The Components of an α -Glycerophosphate Cycle and their Relation to Oxidative Metabolism in the Lens

By M. H. GRIFFITHS*

Nuffield Laboratory of Ophthalmology, University of Oxford

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1. The concentration of ATP in a lens brei is maintained when the brei is incubated in oxygen with α -glycerophosphate. Lack of α -glycerophosphate or incubation in nitrogen causes the concentration to decrease. α -Glycerophosphate has some effect under anaerobic conditions but this is not sufficient to account for the maintenance in oxygen. 2. Manometric experiments show that α -glycerophosphate enhances the respiration of lens preparations. This respiration can be further increased by the addition of ADP and is abolished by cyanide and antimycin. The inference from these experiments is that a mitochondrial system able to oxidize α -glycerophosphate is present, i.e. the particulate half of the α -glycerophosphate cycle. 3. More than the calculated proportion of NADH is used when limiting amounts of dihydroxyacetone phosphate are added to lens tissue in spectrophotometric experiments. Dihydroxyacetone phosphate is therefore regenerated and an α -glycerophosphate cycle is operative. 4. A preparation of a particulate α -glycerophosphate dehydrogenase that takes up oxygen with methylene blue as electron acceptor is described. 5. Methods for obtaining mitochondria from lens are compared, and a useful extraction medium is defined. 6. Mitochondria with activities of the same order of magnitude as those obtained from liver, with α -glycerophosphate and glutamate as substrates, are prepared from epithelium detached from the capsule; some respiratory control is observed.

The lens has a small but significant uptake of oxygen. For example, Field, Tainter, Martin & Belding (1937) found an uptake by rabbit lenses of 0.03 μ l./mg. wet wt./hr. in a Barcroft respirometer, and Sippel (1962), using a flow respirometer, found that rat lenses took up oxygen at a similar rate. In part, this uptake is coupled to energy-producing reactions, the energy being used, for example, for the incorporation of amino acids into protein (Kinoshita, Kern & Merola, 1961). Interest in the oxidative metabolism of the lens centres on possible substrates for oxidation and on the mechanism by which the lens uses NADH formed outside the mitochondria.

A possible mechanism for the oxidation of NADH is the α -glycerophosphate cycle (Scheme 1), as suggested by work on rat skeletal muscle (Bücher & Klingenberg, 1958), rat brain (Sacktor, Packer & Estabrook, 1959) and rat liver and kidney (Boxer & Shonk, 1960). α -GP, $\dagger$ formed in the cytoplasm by the reduction of DHAP with NADH through

the action of a soluble α -GP dehydrogenase (L-glycerol 3-phosphate-NAD⁺ oxidoreductase, EC 1.1.1.8), diffuses into mitochondria and is re-oxidized to DHAP by another α -GP dehydrogenase [L-glycerol 3-phosphate-cytochrome *c* (acceptor) oxidoreductase, EC 1.1.2.1] reacting directly with the cytochrome chain and oxygen. DHAP diffuses out of the mitochondrion either to be metabolized to lactic acid or to be reduced again to α -GP. NADH, which has been shown to be unable to cross the mitochondrial membrane (Lehninger, 1951; Purvis & Lowenstein, 1961), thus passes electrons to the cytochrome system. The possible presence of an α -GP cycle in the lens has been suggested by Pirie (1962).

The soluble enzyme was first found in rabbit muscle by Baranowski (1949), where the particulate enzyme had been found by Green (1936). The mitochondrion was regarded as the site of the insoluble α -GP dehydrogenase by Estabrook & Sacktor (1958), who oxidized α -GP with sarcosomes obtained from fly flight muscle. Sacktor & Cochran (1958) and Klingenberg & Bücher (1961) found ATP synthesis to be coupled to this oxidation. Borst (1962) combined mitochondria from Ehrlich

* Present address: Tunstall Laboratory, Shell Research Ltd., Sittingbourne, Kent.

$\dagger$ Abbreviations: α -GP, α -glycerophosphate; DHAP, dihydroxyacetone phosphate.

Scheme 1. α -Glycerophosphate cycle.

ascites-tumour cells, which do not contain a soluble α -GP dehydrogenase, with a purified soluble enzyme to form a functioning α -GP cycle.

Soluble α -GP dehydrogenase in lens has been shown by van Heyningen (1958) and Pirie (1962). Pirie (1963) has also shown that the α -GP concentration in the intact lens decreases in oxygen and remains constant or increases in nitrogen. Electron micrographs taken by François, Rabaey & Vandermeerssche (1955), Wanko & Gavin (1958, 1959), Jakus (1964) and M. H. Griffiths (unpublished work) show mitochondria-like particles, possessing internal structure, in the cells of the lens epithelium but not in the fibres of the cortex. Virmaux, Porte & Brini (1961) and Virmaux & Mandel (1963) have obtained fractions from the lens that oxidize succinate in the presence of cytochrome *c* (uptake of oxygen $14.5 \mu\text{l./g. wet wt./hr.}$). Berkow & Patz (1964) have studied the rat eye by histochemical techniques and have confirmed the presence of the mitochondrial enzymes malate dehydrogenase (L-malate-NAD⁺ oxidoreductase, EC 1.1.1.37) and succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1] in the lens epithelium.

The results reported in the present paper show that α -GP is a substrate for NAD⁺-independent oxidation, thus indicating the presence of the particulate enzyme, and that extramitochondrial NADH can be oxidized by lens preparations in the presence of limiting amounts of DHAP. This paper also describes the preparation of the particulate enzyme from lens by the method of Green (1936), and the preparation of homogeneous fractions of mitochondria, having activities comparable with those of similar particles from other tissues.

MATERIALS

DL- α -Glycerophosphate was obtained from Boots Pure Drug Co. Ltd. (Nottingham) and Koch-Light Laboratories Ltd. (Colnbrook, Bucks.); 2,2,4-trimethylpentane, methylene blue, EDTA, polyvinylpyrrolidone, 2,4-dinitrophenol, L-glutamic acid and ADP were from British Drug Houses Ltd. (Poole, Dorset); DHAP, ATP and NADH were from

Sigma Chemical Co. (London) Ltd.; phenazine methosulphate (*N*-methylphenazonium methosulphate) and antimycin were from Koch-Light Laboratories Ltd.; crystalline α -GP dehydrogenase was from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); Scarlet Sudan IV and nitro blue tetrazolium were from George T. Gurr Ltd. (London, S.W. 6). All other organic and inorganic reagents used were AnalaR reagents where possible and were obtained from British Drug Houses Ltd.

DHAP was obtained as the dimethyl ketal, di-monocyclohexylamine salt. This was converted into the acid by dissolving 25 mg. in 2 ml. of water and agitating for about 30 sec. with 0.5 g. of Dowex 50 resin (H⁺ form) (Sigma Chemical Co.). The solution was filtered off and incubated at 37° for 4 hr. to hydrolyse the ketal, and was then adjusted to pH 4.5 with KHCO₃. It was stored at -30°.

METHODS

Ox eyes were removed from the heads at the slaughter-house within $\frac{1}{2}$ -2 hr. of death. Rabbit (Dutch) lenses and guinea-pig lenses were obtained from the laboratory stock after killing with intravenous or intraperitoneal Nembutal.

The capsule was removed from the lens by cutting round the lens equator and peeling off both portions. The anterior capsule had the epithelium attached to it and the posterior capsule was discarded. The ring of tissue round the equator and the soft cortex of the lens were easily cut away from the hard central core of lens nucleus. Epithelium was obtained by taking the anterior capsule with epithelium attached, placing it capsule-side down on a glass slide on a tray of ice and scraping it gently with a curved scalpel blade after first removing any adhering fibres. All preparative centrifugation was done in an MSE Highspeed 18 refrigerated centrifuge with the 11 cm.-radius rotor. All preparative operations were carried out on trays of ice or in a cold-room at 2°.

Estimation of ATP maintenance. The whole lens, excluding the posterior capsule and the nucleus, was made into a brei by grinding without abrasive in a chilled mortar. The brei was divided into several portions, one of which was put into a pre-weighed centrifuge tube and weighed. Sufficient 6% (w/v) HClO₄ was added to give a 1:4 (w/v) concentration of lens (wet wt.). The other portions were transferred to pre-weighed 50 ml. beakers, which were weighed, stoppered, incubated and afterwards treated similarly. The protein precipitated by the acid was spun down at about 3000 rev./min. for 10 min., and inorganic

phosphate and 7 min. acid-labile phosphate were estimated in the supernatant by the method of Fiske & Subbarow (1925) and LePage (1949). Neutralization of the mixture and removal of the HClO_4 before estimation were found to be unnecessary. α -GP was always assayed for inorganic phosphate and the value so obtained was subtracted when calculating the results.

The brei was incubated with 0.154 M-KCl buffered with 33 mM-triethanolamine hydrochloride adjusted to pH 7.4 with 0.1 N-NaOH, and in water. NaF, α -GP and penicillin plus streptomycin were also added. The beakers were equipped with tubes through their stoppers for passing O_2 or N_2 over the surface of the contents. A continuous stream of gas from cylinders was passed through wash-bottles immersed in the thermostatically controlled bath to the beakers throughout the incubation.

Estimation of oxygen uptake. Manometric experiments were performed in micro-Warburg cups (fluid volume 1 ml.) with, as manometer fluid, trimethylpentane (sp. gr. 0.692), as suggested by Pennington (1961), thus increasing the sensitivity of the manometers 1.5-fold. The fluid was coloured red with Scarlet Sudan IV. The cups were immersed in a thermostatically controlled bath at 37° and were shaken 100 times/min.

The tissue used in these experiments was normally the anterior capsule with attached epithelium plus the equatorial tissue from ox lenses. This preparation was chopped fine with scissors, ground in a chilled mortar with a little washed sand and washed by spinning in 10 vol. of 0.25 M-sucrose or 0.154 M-KCl at 10000g for 10 min. at 2°. As shown in Table 3, estimations were made on this material at different stages of preparation. The material was incubated in 0.2 M-sodium phosphate buffer, pH 7.4; ADP and inhibitors were put into the main compartment of the cup, and the substrate was added from the side arm. The gas phase was initially O_2 but no difference was found when air was used.

The O_2 uptake was also measured polarographically with a Clark-type micro-electrode (Keilley & Bronk, 1958) in a water-jacketed cell. A Polariter PO4 polarograph was used as a variable voltage source and as a recorder and was operated at a plateau of -0.65 v. The medium used was 0.130 M-KCl, as used by Fletcher, Myant & Tyler (1962), buffered with 0.02 M-sodium phosphate (final concn.), pH 7.4, which had an O_2 concentration of 265 μM at 25°, or 9.5 μl . of O_2 in a 1.5 ml. cell. Mitochondrial preparations were added to the medium in the cell after equilibrating the latter with air.

Experiments on the operation of the α -GP cycle. Rabbit and guinea-pig equatorial tissues were teased in spectrophotometer cuvettes with a small amount of phosphate buffer, pH 7.4, with a polythene rod. Triethanolamine hydrochloride buffer, pH 7.4, was added, together with NADH, and, where specified, substrate and antimycin. The concentration of NADH was followed by measuring the extinction at 340 m μ at regular intervals with a Beckman model DU spectrophotometer; 0.1 μmole of NADH in 3 ml. of buffer in a 1 cm. light-path has an extinction at 340 m μ of 0.207.

Preparation of particulate α -GP dehydrogenase. Capsule and epithelium plus cortex from ox lenses (50g.) were chopped with scissors and the enzyme was prepared by the method of Green (1936), after washing the chopped tissue at 7000g at 0° for 15 min. with 100 ml. of ice-cold water.

The pellet finally obtained by this method was resuspended in 15 ml. of 0.2 M-phosphate buffer, pH 7.4. This method was modified in a later experiment by using less cortex and more epithelium and equatorial tissue, by using 0.154 M-KCl to wash and resuspend the ground material and by spinning the phosphate suspension at 400g for 10 min. and taking the supernatant.

Preparation of mitochondria. (a) In 0.25 M-sucrose. The method of Hogeboom, Schneider & Palade (1947) was used: capsule and epithelium, equatorial tissue and cortex were taken and homogenized in 4 vol. of ice-cold sucrose in a stainless-steel Potter-Elvehjem homogenizer. The pellet obtained after centrifugation was resuspended in 1 vol. of sucrose. Homogenization was later abandoned as the homogenate foamed badly before the lens fibres were broken. Instead, the material was ground, after being chopped, in a chilled mortar with a little sand, and was resuspended with 4 vol. of sucrose. The method was further modified by spinning the suspension, after grinding, at 400g for 10 min., regrounding the sediment, suspending this in a further 4 vol. of sucrose, spinning at 400g for 10 min. and combining the two supernatants. These were then centrifuged at 10000g for 15 min.

(b) In 0.88 M-sucrose (Wanko & Gavin, 1959). Capsule, epithelium and equatorial tissue were ground as above, suspended in 4 vol. of 0.88 M-sucrose, and cell debris was spun down at 7000g for 15 min. Mitochondria were obtained by spinning the supernatant at 18000g for 20 min. The pellet was rubbed up in 1 vol. of sucrose.

(c) In 0.154 M-KCl (van den Bergh & Slater, 1962). Lens material was ground and washed twice with 9 vol. of KCl buffered with 0.2 M-phosphate buffer, pH 7.4, by spinning at 11000g for 10 min. The washed tissue was suspended in 1 vol. of 0.2 M-phosphate buffer, spun at 400g for 10 min., the sediment discarded and the supernatant spun at 11000g for 15 min. The resulting pellet was suspended in 1 ml. of phosphate buffer.

Preparation from epithelium alone. Epithelial cells scraped from the capsule were transferred to a small tared tube in ice and weighed. The slide on which they were prepared was washed with 0.154 M-KCl, and 5 vol. of KCl solution and the washings were added. The suspension was homogenized in a chilled glass micro-homogenizer of the Potter-Elvehjem type in an ice bath for 1 min. at about 1500 rev./min. The homogenate was spun at 400g and 2° for 10 min. and the sediment discarded. The supernatant was spun at 10000g for 15 min. and the pellet so obtained, though often too small to see, was suspended by washing the sides of the polypropylene tubes used with 0.4 ml. (for 40 eyes) or 0.2 M-phosphate buffer by using the homogenizer pestle. In some experiments, 1 mM-EDTA (final concn.) or 2% (w/v) of polyvinylpyrrolidone or both were added to the extraction medium.

Phenazine methosulphate (E'_0 -0.04 v at pH 7) was used as an electron acceptor for the assay of particulate α -GP dehydrogenase (Estabrook & Sacktor, 1958; Ringler & Singer, 1958, 1959). After trial on the preparations described, this compound was used only with mitochondria because it was found that it could accept electrons from the soluble α -GP dehydrogenase in the presence of α -GP and NAD^+ , and also appeared to have an unspecified reaction with lens tissue. On two occasions $\text{K}_3\text{Fe}(\text{CN})_6$ was also used as an acceptor.

The standard assay of oxidation by the particulate

enzyme used methylene blue ($E'_{0}+0.011$ v) as electron acceptor. Green (1936) used this compound to assay his particulate enzyme, and Ely (1951) used it with other lens enzymes. Methylene blue, which has no reaction with the soluble α -GP dehydrogenase, was added from the side arm of micro-Warburg cups to the mitochondrial preparation in phosphate buffer plus substrate in the main compartment. Incubation was in air at 37°.

Miscellaneous methods. Measurement of oxidative phosphorylation was by the method of Chance & Williams (1955).

Measurement of protein concentration was by the method of Folin & Ciocalteu (1927).

Histochemical staining was by the method of Hess & Pearse (1961).

RESULTS AND DISCUSSION

Effect of incubation with α -GP on the concentration of ATP. Kinoshita & Merola (1961) have studied the effect of various substrates on the concentration of 'high-energy' phosphate in the intact lens.

When a lens brei, chosen because it was thought that α -GP would not enter the intact lens, is incubated in oxygen or nitrogen without added substrate, the concentration of ATP falls (Table 1).

If, however, α -GP is added to the medium, there is maintenance of the ATP concentration in oxygen but not in nitrogen. Table 2 shows that the effect of α -GP in nitrogen is not sufficient to explain the maintenance in oxygen. That is to say, α -GP is also taking part in oxidative reactions leading to phosphorylation as well as in glycolysis.

Oxygen uptake by lens tissue in the presence of α -GP. Oxidation of α -GP by lens tissue has been demonstrated manometrically. Table 3 shows that the addition of α -GP to unwashed or washed preparations of lens capsule and epithelium plus equatorial tissue increases the uptake of oxygen to nearly 4 times the endogenous respiration. Cyanide and antimycin, both of which inhibit the electron-transport system, abolish the endogenous respiration and greatly inhibit that due to α -GP.

These results indicate the involvement of the cytochrome system and confirm the oxidative nature of the reaction as shown with lens brei. Antimycin has been shown to inhibit the transport of electrons from the particulate α -GP in other tissues (Estabrook & Sacktor, 1958).

Table 1. *Effect on the concentration of 'high-energy' phosphate of incubation with and without α -GP and oxygen*

Brei was made from the cortex and epithelium of ox lenses (1-2 g.) (1:1, w/v) with 1.6 mM-NaF either in 0.154 M-KCl buffered with 33 mM-triethanolamine hydrochloride, pH 7.4 (Expts. 1-3), or in water (Expts. 4-7); 0.05 ml. of a streptomycin-penicillin solution (10 mg. each/ml.) was added to inhibit bacterial action. DL- α -GP (66 mM) was added where shown and incubation was in a shaking water bath at 37°. A continuous stream of either O₂ or N₂ was passed over the surface of the incubation mixture. The assay of 'high-energy' phosphate is described in the Methods section.

Expt. no.	Incubation time (hr.)	Gas phase	Addition	Concn. of 7 min. acid-labile phosphate (μ moles/g. wet wt.)		
				Initial	After incubation	Decrease
1	5	O ₂	—	6.9	0.60	6.3
		O ₂	α -GP	6.9	4.1	2.8
		N ₂	—	6.9	0.5	6.4
		N ₂	α -GP	6.9	1.5	5.4
2	5	O ₂	—	7.7	2.2	5.5
		O ₂	α -GP	7.7	5.8	1.9
		N ₂	—	7.7	1.3	6.4
		N ₂	α -GP	7.7	3.0	4.7
3	5	O ₂	—	7.6	3.2	4.4
		N ₂	—	7.6	1.3	6.3
		N ₂	α -GP	7.6	2.6	5.0
4	3	O ₂	—	7.1	6.1	1.0
		O ₂	α -GP	7.1	7.1	—
		N ₂	α -GP	7.1	5.0	2.1
5	3	O ₂	—	7.0	3.9	3.1
		O ₂	α -GP	7.0	5.1	1.9
6	5	O ₂	—	5.7	1.7	4.0
		O ₂	α -GP	5.7	4.8	0.9
7	6	O ₂	α -GP	7.0	6.9	0.1
		N ₂	α -GP	7.0	4.7	2.3

Table 2. *Percentage maintenance of initial concentration of 'high-energy' phosphate in incubated ox-lens brei*

Conditions were as given in Table 1. Results are given as means $\pm$ S.E.M., with the numbers of experiments in parentheses. *P* indicates significance of difference.

Addition	Concn. of 7 min. acid-labile phosphate (% of initial concn.)		
	Aerobic	Anaerobic	Difference aerobic—anaerobic
α -GP	88.6 $\pm$ 5.03 (10)	42.5 $\pm$ 7.84 (6)	46.1 (<i>P</i> < 0.001)
—	33.9 $\pm$ 7.09 (7)	14.3 $\pm$ 2.05 (4)	19.6
Difference $\pm$ α -GP	54.7 (<i>P</i> < 0.001)	28.2	

Table 3. *Respiration of lens epithelium and equatorial tissue*

Micro-Warburg manometers (fluid volume 1 ml.) were used at 37° and a shaking rate of 100/min. Approx. 0.25 g. of tissue from ox lenses was incubated with 0.2 M-phosphate buffer, pH 7.4. Substrate was added from the side arm; inhibitors and ADP were in main compartment. Tissue was washed by spinning through 10 vol. of 0.25 M-sucrose or 0.154 M-KCl as described in the Methods section.

Type of preparation	No. of expts.	Addition	<i>Q</i> _{O₂} (μl. uptake/mg. wet wt./hr.)	
			Mean	Range
Epithelium + equatorial tissue, chopped	4	—	0.012	0.010–0.014
		DL- α -GP (10 mm)	0.021	0.019–0.025
Epithelium + equatorial tissue, chopped	5	—	0.014	0.009–0.020
		ADP (5 mm)	0.019	0.016–0.024
		α -GP (10 mm)	0.021	0.018–0.024
		α -GP (10 mm) + ADP (5 mm)	0.032	0.028–0.036
Epithelium + equatorial tissue, ground and washed	6	—	0.004	0.004–0.005
		Antimycin (50 μg.)	0.000	—
		α -GP (10 mm)	0.015	0.013–0.016
		α -GP (10 mm) + antimycin (50 μg.)	0.007	0.005–0.008
Epithelium + equatorial tissue, ground	5	—	0.023	0.008–0.032
		Antimycin (50 μg.)	0.000	—
		α -GP (100 mm)	0.045	0.013–0.070
		α -GP (100 mm) + antimycin (50 μg.)	0.008	0.007–0.009
Epithelium + equatorial tissue, ground	2	—	0.014	0.011, 0.017
		KCN (1 mm)	0.003	0.003, 0.004
		α -GP (100 mm)	0.038	0.035, 0.042
		α -GP (100 mm) + KCN (1 mm)	0.004	0.004, 0.004

Table 3 shows that the addition of ADP stimulates the endogenous respiration and, to a greater extent, that with α -GP, thus confirming the occurrence of oxidative phosphorylation.

A possible α -GP cycle in lens. The experiments so far show that the lens has an α -GP dehydrogenase which reacts with oxygen, and, since the soluble enzyme has previously been demonstrated, it should be possible to show the α -GP cycle in operation.

Sacktor & Dick (1962), working with house-fly flight muscle, have followed spectrophotometrically

the disappearance of added NADH when limited amounts of DHAP were added. If the α -GP cycle is active, the α -GP formed from DHAP with NADH will be reoxidized to DHAP by the mitochondrial enzyme and so be available to oxidize further NADH. In the experiments recorded here, their procedure was copied as far as possible. Since capsule and epithelium do not remain suspended in the spectrophotometer cuvettes, the softer equatorial tissue from rabbit and guinea-pig lenses was chosen as this remained dispersed throughout the medium after teasing. The existence of a func-

Table 4. Utilization of NADH by lens tissue with limiting amounts of DHAP added

Approx. 25 mg. of lens equatorial tissue was teased in a cuvette in 0.2 ml. of 50 mM-phosphate buffer, pH 7.4; 2.4 ml. of triethanolamine hydrochloride buffer, pH 7.4, and 0.4 ml. of 0.5 mM-NADH were added. The cuvette with enzyme contained 10 μ l. of a solution of crystalline α -GP dehydrogenase (10 mg./ml.) instead of tissue. The blank cuvette contained water instead of NADH, and the control for endogenous substrate had NADH but no DHAP added. Portions (10 μ l., 0.05 μ mole) of DHAP, equivalent to a decrease in extinction at 340 m μ of 0.103, were added at intervals. Expt. 1 was performed on rabbit tissue and Expts. 2 and 3 were on guinea-pig tissue. Results are given as means $\pm$ S.E.M. *P* indicates significance of difference.

Expt. no.	No. of expts.	Average decrease in E_{340}			
		Purified α -GP dehydrogenase	Lens tissue	Lens + antimycin	Difference
1	6	0.111 $\pm$ 0.002	0.146 $\pm$ 0.009	—	0.035 (<i>P</i> < 0.001)
2	12	0.096 $\pm$ 0.001	0.112 $\pm$ 0.002	—	0.018 (<i>P</i> < 0.001)
3	6	0.104 $\pm$ 0.006	0.136 $\pm$ 0.012	—	0.032 (<i>P</i> < 0.01)
		0.104 $\pm$ 0.006	—	0.098 $\pm$ 0.005	0.006 (<i>P</i> < 0.25)
		—	0.136 $\pm$ 0.012	0.098 $\pm$ 0.005	0.038 (<i>P</i> < 0.002)

Fig. 1. Use of NADH by guinea-pig equatorial tissue as compared with the purified α -GP dehydrogenase. Experimental details were as given in Table 4. DHAP was added at the curve discontinuities. Δ , Control lacking substrate; $\bullet$, purified enzyme; $\circ$, lens tissue. The rate was measured between the arrows for Table 5.

tioning α -GP cycle was examined by comparing the amount of NADH used by lens tissue with that used by purified α -GP dehydrogenase when equal amounts of DHAP were added to both. Table 4 shows the results of these experiments, and Fig. 1 is a graph of a typical experiment. Table 4 indicates that more NADH is used by lens tissue than by α -GP dehydrogenase alone. In calculating the results, the amount of NADH which disappears in the absence of substrate has been subtracted from the fall when substrate is present. There is no increased use of NADH by lens over that by purified enzyme when antimycin is added to the lens. Antimycin has no effect on the purified α -GP dehydrogenase.

Since more NADH is used by lens than expected it is clear that DHAP is being regenerated. The reaction slows down as DHAP is drawn off into other reactions.

Since the difference in NADH utilization between lens and α -GP dehydrogenase, though consistent, is small in comparison with the total NADH used, it was thought that more significant results might be obtained by measuring the rate of use of NADH by the lens after the amount stoichiometrically equivalent to the added DHAP ($\text{NADH} + \text{DHAP} = \text{NAD}^+ + \alpha\text{-GP}$) has disappeared. If no reoxidation of α -GP takes place the use of NADH should return to the endogenous rate, but if the α -GP cycle is operating the use of NADH should continue at a higher rate. Table 5 shows that the rate of disappearance of NADH is in all cases higher than the control rate, in the presence of DHAP.

Table 5. *Rate of utilization of NADH by equatorial tissue from guinea-pig lenses with limiting amounts of DHAP added*

Tissue and conditions were as given in Table 4; 0.05 μ mole of pyruvate or DHAP was used as substrate, and 50 μ g. of antimycin as inhibitor. The rate was measured for 10 min. after the point on the graph where 0.05 μ mole of NADH has disappeared (see arrows 10 min. apart in Fig. 1).

Expt. no.	Addition	Change in E_{340} in 10 min.		
		Endogenous	Substrate added	Substrate + inhibitor added
1	DHAP	0.006	0.008	—
2		0.006	0.008	—
3		0.015	0.026	—
4		0.040	0.060	—
5	DHAP + antimycin	0.040	—	0.035
6		0.005	—	0.000
7		0.010	—	0.004
8	Pyruvate	0.006	0.001	—
9		0.006	0.005	—
10		0.006	0.004	—
11		0.015	0.004	—

Pyruvate, used as a comparison substrate, shows no such increase. Once again, antimycin abolishes this increase, confirming the link with the cytochrome system.

In a single experiment the presence of α -GP hastened the appearance of a purple colour when lens epithelium was treated with nitroblue tetrazolium and cobalt chloride (method of Hess & Pearce, 1961). Examination under the microscope showed that the colour due to the formation of cobalt formazan by reduction of the tetrazolium by an oxidative enzyme, with in this case α -GP as substrate, is associated with granules in the epithelial cells. The cell wall, nucleus and adhering lens fibres remain uncoloured.

This result has a bearing on previous experiments by suggesting the particulate nature of the α -GP dehydrogenase.

Particulate α -GP dehydrogenase of lens. To locate the enzyme more precisely, Green's (1936) method was used to prepare a washed insoluble tissue residue from lens tissue. The material obtained, when examined by phase-contrast microscopy, showed intact lens fibres, fragments of capsule, and a few small dense particles. The whole preparation, and the particles separated by centrifugation from the remainder, were incubated in Warburg cups and their oxygen uptake with methylene blue is shown in Table 6. The particles take up oxygen at a faster rate than the whole of the insoluble material and are therefore the site of α -GP dehydrogenase. These particles were about the same size as retinal mitochondria prepared at the same time, and, since electron micrographs show mitochondria-like par-

ticles in the epithelium and α -GP dehydrogenase has been found in mitochondria in other tissues, it is reasonable to suppose that these are at worst mitochondrial fragments.

Preparation and α -GP-dehydrogenase activity of lens mitochondria. A search for methods of preparing mitochondria from lens was carried out, the criteria of success being homogeneity with respect to the particles under the microscope and rate of oxygen uptake with methylene blue as intermediate acceptor. In a few cases phenazine methosulphate and potassium ferricyanide were also used as acceptors. Sucrose (0.25M) was first used as an extraction medium, and capsule and epithelium, equatorial tissue and cortex were taken. Variation in the number of times the tissue was ground and the speed at which the fibrous material and capsule could be spun down without trapping the particles in the pellet eventually produced a suspension containing little other than particles and had Q_{O_2} (μ l. uptake/mg. of protein/hr.) (with α -GP as substrate) between 1.9 and 2.6. Use of 0.88M-sucrose as extracting medium gave preparations with similar Q_{O_2} values but which were more heterogeneous. These preparations incubated with nitroblue tetrazolium plus glycerophosphate gave a purple colour within 5–10 min. The colour was more intense in the presence of α -GP than in its absence.

When prepared in buffered potassium chloride the particle suspensions were considerably more homogeneous but Q_{O_2} still did not exceed 4, an order of magnitude less than that of mitochondria from other tissues.

Table 6. *Stages in purification of particulate α -GP dehydrogenase*

Various preparations of α -GP dehydrogenase and mitochondria were incubated in micro-Warburg manometers or spectrophotometric cuvettes whose contents, with minor variations, were as follows. For manometric estimations the contents were: 0.4 ml. of 0.2 M-phosphate buffer, pH 7.4, 0.2 ml. of tissue preparation, 0.2 ml. of 0.1 M-DL- α -GP, or 0.1 M-L-glutamate, or 0.1 M-succinate or buffer in the main compartment; 0.2 ml. of 2 mM-methylene blue (MeB) or 5 mg. of phenazine methosulphate (PMS)/ml. in the side arm. Where the acceptor was O_2 , substrate was added from the side arm. The gas phase was air, the temperature of incubation was 37° and the shaking rate was 100/min. For spectrophotometric estimations the contents were: 1.7 ml. of 0.2 M-phosphate buffer, pH 7.4, 0.2 ml. of tissue preparation, 0.5 ml. of 0.1 M-succinate or buffer, 0.3 ml. of 10 mM- $K_3Fe(CN)_6$, 0.1 ml. of 10 mM-KCN and 0.2 ml. of 2% serum albumin. Q_{O_2} is defined as μ l./mg. wet wt./hr. in experiments with chopped epithelium, and as μ l./mg. of protein/hr. in the remainder. The value given is a mean (arithmetic) when different substrates are used.

No. of expts.	Type of preparation	No. of eyes	Wt. of material (mg.)	Q_{O_2}		Substrate	Electron acceptor
				Endogenous	Substrate added		
11	Epithelium + equatorial tissue, chopped	20	1400	0.015	0.038	Succinate, glutamate or α -GP	O_2
3	Insoluble lens residue	22	480	0.07	0.16	α -GP	MeB + O_2
2	Particles from epithelium + equatorial tissue and cortex	22	65	0.26	0.44	α -GP or succinate	MeB + O_2
11	Mitochondria from epithelium and equatorial tissue	21	7	0.5	2.1	α -GP or succinate	O_2 or MeB + O_2 or $K_3Fe(CN)_6$
3	Epithelial mitochondria	15	0.7	0 4.9 39	3.9 11.6 64	α -GP	O_2 MEB PMS

There are two possible reasons for this: either lens mitochondria are different in kind from others or they are heavily contaminated with inactive material of similar density. If the latter is the case, then the inactive material may be derived from the equatorial tissue and cortex, and therefore preparations made from the epithelium alone should be more active. This proved to be the case and Table 6 shows that the Q_{O_2} of preparations increases as the purification of mitochondria, as inversely measured by the amount of material obtained per lens, proceeds. Few manometric experiments were performed on these last preparations because the amounts available were at the limit of the sensitivity of the method.

Polarographic estimation of oxidation of α -GP. Epithelial mitochondria did not take up oxygen in the absence of substrate. They had some respiratory activity with α -GP and glutamate, of the same order of magnitude as similar particles from other tissues and comparing favourably with the succinate-cytochrome oxidoreductase preparations of Virmaux *et al.* (1961) and Virmaux & Mandel (1963), and exhibited a certain amount of respiratory control since oxygen uptake with these sub-

strates increased up to fourfold when ADP was added (Table 7). A typical trace of oxygen uptake with α -GP and ADP additions is shown in Fig. 2. The gradual increase in respiration that was sometimes seen when ADP was added, which has been ascribed by Weinbach (1959, 1961) to damage of the mitochondria during preparation and storage, prevented the measurement of oxidative phosphorylation, as very high P/O ratios were obtained. Myers & Slater (1957) have shown that adenosine-triphosphatase activity increases under these conditions. Dinitrophenol, an uncoupling agent for oxidative phosphorylation, slightly increases the respiration with α -GP as substrate, and both antimycin and cyanide greatly inhibit (Table 7).

Weinbach, Sheffield & Garbus (1963) restored coupled phosphorylation to uncoupled mitochondria by adding bovine serum albumin and ATP to the medium. These substances had no effect on epithelial mitochondria, and the addition of polyvinylpyrrolidone or EDTA or both to the extraction medium, as suggested by Chappell & Greville (1963), did not improve the performance.

In general, the scrupulous techniques described by Weinbach (1961) have been adhered to as closely

Table 7. *Polarographic estimation of oxygen uptake by lens epithelial mitochondria*

Mitochondria from epithelium and equatorial tissue (Expts. 1 and 6) and from epithelium alone (Expts. 2-5 and 7) were incubated at 25° in 0.130M-KCl buffered with 0.2M-phosphate, pH 7.4; approx. 0.05-0.2 mg. of mitochondrial protein was added. Additions were by Agla micro-syringe through inlets in the cell cap. Substrates were 40 mM-DL- α -GP or 20 mM-L-glutamate, and these were added to the medium before the preparation in the interests of enzyme stability after it had been found in preliminary experiments that there was little or no uptake by the preparations in the absence of substrate. Final concentration of the other additions were: ADP, 2 mM; dinitrophenol, 0.1 mM; antimycin, 30 μ g./ml.; KCN, 5 mM.

Expt. no.	Addition	Substrate	No. of expts.	Q_{O_2} (μ l. uptake/mg. of protein/hr.)
1	—	α -GP	2	1.8, 2.4
	ADP	α -GP	2	7.6, 8.6
2	—	α -GP	7	9.7 (4.5-13.1)
	ADP	α -GP	7	32.7 (14.2-69)
3	—	α -GP	2	8.3, 8.4
	Dinitrophenol	α -GP	2	11.6, 12.1
4	—	α -GP	2	11.6, 21.0*
	Antimycin	α -GP	2	0, 4.2*
5	—	α -GP	2	14.2, 15.4
	KCN	α -GP	2	3.2, 1.3
6	—	Glutamate	2	0, 0.61
	ADP	Glutamate	2	6.7, 8.15
7	—	Glutamate	4	13.3 (6.9-25)
	ADP	Glutamate	4	62.3 (57-69)

* ADP added also.

Fig. 2. Uptake of O_2 by lens epithelial mitochondria measured polarographically. Approx. 0.2 mg. of mitochondrial protein in 0.15 ml. of 0.2M-phosphate buffer, pH 7.4, was added (A) to 1.4 ml. of 0.130M-KCl-20 mM-DL- α -GP. Two additions of 2 mM-ADP were made at the curve discontinuities (B and C). The incubation temperature was 25°. Rates of O_2 uptake (μ l./mg. of protein/hr.) at different places on the graph were: a, 6.9; b, 6.9; c, 15; d, 6.9; e, 25.

Metabolic role of α -GP in the lens. α -GP is rapidly labelled when the lens is incubated with uniformly labelled glucose (R. van Heyningen, unpublished work), and Pirie (1962) has shown that the concentration of α -GP goes up when the intact lens is incubated with glucose. Mann (1958) and Hockwin (1960) have shown that α -GP has the highest specific activity of any non-nucleotide phosphate compound so far examined when the lens is incubated with [32 P]phosphate. Its importance as a possible substrate for oxidation is increased by the feebleness of tricarboxylic acid-cycle activity (Kinoshita, 1955; Kinoshita & Merola, 1961) and the doubtful importance of the direct oxidation pathway for producing metabolic energy (Hockwin, Kleinfeld & Arens, 1956). The establishment of a route for passing electrons from α -GP to oxygen does not confirm that α -GP oxidation is quantitatively important but allows this possibility. Though both enzymes of the α -GP cycle are present in the lens, it is uncertain whether the cycle operates to oxidize NADH with α -GP acting in a catalytic capacity or the enzymes are merely part of a chain by which glucose provides metabolic energy oxidatively.

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as possible. However, the times required for preparation (1½ hr. for rat-liver mitochondria and 4 hr. for lens particles) may be regarded as a measure of the relative severity of the two methods.

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