- Fulton, J. D. & Spooner, D. F. (1959). *Exp. Parasit.* **8**, 137.
 Grant, P. T. & Fulton, J. D. (1957). *Biochem. J.* **66**, 242.
 Grant, P. T. & Sargent, J. R. (1960). *Biochem. J.* **76**, 229.
 Grant, P. T. & Sargent, J. R. (1961). *Biochem. J.* **81**, 206.
 Hoare, C. A. (1957). *Vet. rev. annot.* **3**, 1.
 Johnson, E. M. (1947). *J. Parasit.* **33**, 85.
 Kearney, E. B. & Singer, T. P. (1956). *J. biol. Chem.* **219**, 963.
 Keilin, D. & Hartree, E. F. (1939). *Proc. Roy. Soc. B*, **127**, 167.
 Kersten, H., Kersten, W. & Staudinger, H. (1958). *Biochim. biophys. Acta*, **27**, 598.
 Lenhoff, H. M., Nicholas, D. J. D. & Kaplan, N. O. (1956). *J. biol. Chem.* **220**, 983.
 Pappenheimer, A. M. & Williams, C. M. (1954). *J. biol. Chem.* **209**, 915.
 Ringler, R. L. & Singer, T. P. (1959). *J. biol. Chem.* **234**, 2211.
 Robbie, W. A. (1946). *J. cell. comp. Physiol.* **27**, 181.
 Ryley, J. F. (1951). *Biochem. J.* **49**, 577.
 Ryley, J. F. (1955). *Biochem. J.* **59**, 353.
 Ryley, J. F. (1956). *Biochem. J.* **62**, 215.
 Thorn, M. B. (1956). *Biochem. J.* **63**, 420.
 Tobie, E. J., Brand, T. von & Mehman, B. (1950). *J. Parasit.* **36**, 48.
 Trager, W. (1959). *Ann. trop. Med. Parasit.* **53**, 473.
 Voegtlin, C. & Smith, H. W. (1920). *J. Pharmacol.* **15**, 453.

Biochem. J. (1961) **81**, 206

L- α -Glycerophosphate Dehydrogenase, a Component of an Oxidase System in *Trypanosoma rhodesiense*

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The oxidation of α -glycerophosphate to dihydroxyacetone phosphate with concomitant hydrogen transfer to molecular oxygen may be catalysed by various enzymes or enzyme systems which do not contain pyridine nucleotides. An aerobic α -glycerophosphate dehydrogenase from *Streptococcus faecalis* has been identified as an auto-oxidizable flavoprotein (Jacobs & Van Demark, 1960) and an enzyme of similar properties has also been found in *Escherichia coli* (Strittmatter, 1960). Rabbit muscle (Green, 1936), mitochondria of pig brain (Ringler & Singer, 1959), sarcosomes of insect flight muscle (Estabrook & Sacktor, 1958; Klingenberg & Bücher, 1959) and mitochondria of the mid-gut of the silkworm (Ito & Horie, 1959) all contain an anaerobic α -glycerophosphate dehydrogenase that is structurally and functionally linked to a respiratory chain containing cytochrome pigments. The complete system catalyses the reaction:

Another oxidase system which catalyses the same reaction is present in cell-free preparations of blood-stream trypanosomes of the *brucei* group (Grant & Sargent, 1960a; Grant, Sargent & Ryley, 1961) but differs from that of mammalian and insect tissue in that the reaction with oxygen is not mediated by cytochrome pigments. Moreover, preparations of these protozoa exhibit no appreci-

able oxidase activity towards either succinate or reduced pyridine nucleotide as substrate.

The present work is concerned with some properties of an anaerobic L- α -glycerophosphate dehydrogenase, which is the first component of the trypanosomal oxidase system to be identified.

Some of these results have been reported by Grant & Sargent (1960b).

EXPERIMENTAL

Organism. The Liverpool strain of *Trypanosoma rhodesiense*, its maintenance in rats and the isolation of these cells from infected rat blood have been described by Grant & Fulton (1957). Microscopic examination was made on smears treated with Giemsa stain and the preparation was retained for experimental use if the content of platelets and red and white cells was negligible.

Preparation of cell-free extracts. Cell-free particulate preparations of *T. rhodesiense* containing L- α -glycerophosphate oxidase activity were obtained as described by Grant & Sargent (1960a).

In most experiments, the particulate material was dried from the frozen state immediately after preparation in a centrifugal freeze-drier (Edwards High Vacuum Ltd., Sussex) with P_2O_5 as desiccant. The dried material was stored *in vacuo* at 2°. Immediately before use, it was re-suspended in distilled water (2 mg./ml.) with the aid of either an all-glass homogenizer of the Potter-Elvehjem type (Loughborough Glass Co., Loughborough, Leics.), or a 60 w ultrasonic vibrator of the magnetostriector type with a

stainless-steel resonating probe of end ratio 9:1 (Measuring and Scientific Equipment Ltd., London, S.W. 1).

Measurement of enzyme activity. Consumption of O_2 was measured in a conventional Warburg apparatus at 37° in air. The total fluid volume added to each flask was 3.0 ml. In all cases, flasks were temperature-equilibrated for 10 min. before the start of the reaction. Cell-free preparations did not produce detectable CO_2 and, accordingly, KOH was not added to the centre well in these experiments.

The enzyme reduction of 2:6-dichlorophenol-indophenol was measured by following the extinction changes at 600 $m\mu$ of the reaction mixture contained in a 1 cm.² quartz cell fused to a standard Thunberg attachment (Thermal Syndicate Ltd., Wallsend, Northumberland). The cell, containing the reaction mixture with enzyme suspension in the stopper of the Thunberg tube, was evacuated four times and flushed each time either with O_2 -free N_2 or with air. The compartment of the spectrophotometer containing the cell was covered with a light-tight box. Time was allowed for equilibration of temperature (about 20°), the enzyme was then added from the stopper and readings were commenced 30 sec. later. The amount of dye reduced was calculated from the molar extinction coefficient: ϵ 21×10^3 , at 600 $m\mu$ (Savage, 1957).

Extinction changes in reactions involving DPN were measured at 340 $m\mu$ in a 1 cm.² quartz cell.

All optical measurements were made in a Hilger Uvispek spectrophotometer.

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

Total phosphorus was determined as orthophosphate after digestion with $HClO_4$ (sp.gr. 1.7) at 160°, by the method of Berenblum & Chain (1938) with the micro-modification of Long (1943).

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

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

In one group of experiments (Table 2) both triose phosphate and α -glycerophosphate were determined in the same sample as described by Grant & Sargent (1960a).

Materials. DL- and L- α -Glycerophosphate were synthesized and solutions containing the sodium salts were stored as described by Grant & Sargent (1960a).

2:6-Dichlorophenol-indophenol was purified by the method of Savage (1957). 8-Hydroxyquinoline was recrystallized twice from aqueous 50% (v/v) methanol. Phenazine methosulphate, obtained from the Aldrich Chemical Corp. Inc., Milwaukee, Wis., U.S.A., was recrystallized twice from ethanol.

The following materials were used without additional purification: oxidized glutathione, DPN, DPNH, tris, mammalian cytochrome c, crystalline catalase and crystalline DPN-linked L- α -glycerophosphate dehydrogenase were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany; the dried venom of the cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*) was obtained from Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A., and was used as a source of phospholipase A (cf. Long & Penny, 1957).

Pure H_2O_2 containing no added stabilizing agent was a gift from Laporte Chemicals Ltd., Luton, Beds. Melarsen oxide [*p*-(2:4-diamino-1:3:5-triazin-6-ylaminophenyl)arsenoxide] was a gift from May and Baker Ltd., Dagenham, Essex. Propane-1:2-diol 1-phosphate was a gift from Dr E. Huff and had been prepared by the method of Huff & Rudney (1959).

RESULTS

The activity of the L- α -glycerophosphate oxidase present in particulate preparations of *T. rhodesiense* was completely lost after freeze-drying or by treatment with acetone at -15°. The residual material, however, was capable of oxidizing L- α -glycerophosphate to dihydroxyacetone phosphate in the presence of a suitable electron acceptor such as phenazine methosulphate or 2:6-dichlorophenol-indophenol (Tables 1 and 2). This activity was essentially unchanged after 1 year's storage at 2°, and will be subsequently referred to as L- α -glycerophosphate dehydrogenase.

Freeze-dried preparations were suspended in

Table 1. Activity of L- α -glycerophosphate dehydrogenase with various electron acceptors

Reaction mixtures contained tris buffer, pH 7.6 (0.14M), DL- α -glycerophosphate (33 mM), freeze-dried enzyme preparation and the other additions shown below in a total volume of 3.0 ml. The reduction of 2:6-dichlorophenol-indophenol and cytochrome c were measured spectrophotometrically at 600 and 550 $m\mu$ respectively as described in the Experimental section. Manometric procedures were used for other assays. In all cases the reaction was started by the addition of electron acceptor and the activity was calculated from either the uptake of O_2 in 15 min., or the degree of reduction of the acceptor in 3 min.

Addition	Enzyme added (mg. of N)	Activity (μ moles of substrate utilized/10 min./ mg. of N)
Phenazine methosulphate (4.6 mM) and 8-hydroxyquinoline (mM)	0.14	21.5
2:6-Dichlorophenol-indophenol (0.13 $m\mu$ M)	0.05	3.0
Methylene blue (3.5 mM)	1.00	1.5
Brilliant cresyl blue (4.2 mM)	1.00	Nil
Cytochrome c (1.0 mg.) and cyanide (mM)	1.00	Nil

0.02 M-sodium pyrophosphate buffer, pH 8.6 (5 mg./ml.), and subjected to ultrasonic vibration for 6 min. Essentially all the L- α -glycerophosphate-dehydrogenase activity was present in the residue after centrifuging at 104 000*g* for 60 min., the specific activity (about 1900 μ l. of oxygen utilized/60 min./mg. dry wt.) being about four times that of the starting material. Further purification of this enzyme was difficult because no procedure was found which would release the active enzyme from the particulate material; treatment with salt solutions of various pH values and ionic strengths, trypsin, phospholipase A, butan-1-ol and deoxycholate was not effective.

Assay of L- α -glycerophosphate dehydrogenase with phenazine methosulphate

Kinetic aspects. The following results characterize the enzyme and define the optimum conditions for its assay.

The effect of pH on the enzyme activity in both phosphate and tris buffers in the range pH 6–9 is shown in Fig. 1. The pH optima were about pH 7.2 and 8.2 respectively, the enzyme being appreciably more active when tris buffer was used in the assay system. This result is in marked contrast with the α -glycerophosphate dehydrogenase in the mitochondria of pig brain, which has a maximum activity in phosphate buffer with the same electron acceptor (Ringler & Singer, 1959).

Enzyme activity was determined with different concentrations of substrate and electron acceptor (Fig. 2). From these results the K_m values for substrate and acceptor were calculated by the reciprocal-plot method (Lineweaver & Burk, 1934) to be 9.0 and 0.34 mM respectively.

With saturating concentrations of substrate (40 mM) and electron acceptor (4.6 mM), the activity of the enzyme was proportional to the amount of enzyme added up to about 300 μ g. of N. When greater amounts of enzyme were used the rate was limited by the diffusion of oxygen into the reaction medium, and, for this reason, about 140 μ g. of N were used in subsequent determinations of enzyme activity (140 μ g. of N $\equiv$ 1.0 mg. dry wt. of freeze-dried material).

In this assay system the addition of 8-hydroxyquinoline was essential for the maximum reaction rate (Fig. 3), an effect which was independent of the concentration of 8-hydroxyquinoline added in the range 0.6–3.0 mM.

Stoichiometric relationships and substrate specificity. Glycerol, β -glycerophosphate and propane-1:2-diol 1-phosphate neither served as substrates for the enzyme nor, when added singly in equimolar amounts with L- α -glycerophosphate, had any effect on the oxidation of the latter.

The quantitative relationship between reactants

Fig. 1. Effect of pH on activity. Reaction mixtures contained freeze-dried preparation (140 μ g. of N), buffer (0.14 M), DL- α -glycerophosphate (33 mM), phenazine methosulphate (4.6 mM) and 8-hydroxyquinoline (mM) in a total volume of 3.0 ml. Reaction was started by addition of phenazine methosulphate from the side arm and readings were recorded over a period of 10 min. The pH of the flask contents was subsequently determined by glass electrode at 20°. O, Phosphate buffer; ●, tris buffer.

Fig. 2. Lineweaver-Burk plots {reciprocal of the initial rate, V (μ l. of O₂ utilized/10 min./flask), against the reciprocal of substrate concentration, $[S]$ (mM-substrate), and electron acceptor concentration, $[D]$ (mM-acceptor)} for L- α -glycerophosphate dehydrogenase. Reaction mixtures contained freeze-dried preparation (140 μ g. of N), tris buffer, pH 7.6 (0.14 M), 8-hydroxyquinoline (mM), DL- α -glycerophosphate and phenazine methosulphate. ●, DL- α -glycerophosphate (33 mM) with varied concentrations of phenazine methosulphate; O, phenazine methosulphate (4.6 mM) with varied concentrations of DL- α -glycerophosphate. Reaction was started by the addition of phenazine methosulphate from the side arm and readings were recorded over a period of 10 min.

and products was determined by analysis of the reaction mixture at the beginning and end of each experiment. In the presence of 8-hydroxyquinoline, the results (Expt. 1, Table 2) are consistent with the reaction:

This result is in agreement with that given in Expt. 2, Table 2, in which the addition of an excess of catalase reduced the uptake of oxygen in the presence of 8-hydroxyquinoline to 1 g. atom/mole of substrate utilized.

Dihydroxyacetone phosphate was identified as the triose phosphate present in the reaction mixture. This was shown by the decrease in extinction at 340 m μ of a solution containing DPNH, crystalline DPN-linked L- α -glycerophosphate dehydrogenase and a portion of the reaction mixture obtained at the end of the experiments after removal of protein and phenazine methosulphate. This latter compound catalysed a non-enzymic oxidation of DPNH (Du Buy & Showacre, 1959) and was quantitatively absorbed by percolation of the protein-free reaction mixture through columns (5 cm. $\times$ 1 cm.) of Zeo-Karb 225 (H⁺ form). The enzyme preparations did not contain detectable triose phosphate isomerase (cf. Grant & Sargent, 1960*a*) and for this reason it was concluded that dihydroxyacetone phosphate and not glyceraldehyde 3-phosphate was the product of the dehydrogenase reaction.

Effect of thiol group reagents. The activity of L- α -glycerophosphate dehydrogenase, although unaffected by thiol-alkylating agents such as iodoacetic acid and iodoacetamide, was sensitive to N-ethylmaleimide. *p*-Chloromercuribenzoate, organic trivalent arsenicals, various metal ions (Table 3) and hydrogen peroxide (Table 4), all of which are known to affect thiol groups, inhibited enzyme activity. The effect of *p*-chloromercuribenzoate was particularly marked (Table 3, Fig. 5) and this inhibition could be completely reversed by incubating the treated enzyme with glutathione (mM) at 37° for 10 min.

An enzyme preparation was treated either with potassium cyanide (10 mM) for 12 hr. at 18° or with oxidized glutathione (50 mM) for 6 hr. at 37° exactly as detailed by Keilin & King (1960) in a study of mammalian succinic dehydrogenase. In neither case, however, did the treatment result in any significant decrease in L- α -glycerophosphate-dehydrogenase activity with phenazine methosulphate as electron acceptor.

Effect of metal-chelating and other agents. Chelating agents had no effect on the enzyme activity when included in the assay system involving 2,6-dichlorophenol-indophenol. In contrast, when phenazine methosulphate was used as electron

acceptor, the uptake of oxygen ceased about 6 min. after the addition of acceptor unless either catalase or a chelating agent, preferably 8-hydroxyquinoline, was present (Fig. 3). In the presence of 8-hydroxyquinoline the rate of oxygen uptake was double that observed when both catalase and 8-hydroxyquinoline were present, a result in agreement with the stoichiometry of these reactions (Table 2).

The inhibitory effects of hydrogen peroxide were greatly enhanced by the presence of aqueous extracts of enzyme ash or added metal ions (Table 4). These effects were relieved by 8-hydroxyquinoline and to a lesser extent by cyanide, which

Table 2. *Quantitative relationships between substrate and product of the L- α -glycerophosphate-dehydrogenase reaction*

Reaction mixtures contained freeze-dried preparation (140 μ g. of N), tris buffer, pH 7.6 (0.14M), L- α -glycerophosphate (5.5 mM), phenazine methosulphate (4.6 mM) and either 8-hydroxyquinoline (mM) in Expt. 1, or catalase (5 μ M) and 8-hydroxyquinoline (mM) in Expt. 2, in a total volume of 3.0 ml. Reaction was started in each case by addition of phenazine methosulphate from the side arm and stopped after 12 min. by rapid cooling to 0° with simultaneous addition of 0.5 ml. of 30% (w/v) trichloroacetic acid.

Expt. no.	Uptake of O ₂ (μ moles)	Triose phosphate formed (μ moles)	L- α -Glycerophosphate used (μ moles)
1	4.4	4.2	4.8
2	1.7	3.2	3.4

Table 3. *Effect of inhibitors on L- α -glycerophosphate-dehydrogenase activity*

The main compartment of each flask contained freeze-dried enzyme preparation (140 μ g. of N), tris buffer, pH 7.6 (0.14M), and the additions shown below in a total volume of 2.0 ml. The side arm contained DL- α -glycerophosphate (33 mM), 8-hydroxyquinoline (mM) and phenazine methosulphate (4.6 mM) in a total volume of 1.0 ml. After equilibration for 10 min. at 37° the reaction was started by addition of the side-arm contents.

Addition	Final concn.	Uptake of O ₂ (μ l./15 min.)
Nil	—	108
3-Amino-4-hydroxyphenyl-arsenoxide	10 μ M	67
Melarsen oxide	10 μ M	67
N-Ethylmaleimide	0.1 mM	59
<i>p</i> -Chloromercuribenzoate	1 μ M	58
CuSO ₄	50 μ M	39
ZnSO ₄	0.5 mM	53
CdSO ₄	0.5 mM	103
Iodoacetate* or iodoacetamide*	1 mM	108
Sodium arsenite	0.5 mM	108

* Equilibration for either 10 or 30 min. with enzyme before assay.

Table 4. *Inhibition by oxidizing and mercaptide-forming reagents and the protective effect of other components in the reaction mixture*

Conditions for these experiments are given in Table 3, except that the enzyme was treated as shown below for 15 min. at 37° and the reaction was started by tipping the remaining components of the reaction mixture from the side arm.

Expt. no.	Treatment	Uptake of O ₂ (μl./15 min.)
1	Nil	94
	Phenazine methosulphate (13·8 μmoles)	73
	Enzyme mixed with 8-hydroxyquinoline (3 μmoles) and treated with phenazine methosulphate	75
2	Nil	101
	Unstabilized H ₂ O ₂ (0·4 μmole)	43
	Enzyme mixed with 8-hydroxyquinoline (3 μmoles) and treated with H ₂ O ₂	98
	Enzyme mixed with cyanide (3 μmoles) and treated with H ₂ O ₂	78
	Enzyme mixed with substrate (100 μmoles) and treated with H ₂ O ₂	67
3	Enzyme mixed with phenazine methosulphate (13·8 μmoles) and treated with H ₂ O ₂	56
	Nil	112
	Cu ²⁺ ions (0·05 μmole)	104
	Cu ²⁺ ions and H ₂ O ₂ (0·4 μmole)	21
4	Enzyme mixed with 8-hydroxyquinoline and treated with Cu ²⁺ ions and H ₂ O ₂	105
	Nil	90
	Extract of enzyme ash (equivalent to 300 μg. of enzyme N)	90
	Extract of enzyme ash and H ₂ O ₂ (0·4 μmole)	27
5	Enzyme mixed with 8-hydroxyquinoline (3 μmoles) and treated with extract of enzyme ash and H ₂ O ₂	86
	Nil	104
	<i>p</i> -Chloromercuribenzoate (0·01 μmole)	25
	Enzyme mixed with substrate and treated with <i>p</i> -chloromercuribenzoate	40
	Enzyme mixed with phenazine methosulphate and treated with <i>p</i> -chloromercuribenzoate	59

would indicate that some essential group on the enzyme is sensitive to a metal-catalysed oxidation by hydrogen peroxide. The concentration of hydrogen peroxide added in these experiments was of the same order as that calculated to be present in the assay system after about 6 min., so that the protective effect of 8-hydroxyquinoline, and possibly also that of cyanide, in this system can be attributed to their metal-chelating properties.

Relationship between L-α-glycerophosphate dehydrogenase and oxidase

The effect of aerobic and anaerobic conditions on the rate of reduction of 2:6-dichlorophenol-indophenol by a fresh cell-free preparation containing L-α-glycerophosphate-oxidase activity was compared with that of a freeze-dried sample of the same preparation which contained only L-α-glycerophosphate-dehydrogenase activity (Fig. 4). No significant difference was found between the activity of the fresh preparation and the freeze-dried preparation under anaerobic conditions, thus showing that the L-α-glycerophosphate dehydrogenase originally present had unimpaired activity after desiccation. Nevertheless, the rate of dye

reduction by the fresh preparation under aerobic conditions was about 70 % of that found anaerobically. This difference would suggest that the dehydrogenase is a component of the oxidase system and can transfer electrons either to the terminal part of the respiratory chain or to a suitable acceptor such as 2:6-dichlorophenol-indophenol: the proportion of electrons transferred to either acceptor would be determined by the experimental conditions. These experiments have not been pursued but are in general agreement with those given below.

A comparison was made of the effects of *p*-chloromercuribenzoate on oxidase activity of fresh and aged portions of the same preparation, and of dehydrogenase activity with phenazine methosulphate as acceptor in the same material after freeze-drying. Under these directly comparable conditions (Fig. 5) the dehydrogenase had twice the activity (μmoles of substrate utilized/min./mg. of N) of the freshly prepared oxidase. The dehydrogenase was progressively inhibited by increasing concentrations of *p*-chloromercuribenzoate, whereas the activity of the oxidase was essentially unchanged until a concentration of

Fig. 3. Effect of metal-binding and other agents. Reaction mixtures contained freeze-dried preparation (140 μ g. of N), tris buffer, pH 7.6 (0.14 M), DL- α -glycerophosphate (33 mM), phenazine methosulphate (4.6 mM) and the following additions: $\circ$, 8-hydroxyquinoline (mM); $\bullet$, KCN (mM) or ethylenediaminetetra-acetic acid (mM); Δ , catalase (2.5 mg.) and 8-hydroxyquinoline (mM); $\blacktriangle$, catalase (2.5 mg.); $\square$, no addition. The reaction was started in all cases by the addition of phenazine methosulphate from the side arm.

Fig. 4. Effect of aerobic and anaerobic conditions on fresh and freeze-dried enzyme preparations with 2:6-dichlorophenol-indophenol as electron acceptor. Details of these experiments are given in Table 1 and in the Experimental section, except that in all experiments the same amount of enzyme preparation (30 μ g. of N) was used. $\circ$, Freshly prepared enzyme under aerobic conditions; $\bullet$, freshly prepared enzyme under anaerobic conditions; Δ , freeze-dried sample of fresh preparation under either aerobic or anaerobic conditions.

Fig. 5. Comparison of the effects of *p*-chloromercuribenzoate on the activities of L- α -glycerophosphate oxidase and dehydrogenase. Oxidase activity: the enzyme preparation was incubated at 37° for 10 min. with tris buffer, pH 7.6 (0.14 M), and *p*-chloromercuribenzoate as shown below in a total volume of 2.0 ml. The reaction was started by the addition of DL- α -glycerophosphate (1 ml.; 33 mM) from the side arm and the initial rate of the reaction was measured over a period of 15 min. Dehydrogenase activity: reaction mixtures and procedure were as described in Table 3. $\circ$, Oxidase activity in the fresh preparation (275 μ g. of N); Δ , oxidase activity in the same preparation after 4 hr. at 0°; $\blacktriangle$, oxidase activity in the same preparation after 10 hr. at 0°; $\bullet$, dehydrogenase activity in material (275 μ g. of N) obtained by freeze-drying the fresh preparation immediately, or after 10 hr. at 0°.

inhibitor was added which reduced dehydrogenase activity to about the value that corresponded to the activity of the oxidase. Thereafter higher concentrations of *p*-chloromercuribenzoate resulted in an approximately parallel decrease in activity of both oxidase and dehydrogenase.

The activity of the freshly prepared oxidase was usually about half that of the dehydrogenase. This could indicate that the dehydrogenase is not the rate-limiting reaction of the oxidase system. However, no special precautions were taken to protect the latter during its preparation and, as the activity rapidly decreased on standing at 2° (Fig. 5), it was probable that all oxidase preparations contained some inactivated enzyme in amounts which progressively increased with time after the initial rupture of the intact cell. In contrast, dehydrogenase activity in these preparations measured after the sample had been freeze-dried was essentially the same as that of the intact

cells which were freeze-dried without prior treatment. For these reasons, any consideration of the results given in Fig. 5 must take into account the uptake of *p*-chloromercuribenzoate by inactivated enzyme when the oxidase activity of both fresh and aged samples was being measured.

DISCUSSION

The results show that particulate preparations of *T. rhodesiense* contain an anaerobic L- α -glycerophosphate dehydrogenase that is functionally linked to a terminal respiratory chain. This latter system does not involve cytochrome pigments (Grant & Sargent, 1960*a*), and, although the components have not been identified, the results given in Fig. 5 are consistent with the view that the thiol group, which is essential for the activity of the intact oxidase, is located in the dehydrogenase component.

Although insensitive to iodoacetamide, the marked inhibition of L- α -glycerophosphate-dehydrogenase activity by *N*-ethylmaleimide, trivalent organic arsenicals and *p*-chloromercuribenzoate would indicate that thiol groups have an essential role in the activity of the enzyme. 'Dithiol' groups do not appear to be involved since the enzyme, like that present in mammalian muscle (Hopkins & Morgan, 1938), was not affected by a prolonged incubation with oxidized glutathione. Cyanide was also ineffective in this respect. The L- α -glycerophosphate dehydrogenase studied in these experiments thus differs from succinic dehydrogenase of mammalian heart muscle in that the latter enzyme contains a 'dithiol' group that is inhibited by oxidized glutathione (Hopkins & Morgan, 1938; Singer, Kearney & Massey, 1957; Keilin & King, 1960) and a distinct and additional group that combines slowly and irreversibly with cyanide (Tsou, 1951; Giuditta & Singer, 1959; Keilin & King, 1960). An indication that two spatially adjacent thiol groups are not involved with the activity of L- α -glycerophosphate dehydrogenase is that the enzyme was insensitive to both arsenite and Cd²⁺ ions. At low concentrations these compounds are considered to be specific inhibitors of reactions involving such groups (Sanadi, Langley & White, 1959). Arsenite forms only a weak complex with monothiols, but forms a stable ring structure with dithiol compounds provided that the thiol groups are spatially adjacent (Stocken & Thompson, 1946).

The insensitivity of the L- α -glycerophosphate-oxidase system to concentrations of *p*-chloromercuribenzoate that markedly inhibited the activity of the dehydrogenase (Fig. 5) would suggest that the thiol group does not react so readily with the inhibitor when the dehydrogenase

is functionally linked to the respiratory chain to form the intact oxidase system. All preparations of the oxidase contained some inactivated enzyme and the amount progressively increased with time; in contrast, the dehydrogenase activity measured with phenazine methosulphate as acceptor was essentially unchanged by storage. For these reasons, it had been concluded that the oxidase activity in both fresh and aged preparations would be unaffected by *p*-chloromercuribenzoate until the thiol groups in the dehydrogenase component of the inactivated enzyme had first been saturated with the inhibitor. Moreover, the reaction of the dehydrogenase with *p*-chloromercuribenzoate can be partly prevented by the prior addition of phenazine methosulphate (Table 4), so that the thiol group may be concerned with electron transfer to either the respiratory chain or phenazine methosulphate.

The maximum rate of enzyme activity was obtained with phenazine methosulphate as electron acceptor. The enzyme was appreciably more active in tris than in phosphate buffer and the partly purified particulate material in tris buffer, pH 7.6, catalysed a linear rate of oxygen uptake equivalent to a Q_{O_2} (μ l. of O₂/mg. dry wt./hr.) of about 1900. Phenazine methosulphate is also the preferred electron acceptor for the assay of α -glycerophosphate dehydrogenase of brain mitochondria (Q_{O_2} 170–243 in phosphate buffer, pH 7.6; Ringer & Singer, 1959) and of other cytochrome-linked dehydrogenase (Singer, Kearney & Massey, 1957; Mapson & Breslow, 1958; Rendina & Singer, 1959), but with these enzymes inorganic phosphate was essential for maximum reaction rates.

The assay of anaerobic dehydrogenases with phenazine methosulphate as electron acceptor is complicated by the necessity to protect the enzyme from hydrogen peroxide formed during the auto-oxidation of the reduced acceptor. In the original description of the phenazine methosulphate assay procedure for succinic dehydrogenase (Kearney & Singer, 1956) it was found that cyanide and 8-hydroxyquinoline were the most effective protective agents. Cyanide was assumed to react with disulphide (–S–S–) groups formed by the action of hydrogen peroxide on adjacent thiol groups, the enzyme being active when one member of the pair was converted into a thiocyanate (–SCN) and the other into a free thiol (–SH) group. In the present experiments with an enzyme that apparently does not contain adjacent thiol groups, 8-hydroxyquinoline and cyanide to a lesser extent were effective protective agents in the assay procedure. Fig. 3 would indicate that they inhibit a metal-catalysed oxidation by hydrogen peroxide of an essential thiol group on the enzyme, and the protective effect can thus presumably be attributed to

the metal-chelating properties of these compounds.

The particulate nature of the anaerobic α -glycerophosphate dehydrogenase of mammalian tissue was noted by Green (1936), who first systematically studied this enzyme. Subsequently it was shown that the enzyme could be extracted from tissue preparations by bile salts (Tung, Anderson & Lardy, 1952), bile salts and digestion with trypsin (Ling, Wu, Ting & Tung, 1957), digitonin (Ringler & Singer, 1958) and by treatment with phospholipase A (Ringler & Singer, 1959). These and other procedures were not effective with the particulate enzyme studied here and the nature of the electron pathway between L- α -glycerophosphate and phenazine methosulphate cannot be determined until the active enzyme can be released from the particulate material and purified. Flavin-adenine dinucleotide has been identified in these particulate preparations (P. T. Grant & J. R. Sargent, unpublished observations), but whether this coenzyme is concerned with L- α -glycerophosphate-dehydrogenase activity remains to be established.

SUMMARY

1. An enzyme has been found in particulate preparations of *Trypanosoma rhodesiense* which catalyses the oxidation of L- α -glycerophosphate to dihydroxyacetone phosphate in the presence of either phenazine methosulphate or 2:6-dichlorophenol-indophenol as electron acceptors. The enzyme has been termed an anaerobic L- α -glycerophosphate dehydrogenase.

2. A partly purified particulate fraction under optimum conditions with phenazine methosulphate as electron acceptor catalysed an oxygen uptake which corresponded to a Q_{O_2} of about 1900.

3. In the phenazine methosulphate assay procedure, the optimum enzyme activity in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer was about pH 8. The values of K_m for substrate and electron acceptor were 9.0 and 0.34 mM respectively.

4. L- α -Glycerophosphate-dehydrogenase activity was inhibited by certain thiol-group reagents. Inhibition by *p*-chloromercuribenzoate could be reversed by glutathione. The enzyme was insensitive to arsenite and Cd^{2+} ions. Prolonged incubation with either oxidized glutathione or cyanide had no significant effect on enzyme activity.

5. Inhibition of enzyme activity by hydrogen peroxide was enhanced in a catalytic manner by either extracts of enzyme ash or Cu^{2+} ions. 8-Hydroxyquinoline almost completely protected the enzyme from these agents and this chelating agent

was added as a routine in the assay procedure to protect the enzyme from hydrogen peroxide formed during the auto-oxidation of the reduced acceptor.

6. A comparison of the activities of both L- α -glycerophosphate oxidase and the corresponding dehydrogenase was made on portions of the same preparation. The effects of *p*-chloromercuribenzoate were also studied. The results are consistent with the view that the dehydrogenase is a component of the oxidase system and that the former contains the thiol group that is essential for the activity of the oxidase.

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REFERENCES

- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
- Burmester, C. F. (1946). *J. biol. Chem.* **164**, 233.
- Estabrook, R. W. & Sacktor, B. (1958). *Arch. Biochem. Biophys.* **76**, 532.
- Du Buy, H. G. & Showacre, J. (1959). *J. Histochem. Cytochem.* **7**, 370.
- Giuditta, A. & Singer, T. P. (1959). *J. biol. Chem.* **234**, 662.
- Grant, P. T. & Fulton, J. D. (1957). *Biochem. J.* **66**, 242.
- Grant, P. T. & Sargent, J. R. (1960*a*). *Biochem. J.* **76**, 229.
- Grant, P. T. & Sargent, J. R. (1960*b*). *Biochem. J.* **76**, 48*r*.
- Grant, P. T., Sargent, J. R. & Ryley, J. R. (1961). *Biochem. J.* **81**, 200.
- Green, D. E. (1936). *Biochem. J.* **30**, 629.
- Hopkins, F. G. & Morgan, E. F. (1938). *Biochem. J.* **32**, 611.
- Huff, E. & Rudney, H. (1959). *J. biol. Chem.* **234**, 1060.
- Ito, T. & Horie, Y. (1959). *Arch. Biochem. Biophys.* **80**, 174.
- Jacobs, N. J. & Van Demark, P. J. (1960). *Arch. Biochem. Biophys.* **88**, 250.
- Kearney, E. B. & Singer, T. P. (1956). *J. biol. Chem.* **219**, 963.
- Keilin, D. & King, T. E. (1960). *Proc. Roy. Soc. B*, **152**, 163.
- Klingenberg, M. & Bücher, T. (1959). *Biochem. Z.* **331**, 312.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Ling, K. H., Wu, S. H., Ting, S. M. & Tung, T. C. (1957). *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto*, p. 260. London: Pergamon Press Ltd.
- Long, C. (1943). *Biochem. J.* **37**, 215.
- Long, C. & Penny, I. F. (1957). *Biochem. J.* **65**, 382.
- Mapson, L. W. & Breslow, E. (1958). *Biochem. J.* **68**, 395.
- Meyerhof, P. & Lohmann, K. (1934). *Biochem. Z.* **276**, 89.
- Rendina, G. & Singer, T. P. (1959). *J. biol. Chem.* **234**, 1605.
- Ringler, R. L. & Singer, T. P. (1958). *Fed. Proc.* **17**, 297.

- Ringler, R. L. & Singer, T. P. (1959). *J. biol. Chem.* **234**, 2211.
 Sanadi, D. R., Langley, M. & White, F. (1959). *J. biol. Chem.* **234**, 183.
 Savage, N. (1957). *Biochem. J.* **67**, 146.
 Singer, T. P., Kearney, E. B. & Massey, V. (1957). *Advanc. Enzymol.* **18**, 65.

- Stocken, L. A. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 535.
 Strittmatter, C. F. (1960). *J. biol. Chem.* **234**, 2794.
 Tsou, C. L. (1951). *Biochem. J.* **49**, 512.
 Tung, T. C., Anderson, L. & Lardy, H. A. (1952). *Arch. Biochem. Biophys.* **40**, 194.

Biochem. J. (1961) **81**, 214

The Lipid Composition of Rat-Liver-Cell Sap

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The fatty acid composition of the total lipids of each of the different compartments (nuclei, mitochondria, fluffy layer, microsomes, supernatant and fatty supernatant) of the rat-liver cell was determined by Getz & Bartley (1961). The fatty acid composition of the different lipid classes (neutral lipids, aminophosphatides and choline-phosphatides) within a particular compartment of the liver cell, the cell sap, is described in this study.

METHODS

The cell sap was prepared from 92 g. of rat-liver pulp (obtained from eight male rats of the Wistar strain, average age 8 months and average weight 400 g.). It was obtained as the supernatant fluid that separated after sedimenting the particles from a homogenate of the livers in 0.25M-sucrose solution. The nuclear fraction and the mitochondria were isolated by the procedure of Werkheiser & Bartley (1957) and the fluffy layer was retained and washed in the same way as the mitochondrial sediment. The microsomes were isolated from the combined supernatants of the mitochondrial and fluffy layer fraction by centrifuging for 60 min. at 30 000g in no. 20 rotor of the Spinco centrifuge. A clear supernatant was obtained with a layer of fatty material floating on the surface. The floating fatty material was removed with a Pasteur pipette and the fat on the sides of the centrifuge tubes removed by careful wiping with fat-free filter paper. The combined material ('floating fatty supernatant') was then extracted with 20 vol. of chloroform-methanol (2:1, v/v). The cell sap was removed

from above the microsomes with a Pasteur pipette. To avoid the extraction of large volumes of cell sap with organic solvents, the lipids were first co-precipitated with protein, with trichloroacetic acid as described by Getz & Bartley (1961), and then extracted from the trichloroacetic acid precipitates with 20 vol. of chloroform-methanol (2:1, v/v).

Analysis of cell sap. One portion of the extract was used for the total lipid determination and another was saponified and the fatty acids liberated were esterified with diazomethane, in each case by procedures outlined by Getz & Bartley (1961). The remainder of the lipid extract was chromatographed on silicic acid (Mallinkrodt Chemical Works; mesh 100, prepared according to the method of Hirsch & Ahrens, 1958). The loading procedure and elution sequence were carried out as described by Getz, Bartley, Stirpe, Notton & Renshaw (1961) except that the triglycerides were eluted with 500 ml. of 10% ether in light petroleum (b.p. 60–80°) and the remaining neutral glycerides with 300 ml. of diethyl ether before the first phospholipid fraction was eluted with 100 ml. of chloroform-methanol (7:1, v/v). The elution sequence was as shown in Table 1. Each lipid fraction was subjected to appropriate analysis by methods described by Getz *et al.* (1961). In addition, the methyl esters of the fatty acids of each fraction were prepared by interesterification and microsublimation (Stoffel, Chu & Ahrens, 1959) and the mixtures of methyl esters so obtained were analysed by gas chromatography.

Preparation of lipid extracts for silicic acid chromatography. Insoluble material was deposited from the chloroform-methanol extract of the cell sap during storage and during concentration of the extract for putting on to the silicic acid column. The extract was therefore evaporated to dryness under N₂ and extracted with warm chloroform. The chloroform extract was filtered and the residue on the filter paper washed three times with chloroform. The combined chloroform extracts were evaporated to dryness, dissolved in a small amount of light petroleum (b.p. 60–80°) and applied to the silicic acid column. The residue filtered off from the chloroform solution was saponified and the fatty

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