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TRYPANOSOMA BRUCEI BRUCEI: THE CATABOLISM OF GLYCOLYTIC INTERMEDIATES BY DIGITONIN-PERMEABILIZED BLOODSTREAM TRYPOMASTIGOTES AND SOME ASPECTS OF REGULATION OF ANAEROBIC GLYCOLYSIS

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Abstract—1. The production of pyruvate, glycerol and glycerol-3-phosphate by intact and digitonin-permeabilized *Trypanosoma brucei brucei* has been studied with glucose or the glycolytic intermediates as substrates.

2. Under aerobic conditions hexosephosphates gave maximal glycolysis in the presence of 40–60 μg digitonin/10^8 trypanosomes while the triosephosphates gave it at 20–30 μg digitonin/10^8 trypanosomes.

3. In the presence of salicylhydroxamic acid, and the glycolytic intermediates, permeabilized trypanosomes produced equimolar amounts of pyruvate and glycerol-3-phosphate and no glycerol. Under the same conditions, glucose catabolism produced glycerol in addition to pyruvated and glycerol-3-phosphate.

4. In the presence of salicylhydroxamic acid and ATP or ADP intact trypanosomes produced equimolar amounts of pyruvate and (glycerol plus glycerol-3-phosphate) with glucose as substrate.

5. A carrier for ATP and ADP at the glycosomal membrane is implicated.

6. It is apparent that glycerol formation is regulated by the ATP/ADP ratio and that it needs intact glycosomal membrane and the presence of glucose.

INTRODUCTION

The bloodstream trypomastigotes of *Trypanosoma brucei brucei* rely on glucose catabolism for energy production. This proceeds rapidly and leads to pyruvate production under aerobic conditions (Brohn and Clarkson, 1978). The NADH produced during glycolysis is reoxidized indirectly by oxygen via the coupled action of glycerol-3-phosphate oxidase (GPO) (Grant and Sargent, 1960) and glycerol-3-phosphate dehydrogenase. Under anaerobic conditions or when the GPO is inhibited by salicylhydroxamic acid (SHAM), the end product of glycolysis are pyruvate and glycerol in equimolar amounts (Grant and Fulton, 1957; Brohn and Clarkson, 1978). The reducing equivalents from NADH generated during the oxidation of glyceraldehyde-3-phosphate (Gra-3-P) are transferred to glycerone phosphate (GrnP) and eventually excreted as glycerol.

Kiaira and Njogu (1983) reported that glycolysis in *T.b. brucei* could be studied *in situ* using glycolytic intermediates as substrates after permeabilization with digitonin. ATP and Mg^{2+} were required for the catabolism of glucose-6-phosphate and fructose-6-phosphate as judged by the rate of oxygen consumption. The present investigation was undertaken to study further the mechanism of glycerol production *in situ* during anaerobiosis. In this paper we report the products formed by *T.b. brucei* when incubated with various glycolytic intermediates at different concentrations of digitonin under aerobic and SHAM-simulated anaerobic conditions. The effect of exogenous ATP and ADP on the products of anaerobic glycolysis was also investigated.

MATERIALS AND METHODS

Chemicals and enzymes

Substrates and enzymes were of high purity from Sigma Chemical Co., St Louis, Mo., U.S.A. or from BDH Chemicals, Poole, Dorset, U.K. Salicylhydroxamic acid (SHAM) and digitonin were dissolved in absolute alcohol whenever they were used. Parasites were routinely incubated in 100 mM KCl containing 5.0 mM $MgCl_2$, 2 mM K_2HPO_4 and 90 mM Tris–HCl of pH 7.5 (Buffer A). Phosphate saline sucrose (PSS) buffer (pH 7.5) consisting of 60 mM Sucrose 51 mM NaCl, 40 mM Na_2HPO_4 and 4 mM NaH_2PO_4 was routinely used.

Organisms

Trypanosoma brucei brucei Stock EATRO 1969 was used in the study. The history of the parasites; *in vivo* growth and harvesting methods have been reported before (Njogu and Kiaira, 1982).

Separation of trypanosomes from the blood

This was done by a slight modification of the method of Njogu and Kiaira (1982). The infected rat blood was centrifuged at 1000 *g* for 10 min at 4°C. The trypanosomes were collected from the interface with a Pasteur pipette. The contaminating erthrocytes were removed by hypotonic shock as follows: The trypanosome rich layer was suspended in 100 vol of 1 mM sodium phosphate buffer (pH 7.5) containing 0.3% w/v NaCl and incubated at 37°C. The treatment was stopped after 3 min by addition of 0.1 vol of 10-fold concentrated PSS buffer containing 10 mM D-glucose. The suspension was centrifuged at 1000 *g* for 10 min to obtain a trypanosome pellet. The pellet was suspended in 5 vol buffer A and kept in ice for at most 5 min before use. The trypanosomes were counted on Neubauer haemocytometer.

Metabolic and enzymes assays

When glycolytic end-products were to be determined, incubations were carried out for 1 hr at 25°C unless otherwise stated in appropriate legends. The incubations of 1 ml final volume were carried out in 25 ml Erlenmeyer flasks. The samples were shaken constantly in Dubnoff Metabolic Shaking Incubator. In each incubation there were $1–3 \times 10^8$ trypanosomes/ml. Anaerobiosis was simulated by addition of 2 mM SHAM. The incubations were stopped by deproteinization with perchloric acid, final concentration 7% v/v. Deproteinized samples were neutralized with 6 M KOH. Pyruvate was determined immediately but Gro-3-P and glycerol were usually determined after 24 hr. Metabolite and enzyme assays were performed using an SP 1800 spectrophotometer coupled to a Pye Unicam recorder or Perkin–Elmer 550S UV/VIS spectrophotometer. Whenever SHAM was present in the incubations the assays were carried out at 366 nm instead of 340 nm (Fairlamb and Bowman, 1980). Otherwise assays were performed as described by Bergmeyer (1974). The results presented in this paper have been corrected for the metabolites already present prior to incubation.

RESULTS

Aerobic pyruvate production by T. brucei brucei from glycolytic intermediates: stimulation by permeabilization with digitonin

It has been shown that *T. brucei* can respire on glycolytic intermediates in the presence of sufficient concentration of digitonin (Kiaira and Njogu, 1983). In order to show that glycolytic intermediates can be catabolized via the whole span of the glycolytic pathway in digitonin-permeabilized trypanosomes, pyruvate was determined at various concentrations of digitonin after incubation with glucose-6-phosphate (Glc-6-P), fructose 6-phosphate (Fru-6-P), fructose-1-6-bisphosphate (Fru-1,6-P_2), glyceraldehyde-3-phosphate (Gra-3-P) and glycerone phosphate (GrnP). For glycolytic intermediates that have to be phosphorylated prior to catabolism 2.5 mM ATP was added and those that do not require further phosphorylation 1.5 mM ADP was added.

Figure 1 shows pyruvate and Gro-3-P production from Glc-6-P and GrnP at various concentrations of digitonin. There was no significant pyruvate production from Glc-6-P, Fru-6-P or Fru-1,6-P_2 without digitonin. Increasing concentrations of digitonin increased pyruvate production from the glycolytic intermediates tested. Mg^{2+} was required in all cases involving digitonin permeabilization otherwise the rate of pyruvate production was very low.

There was a low production of pyruvate from the triosephosphates in the absence of digitonin. Concentrations of digitonin $>25–30\ \mu g/10^8$ trypanosomes progressively caused an increase in Gro-3-P but not glycerol production from all the glycolytic intermediates tested. It is apparent that concentrations of digitonin $>30\ \mu g/10^8$ trypanosomes progressively inhibited the GPO. No other enzymes of glycolysis appeared significantly affected by up to $60\ \mu g$ digitonin/10^8 trypanosomes.

Table 1 shows the concentrations of digitonin required for the highest rates of pyruvate production from Glc-6-P, Fru-6-P and Fru-1,6-P_2 which were obtained with $40–60\ \mu g$ digitonin/10^8 trypanosomes whereas Gro-3-P, GrnP, Gra-3-P, glycerate-2-phosphate (Gri-2-P) glycerate-3-phosphate (Gri-3-P) and phosphoenolylpyruvate required $20–30\ \mu g$ digitonin/10^8 trypanosomes.

Digitonin concentration $>60\ \mu g/10^8$ trypanosomes was not exceeded because it inhibits respiration (Kiaira and Njogu, 1983) and pyruvate production.

Figure 2 shows pyruvate and Gro-3-P production from glucose at increasing concentrations of digitonin in the presence and absence of ATP. Digitonin

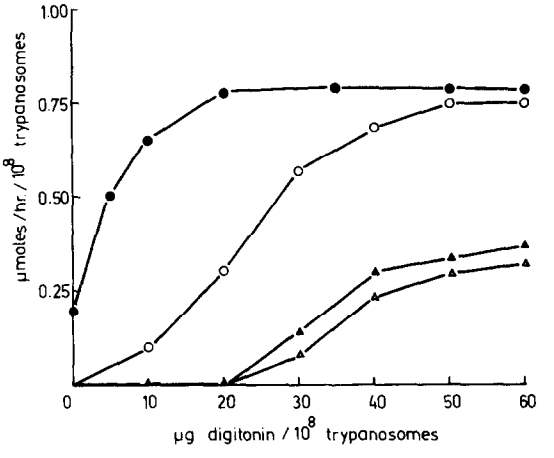

Fig. 1. A comparison of the concentrations of digitonin required for maximum rate of pyruvate production from Glc-6-P and GrnP. The incubations contained 10 mM Glc-6-P or 20 mM GrnP and 2.5 mM ATP (○——○) and (△——△) indicates pyruvate and Gro-3-P production from Glc-6-P, respectively. (●——●) and (▲——▲) indicates pyruvate and Gro-3-P production from GrnP, respectively. A similar pattern of results was obtained when Glc-6-P was replaced by Fru-6-P or Fru-1,6-P_2.

Table 1. Concentrations of digitonin required for maximum pyruvate production from phosphorylated glycolytic intermediates. The incubations contained saturating concentrations of glycolytic intermediates, 2.5 mM ATP or ADP and various concentrations of digitonin

Substrate	Substrate concentration (mM)	µg Digitonin/10^8 trypanosomes	Maximum rate of pyruvate production: µmol/hr/10^8 trypanosomes
Glucose	10	—	3.6 ± 0.8 (4)
Glc-6-P	10	50 ± 8 (4)	0.74 ± 0.11 (4)
Fru-6-P	30	52 ± 8 (4)	1.53 ± 0.32 (4)
Fru-1,6-P_2	15	50 ± 7 (4)	0.73 ± 0.14 (4)
Gra-3-P	20	24 ± 8 (3)	0.70 ± 0.10 (3)
GrnP (DHAP)	20	25 ± 8 (3)	2.40 ± 0.40 (3)
Gri-3-P	10	25 ± 3 (3)	2.40 ± 0.40 (3)
Gri-2-P	10	24 ± 4 (3)	3.40 ± 0.50 (3)
PEPyr	10	24 ± 4 (3)	3.50 ± 0.60 (3)

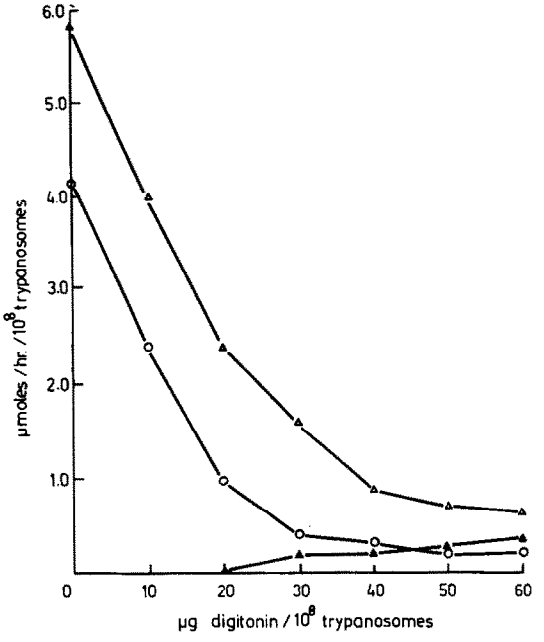

Fig. 2. Effects of increasing the concentration of digitonin on pyruvate and Gro-3-P production by *T. brucei* incubated with glucose in the presence and absence of 2.5 mM ATP. Glucose concentration was 10 mM. (△——△) and (▲——▲) indicate pyruvate and Gro-3-P, respectively, in the presence of ATP. (○——○) indicates pyruvate without ATP.

progressively caused a decrease in pyruvate production while inclusion of ATP caused a significant increase in pyruvate formation. There was also a progressive increase in Gro-3-P levels when digitonin concentration was gradually increased above 30 μg digitonin/10^8 trypanosomes.

Products of glucose catabolism in intact T. brucei under simulated anaerobic conditions: effects of exogenous ATP and ADP

Equimolar amounts of pyruvate and glycerol were formed by *T. brucei* incubated with glucose and SHAM (Brohn and Clarkson, 1978). Transport of exogenous ATP and ADP and AMP in Trypanosoma brucei has been demonstrated (Sanchez *et al.*, 1976). We have studied the effects of ATP and ADP on the products of glucose catabolism in the presence of SHAM.

Figure 3(a) shows that as the concentration of ATP was increased from 0 to 4 mM in the presence of 2.5 mM ADP, pyruvate production was increased by about 30% and Gro-3-P was increased from very small amounts to about 4-fold while glycerol was slightly reduced. In parallel experiments carried in the presence of 2.5 mM ATP, increasing the concentrations of ADP from 0 to 4 mM did not affect the production of pyruvate significantly [Fig. 3(b)]. Gro-3-P production was reduced whereas that of glycerol was increased by approximately the corresponding decrease. In both cases [Fig. 3(a) and (b)] the molar ratios of pyruvate to glycerol plus Gro-3-P remained approx. 1:1. It was not possible to convert all the glycerol to Gro-3-P by adding more exogenous ATP.

To determine whether ADP and ATP permeabilized the plasma and glycosomal membranes, the above experiments were repeated with Glc-6-P or Fru-6-P as substrate instead of glucose. No significant production of pyruvate, Gro-3-P or glycerol was detected and the trypanosomes were immobilized within 5 min. This suggests that although exogenous ATP and ADP have access to the glycolytic enzymes the hexosephosphates cannot replace glucose as substrate in intact trypanosomes due to a permeability barrier. Gro-3-P appears able to leave the parasites.

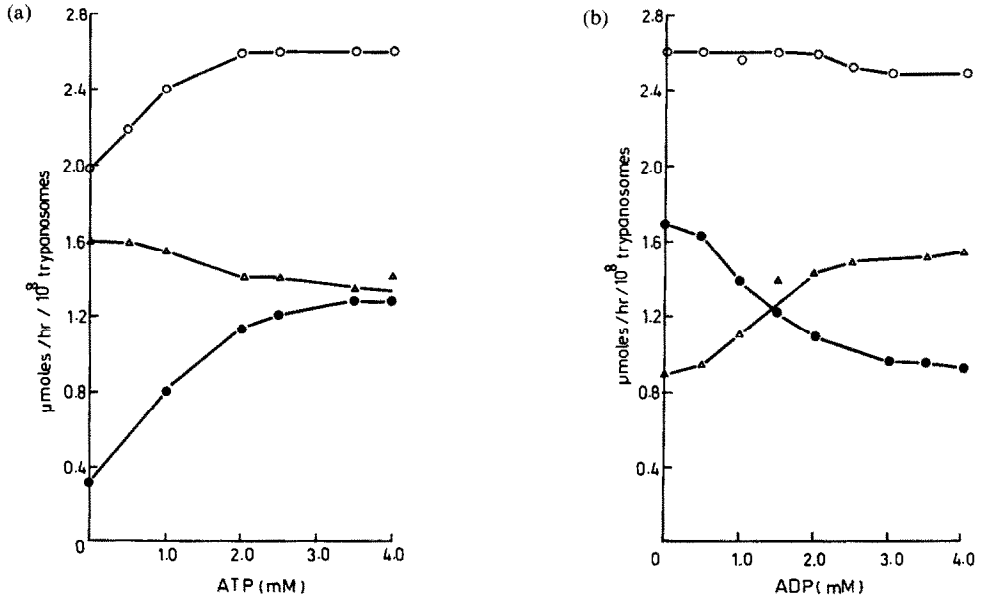

Fig. 3. Effect of increasing ATP or ADP on the production of pyruvate (○——○), Gro-3-P (●——●) and glycerol (△——△). The incubations initially contained 2 mM SHAM, 10 mM glucose and either (a) 2.5 mM ATP or (b) 2.5 mM ADP. ATP or ADP was added in 10 μl portions to make the concentration shown.

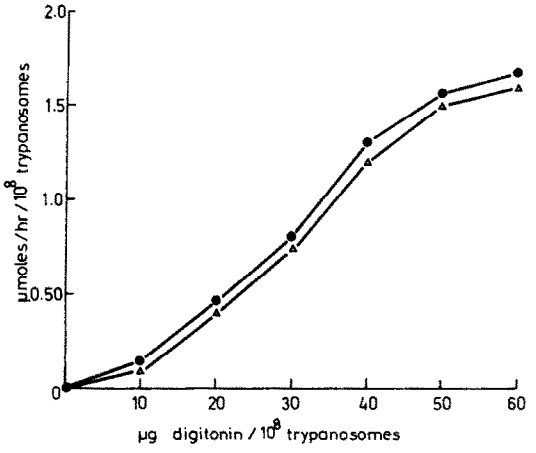

Fig. 4. Stimulation of Glc-6-P catabolism in the presence of SHAM. The incubation contained 10 mM Glc-6-P, 2 mM SHAM and 1.5 mM ADP (●——●) indicates pyruvate and (△——△) Gro-3-P produced.

Products of catabolism of glycolytic intermediates by digitonin permeabilized T. brucei under simulated anaerobic conditions

The possibility that digitonin-permeabilized *T. brucei* might form glycerol and pyruvate anaerobically from glycolytic intermediates was investigated. A parallel control experiment was always run with D-glucose as substrate under similar conditions. Figures 4, 5 and 6 show that increasing concentrations of digitonin resulted in the production of equimolar Gro-3-P and pyruvate from Glc-6-P, Fru-6-P and Fru-1,6-P_2. Glycerol was not detected in any of the incubations containing the hexosephosphates as substrates. When the triosephosphates were used there were small but equimolar amounts of pyruvate and Gro-3-P production by intact trypanosomes. Digitonin lead to an increase in production of pyruvate and Gro-3-P. Again no glycerol was detected (Figs 7 and 8).

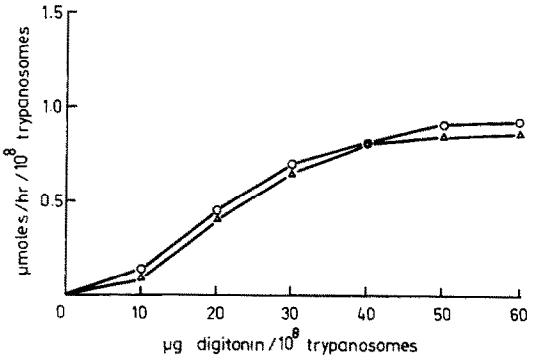

Fig. 6. Fru-1,6-P_2 catabolism at various concentrations of digitonin. Each incubation contained 1.5 mM ADP, 15 mM Fru-1,6-P_2 and 2 mM SHAM. (○——○) indicates pyruvate and (△——△) Gro-3-P produced.

The end products of glucose catabolism were pyruvate, Gro-3-P and glycerol. Increasing concentrations of digitonin progressively caused a decrease in glycerol production. Glycerol was detected in all the incubations containing glucose even at 60 µg digitonin/10^8 trypanosomes. The molar ratio of pyruvate to (glycerol plus Gro-3-P) was always approx. 1:1 (Fig. 9).

Incubation of the trypanosomes with glycolytic intermediates: Gri-3-P, Gri-2-P and PEPyr that do not lead to reduction of NAD^+ led to production of only pyruvate as end product (Fig. 10).

DISCUSSION

The glycolytic enzymes in *T. brucei* are distributed in the glycosome and the cytosol (Opperdoes and Borst, 1977). This is supported by results from latency experiments, differential centrifugation and pulse labelling experiments (Visser and Opperdoes, 1980; Oduro *et al.*, 1980; Visser *et al.*, 1981). We have attempted to investigate the extent of permeabilization of the *T.b. brucei* plasma and glycosomal membranes by digitonin, assessed by how

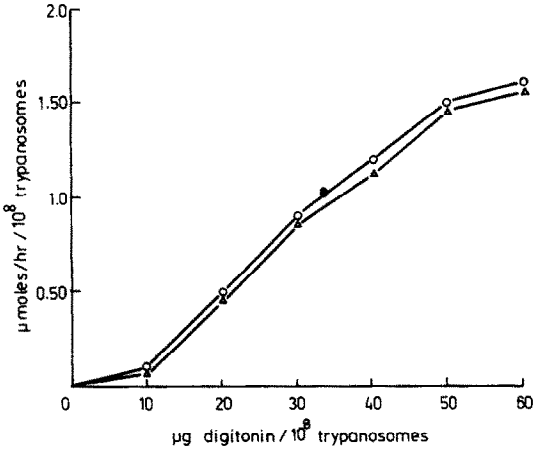

Fig. 5. Stimulation of Fru-6-P catabolism by digitonin in the presence of SHAM. The incubations contained 1.5 mM ADP, 2 mM SHAM and 30 mM Fru-6-P. (○——○) indicates pyruvate and (△——△) Gro-3-P produced.

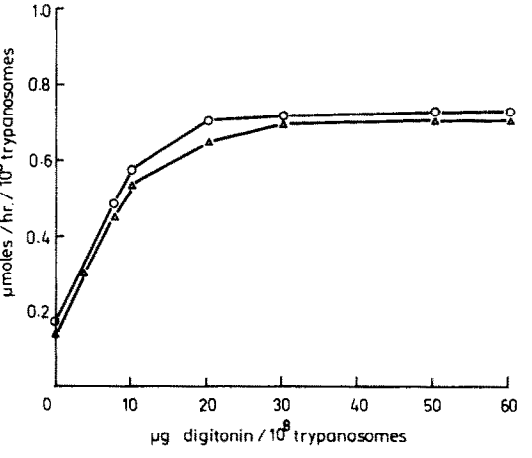

Fig. 7. Stimulation of GrnP catabolism by digitonin in the presence of SHAM. The respective incubations contained 2 mM SHAM, 20 mM GrnP and 1.5 mM ADP. (○——○) indicates pyruvate and (△——△) Gro-3-P produced.

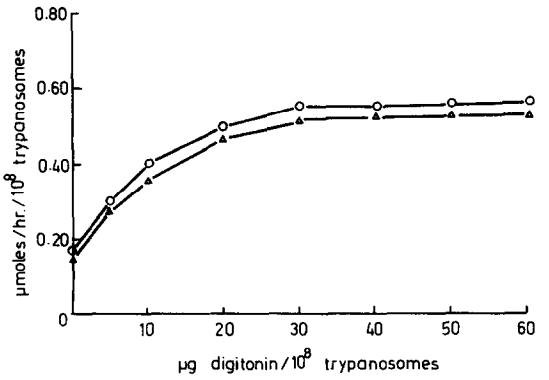

Fig. 8. Gra-3-P catabolism at various concentrations of digitonin. In each incubation mixture was 2 mM SHAM, 1.5 mM ADP and 20 mM Gra-3-P. (○——○) indicates pyruvate and (△——△) Gro-3-P.

much the exogenous non-permanent glycolytic intermediates gain access to the glycolytic enzymes which we quantified in terms of the formation of their end-products. The oxidation of the triosephosphates required 20–30 μg digitonin/10^8 trypanosomes whereas that of the larger hexosephosphates required 40–60 μg digitonin/10^8 trypanosomes. Table 1 shows that the differences in the concentrations of digitonin required for the highest rate of pyruvate production might not be due to only the latency of the glycolytic enzymes but also due to the molecular size of the intermediates. These results point to two compartments in the parasite; one made accessible to triosephosphates by 20–30 μg digitonin/10^8 parasites and second one made accessible to hexosephosphates by 40–60 μg digitonin/10^8 parasites. They can also be interpreted to be in support of the localization of some glycolytic enzmes in the glycosome and others in the cytosol (Visser and Opperdoes, 1980).

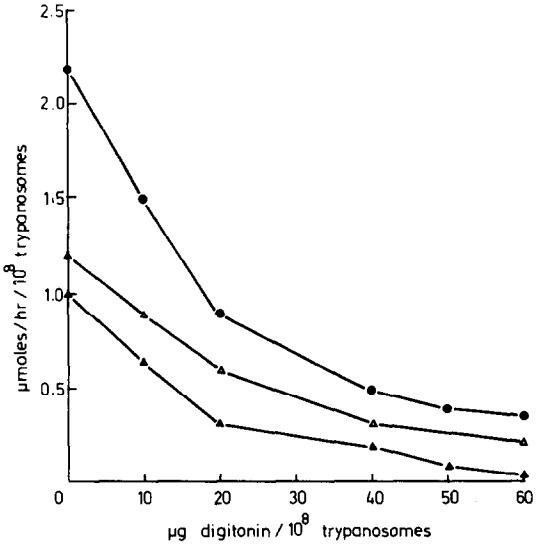

Fig. 9. Effect of increasing concentrations of digitonin on pyruvate (●——●), glycerol (△——△) and Gro-3-P production form D-glucose (▲——▲) by *T. brucei*. The trypanosomes were incubated with 1.5 mM ADP, 2 mM SHAM and 10 mM D-glucose.

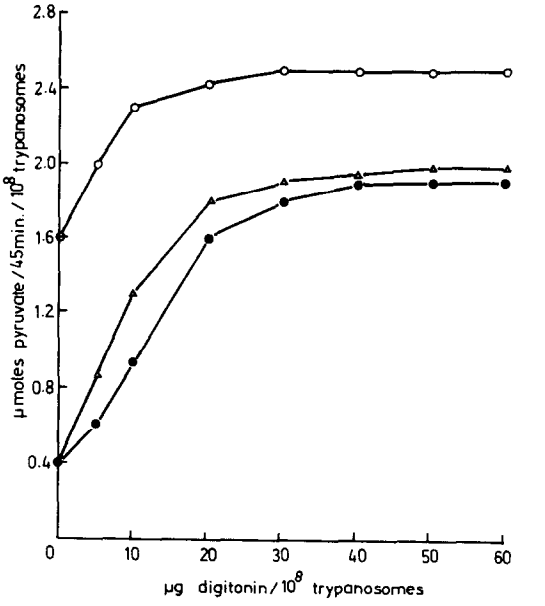

Fig. 10. Stimulation of conversion of Gri-3-P, Gri-2-P and PEPyr to pyruvate by digitonin. In each incubation was 2 mM SHAM, 1.5 mM ADP and either 10 mM Gri-3-P, Gri-2-P or PEPyr. (○——○), (△——△) and (●——●) indicates pyruvate production from PEPyr, Gri-2-P and Gri-3-P, respectively.

Effect of exogenous ATP and ADP on anaerobic glycolysis

The glycosome is a single membrane-bound organelle which contains enzymes that catalyse the conversion of glucose to Gri-3-P and Gro-3-P in *T. brucei* (Opperdoes and Borst, 1977; Oduro *et al.*, 1980). A permeability barrier for the glycolytic intermediates, ATP, ADP, AMP, NAD and inorganic phosphate has been proposed (Opperdoes and Borst, 1977) but has been demonstrated for only the hexosephosphates using isolated glycosomes (Opperdoes and Nwagu, 1980; Hammond *et al.*, 1985).

The transport of exogenous ATP, ADP, AMP and cyclic AMP in intact *T. brucei* has been demonstrated (Sanchez *et al.*, 1976). The maximum rates of uptake were 21.88, 23.81, 146.82 and 29 μmol/10^8 trypanosomes/hr, respectively. No such studies at the glycosomal membrane have been done probably due to the inability to isolate pure absolutely intact glycosomes. In this investigation, a supposedly glycosomal reaction catalysed by the glycerol kinase could be manipulated by exogenous ATP and ADP in intact trypanosomes incubated with SHAM. This observation was unexpected because the glycosome is thought to be impermeable to ATP and ADP (Opperdoes and Borst, 1977). The glycosomal membrane could not have been permeabilized because Glc-6-P or Fru-6-P which are partly catabolized in this organelle (Visser *et al.*, 1981) could not produce pyruvate without the addition of digitonin. A carrier(s) for ATP and ADP at glycosomal membrane may explain these results since increasing exogenous ADP concentration in the incubations at fixed ATP concentration stimulated glycerol production and inhibited that of Gro-3-P. We further propose that the

cellular levels of glycerol and Gro-3-P are controlled by the ratios of ATP/ADP. Under anaerobic condition this ratio is low resulting in more glycerol formation. The increase of Gro-3-P in the incubations due to a high ATP/ADP ratio in the presence of SHAM, suggests either phosphorylation of the preformed glycerol or inhibition of glycerol-3-phosphate dephosphorylation.

Glycerol production from exogenous glycolytic intermediates

The catabolism of exogenous Glc-6-P, Fru-6-P, Fru-1,6-P_2, GrnP or Gra-3-P to pyruvate requires the permeabilization of both the plasma and glycosomal membranes. According to the initial concept of the glycosome (Opperdoes and Borst, 1977) permeabilization of the glycosome would lead to no glycerol production because high optimal local concentrations of substrates and cofactors involved in various reactions cannot be achieved. Our observation of no glycerol production from glycolytic intermediates in digitonin-permeabilized trypanosomes is consistent with this concept. However, it is difficult to explain why glycerol production was observed from glucose as substrate in the presence of the same concentrations of digitonin but not with the glycolytic intermediates. Since permeabilization of the trypanosomes incubated with SHAM and hexophosphates, Gra-3-P or GrnP as substrates stimulated the production of equimolar amounts of pyruvate and Gro-3-P (Figs 4, 5, 6, 7 and 8) and no glycerol formation, we propose that the enzymes that catalyse the dephosphorylation of Gro-3-P requires intact glycosomal membrane and glucose as substrate. It is further proposed that the enzyme which catalyse the formation of glycerol is/are under modulation by ATP/ADP ratio possibly in addition to Gro-3-P accumulation. We are investigating this apparent direct requirement of glucose in order for trypanosomes to form glycerol.

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