THE STABILITY OF OXIDATIVE AND PHOSPHORYLATIVE SYSTEMS IN MITOCHONDRIA UNDER ANAEROBIC CONDITIONS*

by

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During the course of a study on the inactivation of oxidative and phosphorylative systems in mitochondria by preincubation with inorganic orthophosphate[2] it was observed that phosphate failed to show its inactivating effect when 10 or 15 ml of mixture were preincubated in a tube instead of the routine one or two ml. This observation and the report[3] that phosphate-induced swelling of mitochondria was largely prevented by anaerobic conditions prompted the present investigation. The results indicate that both phosphate-induced swelling and loss of DPN (diphosphopyridine nucleotide) are dependent on aerobic conditions.

EXPERIMENTAL METHODS

Rat liver mitochondria were prepared by the general procedure previously described[3] except that a glass-teflon homogenizer was used. In most cases the homogenizing fluid contained 0.002 *M* or 0.01 *M* EDTA (ethylenediaminetetraacetate). The mitochondria were washed with 0.25 *M* sucrose containing 0.002–0.02 *M* Tris buffer (tris (hydroxymethyl) aminomethane) pH 7.4. The final suspension and amount used were the same as in the earlier study.

The preincubation treatments were carried out exactly as previously described except that the anaerobic samples were run in Thunberg tubes and thoroughly evacuated at 0° with frequent shaking for 15–20 minutes before the phosphate was tipped into the main tube. In some experiments the aerobic controls were also subjected to the evacuation procedure and then filled with oxygen.

Swelling of mitochondria was rated + 1 to + 6 from the increase in translucence (decrease in turbidity) of the suspension. These suspensions were too dense to be read directly in the spectrophotometer.

The test incubation was the same as previously described except that Mg^{++} was raised to 0.007 *M*. β-Hydroxybutyrate was the substrate in all cases. All solutions were adjusted to pH 7.4 with HCl or KOH.

RESULTS

Anaerobiosis prevents both the swelling and the inactivation of β-hydroxybutyrate oxidation which occurs rapidly during aerobic preincubation of mitochondria with a number of substances (Table I). The protection by anaerobic conditions is complete for at least 15 minutes over a range of phosphate and arsenite concentrations. In the case of the metal ions (Zn^{++} and Ca^{++}) and the surface active agents (lauryl

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sulfate and saponin) anaerobic conditions prevent the inactivating effects of lower concentrations but give only partial protection against higher concentrations.

TABLE I

ANAEROBIC CONDITIONS PROTECT MITOCHONDRIA AGAINST SWELLING AND INACTIVATION OF β-HYDROXYBUTYRATE OXIDATION

Mitochondria were separated from homogenates containing 0.002 M EDTA except in Expt. 2, where no EDTA was used. Final suspension was in 0.25 M sucrose – 0.02 M TRIS in Expts. 1 and 2, in 0.25 M sucrose – 0.002 M TRIS in Expts. 3 and 4. The preincubation period was 15 minutes. Swelling was rated by the increase in translucence. The oxygen consumption is expressed as microatoms. When added, DPN = $1.3 \cdot 10^{-3} M$.

Expt.	Preincubation		Test incubation		
	Conditions	Swelling	Added	ΔO^1	P:O
1	Control Aerobic, 0°	0	—	6.5	2.9
	Phosphate $2 \cdot 10^{-2} M$, Aerobic, 30°	+3	—	0.7	0
	Phosphate $2 \cdot 10^{-2} M$, Aerobic, 30°	+3	DPN	7.4	2.2
	Phosphate $2 \cdot 10^{-2} M$, Anaerobic, 30°	0	—	5.8	2.0
	Phosphate $2 \cdot 10^{-2} M$, Anaerobic, 30°	0	DPN	7.1	1.8
2	Arsenite $1 \cdot 10^{-4} M$, Aerobic, 30°	+2	—	1.0	0
	Arsenite $1 \cdot 10^{-4} M$, Aerobic, 30°	+2	DPN	9.4	1.6
	Arsenite $1 \cdot 10^{-4} M$, Anaerobic, 30°	0	—	9.9	1.5
	Arsenite $1 \cdot 10^{-4} M$, Anaerobic, 30°	0	DPN	9.5	1.7
3	Control Aerobic, 0°	0		10.1	2.7
	Zn^{++} $5 \cdot 10^{-5} M$, Aerobic, 30°	+1.5		1.7	0
	Zn^{++} $5 \cdot 10^{-5} M$, Anaerobic, 30°	0		9.5	1.9
	Zn^{++} $2 \cdot 10^{-4} M$, Aerobic, 30°	+1.5		2.4	0
	Zn^{++} $2 \cdot 10^{-4} M$, Anaerobic, 30°	0		3.9	1.1
	Ca^{++} $5 \cdot 10^{-4} M$, Aerobic, 30°	+2		0.8	0
	Ca^{++} $5 \cdot 10^{-4} M$, Anaerobic, 30°	0		11.3	1.8
	Ca^{++} $2 \cdot 10^{-3} M$, Aerobic, 30°	+3		0.7	0
	Ca^{++} $2 \cdot 10^{-3} M$, Anaerobic, 30°	0		8.0	2.0
4	Control Aerobic, 0°	0		5.4	2.8
	Lauryl sulfate 0.02%, Aerobic, 30°	+2.5		1.2	0
	Lauryl sulfate 0.02%, Anaerobic, 30°	0		6.4	1.3
	Lauryl sulfate 0.08%, Aerobic, 30°	+6		1.4	0
	Lauryl sulfate 0.08%, Anaerobic, 30°	+5		1.9	0
	Saponin 0.06%, Aerobic, 30°	+2		0.6	0
	Saponin 0.06%, Anaerobic, 30°	0		6.7	1.6
	Saponin 0.2 %, Aerobic, 30°	+4		0.5	0
	Saponin 0.2 %, Anaerobic, 30°	+1		4.1	1.0

Because of the striking protective effect of anaerobic conditions during short periods of preincubation with phosphate, a series of experiments with long preincubation periods was carried out to determine how long anaerobic conditions might protect the oxidative and phosphorylative activities (Table II). There was considerable variation from one experiment to another. For example, in one experiment the oxygen uptake was 85% and the phosphorylation was 40% of the control after 120 minutes anaerobically at 30°. In others cases both figures had fallen to zero in 90 minutes. Since the critical factor appears to be strictly anaerobic conditions, the more rapid fall of oxidative activity in some experiments may have been due to failure to remove every trace of oxygen.

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TABLE II

STABILITY OF OXIDATIVE AND PHOSPHORYLATIVE SYSTEMS IN MITOCHONDRIA PREINCUBATED WITH PHOSPHATE FOR 15–120 MINUTES

The experimental conditions were similar to those given in Table I. All values are expressed as per cent of the corresponding figure for the mitochondrial preparation without any preincubation at 30° and represent the averages for several experiments. The number in parentheses indicates the number of experiments.

Period of preincubation with 0.02 M phosphate 30°	*Addition of DPN during test incubation*	*Preincubation conditions*			
		Aerobic		*Anaerobic*	
		Test incubation			
		ΔO_2	*P:O*	ΔO_2	*P:O*
		Per cent of the control value			
15 minutes	—	16 (10)	39 (10)	82 (6)	83 (6)
	+	93 (6)	88 (6)	—	—
30 minutes	—	0 (3)	0 (3)	82 (10)	74 (10)
	+	89 (7)	90 (7)	79 (2)	75 (2)
60 minutes	—	—	—	71 (11)	26 (11)
	+	104 (7)	72 (7)	98 (6)	56 (6)
90 minutes	—	—	—	40 (2)	11 (2)
	+	110 (1)	36 (1)	113 (2)	17 (2)
120 minutes	—	—	—	20 (6)	7 (6)
	+	99 (6)	33 (6)	98 (6)	34 (6)

Experiments with cyanide present during the preincubation period are presented in Table III. It is apparent that cyanide inhibition of cytochrome oxidase does not produce the same result as anaerobiosis. On the other hand, addition of ferricyanide and other oxidizing agents anaerobically did produce the same result as preincubation aerobically (Table IV).

TABLE III

CYANIDE DOES NOT PROTECT AGAINST PREINCUBATION WITH PHOSPHATE

Experimental conditions were similar to those given in Table I and in the text.

Expt.	*Preincubation*			*Test incubation*	
	Additions	*Temp.*	*Swelling*	ΔO_2	*P:O*
1	None	30°	0	7.5	2.3
	Phosphate 0.02 *M*	30°	+2	0.5	0
	Phosphate 0.02 *M* + CN^- 10^{-3} *M*	30°	+2	0.2	0
	CN^- 10^{-3} *M*	30°	+1	7.4	1.9
2	None	0°	0	6.9	2.7
	Phosphate 0.02 *M*	30°	+2	0	0
	Phosphate 0.02 *M* + CN^- 10^{-3} *M*	30°	+3	0.4	0
	CN^- 10^{-3} *M*	30°	0	4.9	1.2

In these experiments cyanide was added as indicated in the preincubation period. Since oxygen consumption could not be measured subsequently until this cyanide was removed, advantage was taken of the fact that in the Warburg flask cyanide rapidly distills into the alkali of the center well. Oxygen consumption began within five minutes after the ten minute equilibration period and equalled the control rate in two or three minutes.

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TABLE IV

THE EFFECT OF FERRICYANIDE AND HYDROGEN PEROXIDE DURING ANAEROBIC PREINCUBATION

Experimental conditions similar to those in Table I. Preincubation 15 minutes at 30°.

Preincubation		*Test incubation*		
Conditions	*Swelling*		ΔO_2	*P:O*
Phosphate 0.02 *M*	Aerobic	+2	—	0.6	0
Phosphate 0.02 *M*	Aerobic	+2	DPN	5.9	2.2
Phosphate 0.02 *M* + $K_3Fe(CN)_6$ $2 \cdot 10^{-3}$ *M*	Aerobic	+3	DPN	5.8	1.8
Phosphate 0.02 *M* + H_2O_2 $2 \cdot 10^{-2}$ *M*	Aerobic	+3	DPN	4.9	2.1
Phosphate 0.02 *M*	Anaerobic	0	—	5.3	2.1
Phosphate 0.02 *M* + $K_3Fe(CN)_6$ $3 \cdot 10^{-4}$ *M*	Anaerobic	+2	—	0.4	0.1
Phosphate 0.02 *M* + $K_3Fe(CN)_6$ $2 \cdot 10^{-3}$ *M*	Anaerobic	+3	—	0.2	0
Phosphate 0.02 *M* + $K_3Fe(CN)_6$ $2 \cdot 10^{-3}$ *M*	Anaerobic	+3	DPN	5.3	2.0
Phosphate 0.02 *M* + H_2O_2 $2 \cdot 10^{-3}$ *M*	Anaerobic	+1	—	0.8	0
Phosphate 0.02 *M* + H_2O_2 $2 \cdot 10^{-2}$ *M*	Anaerobic	+1.5	—	0.7	0.6
Phosphate 0.02 *M* + H_2O_2 $2 \cdot 10^{-2}$ *M*	Anaerobic	+1.5	DPN	5.0	1.9

The values for the ratio of the inorganic phosphate disappearing to the atoms of oxygen consumed (P:O) seen after the 15 minute anaerobic preincubation at 30° are similar to those seen on restoring the oxygen consumption with DPN in preparations inactivated by aerobic preincubation (Table I and HUNTER AND FORD[2]). Whenever the oxygen consumption is restored by DPN, and especially after longer preincubations, the observed P:O ratios may be considered minimal, for added DPN appears to result in some oxidation by a non-phosphorylating pathway in many mitochondrial preparations even without preincubation. The P:O ratio gradually decreases as the preincubation is lengthened (Table II).

TABLE V

ADDITION OF PHOSPHATE ACCEPTORS OR DNP TO DEPLETE ATP DOES NOT ELIMINATE PROTECTION AFFORDED BY ANAEROBIC CONDITIONS

Experimental conditions similar to those in Table I. Where indicated hexokinase was added in the same amount normally used in a Warburg flask and Mg^{++} was 0.005 *M*. Swelling was observed only with phosphate aerobically.

Preincubation		*Test incubation*	
Addition	*Conditions Time: 15 min*	ΔO_2	*P:O*
None	Aerobic, 0°	5.5	2.7
Phosphate 0.02 *M*	Aerobic, 30°	0.9	0
Phosphate 0.02 *M*	Anaerobic, 30°	4.7	3.0
Phosphate 0.02 *M* + DNP 10^{-4} *M*	Anaerobic, 30°	6.7	0*
Phosphate 0.02 *M* + Hex. + glucose	Anaerobic, 30°	5.3	2.8
Phosphate 0.02 *M* + Hex. + glucose + Mg^{++}	Anaerobic, 30°	5.2	3.5
DNP 10^{-4} *M*	Anaerobic, 30°	7.0	0*
Hexokinase + glucose	Anaerobic, 30°	6.4	2.8
Hexokinase + glucose + Mg^{++}	Anaerobic, 30°	6.3	3.0

* The amount of DNP carried over from the preincubation into the test incubation could account for the complete lack of phosphorylation. Therefore no conclusion can be drawn about the state of the phosphorylating system.

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The addition of DNP (2,4-dinitrophenol) to uncouple phosphorylation and to activate ATPase, or the addition of hexokinase and glucose to deplete ATP, even when combined with anaerobic conditions to decrease ATP formation, did not result in rapid swelling and inactivation of β-hydroxybutyrate oxidation in mitochondria (Table V).

DISCUSSION

The fact that both swelling and loss of DPN from mitochondria can be prevented for considerable periods of time by anaerobic conditions suggests that certain primary changes during ageing of isolated mitochondria are oxidative reactions. These reactions do not involve electron transfer by way of the cytochrome system, for cyanide inhibition does not protect like anaerobiosis. Possibly the oxidation is mediated by other systems, such as flavoproteins which react directly with oxygen. However, it seems more likely that oxygen reacts directly with certain groups to produce the changes which result in swelling and loss of DPN. Certain metal ions, arsenite and phosphate (or trace metal impurities in phosphate) could act by catalyzing the oxidative reaction, perhaps by forming a more readily oxidizable complex with the key chemical group. Support for the oxidative hypothesis is found in the observation that addition of ferricyanide or H_2O_2 under anaerobic conditions results in swelling and loss of DPN exactly like that seen aerobically. A sulfhydryl group répresents a likely possibility for the key group. *p*-Chloromercuribenzoate at $2 \cdot 10^{-4} M$ does produce a small effect qualitatively like phosphate during aerobic preincubation. However, this effect, like that of arsenite, is prevented by anaerobiosis and glutathione does not protect against preincubation with phosphate.

The protection against swelling and loss of DPN which is seen with EDTA, ATP (adenosinetriphosphate), and ADP (adenosinediphosphate)[2] could be due to complex formation with metals which catalyze the oxidative change. Another possibility which would explain the protective effect of Mn^{++} and Mg^{++} as well as EDTA, ATP, and ADP is that these substances all complex with the key chemical group and prevent its oxidation. DICKENS[4] has reported that Mn^{++} and Mg^{++} protect brain cortex slices and minces against the damaging effects of high oxygen. He suggested that the mechanism involves protection of sulfhydryl groups. The partial reversal of mitochondrial swelling produced by Mn^{++} + ATP, as reported by ERNSTER AND LOW[5], may result from some reversal of the oxidative changes.

The question whether the loss of DPN is secondary to swelling and increased permeability has not been conclusively settled. Swelling always occurs concomitantly with DPN loss under conditions so far studied and every substance which prevents swelling prevents loss of DPN[2]. However, equal degrees of swelling in hypotonic media do not result in loss of DPN**. This suggests that either DPN is bound and not released by hypotonic swelling or that other methods of inducing swelling cause much greater increase in permeability to DPN. The finding of COOPER, DEVLIN AND LEHNINGER[6] that no DPN need be added to their particulate system derived from mitochondria indicates that the DPN is bound.

The inactivation of the oxidative system is the result of splitting of DPN as well as release from the mitochondria*. This splitting appears to be entirely at the nicotin-

* Details of these experiments will be presented in a separate publication with J. F. LEVY.

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amide riboside link (DPNase). Since this enzyme does not split DPNH (reduced diphosphopyridine nucleotide), one possible explanation for the differences aerobically and anaerobically would be that in the latter case DPN becomes all reduced, is not split, and swelling does not occur. However, this is not the primary event, for in aerobic experiments nicotinamide prevents the splitting of DPN but does not prevent swelling or the release of DPN. Therefore swelling is not secondary to splitting of DPN.

The decline of the phosphorylation system during two hours at 30° occurs slowly both aerobically and anaerobically. Thus, the widespread impression that phosphorylating mechanisms are rapidly inactivated during incubation in the absence of substrate does not hold for all conditions. The decline may be slightly more rapid anaerobically. EDTA does not markedly slow deterioration of the phosphorylation mechanism. Loss of phosphorylation appears to be independent of the endogenous DPN, the presence of oxygen, and swelling. This last observation is consistent with the fact that hypotonic swelling does not inactivate phosphorylation**.

Mitochondria can be incubated for 15 minutes anaerobically with an uncoupling and ATPase activating agent like DNP or an ATP utilizing system like hexokinase and glucose without swelling and inactivation. FONNESU AND DAVIES[7] have reported that DNP actually inhibits swelling under some conditions. Since such conditions would be expected to result in a very great fall in the ATP level, these experiments raise some question about the concept that a supply of ATP is essential at all times for maintaining mitochondrial integrity. Using a longer preincubation period with large amounts of hexokinase in a complex preincubation medium, ERNSTER AND LOW[5] have concluded that depletion of ATP by hexokinase does lead to swelling.

SUMMARY

Anaerobic conditions protect mitochondria from the swelling and loss of DPN-dependent oxidations produced by preincubation at 30° with phosphate, arsenite, Ca^{++}, Zn^{++}, and a number of other substances. Cyanide inhibition of cytochrome oxidase does not mimic anaerobiosis. Anaerobic addition of oxidizing agents like ferricyanide does mimic aerobic conditions. It is suggested that oxidation of some key group is responsible for swelling and loss of DPN. The phosphorylating mechanism is not rapidly inactivated during preincubation, but rather declines gradually over several hours, independently of the presence or absence of oxygen. Preincubation anaerobically with DNP, a condition which should deplete ATP, does not result in rapid swelling and loss of DPN.

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