Ion Transport by Heart Mitochondria. X. The Uptake and Release of Zn^{2+} and Its Relation to the Energy-Linked Accumulation of Magnesium*

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ABSTRACT: Isolated heart mitochondria take up Zn^{2+} avidly from dilute solutions. In the absence of phosphate (P_i) this accumulation requires respiration and is abolished by uncouplers of oxidative phosphorylation. It is also strongly inhibited by added Mg^{2+}. As the concentration of Zn^{2+} is increased, a greater proportion of the bound Zn^{2+} results from a passive uptake. Both the energy-linked and the passive uptake reach a limit of about 150 mμmoles of Zn^{2+}/mg of protein. Uptake of Zn^{2+} by either process results in the loss of the endogenous K^+ and a portion of the bound Mg^{2+} of the mitochondrion. In the presence of P_i large amounts of Zn^{2+} appear to be taken up in association with P_i by a process which shows no energy dependence. Zn^{2+} deposited under these conditions results in release of endogenous K^+ and Mg^{2+} only in the presence of respiration and the absence of an uncoupler. Zn^{2+} bound either in the presence or absence of P_i activates the energy-linked accumulation of Mg^{2+}. Zn^{2+} bound in the presence of P_i is more readily removed by EDTA than Zn^{2+} which has been accumulated in the absence of this anion. Removal of bound Zn^{2+} by treatment with EDTA decreases the rate of the energy-linked Mg^{2+} and P_i uptake to the rate observed before the addition of Zn^{2+}. Normal P:O and respiratory control ratios with succinate are also obtained after removal of bound Zn^{2+}. Irreversible membrane changes do not, therefore, appear to be involved in the induction of increased accumulation of Mg^{2+} by Zn^{2+}.

Recent studies in our laboratory have established that the addition of low concentrations of Zn^{2+} to suspensions of heart mitochondria oxidizing ascorbate and TMPD[1] results in marked activation of the energy-linked accumulation of Mg^{2+} (Brierley and Bhattacharyya, 1966; Brierley, 1967). Zn^{2+} also induces a massive uptake of K^+ under similar conditions (Brierley *et al.*, 1966; Brierley and Settlemire, 1967) and in many ways results in responses which resemble those reported for valinomycin (Pressman, 1965), gramicidin (Chappell and Crofts, 1966), parathyroid hormone (Rasmussen and Ogata, 1966), and other inducers of mitochondrial ion transport. A similar enhancement of Mn^{2+} accumulation by Ca^{2+} has been reported by Chance and Mela (1966). Clearly, interaction of certain cations with the mitochondrial membrane can bring about alterations in the ability of the membrane to transport other ions. The present communication examines some of the factors involved in the interaction of Zn^{2+} with the mitochondrial membrane and how this interaction is reflected in the energy-linked uptake of Mg^{2+}.

Methods

Nagarse beef heart mitochondria were prepared by a minor modification of the procedure of Hatefi *et al.* (1961; Brierley, 1967). Suspensions of these particles were incubated under the conditions specified for the individual experiments. The mitochondria were removed from the suspensions either by centrifugation or by filtration through membrane filters (Polypore GM-6, Gelman Instrument Co.). Filtrates were diluted in 0.1 N HCl and analyzed for Zn^{2+} by atomic absorption spectroscopy using a Perkin-Elmer Model 303 spectrophotometer. Mitochondrial pellets were extracted with 0.5 N $HClO_4$ and diluted with 0.1 N HCl before determination of Zn^{2+} as described above. Mg^{2+}, Ca^{2+}, K^+, and Na^+ were also estimated by atomic absorption spectroscopy of acid extracts.

The accumulation of Mg^{2+} in the presence of P_i was determined either by direct analysis of the pellets for Mg^{2+} and P_i or by following the decrease in $^{32}P_i$ in the filtrates during an incubation (Brierley, 1967). Under these conditions the uptake is also reflected in changes in rate of respiration and the pH of the

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[1] Abbreviations used: TMPD, *N,N,N',N'*-tetramethylphenylenediamine; CCP, *m*-Cl-carbonylcyanidephenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ADP, adenosine diphosphate; ATPase, adenosine triphosphatase.

TABLE I: Uptake of Zn^{2+} and Release of Mg^{2+} and K^+ in the Presence of Phosphate.[a]

	Zn^{2+} Added (μM)	Mg^{2+} Added (mM)	Intramitochondrial Cations (mμmoles/mg)		
			Zn^{2+}	K^+	Mg^{2+}
Ascorbate–TMPD	None		3	75	30
Ascorbate–TMPD + CCP	None		2	55	26
Ascorbate–TPMD	100		48	10	18
Ascorbate–TMPD + CCP	100		40	72	30
Ascorbate–TMPD	500		254	16	20
Ascorbate–TMPD + CCP	500		247	60	27
Ascorbate–TMPD	None	10	3	59	246
Ascorbate–TMPD	100	10	41	20	325
Ascorbate–TMPD + CCP	100	10	46	74	85

[a] Mitochondria (5 mg of protein) were incubated for 3 min at 25° in 3 ml of a medium consisting of sucrose (0.25 M), Tris-PO_4 (3 mM, pH 7.0), Tris-HEPES (8 mM, pH 7.0), Tris-ascorbate (5 mM), TMPD (0.1 mM), rotenone (7 μM), and the indicated amount of Zn^{2+} as the acetate salt. Where indicated $MgCl_2$ (10 mM) and CCP (20 μM) were also present. The tubes were then cooled quickly and centrifuged, the supernatants were removed, and the pellets were rinsed rapidly with 10 ml of cold 0.25 M sucrose. The resulting pellets were extracted and analyzed for Zn^{2+}, K^+, and Mg^{2+} by atomic absorption spectroscopy.

medium. These parameters were monitored as described previously (Brierley, 1967).

Density gradient centrifugation was carried out in sucrose gradients using a Spinco SW 25.1 rotor at 20,000 rpm for 45 min. The layers were removed dropwise from a pinhole in the bottom of the tube. To minimize the deposition of Zn^{2+} salts at the bottom of the tube (and possible contamination of successive fractions with Zn^{2+}) the bottom layer was acidified with $HClO_4$.

Results

The Uptake of Zn^{2+} in the Presence of P_i. The activation of the energy-linked accumulation of Mg^{2+} by Zn^{2+} is difficult to demonstrate unless phosphate (or arsenate) is also present in the incubation medium (Brierley, 1967). Under these conditions about 80% of the available Zn^{2+} is recovered from the mitochondrial pellet and no energy requirement for the uptake of Zn^{2+} can be detected (Brierley, 1967). As the concentration of Zn^{2+} in the incubation medium is increased under these conditions (Table I), more Zn^{2+} appears in the centrifuged pellets. The uptake of Zn^{2+} does not require energy and is not inhibited by uncouplers of oxidative phosphorylation. Phosphate is associated with this uptake in a Zn^{2+}:P_i ratio of about 3:2 at higher Zn^{2+} concentrations, and there is no tendency for the uptake to reach a limit, even at very high Zn^{2+} concentrations (2 mM and above).

Since these observations and the slight solubility of Zn^{2+} phosphate suggested the possible presence of a nonspecific deposit of Zn^{2+} salts, a series of density gradient centrifugations was carried out (Figure 1). It is apparent that the bulk of the Zn^{2+} in the incubation mixture is closely associated with the mitochondrial band in the absence of an energy source (Figure 1A) or in the presence of dinitrophenol (not shown). When the mitochondria respire with ascorbate and TMPD as substrate two bands of more dense mitochondria appear in sucrose density gradients. This pattern appears quite analogous to those reported by Greenawalt *et al.* (1964) for liver mitochondria which have accumulated massive amounts of Ca^{2+} and P_i. In the experiment shown (Figure 1B) Zn^{2+} is associated with both the mitochondria in the band corresponding to that in the absence of energy and in the lower bands (which are sedimented to the bottom of the tube in this particular gradient). Control experiments in the absence of mitochondria establish that Zn^{2+} is precipitated and sedimented through the gradient under the conditions of these experiments (Figure 1C).

In the absence of P_i (Figure 1D) much less Zn^{2+} is associated with the mitochondria and the excess Zn^{2+} remains in solution at the top of the gradient. These studies indicate that the large amounts of Zn^{2+} and P_i which are recovered from centrifuged pellets of mitochondria are associated with the mitochondria and do not appear to be a simple coprecipitate of insoluble Zn^{2+} salt.

The deposition of Zn^{2+} and P_i in the absence of energy results in little change in the concentration of the endogenous ions of the mitochondrion. In the presence of energy and a medium containing only Zn^{2+} and Tris as added cations, however, the bulk of the endogenous K^+ and a lesser proportion of the mitochondrial Mg^{2+} are extruded (Table I). When Mg^{2+} is also added to the suspending medium under these conditions, the energy-linked uptake of Mg^{2+} is activated and there is a marked loss of K^+ from the parti-

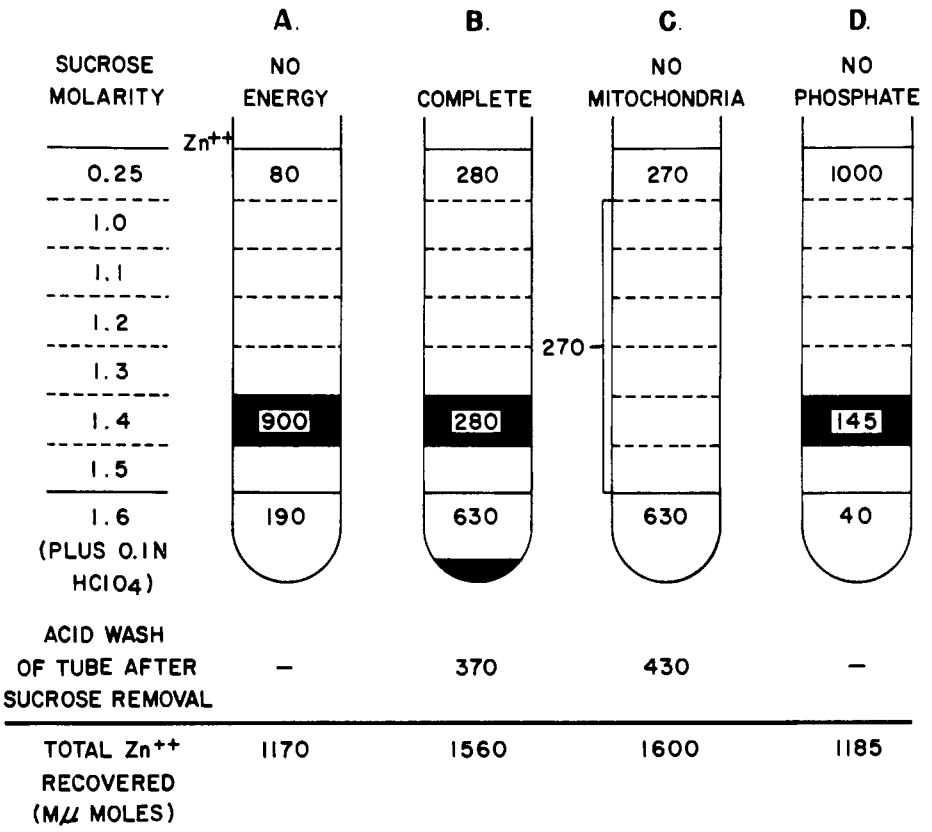

FIGURE 1: Sucrose density gradient study of Zn^{2+} uptake by mitochondria in the presence of phosphate. Mitochondria (5 mg of protein) were incubated for 3 min at 25° in a medium of sucrose (0.25 M), $MgCl_2$ (10 mM), Tris-HEPES (8 mM, pH 7.0), rotenone (7 μM), and Zn^{2+}-acetate (0.5 mM). Flask A had no substrate added; the others contained Tris-ascorbate (5 mM) and TMPD (0.1 mM). All but flask D contained 5 mM Tris-PO_4. The mitochondria were omitted from flask C. The contents of the incubation flasks were then layered in tubes containing sucrose gradients and centrifuged for 45 min at 20,000 rpm in a Spinco SW 25.1 rotor. The gradients were prepared by placing successive 3-ml layers of 1.5–1.0 M sucrose over 3 ml of 1.6 M sucrose containing 0.1 M $HClO_4$. After centrifugation the layers were collected dropwise through a pinhole in the bottom of the tube and, where indicated, the tubes were washed with 0.1 N $HClO_4$. The Zn^{2+} content of the layers was estimated by atomic absorption spectroscopy of appropriate dilutions. The position of bands of mitochondrial protein is indicated as well as the recovery of Zn^{2+} from the various layers.

cles. It appears significant that the spontaneous, energy-linked accumulation of Mg^{2+} which occurs in the absence of Zn^{2+} results in little displacement of the endogenous K^+. Uncouplers of oxidative phosphorylation, such as CCP and dinitrophenol, block the loss of endogenous cations in the presence of Zn^{2+} (Table I). Treatment with CCP in the absence of Zn^{2+} results in the release of the portion of the mitochondrial K^+, but the combination of CCP and Zn^{2+} prevents the release.

The Uptake of Zn^{2+} in the Absence of P_i. In the absence of P_i the uptake of Zn^{2+} shows quite different properties. At concentrations of Zn^{2+} below about 0.3 mM a large portion of the available Zn^{2+} is taken up by an energy-linked reaction (Table II). The uptake is supported by either endogenous substrate or by ascorbate–TMPD oxidation. It is inhibited by inhibitors of respiration and uncouplers of oxidative phosphorylation, but not by oligomycin or by *p*-chloromercuriphenylsulfonate. In each case the uptake of Zn^{2+} is reflected in the loss of most of the endogenous K^+ of the mitochondrion and a portion of the bound Mg^{2+}. When Zn^{2+} uptake is prevented by rotenone or CCP, the loss of endogenous cations is considerably diminished (Table II). The energy-linked uptake is superimposed on a concentration-dependent passive uptake of Zn^{2+}. Both the active and the passive uptakes tend to converge on a limiting value of just over 150 mμmoles of Zn^{2+}/mg of protein (Figure 2). The amount of K^+ lost from the mitochondrion depends closely on the amount of Zn^{2+} accumulated. As the concentration of Zn^{2+} available to the mitochondrion is increased, more Zn^{2+} is taken up by both the active (Figure 3A) and the passive (Figure 3B) processes. Loss of mitochondrial K^+ is nearly complete when 30 mμmoles of Zn^{2+}/mg of protein have been accumulated in the presence of ascorbate–TMPD respiration. The outflow of K^+ is not so precipitous in the case of the passive uptake of Zn^{2+} (CCP present). However, as larger amounts of Zn^{2+} are driven into (or onto)

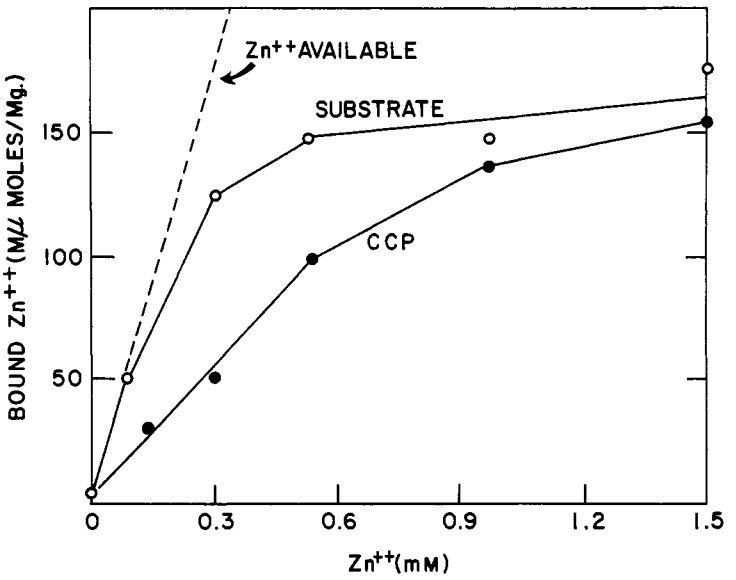

FIGURE 2: The energy-linked accumulation of Zn^{2+} by heart mitochondria and the uptake of Zn^{2+} by uncoupled mitochondria in the absence of added phosphate. Mitochondria were incubated in the medium described in the legend for Table I except that phosphate was omitted. Zn^{2+} was added as the acetate salt to the concentration indicated. The open circles show the Zn^{2+} content of mitochondria incubated in the absence of an uncoupler; the closed circles show the result in the presence of CCP (20 μM).

the mitochondrion by this process, complete release of the endogenous K^+ occurs (Figure 3B). There is little, if any, tendency to release mitochondrial Na^+ as the level of intramitochondrial Zn^{2+} is increased. A portion of the bound Mg^{2+} is released in much the same way as the K^+, but no such trend is observed with the small amount of Ca^{2+} present in these preparations (5–10 mμmoles/mg).

The energy-linked (and to a lesser extent, the passive) uptake of Zn^{2+} in the absence of P_i is inhibited by Mg^{2+}. The study shown in Figure 4 establishes that as the concentration of Mg^{2+} in the suspending medium is increased at a constant level of Zn^{2+} (100 μM) and in the presence of ascorbate–TMPD respiration, the amount of Zn^{2+} recovered from the mitochondrion decreases sharply. Mitochondrial Mg^{2+} increases with increasing Mg^{2+} concentration, but mitochondrial K^+ is not retained even when very little Zn^{2+} is accumulated. If the identical experiment is run with an uncoupler present (Figure 4A, curves marked CCP) the passive uptake of Zn^{2+} is diminished, the uptake of Mg^{2+} is lowered considerably, and a large portion of the mitochondrial K^+ is retained. It should be noted that increasing Mg^{2+} concentrations under these conditions promote the retention of K^+ even in the presence of CCP and Zn^{2+}. For comparison, the effect of increasing Mg^{2+} concentration on mitochondrial cation content in the absence of Zn^{2+} is presented in Figure 4B. It is apparent that the spontaneous uptake of Mg^{2+} under these conditions, as in the presence of P_i, results in little displacement of mitochondrial K^+. Mg^{2+} protects against K^+ loss induced by the presence of CCP. Comparison of the plots for Mg^{2+} uptake in the presence and the absence of CCP in Figure 4B shows a small respiration-dependent uptake which can be regarded as the spontaneous, energy-linked accumulation of Mg^{2+} in the absence of P_i (Brierley *et al.*, 1964). The data of Figure 4A indicate that this increment (Mg^{2+} uptake in the absence of CCP over that in the presence of uncoupler) is much larger in the presence of Zn^{2+}.

The energy-linked uptake of Zn^{2+} is inhibited slightly by K^+ under similar conditions (10 mM KCl inhibits the active increment of Zn^{2+} uptake by 41% as compared with 92% for 10 mM $MgCl_2$), but not by NaCl or choline chloride.

Retention of Bound Zn^{2+} by the Mitochondrion. The above experiments show that the mitochondrion can take up Zn^{2+} by an active process and by a passive mechanism either in the presence or the absence of P_i. Zn^{2+} bound under each of these four conditions is retained upon washing in cold isotonic sucrose (Table III). Zn^{2+} bound in the absence of energy is lost to a greater extent on washing than is Zn^{2+} bound in the presence of respiration. Addition of EDTA causes the loss of the bulk of the bound Zn^{2+} except in the case of Zn^{2+} taken up in the absence of P_i and the presence of respiration. In this latter case more than 50% of the bound Zn^{2+} is retained even in the presence of a large molar excess of EDTA (Table III).

Activity of Bound Zn^{2+}. The question of whether Zn^{2+} firmly bound to the mitochondrion can activate the energy-linked accumulation of Mg^{2+} or K^+ was also investigated. The data presented in Table IV establish that Zn^{2+} taken up by the mitochondrion in the presence of P_i and the absence of energy does

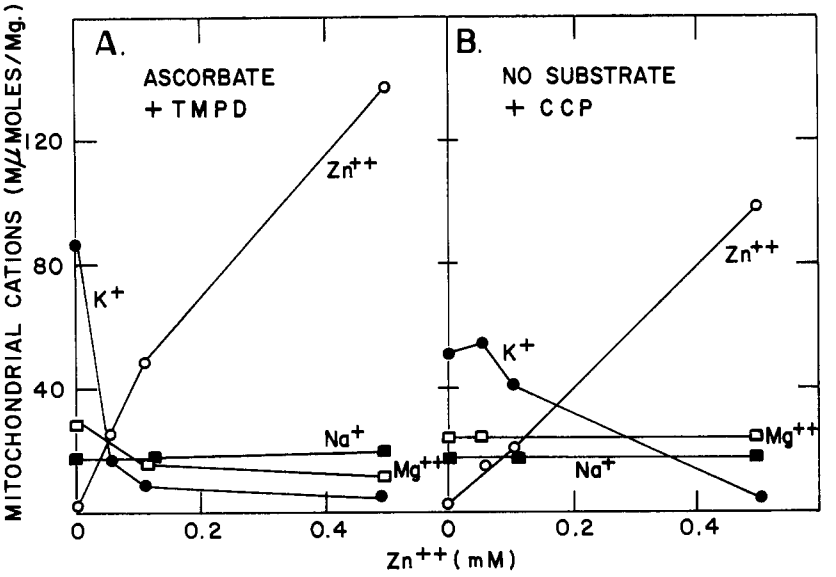

FIGURE 3: Release of endogenous cations from heart mitochondria as a function of Zn^{2+} uptake. (A) Mitochondrial cations following 3 min of incubation at 25° in a medium consisting of sucrose (0.25 M), Tris-HEPES (7 mM, pH 7.0), Tris-ascorbate (5 mM), TMPD (0.1 mM), rotenone (7 μM), and Zn^{2+}-acetate (100 μM). The mitochondria were sedimented (5 min at 15,000*g*), extracted with perchloric acid, and analyzed for Zn^{2+}, Mg^{2+}, K^+, Na^+, and Ca^{2+}. Ca^{2+} values varied from 5 to 10 mμmoles/mg with no trend toward release with increasing Zn^{2+}. (B) Mitochondrial cations following incubation under the conditions of part A with the omission of ascorbate and TMPD and addition of CCP (20 μM).

TABLE II: The Energy-Linked Uptake of Zn^{2+} in the Absence of P_i.[a]

Condition	Mitochondrial Cations (mμmoles/mg)		
	Zn^{2+}	K^+	Mg^{2+}
Endogenous substrate	40	13	14
Endogenous substrate + rotenone	22	36	19
TMPD–ascorbate	55	10	14
TMPD–ascorbate + CCP (20 μM)	20	57	21
TMPD–ascorbate + oligomycin (2 μg/mg)	50	10	14
TMPD–ascorbate + *p*-chloromercuriphenylsulfonate (100 μM)	48	10	23
No Zn^{2+}, endogenous substrate	3	81	25

[a] Mitochondria were incubated at the concentration and in the incubation medium described in the legend for Table I with omission of phosphate. Zinc acetate was present except where indicated at a concentration of 0.1 mM (60 mμmoles/mg).

activate the energy-linked uptake of Mg^{2+} and P_i. It should be noted that these mitochondria take up more Zn^{2+} and the energy-linked uptake of Mg^{2+} is further activated when additional Zn^{2+} is added during the assay.

Mitochondria which have accumulated Zn^{2+} under other conditions do not survive the washing procedure as well as those just considered and consequently, assay of these particles for energy-linked Mg^{2+} uptake gives somewhat equivocal results. Studies in collaboration with Dr. C. T. Settlemire (to be published) indicate that Zn^{2+} bound in the absence of P_i by the energy-linked reaction activates the energy-linked uptake of K^+ to some extent even in the presence of EDTA. A large portion of the Zn^{2+} bound under these conditions is retained (Table III) in the presence of EDTA and the concentration of free Zn^{2+} appears to be negligible. A similar marginal activation of Mg^{2+} uptake by Zn^{2+} bound under these conditions is also observed.

Reversibility of the Interaction of Zn^{2+} with the Mitochondrion. Under the conditions of the energy-linked accumulation of Mg^{2+} and P_i, addition of Zn^{2+} markedly activates the reaction. The study shown in Figure 5 establishes that this activation is reversible. In this study the rate of ion uptake was monitored by the decrease in $^{32}P_i$ in the filtrate following separation of the mitochondria on membrane filters. The Zn^{2+} content of the filtrates was also determined and is shown in Figure 5B. It is apparent that in the

TABLE III: Retention of Zn^{2+} Taken up by Heart Mitochondria under Different Conditions.[a]

No.	Conditions for Zn^{2+} Uptake	Initial Zn^{2+} Content[b]	Zn^{2+} after 2 Washes[b]	% Retained	Zn^{2+} after 1 Wash in EDTA[b]	% Retained
1	Zn^{2+}, TMPD, P_i	44	33	75	11	25
2	Zn^{2+}, P_i	39	22	56	9	23
3	Zn^{2+}, TMPD	53	44	83	31	59
4	Zn^{2+}	65	45	69	10	15

[a] Mitochondria (5 mg of protein) were incubated for 3 min at 25° in the following media. Condition 1: sucrose (0.25 M), Tris-HEPES (5 mM, pH 6.9), Tris-ascorbate (5 mM), TMPD (0.1 mM), Tris-PO_4 (3 mM), rotenone (7 μM), and Zn^{2+} (50 mμmoles/mg); condition 2: as for 1 but with ascorbate and TMPD omitted; condition 3: as for 1 but with phosphate omitted; and condition 4: as for 2 but with P_i omitted and 100 mμmoles of Zn^{2+}/mg. The mitochondria were separated by centrifugation and resuspended in 0.25 M sucrose. A sample was withdrawn for Zn^{2+} analysis, another treated with EDTA (millimolar) and reisolated by centrifugation, and a third was washed twice in 10 ml of cold sucrose (0.25 M). Zn^{2+} was estimated by atomic absorption spectroscopy of acid extracts. [b] In millimicromoles per milligram.

TABLE IV: Activation of Mg^{2+} Accumulation by Bound Zn^{2+} and by Zn^{2+} Added to the Assay.[a]

Treatment of Mitochondria	No Added Zn^{2+}[b]		Zn^{2+} (67 μM) Added to Assay[b]	
	Mg^{2+}	Zn^{2+}	Mg^{2+}	Zn^{2+}
Untreated	80	1	300	25
Untreated, washed once	130	1	200	24
Preincubated with Zn^{2+}, washed once	200	42	360	58
Untreated, washed three times	100	2	300	29
Preincubated with Zn^{2+}, washed three times	197	36	325	58

[a] The accumulation of Mg^{2+} and the Zn^{2+} content of the mitochondria were determined as follows. Mitochondria (5 mg of protein) were incubated for 3 min at 30° in 3 ml of a medium consisting of sucrose (0.25 M), $MgCl_2$ (10 mM), Tris-HEPES (8 mM, pH 7.0), Tris-PO_4 (3 mM), Tris-ascorbate (5 mM), TMPD (0.1 mM), and rotenone (7 μM). The tubes were cooled rapidly and centrifuged. The resulting pellets were extracted with $HClO_4$ and the Mg^{2+} and Zn^{2+} content estimated by atomic absorption spectroscopy. Washed mitochondria were prepared by suspending 50 mg of mitochondrial protein in 25 ml of cold 0.25 M sucrose followed by centrifugation. This process was repeated three times where indicated. Preparations were preincubated with Zn^{2+} by incubating 50 mg of protein in 25 ml of sucrose (0.25 M), Tris-PO_4 (3 mM), Tris-HEPES (8 mM, pH 7.0), and Zn^{2+}-acetate (200 μM) for 3 min at 25°. The mitochondria were isolated by centrifugation and washed as described above. [b] In millimicromoles per milligram of protein.

presence of low concentrations of Zn^{2+} the rate of Mg^{2+} and P_i uptake is greatly enhanced over that seen in the absence of Zn^{2+}. As expected, added Zn^{2+} is present largely in the mitochondria under these conditions. Addition of EDTA removes the bulk of the Zn^{2+} from the pellet (Figure 5B) and lowers the rate of ion uptake to nearly that seen in the absence of Zn^{2+} addition. A second addition of Zn^{2+} causes the reappearance of Zn^{2+} in the mitochondrion and again activates the rate of ion accumulation (Figure 5A).

Two other parameters which are closely associated with the energy-linked accumulation were also monitored during these experiments. Figure 5C shows that the elevated rate of respiration with TMPD–ascorbate which is associated with the Zn^{2+}-activated accumulation of Mg^{2+} (Brierley, 1967) is rapidly inhibited upon the addition of EDTA. The rate of O_2 uptake falls from 0.39 μatom/min per mg of protein in the presence of Zn^{2+} to 0.29 following the addition of EDTA. A second addition of Zn^{2+} elevates the rate to 0.35. Changes in pH also reflect the change in rate of Mg^{2+} accumulation under these conditions (Brierley, 1967). In the presence of Zn^{2+} the increased ion accumulation releases H^+ into the medium which counteracts the alkalinization of the medium due to oxidation of ascorbate, and a low rate of pH change is observed. Addition of EDTA accelerates the pH increase (since

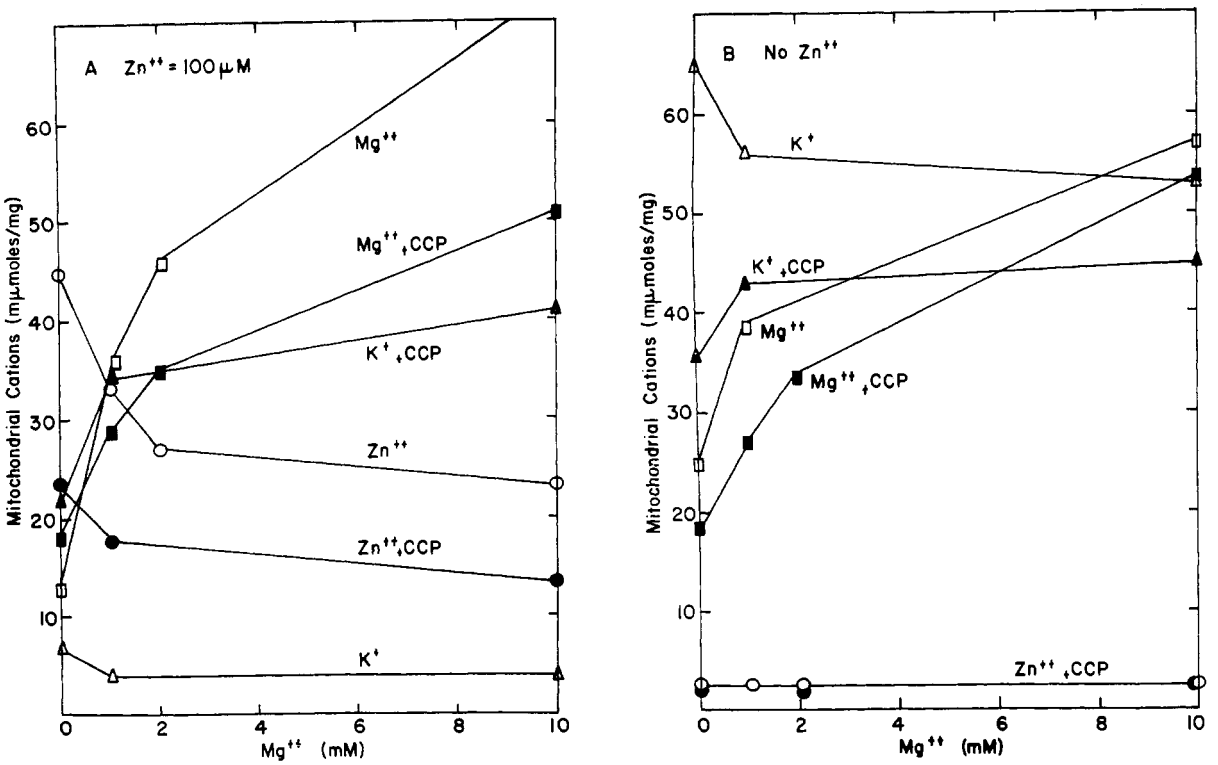

FIGURE 4: Effects of increasing Mg^{2+} on mitochondrial cation content in the presence and absence of Zn^{2+}. (A) The effect of increasing Mg^{2+} on mitochondrial cation content in the presence of Zn^{2+} (100 μM). Two conditions are plotted; the open symbols show the values for mitochondrial Zn^{2+}, Mg^{2+}, and K^+ when mitochondria are incubated with ascorbate, TMPD, and the indicated amount of $MgCl_2$ as described in the legend for Figure 3A. The closed symbols show the mitochondrial cation content under the identical conditions except with the addition of CCP (20 μM). (B) The effect of increasing Mg^{2+} on mitochondrial cations in the absence of Zn^{2+}. Open symbols: values obtained in the presence of ascorbate–TMPD respiration as described in the legend for Figure 3. Closed symbols: values obtained in the presence of ascorbate, TMPD, and CCP (20 μM).

it inhibits Mg^{2+} uptake and H^+ release). A second addition of Zn^{2+} decreases the rate of pH increase as expected. Recordings of the rate of oxidation and of pH change show an almost immediate response to the addition of either Zn^{2+} or EDTA indicating that the binding of Zn^{2+} to the mitochondrion and its removal are both rapid reactions. We are unable to estimate a rate for either the binding or the removal of Zn^{2+} by use of the filtration procedure (Figure 5B), since both reactions appeared to be complete in less than 20 sec. Records of pH changes and respiration indicate that several cycles of Zn^{2+} activation and inhibition by EDTA can be observed and that the reaction appears to be essentially reversible. Cysteine also reverses the effect of Zn^{2+} on Mg^{2+} transport under similar conditions.

Zn^{2+} inhibits the oxidation of succinate and other physiological substrates of mitochondrial respiration (Hunter and Ford, 1955; Skulachev *et al.*, 1967). This property can also be used to demonstrate the ease of reversibility of the interaction of Zn^{2+} with the mitochondrion in the presence of P_i. The study shown in Figure 6 establishes that 60 μM Zn^{2+} rapidly lowers the rate of succinate oxidation in the presence of CCP from 0.55 to 0.21 μatom of O_2/min per mg of protein. Addition of an equal amount of EDTA causes the rate to return to 0.52. This cycle can be repeated several times as shown. In a coupled system the presence of Zn^{2+} inhibits the high rate of respiration expected with succinate on addition of ADP. Addition of EDTA under these conditions (Figure 6B) results in an immediate state 3 rate (Chance and Williams, 1955) with a P:O ratio of 1.5 for succinate and respiratory control ratio of 1.6. These values were not significantly lower than the corresponding controls which were not treated with Zn^{2+}. In another preparation, for example, the state 3 rate of untreated mitochondria with succinate was 0.34 μatom of O_2/min per mg of protein, the P:O ratio was 1.5, and the respiratory control ratio was 3.5. Treatment with Zn^{2+} (50 μM) lowered the initial rate from 0.07 to 0.02 and abolished the response to ADP. Addition of EDTA caused a burst of respiration correspondong to a state 3 rate of 0.34, a P:O ratio of 1.5, and a respiratory control ratio of 3.5. Similar values were obtained when EDTA was added as much as 5 min after Zn^{2+} addition.

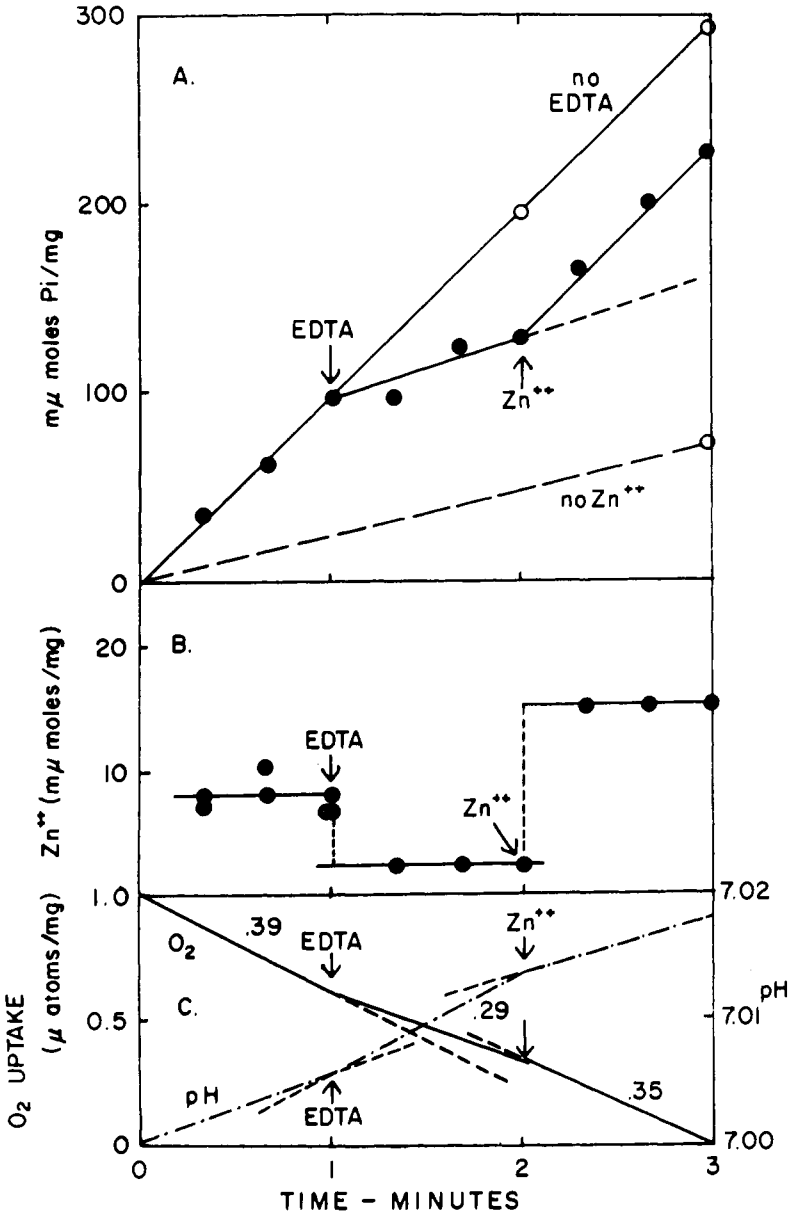

FIGURE 5: Studies of Zn^{2+}. (A) The effect of Zn^{2+} and EDTA additions on the rate of Mg^{2+} accumulation by isolated heart mitochondria. Ion uptake was estimated by the decrease in $^{32}P_i$ in the filtrate following separation of the mitochondria by filtration through AM-6 Polypore filters. The experiment was started by the addition of mitochondria (17 mg of protein) to 10 ml of the following medium: sucrose (0.25 M), $MgCl_2$ (10 mM), Zn^{2+}-acetate (33 μM), Tris-ascorbate (5 mM), and Tris-PO_4 labeled with ^{32}P. The medium was stirred rapidly with a magnetic stirrer. Samples (1 ml) were withdrawn and filtered at the indicated points. The radioactivity of the filtrate was then estimated using a thin end-window counter. After 1 min of incubation EDTA was added to a final concentration of 50 μM. After the second minute of incubation Zn^{2+} acetate was added to a concentration of just over 100 μM. Parallel control incubations in which the addition of Zn^{2+} and of EDTA were omitted are also shown. (B) The Zn^{2+} content of the mitochondria of part A. The filtrates from the experiment shown in part A were diluted with 0.1 N HCl and analyzed for Zn^{2+} by atomic absorption spectroscopy. (C) The effect of the presence and the removal of Zn^{2+} on the rate of respiration and on the pH changes which accompany the accumulation of Mg^{2+} and P_i. Respiration and pH were recorded simultaneously using a YSI 5331 Clark electrode and a Thomas 4858 combination electrode in a 5-ml closed cuvet. The medium was identical with that used in the experiment shown in part A except that Tris-HEPES was omitted and the P_i was not labeled. Omission of Tris-HEPES has little affect on control experiments identical with those of part A. The rate of respiration in microatoms of O_2 per minute per milligram of protein is indicated for each addition. EDTA and Zn^{2+} concentrations and points of addition were identical with those of part A.

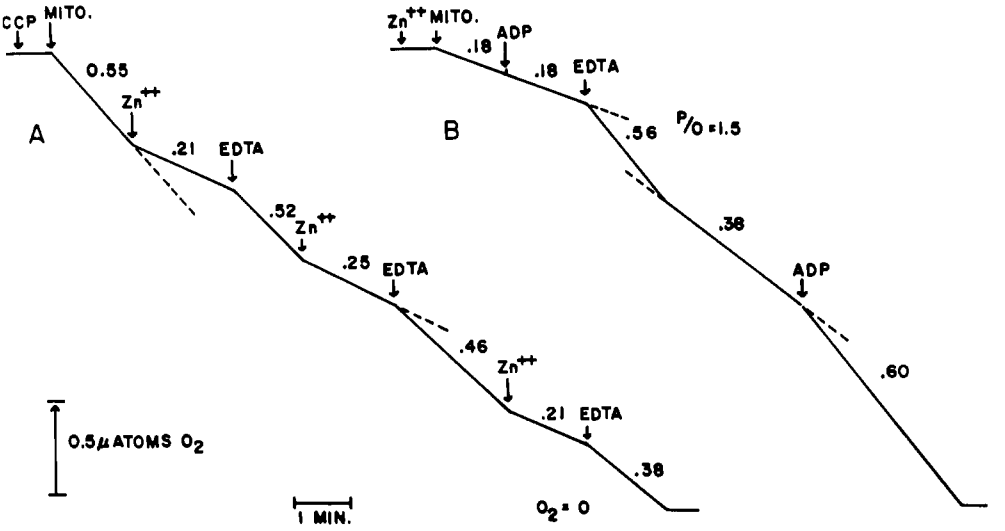

FIGURE 6: Effects of Zn^{2+} and EDTA on respiration with succinate as substrate. (A) The mitochondria (2.5 mg o protein) were uncoupled with CCP (6 μM) and treated with successive additions of 0.3 μmole of Zn^{2+} acetate and 0.3 μmole of Tris-EDTA. Respiration was measured using a Clark electrode at 25° in a closed chamber containing 5 ml of the following medium: sucrose (0.25 M), $MgCl_2$ (5 mM), Tris-PO_4 (5 mM, pH 7.0, Tris-succinate (4 mM), and rotenone (5 μM). (B) Zn^{2+} was present initially and the uncoupler was omitted. ADP (0.75 μmole) was added before the addition of EDTA (0.3 μmole). A second addition of 2.0 μmoles of ADP produced the final deflection.

Discussion

The present study establishes that Zn^{2+} taken up under different conditions produces different responses in the mitochondrion. Endogenous K^+ and Mg^{2+} (but not Na^+ or Ca^{2+}) are lost when Zn^{2+} is taken up in the presence of respiration regardless of whether P_i is present or not. Zn^{2+} bound in the presence of respiration but the absence of P_i is very difficult to remove from the mitochondrion. It appears, therefore, that multiple forms of bound Zn^{2+} may exist and that changes in the conditions of uptake result in alteration in the predominant form of the bound Zn^{2+}.

Previous studies in our laboratory have established that Zn^{2+} markedly activates the energy-linked accumulation of both Mg^{2+} (Brierley, 1967; Brierley and Bhattacharyya, 1966) and K^+ (Brierley *et al.*, 1966; Brierley and Settlemire, 1967) under conditions in which the supply of energy is maintained. The accelerated accumulation of these ions requires the presence of P_i or arsenate. Studies of the induction of mitochondrial ion transport by valinomycin (Pressman, 1965), gramicidin (Chappell and Crofts, 1966), and parathyroid hormone (Rasmussen and Ogata, 1966) have indicated that P_i accelerates the rate of ion uptake in the presence of these inducers but is not essential for the process. The present study indicates that the efficacy of P_i in the presence of Zn^{2+} can probably be ascribed to its ability to control the amount of free Zn^{2+} available to the system while delivering Zn^{2+} as a phosphate salt to the membrane.

In the presence of P_i and the absence of respiration the bulk of the available Zn^{2+} sediments with the mitochondrial band. This association shows no energy dependence and appears to be the result of the insolubility of Zn^{2+} phosphate and adsorption of the precipitate to the mitochondrion. In this form Zn^{2+} causes little change in the endogenous ion content and is more susceptible to removal by washing than other forms of bound Zn^{2+}. It appears possible that in this condition the Zn^{2+} has not interacted with the membrane to bring about the changes which result in induced ion uptake, but is poised in close juxtaposition to these sites. If respiration is then initiated by the addition of ascorbate and TMPD (respiration with this substrate is largely insensitive to Zn^{2+}) membrane changes become apparent. The mitochondria lose endogenous K^+ and Mg^{2+} if neither of these cations is supplied in the incubation medium. If K^+ or Mg^{2+} is present at sufficient concentration, the energy-linked uptake is activated.

In the absence of P_i the amount of Zn^{2+} available to the system is greatly increased for any given concentration. Since the nonspecific (and apparently inactive) deposition of the phosphate salt does not occur, it is possible to observe an energy-linked accumulation Zn^{2+} under these circumstances. The uptake of Zn^{2+} stops after about 150 mμmoles/mg have been accumulated, indicating that a fixed number of binding sites are available. In the absence of an energy source, higher concentrations of Zn^{2+} also cause the saturation of these sites. As in the presence of P_i, uptake of Zn^{2+} in the absence of P_i is reflected in extensive loss of endogenous K^+ and a lesser loss of Mg^{2+} when these cations are not supplied in the incubation medium. An additional feature of the inter-

relationship of these cations is apparent in the absence of P_i in that the addition of Mg^{2+} to the medium results in marked inhibition of Zn^{2+} uptake. A lesser inhibition of Zn^{2+} uptake is seen in the presence of K^+, but not with Na^+ or choline$^+$. The energy-linked uptake of Na^+ and choline$^+$ is not activated by Zn^{2+} (Brierley and Settlemire, 1967). In the presence of Zn^{2+}, Mg^{2+}, and an energy source, despite the low Zn^{2+} content of isolated mitochondria, two major changes in mitochondrial cations occur. The energy-linked increment in bound Mg^{2+} increases markedly and there is an almost quantitative release of mitochondrial K^+. Mitochondrial Na^+ is not released under these conditions. These results suggest a close correspondence between the events involved in the uptake, transport, and retention of Zn^{2+}, Mg^{2+}, and K^+. The results, in conjunction with the earlier report of Gamble (1963), also support the suggestion that the uptake and retention of Na^+ by the mitochondrion involves factors different from those which affect mitochondrial K^+.

The nature of the binding sites for Zn^{2+} in the mitochondrion is not yet certain. The insensitivity of Zn^{2+} uptake to mercurials (*p*-chloromercuriphenylsulfonate and mersalyl) makes it unlikely that SH groups are directly involved in the accumulation. Subsequent interaction of accumulated Zn^{2+} with SH groups within the mitochondrion cannot be excluded, however. Interaction with the phosphate moiety of mitochondrial phospholipids appears to account satisfactorily for the observed limit of Zn^{2+} uptake in the absence of P_i (*cf.* Chappell *et al.*, 1964). Zn^{2+} bound in the absence of P_i appears to involve additional binding sites or otherwise interacts with components not available in the presence of P_i since the ability of EDTA to remove the bound Zn^{2+} is considerably less under these conditions.

Earlier experiments in our laboratory have established that mitochondria with low respiratory control ratios accumulate Mg^{2+} more effectively than tightly coupled preparations (Brierley, 1967). The latter preparations show much greater increases in Mg^{2+} uptake following treatment with Cd^{2+} or Zn^{2+}. The possibility was entertained, therefore, that relatively nonspecific membrane lesions might result either from mechanical damage or from interaction with a toxic heavy metal. Treatment with heavy metals has been shown to inhibit mitochondrial respiration (Hunter and Ford, 1955; Skulachev *et al.*, 1967), decrease oxidative phosphorylation (Jacobs *et al.*, 1956), activate ATPase (Lehninger, 1962), promote large amplitude swelling (Tapley, 1956), and generally result in degeneration of mitochondrial activities. The present studies indicate that, at least in the case of Zn^{2+} treatment in the presence of P_i, the increased accumulation of Mg^{2+} is a result of the reversible combination of the heavy metal with a component of the membrane. The ease of reversibility by EDTA and lack of permanent damage support the argument that a nonspecific inactivation or rearrangement of membrane components by the presence of the heavy metal is not involved under these conditions.

The mechanism of mitochondrial ion transport and the relation of this activity to oxidative phosphorylation have yet to be firmly established. Lehninger *et al.* (1967) have recently reviewed this field and summarized a number of suggested mechanisms. In view of the many basic uncertainties regarding energy coupling mechanisms in mitochondria we feel that detailed speculation of the mechanisms by which Zn^{2+} activates the energy-linked uptake of ions by the mitochondrion would be premature. However, the observations reported here are compatible with the suggestion that Zn^{2+} interacts with the membrane or a component of the membrane in such a way as to increase the permeability to K^+ and Mg^{2+}. The available experiments do not yet permit a firm choice between any of the suggested mechanisms available in the literature (*cf.* Chance and Mela, 1966; Rasmussen and Ogata, 1966; Chappell and Crofts, 1966).

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Ion Transport in Liver Mitochondria. V. The Effect of Anions on the Mechanism of Aerobic K^+ Uptake*

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ABSTRACT: The effect of succinate on the stoichiometry of proton translocation and on mitochondrial swelling during K^+ uptake has been studied. The decrease of the H^+:K^+ ratio from 1 to 0.2 is accounted for quantitatively by the intramitochondrial accumulation of succinate.

When the accumulation of succinate is inhibited at alkaline pH the H^+:K^+ ratio increases. When mitochondria oxidize endogenous substrate the decrease of the H^+:K^+ ratio is due to the reuptake of endogenous anions released during storage. Mitochondrial swelling parallels the accumulation of succinate. When K^+ is taken up by the mitochondria in the absence of anions no swelling is observed. The binding of K^+ to the mitochondria in the absence of anions is dependent on metabolism.

It is generally agreed that a rapid and large uptake of univalent cations and release of protons occurs after addition of valinomycin or gramicidin to liver mitochondria incubated in media supplemented with univalent cations under aerobic conditions. The uptake of cations is accompanied by a reversible mitochondrial swelling. However several problems remain still unsolved, among which are the effect of the anions on the stoichiometry of proton translocation and on the mitochondrial swelling and the identification of the energy-dependent step for cation uptake.

Moore and Pressman (1964) and Chappell and Crofts (1965) reported that stimulation of respiration and swelling occurred only in the presence of anions such as phosphate and arsenate. Azzi and Azzone (1965, 1966), Azzone and Azzi (1966), and Ogata and Rasmussen (1966), on the other hand, observed stimulation of respiration and swelling also in the absence of phosphate when succinate or acetate were present. According to Pressman (1965) the uptake of cations did not account for the osmotic movement of water, whereas a correlation between ion and water movements was observed in other laboratories (Chappell and Crofts, 1966; Azzi and Azzone, 1966; Azzone and Azzi, 1966; Ogata and Rasmussen, 1966).

Moore and Pressman (1964) reported a H^+:K^+ ratio of 0.7–0.9, whereas Azzi and Azzone (1966) reported a H^+:K^+ ratio of 0.25. Both results were obtained in media devoid of anions (added) such as phosphate or acetate. Lynn and Brown (1966) and Ogata and Rasmussen (1966) have reported accumulation of anions during K^+ uptake. Harris *et al.* (1966) also observed a H^+:K^+ ratio as low as 0.2 at pH below 7, whereas the ratio was higher at alkaline pH. Chance and Mela (1966a,b) have proposed that the Ca^{2+} translocation across the membrane is dependent on energy supply and not the binding of Ca^{2+} to the mitochondrial membrane.

In the present report it will be shown that the H^+:K^+ ratio decreases from 1 to 0.1 owing to the influx of anions. The anions take up are either those added to the medium, such as succinate or acetate, or those released from the mitochondria during storage. When K^+ is taken up by the mitochondria together with anions an osmotic uptake of water is observed. The binding of K^+ to the mitochondrial membrane, under conditions where the K^+ is not osmotically active, is dependent on energy supply.

Experimental Section

Rat liver mitochondria prepared in 0.25 M sucrose–0.5 mM EGTA[1] were used throughout and all the experiments were conducted at 20–22°. K^+ accumulation and pH were monitored continuously by means of Beckman 39047 and 39030 combination electrodes, respectively (Azzi and Azzone, 1966; Rossi *et al.*,

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[1] Abbreviations used: EGTA, ethylene glycolbis(β-aminoethyl ether)-*N,N*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.