ION ACCUMULATION IN HEART MITOCHONDRIA SUPPORTED BY THE OXIDATION OF REDUCED CYTOCHROME c

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The accumulation of high concentrations of Mg^{++}, Ca^{++}, and Mn^{++} by isolated mitochondria is an energy-linked process (Brierley et al., 1963 a, b, c; Vasington and Murphy, 1962; Chappell et al., 1963; Lehninger et al., 1963; DeLuca and Engstrom, 1961). The process appears to be supported by a high energy intermediate ($\sim$) of oxidative phosphorylation which can be generated by either of the two following reactions:

(1) A respiration-dependent reaction which is insensitive to oligomycin;

(2) An ATP-dependent process which is independent of respiration and inhibited by oligomycin.

Little is known of the subsequent steps leading to the accumulation of ions in the mitochondrion except that the high energy compound ($\sim$) formed in reaction (1) or (2) can be utilized in a system which moves ions against an apparent concentration gradient (reaction 3).

It is well known that electron flow from reduced cytochrome c to oxygen is coupled to the synthesis of ATP. This terminal site of oxidative phosphorylation is currently under intensive investigation in our laboratory (Webster, 1963). Since it now appears possible to define with considerable precision the sequence of events during this phosphorylation reaction, we have initiated a study of the ion accumulation which is supported by electron transfer in this region of

the electron transfer chain in the hope of identifying the intermediate which supports the observed ion accumulation. The present communication deals with some aspects of the uptake of ions which is supported by oxidation in the terminal segment of the electron transfer chain.

A large accumulation of Mg^{++} and P_i can be supported by this segment of the chain when reducing equivalents are supplied by ascorbate plus N,N,N',N' tetramethylphenylenediamine (TMPD) (Jacobs, 1960; Packer and Jacobs, 1962; Packer, 1963) in the presence of antimycin. As much as 0.5 μmole of P_i per mg of protein can be accumulated in a 10 min incubation at 30°, the averaged Mg^{++}/P_i ratio being 1.7. The accumulation reaction supported by oxidation in this portion of the respiratory chain exhibits the same features as does the accumulation supported by oxidations involving the entire chain (Brierley et al., 1963b). These features are as follows: (a) The reaction is abolished by cyanide and other inhibitors of cytochrome oxidase; (b) The uptake is not inhibited, and is occasionally stimulated, by oligomycin; (c) The accumulation is inhibited by dinitrophenol and other uncouplers of oxidative phosphorylation at the same concentrations as those which affect phosphorylation; (d) The uptake is inhibited by AMP and ADP and the inhibition by adenine nucleotides is abolished by the addition of oligomycin; (e) There is a release of H^+ into the incubation medium which parallels the uptake of Mg^{++} and P_i. The accumulation of Ca^{++} and P_i can also be energized by the ascorbate-TMPD system (Rossi and Lehninger, 1963), (Table I of this paper).

A unique feature of the ascorbate-TMPD respiration which is of value in the study of ion accumulation is its relative insensitivity to reagents reacting with the sulfhydryl group, such as p-chloromercuri-benzene sulfonate (p-CMS) and Cd^{++}. Since both of these reagents at low levels inhibit the oxidation of succinate and of DPNH-linked substrates, it has hitherto not been possible to assess their effect on the ion accumulation reaction. Table I shows that the accumulation of

both Mg and Ca phosphates, supported by the ascorbate-TMPD system, was inhibited strongly by p-CMS (both reactions being inhibited about 50% by 10^{-5} M p-CMS). Respiration was inhibited less than 10% by p-CMS under these conditions. The accumulation of both Mg^{++} and Ca^{++} in the presence of ATP and antimycin (reaction 2) was also inhibited by low levels of p-CMS (Table I); however, the sensitivity of the ATP-supported accumulation of Mg^{++} declined when the concentration of p-CMS was increased. This biphasic effect of p-CMS was not observed with the ATP-supported accumulation of Ca^{++}.

Table I

The Effect of p-CMS and Cd^{++} on Ion Accumulation

	P/O ratio	Mg^{++} accumulation (mμmoles P_i per mg of protein)		Ca^{++} accumulation (mμmoles Ca^{++} per mg of protein)	
	Ascorbate + TMPD	Ascorbate + TMPD	ATP	Ascorbate + TMPD	ATP
No addition	0.73	385	162	1067	1300
p-CMS (2×10^{-5} M)	0.27	110	103	291	720
Cd^{++} (10^{-4} M)	0.40	778	225	382	284

The accumulation of Mg^{++} and P_i was measured as described by Brierley et al. (1963b) following a 10 min incubation at 30° in a medium containing $MgCl_2$ (17 mM), P_i (3.3 mM, pH 7.4), and sucrose (0.25 M). Heart mitochondria were incubated with antimycin (0.4 μg/mg of protein) for 5 min at 0°. The reaction was supported either by ascorbate (5 mM) plus TMPD (0.2 mM) or by ATP (3.3 mM) as indicated. P/O ratios were determined in the identical medium by standard manometric procedures. The uptake of Ca^{++} was determined as described by Brierley et al. (1963c) following a 5 min incubation at 30°. The reaction was supported either by a mixture consisting of ascorbate (5 mM), TMPD (0.2 mM), and ATP (3.3 mM) in the presence of antimycin (0.4 μg/mg of protein) and oligomycin (1.0 μg/mg of protein) or by ATP (3.3 mM) in the presence of antimycin.

Cd^{++} is known to uncouple oxidative phosphorylation (Jacobs et al., 1956), to cause swelling of mitochondria, and to stimulate ATPase activity (Fluharty and Sanadi, 1962). It was, therefore, somewhat unexpected to find that low concentrations of Cd^{++} produced a striking

stimulation of the accumulation of Mg^{++} and P_i induced by oxidation of reduced TMPD (Table I). Two- to three-fold increases in the amount of Mg^{++} and P_i bound were observed in the presence of 10^{-4} M Cd^{++}. Both the rate of uptake and the extent of the accumulation were increased by the addition of Cd^{++} (Fig. 1). The uptake was inhibited by cyanide, but was not sensitive to oligomycin. Table I also shows that the accumulation of Mg^{++} and P_i, energized by ATP in the presence of antimycin, was stimulated by Cd^{++}. In marked contrast to the accumulation of Mg^{++}, the accumulation of Ca^{++}, when supported either by ascorbate plus TMPD or by ATP, was inhibited by added Cd^{++}. The response to Cd^{++} appears to be a point of departure between the otherwise similar reactions by which Mg^{++} and Ca^{++} are accumulated (Brierley et al., 1963c).

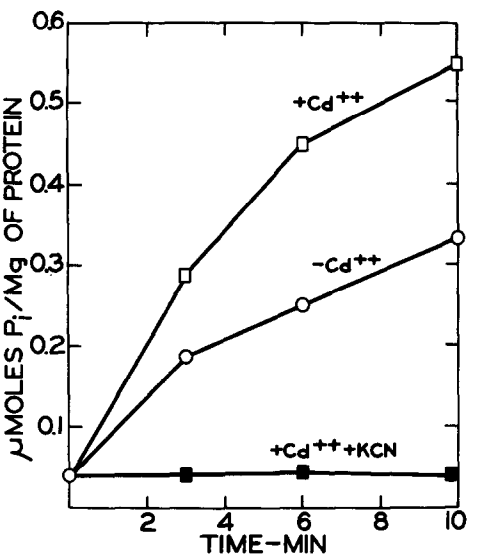

Fig. 1 - The effect of Cd^{++} on the accumulation of Mg^{++} and P_i supported by the oxidation of ascorbate-TMPD. The conditions are described in the legend for Table I. The Cd^{++} concentration was 10^{-4} M, the cyanide concentration 5×10^{-4} M. The Mg^{++} to P_i ratio for the points shown in this experiment averaged 1.7.

The effect of Cd^{++} on the accumulation of Mg^{++} was duplicated by Zn^{++} and perhaps by Fe^{++}, but not by Mn^{++}, Ca^{++}, Hg^{++}, or Ni^{++}. The

time of contact between the mitochondrion and the Cd^{++} ion was extremely important. Cd^{++} (10^{-4} M) virtually abolished phosphorylation associated with the terminal site of phosphorylation when preincubated (10 min at 0^{o}) with the enzyme prior to assay; under these conditions Mg^{++} accumulation was inhibited. Maximal stimulation of ion uptake by Cd^{++} was obtained when Cd^{++} was added to the suspension without prior incubation. This treatment reduced the P/O of the terminal site by 40 - 50% (Table I). The effect of Cd^{++} on the accumulation of Mg^{++} and P_i resembles in many ways the action of parathyroid hormone (Sallis et al., 1963).

The data presented here indicate that functional sulfhydryl groups may be involved in the accumulation of ions by heart mitochondria. The substrate-supported accumulation [reactions (1) + (3)], the ATP-supported accumulation [(2) + (3)] and oxidative phosphorylation [(1) + (2)] were all inhibited by p-CMS. Therefore, it appears that at least two sites in the energy-transfer system are sensitive to p-CMS, although it is not apparent whether reaction 3 is sensitive to this reagent. The facts that Cd^{++} depressed oxidative phosphorylation (indicating that either (1) or (2) is sensitive to this reagent under these conditions), but stimulated both the ATP-supported and the substrate-supported accumulation of Mg^{++} and P_i appear to localize the site of action of Cd^{++} at the point at which the accumulation reaction occurs (reaction 3). Since the accumulation of Ca^{++} under these conditions is inhibited by Cd^{++} it might appear that the reaction (3) for Ca^{++} is not the same as for Mg^{++}. However, it is also possible to interpret the enhancement of the Mg^{++} uptake induced by Cd^{++} in terms of an "opening" of the mitochondrion with the result that Mg^{++} is more readily available to the sites involved in the transport reaction. No such opening would be required for Ca^{++} which itself causes rapid swelling of mitochondria. If this were true, the only observable effect of Cd^{++} on Ca^{++} uptake

would be inhibition due to the effect of Cd^{++} on the energy-transfer system. Further studies are now in progress to test these possibilities.

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