Effects of Oligomycin on Glucose Utilization and Calcium Transport in African Trypanosomes

By PETER G. G. MILLER† AND ROGER A. KLEIN*

Medical Research Council Biochemical Parasitology Unit, Molteno Institute, University of Cambridge, Downing Street, Cambridge CB2 3EE

(Received 29 May 1979; revised 21 June 1979)

Oligomycin (3 μg ml⁻¹) inhibited glucose utilization in *Trypanosoma brucei* S42 as shown by measurements of oxygen uptake and pyruvate production. Carbonyl cyanide 3-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation, did not relieve this inhibition, although some relief was afforded by the alternative substrate glycerol. Naturally dyskinetoplastic *Trypanosoma evansi* MIAG 105 was less sensitive to inhibition by oligomycin although glycerol relief was still observed, reflecting the differential sensitivity of the two pathways. With glucose present as the substrate, ⁴⁵Ca²⁺ transport was inhibited in *T. brucei*, but was stimulated in *T. evansi*. These results are discussed in terms of alternative systems for maintaining cytoplasmic Ca²⁺ concentrations in normal and dyskinetoplastic strains of trypanosome.

INTRODUCTION

Parasitic protozoa of the genus *Trypanosoma* cause important tropical diseases including sleeping sickness in man and nagana in livestock. They are characterized by possession of a complex, high molecular weight mitochondrial DNA localized in a specialized region of the mitochondrion, termed the kinetoplast. Members of the *T. brucei* complex undergo a cycle of mitochondrial repression and derepression related to the phase of the infection (Vickerman, 1965; Newton et al., 1973). In the midgut of the tsetse fly, the invertebrate vector, the mitochondrion becomes fully derepressed, mitochondrial oxidation of amino acids takes place and terminal oxidation is mediated by cytochromes. By contrast, in the vertebrate host the mitochondrion is acristate and devoid of cytochromes, the parasite being almost exclusively glycolytic. NAD generated by triose phosphate oxidation is oxidized via a glycerol 3-phosphate shuttle and a mitochondrial oxidase not coupled to ATP synthesis (Grant & Sargent, 1960; Opperdoes et al., 1977). The similarity between the mitochondrial development of trypanosomes and yeast includes the retention of an oligomycin-sensitive Mg²⁺-ATPase in the promitochondrion of the bloodstream form (Opperdoes et al., 1976b). Furthermore, certain strains of trypanosomes resemble petite mutants in being incapable of full mitochondrial derepression, having lesions in their mitochondrial DNA and lacking the putative mitochondrial gene product, the membrane sector of the Mg²⁺-ATPase (Opperdoes et al., 1976b). These strains, termed dyskinetoplastic, may be induced by mitochondrial mutagens such as the trypanocid eithidium bromide or may occur naturally, in which case the infection must be transmitted mechanically, in the case of *T. evansi* (a species closely related to *T. brucei*) on the insect mouthparts (Vickerman, 1977).

We sought to extend this work by studying the effects of oligomycin on living trypanosomes *in vitro* with regard to mitochondrial function, particularly respiration and calcium transport. A strain of *T. evansi* lacking a mitochondrial Mg²⁺-ATPase sensitive to oligomycin was included as a control.

† Present address: School of Biological Sciences, University of East Anglia, Norwich NR4 7TH.
METHODS

Organisms. Trypanosomes were grown in male CFY rats, harvested after 3 d by aortic puncture and purified by the method of Lanham (1968), using procedures previously described (Linstead et al., 1977).

Trypanosoma brucei S42 clone D10 has normal mitochondrial DNA, is transmissible by tsetse fly and can be cultured at 25 °C (Taylor, 1978). This strain was isolated by Dr J. R. Baker in 1966 from an adult female warthog in Tanzania and was obtained from the Nuffield Institute of Comparative Medicine, London, in 1969 (Taylor & Cross, 1977). Clone D10 was prepared at this Institute by Dr D. W. Taylor from the blood of an infected rabbit. The stabilates of this clone were all of intermediate morphology with a low incidence of frank short, stumpy forms.

Trypanosoma evansi MIAG 105 was isolated from a South American capybara and was cloned by Dr N. Van Meirvenne of the Prince Leopold Institute for Tropical Medicine, Antwerp, Belgium (from whom the strain was obtained) as ITMAS 301274A/AnTat3/Antademe IV. Stabilates were transferred to the Molteno Institute in 1975. This strain is monomorphic and relatively slow growing in laboratory rodents when compared with T. brucei S42. Kinetoplast DNA cannot be demonstrated using fluorescent intercalating dyes such as acridine or 4,6-diamidino-2-diphenylindole (Vickerman, 1977).

Pyruvate production. Pyruvate production was estimated using the lactate dehydrogenase method of Czok & Lamprecht (1974). Cells were incubated in a final volume of 1 ml at 25 °C with shaking, separated using a Quickfit Minifuge and the supernatant was assayed in a mixture containing 0.12 mM-NADH, 4.5 mM-EDTA and 0.45 M-triethanolamine hydrochloride, pH 7.6. The absorbance was read at 340 nm using a Quickfit Minifuge and the supernatant was assayed in a mixture containing 0.12 mM-NADH, 4.5 mM-EDTA and 0.45 M-triethanolamine hydrochloride, pH 7.6. The absorbance was read at 340 nm against air; 10 μg lactate dehydrogenase (Boehringer) was then added and the was read again after 2 min. The assay was linear up to 50 nmol pyruvate.

Oxygen uptake. Oxygen uptake was monitored with a Clark-type electrode (Estabrook, 1967). Measurements were made in a modified Krebs–Ringer phosphate buffer containing (mm): D-glucose, 20; KCl, 5; NaCl, 80; MgSO₄, 1; Na₂HPO₄, 20; NaH₂PO₄, 2; adjusted to pH 7.4.

Calcium transport. The assay was linear up to 50 nmol pyruvate.

RESULTS AND DISCUSSION

Oligomycin (above 3 μg ml⁻¹) inhibited O₂ uptake and pyruvate production by T. brucei (Fig. 1 and 2, Table 1), leading to cell death as observed microscopically. Carbonyl cyanide 3-chlorophenylhydrazone did not relieve this inhibition and was itself inhibitory (Table 2). Some relief from oligomycin inhibition was obtained when glycerol, an alternative energy source, was present (Fig. 1). Salicylhydroxamic acid, an inhibitor of the terminal oxidase, did not prevent oligomycin inhibition and ouabain, which inhibits the Na⁺/K⁺-ATPase, was less sensitive to oligomycin (Fig. 2), although inhibition was again relieved by glycerol (results not shown).

Transport of ⁴⁰Ca²⁺ was negligible at 4 °C and the background incorporation at this temperature was used to correct for the passive carry-over of label (Fig. 3). Mean values for the rates of transport observed at 25 °C were 1.6 and 0.4 nmol Ca²⁺ (5 × 10⁶ cells⁻¹) (10 min⁻¹) for T. brucei and T. evansi, respectively. The calcium ionophore A23187 greatly enhanced transport (Fig. 3), while the substitution of glycerol for glucose as energy substrate halved the rate (Table 2). Under these conditions oligomycin decreased ⁴⁰Ca²⁺ transport in the presence of glycerol to near background rates although the trypanosomes remained motile. With glucose as substrate, oligomycin inhibited ⁴⁰Ca²⁺ transport in the
Effects of oligomycin on trypanosomes

Fig. 1. Effect of oligomycin on the respiration of *Trypanosoma brucei* S42 D10. The polarographic traces were obtained from $2 \times 10^7$ trypanosomes maintained at 37 °C in 1.5 ml modified Krebs-Ringer phosphate buffer containing 20 mM-glucose. Additions (5 μl) were made at the times indicated by the arrows: Oligo, oligomycin (3-3 μg ml$^{-1}$); Gly, glycerol (5 mM); CCCP, carbonyl cyanide 3-chlorophenylhydrazone (16 μM).

Fig. 2. Effects of oligomycin on $^{45}$Ca$^{2+}$ uptake (○) and pyruvate production (●) by (a) *Trypanosoma brucei* S42 D10 and (b) *T. evansi* MIAG 105. Control rates were the same as those given in Table 2. Reaction mixtures contained Tris/HCl buffer, pH 7.4, and 20 mM-glucose at 25 °C.

*T. brucei* strain whereas in the *T. evansi* strain a stimulation was observed (Fig. 2). Carbonyl cyanide 3-chlorophenylhydrazone and 2-deoxyglucose had effects similar to oligomycin (Table 2).

Oligomycin is widely regarded as a specific inhibitor of the energy-transducing mitochondrial Mg$^{2+}$-ATPase (Slater, 1967). Our results suggest, however, that this is not the principal site resulting in an inhibition of glycolysis in the bloodstream form of *T. brucei*. Unlike other systems the inhibition cannot be reversed by an uncoupler and it occurs in a strain lacking this enzyme. Moreover, the alternative terminal oxidase of bloodstream forms is probably not coupled to ATP synthesis and thus to the Mg$^{2+}$-ATPase (Grant & Sargent, 1960). Oligomycin is also known to inhibit the ouabain-sensitive plasma membrane Na$^+$/K$^+$-ATPase but glycolysis in *T. brucei* is insensitive to ouabain. The ability of glycerol to support respiration in the presence of oligomycin suggests that the function affected is not
Table 1. Effect of oligomycin on pyruvate production by Trypanosoma brucei S42

Incubations were done in a modified Krebs–Ringer phosphate buffer as described in Methods, in the absence or presence of oligomycin (5 μg ml⁻¹). Results show the mean values for four replicates ± standard deviations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate production [μmol (5 x 10⁸ cells⁻¹ (30 min)⁻¹]</th>
<th>Percentage of control rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (20 mM)</td>
<td>3·06±0·17</td>
<td>-0·02±0·05 -0·7±1·7</td>
</tr>
<tr>
<td>+ Oligomycin</td>
<td>1·78±0·03</td>
<td>0·79±0·11 44±6</td>
</tr>
<tr>
<td>Glycerol (20 mM)</td>
<td>2·30±0·18</td>
<td>1·07±0·18 47±8</td>
</tr>
<tr>
<td>Glucose (10 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ glycerol (10 mM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of substrates and inhibitors on the rates of pyruvate production and ⁴⁵Ca²⁺ uptake by Trypanosoma brucei S42 D10 and T. evansi MIAG 105

Results show the mean values of a number of separate experiments, with the range and number of experiments in parentheses. Control rates of ⁴⁵Ca²⁺ uptake were 1·6 and 0·4 nmol Ca²⁺ (5 x 10⁸ cells⁻¹ (10 min)⁻¹ for T. brucei S42 D10 and T. evansi MIAG 105, respectively. Control rates of pyruvate production were 0·55 and 0·52 μmol pyruvate (5 x 10⁸ cells⁻¹ (10 min)⁻¹ for T. brucei S42 D10 and T. evansi MIAG 105, respectively.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>⁴⁵Ca²⁺ uptake (nmol (5 x 10⁸ cells⁻¹ (10 min)⁻¹)</th>
<th>Pyruvate production (μmol (5 x 10⁸ cells⁻¹ (30 min)⁻¹)]</th>
<th>⁴⁵Ca²⁺ uptake (nmol (5 x 10⁸ cells⁻¹ (10 min)⁻¹)</th>
<th>Pyruvate production (μmol (5 x 10⁸ cells⁻¹ (30 min)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (20 mM)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glucose (20 mM) +</td>
<td>67·4</td>
<td>63</td>
<td>260·3</td>
<td>85</td>
</tr>
<tr>
<td>CCCP (5 μM)</td>
<td>(62·5–71·2, 2)</td>
<td>(61–65, 2)</td>
<td>(206–289, 3)</td>
<td>(83–87, 2)</td>
</tr>
<tr>
<td>Glucose (20 mM) +</td>
<td>53·8</td>
<td>90</td>
<td>317·2</td>
<td>92</td>
</tr>
<tr>
<td>2-deoxyglucose (10</td>
<td>(45·9–61·7, 2)</td>
<td>(89–91, 2)</td>
<td>(250–360, 3)</td>
<td>(83–100, 2)</td>
</tr>
<tr>
<td>Glucose (20 mM)</td>
<td>48·7</td>
<td>*</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>+ oligomycin</td>
<td>(28·0–64·6, 3)</td>
<td></td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Glycerol (20 mM)</td>
<td>2·1</td>
<td>*</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>(5 μg ml⁻¹)</td>
<td>(–13·0–+11·1, 3)</td>
<td></td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

⁴⁵Ca²⁺ uptake or pyruvate production (% of control rate)

essential and that the inhibition occurs primarily prior to triose phosphate oxidation. The decreased rate of glycolysis and the lower ATP concentrations observed with glycerol are not sufficient to explain our results (Opperdoes et al., 1976a). Similar conditions pertain when terminal oxidation is inhibited by salicylhydroxamic acid, although this does not protect the cell from oligomycin. The rapid trypanocidal effect of oligomycin makes its site of action worthy of further study.

The effect of oligomycin on ⁴⁵Ca²⁺ transport indicates that it also inhibits the mitochondrial Mg²⁺-ATPase. In many systems Ca²⁺ enters the cell down a concentration gradient and is then transferred from the cytoplasm into the mitochondrion at the expense of ATP hydrolysis by the oligomycin-sensitive Mg²⁺-ATPase (Bygrave, 1978). Ca²⁺ transport in T. brucei appears to be sensitive to ATP concentrations as glycerol reduces the rate of transport by half, probably reflecting a similar decrease in ATP concentration. Addition of oligomycin under these conditions inhibits Ca²⁺ transport completely although the trypanosomes remain viable. Furthermore, inhibition of transport is more pronounced cedthan
effects of oligomycin on trypanosomes

Fig. 3. Effect of temperature and the calcium ionophore A23187 on \(^{45}\)Ca\(^{2+}\) uptake in *Trypanosoma brucei* S42 D10. Incubations were carried out in Tris/HCl buffer, pH 7.4, containing 20 mM-glucose at 4 °C (■) or 25 °C (○), or at 25 °C with A23187 (4 µg ml\(^{-1}\)) (●).

inhibition of glucose utilization, suggesting that the mitochondrial Mg\(^{2+}\)-ATPase is affected rather than the production of ATP by glycolysis.

By contrast, oligomycin appears to stimulate Ca\(^{2+}\) transport in the dyskinetoplastic *T. evansi*. This may be due to the use of an alternative system for removing cytoplasmic Ca\(^{2+}\) in the absence of the mitochondrial Mg\(^{2+}\)-ATPase. One possibility is the use of a plasma membrane calcium pump requiring ATP to expel Ca\(^{2+}\) from the cell. Inhibition of glycolysis by oligomycin, carbonyl cyanide 3-chlorophenylhydrazone or 2-deoxyglucose would starve the enzyme of ATP, block Ca\(^{2+}\) efflux and thus enhance the net \(^{45}\)Ca\(^{2+}\) influx. An analogous effect occurs in erythrocytes where inhibition of glycolysis and depletion of ATP by iodoacetamide and inosine is used to load the cell with \(^{45}\)Ca\(^{2+}\) (Lew, 1971). Efflux in this case is mediated by a plasma membrane Ca\(^{2+}\)-ATPase. Such an activity has been reported in *T. cruzi* and can be detected in crude sonicates of *T. brucei* (Frasch et al., 1978; P. G. G. Miller, unpublished observations). The ATPase activity is not sensitive to oligomycin and no function can yet be assigned to it.

Our work provides experimental confirmation of earlier speculation that the mitochondrial Mg\(^{2+}\)-ATPase in the bloodstream form of *T. brucei* mediates ion transport including the regulation of cytoplasmic Ca\(^{2+}\) concentrations. Mitochondrial sequestration of Ca\(^{2+}\) may be especially important in trypanosomes as they are completely dependent upon exogenous carbohydrate for their high glycolytic rates, and may thus be particularly sensitive to interference by Ca\(^{2+}\) with glycolytic kinases in the cytosol (Bygrave, 1978). The regulation of Ca\(^{2+}\) levels may also be critical in the control of flagellar reversal as in the non-pathogenic trypanosomatid *Crithidia oncopelti* (Holwill & McGregor, 1975). Survival of dyskinetoplastic trypanosomes lacking the mitochondrial Mg\(^{2+}\)-ATPase can occur only in the bloodstream form where this enzyme is not required for ATP synthesis and its role in calcium regulation can be assumed by a plasma membrane pump. The availability of such mutants makes this system potentially rewarding for the study of intracellular calcium regulation.

P.G.G.M. was in receipt of an MRC Research Studentship.

REFERENCES


