3-PGA production, the direction found in glycolysis.

g) Phosphoglycerate mutase (PGM)

Assay principle :

i) $3\text{-PGA} \xrightarrow{PGM} 2\text{-PGA}$

ii) $2\text{-PGA} \xrightarrow{(ENOL)} PEP + H_2O$

iii) $PEP + ADP \xrightarrow{(PK)} pyruvate + ATP$

iv) $pyruvate + NADH + H^+ \xrightarrow{(LDH)} lactate + NAD^+$

Reaction mixture at pH 7.5	Final Concentration or Activity
TRIS	50 mM
KCl	50 mM
$MgSO_4$	10 mM
ADP	5 mM
EDTA	0.2 mM
NADH	0.16 mM
2,3-diPGA	0.1 mM
Enolase (ENOL)	1.3 U
Pyruvate kinase (PK)	3.3 U
Lactate dehydrogenase (LDH)	5.0 U
The reaction was started by the addition of 3-PGA	10 mM

h) Enolase (ENOL)

Assay principle :

i) $2\text{-PGA} \xrightarrow{ENOL} PEP + H_2O$

ii) $PEP + ADP \xrightarrow{(PK)} pyruvate + ATP$

iii) $pyruvate + NADH + H^+ \xrightarrow{(LDH)} lactate + NAD^+$

Reaction mixture at pH 7.5	Final Concentration or Activity
Tris	50 mM
KCl	50 mM
$MgSO_4$	10 mM
ADP	5 mM
EDTA	0.2 mM
NADH	0.16 mM
Pyruvate kinase	3.3 U
Lactate dehydrogenase	5.0 U
The reaction was started by the addition of 2-PGA	10 mM

i) Pyruvate kinase (PK)

Assay principle :

i) $PEP + ADP \xrightarrow{PK} pyruvate + ATP$

ii) $pyruvate + NADH + H^+ \xrightarrow{(LDH)} lactate + NAD^+$

Reaction mixture at pH 7.5	Final Concentration or Activity
Tris	50 mM
KCI	50 mM
$MgSO_4$	10 mM
PEP	5 mM
EDTA	0.2 mM
NADH	0.16 mM
Lactate dehydrogenase (LDH)	5 U
The reaction was started by the addition of ADP	5 mM

j) Lactate dehydrogenase (LDH)

Assay principle :

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

Reaction mixture at pH 7.5	Final Concentration
Tris	50 mM
KCl	50 mM
$MgSO_4$	10 mM
EDTA	0.2 mM
NADH	0.16 mM
The reaction was started by the addition of pyruvate	10 mM

k) Glycerol-3-phosphate dehydrogenase (G-3-PDH)

Assay principle :

i) $\text{F-D-P} \xrightarrow{(\text{ALD})} \text{GA3P} + \text{DHAP}$

ii) $\text{GA3P} \xrightarrow{(\text{TIM})} \text{DHAP}$

iii) $\text{DHAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{G-3-PDH}} \text{GP} + \text{NAD}^+$

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
F-D-P	10 mM
Iodoacetic acid	0.1 mM
$MgCl_2$	1 mM
NADH	0.16 mM
Aldolase (ALD)	0.7 U
Triosephosphate isomerase (TIM)	20 U

The reaction was started by the addition of the extract, the control was minus F-D-P

1) Glycerokinase (glycerol + ATP ⟶ GP + ADP) (GK)

Assay principle :

i) glycerol + ATP $\xrightarrow{GK}$ GP + ADP

ii) PEP + ADP $\xrightarrow{(PK)}$ pyruvate + ATP

iii) pyruvate + NADH + H^+ $\xrightarrow{(LDH)}$ lactate + NAD^+

Reaction mixture at pH 7.5	Final Concentration or Activity
Tris	50 mM
KCl	50 mM
$MgSO_4$	10 mM
EDTA	0.2 mM
SHAM	0.5 mM
NADH	0.32 mM
ATP	4 mM
PEP	5 mM
Pyruvate kinase (PK)	3.3 U
Lactate dehydrogenase (LDH)	5.0 U
The reaction was started by the addition of glycerol	10 mM

Alternative reaction mixture at pH 7.0	Final Concentration or Activity
Sucrose	250 mM
K_2HPO_4	50 mM
NaH_2PO_4	2 mM
KCl	5 mM
$MgSO_4$	8 mM
SHAM	1 mM
BSA	3 mg/ml
EDTA	0.2 mM
Nicotinamide	0.2 mM

PEP	4 mM
NADH	0.32 mM
ATP	4 mM
Pyruvate kinase (PK)	3.3 U
Lactate dehydrogenase (LDH)	5.0 U
+ melarsen oxide to control	0.03 mM
The reaction was started by the addition of glycerol to the experimental and control cuvettes	10 mM

Glycerokinase (GP + ADP $\longrightarrow$ glycerol + ATP) (GK)

Assay principle :

i) $GP + ADP \xrightarrow[Mg^{2+}]{GK} glycerol + ATP$

ii) $glucose + ATP \xrightarrow{(HK)} G\text{-}6\text{-}P + ADP$

iii) $G\text{-}6\text{-}P + NADP^{+} \xrightarrow{(G\text{-}6\text{-}PDH)} 6\text{-phosphogluconate} + NADPH + H^{+}$

Reaction mixture at pH 7.0	Final Concentration or Activity
Sucrose	250 mM
K_2HPO_4	50 mM
NaH_2PO_4	2 mM
KCl	5 mM
$MgSO_4$	8 mM
SHAM	1 mM
BSA	3 mg/ml
EDTA	0.2 mM
Nicotinamide	0.2 mM
Glucose	55 mM
$NADP^{+}$	0.4 mM
ADP	1 mM

Hexokinase (HK)	1 U
Glucose-6-phosphate dehydrogenase (G-6-PDH)	1 U
+ melarsen oxide to control	0.03 mM
The reaction was started by the addition of GP to both the experimental and control cuvettes	60 mM

m) Glycerol dehydrogenase (GDH)

Assay principle :

dihydroxyacetone + NADPH + H^+ $\xrightarrow{GDH}$ glycerol + $NADP^+$

or glyceraldehyde or NADH or NAD^+

Reaction mixture at pH 7.0	Final Concentration
TEA	200 mM
$MgCl_2$	1 mM
NADPH or NADH	0.16 mM
The reaction was started by the addition of dihydroxyacetone or glyceraldehyde	50 mM

n) Hexose monophosphate aldolase (HPA)

Assay principle :

i) hexose phosphate $\xrightarrow{HPA}$ triosephosphate + triose

ii) triosephosphate $\xrightarrow{(TIM)}$ DHAP

iii) DHAP + NADH + H^+ $\xrightarrow{(G3PDH)}$ GP + NAD^+

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
$MgCl_2$	1 mM
NADH	0.16 mM
Iodoacetic acid	0.1 mM
Triosephosphate isomerase (TIM)	20 U

Glycerol-3-phosphate dehydrogenase (G-3-PDH)	0.13 U
The reaction was started by the addition of various hexose phosphates	100 mM

o) GP : glucose transphosphorylase (TP)

Assay principle :

i) glucose + GP $\xrightarrow{TP}$ G-6-P + glycerol

ii) G-6-P + $NADP^+$ $\xrightarrow{(G\text{-}6\text{-}PDH)}$ 6-phosphogluconate + NADPH + H^+

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	100 mM
$MgCl_2$	1 mM
$NADP^+$	0.5 mM
SHAM	0.5 mM
GP	500 mM
Glucose-6-phosphate dehydrogenase (G-6-PDH)	1.3
The reaction was started by the addition of glucose	500 mM

p) GP:F-6-P transphosphorylase (TP)

Assay principle :

i) F-6-P + GP $\xrightarrow{TP}$ F-D-P + glycerol

ii) F-D-P $\xrightarrow{(ALD)}$ DHAP + GA-3-P

iii) GA-3-P $\xrightarrow{(TIM)}$ DHAP

iv) DHAP + NADH + H^+ $\xrightarrow{(G3PDH)}$ GP + NAD^+

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
Iodoacetic acid	0.1 mM
GP	500 mM

$MgCl_2$	1 mM
NADH	0.16 mM
SHAM	0.5 mM
Aldolase (ALD)	0.7 U
Triosephosphate isomerase (TIM)	20 U
Glycerol-3-phosphate dehydrogenase	0.13 U
The reaction was started by the addition of F-6-P	100 mM

q) GP:Triose transphosphorylase

Assay principle :

i) triose + GP $\xrightarrow{TP}$ triosephosphate + glycerol

ii) triosephosphate $\xrightarrow{(TIM)}$ DHAP

iii) DHAP + NADH + H^+ $\xrightarrow{(G\text{-}3PDH)}$ GP + NAD^+

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
Iodoacetic acid	0.1 mM
GP	500 mM
$MgCl_2$	1 mM
NADH	0.16 mM
SHAM	0.5 mM
Glycerol-3-phosphate dehydrogenase	0.13 U
Triosephosphate isomerase (TIM)	20 U
The reaction was started by the addition of dihydroxyacetone or glyceraldehyde	100 mM

r) Adenylate kinase (AK)

Assay principle :

i) 2 ADP $\xrightarrow{AK}$ ATP + AMP

ii) glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

iii) G-6-P + $NADP^+$ $\xrightarrow{(G6PDH)}$ 6 phosphogluconate + NADPH + H^+

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	100 mM
$MgCl_2$	1 mM
Glucose	55 mM
$NADP^+$	0.5 mM
Hexokinase (HK)	1 U
Glucose-6-phosphate dehydrogenase (G-6-PDH)	1 U
The reaction was started by the addition of ADP	1 mM

s) Glycerol-3-phosphate oxidase (GPO)

Assay principle :

GP + $\frac{1}{2}O_2$ $\xrightarrow{GPO}$ DHAP + H_2O

Reaction mixture at pH 8.0	Final Concentration
Sucrose	250 mM
Tris	20 mM
BSA	3 mg/ml
The reaction was started by the addition of GP	20 mM

6.3 Substrate Assay Procedures

a) D-glucose

The assay principle and reaction conditions are identical to that of the hexokinase assay except that the addition of 0.13 U hexokinase, not glucose was used to start the reaction, see 5.1 a.

A second method for the determination of glucose concentrations was based on the method of Werner et al. (1970) and was used in preference to the above method when many estimations were required. The assay principle has been described in the methods (2.17) and the assay mixture and standards were purchased in their made-up form from Boehringer Mannheim GmbH.

b) D-glucose-6-phosphate

Assay principle :

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{(G6PDH)}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
$MgCl_2$	1 mM
$NADP^+$	1 mM
The reaction was started by the addition of glucose-6-phosphate dehydrogenase (G-6-PDH)	0.13 U

c) D-fructose-6-phosphate

On completion of the G-6-P assay 0.1 U of phosphoglucose isomerase were added.

d) Dihydroxyacetone phosphate

Assay principle :

$$\text{DHAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{(G3PDH)}} \text{GP} + \text{NAD}^+$$

Reaction mixture at pH 7.0	Final Concentration or Activity
TEA	200 mM
$MgCl_2$	1 mM
NADH	0.16 mM
The reaction was started by the addition of glycerophosphate dehydrogenase	0.13 U

e) D-glyceraldehyde-3-phosphate

On completion of the DHAP assay, 20 U of triosephosphate isomerase were added.

f) D-fructose-1,6-diphosphate

On completion of the GA-3-P assay, 0.7 U of aldolase were added.

g) sn-Glycerol-3-phosphate

Assay principle :

$$GP + NAD^+ + \text{hydrazine} \xrightarrow{(G3PDH)} DHAP\ \text{hydrazine} + NADH + H^+$$

Reaction mixture at pH 9.5	Final Concentration or Activity
Hydrazine	0.2 M
Glycine	0.5 M
EDTA	0.2 mM
NAD^+	0.5 mM
The reaction was started by the addition of glycerol-3-phosphate dehydrogenase (G-3-PDH)	0.13 U

h) Glycerol

On completion of the GP assay, 4 mM-ATP, 0.5 mM-$MgCl_2$ and 0.6 U of glycerokinase were added.

The above method became impractical when high concentrations of GP were present, consequently the GP concentration was either decreased (2.11) or if the concentration of ADP were low the following method of glycerol determination was employed :

This assay principle and reaction conditions were identical to that of the glycerokinase assay (glycerol + ATP $\longrightarrow$ GP + ADP) except that the addition of 0.6 U of glycerokinase not glycerol started the reaction.

i) Pyruvate

Assay principle :

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{(LDH)}} \text{lactate} + \text{NAD}^+$$

Reaction mixture at pH 7.2	Final Concentration or Activity
TEA	200 mM
NADH	0.16 mM
KCl	1 mM
$MgCl_2$	1 mM
The assay was started by the addition of lactate dehydrogenase (LDH)	5 U

j) Phosphoenol pyruvate

On completion of the pyruvate assay 4 mM-ADP was added followed by 3.3 U /ml pyruvate kinase.

k) 2-Phosphoglycerate

On completion of the PEP assay, 1.3 U /ml of enolase was added.

l) 3-Phosphoglycerate

On completion of the 2-PGA assay, 0.04 mM-2,3DiPGA was added, followed by 0.16 U /ml phosphoglycerate mutase.

m) ATP

On completion of the G-6-P assay, 60 mM-glucose and 0.13 U /ml of hexokinase were added.

n) ADP

On completion of the pyruvate assay, 1 mM-PEP was added, followed by 3.3 U/ml of pyruvate kinase.

o) AMP

Assay principle :

i) $AMP + ATP \xrightarrow{(AK)} 2\ ADP$

ii) $2\ ADP + 2\ PEP \xrightarrow{(PK)} 2\ ATP + 2\ pyruvate$

iii) $2\ pyruvate + 2\ NADH + 2H^{+} \xrightarrow{(LDH)} 2\ lactate + 2\ NAD^{+}$

On completion of the ADP assay, 0.1 mM-ATP was added, followed by 0.5 U/ml adenylate kinase.

Simulation of Metabolic Pathways

Most of the 2 substrate catalysed reactions considered will be described by the following general equation :

$$v = \frac{Vmax.}{1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K^{AB}}{A\ B}}$$ (Alberty, 1953a)

where A and B are the two substrates and K_m^A and K_m^B are their respective Michaelis constants.

The equations in the text represent the situations that would apply if the reactions obeyed the rate laws of simple chemical kinetics; these may be approximately applicable to enzyme-catalysed reactions when the substrate concentrations are below their Michaelis constants and the affinity of one substrate to the relevant enzyme is uninfluenced by the binding of another.

SECTION 7

LIST OF REFERENCES

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