Ca^{++} AND P_i UPTAKE BY NON-PHOSPHORYLATING MITOCHONDRIAL PREPARATIONS

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Intact isolated mitochondria from a variety of tissues accumulate large quantities of inorganic ions such as Ca^{++}, Mg^{++}, Mn^{++} and phosphate (P_i) (Slater and Cleland, 1953; Vasington and Murphy, 1961, 1962; DeLuca and Engstrom, 1961; Vasington, 1963; Lehninger, et al., 1963; Brierley, et al., 1962, 1963; Bartley and Amoore, 1958; Chappell, et al., 1962,1963). The accumulation of Ca^{++}, Mg^{++} and P_i occurs by either a respiration-dependent pathway or an ATP-supported pathway in the absence of respiration (Vasington and Murphy, 1961, 1962; DeLuca and Engstrom, 1961; Brierley, et al., 1962; Lehninger, et al., 1963). It has been suggested that a high energy intermediate formed during electron transport is involved in the uptake of ions by intact mitochondria (Vasington and Murphy, 1961, 1962; Brierley et al., 1962; Vasington, 1963; Lehninger, et al., 1963), and that in the absence of respiration, ATP supports ion uptake by forming the same high energy intermediate through a reversal of the energy transfer mechanism (Brierley, et al., 1963).

This communication reports a study of the uptake of Ca^{++} and P_i by non-phosphorylating mitochondrial preparations. The data suggest that if the ATP-supported uptake of ions is mediated through the same intermediate involved in the respiration-dependent process, then ATP does not form this intermediate through a reversal of the normal energy transfer pathway.

Freshly isolated liver mitochondria (Wistar white rats - Carworth Farms) washed three times in 0.25 M sucrose were uniformly suspended in a volume of cold distilled water equivalent to the grams of tissue from which the mitochondria were prepared. The suspension was centrifuged at 105,000 x g for 30 minutes, the supernatant was decanted and the pellet was washed two to four more times by the same procedure. The final pellet was suspended in cold distilled water for use in the ion uptake and phosphorylation experiments. The washing procedure removes 30 to 40 percent of the mitochondrial protein.

Water-washed mitochondria can be readily distinguished in the electron microscope from mitochondria isolated in 0.25 M sucrose by the presence of only a single surrounding membrane, an increase in diameter, a decrease in the density of the matrix and the disappearance of normal "dense granules" and cristae. Examination of sections of water-treated mitochondria which had accumulated Ca^{++} and P_i showed the presence of large electron dense granules similar to those seen in intact mitochondria (Greenawalt, et al., in press).

The data in Table I show that water-washed mitochondria oxidize succinate and β-hydroxybutyrate (provided DPN is added) approximately twice as rapidly as do intact mitochondria per mg protein; the addition of cytochrome c has little effect on the oxidation of these two substrates. However, the water-washed mitochondria consistently show an 80 to 100 percent loss of the phosphorylating capacity present in untreated mitochondria when either succinate or β-hydroxybutyrate is oxidized. The addition of cytochrome c and/or DPN does not restore phosphorylation during the oxidation of either succinate or β-hydroxybutyrate (Table I). Weinbach, et al., (1963) have shown that mitochondria which had been swollen by pentachlorophenol were completely uncoupled and that contraction of these mitochondria with bovine serum albumin and ATP largely restored their ability to catalyze coupled phosphorylation. However, coupled phosphorylation is

Table I

EFFECT OF WATER-WASHING ON PHOSPHORYLATING CAPACITY OF MITOCHONDRIA

The reaction mixture contained 20 mM sodium phosphate, pH 7.0, 10 mM Tris-maleate, pH 7.0, 10 mM $MgCl_2$, 10 mM succinate or 14 mM DL-β-hydroxybutyrate as indicated, 2 mM ADP, 40 μmoles glucose and 0.2 mg hexokinase (1500 units/mg) and either mitochondria (9.1 mg protein) or water-washed mitochondria (9.1 mg protein) in a final volume of 3.0 ml. Where indicated, 0.1 mM DPN and 0.04 mM cytochrome c were added. Incubations were for 35 minutes at 25°C. Oxygen consumption was measured in a Warburg apparatus.

Enzyme source	Substrate + additions	μatoms oxygen consumed	μmoles P_i esterified	P:O
Untreated* mitochondria	Succinate	7.31	14.9	2.04
	β-hydroxybutyrate	1.86	4.64	2.5
Water-washed mitochondria	Succinate	14.0	0.20	0.014
	Succinate plus cytochrome c	13.2	0	0
	β-hydroxybutyrate	0	0	0
	β-hydroxybutyrate plus DPN	5.48	0	0
	β-hydroxybutyrate plus cytochrome c and DPN	4.50	0	0

* Untreated mitochondria were maintained at 0-2° during the time required to prepare the non-phosphorylating mitochondria.

not restored in water-washed mitochondria by pre-treatment with ATP and bovine serum albumin.

Table II shows that non-phosphorylating mitochondria, like intact mitochondria, take up Ca^{++} and P_i by both substrate- and ATP-supported pathways, and that the requirements for ion uptake by these preparations are essentially the same as those reported for intact mitochondria (Vasington and Murphy, 1962; Lehninger, et al., 1963). The Ca:P_i ratio is similar to the values observed with intact mitochondria (Lehninger, et al., 1963; Rossi and Lehninger, 1963). The substrate-supported uptake shows an absolute requirement for ATP; Mg^{++} and P_i also enhance ion

Table II

REQUIREMENTS FOR Ca^{++} AND P_i UPTAKE BY NON-PHOSPHORYLATING MITOCHONDRIA

The complete reaction mixture for the substrate-supported uptake contained 4 mM sodium phosphate, pH 7.0, 10 mM Tris-maleate, pH 7.0, 10 mM succinate, 10 mM $MgCl_2$, 4 mM $CaCl_2$ (labeled with Ca^{45}), 3 mM ATP and water-washed mitochondria (5 mg protein) in a final volume of 3.0 ml. The complete reaction mixture for the ATP-supported uptake was the same as the one used in the substrate-supported uptake experiment except that succinate was omitted and 15 mM ATP was used instead of 3 mM ATP. The incubation was for 20 minutes at 30°C. Ca^{++} uptake was measured according to Vasington and Murphy (1962) and P_i by the Fiske-Subbarow method in trichloroacetic acid extracts of the washed mitochondria.

System	Ca^{++} uptake atoms	P_i uptake moles	Ca:P_i
Substrate-supported			
complete	7.45	4.71	1.59
complete, minus succinate	1.21	0.545	2.22
complete, minus ATP	0.25	0.181	1.39
complete, minus $MgCl_2$	4.34	1.61	2.70
complete, minus P_i	5.39	2.83	1.91
ATP-supported			
complete	5.89	3.17	1.86
complete, minus P_i	5.22	2.24	2.34
complete, minus Mg^{++}	0	0.108	-

uptake (Table II). The ATP-supported uptake shows an absolute requirement for Mg^{++}; the addition of P_i only slightly enhances this process. It is probable that hydrolysis of ATP yields enough P_i to support Ca^{++} uptake and the addition of external P_i therefore does not show much stimulation.

The water-washed, non-phosphorylating mitochondria respire twice as actively as do untreated mitochondria (Table I), and they also take up more Ca^{++} and P_i, frequently almost twice as much, per mg protein than do untreated mitochondria (Table II).

No correlation between the residual phosphorylating capacity and the ability to accumulate ions has been seen in the water-washed preparations, i.e., on a mg protein basis, water-washed mitochondria which have completely lost their ability to form ATP accumulate just as much Ca^{++} and P_i

as do those preparations which still retain 20 percent of the phosphorylating capacity present in intact mitochondria.

As in intact mitochondrial systems, (DeLuca and Engstrom, 1961; Vasington and Murphy, 1962; Lehninger, et al., 1963; Brierley, et al., 1963) substrate-supported uptake of Ca^{++} and P_i by the non-phosphorylating mitochondria is inhibited by the respiratory inhibitor, antimycin A, but not by oligomycin, whereas the ATP-supported uptake is inhibited only slightly by antimycin but is completely sensitive to oligomycin. Also as observed in intact mitochondria, both substrate- and ATP-supported ion accumulation by non-phosphorylating mitochondria are inhibited by 2,4-dinitrophenol.

The finding that non-phosphorylating mitochondria rapidly take up Ca^{++} and P_i by a process that is inhibited by respiratory inhibitors and uncouplers of oxidative phosphorylation strongly supports the view that respiration-dependent ion accumulation is promoted by some high energy intermediate of the normal oxidative phosphorylation reactions. If this concept of ion accumulation is correct, then our finding that water-washed mitochondria accumulate ions by a respiration-dependent pathway, even though unable to phosphorylate ADP to ATP, indicates that the coupled oxidative phosphorylation reactions are non-functional or lost at some step beyond the high energy intermediate which promotes respiration-dependent ion uptake. Brierley, et al., (1963) have suggested that the same intermediate is involved in respiration-dependent and ATP-supported ion accumulation and that ATP forms this common intermediate by a reversal of the oxidative phosphorylation reactions. Our findings are not compatible with this view. Since one or more of the oxidative phosphorylation reactions are non-functional in the water-washed, non-phosphorylating mitochondria, it would appear unlikely that ATP forms this same intermediate through a reversal of the oxidative phosphorylation reactions. Hence, if the same intermediate does promote both respiration-dependent and ATP-supported ion accumulation, the present data suggest that ATP forms this intermediate by a pathway distinct from the oxidative

phosphorylation reactions. Some support for a separate pathway is provided by the earlier observation that mitochondrial membrane fragments which do phosphorylate ADP to ATP exhibit a respiration-dependent but not an ATP-supported accumulation of Ca^{++} (Vasington, 1963).

The possibility also exists that ATP-supported ion accumulation is promoted by an intermediate different than the one which is involved in respiration-dependent ion uptake. This second intermediate could be formed either by only a partial reversal of the normal oxidative phosphorylation reactions or by a separate oligomycin-sensitive pathway.

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