International Laboratory for Research on Animal Diseases (ILRAD),
P.O.Box 30709, Nairobi, Kenya

Trypanosoma (Nannomonas) congolense: properties of hexokinase and phosphofructokinase from cultured procyclic trypomastigotes and bloodstream forms[1]

M. Nwagwu[2], H. Hirumi

Summary

The distribution and kinetics of two key glycolytic enzymes hexokinase (HK) and phosphofructokinase (PFK) were studied in animal-infective bloodstream forms (haematozoic trypomastigotes) and uninfective procyclic forms (insect trypomastigotes) of *Trypanosoma congolense*. The results show that in both forms of *T. congolense* HK and PFK are particulate and are probably localized in a membrane-delimited organelle, the glycosome. Hexokinases of bloodstream and procyclic forms of *T. congolense* are kinetically similar with respect to their affinity for glucose and ATP, the apparent K_m for glucose being within the range, of 91 μM to 100 μM and that for ATP, 65 μM to 91 μM. Phosphofructokinase of both forms responds to its substrate in a complex manner: a plot of initial velocity versus substrate concentration displays intermediary plateau regions.

Key words: *Trypanosoma congolense;* glycolytic enzymes; glycosome; hexokinase; phosphofructokinase.

Introduction

Significant advance has been made on elucidating glucose metabolism in *T. brucei*. We now know that seven of the glycolytic enzymes, catalyzing the steps from glucose to 3-phosphoglycerate and two enzymes of glycerol meta-

---

Correspondence: Prof. Mark Nwagwu, University of Ibadan, Department of Zoology, Ibadan, Nigeria

bolism namely, glycerol-3-phosphate dehydrogenase and glycerol kinase, are contained in a microbody-like organelle called the glycosome, so far, found only among members of the family Trypanosomatidae (Opperdoes and Borst, 1977; Taylor et al., 1980; Opperdoes et al., 1981; Cannata et al., 1982; Coombs et al., 1982; Hart et al., 1984). These glycosomal enzymes exhibit the phenomenon of latency, that is, their activities are low when measured under conditions in which the glycosomal membrane is maintained intact, since the membrane is impermeable to the substrates and co-factors for the respective enzymes (Opperdoes et al., 1977; Visser and Opperdoes, 1980).

By contrast only scanty biochemical information is available on *T. congolense*. The bloodstream forms contain tubular, cristate mitochondria and some of the enzymes of the Krebs cycle; however a fully functional Krebs cycle or cytochrome system is probably not operative (Fulton and Spooner, 1969; Gutteridge and Coombs, 1977). The major end products of carbohydrate metabolism are acetate and succinate, pyruvate being completely metabolized. Even less is known about the insect stage of *T. congolense:* pyruvate (39%) and acetate (23%) are the major end products of aerobic glucose metabolism, with minor amounts of succinate, glycerol and lactate (Agosin and von Brand, 1954; Bowman and Flynn, 1976; Gutteridge and Coombs, 1977). However, other biochemical properties, particularly the activities and distributions of the glycolytic enzymes are unknown. Furthermore, no information is available on the presence or absence of glycosomes in *T. congolense*. A study of the activities of glycolytic enzymes and their regulation in bloodstream and procyclic forms may provide a better understanding of the mechanism(s) underlying transformation.

In this paper, results of the distribution and kinetics of HK and PFK of in vivo-cultivated bloodstream forms and procylic forms of *T. congolense* are presented.

Materials and Methods

The bloodstream forms (haematozoic trypomastigotes) of *T. congolense* (ILRAD 1678) used in this study were propagated in vivo in irradiated Wistar rats weighing 200–300 g and trypanosomes were obtained free of blood cells by the method of Lanham (1968). *Trypanosoma congolense* procyclic forms ("insect" trypomastigote, ILRAD 20–39), propagated in vitro as previously described (Gray et al., 1981), were harvested in the late log phase of growth, washed three times with STE buffer (0.25 M sucrose – 25 mM Tris–HCl – 1 mM EDTA, pH 7.8) and then stored in liquid nitrogen until used.

The procyclic forms ($0.2–1.0 \times 10^9$) were sonicated in STE buffer with a micro-size probe of a Braun-sonic 1510 homogenizer (B. Braun, Melsungen AG, West Germany) set at 100 watt, for 60 sec in an ice bath. Electron micrographs of the cellular extracts showed that the membrane integrity of the glycosomes was maintained intact. The bloodstream forms were homogenized with silicon carbide as described by Opperdoes et al. (1977) and resuspended in STE buffer.

284

Cell fractionation

All procedures were carried out at 4°C unless otherwise indicated. Homogenates were centrifuged to isolate the following fractions as described by Opperdoes et al. (1977): nuclear, large granule, microsomal and final supernatant except the large granule fraction, which was obtained by spinning the post-nuclear extract at 15,000 rpm for 10 min in a Beckman J-21 B centrifuge with a JA 20 rotor.

Enzyme determination

Hexokinase and PFK were determined as described by Nwagwu and Opperdoes (1982), in an enzyme-coupled reaction assay by following, at A_{340} nm' the reduction of NADP (for HK), or the oxidation of NADH (for PFK), respectively, at 25°C in a mixture containing 100 mM Tris-HCl, pH 7.8, 5 mM $MgSO_4$ and 1 mM EDTA. The reaction mixture contained a large excess of auxiliary enzymes (3 units each) namely, glucose-6-phosphate dehydrogenase (for HK) and aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase (for PFK). Preliminary observations established that the conditions for assaying HK and PFK did not affect the activities of the auxiliary enzymes and the kinetics of HK and PFK of the large granule fraction were similar to those of the same enzymes of purified glycosomes prepared by a modification of the method of Opperdoes (1981). In this modified procedure glycosomes were prepared by spinning a large granule fraction in a 45% Percoll gradient at 30,000×g for 2 h at 4°C. For practical reasons enzyme kinetics were determined with a large granule fraction solubilized in 0.2% Triton X-100. As initial results showed that the optimal conditions for the assay of HK and PFK of *T. brucei* do not significantly differ from those for the enzymes of *T. congolense,* the methods described by Nwagwu and Opperdoes (1982) have been employed throughout the study. The initial velocity of reaction was expressed as μmol of glucose-6-phosphate (for HK) or fructose, 1,6-biphosphate (for PFK) formed per min per mg of large granule protein. The amount of protein in the sample used for enzyme determinations varied from 4 μg for the bloodstream form to 20 μg for the procyclic form. Protein content was determined according to Lowry et al. (1951) with bovine serum albumin as standard.

Enzyme latency

The latency of HK and PFK was determined as previously described (Visser and Opperdoes, 1980; Opperdoes et al., 1981).

All chemicals were of the highest purity available. Aldolase (E.C. 4.1.2.13), glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8), triosephosphate isomerase (E.C. 5.3.1.1), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), ATP, fructose-6-phosphate, NADH, NADP were purchased from Sigma Chemical Co., London, U.K.

Results and Discussion

An important and unique feature of some glycolytic enzymes in trypanosomes, including HK and PFK, is that they are localized in a membrane-delimited, microbody-like organelle called the glycosome (Opperdoes and Borst, 1977). The results presented in Fig. 1 show that HK and PFK of bloodstream and procyclic forms of *T. congolense* are to a high extent localized in the large granule fraction. As shown in Table 1, these enzymes also exhibit a high degree of latency (68–89%) comparable to levels obtained in *T. brucei* (Visser and Opperdoes, 1980) suggesting that they are enveloped by a membrane which is impermeable to their substrates and co-factors. We therefore conclude that HK and PFK of bloodstream and procyclic forms of *T. congolense* are probably localized in the glycosome.
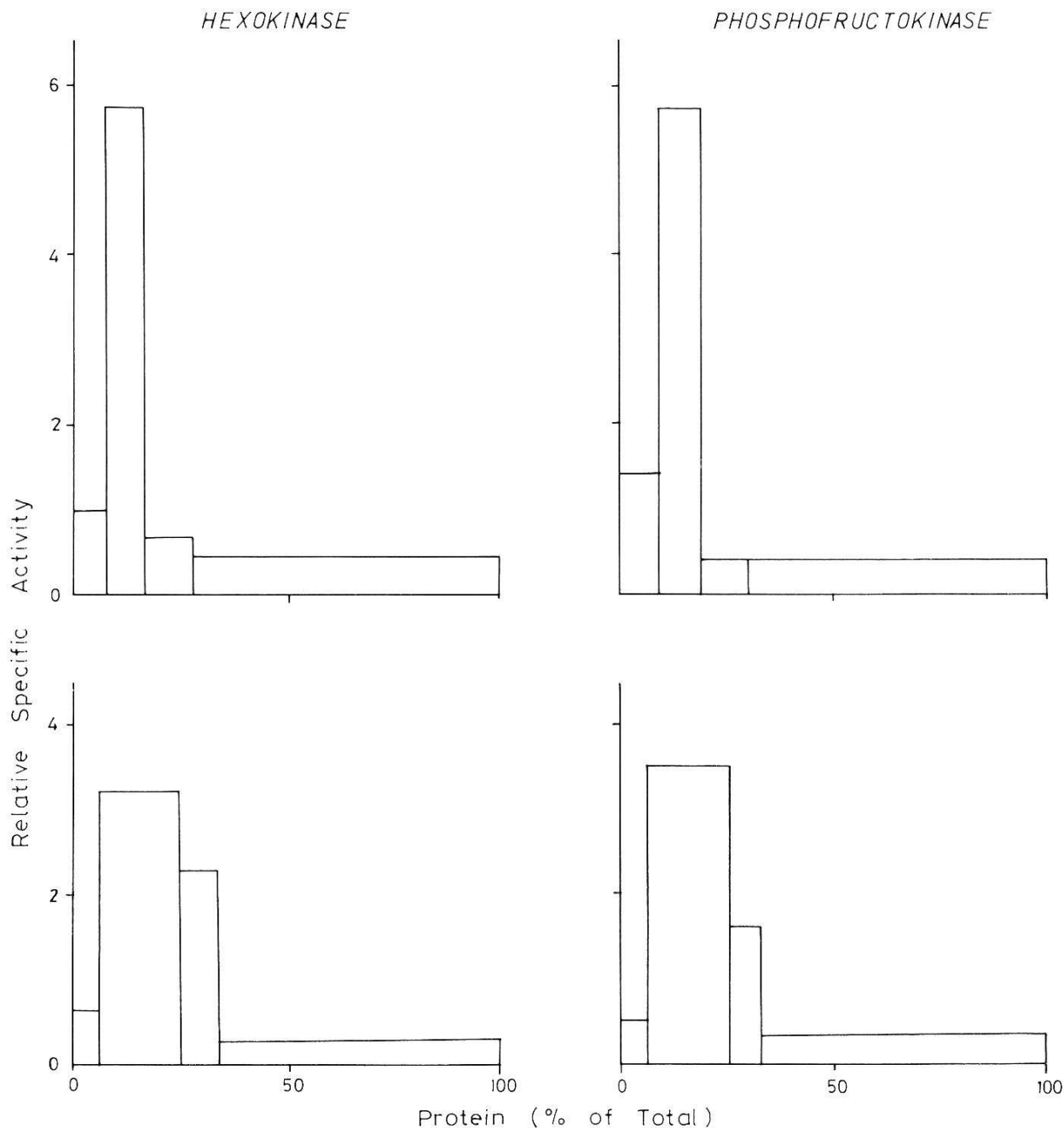
Fig. 1. Distribution of hexokinase and phosphofructokinase of bloodstream and procyclic forms of *T. congolense* in fractions obtained by differential centrifugation. The fractions are plotted in the order of their isolation, i.e. from left to right, nuclear, large granule, microsomal and supernatant fractions. The cumulative protein content is presented in the abscisca; the relative specific activity (percentage of total enzyme activity/percentage of total protein) is presented in the ordinate. The distribution patterns for the in vitro-propagated procyclic forms and bloodstream forms are presented in the upper and lower panels, respectively.

The specific activities of HK of the large granule fraction of both forms of *T. congolense* were similar; however, the specific activity of PFK of the large granule fraction of the bloodstream form of *T. congolense* was approximately 3 times that of the enzyme of the procyclic form (Table 2). This suggests that PFK might play a more significant role in the bloodstream form than in the procyclic form.

286

Table 1. Latency of hexokinase and phosphofructokinase of the large granule fraction of in vivo-grown bloodstream and procyclic forms of *T. congolense*

Enzyme	Latency (%)	
	Bloodstream form	Procyclic form
Hexokinase	80 ± 3.2 (84)[a]	89 ± 4.6 (87)[b]
Phosphofructokinase	72 ± 5.2 (71)[a]	68 ± 3.4 (76)[b]

Free activity and total activity were measured in the absence and presence of Triton X-100 (0.2%), respectively. The large granule fraction was incubated in a reaction mixture with a final sucrose concentration of 250 mM. Latency is expressed as:

$$\frac{\text{total activity} - \text{free activity}}{\text{total activity}} \times 100$$

The results are given as ± standard error and are averages of four experiments:
a) The percentages were taken from Visser and Opperdoes (1980) and apply to in vivo-grown bloodstream forms of *T. brucei.*
b) The percentages were taken from Opperdoes et al. (1981) and apply to procyclic forms of *T. brucei.*

Table 2. Specific activity of hexokinase and phosphofructokinase of large granule fractions of bloodstream and procyclic forms of *T. congolense*

Enzyme	Specific activity (μmol · min^{-1} · mg^{-1})	
	Bloodstream forms	Procyclic forms
Hexokinase	0.39 ± 0.05 (5.0)[a]	0.41 ± 0.11
Phosphofructokinase	1.7 ± 0.21 (5.0)[a]	0.66 ± 0.21

The specific activity of hexokinase is expressed as μmol of glucose-6-phosphate formed per min per mg of large granule protein; that of phosphofructokinase is expressed as μmol of fructose 1,6-diphosphate formed per min per mg of large granule protein. The results are given as ± standard error and represent averages of four experiments.
a) The figures in parenthesis are taken from Nwagwu and Opperdoes (1982) representing the specific activities of the enzymes of the large granule fraction of in vivo-propagated bloodstream forms of *T. brucei.*

The specific activities of HK and PFK of bloodstream forms of *T. brucei* are, approximately 13 and 3 times higher, respectively, than those of the corresponding enzymes of the bloodstream forms of *T. congolense* (Table 2). In *T. brucei* bloodstream forms, glycolysis is the sole source of energy (von Brand, 1951), pyruvate and minute amounts of glycerol being the end products of aerobic glucose metabolism (Grant and Sargent, 1960). In contrast, in bloodstream forms of *T. congolense,* acetate accounts for 33–55% of the end products of aerobic glucose metabolism, with succinate contributing 33%; glycerol 18%; and CO_2 8% (Bowman and Flynn, 1976). The finding that the specific activities
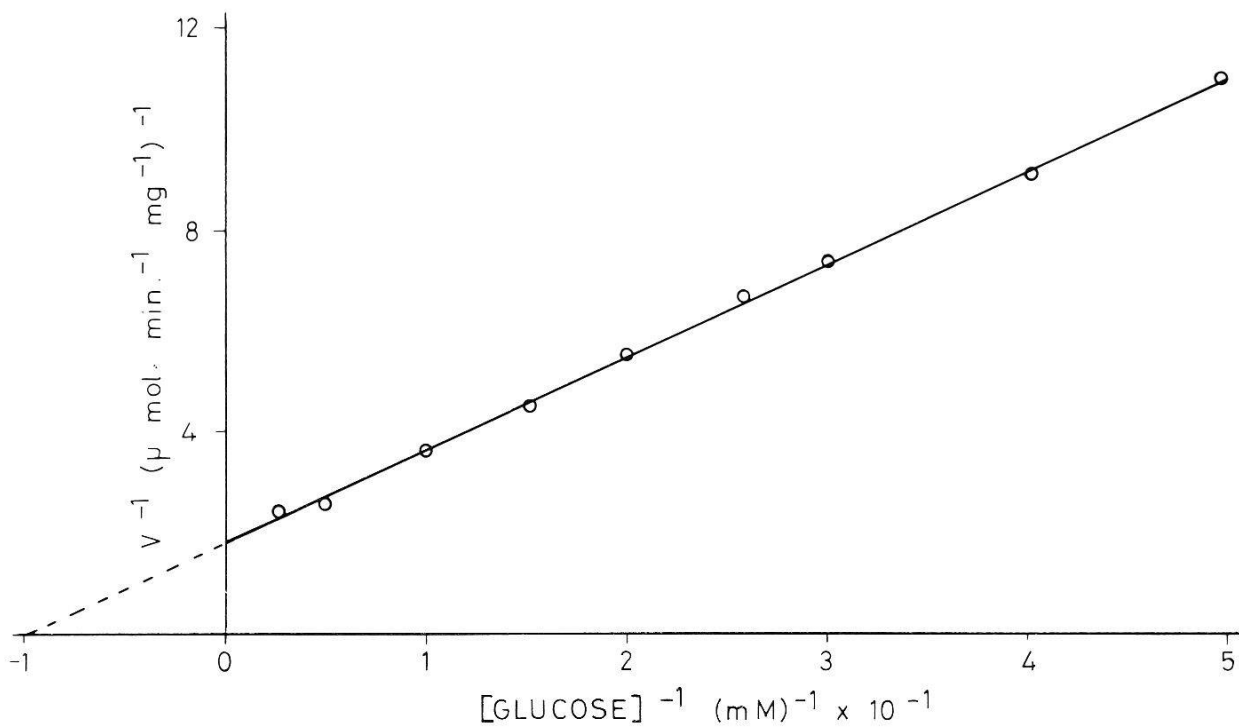
Fig. 2. Hexokinase of cultured, procyclic, trypomastigote forms of *T. congolense*. Lineweaver-Burk plot for glucose. The experiment was carried out under standard conditions at optimal ATP concentration (3.2 mM). A similar plot for hexokinase of the bloodstream form is omitted.
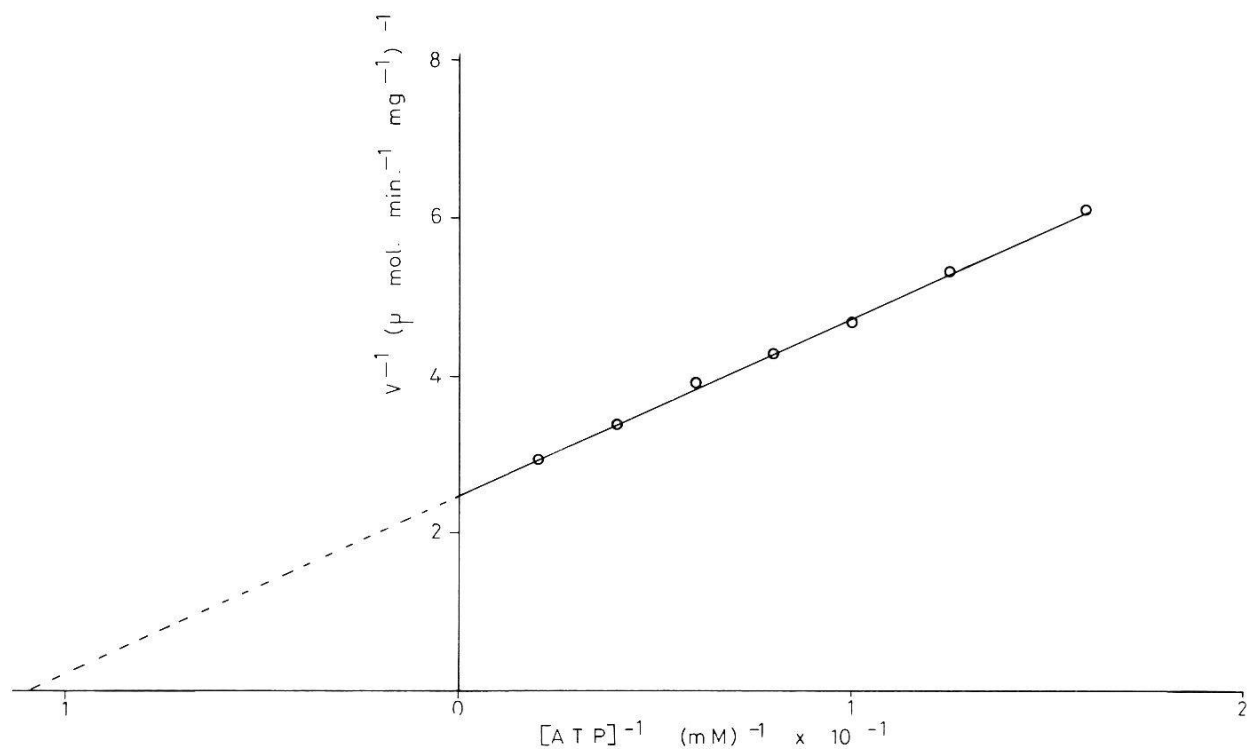

Fig. 3. Hexokinase of cultured, procyclic, trypomastigote forms of *T. congolense*. Lineweaver-Burk plot for ATP. The experiment was carried out under standard conditions at optimal glucose concentration (1 mM). A similar plot for hexokinase of the bloodstream form is not presented.
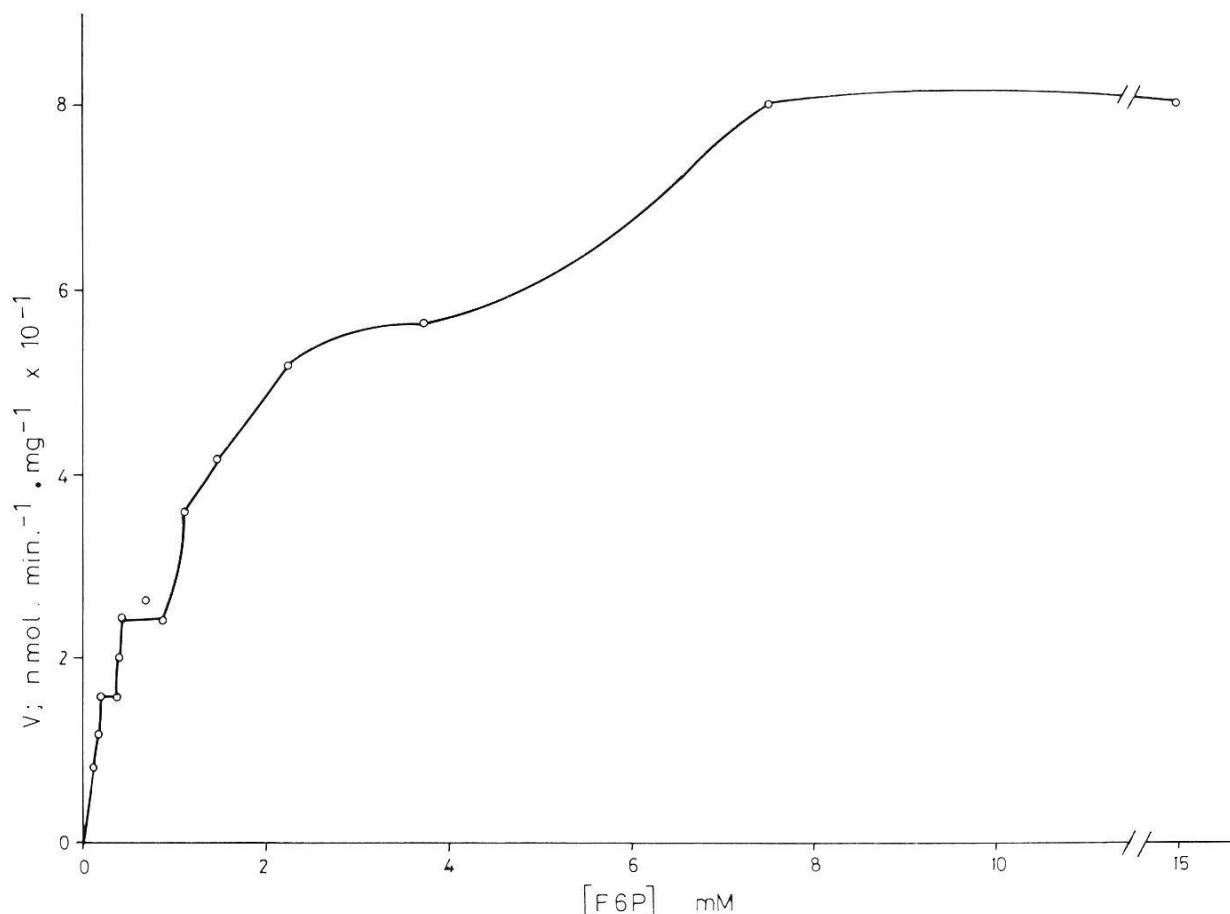
Fig. 4. Phosphofructokinase of the bloodstream form of *T. congolense*. Plot of initial velocity versus fructose-6-phosphate concentration. The reaction mixture contained a saturating concentration of ATP (0.32 mM) and 10 μg of protein from the large granule fraction. Other conditions were as described under "Materials and Methods". The figure is representative of results obtained for procyclic forms which are, therefore, omitted.

of two key glycolytic enzymes, HK and PFK, are higher in *T. brucei* bloodstream forms than in *T. congolense* bloodstream forms might underscore the greater significance of glycolysis in the energy metabolism of *T. brucei* than in that of *T. congolense*.

The apparent K_m values for glucose of HK of the bloodstream and procyclic forms of *T. congolense* were 91 μM and 100 μM, respectively: the K_m values for ATP of HK were 65 μM and 91 μM for the bloodstream and procyclic forms, respectively, suggesting that there are probably no significant, qualitative differences between the hexokinases of both forms (Figs. 2 and 3).

Phosphofructokinase from crude cell extracts of *T. congolense* displayed unusual cooperative kinetics: intermediary plateau regions were observed in plots of initial velocity versus substrate concentration, indicative of negative cooperativity (Fig. 4). Control experiments showed that the plateau regions were not produced by ATP depletion, adenylate kinase activity or low enzyme concentration. In a recent study Nwagwu et al. (1985) have shown that purified PFK from the bloodstream form of *T. brucei* also displayed the characteristic,

289

intermediary plateau regions. Other unpublished data on purified PFK from the procyclic form of *T. brucei* corroborate the above findings.

Nwagwu and Opperdoes (1982) had shown that PFK of *T. brucei* displayed sigmoidal rate dependency with respect to its substrate, F6P. Cronin and Tipton (1985) also reported similar kinetics for purified PFK of *T. brucei*. The assays of PFK in the present study included several substrate concentrations covering a wide range and the plateau regions were reproducible, thus satisfying a condition described by Teipel and Koshland (1969) for detecting "bumpy curves".

Other phosphofructokinase from pea seedlings (Kelly and Turner, 1971), human erythrocytes (Lee et al., 1973), *Ascaris suum* (Hofer et al., 1982) also displayed unusual cooperative kinetics with respect to their substrate.

The display of complex kinetics by PFK of *T. brucei* would render it susceptible to effector modulation. Huth and Menke (1982) have proposed that the product-generated complex kinetics of acetyl-CoA acetyltransferase indicated by negative cooperativity is a potent control mechanism in regulating acetyl-CoA synthesis. Detailed investigations need to be carried out to determine whether or not PFK is susceptible to significant modulation in trypanosomes. So far only AMP and, to some extent ADP have been found to activate PFK in *T. brucei* (Nwagwu and Opperdoes, 1982; Cronin and Tipton, 1985).

It would appear from the present study that transformation of trypanosomes from the bloodstream (mammalian-infective) to the procyclic (insect trypomastigote) form does not involve qualitative changes in the glycolytic enzymes if the results on HK and PFK also apply to other glycolytic enzymes. Hart et al. (1984) have shown that all peptide electrophoretic bands found in glycosomes of bloodstream forms are also present in the glycosomes of insect stages, of *T. brucei*, suggesting that there are no significant qualitative differences between the glycolytic enzymes of both forms. Instead, it seems that upon transformation to the procyclic (insect stage) form, the trypanosome reduces the level of synthesis of glycolytic enzyme as a result of, or in response to, a decreased demand for glycolysis as a sole energy source.

Agosin M., von Brand T.: Studies on the carbohydrate metabolism of *Trypanosoma congolense*. Exp. Parasit. *3*, 517–524 (1954).

Anderson S. A., Purich D. L.: A reinvestigation of dynein ATPase kinetics and the inhibitory action of vanadate. J. biol. Chem. *257*, 6656–6658 (1982).

Bowman I. B. R., Flynn I. W.: Oxidative metabolism of trypanosomes. In: Biology of the Kineto-plastida, ed. by W. H. R. Lumsden and D. A. Evans, vol. 1. p. 435–476. Academic Press, New York 1976.

Cannata J. J. B., Valle I., Decampo R., Cazzuolo J. J.: Subcellular localization of phosphoenolpyru-vate carboxykinase in the trypanosomatids *Trypanosoma cruzi* and *Crithidia fasciculata.* Molec. biochem. Parasit. *6,* 151–160 (1982).

Coombs G. M., Craft J. A., Hart D. T.: A comparative study of *Leishmania mexicana* amastigotes and promastigotes. Enzyme activities and subcellular localization. Molec. biochem. Parasit. *5,* 199–211 (1982).

Cronin C. N., Tipton K. F.: Purification and regulatory properties of phosphofructokinase from *Trypanosoma (Trypanozoon) brucei brucei.* Biochem. J. *227,* 113–124 (1985).

Fulton J. D., Spooner D. F.: Terminal respiration in certain mammalian trypanosomes. Exp. Parasit. *8,* 137–162 (1969).

Grant P. T., Sargent J. R.: Properties of L-glycerophosphate oxidase and its role in respiration of *Trypanosoma rhodesiense.* Biochem. J. *76,* 229–237 (1960).

Gray M. A., Cunnigham T., Gardiner P. R., Taylor A. M., Luckins A. G.: Cultivation of infective forms of *Trypanosoma congolense* from trypanosomes in the proboscis of *Glossina morsitans.* Parasitology *82,* 81–95 (1981).

Gutteridge W. E., Coombs G. N.: Biochemistry of parasitic protozoa. Macmillan, London, U.K. 1977.

Hart D. T., Misset O., Edwards S. W., Opperdoes F. R.: A comparison of the glycosomes (microbo-dies) isolated from *Trypanosoma brucei* bloodstream forms and cultured procyclic trypomasti-gotes. Molec. biochem. Parasit. *12,* 25–35 (1984).

Hofer H. W., Allen B. L., Kaeini M. R., Pette D., Harris B. J.: Phosphofructokinase from *Ascaris suum.* Regulatory kinetic studies and activity near physiological conditions. Biochemistry *257,* 3801–3806 (1982).

Huth W., Menke R.: Regulation of ketogenesis. Mitochondrial acetyl-CoA acetyltransferase from rat liver: initial-rate kinetics in the presence of the product CoASH reveal intermediary plateau regions. Europ. J. Biochem. *128,* 413–419 (1982).

Kelly G. J., Turner J. F.: Cooperativity in pea-seed phosphofructokinase. Biochim. biophys. Acta *242,* 559–565 (1971).

Lanham S. M.: Separation of trypanosomes from the blood of infected rats and mice by anion exchangers. Nature (Lond.) *218,* 1273–1274 (1968).

Lee L. M. Y., Krupka R. M., Cook R. A.: Cooperativity in human erythrocyte phosphofructokinase. Biochemistry *12,* 3503–3508 (1973).

Lowry O. H., Rosebrough N. J., Parr A. L., Randall R. I.: Protein measurement with the folin phonel reagent. J. biol. Chem. *193,* 265–275 (1951).

Nwagwu M., Opperdoes F. R.: Regulation of glycolysis in *Trypanosoma brucei:* hexokinase and phosphofructokinase. Acta trop. (Basel) *39,* 61–72 (1982).

Nwagwu M., Grab D. J., Moi Yoi O., Hirumi H., Watanabe M.: Phosphofructokinase of *Trypano-soma brucei:* purification and properties. Biochem. Soc. Trans. *13,* 890–891 (1985).

Opperdoes F. R.: A rapid method for the isolation of intact glycosomes from *Trypanosoma brucei* by Percoll centrifugation in a vertical rotor. Molec. biochem. Parasit. *3,* 183–186 (1981).

Opperdoes F. R., Borst P.: Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei:* the glycosome. FEBS Lett. *80,* 360–364 (1977).

Opperdoes F. R., Borst P., Spits N.: Particle-bound enzymes in the bloodstream forms of *Trypano-soma brucei.* Europ. J. Biochem. *76,* 21–28 (1977).

Opperdoes F. R., Markos A., Steiger R. F.: Localization of malate dehydrogenase, adenylate kinase and glycolytic enzymes in glycosomes and the threonine pathway in the mitochondrion of cultured procyclic trypomastigotes of *Trypanosoma brucei.* Molec. biochem. Parasit. *4,* 291–309 (1981).

Taylor M. B., Berghausen H., Heyworth F., Messenger N., Roes I. J., Gutteridge W. E.: Subcellular localization of some glycolytic enzymes in parasitic flagellated protozoa. Int. J. Biochem. *11,* 117–120 (1980).

291

Teipel J., Koshland D. E. jr.: The significance of intermediary plateau regions in enzyme saturation curves. Biochemistry *8*, 4656–4663 (1969).

Visser N., Opperdoes F. R.: Glycolysis in *Trypanosoma brucei*. Europ. J. Biochem. *103*, 623–632 (1980).

von Brand T.: Metabolism of Trypanosomatidae and Bodomidae. In: Biochemistry and physiology of protozoa, ed. by A. Lwoff, vol. 1, p. 177–234. Academic Press, New York 1951.