have to be 10^{-15} seconds. A reasonable figure for the collision broadening of the excited level would be given by $\Gamma \sim 10^{13}$ sec^{-1}. Since the chlorophyl molecules are thought to be arranged in a monomolecular layer. we let $n = 2$, then

$$\frac{\Gamma t_1}{n^2} \sim 2 \times 10^{-3}.$$

Therefore the condition for convergence of the perturbation series is by no means fulfilled, and in order to get a valid approximation, one would have to solve the secular equation, which would lead to nonlocalized states. Further work in this direction is in progress.

The above considerations hold also for more extensive systems. It seems possible that they can be applied to biological processes other than photosynthesis. For example, excitation processes in the retina could be mentioned. The light wave focused on one visual rod is coherent within the area of this rod. It seems probable that this collective character of the excitation is important for the formation of the signal in the adjoining nervous system. It also seems possible that collective activities play a role in the function of the central nervous system and relations may be found, say, between conscience and nonlocalized electronic states, or S and storage of memory.

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*CALCIUM UPTAKE BY RAT KIDNEY MITOCHONDRIA**

BY H. F. DELUCA AND G. W. ENGSTROM

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN

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It has become increasingly apparent that an understanding of the biochemical events involved in the transport of calcium across biological membranes is essential to the ultimate elucidation of the mechanisms of action of vitamin D and parathyroid hormone. It is well established that the primary physiological action of vitamin D is to improve calcium absorption by the small intestine[1] and that parathyroid hormone is involved in blood calcium homeostatic mechanisms.[2] The hormone also has been shown to improve intestinal absorption of calcium.[3] *In vitro* experiments with everted intestinal loops or sacks have further demonstrated that vitamin D and parathyroid hormone increase the transport of calcium across the intestinal membrane.[4-7]

Slater and Cleland[8] found that heart muscle sarcosomes could take up large

quantities of calcium. Phosphorylating kidney mitochondria accumulate sodium and potassium ions against a concentration gradient,[9–11] and it has been reported that calcium ions likewise are taken up by kidney mitochondria undergoing oxidative phosphorylation.[12] The process of oxidative phosphorylation appeared essential to the binding of calcium by the mitochondria. Work in this laboratory[13] has shown a striking effect of vitamin D on the structural integrity of isolated kidney mitochondria. It therefore seemed plausible that kidney mitochondria might provide a model membrane system for studies of calcium transport and the mechanisms of action of vitamin D and parathyroid hormone.

This communication indicates that kidney mitochondria take up large quantities of calcium by a process which requires adenosine triphosphate (ATP), an oxidizable substrate, and magnesium ions, but which is not directly dependent upon oxidative phosphorylation.

Methods.—Young male rats of the Holtzman strain weighing 70–80 gm were housed in hanging wire cages and given food and water *ad libitum*. The diet fed was essentially the adequate calcium and phosphorus diet of Steenbock and Herting[14] except that vitamin-free casein plus 0.2 per cent L-cystine replaced egg white as the protein source. In each experiment, the rats were divided into two equal groups. One group received no vitamin D, while in the remaining group each rat received 75 i.u. of crystalline calciferol in cottonseed oil every three days. Retarded growth and reduced serum calcium were evident in the vitamin-D deficient group.[14] After the rats had been on their respective diets for at least 21 days, pairs of vitamin-D deficient and supplemented rats were killed by a sharp blow on the head followed by decapitation. The kidneys were removed quickly and chilled in ice-cold isotonic sucrose. A 10 per cent homogenate of the kidneys was prepared at 0°C with a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Ten ml of homogenate was layered over 10 ml of 0.34 M sucrose and centrifuged at 600 $\times$ g for 10 min to remove nuclei, debris, and unbroken cells. The upper layer was removed carefully and centrifuged at 8,000 $\times$ g for 10 min to sediment the mitochondria. This preparation was washed once with 0.25 M sucrose and finally suspended in 5 ml of the isotonic sucrose. Unless otherwise indicated, 0.5 ml of this suspension was incubated at 30°C in a mixture which contained 0.08 μmoles cytochrome c; 6 μmoles ATP; 45 μmoles succinate or 15 μmoles glutamate; 10 μmoles $MgCl_2$; 0.9 μmoles $CaCl_2$ containing Ca^{45}; and either 40 μmoles Tris buffer, pH 7.4, or 40 μmoles phosphate buffer, pH 7.4; 40 μmoles KCl and 300 μmoles sucrose in a final volume of 3.2 ml. Mixtures were incubated in a Dubnoff-type shaker with air as the gas phase. Aliquots of the reaction mixture were removed, chilled to 0°C and immediately centrifuged at 20,000 $\times$ g for 7–10 minutes. The supernatant was decanted and, when desired, analyzed for calcium by a modification of an ethylenediaminetetraacetate (EDTA) titration method[15] or counted for radioactivity. To measure the radioactivity of the precipitate, the centrifuge cup was drained and wiped dry with tissue paper; the pellet was dissolved in dilute nitric acid and digested or it was dissolved in 0.2 per cent sodium lauryl sulfate, plated on stainless steel planchets, and counted as infinitely thin samples. Measurements of radioactivity were made in the Geiger region with a thin end-window automatic counting device. A sample of each mitochondrial preparation was analyzed routinely for nitrogen by Nesslerization.[16]

When ethylenediaminetetraacetate (EDTA) preparations were made, kidneys were homogenized in 0.25 M sucrose containing 0.001 M sodium EDTA. The isolation was as described previously except that the mitochondria were washed once with the 0.25 M sucrose containing EDTA and then washed in sucrose free of EDTA. The preparations were suspended in isotonic sucrose free of EDTA and used as described previously.

Results and Discussion.—When mitochondria were incubated in the complete medium, calcium was taken up very rapidly (Fig. 1). Even at 0–4°C, approximately 60 per cent of the calcium was taken up by the mitochondria within 5 min. With the complete medium, the calcium remained in the mitochondria for at least 40–50 min at 30°C. When the mitochondria were kept at 0°C following a 10-min

incubation at 30°C in the complete medium, the calcium remained in the mitochondria for at least 3 hours and then was lost slowly. Mitochondria from vitamin-D deficient and vitamin-D supplemented rats took up approximately equal quantities of calcium and at equal rates, and the *in vitro* addition of either vitamin D[17] or of pure parathyroid hormone did not increase the rate of calcium uptake. However, both vitamin D and parathyroid hormone have a dramatic effect on the release of calcium from these particles, a phenomenon which will be described in detail in subsequent communications.

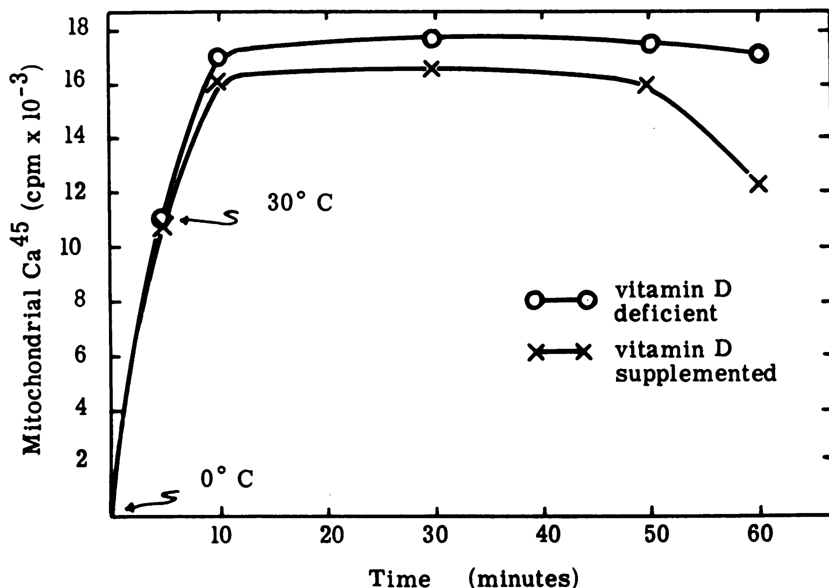

FIG. 1.—Time course of the uptake of calcium by mitochondria. The complete phosphate buffered medium described in the text was used with 0.6 μmole calcium (18,000 cpm). The reaction mixture was incubated at 0–4°C and after 5 min transferred to a 30°C Dubnoff shaker.

TABLE 1

THE UPTAKE OF CALCIUM BY MITOCHONDRIA WITH AND WITHOUT EDTA TREATMENT

Calcium added, μmoles	No EDTA: Mitochondrial Ca,* μmoles	No EDTA: Medium Ca,† μmoles	Plus EDTA: Mitochondrial Ca,* μmoles	Plus EDTA: Medium Ca,† μmoles
0.03	0.03	...	...	...
0.3	0.3	<0.05	0.3	<0.05
0.9	0.9	<0.05	0.9	<0.05
1.5	1.5	<0.05	1.5	<0.05
3.0	1.8	1.1	3.0	<0.05
30.0	...	...	8.5	21.2

* Calcium was determined by following Ca^{45} as described in *Methods* section.
† The calcium was determined directly by the EDTA titration method. The incubation medium was as described in the text, except that the concentration of calcium was changed as shown in column 1. The mitochondria were isolated from a vitamin-D deficient rat in isotonic sucrose with and without 0.001 *M* sodium EDTA.

Kidney mitochondria are capable of taking up relatively large quantities of calcium, as shown in Table 1. Mitochondria isolated from 1 gm of kidney will take up as much as 15 μmoles of calcium or 4.5 μmoles of calcium per mg mito-

chondrial nitrogen. Calcium is lost rapidly from mitochondria containing high concentrations of calcium, perhaps due to excessive mitochondrial damage. Mitochondria isolated from EDTA sucrose solutions had a greater capacity to take up calcium as reported by Slater and Cleland,[8] probably because these mitochondria had lower levels of residual Ca^{40} at the beginning of the incubation. Table 1 indicates a net uptake of calcium and not merely an exchange of Ca^{45} for unlabeled calcium. In subsequent experiments, therefore, only the Ca^{45} measurements are reported. EDTA preparations were not used routinely, because this treatment may remove a metal ion of importance in the over-all process. In routine work, the larger concentrations of calcium also were avoided because of the possibility of damaging the mitochondria.

The next experiments were designed to determine what supplements were required by the mitochondria to support their uptake of calcium. Table 2 clearly reveals that ATP and magnesium ions are requirements for the uptake of calcium. The requirement for an oxidizable substrate is not demonstrated so readily, presumably because of the presence of endogenous substrates.[18] If the calcium and ATP were added initially, added substrate often had little effect. However, if substrate, ATP, and calcium were added after the mitochondria had been incubated for 15 min. in their absence, a requirement for substrate was apparent.

TABLE 2

MATERIALS REQUIRED BY MITOCHONDRIA FOR CALCIUM UPTAKE*

Reaction medium	Mitochondrial Ca, cpm
Complete (PO_4^{---}, 40 μmoles + Tris, 40 μmoles)	66,000
No PO_4^{---}	66,000
No ATP	2,400
ADP, 6 μmoles; PO_4^{---}, 40 μmoles; no ATP	66,000
No Mg^{++}	2,290
Complete (Tris buffer)	80,750
No cytochrome c	80,750
No Mg^{++}	10,800
No ATP	5,825
No succinate	69,500
Complete (Tris buffer)	73,500
No K^{+}	70,250
No K^{+} or sucrose	68,300
Complete (preincubated 15 min without succinate, ATP, and calcium)	73,000
No succinate (preincubated 15 min without calcium and ATP)	23,800

* The incubation medium was described in the text. Mitochondria were prepared from vitamin-D deficient rats; those prepared from vitamin-D supplemented rats gave the same results. Reaction mixtures were incubated for 10 min at 30°C.

It is noteworthy that inorganic phosphate is not necessary for calcium uptake. Nevertheless, in all subsequent experiments, the results have been checked with both Tris and phosphate buffered media, because phosphate has a pronounced effect on calcium metabolism. Table 2 also indicates that cytochrome c, potassium chloride, and sucrose are not necessary for the uptake of calcium. The low osmolarity of the medium devoid of these components had no adverse effect on the calcium binding process, suggesting that mitochondrial swelling plays little or no role in the phenomenon. It also is unlikely that oxidative phosphorylation *per se* is required, since maximal calcium uptake occurs in the absence of inorganic phosphate. Furthermore, it is apparent that ATP can completely replace ADP plus

inorganic phosphate in the process of calcium uptake, which explains the requirement for oxidative phosphorylation reported earlier.[12]

These results prompted a detailed examination of the quantity of calcium bound per μmole of added ATP. Figure 2 reveals a nonlinear relationship between added ATP and the calcium taken up. The initial portion of the curve indicates that approximately 1 μmole of ATP is required per 10–15 μmoles of calcium bound. This is only an estimate, because it was not possible to determine synthesis and destruction of ATP by nonrelated processes.

Succinate, α-ketoglutarate, glutamate, DPNH, and malate were found about equally effective substrates in supporting the uptake of calcium. TPNH and β-hydroxybutyrate at the levels tried gave minimal calcium uptake. As was

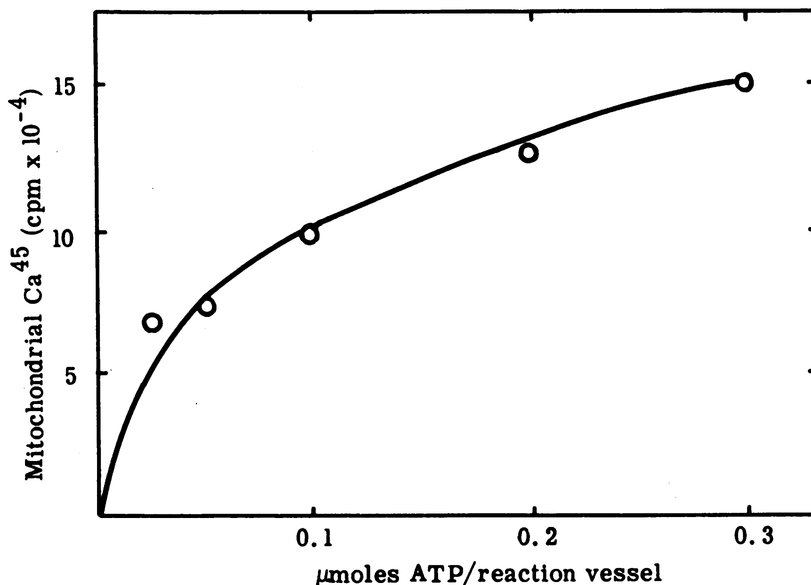

FIG. 2.—Dependence on ATP for calcium uptake by kidney mitochondria. The mitochondria were isolated from a vitamin-D deficient rat and incubated for 5 min at 30°C in the Tris buffered medium as described in the text, except that the ATP concentration was varied as shown.

expected, citrate interfered with calcium uptake, presumably by virtue of its chelation abilities.

Metabolic inhibitors provided useful information on factors involved in calcium uptake. Mitochondria were incubated with the inhibitor plus all the components of the reaction medium except ATP and calcium for 5 min, and then ATP and calcium were added. This technique prevented the destruction of ATP prior to the addition of calcium and provided time for the inhibitor to act. Dinitrophenol and sodium azide, potent uncouplers of oxidative phosphorylation, were without effect on the calcium uptake process; this apparently eliminates a direct involvement of oxidative phosphorylation. Oligomycin A, also an inhibitor of oxidative phosphorylation,[19, 20] was without effect. Inhibition of cytochrome oxidase by cyanide or sodium azide had little or no effect on the uptake of calcium. However, antimycin A, dicumarol, and 2,3-dimercaptopropanol (BAL) dramatically blocked

TABLE 3

The Effectiveness of Various Substrates in Supporting Calcium Uptake by Mitochondria*

Substrate	Calcium in mitochondria, cpm
None	11,700
Succinate, 45 μmoles	86,000
Glutamate, 15 μmoles	81,250
α-Ketoglutarate, 15 μmoles	84,000
Malate, 15 μmoles	83,250
DPNH, 10 μmoles	75,200
TPNH, 2.5 μmoles	17,250
β-Hydroxybutyrate, 15 μmoles	15,150
Citrate, 45 μmoles	9,350

* Mitochondria from 100 mg of a vitamin-D deficient kidney were incubated in the complete medium without substrate, ATP, or calcium for 15 min. The substrate, ATP, and 0.9 μmoles of calcium (86,200 cpm) were then added and the incubation continued for 10 min.

the uptake of calcium. Antimycin A is believed to inhibit the electron transport chain between cytochrome b and cytochrome c_1[21–23] whereas BAL inhibits "Slater's factor," which is in the same section of the electron transport chain.[24] Dicumarol, besides uncoupling phosphorylation, is believed to inhibit quinone reductase[25, 26] which places its effect in a region near that of antimycin A and BAL. It seems unlikely that the uncoupling effect of dicumarol is responsible for its inhibition of calcium uptake, because other inhibitors of oxidative phosphorylation, such as dinitrophenol, sodium azide, and oligomycin A, are without effect. Warfarin, unlike dicumarol, has no effect on either quinone reductase[25] or on calcium uptake. These results suggest that some component of the electron transport chain between coenzyme Q and cytochrome oxidase must be in the reduced form to support calcium uptake, since oxidizable substrate is required. Malonate showed some inhibition; this was not surprising for succinate was furnished as a substrate. p-Chloromercuribenzoate at a concentration of 10^{-5} M, and 10^{-3} M phloridzin were about equally inhibitory, whereas 10^{-4} M iodoacetate was without effect.

TABLE 4

The Effect of Metabolic Inhibitors on Calcium Uptake by Mitochondria*

Addition	Mitochondrial calcium, cpm
None	94,500
Sodium azide, 10^{-3} M	91,000
Sodium cyanide, 10^{-3} M	93,500
Dinitrophenol, 2×10^{-5} M	93,500
p-Chloromercuribenzoate, 10^{-5} M	43,350
Iodoacetate, 10^{-4} M	94,000
Phloridzin, 10^{-3} M	41,150
Oligomycin, 10^{-6} M	93,500
Malonate, 0.01 M	36,500
Antimycin A, 10^{-7} M	1,775
Dicumarol, 7×10^{-5} M	2,180
2,3-Dimercaptopropanol (BAL), 10^{-3} M	28,000
Warfarin, 7×10^{-5} M	91,000

* Mitochondria were incubated with inhibitor; 40 μmoles PO_4^{---}, pH 7.4; 300 μmoles sucrose; 40 μmoles KCl; 0.08 μmoles cytochrome c; 45 μmoles succinate, and 20 μmoles $MgCl_2$ in 3 ml for 10 min, at which time 6 μmoles ATP, and 0.9 μmoles Ca^{40}-Ca^{45} were added. After 10 min the mitochondria were analyzed for Ca^{45} as described in the text.

The relationship between uptake of calcium by mitochondria and calcium transport in intact cells is not clear. However, the mitochondrial membrane system

may serve as a model in the investigation of calcium transport mechanisms. Apparently vitamin D and parathyroid hormone do not affect the uptake process, but evidence will be presented elsewhere to show that they dramatically influence the release of calcium from mitochondria.

Summary.—Rat kidney mitochondria take up large quantities of calcium by a process requiring ATP, magnesium ions, and an oxidizable substrate. Although the process requires ATP, it is not directly dependent upon oxidative phosphorylation or upon the operation of the entire electron transport chain. The rate of calcium uptake is not enhanced by vitamin D. It is inhibited strongly by dicumarol, antimycin A and 2,3-dimercaptopropanol and less strongly by phloridzin and p-chloromercuribenzoate, but not by dinitrophenol, azide, cyanide, oligomycin, and iodoacetate at the concentrations tested. The mitochondria apparently can bind about 15 calcium ions per molecule of ATP utilized.

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