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Specificity and Locale of the L-3-Glycerophosphate–Flavoprotein Oxidoreductase of Mitochondria Isolated from the Flight Muscle of *Sarcophaga barbata* Thoms.

By J. F. DONNELLAN, M. D. BARKER, J. WOOD and R. B. BEECHEY
Shell Research Ltd., Woodstock Agricultural Research Centre, Sittingbourne, Kent, U.K.

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1. The oxidation of L-3-glycerophosphate by flight-muscle mitochondria isolated from the flesh fly *Sarcophaga barbata* has been studied. Use of substrate analogues indicates that the catalytic and effector L-3-glycerophosphate binding sites on the allosteric L-3-glycerophosphate–flavoprotein oxidoreductase differ markedly in specificity. 2. The L-3-glycerophosphate–cyanoferrate oxidoreductase system in these mitochondria is antimycin-insensitive whereas the corresponding NADH–cyanoferrate oxidoreductase is extremely sensitive to this respiratory-chain inhibitor. Also no swelling is observed when these mitochondria are suspended in iso-osmotic solutions of ammonium glycerophosphate in contrast with the extensive swelling seen in similar solutions of ammonium pyruvate. These observations indicate that L-3-glycerophosphate does not penetrate the mitochondrial matrix whereas pyruvate does. 3. Submitochondrial particles catalyse the ATP-driven reduction of NAD^+ by L-3-glycerophosphate but at a far lower rate than that seen when succinate is the electron donor. These particles do not have an energy-linked pyridine nucleotide transhydrogenase activity. 4. We conclude that the L-3-glycerophosphate–flavoprotein oxidoreductase is located on the outer surface of the inner membrane of the flight-muscle mitochondria.

In describing previous work (Donnellan & Beechey, 1969; Donnellan, Barker & Beechey, 1970) we have followed the nomenclature of Fischer & Baer (1937) in naming the isomer of α-glycerophosphate oxidized by flight-muscle mitochondria L-glycerol 1-phosphate. In this paper the name recommended in the document on the nomenclature of lipids [*Biochem. J.* (1967), **105**, 897] is used, i.e. L-3-glycerophosphate.

The L-3-glycerophosphate oxidase activity of insect flight-muscle mitochondria when coupled with the cytoplasmic L-3-glycerophosphate–NAD^+ oxidoreductase (EC 1.1.1.8) provides insect flight muscle with a system with a high capacity for the oxidation of the extramitochondrial NADH generated during glycolysis (Zebe, Delbruck & Bücher, 1957). Thus the L-3-glycerophosphate cycle or shuttle assumes a highly significant role in the overall metabolism of flight muscle compensating for the low amount of lactate dehydrogenase activity present in this tissue (Sacktor, 1955; Zebe & McShan, 1957).

The L-3-glycerophosphate oxidase system is bound firmly to the inner membrane of the flight-muscle mitochondrion (Zebe & McShan, 1957). The question now arises, on which side of the inner mitochondrial membrane, i.e. the cristal membrane, is the L-3-glycerophosphate–flavoprotein oxidoreductase (EC 1.1.99.5) located? If it lies on the exterior of the cristal membrane there will be no permeability barrier between the substrate and the enzyme. However, if the L-3-glycerophosphate–flavoprotein oxidoreductase lies on the inner, i.e. the matrix, side of the cristal membrane, the substrate molecules will have to traverse this membrane before oxidation can proceed. The cristal membrane of flight-muscle mitochondria is peculiarly impermeable. Thus most of the substrates that are readily oxidized by mammalian liver or heart mitochondria are not oxidized by flight-muscle mitochondria (Van den Bergh & Slater, 1962). The existence of porter systems to transport ionic substances across the inner mitochondrial membrane has been proposed by Mitchell (1966) and experimental data in support have been presented (Chappell, Henderson, McGivan & Robinson, 1968; De Haan & Tager, 1966) for carboxylic acid transport. Also, atractylate (Bruni, Luciani & Contessa, 1964) has been shown to inhibit the exchange of adenine nucleotides across the inner membrane,

whereas mersalyl and *p*-chloromercuribenzoate have been used to indicate the presence of a phosphate porter in liver mitochondria (Fonyo & Bessman, 1968; Tyler, 1969). The location of the L-3-glycerophosphate–flavoprotein oxidoreductase on the matrix side of the cristal membrane would imply the existence of a porter system for L-3-glycerophosphate, as has been demonstrated in *Escherichia coli* (Hayashi, Koch & Lin, 1964).

The experiments described here were designed initially to investigate the presence of an L-3-glycerophosphate porter in the inner membrane of insect flight-muscle mitochondria. The results suggest that the membrane-bound L-3-glycerophosphate–flavoprotein oxidoreductase is located on the external surface of the inner mitochondrial membrane, thus dispensing with the need for such a porter, in agreement with the data and conclusions of Klingenberg & Buchholz (1970).

Estabrook & Sacktor (1958) demonstrated that multivalent metal ions at millimolar concentrations were important in the oxidation of L-3-glycerophosphate by flight-muscle mitochondria isolated from *Musca domestica*. They also showed that this requirement could be overcome by increasing the concentration of substrate. The allosteric nature of the L-3-glycerophosphate–flavoprotein oxidoreductase has now been demonstrated, with L-3-glycerophosphate as a homotropic effector and multivalent metal ions acting as heterotropic effectors, Ca^{2+} being effective at 0.1 μM (Hansford & Chappell, 1967). We have shown that this allosteric behaviour is characteristic for the enzyme present in flight-muscle mitochondria isolated from a range of insects and that it is also seen in submitochondrial particles (calcium-particles) prepared by the sonication of flight-muscle mitochondria in the presence of 10 μM-Ca^{2+} (Donnellan & Beechey, 1969). The results presented below show that the enzymic and effector L-3-glycerophosphate-binding sites have very different specificity requirements. Some of these data have been communicated to the Biochemical Society (Donnellan *et al.* 1970).

METHODS

Preparation of flight-muscle mitochondria. Mitochondria were prepared from the thoraces of *S. barbata* Thoms. by the method described by Donnellan & Beechey (1969). This is based on the Nagarse proteinase method of Chance & Hagihara (1961). The isolated mitochondria were finally suspended in a solution containing 0.25M-sucrose, 5mM-tris chloride and 1mM-ethylenedioxybis(ethylamine)tetraacetate adjusted to pH7.1. The final protein concentration was approximately 10mg/ml.

Preparation of calcium-particles. These are submitochondrial particles prepared by subjecting a freshly prepared mitochondrial suspension, 5mg of protein/ml, to ultrasonic radiation with a MSE 100W ultrasonicator. Before sonication, the final suspension medium was supplemented with 200mM-$CaCl_2$ to give a final buffered free Ca^{2+} concentration of 10 μM (Portzehl, Caldwell & Rüegg, 1964). The sonicated suspension was centrifuged for $1.2\times10^5 g$-min. The resulting supernatant was then centrifuged for $2\times10^6 g$-min giving a pellet which was suspended in the sucrose solution described above. All these operations were performed at 0–4°C. The yield of calcium-particles was 30–40% on a protein basis. The characteristic property of calcium-particles is that in the presence of 0.1 μM-free Ca^{2+} the oxidation of L-3-glycerophosphate follows Michaelis–Menten kinetics whereas in the absence of free Ca^{2+} the velocity–substrate concentration plot is sigmoidal (Donnellan & Beechey, 1969). Freshly prepared mitochondria were used as a routine since it was observed that calcium-particles prepared from mitochondria stored at −20°C did not show this characteristic pattern of L-3-glycerophosphate oxidation.

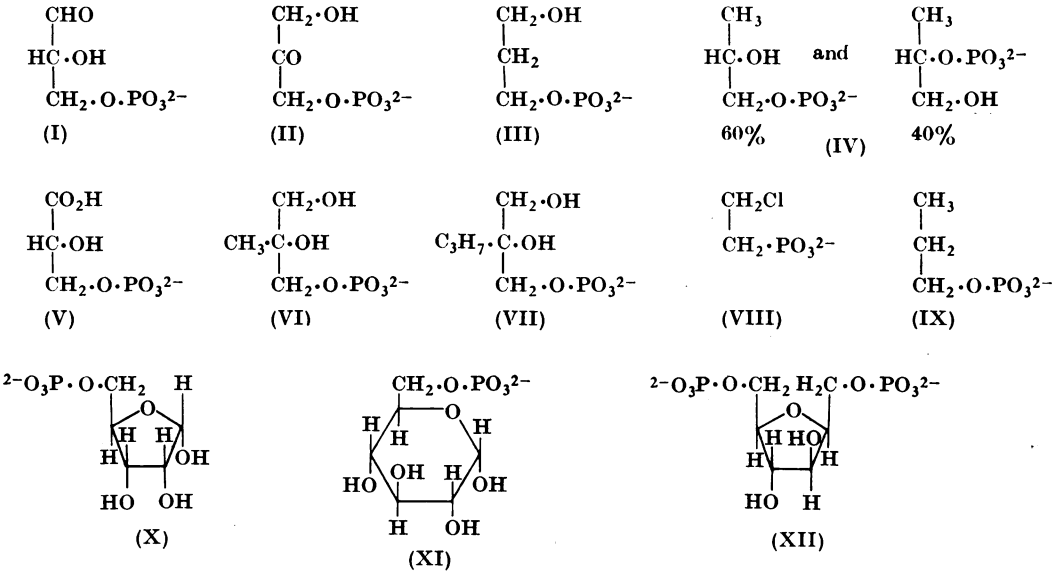
Assay of protein. Protein was measured colorimetrically by the biuret method of Gornall, Bardawill & David (1949), after solubilization of the protein with 1.5% (w/v) potassium cholate.

Assay of respiration rates. Oxygen concentrations were measured with a Beckman oxygen electrode inserted into a Perspex cell, volume 2ml. Mitochondrial suspension (50 μl, containing approx. 0.5mg of protein) was added to 1.95ml of a reaction mixture (pH7.1) that contained 0.154M-KCl, 10mM-orthophosphate, 25 μM-2,4-dinitrophenol and 2mM-ethylenedioxybis(ethylamine)tetraacetate. When indicated 10 μl of a 200mM-$CaCl_2$ solution was added to give a final buffered free Ca^{2+} concentration of 0.1 μM. 2,4-Dinitrophenol was included in the reaction mixture to avoid complications in measurements of rates of respiration due to respiratory control. When the effect of a compound on the oxidation of L-3-glycerophosphate was studied, a solution of the compound (not greater than 60 μl, pH7.1) was added to the reaction vessel. DL-3-Glycerophosphate (1M) was then added with a microsyringe to give the required concentration. Respiration rates were measured by following the change in oxygen concentration with time.

Assay of energy-dependent reactions. The ATP-driven reduction of NAD^+ by either succinate or L-3-glycerophosphate and the aerobic energy-linked nicotinamide nucleotide transhydrogenase were assayed at 30°C with the conditions described by Beechey, Roberton, Holloway & Knight (1967).

Preparation of ammonium salt of DL-*3-glycerophosphoric acid.* A 1mmol portion of disodium DL-3-glycerophosphate hexahydrate was dissolved in 7.8ml of methanol at room temperature (heating causes the salt to come out of solution). An equal volume of methanol saturated with HCl was added, together with 14ml of diethyl ether to ensure complete precipitation of the NaCl. After filtration the solvent and HCl were removed *in vacuo*. The resultant free acid was neutralized with NH_3. This solution was diluted and used directly in experiments. No attempt was made to estimate the amount of the 2-glycerophosphate present since at equilibrium there is only 13.5% of the latter (Kugel & Halmann, 1966).

Preparation of ammonium pyruvate. A solution of sodium pyruvate was adjusted to pH1 and the free acid was extracted with diethyl ether. Excess of solvent was



removed *in vacuo* and the resulting free acid was neutralized with NH_3.

Structure, nomenclature and synthesis of L-3-*glycerophosphate analogues used.* The following compounds were either synthesized or purchased as indicated below.

(*a*) Compound (I), DL-glyceraldehyde 3-phosphate. An aqueous solution of the free acid was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. It was adjusted to pH 7.1 with NaOH.

(*b*) Compound (II), dihydroxyacetone phosphate. The dimethylketal dimonocyclohexylamine salt monohydrate was purchased from the Sigma Chemical Co. The ketal was prepared by ion-exchange chromatography with Dowex 50 (X4; H^+ form) and then hydrolysed by heating the resultant aqueous solution at 40°C for 3h as directed by the suppliers. The pH was adjusted to 7.1 with K_2CO_3.

(*c*) Compound (III), 3-hydroxypropyl phosphate. Propane-1,3-diol was phosphorylated and the product isolated as the dicyclohexylamine salt by the method of Kirby (1963). This was converted into the sodium salt by stirring a solution of the dicyclohexylamine salt with a large excess of Dowex 50 (Na^+ form), followed by filtration and evaporation.

(*d*) Compound (IV), a mixture of 2-hydroxypropyl phosphate and 2-hydroxyisopropyl phosphate. Propane-1,2-diol was phosphorylated and the sodium salt of the product isolated by the methods described for the preparation of compound (III). From the proton-magnetic-resonance spectrum it was shown that the product contained 60% of 2-hydroxypropyl phosphate and 40% of 2-hydroxyisopropyl phosphate.

(*e*) Compound (V), D-glyceric acid 3-phosphate. The sodium salt was purchased from the Sigma Chemical Co.

(*f*) Compound (VI), 2-methylglycerol 1-phosphate. This was prepared from β-methallyl alcohol exactly as described for the preparation of compound (VII). The barium salt was isolated in 30% yield (Found: C, 14.1; H, 3.0; P, 8.5; $C_4H_9BaO_6P$ requires: C, 15.0; H, 2.8; P, 9.6%).

(*g*) Compound (VII), 2-*n*-propylglycerol 1-phosphate. Pentan-1-al was converted into 2-methylenepentan-1-al by the method of Marvel, Myers & Saunders (1948). The crude product was used as it tends to polymerize on distillation.

Crude 2-methylenepentan-1-al (approx. 137g) was stirred into methanol (200ml) while a solution of $NaBH_4$ (14.4g) in aq. 66% (v/v) methanol (100ml) was added dropwise at 35–40°C (Chaikin & Brown, 1949). The solution was stirred for a further 90min, NaOH (32g) in water (800ml) was added and the solution was heated under reflux for a further 1h. The methanol was removed under reduced pressure, the residual aqueous layer extracted with diethyl ether and the ether layer dried over $MgSO_4$. After removal of the diethyl ether the residual oil was distilled to give 2-methylenepentan-1-ol (64g), b.p. 49–50.5°C/5mmHg. The yield was 47.3%.

2-Methylenepentan-1-ol (64g) was dissolved in water (150ml) and 50ml of catalyst (prepared by adsorption of $NaHWO_4$ on 80ml of granular charcoal) added (Gable, 1959). The pH of the solution was adjusted to 4.5–5.0. A solution of H_2O_2 (78ml of 100vol. H_2O_2 in 200ml of water) was added dropwise at 50°C and the solution stirred at this temperature for 2h, after which the solution was heated under reflux for a further 2h to complete hydration of the epoxide. The glycerol was continuously extracted from the aqueous solution with diethyl ether (24–48h). The ether solution was shaken once with water, the glycerol dissolving in the water and leaving impurities in the ether. The aqueous solution was evaporated to leave 2-*n*-propylglycerol (41g, 49% yield) (Found: C, 53.4; H, 10.4; $C_6H_{14}O_3$ requires: C, 53.7; H, 10.5%). 2-*n*-Propylglycerol (34.1g), anhydrous acetone (60g), light petroleum (b.p. 40–60°C, 150ml) and toluene-*p*-sulphonic acid (1g) were stirred together under reflux by using a Vigreux

column (2cm × 40cm) and a Dean & Stark trap for removal of water formed in the reaction. After 24h, when the reaction was essentially complete, fused sodium acetate (1g) was added and the suspension stirred at room temperature for 30min. After filtration of the solids, the solvents were removed and the residual oil was distilled at reduced pressure to give 2-*n*-propylisopropylideneglycerol (36.7g, 83% yield), b.p. 84–85°C/3mmHg (Found: C, 62.1; H, 10.4; $C_9H_{18}O_3$ requires: C, 62.0; H, 10.4%).

2-*n*-Propylisopropylideneglycerol (7.4g) was dissolved in dry quinoline (40ml) and added dropwise to a solution of phosphorus oxychloride (15.3g) dissolved in dry quinoline (50ml) at a temperature between −15° and −20°C. The suspension was stirred at −20°C for 30min after the addition, then the temperature was allowed to rise to ambient and stirring was continued for 1h. It was then poured into ice–water (1litre) containing conc. H_2SO_4 (39g). The HCl liberated was removed by addition of Ag_2CO_3 (45g), filtered and excess of silver removed by passage of H_2S gas and filtration. The acid filtrate was heated on a boiling-water bath for 15min, cooled to ambient temperature, $Ba(OH)_2,8H_2O$ (173g) was added and the solution stirred for 1h. It was then centrifuged and the clear supernatant was extracted with ether. Passage of CO_2 gas through the residual aqueous solution removed excess of Ba^{2+} and after filtration water was evaporated off until the volume was approx. 70ml. This was heated on a water bath and the precipitated barium salt was filtered from the hot suspension. After drying 13.3g (38%) was obtained (Found: C, 22.5; H, 4.2; P, 9.5; $C_6H_{13}BaO_6P$ requires: C, 20.6; H, 3.7; P, 8.9%).

The barium salt was converted into the sodium salt by treatment of an aqueous solution with an excess of Amberlite IR-120 (Na^+ form), filtration and evaporation of the aqueous solution.

(*h*) Compound (VIII), 2-chloroethyl phosphonate. Commercial material, purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., was dissolved in acetone and the solution treated with excess of dicyclohexylamine. The precipitated salt was filtered off, recrystallized from ethanol and converted into the sodium salt with an excess of Dowex 50 (Na^+ form).

(*j*) Compound (IX), *n*-propyl phosphate. *n*-Propanol was phosphorylated by the method of Kirby (1963). The product was isolated as the dicyclohexylamine salt which was then converted into the sodium salt with Dowex 50 (Na^+ form).

(*k*) Compound (X), D-ribose 5′-phosphate. The disodium salt was purchased from the Sigma Chemical Co.

(*l*) Compound (XI), α-D-glucose 6-phosphate. The disodium salt was purchased from the Sigma Chemical Co.

(*m*) Compound (XII), D-fructose 1,6-diphosphate. The sodium salt, 98–100% pure, was purchased from the Sigma Chemical Co.

RESULTS

Oxidation of various substrates by flight-muscle mitochondria. The limited ability of insect flight-muscle mitochondria to oxidize intermediates of the tricarboxylic acid cycle and related amino acids has been long appreciated. The results listed in Table 1 show that the flight-muscle mitochondria isolated from *S. barbata* are no exception. Succinate, L-malate, L-proline, L-glutamate, 2-oxoglutarate, L-alanine and pyruvate are all oxidized at rates below 30nmol min^{-1} mg^{-1} of protein. However, when a secondary substrate is available to generate

Table 1. *Oxidation of various substrates by mitochondria isolated from the flight muscle of* S. barbata

The respiration rates were measured with an oxygen electrode as described in the Methods section except that 2,4-dinitrophenol was omitted from the reaction mixture. Substrates were added as molar solutions at pH7.1. ADP-stimulated rates were measured after the addition of 10μl of 40mM-ADP, pH7.1. Mitochondria added: 0.27mg of protein when L-3-glycerophosphate was the substrate; 0.67mg of protein for other substrates.

Substrate	Concn. (mM)	ADP-stimulated respiration rate (nmol of oxygen min^{-1} mg^{-1} of protein)	ADP/O ratio	Respiratory-control ratio
L-3-Glycerophosphate	25	400	1.5	2.3
L-3-Glycerophosphate +0.1 μM-Ca^{2+}	5	420	1.5	2.3
Succinate	10	21	—	—
L-Malate	10	11		
+pyruvate	10	183	2.8	6.0
L-Proline	25	31		
+pyruvate	5	219	2.7	6.0
L-Glutamate	10	7		
+L-malate	2.5	19	—	—
Pyruvate	10	12		
+L-proline	2.5	252	2.9	12.5
2-Oxoglutarate	10	9		
+L-alanine	10	57	3.0	3.0
L-Alanine	10	7		
+2-oxoglutarate	10	64	2.7	2.0

oxaloacetate then pyruvate is oxidized at high rates with ADP/O ratios close to 3 and respiratory control ratios in the range 5–14. Other experiments have shown that L-malate can act in this capacity but L-proline is far more effective, as first shown by Childress & Sacktor (1966) with flight-muscle mitochondria isolated from *Phormia regina*. A significant rate of oxygen consumption is found with L-alanine plus 2-oxoglutarate as mixed substrate, but singly these are poor substrates. The L-alanine–2-oxoglutarate aminotransferase (EC 2.6.1.2) present in these mitochondria (sp. activity 100–150 nmol min^{-1} mg^{-1} of protein) presumably forms pyruvate, which is then oxidized. L-3-Glycerophosphate is oxidized at a rate in excess of all the other substrates, maximum rates of oxidation (about 400 nmol min^{-1} mg^{-1} of protein) being seen either at high concentrations (25 mM) of L-3-glycerophosphate or at low concentrations (5 mM) in the presence of 0.1 μM-Ca^{2+}. The ADP/O ratio is in the range of 1.5–1.9 and the respiratory control ratio is low (2–4) as noted by other workers (Hansford & Chappell, 1967).

Effects of organophosphates and phosphonates on the oxidation of L-*3-glycerophosphate by flight-muscle mitochondria.* The effects of compounds (I)–(XII) on the oxidation of L-3-glycerophosphate were studied, both in the presence and absence of 0.1 μM-Ca^{2+}. None of these compounds were oxidized by flight-muscle mitochondria.

(*a*) In the presence of 0.1 μM-Ca^{2+}. Compound (I) at concentrations of 0.8 mM and below proved to be a potent competitive inhibitor of the oxidation of L-3-glycerophosphate, as is shown by the results illustrated in Fig. 1. This is a double-reciprocal plot of L-3-glycerophosphate concentration against the rate of oxygen consumption at different concentrations of compound (I). The calculated K_i is 70 μM. The following compounds also inhibited by a similar mechanism: (II) K_i 7 mM; (III) K_i 12 mM; (IV) K_i 15 mM; (V) K_i 20 mM. At concentrations of compound (I) greater than 0.8 mM the mechanism of inhibition became more complex; the reciprocal plots were not typical of competitive inhibition and may indicate a non-competitive inhibition.

Compounds (VI)–(XII) had no effect on the oxidation of L-3-glycerophosphate under these conditions, indicating that these compounds do not bind at the catalytic substrate-binding site of the L-3-glycerophosphate–flavoprotein oxidoreductase. As compounds (VI) and (VII) only differ from the substrate by substitutions of methyl and *n*-propyl residues for the hydrogen atom on C-2, this suggests that there are severe steric limitations to the binding

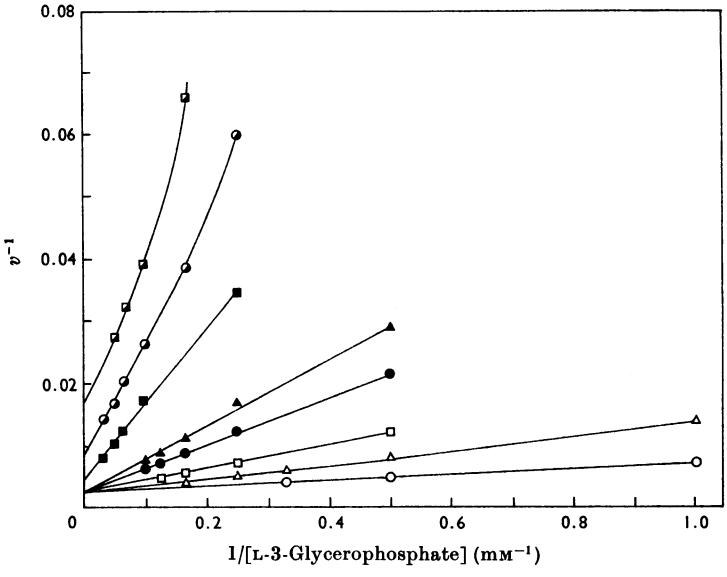


Fig. 1. Competitive inhibition by DL-glyceraldehyde 3-phosphate (compound I) of the oxidation of L-3-glycerophosphate by flight-muscle mitochondria in the presence of 0.1 μM-Ca^{2+}. The respiration rates were measured with an oxygen electrode as described in the Methods section. A portion of 0.163 M-DL-glyceraldehyde 3-phosphate was added to the reaction mixture to give the required concentration. Flight-muscle mitochondrial suspension (0.53 mg of protein) was then added. Samples of M-DL-3-glycerophosphate were added successively and the respiration rates measured after each addition. Concentrations of DL-glyceraldehyde 3-phosphate: ○, nil; △, 81 μM; □, 204 μM; ●, 408 μM; ▲, 815 μM; ■, 1.63 mM; ◑, 3.27 mM; ◪, 6.54 mM.

of compounds at that region of the active site which is adjacent to C-2 of bound L-3-glycerophosphate.

The absence of inhibition by compound (IX) suggests that the hydroxyl groups on C-2 and C-3 of L-3-glycerophosphate are involved in substrate binding at the catalytic site. However, the presence of hydroxyl groups on both C-2 and C-3 is not essential because compound (III) and the mixture of compounds (IV), which are deoxy analogues of L-3-glycerophosphate, are both competitive inhibitors. In view of the steric limitations at the catalytic site in the region of the C-2 of L-3-glycerophosphate revealed by compounds (VI) and (VII) it is probable that it is the 2-hydroxypropylphosphate component of the mixture (IV) which is the inhibitory compound.

The length of the carbon chain seems to be important for binding at the catalytic site. All the competitive inhibitors are C_3 compounds. Increasing the chain length of the most potent inhibitor, compound (I), by the insertion of two or three hydroxymethylene groups to give compounds (X), (XI) and (XII) completely obliterates the inhibitory effect.

The affinity of compound (I) for the catalytic site is some 20-fold greater than the apparent affinity of the substrate. Molecular models show that the carbonyl oxygen atom of L-glyceraldehyde 3-phosphate can occupy a site almost identical with that of the hydroxyl oxygen atom on C-3 of L-3-glycerophosphate, with the common components of these molecules occupying identical conformations. Dawson & Thorne (1969) showed that the L-3-glycerophosphate–flavoprotein oxidoreductase of pig brain mitochondria was inhibited by L-3-glyceraldehyde 3-phosphate (K_i 35μM) but relatively weakly by the D-enantiomorph (K_i 500μM). It would seem that there is some functional group with a high affinity for the substrate carbonyl groups on the enzyme protein near the enzymic site. However, molecular models of compound (V) show that the carboxyl carbonyl group lies some 2Å above this binding site and with this compound the inhibition is relatively weak, K_i 20mM. Whereas the carbonyl oxygen atom of compound (II) can occupy the site occupied by the hydroxyl group on C-2 of L-3-glycerophosphate, the carbon chain is twisted somewhat out of the geometry occupied by compounds (I) and (III)–(V), again resulting in a weak inhibition, K_i 7mM.

(*b*) In the absence of Ca^{2+}. Compounds (VI)–(XII) at concentrations in the range 5–40mM act as homotropic effectors of the L-3-glycerophosphate flavoprotein oxidoreductase. They enhance the velocity of the oxidation of L-3-glycerophosphate and also change the shape of the velocity versus substrate concentration plots from sigmoidal to hyperbolic curves. A typical set of results is shown in Fig. 2. In this experiment the addition of increasing concentrations of compound (VIII) to flight-muscle mitochondria oxidizing L-3-glycerophosphate causes the effects described above.

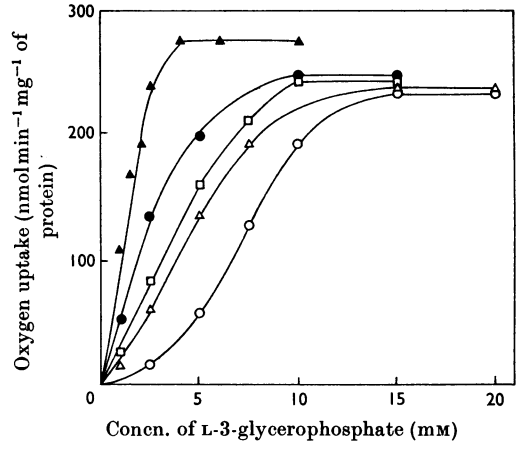


Fig. 2. Effect of 2-chloroethyl phosphonate (compound VIII) on the oxidation of L-3-glycerophosphate by flight-muscle mitochondria in the absence of Ca^{2+}. The respiration rates were measured with an oxygen electrode as described in the Methods section. A sample of M-2-chloroethyl phosphonate was added to the reaction mixture to give the required concentration. Flight-muscle mitochondrial suspension (0.68mg of protein) was then added. Portions of M-DL-3-glycerophosphate were added successively and the respiration rates were measured after each addition. Concentrations of 2-chloroethyl phosphonate: ○, nil; △, 10mM; □, 20mM; ●, 30mM; ▲, nil+0.1μM-Ca^{2+}.

Compounds (VI)–(XII) appear to be acting at the effector L-3-glycerophosphate-binding site inducing co-operative phenomena between protomers of the L-3-glycerophosphate–flavoprotein oxidoreductase, so enhancing the affinity of the enzyme for the substrate. This hypothetical mode of action is supported by the changes in the slope of the Hill plot. In agreement with the results of Hansford & Chappell (1967) for concentrations of L-3-glycerophosphate below 10mM in the absence of 0.1μM-Ca^{2+} the slope is 2, whereas in the presence of 0.1μM-Ca^{2+} it is unity. In the presence of increasing concentrations of compounds (VI)–(XII) there is a graded decrease of the slope from 2 to unity, indicating association of the protomers.

Compounds (I) and (II) which act as competitive inhibitors in the presence of 0.1μM-Ca^{2+} also inhibit the oxidation of L-3-glycerophosphate in the absence of Ca^{2+}. This is illustrated in the experiment shown in Fig. 3. It can be seen that the addition of increasing concentrations of compound (I) causes a decrease in the rate of oxidation of L-3-glycerophosphate. However, the form of the

velocity versus substrate concentration plot changes from a sigmoidal curve in the absence of compound (I) to a hyperbolic curve in the presence of 1.63mM-compound (I). We believe that compounds (I) and (II) are acting simultaneously as homotropic effectors at the non-enzymic L-3-glycerophosphate-binding site and as competitive inhibitors at the enzymic site. The positive action of these compounds at the effector site is again supported by graded changes in the slope of the Hill plot at low L-3-glycerophosphate concentrations from 2 to unity.

Compounds (III), (IV) and (V), which act as competitive inhibitors in the presence of 0.1μM-Ca^{2+}, do not inhibit oxidation in the absence of Ca^{2+} but act as homotropic effectors under these conditions. The pattern of stimulation is similar to that shown by compounds (VI)–(XII) (see Fig. 2).

It is concluded from these results that the substrate specificity of the enzymic and effector L-3-glycerophosphate-binding sites are markedly different.

Reduction of cyanoferrate catalysed by flight-muscle mitochondria and by calcium-particles. If the L-3-glycerophosphate–flavoprotein oxidoreductase is located on the cristal membrane in such a manner that the substrate must approach the enzymic site from the matrix space, this would imply a requirement for a porter system to facilitate the entry of L-3-glycerophosphate into the matrix space. The experiments with analogues of L-3-glycerophosphate showed the ease with which the activity of the L-3-glycerophosphate–flavoprotein oxidoreductase can be modified by a wide range of compounds ranging from C-2 to C-6. These results would also then imply that the specificity of the porter system was very low. In view of the relative impermeability of flight-muscle mitochondria to most substrates (Van den Bergh, 1964; and see Table 1) this implied low specificity seems improbable. The results of the following experiments with cyanoferrate as an impermeant electron acceptor (Estabrook, 1961; Mitchell & Moyle, 1969; unpublished data of F. Urban & M. Klingenberg, quoted by Klingenberg, 1968) suggests the location of the L-3-glycerophosphate–flavoprotein oxidoreductase on the external surface of the cristal membrane.

The results illustrated in Fig. 4(*a*) show that with pyruvate plus proline as mixed substrates the reduction of cyanoferrate catalysed by flight-muscle mitochondria proceeds at the rate of 2.48μmol min^{-1} mg^{-1} of protein. This is in a system where cyanide was present to prevent oxygen acting as an electron acceptor and where excess of ADP was added to ensure that electron transport was not limited by absence of phosphate acceptor. The addition of 0.2μg of antimycin A decreased electron transport to 8% of its former rate. We concluded

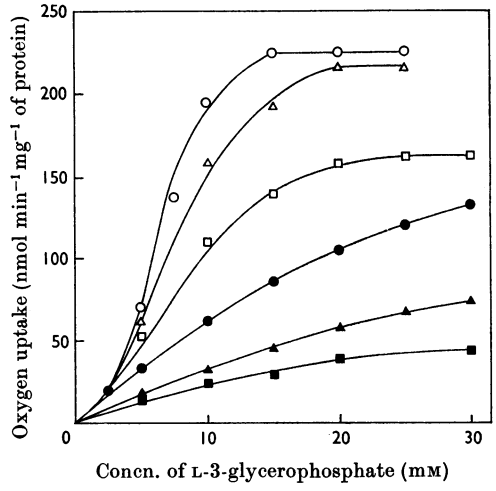


Fig. 3. Effect of DL-glyceraldehyde 3-phosphate (compound I) on the oxidation of L-3-glycerophosphate by flight-muscle mitochondria in the absence of Ca^{2+}. The respiration rates were measured with an oxygen electrode as described in the Methods section. A portion of 0.163M-DL-glyceraldehyde 3-phosphate was added to the reaction mixture to give the required concentration. Flight-muscle mitochondrial suspension (0.53mg of protein) was then added. Portions of a M-DL-3-glycerophosphate solution were added successively and the respiration rates were measured after each addition. Concentrations of DL-glyceraldehyde 3-phosphate: ○, nil; △, 0.41mM; □, 0.81mM; ●, 1.63mM; ▲, 3.27mM; ■, 6.54mM.

from this observation that in the intact mitochondrion there is no component of the operative electron-transfer chain from NADH to cyanoferrate lying on the substrate side of the antimycin A block that can interact with cyanoferrate, if in the absence of antimycin A it is assumed that cyanoferrate interacts with cytochromes $c+c_1$ (which are located on the external surface of the cristal membrane; Tyler, 1970; Klingenberg, 1968).

However, the addition of 5mM-L-3-glycerophosphate (Fig. 4*a*) to the antimycin-inhibited system causes cyanoferrate to be reduced at a rate of 5.1μmol min^{-1} mg^{-1} of protein. This indicates that some component of the electron-transport chain from the L-3-glycerophosphate–flavoprotein oxidoreductase to the antimycin A site can interact with cyanoferrate. The previous result rules out cytochrome *b* and ubiquinone since these are common to the L-3-glycerophosphate and NADH oxidase systems. Thus either the L-3-glycerophosphate–flavoprotein oxidoreductase or a possible associated non-haem iron protein is capable of interacting with cyanoferrate. In agreement with the results of Estabrook & Sacktor (1958), i.e. that iron-chelating agents had little or no effect on the oxidation of

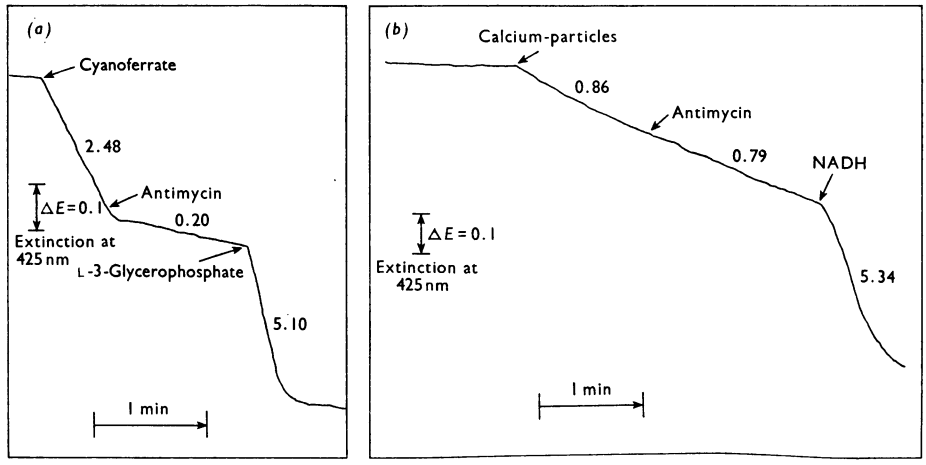


Fig. 4. Reduction of cyanoferrate as catalysed by (*a*) flight-muscle mitochondria and (*b*) calcium-particles. All solutions (pH 7.1) were pre-equilibrated to 25°C and reactions (final volume 2ml) were carried out at this temperature. The numbers quoted give the rates of reduction of cyanoferrate in μmol min^{-1} mg^{-1} of protein. These were calculated by using ϵ_{425}^{mM} 1 (Ibers & Davidson, 1951). (*a*) Flight-muscle mitochondrial suspension (0.78mg of protein) was added to a cuvette containing 133mM-KCl, 10mM-orthophosphate, 2mM-ethylenedioxybis(ethylamine)tetra-acetate, 10mM-pyruvate, 2.5mM-L-proline and 1.2mM-ADP. The contents were incubated for 1min to ensure the onset of a maximum rate of substrate oxidation before the addition of 1mM-KCN. Extinction at 425nm was then followed for 2min to check that the mitochondria were neither swelling nor shrinking before the further addition of 0.75mM-cyanoferrate. Antimycin (0.2μg) and 5mM-L-3-glycerophosphate were added as indicated. (*b*) Calcium-particle suspension (0.3mg of protein) was added to a cuvette containing 143mM-KCl, 10mM-orthophosphate, 2mM-ethylenedioxybis(ethylamine)tetra-acetate, 0.1 μM-free Ca^{2+} and 5mM-L-3-glycerophosphate. KCN (1mM) and cyanoferrate (0.75mM) were then added and the change in extinction at 425nm was monitored. Antimycin (0.2μg) and 32.5μM-NADH were added where indicated.

L-3-glycerophosphate in flight-muscle mitochondria, we noted that concentrations of thenoyltrifluoroacetone [4,4,4-trifluoro-1-(2-thienyl)butane-1,3-dione] up to 0.5mM had little effect on the oxidation of L-3-glycerophosphate by either mitochondria or submitochondrial particles. This concentration is approximately 50-fold greater than that required to inhibit the oxidation of succinate by rat liver mitochondria (Tyler, Gonze & Estabrook, 1966), suggesting the absence of a readily accessible non-haem iron protein associated with the oxidation of L-3-glycerophosphate. The overall conclusion from this experiment is that the L-3-glycerophosphate–flavoprotein oxidoreductase is located on the external surface of the cristal membrane, in the sense that it can interact with the non-permeant anion, cyanoferrate.

A corollary to this conclusion is that submitochondrial particles should have a lower capacity to oxidize L-3-glycerophosphate than intact mitochondria. This point is illustrated in Fig. 4(*b*). In this experiment cyanide-treated calcium-particles were prepared from the same batch of mitochondria used in Fig. 4(*a*). With cyanoferrate as electron acceptor the calcium-particles oxidize L-3-glycerophosphate at a low rate (0.86μmol min^{-1} mg^{-1} of protein) relative to that noted for intact mitochondria (5.1μmol min^{-1} mg^{-1} of protein). This reaction is not inhibited by the addition of antimycin A. Since there is no solubilization of the L-3-glycerophosphate–flavoprotein oxidoreductase on the sonication of mitochondria we propose that this relatively low rate is due to the enzyme being sited on the inner surface of the calcium-particle vesicle and that L-3-glycerophosphate must now enter the lumen of the calcium-particle through leaks in the membrane to reach the enzyme. In direct contrast NADH (which is oxidized at a low rate by intact mitochondria) is now oxidized by the calcium-particles at a rapid rate (5.34μmol min^{-1} mg^{-1} of protein) which is independent of the presence of antimycin. This insensitivity to antimycin is due to the location of NADH dehydrogenase and cytochrome *b* on the outer surface of the calcium-particle, which can now interact with cyanoferrate (Tyler, 1970; Klingenberg, 1968).

Ability of calcium-particles to catalyse partial reactions of energy conservation. Coupled submitochondrial particles prepared from mammalian mitochondria by sonication have the ability to

catalyse the reduction of NAD^+ by succinate and ATP and the nicotinamide nucleotide transhydrogenase reaction with the energy supplied by the operation of the succinate oxidase system. These reactions proceed easily since the inversion of the mitochondrial membrane (Lee & Ernster, 1966) results in the location of both the succinate–flavoprotein oxidoreductase and NADH–flavoprotein oxidoreductase on the outer surface of the submitochondrial particle. Hence there are now no barriers to the access of succinate and NAD^+ to their respective flavoprotein–oxidoreductases.

The inability of intact flight-muscle mitochondria to oxidize succinate and NADH (Tables 1 and 2 and B. Sacktor & C. C. Childress, unpublished work quoted in Childress & Sacktor, 1966) at high rates compared with those measured with calcium-particles (Table 2 and Fig. 4*b*) suggests a similar locale for the succinate–flavoprotein oxidoreductase and NADH–flavoprotein oxidoreductase, i.e. on the inner surface of the cristal membrane. Thus it could be predicted that the ATP-driven reduction of NAD^+ by succinate and the aerobic energy-linked nicotinamide nucleotide transhydrogenase coupled to the oxidation of succinate should proceed rapidly in calcium-particles, but that the corresponding reactions where succinate is replaced by L-3-glycerophosphate should not proceed at all if the membrane is impermeable to L-3-glycerophosphate and the L-3-glycerophosphate–flavoprotein oxidoreductase is located on the inner surface of the calcium-particle vesicle. The results listed in Table 3 show that in calcium-particles the ATP-driven reduction of NAD^+ by succinate can proceed at rates comparable with those measured in submitochondrial particles isolated from ox heart mitochondria. However, when L-3-glycerophosphate is the source of reducing power the reaction proceeds at a rate that is no greater than 29% of the rate with succinate. Whereas the rapid rate of the succinate-driven reaction is in accord with prediction, the lower but significant rate of the L-3-glycerophosphate-driven reaction can only be explained in terms of L-3-glycerophosphate being able to penetrate the membrane through leaks.

The damaged nature of the membrane of calcium-particles is illustrated by the results listed in Table 3. It can be seen that the addition of cytochrome *c* to the reaction mixture enhanced the rates of oxidation of L-3-glycerophosphate and NADH by 52 and 32% respectively. In submitochondrial particles of ox heart, cytochrome *c* is located on the inner surface of the vesicle membrane and added cytochrome *c* has no effect on respiration rates. The ability of cytochrome *c* to enhance respiration rates to such a large extent in calcium-particles suggests that this relatively large molecule is able to penetrate the membrane. Under these circumstances it is not surprising that L-3-glycerophosphate is oxidized. However, the lower rate of the ATP-driven reduction of NAD^+ by L-3-glycerophosphate when compared with that when succinate is the reducing agent might be interpreted as indicating that the calcium-particles are not uniform in nature. Particles with a highly damaged, i.e. permeable, membrane could be responsible for the high rate of oxidation of L-3-glycerophosphate which is stimulated by the addition of cytochrome *c*; these particles would be less able to catalyse the ATP-driven reduction of NAD^+. However, those particles with relatively undamaged membranes, though still somewhat permeable to L-3-glycerophosphate, would be

Table 2. *Comparison of respiration rates of intact flight-muscle mitochondria and calcium-particles*

The respiration rates were measured with an oxygen electrode as described in the Methods section except that 2,4-dinitrophenol was omitted from the reaction mixture; 12.5 μM-cytochrome *c* (horse heart) was added where indicated. Substrates were added as molar solutions at pH 7.1. ADP-stimulated rates were measured after the addition of 10 μl of 40 mM-ADP, pH 7.1. Mitochondria added: 0.13 mg of protein with L-3-glycerophosphate as substrate; 0.27 mg of protein with NADH; 0.5 mg of protein with both pyruvate + proline and succinate. Calcium-particles added: 0.06 mg of protein with NADH as substrate; 0.29 mg of protein with L-3-glycerophosphate or succinate as substrate. When succinate was the substrate the calcium-particles were preincubated with 0.28 M-succinate for 5 min at 30°C before the respiration rate was assayed.

		Respiration rates (nmol of oxygen $min^{-1} mg^{-1}$ of protein)			
		Mitochondria		Calcium-particles	
Substrate	Concn. (mM)	No ADP	+ADP	No cytochrome *c*	+cytochrome *c*
L-3-Glycerophosphate +0.1 μM-Ca^{2+}	10	250	590	372	565
L-Proline	10				
+pyruvate	10	28	273	0	0
Succinate	10	14	14	106	97
NADH	1	38	38	755	1110

Table 3. *Ability of calcium-particles to catalyse the ATP-driven reduction of NAD$^+$ by either succinate or* L-*3-glycerophosphate*

The activity was measured as described in the Methods section. Four different preparations of calcium-particles were used. The amounts of protein/assay were: 1, 0.71mg; 2, 0.57mg; 3, 0.8mg and 4, 0.72mg.

Expt.	Substrate	Concn. (mM)	ATP-driven reduction of NAD$^+$ (μmol reduced min^{-1} mg^{-1} of protein)	$\frac{\text{L-3-Glycerophosphate rate}}{\text{Succinate rate}} \times 100$
1	L-3-Glycerophosphate	1.67	3.4	3.1
	Succinate	5.0	107.4	—
2	L-3-Glycerophosphate	3.1	22.6	18.3
	Succinate	5.0	123.6	—
3	L-3-Glycerophosphate	7.5	1.6	8.4
	Succinate	5.0	19.0	—
4	L-3-Glycerophosphate	8.2	5.2	29.0
	Succinate	5.0	17.9	—

more tightly coupled and able to catalyse the ATP-driven reduction of NAD$^+$ by L-3-glycerophosphate and also oxidize L-3-glycerophosphate at a lower rate.

Even in the presence of oligomycin to inhibit the hydrolysis of high-energy intermediates, it proved impossible to demonstrate the presence of an energy-linked nicotinamide nucleotide transhydrogenase with either succinate or L-3-glycerophosphate as energy sources. Since it has been our consistent experience that this reaction system is the most stable of all the partial reactions of energy conservation in submitochondrial particles isolated by sonication from ox heart (I. G. Knight & R. B. Beechey, unpublished work) and Haas (1964) has shown that this reaction is catalysed by non-phosphorylating ox heart submitochondrial particles, we conclude that the nicotinamide nucleotide transhydrogenase system is absent from flight-muscle mitochondria.

Swelling of flight-muscle mitochondria in iso-osmotic solutions of ammonium salts. When mitochondria are suspended in iso-osmotic solutions of ammonium salts they swell only if the anion is capable of penetrating the inner mitochondrial membrane (Chappell & Haarhoff, 1967). The results presented in Fig. 5 show that flight-muscle mitochondria do not swell in the presence of the ammonium salt of DL-3-glycerophosphoric acid. Ammonium succinate and ammonium L-malate cause the mitochondria to swell to a small extent. However, the swelling in ammonium pyruvate is extensive and continues for 3–4min. The addition of 5mM-orthophosphate does not affect the rates of swelling shown in Fig. 5, in contrast with the results of Chappell & Haarhoff (1967), who showed that, for rat liver mitochondria, swelling in ammonium succinate and ammonium malate was dependent on the presence of orthophosphate. These results

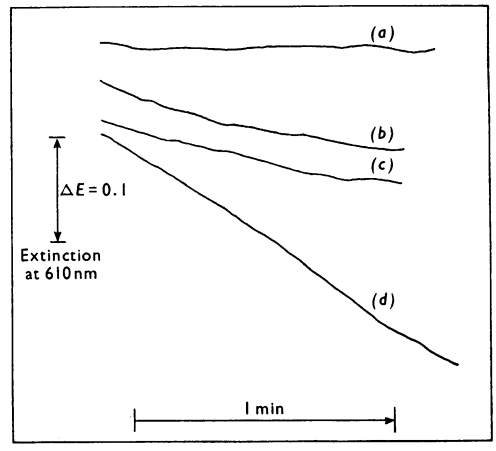


Fig. 5. Light-scattering changes of flight-muscle mitochondria suspended in iso-osmotic solutions of ammonium salts. A 2.5ml portion of a solution containing 1mM-ethylenedioxybis(ethylamine)tetra-acetate, 5mM-tris chloride buffer and the appropriate ammonium salt, pH7.4, at 30°C were added to spectrophotometer cuvette. Mitochondrial suspension, 0.1ml (4mg of protein) containing 1μg of rotenone, was then added. The extinction changes at 610nm were followed with time with a Zeiss PMQII spectrophotometer fitted with a slave recorder. Trace (*a*), 0.08M-ammonium DL-3-glycerophosphate; trace (*b*), 0.08M-ammonium succinate; trace (*c*), 0.08M-ammonium L-malate; trace (*d*), 0.125M-ammonium pyruvate.

suggest that DL-3-glycerophosphate does not enter the mitochondrial matrix space. The slow and limited swelling noted with ammonium L-malate and ammonium succinate suggest that they can enter the matrix space to a limited extent. This observation is in accord with the low rates of respiration noted with these substrates (see Table 1).

The extensive swelling seen with ammonium pyruvate suggests that either the pyruvate anion or the undissociated acid can freely enter the matrix space, thus facilitating its rapid oxidation.

DISCUSSION

The described effects of substrate analogues on the oxidation of L-3-glycerophosphate by flight-muscle mitochondria indicate that the catalytic and allosteric L-3-glycerophosphate-binding sites on the L-3-glycerophosphate–flavoprotein oxidoreductase have markedly different specificities. The specificity of the catalytic site has been studied in the presence of saturating amounts of the heterotropic effector, Ca^{2+}, to fully activate the oxidoreductase and thus allow the exhibiton of Michaelis–Menten kinetics for the oxidation of L-3-glycerophosphate. Under these conditions only compounds (I)–(V) were found to be competitive inhibitors of the oxidation with DL-glyceraldehyde 3-phosphate, compound (I) being by far the most potent, K_i 70 μM. Dawson & Thorne (1969), using a solubilized preparation of the L-3-glycerophosphate–flavoprotein oxidoreductase obtained by detergent treatment of pig brain mitochondria, reported K_i 66 μM for this compound, with the L-isomer (K_i 33 μM) being a more effective inhibitor of L-3-glycerophosphate oxidation than the D-isomer (K_i 500 μM).

Dihydroxyacetone phosphate (compound II) is a competitive inhibitor with K_i 7 mM, which compares with K_i 180 μM reported by Ringler & Singer (1959) using a phospholipase A-solubilized preparation of the oxidoreductase prepared from pig brain mitochondria. In contrast, both Estabrook & Sacktor (1958), using housefly mitochondria, and Dawson & Thorne (1969) reported that dihydroxyacetone phosphate was not an inhibitor of L-3-glycerophosphate oxidation.

D-Glyceric acid 3-phosphate (compound V) was also found by Dawson & Thorne (1969) to be a competitive inhibitor with K_i 20 mM, the same value as reported here. However, Dawson & Thorne (1969) reported that 2-hydroxypropyl phosphate, a constituent of compound (IV), did not inhibit their solubilized preparation of the L-3-glycerophosphate–flavoprotein oxidoreductase, whereas we find the mixture to be a competitive inhibitor.

Thus it would appear that the enzymic sites of the L-3-glycerophosphate–flavoprotein oxidoreductases from different biological sources have different abilities to bind substrate analogues. These may be inherent differences or induced by the method of preparation, e.g. treatment with detergent or phospholipase. We noted that attempts to solubilize the L-3-glycerophosphate–flavoprotein oxidoreductase from flight-muscle mitochondria with detergents resulted in a loss of the allosteric behaviour in that these preparations showed typical Michaelis–Menten kinetics both in the absence and presence of Ca^{2+}. It appears that an intact membrane is required for the oligomeric enzyme to dissociate.

The ease with which the activity of the oxidoreductase has been shown to be modulated by the wide range of phosphate and phosphonate esters would indicate either that there is no L-3-glycerophosphate porter in insect flight-muscle mitochondria or that, if the porter is indeed present, then it has a low specificity. Evidence to support the first postulate is provided by the failure of these mitochondria to swell in iso-osmotic solutions of ammonium DL-3-glycerophosphate whereas extensive swelling is observed in iso-osmotic solutions of ammonium pyruvate (Fig. 5). The absence of swelling in an ammonium glycerophosphate solution could be explained if the porter was a highly specific L-3-glycerophosphate–dihydroxyacetone phosphate exchange system, where L-3-glycerophosphate uptake by the mitochondria was obligatorily linked to the extrusion of dihydroxyacetone phosphate. Under these conditions there would be no net increase of intramitochondrial ion concentrations and hence no swelling.

However, the absence of such a porter is supported by evidence for the location of the L-3-glycerophosphate–flavoprotein oxidoreductase on the outer surface of the inner membrane. Such a locale negates the need for a transport system for L-3-glycerophosphate and would explain the ease with which the oxidoreductase activity can be modified by compounds (I)–(XII). The most compelling evidence for such a location of the oxidoreductase is the insensitivity of the L-3-glycerophosphate–cyanoferrate oxidoreductase system to antimycin A (in agreement with the data of Klingenberg & Buchholz, 1970), coupled with the demonstration that a non-haem iron protein does not appear to be involved in this system. Corraborative evidence is the decrease in activity of this system which is observed after ultrasonication of mitochondria to form calcium-particles, i.e. after inversion of the inner mitochondrial membrane (Figs. 4*a* and 4*b*). Such a process would locate the oxidoreductase on the inner surface of the calcium-particle vesicle of the external location proposed for intact mitochondria. That such a location is the case in calcium-particles in substantiated by the observation that in these particles the ATP-driven reduction of NAD^+ by L-3-glycerophosphate is much slower than the rate observed when the reaction is driven by succinate (Table 3). Theoretically the L-3-glycerophosphate-driven reaction should not take place if the calcium-particle membrane is impermeable to L-3-glycerophosphate, but the relatively high rates of oxidation of the substrate by calcium-particles would indicate

that the membrane is somewhat leaky (Table 2). Such a damaged membrane would explain why the oxidation of L-3-glycerophosphate by calcium-particles can be modified in a similar manner to intact mitochondria, i.e. the catalytic and allosteric sites exhibit similar binding characteristics to those described for mitochondria. However, it has been observed that in these calcium-particles the values of inhibitor constants are higher than those found with mitochondria, e.g. DL-glyceraldehyde 3-phosphate, (compound I): K_I 70 μM for mitochondria, K_I 420 μM for calcium-particles. All these lines of evidence lead us to locate the L-3-glycerophosphate–flavoprotein oxidoreductase on the outer surface of the inner membrane of flight-muscle mitochondria isolated from *S. barbata*. Similar conclusions have been drawn by Klingenberg & Buchholz (1970) with mitochondria isolated from rat liver and the flight muscle of *Locusta migratoria*.

Such a locale would obviate the necessity of a transport or porter system to facilitate substrate penetration of the relatively impermeable inner mitochondrial membrane. However, the presence of a porter system for L-3-glycerophosphate in *E. coli* has been demonstrated (Hayashi *et al.* 1964) but in *E. coli* the metabolic role of such transported L-3-glycerophosphate is either for oxidation or for assimilation into cellular materials. In contrast, in insect flight muscle the mitochondrial L-3-glycerophosphate–flavoprotein oxidoreductase operates in conjunction with the cytoplasmic L-3-glycerophosphate–NAD^+ oxidoreductase to form an efficient shuttle system, whose primary function is to mediate the reoxidation of the NADH generated in aerobic glycolysis.

The location of the L-3-glycerophosphate–flavoprotein oxidoreductase on the outer surface of the mitochondria would ease the operation of such a cyclic process.

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REFERENCES

Beechey, R. B., Roberton, A. M., Holloway, C. T. & Knight, I. G. (1967). *Biochemistry, Easton*, **6**, 3867.

Bruni, A., Luciani, S. & Contessa, A. R. (1964). *Nature, Lond.*, **201**, 1219.

Chaikin, S. W. & Brown, W. G. (1949). *J. Am. chem. Soc.* **71**, 122.

Chance, B. & Hagihara, B. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, vol. 5, p. 3.

Chappell, J. B. & Haarhoff, K. N. (1967). In *Biochemistry of Mitochondria*, p. 75, Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. London: Academic Press (Inc.) Ltd.

Chappell, J. B., Henderson, P. J. F., McGivan, J. D. & Robinson, B. H. (1968). In *The Interaction of Drugs and Subcellular Components of Animal Cells*, p. 71. Ed. by Campbell, P. N. London: J. and A. Churchill Ltd.

Childress, C. C. & Sacktor, B. (1966). *Science, N.Y.*, **154**, 268.

Dawson, A. P. & Thorne, C. J. R. (1969). *Biochem. J.* **111**, 27.

De Haan, E. J. & Tager, J. M. (1966). *Abstr. FEBS 3rd Meet., Warsaw*, p. 159.

Donnellan, J. F., Barker, M. D. & Beechey, R. B. (1970). *Biochem. J.* **116**, 31P.

Donnellan, J. F. & Beechey, R. B. (1969). *J. Insect Physiol.* **15**, 367.

Estabrook, R. W. (1961). *J. biol. Chem.* **236**, 3051.

Estabrook, R. W. & Sacktor, B. (1958). *J. biol. Chem.* **233**, 1014.

Fischer, H. O. L. & Baer, E. (1937). *Naturwissenschaften*, **25**, 589.

Fonyo, A. & Bessman, S. P. (1968). *Biochem. Med.* **2**, 145.

Gable, C. M. (1959). *U.S. Patent* 2870171; cited in *Chem. Abstr.* (1959). **53**, 10250.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.

Haas, D. W. (1964). *Biochim. biophys. Acta*, **89**, 543.

Hansford, R. G. & Chappell, J. B. (1967). *Biochem. biophys. Res. Commun.* **27**, 686.

Hayashi, S., Koch, J. P. & Lin, E. C. C. (1964). *J. biol. Chem.* **239**, 3098.

Ibers, J. A. & Davidson, N. (1951). *J. Am. chem. Soc.* **73**, 476.

Kirby, A. J. (1963). *Chemy Ind.* p. 1887.

Klingenberg, M. (1968). In 19. *Colloq. Gesellschaft für Biologische Chemie: Biochemie des Sauerstoffs*, p. 131. Ed. by Hess, B. & Staudinger, Hj. Berlin: Springer-Verlag.

Klingenberg, M. & Buchholz, M. (1970). *Eur. J. Biochem.* **13**, 247.

Kugel, L. & Halmann, M. (1966). *J. Am. chem. Soc.* **88**, 3566.

Lee, C. P. & Ernster, L. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 218. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.

Marvel, C. S., Myers, R. L. & Saunders, J. H. (1948). *J. Am. chem. Soc.* **70**, 1694.

Mitchell, P. (1966). *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*. Bodmin: Glynn Research Ltd.

Mitchell, P. & Moyle, J. (1969). *Eur. J. Biochem.* **9**, 149.

Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964). *Biochim. biophys. Acta*, **79**, 581.

Ringler, R. L. & Singer, T. P. (1959). *J. biol. Chem.* **234**, 2211.

Sacktor, B. (1955). *J. biophys. biochem. Cytol.* **1**, 29.

Tyler, D. D. (1969). *Biochem. J.* **11**, 665.

Tyler, D. D. (1970). *Biochem. J.* **116**, 30P.

Tyler, D. D., Gonze, J. & Estabrook, R. W. (1966). *Archs Biochem. Biophys.* **115**, 373.

Van den Bergh, S. G. (1964). *Biochem. J.* **93**, 128.

Van den Bergh, S. G. & Slater, E. C. (1962). *Biochem. J.* **82**, 362.

Zebe, E., Delbruck, A. & Bücher, Th. (1957). *Ber. ges. Physiol. exp. Pharm.* **189**, 115.

Zebe, E. & McShan, W. H. (1957). *J. gen. Physiol.* **40**, 779.