have found that carbon dioxide increases the C_s value by up to 50%. We have not yet sufficient results to publish but they do lend support to the concept of an active intracellular transport of oxygen.

SUMMARY

1. The validity of Warburg's equation for diffusion of oxygen through tissue slices is discussed.

2. A method of studying the respiration of tissue slices is described which can be used to measure both their Q_{O_2} and their critical or limiting oxygen concentration.

3. The results obtained with this method support the validity of Warburg's equation.

4. The equation is used to calculate values for the diffusion coefficient of oxygen through respiring liver, heart and kidney slices, and values for the minimum concentration of oxygen necessary to saturate a terminal oxidase in those tissues.

5. Unexpectedly high values for the diffusion coefficients and possibly for the critical oxygen concentration of the terminal oxidase were obtained. Their significance is discussed.

We are indebted to Professor A. Haddow, F.R.S., of the Chester Beatty Research Institute for the supply of rats and to Dr P. Armitage of the School of Hygiene and Tropical Medicine for advice and assistance with the statistical examination of the results.

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Properties of L-α-Glycerophosphate Oxidase and its Role in the Respiration of *Trypanosoma rhodesiense*

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An aspect of host–parasite relationships of significance in chemotherapy concerns the biochemical differences which exist between pathogenic trypanosomes and the tissues of their vertebrate and invertebrate hosts. One important difference is that the blood-stream forms of many trypanosomes do not contain detectable cytochrome pigments (Brand, 1951; Ryley, 1956; Fulton & Spooner, 1959). Despite this, such parasites are notable for their high rates of respiration; for example, it has been calculated that the Q_{O_2} (glucose) of the African trypanosome, *Trypanosoma rhodesiense*, is about 50 times that of the tissues of its mammalian host (Brand, 1951).

The present work deals with hydrogen-transport systems present in a strain of the human parasite, *T. rhodesiense*, which has been maintained by syringe-passage in rats for many years. As far as is known the metabolism of this strain does not differ from that of the parasite freshly isolated from a human patient (Grant & Fulton, 1957; Fulton & Spooner, 1959). The results are mainly concerned with the properties of a L-α-glycerophosphate oxidase whose reaction with oxygen does not appear to be mediated either by pyridine nucleotides or a cytochrome system. The high activity and substrate specificity of this oxidase indicates that it can account for most of the respiration of the intact trypanosome *in vitro*.

A brief account of some of these results has already been presented (Grant & Sargent, 1959).

EXPERIMENTAL

Organism. The 'Liverpool' strain of *T. rhodesiense*, its maintenance in rats and the isolation of the parasite from infected rat blood have been described previously (Grant & Fulton, 1957).

Preparation of cell-free extracts. All procedures were carried out at 2°. A suspension of trypanosomes in iso-osmotic phosphate-buffered medium (Krebs & Eggleston, 1940) containing 0·2% of glucose was centrifuged at 1000 *g* for 10 min. After removal of the supernatant, the sedimented material was used to obtain cell-free preparations as described below.

The sedimented parasites (1 vol.) were suspended in water (4 vol.) with the aid of an all-glass homogenizer of the Potter–Elvehjem type (Loughborough Glass Co., Loughborough). After standing for 30 min. the suspension was centrifuged at 1000 *g* for 10 min. The supernatant, which was optically clear, was removed and the residue, which contained about 90% of the total N originally present in the starting material, was resuspended in water (6·5 vol.) and centrifuged at 1000 *g* for 10 min. The supernatant was removed and the residue resuspended in water as before. This final suspension contained about 1 mg. of N/ml. and will be referred to as the standard 'water-lysed' preparation.

The sedimented parasites (1 vol.) were suspended in 0·25 M-sucrose (4 vol.) containing 5 mM-ethylenediaminetetra-acetic acid (EDTA) previously adjusted to pH 7·4 by the addition of 0·1 N-NaOH. The suspension was then exposed to ultrasonic vibration for 60 sec. (MSE–Mullard ultrasonic disintegrator, stainless-steel vibrator probe with end-ratio 9:1; Measuring and Scientific Equipment Ltd., London). After being centrifuged at 1000 *g* for 10 min. the supernatant was removed and the slight residue discarded. This supernatant contained about 1·2 mg. of N/ml. and will be referred to as the 'ultrasonic' preparation.

Comparison of the oxygen utilization by cell-free preparations with that of intact parasites. In a typical experiment, 10 ml. portions of a trypanosome suspension were centrifuged at 1000 *g* for 10 min. and the supernatants discarded. The sedimented parasites in each tube (about 0·10 ml.) were then resuspended after the addition of one of the following: (*a*) phosphate-buffered medium (14 ml.) containing plasma albumin (140 mg.) and glucose (154 μmoles); (*b*) the same solution except that glucose was replaced by glycerol (216 μmoles); (*c*) water (2 ml.). After standing for 30 min. the three suspensions were centrifuged at 1000 *g* for 10 min. and the supernatants discarded. The 'water-lysed' preparation was resuspended in phosphate-buffered medium (14 ml.) containing plasma albumin (140 mg.) and the sodium salt of L-α-glycerophosphate (224 μmoles). The intact trypanosomes were resuspended in fresh medium (*a*) or (*b*) as described above and the oxygen uptake of each suspension (3 ml. samples) was determined.

Oxygen uptake. Oxygen consumption was measured in a conventional Warburg apparatus at 37° in air. The fluid volume added to each flask was 2·9 ml., except in the one experiment mentioned above. In all cases flasks were temperature-equilibrated for 15 min. before the start of the experiment. Except where otherwise noted, the reaction was started by tipping the substrate from the side arm. Cell-free extracts did not produce detectable CO_2 and, accordingly, KOH was not added to the centre well in these experiments.

Analytical methods. Total N was determined by the micro-Kjeldahl method, the NH_3 being distilled into a boric acid solution containing methyl red–methylene blue indicator.

Total phosphorus was determined as orthophosphate, after digestion with perchloric acid (sp.gr. 1·70) at 160°, by the method of Berenblum & Chain (1938) with the micro-modification of Long (1943).

α-Glycerophosphate (α-GP) was determined as orthophosphate after periodate oxidation followed by acid hydrolysis (Burmaster, 1946).

Total triose phosphate was determined as alkali-labile phosphate (Meyerhof & Lohmann, 1934).

In one group of experiments (Table 3), triose phosphate and α-GP were determined in the same sample. The reaction mixture was deproteinized by the addition of trichloroacetic acid to a final concentration of 5% (w/v) and the filtrate was extracted four times with an equal volume of freshly distilled diethyl ether to remove trichloroacetic acid. Triose phosphate was determined as the increase in orthophosphate observed in the residual solution after treatment with N-NaOH at 23° (alkali-labile phosphate). Under these conditions, it is possible that β-glycerophosphate (β-GP) was formed from the α-isomer originally present (cf. Long & Maguire, 1953), so that the total glycerophosphate in this solution was determined as the further increase in orthophosphate obtained by treatment of the acidified solution with periodic acid at 100° (Burmaster, 1946).

Individual triose phosphate esters were identified by enzymic methods with glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase. The procedure was essentially that described by Racker (1957)

Extinction changes in reactions involving diphosphopyridine nucleotide (DPN^+) were studied in a 1 $cm.^2$ quartz cell at 340 mμ in the Hilger Uvispek spectrophotometer.

Materials. L-α-GP was synthesized as described by Baer (1952) except that quinoline present during the phosphorylation of *iso*propylidene-D-glycerol had to be removed by extraction with diethyl ether before the barium salt of L-α-GP would crystallize from hot water. DL-α-GP was synthesized as described by King & Pyman (1914) and isolated as the barium salt. The sodium salts of these compounds were used in all experiments and were obtained by the addition of a slight excess of sodium sulphate to a solution of the barium salt. After removal of the precipitate by centrifuging, the supernatant was neutralized and stored at −20° for not longer than 1 month before use. In these solutions, 93–96% of the total phosphate present could be accounted for as α-GP.

Triose phosphate isomerase was prepared from rat muscle as described by Beisenherz (1957).

The following materials were obtained commercially: pyridine nucleotides [DPN^+, reduced diphosphopyridine nucleotide (DPNH), triphosphopyridine nucleotide (TPN^+), reduced triphosphopyridine nucleotide (TPNH)], 2-amino-2-hydroxymethylpropane-1:3-diol (tris, recrystallized), mammalian cytochrome *c*, crystalline catalase, crystalline glyceraldehyde 3-phosphate dehydrogenase, from Boehringer und Söhne, Mannheim, Germany; adenosine di- and tri-phosphate (ADP, ATP) from the Sigma Chemical Co., St Louis, Mo., U.S.A.; bovine-plasma albumin fraction V from The Armour Laboratories, Sussex; Amytal (the sodium salt of 5-ethyl-5-*iso*amylbarbituric acid) from Eli Lilly and Co. Ltd., Basingstoke; mapharside (3-amino-4-hydroxyphenylarsenoxide hydrochloride) from Parke Davis and Co. Ltd., London.

The addition compound formed with dioxan and DL-glyceraldehyde-1-bromo-3-phosphoric acid, and the *cyclo*hexylamine salt of the dimethyl ketal of dihydroxyacetone

phosphate were obtained from the Nutritional Biochemical Corp., Calif., U.S.A. DL-Glyceraldehyde 3-phosphate was obtained by water hydrolysis of the former compound (Lardy, 1957) and from the latter dihydroxyacetone phosphate was prepared as described by Ballou & Fischer (1956). Crystalline yeast hexokinase and DL-*iso*citric acid lactone were obtained from L. Light and Co. Ltd., London. DL-*iso*Citric acid was prepared from the lactone as described by Deutsch & Phillips (1957).

Antimycin A was a gift from Dr E. F. Hartree and 2-heptyl-4-hydroxyquinoline *N*-oxide a gift from Dr J. W. Lightbown.

RESULTS

Cell-free preparations obtained from *T. rhodesiense* had a very limited ability to oxidize glucose, intermediates of the tricarboxylic acid cycle and substrate amounts of DPNH and TPNH. In contrast α-GP was rapidly oxidized at a constant rate over a period of 20–25 min., under the conditions given in Table 1. The racemic form was oxidized at half the rate of the L-isomer and in subsequent experiments it was shown that the latter is completely oxidized to dihydroxyacetone phosphate (Table 3 and Fig. 3). Neither glycerol nor β-GP was oxidized to an appreciable extent so that the preparation contains an α-GP oxidase which is stereochemically specific for the L-isomer.

Various means of disrupting the trypanosomes were tried, including the use of ultrasonic vibration, crushing in a press at −20° (Hughes, 1951) and freeze-drying, but the most active preparation was the particulate material obtained after lysis of the parasites in water. For this reason the 'water-lysed' preparation was subsequently used to determine some properties of the oxidase system, and was freshly prepared for each experiment, as some 70% of the original activity was lost after 24 hr. at 2°.

Kinetic aspects of the L-α-*glycerophosphate oxidase.* The effects of pH, enzyme and substrate concentration have been briefly investigated. These results are of interest not only as they help to characterize the system but also because they define the optimum conditions for the assay procedure.

To ensure that the pH of the medium was not affected by the buffering action of L-α-GP at neutral pH values, a high ratio of buffer to substrate was used and at the end of each experiment no appreciable change in pH had occurred, as determined by the glass electrode. It can be seen (Fig. 1) that in phosphate buffer there is an optimum activity at about pH 7·4, an activity of the same order being also found in tris buffer at this pH. For this reason, subsequent assays of enzyme activity were carried out in one or other of these buffers at pH 7·4, although it may be noted that the activity in tris buffer is slightly greater at higher pH values, with an optimum at about pH 8.

The activity of the enzyme with increasing substrate concentration is shown in Fig. 2. From these results the K_m was calculated from the reciprocal-plot method of Lineweaver & Burk (1934) to be 1·3 mM.

Oxidase activity under the experimental conditions given in Table 1 was found to be proportional to enzyme concentration up to 0·6 mg. of trypanosome N/flask.

Influence of various substances on the activity of L-α-*glycerophosphate oxidase. T. rhodesiense* contains no detectable catalase (Fulton & Spooner, 1956) and the addition of catalase had no effect on

Table 1. *Effect of various substances on the uptake of oxygen by cell-free preparation of* Trypanosoma rhodesiense

Each flask contained cell-free preparation (0·5 ml., approx. 0·5 mg. of N), tris buffer, pH 7·4 (0·14 M), and the additions as stated below, in a total volume of 2·9 ml. Oxygen uptakes were measured for 45 min., except with α-GP, when readings were discontinued after 25 min. —, Signifies value not determined.

Additions	Final concn. (mM)	Uptake of O_2 (μl./min./mg. of N)	
		Standard 'water-lysed' preparation	'Ultrasonic' preparation
Nil	—	0	0
DL-α-GP	5·5	3·7	—
L-α-GP	5·5	7·6	7·0
L-α-GP and DPN+	5·5	7·1	—
Glycerol and ATP	6·2	0·9	—
DPNH or TPNH	7·6	0·5	0·5
Glycerol	6·2	<0·5	<0·5
Glucose, ATP, and DPN+	6·9		
Fructose 1:6-diphosphate	5·5		
Pyruvate	7·2		
Succinate	6·2		
DL-*iso*Citrate	9·0		

the activity of the enzyme (Table 2). Hydrogen peroxide, which is highly toxic to the enzyme, is thus unlikely to be a product, a view supported by the stoicheiometry of the reaction (Table 3).

The oxidase was markedly affected by *p*-chloromercuribenzoate, hydrogen peroxide and the trypanocidal drug, mapharside, which would indicate that one or more free thiol groups are essential for enzyme activity. In contrast, neither iodoacetic acid nor iodoacetamide was effective, although both of these compounds inhibit the oxygen uptake of intact trypanosomes (Brand, Tobie & Mehlman, 1950). This apparent anomaly will be referred to subsequently.

The oxidase was not significantly affected by compounds which normally inhibit the cytochrome systems of mammalian tissues (cf. Chance, 1957), an observation in agreement with the fact that *T. rhodesiense* does not contain detectable cytochrome pigments. The effect of azide was tested at pH 6·5

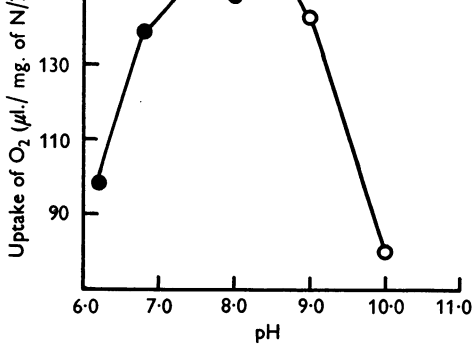

Fig. 1. Effect of pH on the activity of L-α-GP oxidase. Each flask contained the standard 'water-lysed' preparation (0·5 ml., approx. 0·5 mg. of N), buffer (0·14 M) and L-α-GP (5·5 mM), in a total volume of 2·9 ml. pH of flask contents was determined by glass electrode at about 20°. ●, Phosphate buffer; ○, tris buffer.

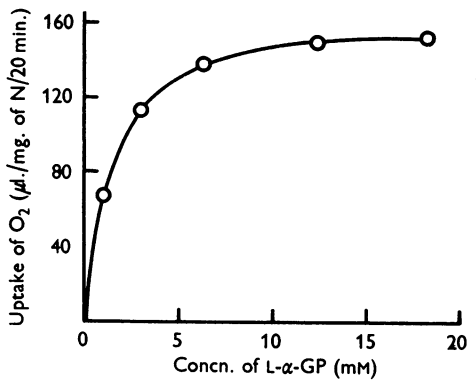

Fig. 2. Effect of substrate concentration on activity of L-α-GP oxidase. Each flask contained 'water-lysed' preparation (0·5 ml., 0·24 mg. of N), tris buffer, pH 7·4 (0·14 M), and L-α-GP, in a total volume of 2·9 ml.

Table 2. *Effect of various substances on the activity of* L-α-*glycerophosphate oxidase*

Each flask contained 'water-lysed' preparation (0·5 ml., approx., 0·5 mg. of N), tris buffer, pH 7·4 (0·14 M), and L-α-GP (5·5 mM) and the substance under test (0·1 ml.) in a total volume of 2·9 ml. All substances were in aqueous solution, except *p*-chloromercuribenzoate and 2-heptyl-4-hydroxyquinoline *N*-oxide, which were dissolved in 0·05 N-NaOH, and antimycin A, which was dissolved in aqueous 50% (v/v) ethanol. After equilibration for 15 min. the reaction was started by the addition of L-α-GP from the side arm. Oxidase activities are expressed as µl. of O_2/ 0·5 mg. of N in 25 min.

Additions	Final concn. (mM)	Oxidase activity
Nil	0	100
Hydrogen peroxide	1·0	0
p-Chloromercuribenzoate	0·1	2
N-Ethyl maleimide	1·0	8
Mapharside	0·1	41
Iodoacetate*	1·0	100
Iodoacetamide*	1·0	96
NaCN	10	96
NaN_3†	10	94
NaF	1·0	100
Amytal	1·0	95
Antimycin A	5 µg./flask	98
2-Heptyl-4-hydroxyquinoline *N*-oxide	10 µg./flask	93
Cytochrome *c*	1 mg./flask	100
Catalase	0·0005	100
EDTA	1·0	110
Plasma albumin	29 mg./flask	115

* Either 15 or 30 min. equilibration with enzyme before assay.

† Assayed in both tris buffer, pH 7·4, and phosphate buffer, pH 6·5.

Table 3. *Quantitative relationship between substrate and product of the* L-α-*glycerophosphate-oxidase reaction*

Each flask contained water-lysed preparation (0·5 ml., 0·3 mg. of N in Expts. 1 and 2, and 0·5 mg. of N in Expt. 3), tris buffer, pH 7·4 (0·14 M), KCN (10 mM) and L-α-GP (15·5 µmoles), in a total volume of 2·9 ml. In Expts. 1 and 2 the reaction was stopped, after 18 min., by rapid cooling to 0° with the simultaneous addition of 0·5 ml. of 30% (w/v) trichloroacetic acid. In Expt. 3 the reaction was stopped in a similar manner after 25 min. —, Signifies value not determined.

Expt. no.	L-α-GP used (µmoles)	Uptake of O_2 (µg.atoms)	Triose phosphate formed (µmoles)
1	8·6	9·0	8·2
2	7·9	8·5	7·7
3	—	14·6	14·2

as well as at pH 7·4 since it has been stated (Keilin, 1936) that azide does not significantly affect cytochrome systems at neutral or slightly alkaline pH values.

Stoicheiometric relationships of the L-α-glycerophosphate-oxidase reaction. To determine the quantitative relationship between reactants and products, enzyme and enzyme–substrate suspensions were analysed at the beginning and end of each experiment. The net decrease in the amount of L-α-GP present and the corresponding increase in triose phosphate for each experiment are given in Table 3. It can be seen that when nearly all or only half of the substrate has been utilized the results are consistent with the reaction:

$$\text{L-α-GP} + \tfrac{1}{2}O_2 \rightarrow \text{triose phosphate} + H_2O. \quad (1)$$

The equilibrium condition of the reaction clearly favours the oxidation of L-α-GP, although cyanide which was added to stabilize triose phosphate as the cyanohydrin may have affected the equilibrium. This latter possibility is, however, unlikely. since the complete oxidation of L-α-GP in accordance with reaction (1) has been consistently observed in the absence of cyanide (Fig. 5).

It was found (Fig. 3) that dihydroxyacetone phosphate was the only triose phosphate ester present in the protein-free reaction mixture at the end of each experiment. The oxidase preparation itself did not contain detectable triose phosphate isomerase since it did not catalyse the reduction of DPN^+ on the addition of glyceraldehyde 3-phosphate dehydrogenase and synthetic dihydroxyacetone phosphate, as judged from the extinction of the solution at 340 mμ. These results show that the product of the oxidase reaction is dihydroxyacetone phosphate and not glyceraldehyde 3-phosphate.

In order to determine whether oxidation of L-α-GP was coupled to phosphorylation, the following additions were made to each of the reaction mixtures containing L-α-GP that were described in Table 1: glucose (1·7 mM), ATP (1·0 mM), hexokinase (152 units, Berger, Slein, Colowick & Cori, 1946), magnesium chloride (3·4 mM) and orthophosphate (9·5 mM). In no case, however, was there any significant decrease in the concentration of orthophosphate during the experimental period.

Enzyme and enzyme sequences of the Embden–Meyerhof glycolytic pathway present in T. rhodesiense. The supernatant obtained by lysis of *T. rhodesiense* with water gave no significant oxygen uptake with L-α-GP or substrate amounts of DPNH. Nevertheless, added DPNH was rapidly oxidized, as judged by the decrease in extinction of the solution at 340 mμ (Fig. 4). This effect was abolished by dialysis, but the activity could be restored by the addition of dihydroxyacetone phosphate, indicating the presence of a soluble

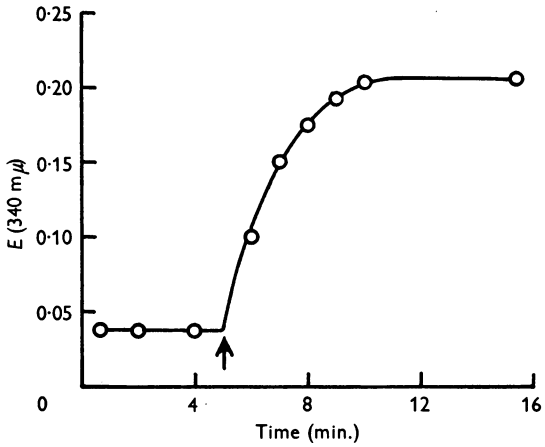

Fig. 3. Dihydroxyacetone phosphate as the product of L-α-GP-oxidase reaction. Cuvettes contained protein-free incubation medium from experiments described in Table 3, pyrophosphate buffer, pH 8·6 (120 μmoles), sodium arsenate (15 μmoles), DPN^+ (0·7 μmole) and crystalline glyceraldehyde 3-phosphate dehydrogenase (0·1 mg.). Triose phosphate isomerase (2 units, Beisenherz, 1957) was added as shown by the arrow. Total volume, 2·9 ml. Control cuvette: protein-free incubation medium omitted.

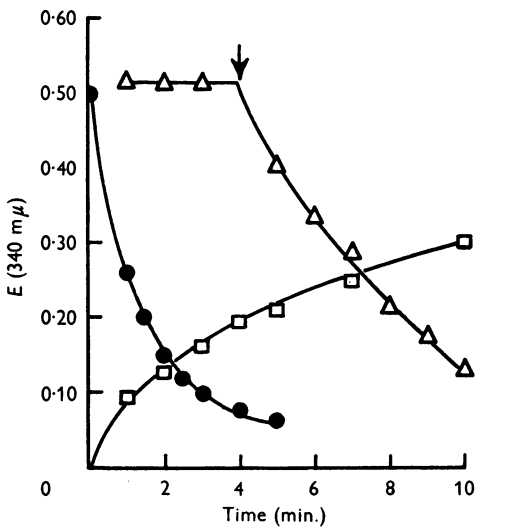

Fig. 4. L-α-GP-dehydrogenase activity in cell-free extracts of *T. rhodesiense*. Cuvettes contained 2·7 ml. of reaction mixture. ●, Phosphate buffer, pH 7·4 (200 μmoles), $MgCl_2$ (10 μmoles) and DPNH (0·3 μmole). The reaction was started by addition of the supernatant from a 'water-lysed' preparation (0·2 ml., 0·1 mg. of N). △, Mixture as given above except that the supernatant had been dialysed. Dihydroxyacetone phosphate (1 μmole) was added at the time shown by the arrow. □, Hydrazine–HCl buffer, pH 9·2 (1 m-mole), $MgCl_2$ (10 μmoles), L-α-GP (16 μmoles), DPN^+ (0·5 μmole) and dialysed supernatant. Control cuvette: pyridine nucleotide omitted.

DPN-linked L-α-GP dehydrogenase. Reduction of DPN^+ in the presence of L-α-GP could be achieved only at a higher pH and in the presence of hydrazine to trap the triose phosphate. The equilibrium of the reaction thus favours the formation of L-α-GP from dihydroxyacetone phosphate (cf. Baranowski, 1949). This enzyme, like the particulate L-α-GP oxidase, was inhibited by *p*-chloromercuribenzoate (0·1 mM), but not by iodoacetate (1·0 mM). Aldolase and triose phosphate isomerase were shown to be present in the supernatant by the oxidation of DPNH in the presence of fructose 1:6-diphosphate or glyceraldehyde 3-phosphate. Triose phosphate isomerase was insensitive to iodoacetate (1·0 mM). Glyceraldehyde 3-phosphate dehydrogenase could not be detected by the usual spectrophotometric method, presumably because DPNH formed in the reaction was immediately reoxidized by the very active DPN-linked L-α-GP dehydrogenase. This explanation is supported by the following experiment, the results of which are given below.

Trypanosomes were lysed in water and the complete cell-free suspension was dialysed. The non-diffusible suspension utilized added L-α-GP with an uptake of 1 g.atom of oxygen/mole of L-α-GP, the oxygen uptake being doubled when DPN^+ and L-α-GP were added together (Fig. 5). This latter result is in contrast with the lack of stimulation of added DPN^+ on L-α-GP oxidation by the washed particulate preparation (Table 1). It is thus probable that triose phosphate formed in the oxidation of L-α-GP (reaction 1) reduces DPN^+ in the glyceraldehyde 3-phosphate-dehydrogenase reaction (reaction 6 below) and DPNH thus formed is reoxidized by the soluble L-α-GP dehydrogenase with the formation of L-α-GP from triose phosphate (reaction 3 below). The overall result of these coupled reactions would be that found experimentally, i.e. 1 mole of oxygen consumed/mole of L-α-GP oxidized. This interpretation is supported by the finding that iodoacetate decreases the uptake of oxygen to approximately that found for the oxidase reaction alone (Fig. 5). In the present results the triose phosphate isomerase and the L-α-GP oxidase and dehydrogenase of *T. rhodesiense* have all been shown to be insensitive to iodoacetate, whereas it is known that glyceraldehyde 3-phosphate dehydrogenases of mammalian tissues, yeasts and trypanosomes closely related to *T. rhodesiense* (Marshall, 1948; Harvey, 1949) are all strongly inhibited by iodoacetate.

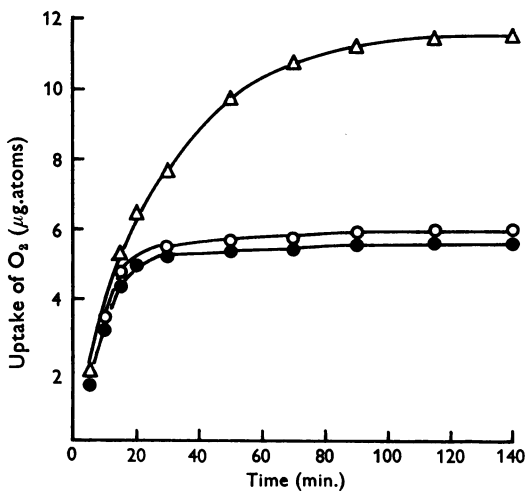

Fig. 5. Oxidation of L-α-GP by dialysed cell-free suspension of *T. rhodesiense*. Trypanosomes were lysed in water and the complete suspension was dialysed for 6 hr. at 2° against a mixture of 50 vol. of phosphate-buffered medium (Krebs & Eggleston, 1940) and 450 vol. of water. ●, Flasks contained non-diffusible suspension (1 mg. of N), phosphate-buffered medium, pH 7·4 (2 ml.), plasma albumin (29 mg.), reduced glutathione (0·15 μmole) and L-α-GP (5·6 μmoles), in a total volume of 2·9 ml. △, System, as described above, DPN^+ (1 μmole) and NaF (2·9 μmoles); ○, as described above, DPN^+ (1 μmole) and iodoacetate (2·9 μmoles). Oxygen uptake was negligible on omission of L-α-GP.

Relationship between the activity of L-α-glycerophosphate oxidase and the respiration of the intact trypanosome. In a series of four experiments, the rate of oxygen uptake of intact trypanosomes has been directly compared with the activity of the oxidase isolated from an equivalent number of parasites. Maximum initial rates were obtained by the use of substrate concentrations so high that substrates did not become rate-limiting over the experimental period (see Fig. 2). A typical result is given in Fig. 6, where it can be seen that the oxygen uptake of the preparation was about 60 and 110 % of that of the intact trypanosome utilizing glycerol and glucose respectively. This would suggest that the rate of conversion of glycerol into L-α-GP is faster than that of glucose. Indeed under these conditions only trace amounts of L-α-GP were detectable in the trypanosome when glucose was used as substrate. In contrast, the parasite respiring in glycerol contained 2·1 μmoles of α-GP/mg. of trypanosome N. On the assumption that there is only one hydrogen-transport system in *T. rhodesiense*, the oxidase must be a rate-limiting step in the metabolism of glycerol and must function at near its maximum activity. It is thus probable that the correspondence between the respiration rate of the intact trypanosome metabolizing glucose and the oxidase activity is fortuitous and that the isolated enzyme system contains only about 60 % of the oxidase activity present in the intact trypanosome.

The isolated oxidase system was found to be

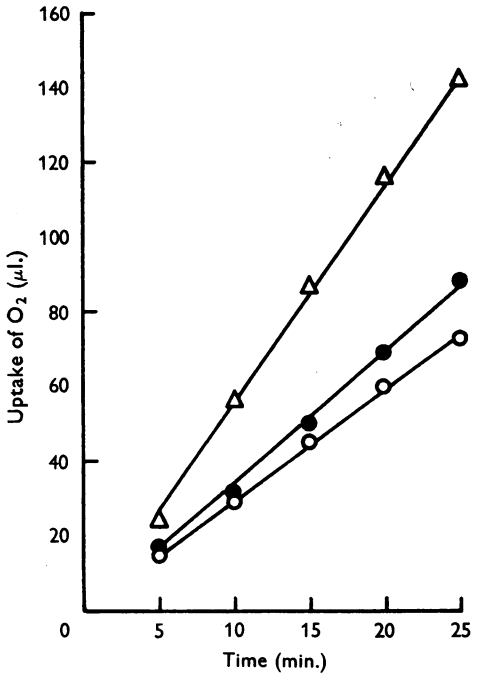

Fig. 6. Rate of oxygen uptake by a suspension of *T. rhodesiense* compared with that by the L-α-GP oxidase isolated from the same suspension. Details are given in the Experimental section. ●, Oxidase preparation (isolated from intact trypanosome suspension containing 0·29 mg. of N) with L-α-GP (16 mM) as substrate. ○, Intact organism (0·29 mg. of N) with glucose (11 mM) as substrate. △, Intact organism (0·29 mg. of N) with glycerol (15·4 mM) as substrate.

very unstable in water and accordingly attempts were made to obtain enhanced enzyme activity by decreasing the standard time of water-lysis of the intact parasite. Unfortunately, water-lysis for periods of 20 min. or less resulted in the presence of intact trypanosomes in the preparation and this approach was accordingly abandoned in the present experiments.

DISCUSSION

Many tissues contain both a cytoplasmic DPN-linked α-GP dehydrogenase and a particulate α-GP-oxidase system which reacts directly with oxygen. These enzymes have been found in mammalian tissue (Green, 1936; Green, Needham & Dewan, 1937; Young & Pace, 1958), in insect muscle (Bücher & Klingenberg, 1958; Estabrook & Sacktor, 1958*a*) and in the midgut of the silkworm (Ito & Horie, 1959). The present results show that enzymes of both types are present in the protozoon *T. rhodesiense*.

The L-α-GP oxidase studied in these experiments differed from the oxidase present in mammalian-brain mitochondria (Sacktor, Packer & Estabrook, 1959; Ringler & Singer, 1959) and insect-muscle sarcosomes (Estabrook & Sacktor, 1958*a*, *b*) in that its reaction with oxygen was not mediated by a cytochrome system and its activity was unaffected by either EDTA or sodium fluoride. Nevertheless, the oxidase was associated with the particulate material of the cell, but no attempt was made to determine its distribution in subcellular particles. There is no evidence that trypanosomes contain mitochondria (Anderson, Saxe & Beams, 1956), although such structural features are present in larger protozoa (Wolken & Palade, 1953).

Apart from the sensitivity to either alkylating or mercaptide-forming reagents, which would suggest that one or more thiol groups are essential for enzyme activity, virtually nothing is known about the mechanism of hydrogen transport in the L-α-GP oxidase. Pyridine nucleotides do not appear to be involved since neither DPN^+ nor TPN^+ enhances oxidase activity. The possibility that the system is already saturated with firmly bound coenzyme cannot be excluded, but it may be noted that the enzyme was insensitive to Amytal, a compound known to inhibit the reaction of some DPN-linked dehydrogenases with flavoproteins of mitochondria and sarcosomes (Chance & Baltscheffsky, 1958). Experiments by Ryley (1956) and Fulton & Spooner (1959) would suggest that cell-free preparations of *T. rhodesiense* contain a cytochrome *c* oxidase. However, the intact parasite does not contain detectable cytochrome pigments and its respiration is insensitive to cyanide, azide and carbon monoxide (Fulton & Spooner, 1959), so that the significance of these experiments to the mechanism of hydrogen transport is obscure at the present moment.

The respiration and motility of the trypanosome isolated from rat blood is entirely dependent on an extracellular supply of glucose or other substrate such as glycerol. Under these conditions, glucose is metabolized by the Embden–Meyerhof glycolytic pathway to pyruvate and a trace of glycerol (Grant & Fulton, 1957), about 1 mole of oxygen being taken up/mole of glucose utilized (Ryley, 1956). Pyruvate is not metabolized further (Grant & Fulton, 1957) and it has been assumed that DPNH formed in the glyceraldehyde 3-phosphate-dehydrogenase reaction was reoxidized by direct reaction with a hydrogen-transport system. The finding that undialysed crushed-cell suspensions of *T. rhodesiense* had a very low DPNH-oxidase activity (Fulton & Spooner, 1959) is in agreement with the present results, in which a variety of possible hydrogen donors, including DPNH, were utilized at about 5% of the rate found for L-α-GP. These results, together with the fact that the activity of the isolated oxidase can account for the

major part of the respiration of the intact trypanosome, would indicate that this enzyme system must play an important role in the oxidative metabolism of the organism. On the evidence available, it is therefore suggested that the reactions catalysed by the particulate oxidase (2) and the cytoplasmic DPN-linked L-α-GP dehydrogenase (3) are coupled and constitute a hydrogen carrier system for the reoxidation of DPNH.

$$\text{L-α-GP} + \tfrac{1}{2}O_2 \rightarrow \text{dihydroxyacetone phosphate} + H_2O \quad (2)$$

$$\text{Dihydroxyacetone phosphate} + \text{DPNH} + H^+ \rightleftharpoons \text{L-α-GP} + \text{DPN}^+ \quad (3)$$

Sum: $$\text{DPNH} + H^+ + \tfrac{1}{2}O_2 \rightarrow \text{DPN}^+ + H_2O.$$

This scheme is feasible since the equilibrium condition of reaction (2) results in essentially complete oxidation of L-α-GP (Table 3). Moreover, the reaction catalysed by the dehydrogenase favours L-α-GP formation (Fig. 4), a result in agreement with the equilibrium constant of the reaction $K_c = 5{\cdot}5 \times 10^{-12}$ M at 25° and at an ionic strength of about 0·03 (Burton & Wilson, 1953). A similar scheme has been suggested by Bücher & Klingenberg (1958) and Estabrook & Sacktor (1958*a*) to account for the respiration of insect flight muscle under certain conditions. In the present experiments, however, the coupling of these reactions has been demonstrated in a cell-free suspension of *T. rhodesiense*, the constitutive glyceraldehyde 3-phosphate dehydrogenase being used to generate reducing equivalents of DPNH. In this experiment (Fig. 5), about 1 mole of oxygen was utilized/mole of L-α-GP added (reactions 4, 5 and 6).

This scheme can satisfactorily account for the oxidative catabolism of glucose or glycerol to pyruvate. The essential details of these reactions are illustrated in Fig. 7, where it may be noted that the oxidation of DPNH involves indirect hydrogen transfer to the respiratory chain via L-α-GP.

It has been repeatedly observed that the respiration of the blood-stream form of *T. rhodesiense* was extremely sensitive to alkylating and mercaptide-forming reagents (Brand *et. al* 1950; Ryley, 1956) and to oxidizing agents such as hydrogen peroxide (Fulton & Spooner, 1959). The present results indicate that these compounds, with the exception of iodoacetate, can act directly on the hydrogen-transport system. Iodoacetate has been shown to inhibit the glyceraldehyde 3-phosphate dehydrogenase of *T. rhodesiense* (Fig. 5) and of other closely related trypanosomes (Marshall, 1948; Harvey, 1949), so that inhibition of this enzyme could account for its effect on the respiration of the intact organism.

Further discussion on the results presented here may be more usefully undertaken when the components of the hydrogen-transport system have been identified and when more is known about the possible changes that may be brought about in the structure and function of the system during its isolation from the organism.

$$2\ \text{L-α-GP} + O_2 \rightarrow 2\ \text{triose phosphate} + 2H_2O \quad (4)$$

$$\text{Triose phosphate} + \text{DPNH} + H^+ \rightarrow \text{L-α-GP} + \text{DPN}^+ \quad (5)$$

$$\text{Triose phosphate} + \text{DPN}^+ + H_3PO_4 \rightarrow \text{1:3-diphosphoglyceric acid} + \text{DPNH} + H^+ \quad (6)$$

Sum: $$\text{L-α-GP} + O_2 \rightarrow \text{1:3-diphosphoglyceric acid} + 2H_2O$$

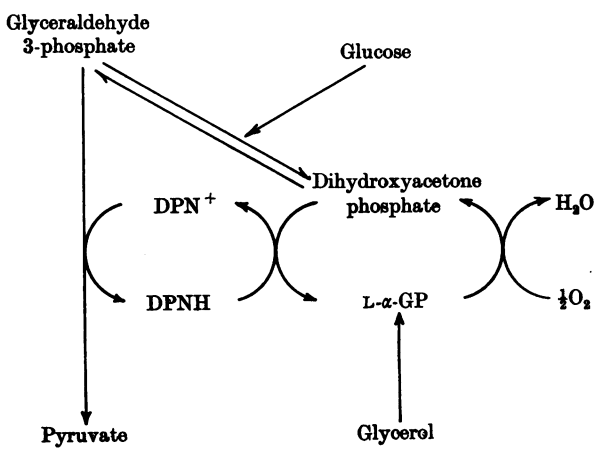

Fig. 7. Suggested role of L-α-GP dehydrogenase and oxidase in the oxidative catabolism of glucose or glycerol to pyruvate by *T. rhodesiense*

SUMMARY

1. Cell-free extracts of *Trypanosoma rhodesiense* have been found to contain a cytoplasmic L-α-glycerophosphate dehydrogenase and a particulate hydrogen-transport system whose rate of reaction with L-α-glycerophosphate was about 20 times that of other possible hydrogen donors such as reduced diphosphopyridine nucleotide or succinate.

2. The latter enzyme system has been termed L-α-glycerophosphate oxidase, as 1 g.atom of oxygen was utilized in the oxidation of L-α-glycerophosphate to dihydroxyacetone phosphate.

3. The optimum activity of the oxidase in phosphate buffer and in 2-amino-2-hydroxymethylpropane-1:3-diol buffer was about pH 7·4 and pH 8·0 respectively. At pH 7·4 the K_m was calculated to be 1·3 mM.

4. The activity of the oxidase was inhibited by certain thiol reagents but not at all by compounds which inhibit systems containing cytochrome pigments. There was no evidence that pyridine nucleotides are involved in the oxidase reaction.

5. Under conditions where the substrate concentration was not a limiting factor over the experimental period, the rate of uptake of oxygen by the oxidase was about 60 and 110% of that found for the intact trypanosome respiring in either glycerol or glucose respectively.

6. A dialysed cell-free suspension of *Trypanosoma rhodesiense* utilized 1 g.atom of oxygen/mole of L-α-glycerophosphate added but this uptake of oxygen could be doubled when oxidized diphosphopyridine nucleotide was also added.

7. It is suggested that the reactions catalysed by the diphosphopyridine nucleotide-linked dehydrogenase and the oxidase are coupled and constitute a hydrogen-transport system for the oxidation of reduced diphosphopyridine nucleotide. The compatability of this suggestion with previous work on the oxidative catabolism of glucose by *Trypanosoma rhodesiense* has been discussed.

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