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Ion Transport in Liver Mitochondria

I. METABOLISM-INDEPENDENT Ca^{++} BINDING AND H^+ RELEASE*

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SUMMARY

1. Liver mitochondria are able to bind large amounts of Ca^{++} through a process which is independent of metabolism. The amount of Ca^{++} bound is increased by increasing the pH of the medium and is decreased by the addition of univalent cations. The binding of Ca^{++} to the mitochondria is a function of the concentration of Ca^{++} in the medium and is affected by the Ca^{++} to protein ratio.

2. A large part of the metabolism-independent binding of Ca^{++} occurs in a space which is rendered accessible to univalent cations by valinomycin or gramicidin. In fact when the mitochondria are pretreated with valinomycin or gramicidin, the binding of Ca^{++} is increased in a sucrose medium and decreased in a KCl or NaCl medium. The competition between Ca^{++} and univalent cations has been studied.

3. The metabolism-independent binding of Ca^{++} is coupled to a release of H^+ or of K^+. The conditions affecting the exchange between divalent and univalent cations are studied and data are reported on the stoichiometry of H^+: Ca^{++} and K^+: Ca^{++} in normal and valinomycin-treated mitochondria.

Chappell, Cohn, and Greville (1) have observed a rapid binding of Mn^{++} to mitochondria which is not inhibited by uncouplers or respiratory chain inhibitors. It was suggested that this stage probably represented "a nonspecific surface binding of Mn^{++}, possibly not associated with the accumulation process per se." O'Brien and Brierley (2) have observed a "passive binding" of about 60 mμmoles of Mg^{++} per mg of protein at saturating concentrations of Mg^{++} in the medium. Rasmussen, Chance, and Ogata (3) have denoted the dinitrophenol-insensitive binding of Ca^{++} as "unspecific absorption."

In the present paper, data relevant to the mechanism of the metabolism-independent binding of Ca^{++} to liver mitochondria are reported. It is shown that the metabolism-independent binding of Ca^{++} is influenced by the pH of the medium and the presence of univalent cations, and is accompanied by a release of H^+. Furthermore, strong inhibition of the binding is given by valinomycin or gramicidin in KCl or NaCl media. A preliminary report of the present studies has already been presented (4).

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EXPERIMENTAL PROCEDURE

Rat liver was homogenized in 0.25 M sucrose, 5×10^{-4} M EDTA, or ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid. EDTA was omitted in all subsequent steps. The binding of Ca^{++} was measured by incubating the mitochondria with $^{45}CaCl_2$ and then by separating the mitochondria from the incubation medium either by rapid filtration through membrane filters (average diameter of the pores, 0.6 μ) or, in most cases, by centrifugation at 23,000 $\times$ *g* for 5 min. The mitochondrial pellet was dissolved in 1 N formic acid and the number of counts was measured on an aliquot of the formic acid solution. The amount of water present in the pellet was calculated by difference between the wet and dry weight. The amount of Ca^{++} found in the pellet was corrected for the Ca^{++} present in the total water content of the pellet.

In the equilibrium dialysis experiment, 1 ml of the rotenone-treated mitochondrial suspension (12.9 mg of protein), supplemented with 1×10^{-3} M $CaCl_2$, was kept in a dialysis tube (1 cm diameter). The tube was maintained in rapidly stirred 0.1 M NaCl (250 ml). Aliquots were taken from the outer solution at fixed times. The concentration of soluble Ca^{++} at the end of the experiment was equal in the inner and outer phase, about 2 μM at pH 8 and 3.4 μM at pH 6.5.

pH was recorded with a Beckman pH meter (expanded scale) and a Beckman glass electrode.

K^+ was measured with a Beckman cationic electrode attached to a Beckman pH meter. Calibration of the K^+ electrode was made by adding, in separate samples and under identical experimental conditions, amounts of a standard KCl solution to obtain the same potential change as observed in the experiments with mitochondria.

RESULTS

Effect of pH and Univalent Cations on Metabolism-independent Binding of Ca^{++}—The binding of Ca^{++} in a sucrose medium was measured at various Ca^{++} concentrations. Mitochondria were pretreated for 3 min with a respiratory chain inhibitor, such as

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rotenone, antimycin, or KCN. Ca^{++} was then added and the mitochondria were centrifuged immediately. When the amount of Ca^{++} bound per mg of mitochondrial protein was plotted against the concentration of Ca^{++} in the medium, a "saturation" curve was obtained (Fig. 1). The amount of Ca^{++} bound per mg of protein was slightly increased by raising the pH of the medium. The amount of Ca^{++} bound was practically unchanged when dinitrophenol was added to mitochondria incubated in 0.25 M sucrose. At each pH the amount of Ca^{++} bound was always lower in 0.10 M NaCl than in sucrose media.

The effect of the protein concentration in the medium on the amount of Ca^{++} bound is analyzed in Fig. 2, *A* and *B*. The amount of Ca^{++} bound per mg of protein was lowered by increasing the protein concentrations, and the effect of increasing protein concentrations was more evident at the lower Ca^{++} concentrations (Fig. 2*A*). When extrapolated to zero protein concentration, however, the amounts of Ca^{++} bound per mg of protein were very similar at 0.5 and 1 mM Ca^{++}. Probably, an increase of protein in the medium reduces the actual Ca^{++} concentration and this lowers the amount of Ca^{++} bound if the concentration of Ca^{++} is below the binding constant. A similar dependence of the amount of Ca^{++} bound on the Ca^{++} to protein ratio is seen in Fig. 2*B*. In this experiment, the mitochondria were incubated with the same amount of Ca^{++} at three different pH values. The increase of the Ca^{++}-binding capacity of the mitochondria induced by the increase of pH of the medium was much more evident at the higher than at the lower Ca^{++} to protein ratios. From these experiments, we conclude that the binding of Ca^{++} is a function of the actual concentration of Ca^{++} in the medium and of the binding capacity of the mitochondria for Ca^{++}.

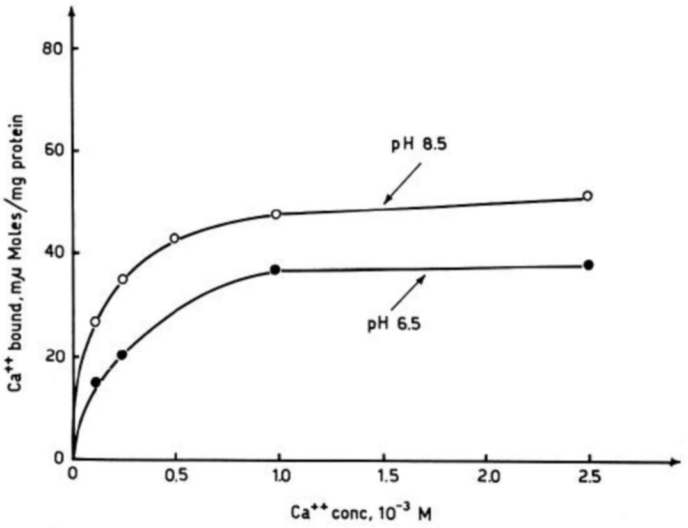

FIG. 1. Binding of Ca^{++} with respect to Ca^{++} concentrations in sucrose. The incubation medium contained, in a final volume of 2 ml, 0.25 M sucrose, 0.025 M Tris-HCl at the pH indicated, 2 μg of antimycin A, and various $CaCl_2$ concentrations as indicated in the figure. Amount of mitochondrial protein was 4.5 mg. Time of incubation was 1 min at 0°.

In Fig. 3 are reported the figures of an equilibrium dialysis experiment. At equilibrium, the amount of Ca^{++} bound to the mitochondria was a function of the pH of the medium.

Sites of Metabolism-independent Ca^{++} Binding and Competition between Univalent and Divalent Cations—The antibiotics valinomycin and gramicidin were used in order to investigate whether the metabolism-independent Ca^{++} binding involves sites which are localized only on the mitochondrial surface or whether it also involves sites localized in the space which is rendered rapidly accessible for univalent cations by valinomycin and gramicidin (5–7). The metabolism-independent binding of Ca^{++} was therefore measured in sucrose and KCl media with mitochondria which had been pretreated with valinomycin. Incubation in a sucrose medium reveals the competition between Ca^{++} and the intramitochondrial K^+. Incubation in a KCl medium reveals the competition between Ca^{++} and extramitochondrial K^+.

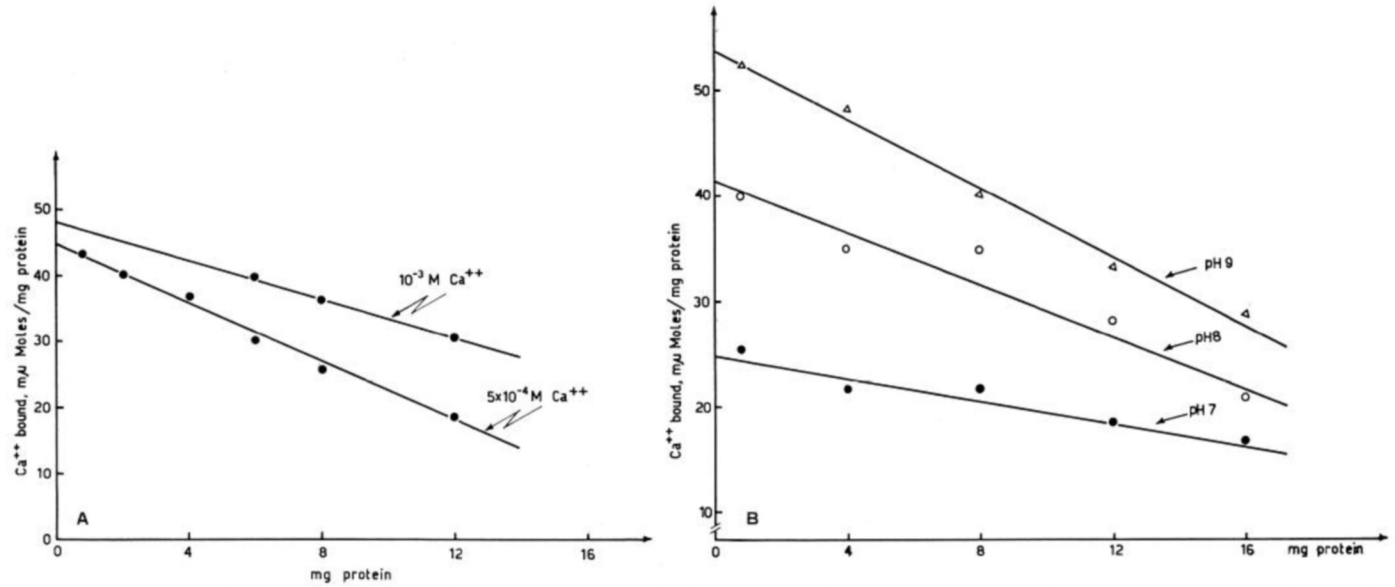

FIG. 2. *A* and *B*, binding of Ca^{++} at different protein and Ca^{++} concentrations and at various pH values. Mitochondria were incubated at pH 8 in 0.1 N NaCl as described in Fig. 1, 2 μM rotenone. In *B*, concentration of $CaCl_2$ was 1×10^{-3} M and the pH indicated was obtained with Tris-HCl.

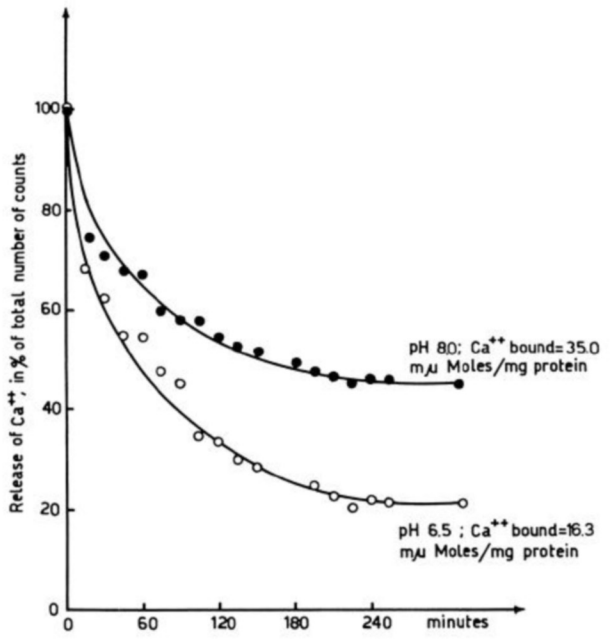

FIG. 3. Binding of Ca^{++} as measured by equilibrium dialysis. One milliliter of mitochondrial suspension, corresponding to 12.9 mg of protein, was pretreated with rotenone and 1×10^{-3} M $^{45}CaCl_2$ for 10 min at 0°. The mitochondrial suspension was then dialyzed against a large volume of 0.1 M NaCl at the pH indicated. At the times indicated in the figures, the number of counts in the dialyzing medium was measured.

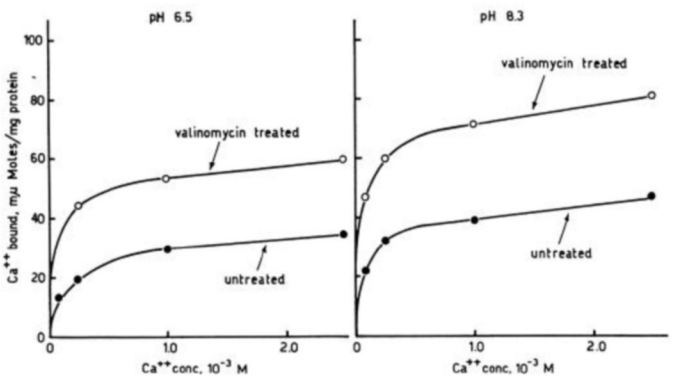

FIG. 4. Binding of various Ca^{++} concentrations to valinomycin-treated mitochondria in sucrose media. Mitochondrial protein, 4.5 mg treated with valinomycin and antimycin, was incubated for 30 min at 0° in a medium containing 25 M Tris-HCl, 0.25 M sucrose, and various $CaCl_2$ concentrations as indicated in the figure. Untreated mitochondria were incubated for 1 min in the same medium in the presence of 2 μg of antimycin. For treatment with valinomycin, the mitochondria were incubated at pH 6.5 in 0.25 M sucrose in the presence of valinomycin and antimycin. The mitochondrial suspension was diluted with 0.25 M sucrose, centrifuged, and resuspended in 0.25 M sucrose.

In Fig. 4 it is seen that the binding of Ca^{++} was considerably higher in valinomycin-treated than in fresh mitochondria when the incubation was carried out in 0.25 M sucrose. The plot of the amount of Ca^{++} bound per mg of protein with respect to the concentration of Ca^{++} in the medium indicated a "saturation" curve in fresh as well as in valinomycin-treated mitochondria. The experiments reported in Fig. 4 show that the initial K^+ content of fresh mitochondria lowers the binding of Ca^{++}. They also suggest that the metabolism-independent binding of Ca^{++} occurs in the same space in which the intramitochondrial K^+ is present. This suggestion is further strengthened by the experiments in which the binding of Ca^{++} in valinomycin-treated mitochondria is measured in KCl media. Valinomycin makes the membrane more permeable to K^+, thus rendering the extramitochondrial K^+ able to compete with Ca^{++} for the intramitochondrial binding sites. As seen in Fig. 5*A*, the binding of Ca^{++} to valinomycin-treated mitochondria was strongly reduced when the incubation was carried out in the presence of KCl. In Fig. 5*B* is reported the binding of Ca^{++} to fresh mitochondria and to valinomycin-treated mitochondria in a KCl medium at pH 6.5. In Fig. 6 a comparison is shown between the binding of Ca^{++} to valinomycin-treated mitochondria in a sucrose and in a KCl medium at various pH values. The increase of pH stimulated considerably the binding of Ca^{++} in sucrose but not in 0.15 M KCl. The above data thus indicate that Ca^{++} is bound by means of a

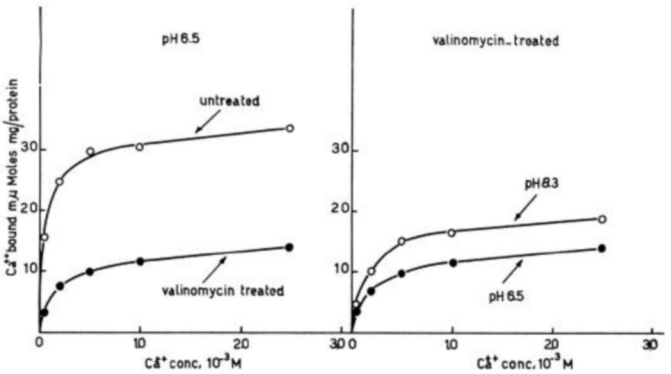

FIG. 5. *A* and *B*, inhibition by K^+ of the binding of various Ca^{++} concentrations in valinomycin-treated mitochondria. Experimental conditions were described in Fig. 4 except that 0.25 M sucrose was replaced by 0.15 M KCl. In *A*, the pH was 6.5.

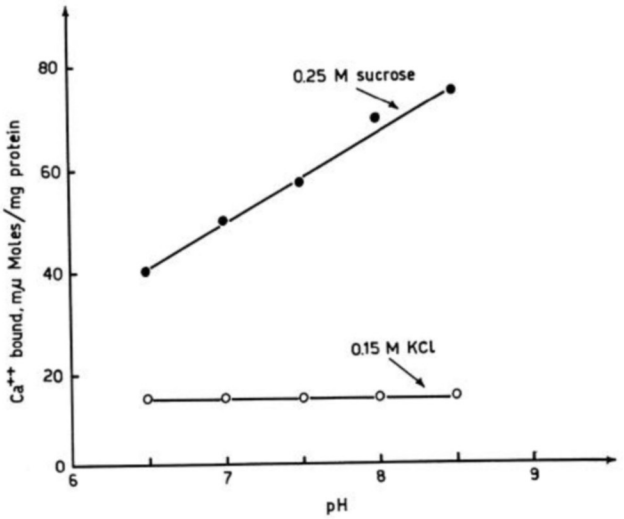

FIG. 6. Binding of Ca^{++} at various pH values to valinomycin-treated mitochondria in sucrose and KCl media. Experimental conditions for valinomycin-treated and untreated mitochondria were as described in Fig. 4.

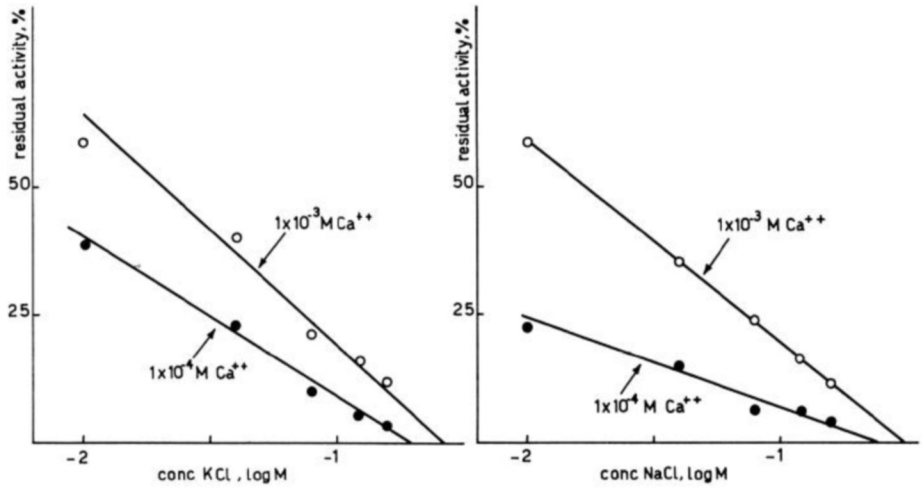

FIG. 7. *A* and *B*, competition between K^+ and Ca^{++} or Na^+ and Ca^{++} in K^+- and Na^+- permeable mitochondria. Mitochondria were pretreated with gramicidin in the presence of antimycin as described in Fig. 4. Incubation was then carried out at various CaCl and KCl or NaCl concentrations for 30 min at 0°. Final osmolarity of 0.25 was obtained with sucrose and 0.025 M Tris-HCl, pH 8.5

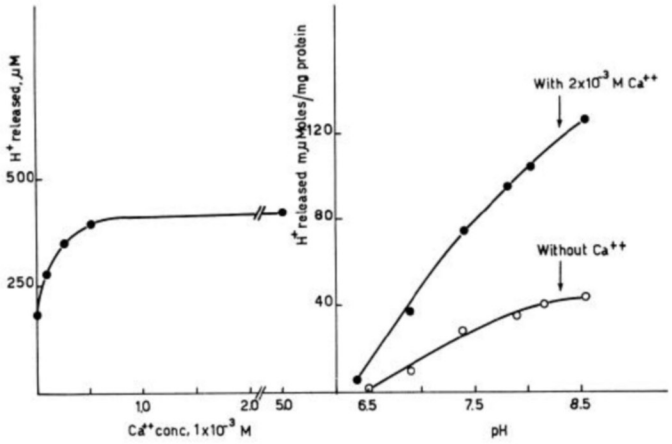

FIG. 8. *A* and *B*, H^+ release at various Ca^{++} concentrations and at various pH values. In *A*, rotenone-treated mitochondria, corresponding to 11.8 mg of protein, were added to an incubation medium containing 0.25 M sucrose, 5×10^{-3} M Tris-HCl (pH 8), 0.5 µg of antimycin, and, when indicated, various Ca^{++} concentrations. In *B*, antimycin-treated mitochondria, corresponding to 5 mg of protein, were added to an incubation medium containing 0.25 M sucrose and 5×10^{-3} M Tris-HCl at the pH indicated. $CaCl_2$, 2×10^{-3} M, was also present when indicated.

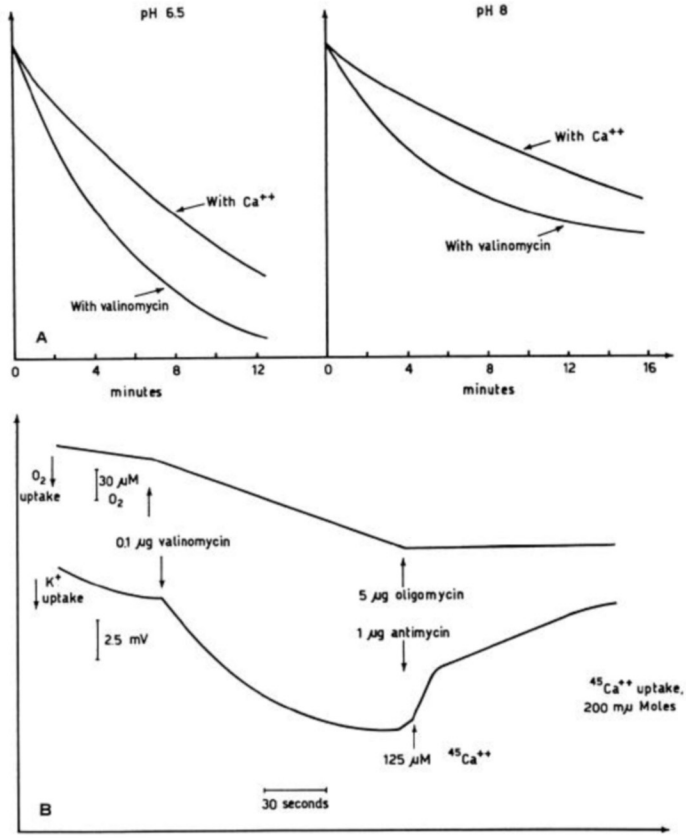

FIG. 9. *A* and *B*, K^+ release and Ca^{++} uptake. In *A*, rotenone-treated mitochondria, corresponding to 5 mg of protein, were incubated in 0.25 M sucrose and 5×10^{-3} M Tris-HCl. The release of K^+ was started by the addition of 1×10^{-3} M $CaCl_2$ or 0.2 µg of valinomycin. In *B*, mitochondria, corresponding to 6 mg of protein, were incubated aerobically in the presence of 0.25 M sucrose, 2×10^{-3} M KCl, 1.5×10^{-3} M P_i, 3×10^{-3} M succinate, and 5×10^{-3} M Tris-HCl (pH 7.5). After oligomycin and antimycin, 125 µM $CaCl_2$ was added and the amount of Ca^{++} taken up was measured by filtration through membrane filters. Oxygen uptake was measured polarographically.

metabolism-independent process in a mitochondrial space which is rendered accessible for K^+ by the addition of valinomycin.

In Fig. 7 is reported the inhibition of Ca^{++} binding to gramicidin-treated mitochondria by K^+ and Na^+ salts. It is seen that the inhibition of Ca^{++} binding was practically complete at concentrations of K^+ and Na^+ of 0.25 to 0.3 M. It also appears from Fig. 7 that at the lower salt concentrations the inhibitory effect of Na^+ is higher than that of K^+.

Metabolism-independent Release of H^+ and K^+—Addition of respiratory-inhibited liver mitochondria to an incubation medium at a pH above 7 to 7.2 caused an acidification of the medium, which in part was accounted for by a release of H^+ due to dissociation of mitochondrial anionic groups. The amount of H^+ release considerably increased when Ca^{++} was present

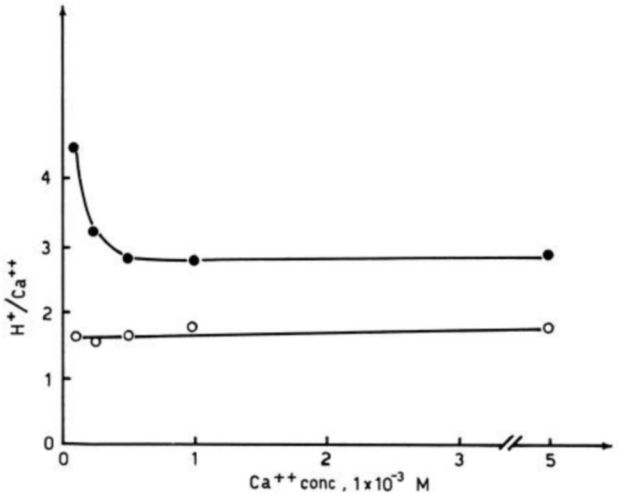

FIG. 10. Stoichiometry between H^+ release and Ca^{++} binding at various Ca^{++} concentrations. H^+ release was measured as described in Fig. 8; 12 mg of mitochondrial protein. The pH of the medium was 8. ○, total H^+ release; ●, extra H^+ release.

in the incubation medium up to a concentration of Ca^{++} of 1 mM (Fig. 8*A*). The amount of H^+ release increased parallel to the increase of pH of the incubation medium (Fig. 8*B*). Also the H^+ release occurring in the presence of Ca^{++} increased parallel to the alkalinization of the medium.

Addition of Ca^{++} to respiratory-inhibited, fresh, liver mitochondria resulted in a slow release of K^+ (Fig. 9*A*). The release of K^+ was faster at pH 6.5 than at pH 8.5. At both pH values, however, the rate of K^+ release caused by Ca^{++} was lower than the rate of K^+ release observed in the presence of valinomycin. In the experiment reported in Fig. 9*B*, liver mitochondria were incubated aerobically in the presence of valinomycin and of 4 mM K^+. A large uptake of K^+ occurred on the expense of respiration. Addition of antimycin and oligomycin initiated an efflux of K^+, driven by the K^+ concentration gradient. Addition of Ca^{++} accelerated the efflux of K^+. During the interval of accelerated K^+ efflux, Ca^{++} was taken up by the mitochondria.

TABLE I

H^+ and K^+ release during Ca^{++} binding in fresh mitochondria

Experimental conditions were as in Fig. 8; 10 mg of mitochondrial protein.

pH	Ca^{++} present	Ca^{++} bound	H^+ released	K^+ released	$H^+:Ca^{++}$ ratio	$H^+ + K^+:Ca^{++}$ ratio
			μmoles/mg protein			
6.6			11.4	56.2		
	+	32.2	27.6	84.5		
			ΔH^+ 16.2	ΔK^+ 28.3	0.5	1.38
7.7			20.7	64		
	+	38.5	78	84.5		
			ΔH^+ 49.3	ΔK^+ 20.5	1.28	1.81

The stoichiometric relationship between K^+ efflux and Ca^{++} influx was 2 K^+ per Ca^{++} (8).

The quantitative relationship between H^+ release and Ca^{++} binding is studied in the experiments of Figs. 10 and 11. In Fig. 10 are reported the $H^+:Ca^{++}$ ratios calculated both on the "total" and "extra" H^+ release at various Ca^{++} concentrations. When calculated on the total H^+ release, the $H^+:Ca^{++}$ ratio was very high at low Ca^{++} concentrations and declined at about 2.6 above 200 μM Ca^{++}. When calculated on the extra H^+ release the $H^+:Ca^{++}$ ratio was constant in the region of 1.6 to 1.7 at all Ca^{++} concentrations. In the experiment of Fig. 11*A*, the mitochondria were depleted of K^+ by treatment with valinomycin at acidic pH. It is seen that the increase of pH of the medium caused almost a parallel increase in the amount of Ca^{++} bound and of H^+ released. The stoichiometry between H^+ release (total) and Ca^{++} bound was about 2.2 at pH 6.3 and tended to increase slightly at the higher pH (Fig. 11*B*). For comparison, the $H^+:Ca^{++}$ ratio in fresh mitochondria is reported in Fig. 1*B*. The $H^+:Ca^{++}$ ratio was particularly low at the acidic pH and tended to increase at the alkaline pH.

That the lower $H^+:Ca^{++}$ ratio in fresh mitochondria is largely due to an exchange of Ca^{++} with intramitochondrial K^+ is indicated by the experiment of Table I. It is seen that a large amount of K^+ was released by the mitochondria parallel to the

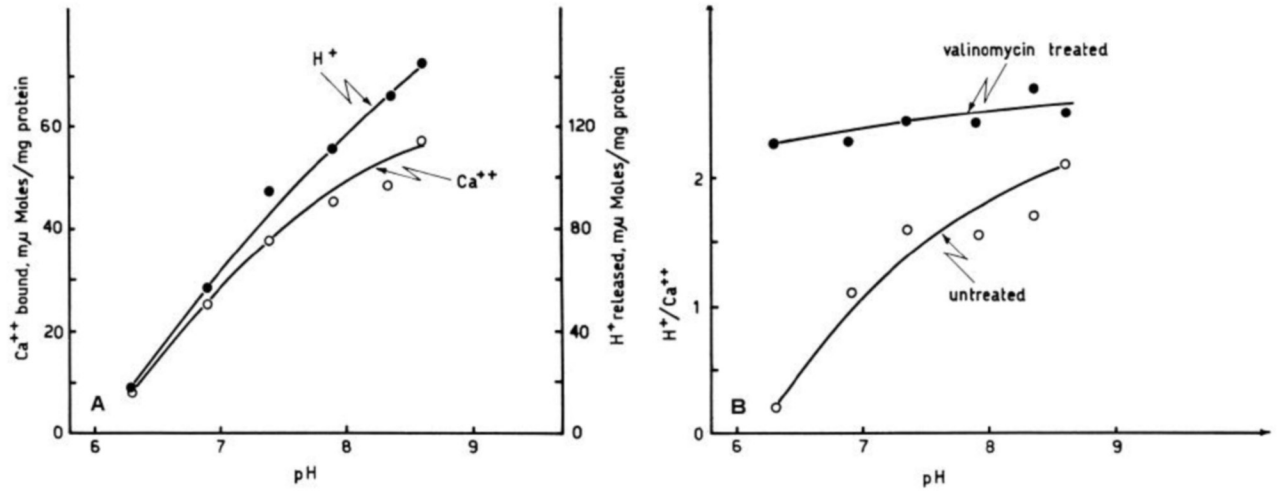

FIG. 11. *A* and *B*, stoichiometry between H^+ release and Ca^{++} binding at various pH values. H^+ release was measured as described in Fig. 8; 2.5×10^{-3} M $CaCl_2$. The binding of $^{45}Ca^{++}$ was measured in parallel samples by centrifugation at 20,000 × *g* after 1 min of incubation; 5 mg of mitochondrial protein. When indicated, mitochondria were pretreated with valinomycin as described in Fig. 4.

binding of Ca^{++}. At pH 6.6 the H^+:Ca^{++} ratio was low, about 0.5, whereas the $H^+ + K^+$:Ca^{++} ratio was about 1.4. At pH 7.7, the H^+:Ca^{++} ratio was 1.28, whereas the $H^+ + K^+$:Ca^{++} ratio was 1.8. Thus at the higher pH, because of the large increase of H^+ release, the contribution of K^+ to the stoichiometry of Ca^{++} binding was considerably lower.

DISCUSSION

Mechanism of Metabolism-independent Binding of Ca^{++}—The following results rule out the possibility that the metabolism-independent binding of Ca^{++} reported here is due to exchange of added $^{45}Ca^{++}$ with intramitochondrial Ca^{++}. First, the amount of Ca^{++} bound per mg of protein was not decreased when mitochondria were incubated in the presence of 35 mM Ca^{++}. Under these conditions, since the Ca^{++} content of our mitochondrial preparations was lower than 10 μmoles per g of protein, the initial ratio between intra and extramitochondrial Ca^{++} is below 1:1000. Second, the metabolism-independent binding of Ca^{++} was coupled to a release of H^+ or K^+, or both.

On the other hand, the present data suggest that Ca^{++} is bound to mitochondrial anions by means of a process which is independent of metabolism but influenced by several physical parameters, namely the pH of the medium and the presence of other cations. The following results appear significant for the characterization of the process. (*a*) The binding of Ca^{++} follows a "saturation" curve; the number of binding sites is constant among various mitochondrial preparations and it is markedly influenced by the pH of the incubation medium. (*b*) The binding of Ca^{++} is accompanied by H^+ release. (*c*) Univalent cations compete with Ca^{++} for the mitochondrial binding sites. We assume therefore that Ca^{++} becomes bound to mitochondrial anions which either are dissociated during the increase of pH of the medium or undergo a decrease of their pK because of the presence of Ca^{++}.

Chappell *et al.* (1) have reported that liver mitochondria bind 20 to 30 μmoles of Mn^{++} per g of protein. Mg^{++}, 60 μmoles per g of protein was bound to heart mitochondria in the experiments of O'Brien and Brierley (2). The data reported in the present paper are in a substantial agreement with those of Chappell *et al.* (1) and of O'Brien and Brierley (2).

The extent of metabolism-independent Ca^{++} binding can be diminished for two reasons, first, because of an increase of the amount of Ca^{++} bound during isolation of mitochondria and, second, because of an increase of intramitochondrial K^+ content (see below). The low figures for Ca^{++} binding observed by Rasmussen *et al.* (3) may be due to either of these causes; at present, it is impossible to say which is responsible.

Sites of Metabolism-independent Binding of Ca^{++}—That the metabolism-independent binding of Ca^{++} involves partly sites localized on the mitochondrial surface appears probable. The following results, however, are relevant for the localization of another part of the mitochondrial sites involved in the metabolism-independent binding of Ca^{++}. (*a*) The amount of Ca^{++} bound by the metabolism-independent process is not additive with the amount bound by the metabolism-dependent process. (*b*) Depletion of intramitochondrial K^+ by treatment with valinomycin is followed by an increase of the metabolism-independent binding of Ca^{++}. (*c*) Permeabilization of the mitochondrial membrane to K^+ by means of valinomycin causes an inhibition of the metabolism-independent Ca^{++} binding in KCl medium; similar results are obtained with gramicidin in a NaCl medium. (*d*) Valinomycin- or gramicidin-treated mitochondria can rapidly accumulate, at the expense of metabolism, concentration gradients of univalent cations (5–8); Ca^{++} can be taken up in exchange with the aerobically accumulated, osmotically active, K^+. We suggest, therefore, that part of Ca^{++} becomes bound by means of a metabolism-independent process to sites which are localized in the same space in which univalent and divalent cations are accumulated by means of a metabolism-dependent process.

The extent to which the H^+ released by the mitochondria in the presence of Ca^{++}, during the metabolism-independent binding, is dissociated from the intramitochondrial anionic groups, cannot be assessed at present. However, it appears likely that, after the mitochondria have been depleted of K^+ and protonated during treatment with valinomycin, the increase of H^+ release observed in the presence of Ca^{++} (*cf.* Fig. 11) comes from the intramitochondrial anionic groups.

Therefore, the metabolism-independent binding of Ca^{++} to the mitochondria can be coupled to a release of either H^+ or K^+. The extent to which intramitochondrial H^+ or K^+ are released from the mitochondria during Ca^{++} binding depends on the permeability of the mitochondrial membrane to K^+ and on the intramitochondrial K^+ content. When the mitochondria have a high K^+ content and the membrane is highly permeable to K^+, the binding of Ca^{++} is coupled mostly to a release of K^+. When the mitochondria have a low K^+ content, the binding of Ca^{++} is coupled mostly to a release of H^+. As shown in Fig. 11 and Table I, the contribution of K^+ to the stoichiometry was always higher at pH 6.5 to 7.0 than at pH 8.0 to 8.5.

Addition of mitochondria to an incubation medium containing Ca^{++} results in a H^+ release from the mitochondria which is higher than that observed in the absence of Ca^{++}. The larger H^+ release due to Ca^{++} can be explained in two ways: (*a*) Ca^{++} lowers the pK of mitochondrial anions; (*b*) equilibration of intra- with extramitochondrial pH can occur only when permeant cations, such as Ca^{++}, are present, because the exchange of ions through the mitochondrial membrane requires maintenance of electrical neutrality. The two possibilities are not mutually exclusive but may coexist. Indication that electrical neutrality is maintained during the exchange of ions through the membrane is given by the observation that a release of 2 K^+ accompany the binding of 1 Ca^{++} through the metabolism-independent process.

The penetration of Ca^{++} in the intramitochondrial spaces in the absence of metabolism classifies Ca^{++} as a permeant cation. Presumably only the distribution of Ca^{++} at the two sides of the membrane, but not the transfer of Ca^{++} through the membrane, is affected by mitochondrial metabolism.

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