The Nature of Ca^{++} Binding by Kidney Mitochondria*

GEORGE W. ENGSTROM AND HECTOR F. DELUCA

From the Department of Biochemistry, University of Wisconsin, Madison

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The uptake of calcium by isolated kidney mitochondria can be supported by either ATP or oxidative phosphorylation. However maximal binding rates are obtained when an oxidizable substrate, ATP, and magnesium ions are present. Approximately one H^+ ion is released for every calcium ion bound. Inorganic phosphate as such is required only in the absence of exogenous ATP and when succinate is used as the substrate. With glutamate as the substrate, inorganic phosphate failed to stimulate calcium uptake. Cyanide (10^{-3} M) and dinitrophenol (2×10^{-5} M) had only a slight inhibitory effect when both succinate and ATP were present, but they strongly inhibited calcium binding in the absence of exogenous ATP. Oligomycin, on the other hand, had little or no effect under these conditions. In the absence of added substrate but in the presence of ATP, cyanide (10^{-3} M) and dinitrophenol (2×10^{-5} M) partially inhibited calcium uptake while oligomycin (10^{-6} M) completely inhibited uptake under these conditions. These findings are consistent with the suggestion that an intermediate of oxidative phosphorylation is involved in the translocation of calcium phosphate into mitochondria. Of special note is that Dicumarol (7×10^{-5} M) and antimycin A (5×10^{-6} M) blocked calcium binding under all conditions employed. Vitamin D had no effect on the rate of calcium uptake in either the medium containing ATP and substrate or in a medium containing ATP and no substrate. When substrate was present but ATP was absent, mitochondria from vitamin D–fed rats accumulated less calcium and released it more rapidly than did those from vitamin D–deficient rats. These findings are consistent with the belief that vitamin D stimulates the release of calcium from isolated mitochondria.

The binding of calcium by mitochondria was first reported by Slater and Cleland in 1953. This important observation went largely unnoticed until recently when it has become an extremely popular subject of investigation. Vasington and Murphy (1961) were the first to report an active uptake of calcium coupled to oxidative phosphorylation of mitochondria. DeLuca and Engstrom (1961) demonstrated that oxidative phosphorylation was not an absolute requirement for calcium uptake by mitochondria provided that an external source of ATP was supplied. Vasington and Murphy (1962) then reported that respiration and ATP were both required for maximum binding of calcium. Simultaneously Brierley *et al.* (1963) and Lehninger *et al.* (1963) demonstrated that calcium binding could be supported by either ATP or respiration. Although the source of energy required for the translocation of calcium is becoming clarified, many other details of this process remain to be resolved. Vasington and Murphy (1962) reported that inorganic phosphate, magnesium ions, and substrate were required for maximal calcium binding, while DeLuca and Engstrom (1961) found no requirement for inorganic phosphate provided that ATP was added externally. Furthermore many discrepancies appear to exist with regard to the sensitivity of the calcium-transport system to various metabolic inhibitors. It therefore appeared imperative to re-examine the binding of calcium by mitochondria using low concentrations of calcium and studying the early events in the binding process. It is the purpose of this paper to report these experiments. Data are also presented which demonstrate the importance of the vitamin D status of the animal to results obtained from calcium-binding studies.

METHODS

Young male rats of the Sprague Dawley strain weighing 60–80 g were fed a diet adequate in calcium and phosphorus for 21–28 days as described previously except that casein plus 0.2% L-cystine served as the protein source (Steenbock and Herting, 1955). In most experiments the rats were divided into two groups; those in one group received 75 IU of vitamin D_2 every third day while those in the other group received no vitamin D. The animals not receiving vitamin D showed reduced growth and lowered serum calcium as reported by Steenbock and Herting (1955). After the rats had been on their respective diets for at least 21 days, the animals were killed by a sharp blow on the head followed by decapitation. Kidney mitochondria were prepared in 0.25 M sucrose at 0° according to a modification of the method of Schneider as described in an earlier report (DeLuca and Engstrom, 1961). The mitochondria isolated from 200 mg of kidney were incubated in a Dubnoff shaker at 30° with 9.4 mM glutamate or 14 mM succinate, 1.9 mM ATP, 0.025 mM cytochrome c (Sigma Co., horse heart type II), 3 mM $MgCl_2$, 15.6 mM imidazole-HCl buffer (*p*H 7.0–7.4) or other buffer as indicated, 145–165 mM sucrose, and 0.3 mM ^{40}Ca (labeled with ^{45}Ca) unless otherwise indicated in a total volume of 6.4 ml. Samples were removed at the indicated times to determine bound calcium and in some cases phosphate.

Two methods of processing the samples were used in these experiments. In the first method, samples were pipetted into chilled tubes and centrifuged at 20,000 $\times$ *g* for 7 minutes. The supernatant fluid was discarded and the pellet was dissolved in 5.0 ml of a 0.2% solution of sodium lauryl sulfate. An aliquot was plated on aluminum planchets and radioactivity was counted as infinitely thin samples in the Geiger region. The second method was a rapid sampling procedure in which perforated stainless steel planchets were used. A filter paper disk was placed over the holes and 2 ml of a 10% aqueous suspension of Hyflo Super-Cel (Johns Manville Co.) was pipetted into the planchet, forming a filter mat after being pulled dry by suction. The samples were pipetted onto the filter-mat planchets, washed twice with 1-ml aliquots of ice cold 0.25 M sucrose, and pulled dry by suction. These planchets were dried in an 80° oven and radioactivity

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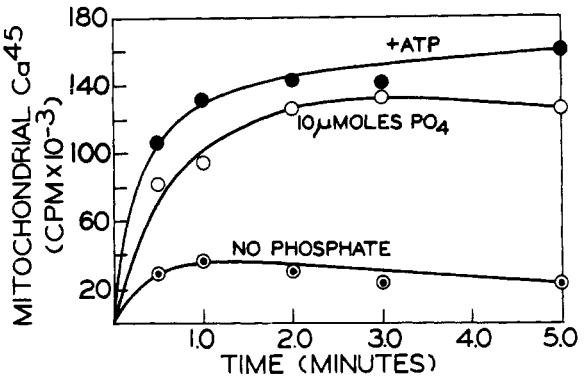

FIG. 1.—Phosphate requirement for maximum calcium binding with succinate as the substrate in the absence of ATP. The reaction mixture described in the text included 14 mM succinate, 15.6 mM imidazole buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with ^{45}Ca), and kidney mitochondria from vitamin D–deficient rats (4.7 mg protein) in a volume of 6.4 ml.

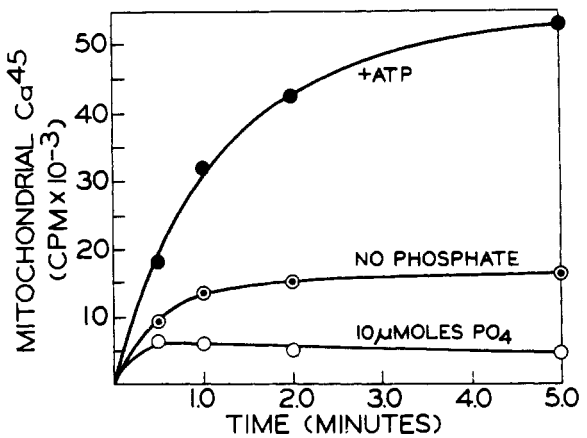

FIG. 2.—Effect of phosphate on calcium binding with glutamate as the substrate in the absence of ATP. The reaction mixture described in the text included 9.4 mM glutamate, 15.6 mM imidazole buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with ^{45}Ca), and kidney mitochondria from vitamin D–deficient rats (4.6 mg protein) in a volume of 6.4 ml.

was counted in the Geiger region. Inorganic phosphate was determined by the method of Chen *et al.* (1956) and calcium by the method of Harrison and Harrison (1955). Acid production was followed by means of a Radiometer automatic titration unit using the standard incubation medium without the imidazole buffer.

RESULTS

In view of the report by Vasington and Murphy (1962) that inorganic phosphate was required for maximal calcium binding and that this was in conflict with our findings, we repeated their experiments using their incubation conditions. The results in Table I demonstrate that addition of phosphate resulted in increased calcium binding relative to that obtained in its absence, in agreement with Vasington and Murphy (1962). However, since Tris buffers poorly in the range in which it was used (*p*H 7.0), the question arose whether the phosphate requirement was valid or whether it was due to buffering by this anion. When *p*H 7.0 imidazole buffer (6.2–7.8) replaced the entire buffer system or only the phosphate portion of the medium of Vasington and Murphy, calcium binding was maximal and unaffected by inorganic phosphate, thereby demonstrating a requirement for adequate buffering rather than phosphate per se.

TABLE I

CALCIUM BINDING BY MITOCHONDRIA IN VARIOUS BUFFERS[a]

Buffer	Concn (mM)	*p*H of Buffer (before)	Mitochondrial ^{45}Ca (cpm)	*p*H of Medium (after)
Tris + phosphate	5.0 4.0	7.0 7.0	155,000	6.1
Tris	5.0	7.0	93,000	5.7
Tris + imidazole	5.0 16.6	7.0 7.0	162,000	6.5
Imidazole	16.6	7.0	163,000	6.5

[a] The 6.0-ml reaction mixture included the buffer or buffers listed above, 80 mM NaCl, 6.7 mM succinate, 10 mM $MgCl_2$, 3 mM ATP, 2.5 mM $CaCl_2$ (labeled with ^{45}Ca), and mitochondria (4.75 mg of protein). The incubations were continued for 15 minutes at 30°.

TABLE II

CALCIUM UPTAKE AND ACID PRODUCTION BY KIDNEY MITOCHONDRIA[a]

Calcium Bound (μmoles)	ATP Split (μmoles)	Total Acid (μeq H^+)	Total Acid − ATP Split (μeq H^+)	μeq H^+ / μmole Ca^{++}
1.8	1.76	3.63	1.87	1.04
2.7	0.75	3.24	2.49	0.92
3.6	1.12	4.41	3.29	0.91
4.5	1.38	5.20	3.87	0.86
5.4	1.12	5.49	4.37	0.81

[a] The 9.6-ml reaction mixture included 3 mM $MgCl_2$, 14 mM succinate, 0.01 mM cytochrome c, 165 mM sucrose, $CaCl_2$ (labeled with ^{45}Ca) as indicated above, 0.1–0.23 mM ATP, no buffer, and mitochondria (7.20 mg protein).

Bartley and Amoore (1958) first reported that manganese-ion (Mn^{++}) binding was accompanied by ejection of H^+ ions into the medium and Chappell *et al.* (1962) have more recently confirmed this. They reported acid production of about 0.9 H^+ ions per manganese ion bound. Thus *p*H and adequate buffering most certainly are important factors in this system especially when such large amounts of calcium are added. After a correction for acidity due to ATP hydrolysis, results in Table II demonstrate that about 0.9 H^+ ion is ejected for every Ca^{++} ion bound over a range of calcium concentration, in agreement with the findings of Chappell *et al.* (1962).

The question of a phosphate requirement was re-examined using the medium described under Methods in which adequate buffering was present. Rapid sampling techniques and low concentration of calcium were also employed in this study. In the absence of ATP (substrate-dependent binding), a phosphate requirement for maximal rate of calcium binding was demonstrated with succinate (Fig. 1) but not with glutamate as the substrate (Fig. 2). There was no phosphate requirement with either substrate when ATP was present, at least at the lower calcium concentrations used.

In view of conflicting reports on the requirement for oxidative phosphorylation for calcium binding it became necessary to re-examine the question under our experimental conditions. Through the use of rapid sampling techniques it was possible to study rates of calcium binding with both ATP and substrate present. It should be noted that inorganic phosphate was used in all experiments dealing with the effect of inhibitors because a comparison was made under three sets of conditions, and in one of those (succinate, no ATP) inorganic phosphate is required for calcium binding.

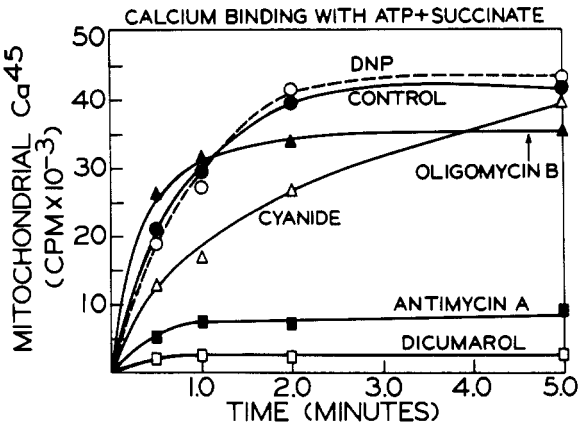

FIG. 3.—Effect of inhibitors on the rate of calcium binding with both ATP and succinate present. The concentrations of the inhibitors were 2×10^{-5} M (DNP), 10^{-3} M (cyanide), 10^{-6} M (oligomycin B), 5×10^{-6} M (antimycin A), and 7×10^{-5} M (Dicumarol). The reaction mixture described in the text included 14 mM succinate, 1.9 mM ATP, 15.6 mM phosphate buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with ^{45}Ca), and kidney mitochondria from vitamin D–deficient rats (4.9 mg protein) in a volume of 6.4 ml.

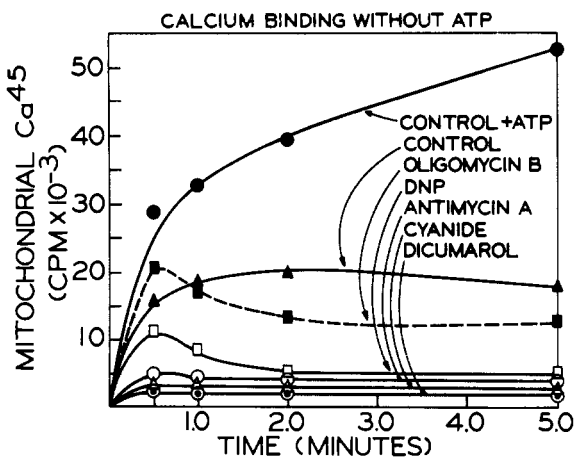

FIG. 4.—Rate of calcium binding by vitamin D–deficient rat kidney mitochondria in the presence of various inhibitors in the absence of ATP. The inhibitor concentrations were 10^{-6} M (oligomycin B), 2×10^{-5} M (DNP), 5×10^{-6} M (antimycin A), 10^{-3} M (cyanide), and 7×10^{-5} M (Dicumarol). The reaction mixture described in the text included 14 mM succinate, 15.6 mM phosphate buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with ^{45}Ca), and mitochondria (4.8 mg protein) in a volume of 6.4 ml.

In 1961, DeLuca and Engstrom first reported that oligomycin did not block calcium binding when both ATP and substrate were present. In the current experiments (Fig. 3) oligomycin had a small effect, much less than that of antimycin A or Dicumarol and slightly less than that of cyanide. When ATP was omitted from the medium, inhibitors of respiration (cyanide) and uncouplers of oxidative phosphorylation (DNP) blocked calcium binding while oligomycin had only a slight effect (Fig. 4). However, when energy was supplied by ATP (Fig. 5) calcium binding was blocked by oligomycin in agreement with Brierley *et al.* (1963) while cyanide (10^{-3} M) and dinitrophenol (2×10^{-5} M) had only a partial effect. In results not shown here, dinitrophenol at 10^{-4} M inhibited to the same degree as did oligomycin in the absence of substrate, possibly through the stimulation of adenosine triphosphatase activity. It should be noted however that antimycin A (5×10^{-6} M) and Dicumarol (7×10^{-5} M) strongly inhibit calcium binding in the absence of either ATP or substrate as well as in the presence of both, in agreement with previous results (DeLuca and Engstrom, 1961). Thus it appears that the energy necessary for calcium binding may be supplied by either substrate (respiration) or exogenous ATP although both are necessary for maximal binding. A schematic representation of the energy and other requirements for calcium binding similar to that of Brierley *et al.* (1963) is shown in Figure 6.

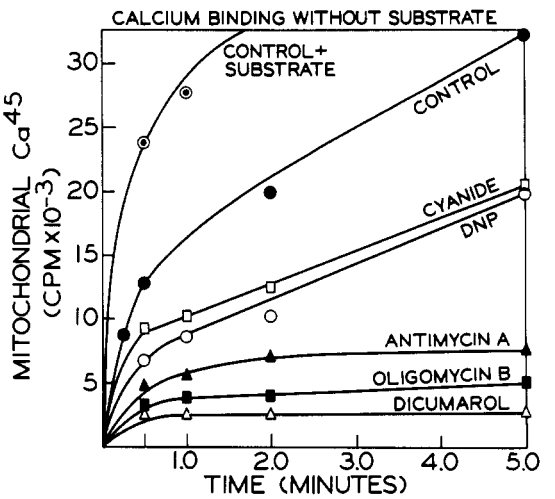

FIG. 5.—Calcium binding by kidney mitochondria in the presence of the various inhibitors and in the absence of substrate. The inhibitor concentrations were 10^{-3} M (cyanide), 2×10^{-5} M (DNP), 5×10^{-6} M (antimycin A), 10^{-6} M (oligomycin B), and 7×10^{-5} M (Dicumarol). The reaction mixture described in the text included 1.9 mM ATP, 15.6 mM phosphate buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with ^{45}Ca), and kidney mitochondria from vitamin D–deficient rats (4.6 mg protein) in a volume of 6.4 ml.

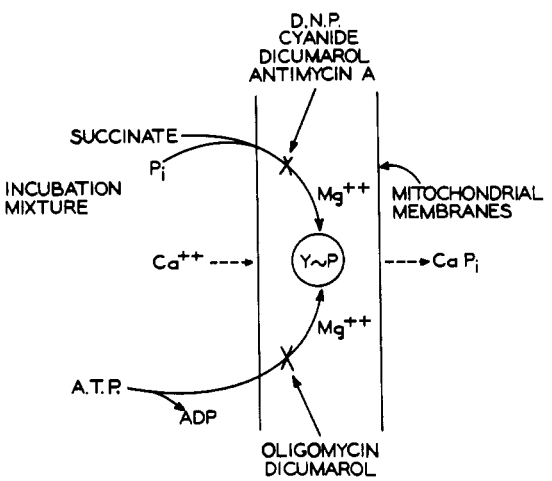

FIG. 6.—A schematic representation of calcium binding by mitochondria.

In a recent publication (Engstrom and DeLuca, 1964) it was reported that the phosphate which is deposited with the calcium originates from ATP and endogenous sources since no inorganic phosphate was added and that this phosphate was released simultaneously with the calcium. The mitochondrial ratio of calcium to phosphate under the present conditions has been found to be about 1.0. This might suggest that the mitochondrial calcium phosphate under our experimental conditions is in the form of $CaHPO_4$. When variations in *p*H or in phosphate or calcium concentration occur, as reported from other laboratories

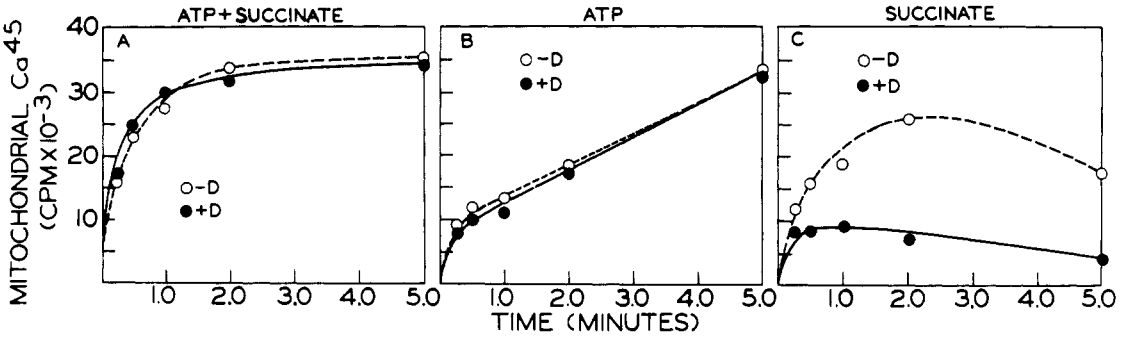

FIG. 7.—Effect of the vitamin D status of the animal on calcium binding rates of rat kidney mitochondria incubated in the complete medium (A), complete medium minus substrate (B), and complete medium minus ATP (C). The open circles (O) represent values from incubations with mitochondria from vitamin D–deficient rats (−D) and the closed circle (●) from vitamin D–fed rats (+D).

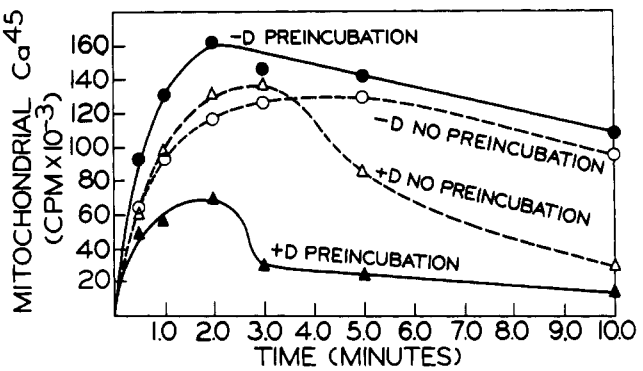

FIG. 8.—Effect of preincubation on the vitamin D–stimulated release of calcium from rat kidney mitochondria in the complete incubation medium minus ATP. Mitochondria were preincubated for 3 minutes at 30° in the absence of succinate and calcium, after which they were added and the incubation was continued. The reaction mixture described in the text included 14 mM succinate, 15.6 mM phosphate buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with ^{45}Ca), and mitochondria (4.6 mg protein) in a volume of 6.4 ml. The circles (open and solid) represent values from incubations with mitochondria from vitamin D–deficient rats (−D), and the triangles (open and solid) from vitamin D–fed rats (+D).

(Brierley *et al.*, 1963; Lehninger *et al.*, 1963; Rossi and Lehninger, 1963), other ratios are possible.

It was of particular interest to determine what effect the vitamin D status of the animal would have on calcium binding in the three different systems. The results of these experiments (Fig. 7) demonstrate quite clearly that the vitamin D status is very important in rate studies on calcium binding by kidney mitochondria in the absence of ATP. Previously it was reported from this laboratory that vitamin D, whether added *in vivo* or *in vitro*, stimulates the release of calcium from kidney mitochondria (Engstrom and DeLuca, 1962). Since the over-all rate is a reflection of the balance between binding and release reactions, consideration must be given to the release process when calcium binding is being studied, especially in the absence of exogenous ATP. In no case does vitamin D cause an increase in rate of binding above that of the control (vitamin D deficient). In all cases to date, the effect of vitamin D in stimulating the release of calcium from the mitochondria is apparent only in the absence of external ATP and in the presence of oxidizable substrate (Engstrom and DeLuca, 1964).

An earlier report from this laboratory (DeLuca and Engstrom, 1961) stated that it was necessary to preincubate the mitochondrial incubation mixture for 10–15 minutes in order to demonstrate a substrate requirement for calcium binding. The purpose in this case was to eliminate endogenous substrate prior to the addition of calcium. The fact that cyanide (10^{-3} M) partially inhibits calcium uptake in the absence of added substrate is in agreement with this belief (Fig. 5). In other cases preincubation with hexokinase-glucose has been used in an effort to reduce endogenous ATP. During investigations of substrate-dependent binding of calcium (no ATP) it was determined that a short 3-minute preincubation without the substrate or calcium markedly accentuated but was not essential to a demonstration of a vitamin D effect (Fig. 8).

It is interesting to note that the mitochondria from vitamin D–fed rats bound very much less calcium under these conditions than did the vitamin D–deficient controls. This result may well be due to an increased rate of release as reported previously (Engstrom and DeLuca, 1964) or, less likely, it may be due to an impaired substrate-dependent binding.

DISCUSSION

The results reported here demonstrate the importance of the vitamin D status of the animal to rate studies of calcium binding by mitochondria when no exogenous ATP is present. As seen in Figure 7, when ATP was added there was essentially no difference in the rate of calcium binding between kidney mitochondria from vitamin D–deficient and vitamin D–fed rats whether the substrate was present or not. However, when ATP was not added a reduction in apparent rate of calcium binding was noted with mitochondria from vitamin D–fed rats especially following a 3-minute preincubation. This is probably due to the stimulation of calcium release by vitamin D as reported previously (Engstrom and DeLuca, 1964).

In Figures 3, 4, and 5 results are presented from studies of inhibitors, which help explain previously published findings from this laboratory and elsewhere (DeLuca and Engstrom, 1961). Through the use of rapid sampling techniques it was possible to study the effects of various inhibitors on the rate of calcium binding. When both ATP and substrate were added there was some inhibition with cyanide as indicated by a reduction in rate of calcium binding. However, after 5 minutes as well as later, the total amount of calcium was bound whether cyanide was present or not. Dinitrophenol (2×10^{-5} M) also produced little or no effect on the rate of binding in the presence of added ATP unless the concentration of dinitrophenol was increased to 10^{-4} M or higher, which gives maximal adenosine triphosphatase activity. However, when ATP was absent, cyanide (10^{-3} M) and dinitrophenol (2×10^{-5} M) completely blocked calcium uptake, which

demonstrates that oxidative phosphorylation supported the uptake of calcium. Oligomycin strongly inhibited the rate of calcium binding in the absence of substrate and presence of ATP; it had only a slight effect in the absence of ATP and no effect when both ATP and substrate were added. The partial inhibition of calcium binding by cyanide and dinitrophenol in the absence of added substrate may be a reflection of endogenous substrates. Clearly, energy for calcium binding can come from ATP or from oxidative phosphorylation. However, there was considerably less binding in the absence of either ATP or substrate than when both were present. In all three systems antimycin A and Dicumarol caused maximal or near-maximal inhibition of calcium binding with our experimental conditions. The discrepancy between the effect of antimycin A reported here and that of Brierley *et al.* (1963) may be due either to a difference in inhibitor concentration used or to a difference in sensitivity in kidney vs. heart mitochondria. The ion and energy requirements for calcium binding by mitochondria are diagrammatically shown in Figure 6. Because of the discrepancy between heart and kidney mitochondria with regard to the effect of antimycin A on the ATP-supported binding of Ca^{++}, this effect is omitted from that portion of Figure 6.

It has been reported that ATP formation does not occur in intact mitochondria which are treated with 3 mM calcium (Brierley *et al.*, 1963). In an earlier publication from this laboratory it was reported that maximum calcium binding did occur when ADP plus inorganic phosphate were substituted for ATP, thus demonstrating that under conditions of low calcium concentration (0.3 mM) some ATP was being generated. It has also been reported from this laboratory (Engstrom and DeLuca, 1964) that the effects of vitamin D on calcium metabolism are no longer apparent when very high calcium concentrations are used in contrast to lower concentrations (0.3 mM). These are two important findings which suggest that care should be exercised in the interpretation of results when large and inhibitory concentrations of calcium are employed. In addition Siekevitz and Potter (1953) and Potter *et al.* (1953) have reported that low levels of calcium (approximately 10^{-4} to 5×10^{-4} M) stimulate respiration whereas higher concentrations (10^{-3} M and higher) markedly inhibit respiration (Slater and Cleland, 1953; Siekevitz and Potter, 1953; Potter *et al.*, 1953; Hunter and Ford, 1955).

The question of an inorganic phosphate requirement for maximum calcium binding has been far from resolved. There is no doubt that phosphate is deposited with calcium in the mitochondria (Brierley *et al.*, 1963; Lehninger *et al.*, 1963; Engstrom and DeLuca, 1964). However, the source of the phosphate may be another matter. Certainly in the absence of exogenous ATP, phosphate is required for maximal calcium binding rates. Nevertheless when ATP is present together with sufficient buffering no inorganic phosphate requirement is found even in the presence of massive amounts of calcium. The failure to demonstrate a phosphate requirement with glutamate and no ATP must remain unanswered at the present time. Certainly inorganic phosphate could not replace ATP in this system.

Acknowledgment

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