The Effect of Temperature and Anoxia of Kidney on the Subsequent Oxidative Phosphorylation of Mitochondria

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1. Kidneys were kept anoxic at 4°, 20° and 38°. Mitochondria were then isolated and their oxidative phosphorylation and respiration were determined. 2. Under all conditions the rate of phosphate esterification was affected to a greater extent, or earlier, than oxygen consumption. 3. Glutamate and succinate were used as substrates. The depression of P/O ratio was greater for glutamate at 4°, and for succinate at 20°. 4. Anoxia abolished the inhibiting effect of fluoride on respiration. 5. Phosphate esterification, after anoxia, was higher in the presence of fluoride than its absence, whereas in control preparations they were the same. 6. The decrease in P/O ratio did not appear to be due to activation of adenosine triphosphatase, as activities of both Mg^{2+} - and dinitrophenol-activated adenosine triphosphatases were decreased after anoxia.

The capacity of kidney slices to consume oxygen is affected by periods of time in the cold (Rochman, Clark, Lathe & Parsons, 1967), but the effects are not very large. After 12 hr. at 4°, oxygen consumption at 38° was 22% less than in controls studied immediately. When kept at 20° without oxygen for 4 hr., subsequent respiration was almost unaffected. After 6 hr. the decrease was only 27%. This led us to examine the efficiency with which oxidation can be used for synthetic purposes, as measured by oxidative phosphorylation. Isolated rat kidneys were kept intact, and anoxic, at 4°, 20° and 38° for various periods; the mitochondria were then isolated and examined at 38°. Oxidative phosphorylation was more affected by this treatment than was oxygen consumption.

METHODS AND MATERIALS

Animals. Adult male Wistar rats weighing 250–350 g. were killed by stunning and cervical dislocation.

Treatment of kidneys. Kidneys were kept at 4°, 20° and 38°. The renal capsule, perirenal fat and kidney were removed in one piece and stored in a screw-top bottle containing 0.25 M-sucrose that had been gassed with N_2 . Kidneys to be kept at 20° were immersed in sucrose at 4° for 2 min. and then transferred to sucrose at 20°. Kidneys to be stored at 4° and 38° were placed directly into sucrose at the appropriate temperature.

Preparation of mitochondria. At the end of the storage period the kidneys were transferred to ice-cold 0.25 M-sucrose after stripping the capsule and fat and excising a small part of the pelvis (to exclude fat). Mitochondria were prepared by following the technique of Schneider (1948) in

all essential details. The kidneys were cut into small pieces and homogenized in 0.25 M-sucrose with a Teflon pestle and a modified Potter–Elvehjem homogenizer. The homogenate was centrifuged for 10 min. at 680 g. The supernatant was centrifuged at 8000 g for 10 min. in an MSE Magnum refrigerated centrifuge with a high-speed head attachment (Measuring and Scientific Instruments Ltd., London). The resultant mitochondrial pellet was resuspended in 0.25 M-sucrose and recentrifuged. The pellet was resuspended in 0.25 M-sucrose.

Measurement of oxidative phosphorylation. Warburg flasks for measuring oxidative phosphorylation contained 30 μ moles of L-sodium glutamate or sodium succinate, 60 μ moles of tris-HCl buffer, pH 7.4, 5 μ moles of ATP (disodium salt), 200 units of hexokinase, 60 μ moles of glucose, 20 μ moles of $MgCl_2$, 0.03 μ mole of cytochrome c, 125 μ moles of sucrose, 30 μ moles of NaF (in some experiments) and mitochondria, in a final volume of 3.0 ml. The centre well contained 20% (w/v) KOH and a piece of filter paper. The amount of mitochondria was varied from 0.2 to 1.0 mg. of N, depending on the conditions of storage, with a view to maintaining the O_2 consumption at 10–15 μ g.atoms/flask/23 min.

After equilibration for 10 min. at 38° the O_2 consumption was determined manometrically during 13 min. The reaction was stopped by plunging the flask into crushed ice and adding 3 ml. of 20% (w/v) trichloroacetic acid. Inorganic phosphate was determined by the method of Martin & Doty (1949). For the estimation of P/O ratios the O_2 uptake was calculated for a total of 23 min. and phosphate uptake was calculated as the difference between flasks with and without mitochondria. The N was determined, after digestion, by distillation into alkali in a Markham apparatus.

Measurement of ATPase* activity. The reaction was carried out in a Dubnoff shaker at 28° for 30 min. Mito-

* Abbreviation: ATPase, adenosine triphosphatase.

chondria equivalent to 30 mg. wet wt. of kidney were added in 0.5 ml. of 0.25 M-sucrose to flasks to give a final volume of 2 ml. containing tris-HCl buffer, pH 7.4 (0.05 M), and ATP (disodium salt) (4 mM). In some experiments MgCl_2 (4 mM), 2,4-dinitrophenol (0.1 mM) and NaF (20 mM) were present. The reaction was stopped by the addition of 2 ml. of 20% (w/v) trichloroacetic acid. Inorganic phosphate was determined by the method of Martin & Doty (1949).

Reagents. ATP (disodium salt) and cytochrome *c* were from Sigma Chemical Co., St Louis, Mo., U.S.A. Hexokinase was from Seravac Laboratories, Maidenhead, Berks. All the other reagents used were laboratory or AnalaR reagents from British Drug Houses Ltd., Poole, Dorset.

Statistical analysis. Fisher's *P* values were obtained by an analysis of variance as given by Bailey (1959).

RESULTS

The oxygen consumption of mitochondria, isolated from kidneys that were stored anaerobically for various periods at 4°, 20° and 38°, is shown in Figs. 1(a), 1(b) and 1(c) respectively, together with the P/O ratios [Figs. 2(a), 2(b) and 2(c) respectively], with glutamate as substrate.

The general features of inhibition of P/O ratio obtained with glutamate were confirmed with succinate (Table 1). To examine the extent to which the fall in P/O ratio was dependent on the emergence of ATPase systems we determined the P/O ratio in the presence and absence of fluoride

Fig. 1. Oxygen consumption of mitochondria isolated from kidneys kept intact and anoxic at (a) 4°, (b) 20° and (c) 38° for various periods. Each point is the mean value of five experiments and the verticals represent the S.E.M., except for the 2 hr. result in (c), which is of one result only. Conditions of the determination are given in the Methods and Materials section. Fluoride was present.

Fig. 2. P/O ratios of mitochondria isolated from kidneys kept intact and anoxic at (a) 4°, (b) 20° and (c) 38° for various periods. Each point is the mean value of five experiments and the verticals represent the S.E.M., except for the 2 hr. result in (c), which is of one result only. Conditions of the determination are given in the Methods and Materials section. Fluoride was present.

Table 1. *Effect of fluoride on the oxidative phosphorylation of kidney mitochondria with succinate as substrate*

The kidneys were kept intact and anoxic for various times and the mitochondria were isolated and examined. Fluoride was 10 mM, when present. Each result is the average of two experiments. Experimental details are given in the Methods and Materials section.

Conditions of storage		Average mitochondrial N/flask (mg.)	Fluoride present			Fluoride absent		
			P _i esterified (μmoles)	O ₂ consumed (μg.atoms)	P/O ratio	P _i esterified (μmoles)	O ₂ consumed (μg.atoms)	P/O ratio
Control	0	0.27	26.3	11.6	2.3	29.3	16.4	1.7
4°	24	0.32	29	13.8	2.1	27.8	18.8	1.5
4	48	0.40	13.5	12.4	1.1	11.8	13.9	0.9
4	72	0.50	9.4	10.4	0.9	7.0	11.6	0.6
20	2	0.27	17.8	11.5	1.5	18.4	14.2	1.3
20	5	0.50	16.5	12.5	1.3	16.1	13.6	1.2
38	1	0.54	8.1	12.7	0.6	2.7	12.5	0.2

Table 2. *Effect of fluoride on the oxidative phosphorylation of kidney mitochondria with glutamate as substrate*

The kidneys were kept intact and anoxic for various times and the mitochondria were isolated and examined. Fluoride was 10 mM, when present. Each result is the average of two experiments. Experimental details are given in the Methods and Materials section.

Conditions of storage		Average mitochondrial N/flask (mg.)	Fluoride present			Fluoride absent		
			P _i esterified (μmoles)	O ₂ consumed (μg.atoms)	P/O ratio	P _i esterified (μmoles)	O ₂ consumed (μg.atoms)	P/O ratio
Control	0	0.45	28.2	10.1	2.8	33.2	12.5	2.7
4°	24	0.55	20.9	11.6	1.8	19.8	13.8	1.4
4	48	0.91	16.2	12.0	1.4	11.7	13.6	0.9
20	2	0.47	26.4	11.1	2.4	27.0	12.0	2.2
20	5	0.90	17.3	11.0	1.6	16.0	11.5	1.4
38	1	0.78	7.0	7.4	0.9	2.7	7.6	0.4

Table 3. *Comparison of the fall in oxidative phosphorylation, due to kidneys being kept intact and anoxic, with 10mM-succinate or 10mM-glutamate as substrate*

Fluoride was present in all flasks. The conditions for determination of oxidative phosphorylation are given in the Methods and Materials section.

Conditions of storage		Fall in P/O ratio (%)	
Temp.	Time (hr.)	With succinate	With glutamate
4°	24	9	33
4	48	52	48
4	72	61	59
20	2	35	13
20	5	43	44
38	1	74	63

ATPase activity was also measured directly in the presence and absence of stimulators (Mg²⁺ and dinitrophenol), as shown in Table 4. The rate of appearance of ATPase activity at 4° was defined (Fig. 3). Since the determinations of ATPase were carried out in the presence of 62mM-sucrose, the effect of an iso-osmotic medium on the Mg²⁺- and dinitrophenol-stimulated ATPase activity was also examined (Table 5).

An attempt was made to stabilize the P/O ratio by administering a large amount of substrate intraperitoneally 5–30 min. before killing the animal. The following were given without producing any apparent effect on the P/O ratio: 1 g. of glutamate/kg. (two experiments); 1 g. of glucose/kg. (five experiments).

DISCUSSION

with glutamate (Table 2) and succinate (Table 1) as substrates. The percentage falls in P/O ratios with the two substrates are compared in Table 3. The

Although the stability of oxygen consumption in the present study may be compared with that observed previously (Rochman *et al.* 1967), strict agreement is not expected as the treatments

Table 4. *Effect of stimulating and inhibiting substances on the amount of inorganic phosphate released from ATP by mitochondria isolated from kidneys before and after 72 hr. at 4°*

Stimulating and inhibiting substances were at the following concentrations: 4 mM-MgCl₂; 0.1 mM-dinitrophenol; 20mM-NaF. ATPase activity was determined as described in the Methods and Materials section, in the presence of 0.3M-sucrose. The values given are the means of two experiments.

Added substance ...	ATPase activity (μmoles of P _i released)					
	None	Mg ²⁺	Dinitrophenol	NaF	Mg ²⁺ + NaF	Dinitrophenol + NaF
Fresh mitochondria	1.3	2.8	3.3	1.1	1.8	1.4
Mitochondria after 72 hr. at 4°	0.4	1.8	0.5	0.3	0.9	0.2

Fig. 3. ATPase activity of mitochondria isolated from kidney kept intact and anoxic at 4° for various periods, estimated in the presence of 0.1 mM-dinitrophenol (▲—▲) or 4 mM-MgCl₂ (●---●). Conditions of the determinations are given in the Methods and Materials section.

differed in two ways: the state of the tissue when exposed to anoxia, and the material examined for oxygen consumption. In the preceding paper anoxic kidneys were stored as slices and the oxygen consumption of the slices was determined at 38°. In the present paper anoxic kidney was kept intact for the period of storage, after which mitochondria were isolated and their oxygen consumption and oxidative phosphorylation were determined at 38°. Differences in the stability of respiration were noted. Mitochondria from kidneys kept at 4° for 24 hr. (Fig. 1a) showed a 9% decrease in oxygen consumption, whereas previously slices showed a 22% decrease after 12 hr. Storage intact at 20° (Fig. 1b) produced a fall that only became significant ($P < 0.001$) after 3 hr. of storage. By 5 hr. the effect was much greater than found previously with slices. Storage at 38° for 1 hr. produced a 40–60% decrease (Fig. 1c) similar to that obtained with slices. Thus

at 4° there was less indication of damage if the kidney was kept intact, whereas at 20° the intact organ may have been slightly more susceptible.

The primary purpose of the present study was to see whether oxidative phosphorylation was depressed more than was oxygen consumption. A low temperature (4°) for periods up to 24 hr. had little effect on the subsequent oxygen consumption of mitochondria (Fig. 1a), whereas the P/O ratio (Fig. 2a) appeared to fall immediately, becoming significantly lower by 12 hr. Similarly the P/O ratio of mitochondria isolated from kidneys stored at 20° (Fig. 2b) fell before the oxygen consumption (Fig. 1b), though the difference was less striking. At 38° the effect on P/O ratio was significant at 30 min. (Fig. 2c), but 1 hr. was required to lower the oxygen consumption significantly ($P < 0.001$) (Fig. 1c). Thus at 4°, 20° and 38° the P/O ratio was less well maintained than was oxygen consumption. The P/O ratio after storage of the intact organ at 4° for 24, 48 and 72 hr. was similar to that obtained by Weinbach (1959), who stored isolated liver mitochondria in 0.25 M-sucrose for up to 72 hr. It would seem therefore that the observed changes are an inherent property of the mitochondria themselves rather than the result of interaction with other cellular constituents.

The experiments of Figs. 1 and 2 were carried out with glutamate as substrate. The main features of the fall of P/O ratio with storage also occurred when succinate (Tables 1 and 3) was used. Some differences in the effect of anoxia were noted. At 4° for 24 hr. the effect on phosphorylation with succinate was insignificant whereas that with glutamate was considerable. In contrast, at 20° the larger effect after 2 hr. was obtained with succinate (Table 3). At the present time these differences are unexplained.

The possibility that the lowered P/O ratios after keeping the kidneys were due to development of ATPase activity, as suggested by Cooper & Lehninger (1957) to explain the lower P/O ratios of digitonin-treated mitochondria, was considered. As fluoride inhibits Mg²⁺-activated systems, and

Table 5. *Effect of sucrose concentration on the ATPase activity of fresh mitochondria and of those from kidneys kept intact and anoxic at 4° for 72 hr.*

The assay medium contained tris-HCl buffer (0.05M), ATP (disodium salt) (4mM), $MgCl_2$ (4mM, when added) and dinitrophenol (0.1mM, when added). Mitochondrial suspension (0.5ml, equivalent to 30mg. wet wt. of kidney, in 0.25M-sucrose, was added to each flask. Additional sucrose was added to iso-osmotic flasks to give a final 0.3M concentration. Incubation was for 30 min. at 28° and pH 7.4. The results are the means of two experiments.

		ATPase activity (μ moles of P_i released)					
		Hypo-osmotic medium			Iso-osmotic medium		
Added substance	...	None	Mg^{2+}	Dinitrophenol	None	Mg^{2+}	Dinitrophenol
Conditions of storage							
Control		1.5	2.8	3.6	1.4	2.8	3.5
72 hr. at 4°		0.7	2.1	1.0	0.4	1.8	0.5

has been used to increase phosphorylation efficiency of heart homogenates (Ochoa, 1943), a comparison of P/O ratios was made in the presence and absence of 20mM-fluoride.

In the presence of fluoride the P/O ratios were increased under all conditions of aging (Tables 1 and 2). Oxygen uptake was inhibited by fluoride, but the degree of inhibition diminished in the aged preparations. Phosphate esterification fell on aging both in the presence and absence of fluoride and, although there was a greater fall in the absence of fluoride, this may be accounted for, at least in part, by the greater fall in oxygen uptake. Had the fall in P/O ratio on aging been due to the appearance of ATPase activity capable of partial inhibition by fluoride, a much greater fall of P/O ratio would have been expected in the flasks lacking fluoride. There was thus no consistent indication of the development of ATPase systems. This was also indicated by the direct measurement of the rate of development of ATPase activity (Fig. 3). This activity decreased rather than increased. The initial state, relatively high activity in the presence of Mg^{2+} (Table 3), is unlike that of fresh liver mitochondria (Potter, Siekevitz & Simonson, 1953), in which most of the Mg^{2+} -stimulated activity is latent. Sucrose (0.3M) gave less inhibition of the dinitrophenol-stimulated ATPase (Table 5) than was found by Cooper & Lehninger (1957) with digitonin-treated liver mitochondria; the Mg^{2+} -stimulated ATPase showed a similar slight inhibition. One property of kidney mitochondria was similar to that of muscle:

high P/O ratios were obtained in the presence of high Mg^{2+} -activated ATPase activity, and treatment that lowered the P/O ratio (anoxia or deoxycholate) did not cause a concomitant rise in ATPase activity (Azzone, 1961).

These findings indicate that ATPase activity is not the explanation for the fall in P/O ratio when intact anoxic kidney is kept. They suggest too that fundamental study of the differences between ATPase activities of kidney, liver, muscle and other organs would be rewarding, and might contribute to an understanding of the different behaviour of these organs in relation to anoxia.

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