The Role of Compartmentation and Glycerol Kinase in the Synthesis of ATP within the Glycosome of Trypanosoma brucei*

David J. Hammond, Rashid A. Aman, and Ching C. Wang‡

From the Department of Pharmaceutical Chemistry, University of California, School of Pharmacy, San Francisco, California 94143

Glycosomes, purified from trypanomastigote forms of Trypanosoma brucei, contained all the enzymes necessary to convert glucose to a-glycerophosphate and 3-phosphoglycerate. The multienzyme reaction which produces a-glycerophosphate and ATP catalyzed by glycosomal hexokinase and either hexokinase or phosphofructokinase in glycosomal enzyme complexes cross-linked by dimethyl suberimidate treatment of intact glycosomes prior to solubilization of their membrane. Compartmentation of glycolytic intermediates, enzymes, and ATP inside isolated glycosomes was demonstrated by their inaccessibility to exogenous enzymes. We conclude that the compartmentation of the glycosome and the efficient production of ATP in the glycosome from whole cell concentrations of sn-glycerol 3-phosphate and ADP accounts for ATP production in whole cell production of equimolar glycerol from glucose with net ATP synthesis by T. brucei under anaerobic conditions.

Many unusual biological features, including the presence of glycosomes, have been found in species of pathogenic protozoa belonging to the family Trypanosomatidae (1-4). The peroxisome-like glycosomes contain enzymes of glucose metabolism (1-7), purine (8), pyrimidine (9), and alkoxyphospholipid (10) synthesis, oxidation of fatty acids (11), and adenylate kinase (12-13). They have been identified in all trypanosomatids studied to date, although differences are known to exist between glycosomes from different species and from different stages in the life cycle of Trypanosoma brucei (see 14).

Long slender bloodstream forms of T. brucei, the causative agent of nagana in cattle, are completely dependent on a continuous supply of carbohydrate for energy metabolism. Fructose, glucose, glycerol, and mannose may all be utilized in vitro, but the low serum concentrations of fructose, <0.1 mM (e.g. Ref. 15), and glycerol, 0.1 mM (16), compared to glucose in particular, 5 mM, and mannose, 1.5 mM (17), suggest that glucose and mannose are the more likely substrates in vivo. Catabolism of 1 mol of glucose under aerobic conditions proceeds at a rate of about 0.08 μmol/min/mg protein through the Embden-Meyerhof pathway to produce 2 mol of pyruvate as the only end product (18). All the enzymes necessary to convert glucose to 3-phosphoglycerate and a-glycerophosphate (aGP) are found within the glycosome, but aGP must leave the organelle to form dihydroxyacetone phosphate (DHAP) catalyzed by the mitochondrial (19) aGP oxidase. DHAP then enters the glycosome to form additional aGP or via glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate to 3-phosphoglycerate. When aGP oxidase is not operative, equimolar amounts of glycerol and pyruvate are produced from 1 mol of glucose with net synthesis of 1 mol of ATP (20, 21). This stoichiometry has been explained by postulating that very high concentrations of aGP accumulate within the glycosome. Under this special condition, glycerol and ATP could be produced from aGP and ADP catalyzed by the extremely active glycerol kinase, despite the apparently unfavorable free energy change for this reaction (22). ATP is then used to phosphorylate hexoses or hexose-6-phosphates catalyzed by glycosomal hexokinase or phosphofructokinase kinase. Thus, under both aerobic and anaerobic conditions, glycosomal ATP synthesis balances its utilization, while NAD⁺ reduction equals its reoxidation. Net ATP is synthesized in the cytoplasm catalyzed by pyruvate kinase as 3-phosphoglycerate is converted to phospho-enolpyruvate and then to pyruvate.

Despite this most unusual function attributed to glycerol kinase and the glycosome in the anaerobic metabolism of hexoses by T. brucei, its enzymology has been little studied. However, now that a procedure has been established for the purification of glycosomes from bloodstream trypanomastigote forms of T. brucei, it is possible to demonstrate the glycosomal compartmentation in vitro and to assess the ability of glycosomal glycerol kinase to provide ATP for anaerobic glycolysis in purified, intact glycosomes. Moreover, since we are also able to cross-link glycosomal enzymes with the membrane-permeable bifunctional reagent dimethyl suberimidate (DMSI) (22), we can trap the enzymes in a, hopefully, native state of aggregation prior to solubilization of the glycosomal membrane. In this way, it may be possible to evaluate whether the organization of the cross-linked enzymes contributes to the efficiency of ATP production by glycerol kinase.

In this present report, we have studied the carbohydrate metabolism by the cross-linked glycosomal enzyme complex,
the intact glycosomes, and the Triton X-100-solubilized glycosomes. In particular, we describe properties of the reaction catalyzed by glycosomal glycerol kinase:

\[ \alpha G P + ADP \rightarrow \text{glycerol} + \text{ATP} \]

(1)

and the multienzyme reaction catalyzed by the glycosomal enzymes hexokinase, phosphoglucone isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, and \( \alpha G P \) dehydrogenase:

\[ \text{glucose} + 2 \text{NADH} + 2 \text{H}^+ + 2 \text{ATP} \rightarrow \text{glycerol} + 2 \text{NAD}^+ \]

(2)

Moreover, when ATP generated from \( \alpha G P \) and ADP intraglycosomally by glycerol kinase phosphorolyses glucose to produce 2ATP, both \( \alpha G P \) and ADP will be recycled in the glycosome. This is summarized in Equation 3 where Equations 1 and 2 are combined in the ratio 2:1 to produce:

\[ \text{glucose} + 2 \text{NADH} + 2 \text{H}^+ \rightarrow 2 \text{glycerol} + 2 \text{NAD}^+ \]

(3)

As glucose and glycerol must both be permeable to the glycosome (1), and intact peroxosomes are permeable to NAD (28), it is possible to study a major part of the glycolysis in intact glycosomes without the intervention of a rate-limiting membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—All substrates, cofactors, and coupling enzymes were from Sigma or Calbiochem-Behring. [5-3H]Orotate, [8-3H]hypoxanthine, and [U-14C]glucose were from ICN, Irvine, CA. All reagents were from Sigma or Bio-Rad.

**Glycosome Preparation**—A monomorphic line of \( T. \) brucei, EATRO 110, was maintained as stabiles at \(-80^\circ C\). About \( 10^6 \) trypanosomes were injected intraperitoneally into each 300-g male Wistar rat. The parasites were harvested 48 h later and isolated free from blood elements (29). The glycosomes were then purified by a previously described procedure (14, 27). In brief, purified trypanosomes were washed in 55 mM glucose, 50 mM NaHPO₄, 3 mM NaH₂PO₄, and 45 mM NaCl (PSG), pH 8.0, and were either used immediately or were first diluted with PSG containing 20% (v/v) glycerol and then stored at \(-20^\circ C\). The glycosomes were then purified by a previously described procedure (14, 27). In brief, purified trypanosomes were centrifuged at 1,000 × 100g for 10 min at 4 °C in 20 mM Tris, pH 7.4, 250 mM sucrose, 0.2 mM EDTA, 0.1 mM dithiothreitol (TEDS), and 5.5 mM glucose. The pellets were resuspended in an equal volume of TEDS and centrifuged at 100,000 × 100g for 1 min at 100,000 × 100g for 1 min at 4 °C, and pelleted at 33,000 × 100g for 1 min at 37 °C. The pellets were resuspended in TEDS and stored at \(-80^\circ C\). The DMSI-cross-linked glycosomal enzyme preparations were used in the following medium: 75 mM triethanolamine, pH 7.4, 37 mM KCl, 10 mM MgCl₂, 4 mM dithiothreitol, 1.1 mM D-[U-14C]glucose (0.2 μCi/μmol), 5 mM P₃P₃-di(adenosine 5')pentaphosphate, 25 mM GTP, 0.1% (w/v) Triton X-100, and 35 μg of DMSI-cross-linked enzyme complex. 5 mM Fru-6-P and 53 units of purified murine phosphofructokinase were added to an identical mixture to compete with glycosomal hexokinase for the synthesized ATP. These two conditions were repeated with the inclusion of 15 μg of solubilized glycosomal enzyme mixture instead of cross-linked protein. The reactions were started by the addition of 2 mM ATP, and 60-μl samples were taken after 0, 5, 10, 15, 20, 25, and 30 min incubation at 37 °C. The reactions were stopped by heating at 95 °C for 3 min. Glucose was separated from the phosphorylated products by thin layer chromatographic methods as described for the assay of orotate phosphoribosyltransferase (32). The RF for glucose was 0.9, and those for the mixture of phosphorylated products were between 0.1 and 0.4. The radioactivity was counted as described (32).

Possible direct channeling of ATP between glycerol kinase and phosphofructokinase was assessed in the following medium: 75 mM triethanolamine, pH 7.4, 37 mM KCl, 10 mM MgCl₂, 4 mM dithiothreitol, 5.5 mM Fru-6-P, 0.1% (w/v) Triton X-100, and 25 μg/ml DMSI-cross-linked glycosomal enzyme complex. 5.5 mM glucose and 66 units/ml yeast hexokinase were added to an identical mixture to compete with glycosomal phosphofructokinase for the synthesized ATP. These two conditions were repeated with the inclusion of 20 μg/ml solubilized glycosomal enzyme mixture instead of cross-linked enzyme complex. The reactions were started at 37 °C, and after 10 min incubation at 37 °C, the reaction mixture was divided into two parts: one was added to ADP and were followed spectrophotometrically at 366 nm and 37 °C. The production of ATP from \( \alpha G P \) plus ADP, catalyzed by glycerol kinase, was assayed by linking ATP production with glucose phosphorolysis by endogenous glycosomal hexokinase and exogenous NADP" reduction in the presence of 5 units/ml of added yeast Glu-6-P dehydrogenase. Orotate and hypoxanthine phosphoribosyltransferases were assayed by described radioactive methods (8, 32). The amount of L-GP in the commercial mixture of DL-GP was determined using \( \alpha G P \) dehydrogenase as described (24).

ATP Synthesis in \( T. \) brucei Glycosome

**ATP Channeling**—Possible direct channeling of ATP between glycerol kinase and hexokinase was assessed in the following medium: 75 mM triethanolamine, pH 7.4, 37 mM KCl, 10 mM MgCl₂, 4 mM dithiothreitol, 1.1 mM D-[U-14C]glucose (0.2 μCi/μmol), 5 mM P₃P₃-di(adenosine 5')pentaphosphate, 25 mM GTP, 0.1% (w/v) Triton X-100, and 35 μg of DMSI-cross-linked enzyme complex. 5 mM Fru-6-P and 53 units of purified murine phosphofructokinase were added to an identical mixture to compete with glycosomal hexokinase for the synthesized ATP. These two conditions were repeated with the inclusion of 15 μg of solubilized glycosomal enzyme mixture instead of cross-linked protein. The reactions were started by the addition of 2 mm ATP, and 60-μl samples were taken after 0, 5, 10, 15, 20, 25, and 30 min incubation at 37 °C. The reactions were stopped by heating at 95 °C for 3 min. Glucose was separated from the phosphorylated products by thin layer chromatographic methods as described for the assay of orotate phosphoribosyltransferase (32). The RF for glucose was 0.9, and those for the mixture of phosphorylated products were between 0.1 and 0.4. The radioactivity was counted as described (32).

**Protein**—This was determined by using the Bio-Rad protein assay reagent according to the manufacturers' instructions (33). Bovine serum albumin, fraction V, from Sigma was used as standard.

**SDS-Polyacrylamide Gel Electrophoresis**—Gel electrophoresis was performed exactly as previously described (27).

**Kinetic Analysis**—Enzyme kinetic data were analyzed using Wilkinson's method (34), and the calculation of transient times and steady state intermediate concentrations were based on the theory of Esterby (35). Structure-linked latency of glycosomal enzymes was defined by the following relationship:

\[
(\text{activity in presence of 0.1% (w/v) Triton X-100) - (activity in absence of Triton X-100)} \times 100
\]

(activity in presence of 0.1% (w/v) Triton X-100)

\[
\text{RESULTS}
\]

**Constituents of Purified Glycosomes**—The results of a representative experiment in which the activities of most of the known glycosomal enzymes of \( T. \) brucei were determined for the homogenate and for purified glycosomes are given in Table I. These data were obtained from freshly purified trypanosomes, although storage of the parasites at \(-80^\circ C\) before...
fractionation did not alter the resulting activities, latencies, or subcellular distribution of the enzymes. The overall recovery of most glycosomal enzymes in purified glycosomes was routinely 10–25%, and a 10-fold increase in specific activity was usually seen in the extract of purified glycosomes compared to that in the homogenate. The exceptions were hypoxanthine phosphoribosyltransferase, where only a 2–3-fold increase in specific activity was seen, and adenylation kinase, where a decrease was found. Phosphomannose isomerase showed low activity compared to the other catabolic enzymes. Triton X-100 at 0.1% (w/v) was included in the assay media for all the enzymes given in Table I except hypoxanthine and orotate phosphoribosyltransferase which were partially inhibited by this detergent.

The latencies of the glycolytic enzymes after storage at −80 °C are given in Table I. One cycle of freezing and thawing caused <10% decrease in the latency of hexokinase compared to prefrozen values. The glycosome-enriched fraction from Percoll gradients always showed similar latency to the starting homogenate, but organelles obtained following centrifugation in linear sucrose gradients had usually decreased latency. No latency was found for glycerol kinase assayed in the direction leading to ATP synthesis.

Fig. 1 shows the protein bands of glycosomes (lanes 2, 3, and 4) and homogenate (lane 5), following SDS polyacrylamide gel electrophoresis and staining with Coomassie Blue. The identification of bands was based on previous results (14, 27). The dense band at about a M, of 68,000 in the homogenate (lane 5) is most probably the variable surface glycoprotein (27) and is only a minor contaminant in the glycosomal preparations.

Steady State Production of αGP from Glucose and ATP—

The first enzyme of a sequence of enzymes must be rate-limiting before a steady state flux of metabolites through a multienzyme sequence can be achieved. However, since the first enzyme of glycolysis, hexokinase, in Triton X-100-lysed T. brucei glycosomes is present at high activity (e.g. see Table I), this enzyme was rendered rate-limiting by lowering the concentration of ATP. An apparent Kₗ for ATP of 80 ± 11 μM was found for glycosomal hexokinase in the presence of 0.1% (w/v) Triton X-100, while this enzymes’ activity with 20 μM ATP as shown in Fig. 2, line A, by the increase in absorption at 366 nm as NADP⁺ is reduced by the coupling enzyme Glu-6-P dehydrogenase. In this assay, the concentration of glucose was saturating at 5.5 mM, and ATP was regenerated by the inclusion of 2 mM phosphoenolpyruvate and 5 units/ml pyruvate kinase. A maximum and constant rate of NADP⁺ reduction of 1.36 ± 0.05 μmol/min/mg protein was found for line A (1.3–2.0 μmol/min/mg protein in other preparations).

When Glu-6-P production from glucose (measured by NADP⁺ reduction with Glu-6-P dehydrogenase) and ATP utilization (measured by NADH oxidation with lactate dehy-
The reaction catalyzed by Triton X-100-lysed glycosomes remained constant at about 0.35 mM, while the transient time was inversely proportional to and the steady state rate was directly proportional to the glycosomal protein concentration over the range 3.3–129 μg/ml.

The profile of glucose- and ATP-dependent oxidation of NADH by DMSI-cross-linked enzymes is compared with those from Triton X-100-treated native glycosomes and intact glycosomes in Fig. 3. A steady state concentration of glycolytic intermediates of 0.38 ± 0.09 mM was found for the cross-linked enzymes from three different determinations (Fig. 3, line A) and 0.36 ± 0.08 mM for Triton X-100-treated glycosomes (line D). The rate of oxidation of exogenously added NADH by purified intact glycosomes, which varied between preparations, was 3–15% that found for lysed glycosomes, as indicated by line B. Fig. 3, line C, gives the profile of NADH oxidation when the density of intact glycosomes was 5-fold higher than in lines A or B, and unlike B, a steady state rate of NADH oxidation was achieved with an intermediate concentration of 0.32 mM.

The similar steady state intermediate concentrations obtained for the three different enzyme preparations in the presence of 20 μM ATP suggest that there is no compartmentation in intact glycosomes or channeling in cross-linked enzymes. This observation and the low steady state rate of NADH oxidation for intact glycosomes compared to lysed glycosomes can be explained by a small percentage, 15%, of glycosomes being damaged during isolation.

Alternative Substrates to Glucose for ATP-dependent NADH Oxidation—Fructose, mannose, Glu-6-P, Fru-6-P, and Man-6-P, but not fructose-1-phosphate, glucose 1-phosphate, or mannose 1-phosphate, can substitute for glucose as substrates for ATP-dependent NADH oxidation by lysed glycosomes, as seen in Fig. 4. At an ATP concentration of 20 μM, the rate of NADH oxidation with mannose (Fig. 4C) was barely detectable, and even with 2 mM ATP, it was low (0.4 μmol/min/mg protein) compared to glucose (4–6 μmol/min/mg protein). The low activity and long transient time (not shown) observed with mannose as substrate can be attributed to the presence of a low activity, low affinity enzyme, phosphomannose

drogenase, phosphoenolpyruvate, and pyruvate kinase) in the Triton X-100-lysed glycosomes are followed simultaneously, a slow decrease in absorbance at 366 nm of 0.05 pmol/min/mg protein is seen, Fig. 2, line B. Since this value is comparable to the rate of spontaneous NADH oxidation in the absence of either glucose (line C) or ATP (line D), the reduction of NADP⁺ by Glu-6-P dehydrogenase and the oxidation of NADH by lactate dehydrogenase in the assay mixture must have been equal. Thus, ATP utilization balances the measured production of Glu-6-P. Line A, therefore, does not underestimate Glu-6-P production by hexokinase due to the presence of other competing glycosomal enzymes.

The rate of the hexokinase-catalyzed reaction in line A (1.36 ± 0.05 μmol/min/mg protein) was equal to half of the maximum rate of NADH oxidation of 2.64 ± 0.05 μmol/min/mg protein in line E, which accompanies the production of αGP from glucose and 20 μM ATP in the multienzyme-catalyzed reaction. Since 2 mol of αGP are produced per mol of glucose phosphorylated, the glycolytic flux through the multienzyme system must achieve a steady state equilibrium. Consequently, the kinetics of the multienzyme reaction can be studied under steady state conditions.

Transient Time and Steady Intermediate Concentration—Determination of these values is obtained by extrapolating the steady state rate of NADH oxidation in line E of Fig. 2 to intercept with “time 0” the start of the reaction to give the coordinate “o, y” and, then with the starting absorption, to give “oXy.” The steady state intermediate concentration can then be obtained from y to y’, and the transient time is t. Our data indicate that, at an ATP concentration of 20 μM, the steady state concentration of glycolytic intermediates in
isomerase being involved within the multi-enzyme reaction sequence.

*Synthesis of ATP from αGP and ADP Catalyzed by Glycerol Kinase*—In the assays described in Figs. 2–4, 20 μM ATP was present as the phosphate donor and was regenerated extraglycosomally by adding pyruvate kinase and phosphoenolpyruvate. An alternative system for ATP production is through the synthesis of glycerol plus ATP from exogenously added αGP plus ADP, catalyzed by the glycosomal glycerol kinase. When this enzyme was assayed by linking ATP production to glycosomal hexokinase, exogenous Glu-6-P dehydrogenase, and NADP+ reduction, e.g. Table I. This difference is explained by the greater accumulation of glycerol which accompanies ATP production from αGP and ADP in the multi-enzyme reaction prior to reaching a maximum rate of NADH oxidation as DHAP is converted to αGP. The accumulated glycerol inhibits the production of ATP from glycerol kinase and so inhibits the multi-enzyme reaction.

*Inhibition of ATP Production from αGP Plus ADP by Glycerol*—The effect of glycerol on the production of ATP by glycerol kinase was evaluated using two indirect methods. First, its effect on αGP-, ADP- and glucose-dependent NADH oxidation was studied in the multi-enzyme reaction of lysed glycosomes (Fig. 5, line A). Second, its effect on αGP-, ADP-, and glucose-dependent NADP+ reduction with Glu-6-P dehydrogenase was studied using lysed glycosomes (Fig. 5, line B). The concentration of glycerol required for 50% inhibition (I50) was about 0.5 mM in line A and 0.9 mM in line B when the concentration of αGP was 23 mM. Lower concentrations of αGP produced concomitantly lower I50 values for glycerol. The rate of NADH oxidation was optimal with αGP concentrations of 11 mM, but was decreased by about 10% at both higher (23 mM) or lower (5.6 mM) concentrations. An αGP concentration of 23 mM was used routinely as a compromise between the need to record high multi-enzyme activities and to minimize the inhibition caused by the accompanying production of glycerol.

*Apparent Steady State Production of αGP from Glucose, αGP, and ADP*—The profile of glucose-dependent NADH oxidation by intact glycosomes when 23 mM αGP plus 2 mM ADP were present to synthesize ATP is given in Fig. 6, line A. This reaction approaches a steady state, and a constant rate of NADH oxidation, as described for Fig. 2, line 6.

![Graph](image-url)

**Fig. 4.** The effect of different substrates on ATP-dependent oxidation of NADH by lysed glycosomes. The assay conditions were as described in Fig. 3 except that the glycosome protein concentration was 5 μg/ml and instead of glucose, 5.5 mM fructose was added to line A, 5.5 mM mannose to line C, 5.5 mM Fru-6-P to line D, 5.5 mM Glu-6-P to line E, 5.5 mM Man-6-P to line F, while 5.5 mM glucose was present in line B.

**TABLE II**

The effect of pH on αGP-, ADP-, and glucose-dependent oxidation of NADH by lysed glycosomes

The multi-enzyme reaction medium was as described under "Experimental Procedures" except that 400 mM triethanolamine was used. Other additions were 2 mM ADP, 23 mM αGP, and 1 mM NADH; 0.1% (w/v) Triton X-100 was included to lyse the organelles which were present at a concentration of 5 μg of protein/ml.

<table>
<thead>
<tr>
<th>pH</th>
<th>Apparent transient time</th>
<th>Apparent transient</th>
<th>Rate μmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>8.8</td>
<td>0.022</td>
<td>0.21</td>
</tr>
<tr>
<td>6.5</td>
<td>33</td>
<td>0.17</td>
<td>0.46</td>
</tr>
<tr>
<td>7.0</td>
<td>39</td>
<td>0.26</td>
<td>0.63</td>
</tr>
<tr>
<td>7.5</td>
<td>29</td>
<td>0.22</td>
<td>0.70</td>
</tr>
<tr>
<td>8.0</td>
<td>28</td>
<td>0.14</td>
<td>0.45</td>
</tr>
<tr>
<td>8.5</td>
<td>26</td>
<td>0.049</td>
<td>0.17</td>
</tr>
</tbody>
</table>

![Graph](image-url)

**Fig. 5.** The effect of glycerol on the rate of ATP synthesis from αGP plus ADP catalyzed by glycerol kinase. The production of ATP by glycosomes lysed with 0.1% (w/v) Triton X-100 was measured either by coupling to the reduction of 2 mM NADP+ with glycosomal hexokinase and 5 units/ml Glu-6-P dehydrogenase (○) or by the inhibition of glucose-dependent oxidation of 1 mM NADH (●). In both cases, 23 mM αGP and 2 mM ADP were present. Other additions are as described under "Experimental Procedures."
Compartmentation of Glycolytic Intermediates and Enzymes in Isolated Glycosomes—To evaluate whether intermediates of the multienzyme reaction are being compartmentalized within intact glycosomes only when aGP and ADP are used as the phosphate donor for glucose catalysis.

Fig. 6. The profile of glucose-, aGP-, and ADP-dependent oxidation of NADH by line A, 14 mg/ml intact glycosomes; line B, 14 mg/ml glycosomes lysed with 0.1% (w/v) Triton X-100, and line C, 38 mg/ml DMSI-cross-linked protein. The assay conditions were as described under "Experimental Procedures," but with the following additions: 23 mM aGP, 2 mM ADP, and 1 mM NADH.

E, produces an apparent steady state intermediate concentration of 0.04 mM and an apparent transient time of 7 min. These values for intact glycosomes were found to be reproducibly only 25% of those obtained for lysed glycosomes, e.g. Fig. 6, line B. The cross-linked glycosomal enzymes (Fig. 6, line C) gave a response comparable to that of lysed glycosomes. Significantly, unlike the situation described in Fig. 2 for exogenously added ATP where intact glycosomes showed decreased activity compared to the lysed glycosomes, the rates of oxidation of NADH for intact and lysed glycosomes were always found to be similar. Thus, there is a rate-limiting permeability barrier for aGP and ADP. These observations also suggest that intermediates of the multienzyme reaction are being compartmentalized within intact glycosomes only when aGP and ADP are used as the phosphate donor for glucose catalysis.

**Experimental Procedures**... for Glu-6-P. These observations agree with Fig. 5. The profile of glucose-, aGP-, and ADP-dependent oxidation of NADH by line A, 14 mg/ml intact glycosomes; line B, 14 mg/ml glycosomes lysed with 0.1% (w/v) Triton X-100, and line C, 38 mg/ml DMSI-cross-linked protein. The assay conditions were as described under "Experimental Procedures," but with the following additions: 23 mM aGP, 2 mM ADP, and 1 mM NADH.

Compartmentation of Glycolytic Intermediates and Enzymes in Isolated Glycosomes—To evaluate whether intermediates of the multienzyme reaction are being compartmentalized, within glycosomes, the effect of exogenously added enzymes on the rate of NADH oxidation was tested. Exogenous hexokinase was added at very high specific activity with exogenously added 5.5 mM fructose, 5.5 mM glucose, and 5.5 mM mannose were 0.67, 0.70, and 0.49 pmol/min/mg respectively and preincubation with trypsin and pronase (not shown) had no significant effect. These experiments were performed using purified glycosomes. When glycosome-enriched fractions obtained following isopycnic centrifugation in Percoll were studied, very similar results were obtained. Consequently, these results strongly suggest that all the enzymes required for aGP production and the metabolites Glu-6-P, fructose 1,6-diphosphate, and ATP are at least partially accessible to the extraglycosomal environment, i.e. they are compartmentalized within the glycosome. Since oxidation of NADH must also occur in the glycosome catalyzed by proteinase-insensitive, aGP dehydrogenase, glycosomes must also be partially permeable to NADH. No compartmentation was seen when 20 mM or 2 mM ATP was present with either intact or lysed glycosomes (Table III) suggesting, in agreement with Fig. 2, that at least most of the exogenous ATP-dependent oxidation of NADH was due to broken glycosomes.

**The Lack of an Apparent Transient Time for aGP Production from Glucose, aGP and ADP**—An apparent, but relatively short, transient time leading to the maximum rate of NADH oxidation by intact glycosomes was noted in Fig. 5. Its duration was inversely proportional to the amount of glycosomes added, but was unaffected by the order to adding hexose, aGP, or ADP, suggesting that it was due to a permeability barrier of glucose, aGP, or ADP, but was caused by the necessary build up of an intermediate. It was not detectable, however, when 10 units/ml aGP dehydrogenase was included in the assay media, e.g. Fig. 7. Since the addition of aGP dehydrogenase would effectively decrease the extraglycosomal accumulation of DHAP with concomitant oxidation of NADH, this suggests that most, if not all, of the transient in Fig. 6 was due to an extraglycosomal accumulation of DHAP; consequently, there must be at least some leakage of DHAP from the glycosome before it can be reduced by glycosomal aGP dehydrogenase and exogenous NADH. The lack of a detectable lag time for DHAP production from added glucose, aGP, and ADP by intact glycosomes strongly indicates that the intermediates of this overall reaction sequence are being sequestered in the intact glycosomes.

**Alternative Substrates for Intact Glycosomes**—The maximum rates of aGP plus ADP-dependent NADH oxidation with exogenously added 5.5 mM fructose, 5.5 mM glucose, and 5.5 mM mannose were 0.07, 0.70, and 0.49 μmol/min/mg
protein, respectively, from Fig. 7. These rates were all measured in intact glycosomes without a detectable lag time when 10 units/ml aGP dehydrogenase was present. The maximum rates for 5.5 mM Fructose-6-P, 5.5 mM Glucose-6-P, and 5.5 mM Mannose-6-P were 0.96, 0.86, and 0.30 μmol/min/mg protein, but, in contrast to the situation described for the hexoses (e.g. Fig. 8, lines A and B), these were achieved after a lag time, e.g. Fig. 8, line D. Fig. 8 also shows that the maximum rate of NADH oxidation was increased by 43% when Triton X-100 was added to glycosomes in the presence of aGP, ADP, and Fru-6-P, e.g. see lines C and D. No latency was detected when fructose, lines A and B (mannose or glucose), was substrate even though this particular glycosome preparation showed 70% latency for hexokinase when ATP was the phosphate donor (not shown).

The rate of hexose 6-phosphate-dependent NADH oxidation is higher over the first 3 min for intact than for Triton X-100-treated organelles, e.g. 1.0 and 0.65 μmol/min/mg protein with Fru-6-P as substrate, Fig. 8, lines C and D. The higher initial rate of aGP production (measured by NADH oxidation) from the intact glycosomes suggests that some degree of compartmentation of glycolysis may also occur in intact glycosomes when Fru-6-P is the substrate. This postulate is strengthened by the fact that exogenously added fructose-1,6-diphosphatase inhibits NADH oxidation by Fru-6-P, aGP, and ADP in Triton X-100-lysed glycosomes (line F), but has significantly less effect on intact glycosomes (line E), suggesting inaccessibility due to compartmentation of fructose-1,6-diphosphatase inside the glycosome.

Possible Channeling of ATP between Glycerol Kinase and Either Hexokinase or Phosphofructokinase—The possibility that ATP may be directly channeled between glycerol kinase and hexokinase in DMSI-cross-linked enzymes was assessed by competition experiments. The efficiencies of aGP- plus ADP-dependent phosphorylation of d-[U-14C]glucose in the presence and absence of competition for ATP
from exogenously added phosphofructose kinase and Fru-6-P were compared. The results in Table IV show that competition for ATP by phosphofructose kinase produced a 93% inhibition of glucose phosphorylation. This degree of inhibition was similar to that obtained for glycosomes lysed with 0.1% (w/v) Triton X-100 (89%) and indicates that the ATP synthesized from added ADP and αGP is equally accessible to the exogenous enzyme in cross-linked and lysed glycosomes. Consequently, there is no evidence for ATP being channeled between glycerol kinase and hexokinase. Using a similar rationale, possible ATP channeling between glycerol kinase and phosphofructose kinase was assessed by comparing the efficiency of αGP- plus ADP-dependent NADH oxidation by Fru-6-P in the presence and absence of competition from exogenous hexokinase and glucose, Table IV. The addition of hexokinase produced similar inhibition of the phosphofructose kinase-catalyzed reaction in cross-linked and solubilized glycosomes (94 and 97%, respectively), again failing to produce any evidence for ATP channeling between glycerol kinase and the ATP-requiring enzyme, phosphofructose kinase.

**DISCUSSION**

Glycosomes have been purified from long slender trypanomastigote forms of *T. brucei* by techniques described previously (14, 27). Their purity is indicated by the highly reproducible glycosomal protein pattern (27, 36) following gel electrophoresis (Fig. 1) and the reproducible 10-fold increase in the specific activities of most of the glycosomal enzymes (Table I); the exceptions were adenylate kinase, which is also found in other subcellular fractions (12, 13), and hypoxanthine phosphoribosyltransferase.

In agreement with previous observations (37), isopycnic centrifugation in sucrose, but not Percoll, decreased the latency of glycosomal enzymes, e.g. hexokinase from about 80% to between 30 and 70%, although additional storage at −80 °C had little effect. All glycolytic enzymes tested showed latencies ranging from 48 to 83% in homogenates although, when measured in the direction leading to ATP synthesis, glycerol kinase showed little or no latency (Table I).

The multienzyme-catalyzed reaction from glucose to αGP has been studied by following the oxidation of NADH when 20 μM ATP was supplied as the phosphate donor. This low ATP concentration, coupled with a regeneration system, makes hexokinase (apparent *Km* ATP of 80 ± 11 μM) the rate-limiting enzyme in the sequence and produces a steady state rate of NADH oxidation of 2.6 μmol/min/mg protein (Fig. 2). Under these conditions of glucose catabolism in Fig. 3, the steady state concentrations of glycolytic intermediates from DMSI-cross-linked glycosomal enzymes, intact glycosomes, and Triton X-100-treated glycosomes were all about 0.05 mM, suggesting that the kinetic behaviors of the different enzyme preparations may be similar. Intact glycosomes showed very-low glucose-dependent NADH oxidation activity when compared to the lysed glycosomes, demonstrating the presence of a glycosomal membrane barrier to ATP. The intermediates Glu-6-P, and fructose 1,6-diphosphate were fully accessible to exogenous enzymes (Table III), further showing that the oxidation of NADH with ATP supplied as phosphate donor activity was due mainly to noncompartmentalized reactions, presumably due to damage of a low percentage, <15%, of organelles during isolation.

In Table II and Figs. 5–8, there is ample evidence that despite the unfavorable thermodynamics, the ATP for hexose phosphorylation can be readily produced from ADP plus αGP catalyzed by the glycosomal glyceral kinase. The rate of ATP production by glyceral kinase is 1.2 μmol/min/mg protein with 33 mM αGP and 2 mM ADP (Table I). The apparent *Km* of αGP is 5.1 ± 0.7 mM, and the *Km* of ADP is 0.49 ± 0.05 mM when the reaction is coupled to glycosomal hexokinase plus yeast Glu-6-P dehydrogenase. This rate is faster than that necessary for NADH oxidation in the presence of glucose, αGP, and ADP (Table II and Fig. 7), which nonetheless has a maximum activity of 0.8 μmol/min/mg protein when αGP is present at concentrations between 5.6 and 23 mM. Since glycosomal protein makes up 10% of the whole cell protein, this rate can be extrapolated to the whole cell rate of ATP production from glyceral kinase under anaerobic conditions of 0.08 μmol/min/mg protein. Moreover, because the whole cell concentration of αGP is about 10 mM (23, 25), it is not even necessary to propose an accumulation of αGP within the glycosome as suggested in (1) to account for anaerobic glucose metabolism via the action of glyceral kinase. Furthermore, at an αGP concentration of 23 mM, glyceral inhibits the multienzyme reaction with an *Iₐ₅₀* of about 0.9 mM, Fig. 5, which is comparable to that found for whole cells (24).

The rate of αGP-, ADP-, and glucose-dependent oxidation of NADH was similar for intact and Triton X-100-treated glycosomes, demonstrating the absence of a rate-limiting permeability barrier (Fig. 6). This contrasts with the situation described in Fig. 3 where 20 μM ATP was supplied as phosphate donor. Thus, ATP for glucose phosphorylation may be generated from αGP and ADP intraglycosomal. Significantly, when ATP is produced from αGP and ADP catalyzed by glyceral kinase in the glycosome, the subsequent catabolism of glucose, fractose, or mannose to αGP results in the regeneration of intraglycosomal αGP and ADP (see Equation 3). Consequently, both αGP and ADP are recycled in the glycosome, and since glucose and NADH must be at least partially permeable to the glycosome (Table III), it is possible to study a major part of the glycolysis within intact glycosomes without the interference of a limiting membrane.

Compartmentation of glycolysis in vivo has been concluded from whole cell metabolite labeling (26) and is supported by subcellular fractionation studies (e.g. Ref. 1). The possibility that compartmentation of glycolytic intermediates can be studied in vitro is indicated by the observation that a 75% lower apparent steady state intermediate concentration is obtained for intact glycosomes than for either lysed glycosomes or cross-linked glycosomal enzymes (Fig. 6). It is also supported by the demonstrated high degree of inaccessibility of intraglycosomal ATP, Glu-6-P, and fructose 1,6-diphosphate produced during the reaction sequence to extraglycosomal competing enzymes.
ATP Synthesis in T. brucei Glycose

Despite this evidence for compartmentation of glucose metabolism to αGP by intact glycosomes using αGP plus ADP as phosphate donor, an intermediate concentration could not be detected (Fig. 6). This was eliminated by the addition of 10 units/ml muscle αGP dehydrogenase to the assay medium, e.g. Figs. 7 and 8, line A, for fructose. The effectiveness of this enzyme in reducing the intermediate concentration suggests that some DHAP must leave the glycosome before being reduced by NADH to αGP. The metabolism of mannose to DHAP also proceeded without a detectable lag time in the intact glycosome, Fig. 7, showing that all the reactions of this sequence must be contained within the glycosome, so confirming the glycosomal location of phosphomannose isomerase which is also suggested by the results of Table I.

Unlike the situation for fructose, glucose, and mannose, a lag time, latency, and a high sensitivity to fructose-1,6-diphosphatase were observed when Fru-6-P, Glu-6-P, and Man-6-P were the exogenous substrates for purified glycosomes in the presence of αGP, ADP, NADH, and 10 units/ml αGP dehydrogenase. These findings suggest the presence of a membrane barrier against the sugar phosphates. However, limited permeability of Fru-6-P and partial glycosomal compartmentation of fructose 1,6-diphosphate are implicated by the residual fructose-1,6-diphosphatase-insensitive NADH oxidation (Fig. 8).

Thus, compartmentation of ATP and some glycolytic intermediates can be demonstrated in isolated glycosomes, but some permeability to the intermediates could also be detected (Table II and Fig. 8). This permeability would account for the slow labeling of the putative cytosolic pool of glycolytic intermediates by [14C]glucose observed by Visser et al. (26) for whole cells.

In our assay system, glyceraldehyde must produce ATP from αGP and ADP for the phosphorylation of both hexose and Fru-6-P, since glyceraldehyde-3-phosphate dehydrogenase and, consequently, 3-phosphoglycerate kinase, the other ATP producing enzyme in the glycosome, are inhibited. However, under anaerobic conditions, glyceraldehyde kinase and 3-phosphoglycerate kinase are active, and so it is possible that a fraction of ATP produced from glyceraldehyde kinase is used to phosphorylate the intermediates. Experiments were, therefore, performed to establish whether the ATP produced from glyceraldehyde kinase in the cross-linked glycosomal enzyme complex was freely accessible to the assay medium, and thus freely available to exogenous enzymes, or whether it was channeled directly to either hexokinase or phosphofructokinase. We therefore cross-linked glycosomal enzymes with DMSI and, therefore, cross-linked glycosomal enzymes with DMSI and, consequently, 3-phosphoglycerate kinase, the other ATP producing enzyme in the cross-linked glycosomal enzyme complex was freely accessible to the assay medium.

REFERENCES