The Tricarboxylic Acid Cycle and Glycolysis in Relation to Ion Transport by the Ciliary Body

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1. The respiration and aerobic glycolysis of pig ciliary processes in oxygenated phosphate and bicarbonate buffers have been investigated. 2. Significant amounts of lactic acid are produced only in the presence of added glucose, but this does not change the endogenous respiration rate. 3. Succinate and citrate increase the oxygen uptake considerably, but pyruvate has almost no effect; oxaloacetate and fumarate stimulate slightly in the presence of glucose. Aspartate and fumarate together stimulate pyruvate utilization and are oxidized as fast as citrate. 4. Ouabain inhibits the oxidation of glucose and other substrates by limiting the ADP supply from the sodium transport system. Cyanide and azide inhibit respiration and stimulate glycolysis. 5. The transport mechanism depends largely on ATP from oxidative phosphorylation and regulates the rate of respiration and glycolysis by controlling ADP production from the Na^+ - K^+ -activated adenosine triphosphatase.

The formation of aqueous humour depends on the movement of Na^+ ions from the blood capillaries, against an electrochemical gradient across the ciliary epithelium, into the posterior chamber of the eye (Cole, 1961a). It has been shown by de Roeth (1953) that the ciliary body has a high rate of aerobic glycolysis, a characteristic apparent elsewhere among normal adult animal tissues only in the retina. It seemed therefore that ion transport in this tissue might be geared to ATP generated by glycolysis, or by oxidative phosphorylation, or both. However, experiments *in vitro* with cyanide, 2,4-dinitrophenol and under anaerobic conditions suggested that sodium transport across the epithelium depended very largely on oxidative metabolism (Cole, 1961b), and that energy derived solely from glycolysis was unable to support a high rate of sodium movement.

In erythrocytes, where sodium transport depends entirely on glycolysis, it has been shown that inhibition of transport by ouabain causes a marked decrease in the rate of glycolysis (Whittam, Ager & Wiley, 1964). A similar and even more marked inhibition can be demonstrated in the ciliary body, indicating a definite relation between the glycolytic and transport processes (Riley, 1965). It is therefore of interest to determine whether the unusual metabolism of the ciliary body is directly related to its function of aqueous humour formation. Preliminary accounts have already been published (Riley & Baroncelli, 1964; Riley, 1964).

MATERIALS AND METHODS

Pig eyes were obtained from freshly killed animals; the eyes were transported in crushed ice and used within 4 hr. of death. The ciliary body and iris were removed together from the anterior segment and then the iris and most of the posterior part of the ciliary body were cut away, leaving a thin circle of tissue about 2 mm. thick consisting largely of ciliary processes, of about 70 mg. wet wt. These preparations were immersed in ice-cold 0.3 M-mannitol until required.

The incubating medium was of pH 7.4 and contained: PO_4^{3-} , 21 mM; Cl^- , 117 mM; Na^+ , 125 mM; K^+ , 30 mM; Mg^{2+} , 1.2 mM. Glucose was added to give 3.3 mM final concentration and other substrates were 10 mM. In some experiments Krebs-Ringer bicarbonate medium (Umbreit, Burris & Stauffer, 1957) was used, uptake of O_2 being measured by the indirect method of Warburg. Ouabain, used at 0.1 mM, was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Sodium-free media were prepared by using tris-HCl in place of NaCl-KCl and K_2HPO_4 in place of Na_2HPO_4 ; the final composition was: PO_4^{3-} , 21 mM; Cl^- , 117 mM; tris, 117 mM; K^+ , 32 mM; Mg^{2+} , 1.2 mM; pH 7.4. Tissues were incubated individually in 1.5 ml. of medium in 10 ml. Warburg flasks for a period of 40 min. at 37°, with a gas phase of 100% O_2 , or $\text{O}_2 + \text{CO}_2$ (95:5) when bicarbonate buffer was used. Glucose was measured by the glucose oxidase method (Marks, 1959) and lactate by the method of Barker & Summerson (1941). The glucose and lactate content of aqueous extracts of the tissue were measured in some experiments, but there was no appreciable change in either quantity during incubation, and results presented refer only to quantities found in the medium.

Extracts of tissue incubated in the presence of [^{14}C]-

Table 1. *Oxygen uptake, glucose utilization and lactic acid production by pig ciliary processes in the presence of glucose and tricarboxylic acid-cycle intermediates*

Experimental details are given in the text. Results are presented as means $\pm$ S.E.M. with the numbers of experiments in parentheses.

Substrate added	Uptake of O ₂ (μ l./100mg. wet wt./hr.)	Glucose utilized (μ moles/100mg. wet wt.)	Lactate produced (μ moles/100mg. wet wt.)	Ratio lactate produced: glucose utilized
None	93 $\pm$ 2.5 (4)	< 0.1	0.2 $\pm$ 0.1 (4)	—
Glucose	91 $\pm$ 3.3 (6)	2.8 $\pm$ 0.2 (6)	4.9 $\pm$ 0.3 (6)	1.7
Succinate	174 $\pm$ 8.6 (6)	< 0.1	0.3 $\pm$ 0.1 (4)	—
Citrate	144 $\pm$ 4.9 (6)	—	—	—
Glucose + succinate	177 $\pm$ 3.9 (8)	2.3 $\pm$ 0.1 (4)	3.5 $\pm$ 0.5 (4)	1.5
Glucose + citrate	149 $\pm$ 2.0 (6)	2.6 $\pm$ 0.2 (4)	3.6 $\pm$ 0.2 (4)	1.4
Glucose + pyruvate	103 $\pm$ 4.9 (5)	1.2 $\pm$ 0.2 (6)	3.9 $\pm$ 0.2 (6)	3.3
Glucose + oxaloacetate	129 $\pm$ 2.9 (6)	2.2 $\pm$ 0.1 (4)	2.5 $\pm$ 0.2 (4)	1.1
Glucose + pyruvate + oxaloacetate	130 $\pm$ 3.1 (9)	2.0 $\pm$ 0.1 (4)	2.7 $\pm$ 0.4 (4)	1.4
Glucose + fumarate	118 $\pm$ 3.4 (5)	1.8 $\pm$ 0.2 (6)	3.2 $\pm$ 0.3 (4)	1.8
Glucose + pyruvate + fumarate	114 $\pm$ 3.1 (5)	1.2 $\pm$ 0.2 (5)	3.7 $\pm$ 0.3 (5)	3.1
Glucose + fumarate + aspartate	145 $\pm$ 3.3 (6)	2.4 $\pm$ 0.2 (4)	3.2 $\pm$ 0.4 (4)	1.3
Glucose in Krebs-Ringer bicarbonate buffer	94 $\pm$ 5.1 (3)	2.9 $\pm$ 0.4 (3)	5.1 $\pm$ 0.6 (3)	1.8

glucose (2 μ C/flask) were made by homogenizing in 2 ml. of 30% (v/v) ethanol. These extracts were freeze-dried and resuspended in 0.1 ml. of water. Spots (50 μ l.) were applied to Whatman no. 1 papers (40 cm. $\times$ 40 cm.), which were then developed in butan-2-ol-formic acid-water (5:1:1, by vol.) for 14 hr., followed in the second dimension by phenol-water-aq. NH₃ soln. (80:20:1, by vol.) for 16 hr. After drying the papers for 48 hr. radioautographs were made by using Kodak Industrex D X-ray film, with an exposure of 10–12 days.

RESULTS

Oxidation of substrates in phosphate and bicarbonate buffers. The respiratory activity of ciliary body in the presence of glucose and several substrates of the tricarboxylic acid cycle is shown in Table 1. Although glucose did not increase the oxygen uptake above that found in the absence of added substrates, it greatly increased the amount of lactic acid produced. It can be seen that about 85% of the glucose utilized was converted into lactate, whereas the remaining 15%, which by the use of [¹⁴C]glucose was shown to be metabolized beyond pyruvate to carbon dioxide, would account for the observed oxygen uptake. The endogenous substrates have not been identified but it appears that in the presence of other substrates they were not preferentially utilized.

Citrate and succinate produced a large stimulation of respiration whether glucose was present or not. Glucose utilization was depressed only slightly and 70–80% was still converted into lactate. Pyruvate stimulated oxygen uptake only slightly but depressed glucose utilization very strongly. The lactate:glucose ratio was high, as a result of conversion of exogenous pyruvate into lactate by

NADPH formed in the hexose monophosphate pathway, which is stimulated under these conditions (Kinoshita, 1957; Riley & Baroncelli, 1964). Fumarate and oxaloacetate both stimulated oxygen uptake but not to the extent of citrate. The addition of pyruvate with either of these two substrates in the presence of glucose caused no appreciable change in the rate of oxygen uptake. It is apparent from the lactate:glucose ratios that fumarate did not cause the pyruvate resulting from glycolysis to be diverted into the cycle, whereas oxaloacetate, although depressing glucose utilization almost as much as fumarate, caused a much greater amount of pyruvate to be oxidized via the cycle. Therefore it seemed that although fumarate was oxidized, the product was not able to stimulate pyruvate utilization. Each of the substrates added to the medium does cause a large fall in the amount of ¹⁴CO₂ produced from [¹⁴C]glucose (Riley, 1964), indicating that all substrates entered the cells in the present experiments.

On incubation of the ciliary body with [U-¹⁴C]-glucose, a considerable quantity of the label was found in aspartate, only lactate and glutamate showing higher radioactivities on the chromatogram. Specific activities were not measured, but it appears that appreciable amounts of cycle intermediates may be converted into their corresponding amino acids. On addition of aspartate to the ciliary body oxidizing fumarate and glucose, it was found that the rate of oxygen uptake was equal to that of citrate, and that endogenous pyruvate utilization was increased in a manner similar to that when oxaloacetate was present (Table 1). Therefore there can be no limitation to the oxidation of pyruvate by pyruvate oxidase or the condensing

enzyme, the rate being determined by the amount of cycle intermediates being made available as oxaloacetate. Exogenous oxaloacetate was not as effective as aspartate in supporting a high rate of oxidation because at the concentration used, 10mM, it caused a small inhibition of citrate oxidation.

Incubation of ciliary body in Krebs-Ringer bicarbonate with a gas phase oxygen + carbon dioxide (95:5) did not increase the rate of oxygen uptake with glucose, or other substrates, above that observed in the phosphate buffer (Table 1). Thus it seems unlikely that ciliary body is capable of increasing the supply of cycle intermediates by means of the malate reaction, as has been postulated for the retina (Kornblueth, Yardeni-Yaron & Wertheimer, 1953).

Effects of cyanide and azide. The effects of cyanide and azide on the respiration and glucose utilization of the ciliary body are shown in Table 2.

About 20% of the respiration was found to be cyanide-insensitive, in agreement with de Roethth (1953); azide inhibited up to 55% at a concentration of 10mM, and the amount of glucose converted into lactate was greatly increased by both inhibitors at 1mM. If the sum of ATP synthesized by glycolysis and oxidative phosphorylation is calculated by the method of Quastel & Bickis (1959), with 1mM-azide the value is the same as the controls, whereas with 1mM-cyanide the value is much lower than the controls (Table 2).

Omission of sodium from the medium. The replacement of Na^+ ions in the incubating medium by tris caused a marked inhibition of respiration and glycolysis of ciliary body utilizing glucose (Table 3). Similar results were obtained when ouabain was added to the medium instead of removing Na^+ ions, but no further inhibition of respiration or glycolysis could be obtained by adding ouabain to the medium lacking Na^+ ions. Under anaerobic

Table 2. *Effect of cyanide and azide on the respiration and aerobic glycolysis of pig ciliary processes incubated with glucose*

Experimental details are given in the text. Yield of ATP is calculated by the method of Quastel & Bickis (1959). Each value is the mean of at least three experiments.

Inhibitor	Uptake of O_2 ($\mu\text{moles}/100\text{ mg.}$ wet wt./hr.) (a)	Lactate produced ($\mu\text{moles}/100\text{ mg.}$ wet wt./hr.) (b)	Calc. yield of ATP (6a + b)
None	3.8	7.6	30.4
CN^- (1 mM)	0.7	12.6	16.8
N_3^- (1 mM)	2.8	12.5	29.3
N_3^- (2.5 mM)	2.1	—	—
N_3^- (10 mM)	1.7	—	—

Table 3. *Effect of ouabain or absence of Na^+ ions on respiration and glycolysis of pig ciliary processes under different conditions*

Experimental details are given in the text. Each value is the mean of at least four experiments except where indicated by numbers in parentheses. C, Control flasks; E, flasks with ouabain present or with sodium replaced by tris; I, percentage inhibition found in the experimental flasks.

Conditions		Uptake of O_2 ($\mu\text{l.}/100\text{ mg.}$ wet wt./hr.)			Glucose utilized ($\mu\text{moles}/100\text{ mg.}$ wet wt./hr.)			Lactate formed ($\mu\text{moles}/100\text{ mg.}$ wet wt./hr.)		
		C	E	I (%)	C	E	I (%)	C	E	I (%)
Aerobic	Ouabain	85	31	63	4.6	2.2	52	7.1	2.6	63
	Zero Na^+	85	33	61	4.6	2.4	48	7.1	3.0	58
	Zero Na^+ + ouabain (1)	88	35	60	—	—	—	7.4	2.9	61
Anaerobic	Ouabain	—	—	—	5.5	3.2	42	10.4	5.8	44
	Zero Na^+	—	—	—	5.5	3.5	36	10.4	6.3	39
With dinitrophenol (20 μM)										
Aerobic	Ouabain	147	72	51	5.3	3.0	43	7.7	4.9	36
	Zero Na^+ (2)	139	67	52	5.0	3.0	40	7.0	4.7	33
Anaerobic	Ouabain	—	—	—	6.0	4.4	27	11.2	9.2	18

Table 4. *Effect of ouabain on respiration of pig ciliary processes oxidizing succinate or citrate in the presence or absence of glucose*

Experimental details are given in the text. Results are presented as means $\pm$ S.E.M. with the numbers of experiments in parentheses.

Substrate	Uptake of O ₂ (μ l./100 mg. wet wt./hr.)		Inhibition due to ouabain (%)
	Control	With 0.1 mm-ouabain	
Succinate	174 $\pm$ 8.6 (6)	67 $\pm$ 3.2 (6)	61
Succinate + glucose	175 $\pm$ 4.5 (6)	77 $\pm$ 2.7 (6)	56
Citrate	144 $\pm$ 4.9 (6)	33 $\pm$ 2.0 (6)	77
Citrate + glucose	149 $\pm$ 2.0 (6)	38 $\pm$ 1.6 (6)	74

conditions or in the presence of 2,4-dinitrophenol the inhibition of glycolysis is considerably less than under aerobic conditions. Table 4 shows that the oxidation of succinate and citrate is severely inhibited by ouabain. As with glucose oxidation, removal of Na⁺ ions from the medium caused an effect comparable with that of the cardiac glycoside. The smaller inhibition of succinate oxidation as compared with citrate oxidation under these conditions reflects the lower degree of respiratory control that is always observed in succinate metabolism (Hatefi, Jurtshuk & Haavik, 1961). The results have been presented to show the small but significant difference ($P < 0.05$, Student's *t* test) between the ouabain-inhibited succinate oxidation rate in the presence and absence of glucose. The same effect, but smaller and not significant in the samples tested, can be seen with citrate oxidation.

DISCUSSION

The results presented in Table 1 show that the rate of pyruvate oxidation by the ciliary body is not limited by its rate of oxidation to acetyl-CoA, nor by the rate of reaction after condensation with oxaloacetate to form citrate. Pyruvate must be made available to the site of the tricarboxylic acid cycle in excess of its rate of removal by this path, the excess being converted into lactate. As each of the intermediate substrates between citrate and oxaloacetate has been found to be oxidized at a higher rate than pyruvate, the limiting factor in pyruvate oxidation must be the availability of oxaloacetate. From the experiments with [¹⁴C]-glucose it appears that cycle intermediates can be transaminated to α -oxoglutarate and oxaloacetate. When aspartate is provided in the incubation medium to prevent this 'loss' of oxaloacetate, then pyruvate is metabolized as fast as citrate, and the turnover of the cycle must be determined by the availability of ADP, as dinitrophenol has been shown to almost double the rate of oxygen uptake by the ciliary body utilizing glucose (Table 3). The 'loss' of oxaloacetate to form aspartate is sig-

nificant only when the rate of formation of oxaloacetate is low, as when ADP is limiting. Dinitrophenol stimulates turnover of the cycle to such an extent that additional oxaloacetate is not necessary to ensure maximum rate of condensation with acetyl-CoA.

de Roeth (1953) found a respiratory quotient 0.95 for ox ciliary body, indicating a predominantly carbohydrate source of metabolites and, as the ciliary body has an abundant blood supply extending right to the tips of the processes (Scullica, 1962), it is probable that glucose is the normal substrate of the epithelial cells. On the basis of the present experiments *in vitro* therefore it can be calculated that about 75% of the ATP produced in the ciliary epithelium arises from oxidative metabolism in the mitochondria (ratio of glucose oxidized to that converted into lactate, 1:6; ratio of aerobic to anaerobic ATP yield, 18:1; therefore the ratio of aerobic ATP to glycolytic ATP is 3:1). Does the remaining 25% of available energy play a specific role in sodium transport and, if not, is the high rate of aerobic glycolysis essential for the formation of aqueous humour?

Ouabain, which inhibits Na⁺-K⁺-activated adenosine triphosphatase (Skou, 1960), gives some measure of the fraction of metabolic activity which depends on a supply of ADP via this enzyme, and how much is provided by other reactions resulting in ADP formation; the stimulation of oxidation of succinate in the presence of ouabain by addition of glucose (Table 4), thereby causing utilization of ATP, indicates the importance of other sources of ADP when the Na⁺-K⁺-activated adenosine triphosphatase is inhibited. Thus, in the ciliary body, where sodium transport may be assumed to be a major energy-requiring role of the tissue, inhibition of oxygen uptake is about 60% as compared with 40% and 30% respectively in the liver and brain, in which both synthetic reactions and transport processes may be expected to have a need for ATP (Whittam & Willis, 1963; Elshove & van Rossum, 1963).

The inhibition of lactate formation by ouabain

in the ciliary body under aerobic conditions is much higher than that observed in erythrocytes. However, under anaerobic conditions the degree of inhibition is considerably decreased, from 63% to 44%, and in the presence of dinitrophenol the effect of ouabain is diminished even more under both aerobic and anaerobic conditions. These observations suggest that a competition exists between the mitochondria and soluble fraction of the epithelial cell for the available ADP, resulting, under aerobic conditions, in a more severe inhibition than is observed in erythrocytes which have no mitochondria to compete for ADP.

Glycolysis in the ciliary body appears to depend on a supply of ADP from the $\text{Na}^+\text{-K}^+$ -activated adenosine triphosphatase. Similar observations have been made in erythrocytes (Whittam *et al.* 1964) and in freshwater-turtle bladder (Klahr & Bricker, 1965) and in each of these cases it has been shown that sodium transport can be maintained by glycolytic ATP. This is not true for the ciliary body, where oxidative metabolism is obligatory for a normal rate of transport (Cole, 1961*a,b*). However, the experiments of Cole (1961*b*) indicated that transport processes were depressed to a lesser extent by dinitrophenol, cyanide and anoxia than by ouabain, suggesting that a diminished rate of sodium transport can be supported by glycolytic ATP. That anoxia causes a greater fall in the epithelial potential than cyanide (Cole, 1961*b*) is in agreement with this concept, as the greater stimulation of glycolysis by cyanide provides more ATP to support transport than is available under anoxic conditions (Tables 2 and 3).

Azide also stimulates glycolysis, but does not depress the oxidative metabolism to the same extent as cyanide, so that the calculated supply of ATP is maintained at the control value. Under these conditions Cole (1961*a*) showed that sodium transport was markedly stimulated but that the effect could be abolished by dinitrophenol. An essential difference between the effects of cyanide and azide appears to be that for azide an adequate supply of energy is available from oxidative sources, the increased glycolysis in itself being unable to

support the higher sodium transport. It remains to be determined whether the metabolic changes are responsible for the total effects of azide or whether changed membrane properties permit the increased transport so long as a minimum energy supply is maintained.

Glucose metabolism in the ciliary body is thus controlled in part at the tricarboxylic acid cycle level and in part by the transport system. Glycolysis, although not essential for sodium transport in terms of energy supply, is maintained at a high rate by interaction with the transporting mechanism, and the lactate produced constitutes a significant part of the secreted aqueous humour.

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