Vitamin D-Stimulated Release of Calcium from Mitochondria*

GEORGE W. ENGSTROM AND HECTOR F. DELUCA

From the Department of Biochemistry, University of Wisconsin, Madison

Received August 12, 1963

The administration of vitamin D to rats stimulates the release of calcium, strontium, and barium ions which have been actively bound by isolated kidney mitochondria. The effect of vitamin D was greatest with calcium and much less with strontium and barium. The effect of vitamin D on calcium release is observed only in the presence of an oxidizable substrate and is accompanied by a simultaneous release of inorganic phosphate. In the absence of oxidizable substrate (succinate) calcium is released rapidly from mitochondria by a process which is not dependent upon vitamin D. Further, inhibitors of respiration or uncouplers of phosphorylation also cause an immediate release of calcium independent of vitamin D. The stimulation of calcium release by vitamin D can be observed with mitochondria from kidney, liver, and intestine but not from heart or brain. This stimulation is also found with kidney mitochondria from rats fed diets varying widely in calcium and phosphorus content. Large quantities of calcium in the incubation medium inhibit or mask the effect of vitamin D presumably because of its uncoupling and inhibitory action on respiration.

In recent years the belief that vitamin D has a direct action on bone mineralization has given way to the concept that the vitamin functions to elevate serum calcium and/or phosphate which in turn are required for normal calcification. This is brought about primarily by an increased calcium and (secondarily) by phosphate absorption in the small intensine (Nicolaysen and Eeg-Larsen, 1953), an increased bone-mineral mobilization (Carlsson, 1952), and perhaps by an increased reabsorption of calcium (Gran, 1960) and phosphate (Harrison and Harrison, 1941) by the renal tubules.

More recently Schachter and co-workers and Harrison and Harrison have focused attention anew on the role of vitamin D in the intestinal absorption of calcium. They have demonstrated with everted intestinal loops or sacs *in vitro* that vitamin D increases an active as well as passive transport of calcium (Schachter and Rosen, 1959; Dowdle *et al.*, 1960; Harrison and Harrison, 1960) and, secondarily, phosphate (Harrison and Harrison, 1961) across intestinal membranes. These results therefore leave little doubt that vitamin D plays an important role in the transport of calcium and, secondarily, phosphate across biological membranes.

Work in this laboratory has demonstrated that, at the subcellular level, the vitamin has a marked effect on the physiology and structure of isolated kidney mitochondria (DeLuca *et al.*, 1957; DeLuca *et al.*, 1960). These findings prompted our interest in a possible effect of vitamin D on calcium exchange by mitochondria. Calcium uptake by mitochondria from many sources has been the subject of numerous recent reports which are reviewed in another manuscript (Engstrom and DeLuca, 1964). The uptake process is not affected by vitamin D or parathyroid hormone; however, both stimulate the release of calcium from these organelles (Engstrom and DeLuca, 1962; DeLuca *et al.*, 1962).

It is the purpose of this paper to report this action of vitamin D in detail, its specificity, nature, and the extent of its occurrence.

METHODS

Young male rats of the Sprague-Dawley strain weighing 60–80 g were fed an adequate calcium (0.47% Ca) and phosphorus (0.3% P) diet with and without vitamin D for 21–28 days as described previously (DeLuca and Engstrom, 1961). The animals not receiving vitamin D showed reduced growth and lowered serum calcium as reported by Steenbock and Herting (1955). In all experiments, the vitamin D (25 IU/day in Wesson oil) was administered orally to the indicated rats rather than added *in vitro* to isolated mitochondria (DeLuca *et al.*, 1962). The rats were killed by a sharp blow on the head followed by decapitation except when brain mitochondria were to be prepared, in which case the animals were killed by decapitation. Mitochondria were prepared in 0.25 M sucrose at 0° from the tissues of vitamin D–deficient and vitamin D–fed rats according to a modification of the method of Schneider as described in a preceding report (DeLuca and Engstrom, 1961). The mitochondria from 200 mg of kidney or other tissue (4.4 mg protein) were incubated in a Dubnoff shaker at 30° with 14 mM succinate or 9.4 mM glutamate, 0.125 mM ATP, 0.025 mM cytochrome c (Sigma Company, horse heart, type II), 3 mM $MgCl_2$, 12.5 mM KCl, 15.6 mM imidazole hydrochloride buffer *p*H 7.0–7.4 or other buffer as indicated, 115 mM sucrose, and 0.3 mM Ca^{40} containing carrier-free Ca^{45} in a total volume of 6.4 ml. Air was used as the gas phase in all incubations.

To study the release of calcium from the mitochondria, two methods were used. In the first method the mitochondria were incubated in the medium described above for 1 hour and the samples were removed at the indicated times (method 1). In this procedure only enough ATP was added to induce complete binding of the calcium and, since no inorganic phosphate was present, further generation of ATP was not possible. It is essential to note that this system requires ATP since there is no inorganic phosphate added to support substrate-dependent calcium binding (Engstrom and DeLuca, 1964; Brierley *et al.*, 1963). It is possible to consider that the ATP serves as a source of phosphate since it is known that calcium is deposited as a phosphate salt. However, the role of ATP in this system is undoubtedly more complex than this as indicated in another report (Engstrom and

* A preliminary report of this work has already appeared (DeLuca and Engstrom, 1962). The following abbreviations will be used: −D = mitochondria isolated from vitamin D deficient rats; +D = mitochondria isolated from rats fed vitamin D; ATP = adenosine triphosphate. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported by grant No. AM 05800-02 NTN from the U. S. Public Health Service and by the Wisconsin Alumni Research Foundation.

TABLE I

EFFECT OF INCUBATION MEDIUM COMPONENTS ON RELEASE OF CALCIUM[a]

Release-Medium Constituents	Mitochondrial Ca			
	2 Minutes		20 Minutes	
	−D (μmoles)	+D (μmoles)	−D (μmoles)	+D (μmoles)
Complete[b]	1.80	1.76	1.80	1.70
Complete minus ATP	—	—	1.80	1.80
Complete minus Mg^{++}	—	—	1.80	0.88
Complete minus Succinate	—	—	0.65	0.68
15.6 mM Imidazole buffer, *p*H 7.0	0.29	0.31	0.05	0.14
14 mM Succinate	1.30	0.92	0.36	0.25
15.6 mM Imidazole, *p*H 7.0, 164 mM sucrose	1.80	1.71	0.16	0.16
15.6 mM Imidazole, *p*H 7.0, 14 mM succinate	1.10	0.43	0.92	0.11
164 mM Sucrose, 14 mM succinate	1.58	1.26	1.62	0.59
164 mM sucrose, 15.6 mM imidazole, *p*H 7.0, 14 mM succinate	1.71	1.37	1.69	0.36

[a] Initially the mitochondria (from 200 mg kidney) were incubated with 14 mM succinate, 0.125 M ATP, 0.025 M cytochrome c, 3 mM $MgCl_2$, 12.5 mM KCl, 15.6 mM imidazole buffer, *p*H 7.3, 115 mM sucrose, and 0.3 mM Ca^{40}-Ca^{45} in a volume of 6.2 ml for 10 minutes at 30°, at which time all mitochondrial samples had taken up 1.8 μmoles calcium. The mitochondria were then removed by centrifugation, resuspended in the indicated media, and incubated at 10° for 20 minutes (method 2) [b] As described above, but with no calcium. [c] This is the time elapsed during resuspension at 0°.

DeLuca, 1964). Furthermore, the rate of calcium binding is maximal only when both substrate and ATP are present although either can independently support the binding of calcium (Engstrom and DeLuca, 1964; Brierley *et al.*, 1963; Lehninger *et al.*, 1963). For these reasons, ATP was always included in the calcium-uptake medium. After the ATP of the medium was consumed by the uptake reaction the bound calcium was released into the medium. It is this process that was studied. In the second method the mitochondria were incubated in the described medium for 10 minutes at 30°. The mitochondria were then removed by centrifugation at 20,000 × *g* for 7 minutes at 0° and then resuspended in fresh medium as indicated by means of withdrawal and expulsion several times through a serological pipet. This suspension was then incubated in a Dubnoff shaker at 10° and sampled at the indicated times (method 2).

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 × *g* for 7 minutes. The supernatant fluid was discarded and the pellet was dissolved in 5.0 ml of 0.2% sodium lauryl sulfate. An aliquot was plated on aluminum planchets and radioactivity was determined using infinitely thin samples. 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 mats, washed twice with 1-ml aliquots of ice-cold 0.25 M sucrose, and pulled dry by suction. These were dried at 90° and radioactivity was determined. The two techniques gave comparable results except that radioactive measurements on the samples prepared by the second method were corrected for self-absorption and Ca^{45} adsorption to the Celite pads. The number of micromoles calcium in the mitochondria at any time was calculated from the Ca^{45} data. This had previously been shown to agree closely with absolute calcium measurements (DeLuca and Engstrom, 1961).

When the radioisotopes Ba^{133}, Zn^{65}, and Mg^{28}, which are gamma emitters, were used, the samples were placed in low K^{40} glass tubes and radioactivity was counted in a gamma-ray spectrometer. Inorganic phosphate was determined by the method of Chen *et al.* (1956) and calcium by the method of Harrison and Harrison (1955). Protein content of the mitochondrial suspensions was estimated on the basis of nitrogen content determined by the method of Johnson (1941).

RESULTS

In previous experiments from this laboratory it appeared likely that at least two processes were taking place in mitochondrial exchange of calcium: an uptake process requiring ATP, Mg^{++}, and an oxidizable substrate (DeLuca and Engstrom, 1961), and a calcium-release process stimulated by vitamin D and parathyroid hormone (Engstrom and DeLuca, 1962; DeLuca *et al.*, 1962). It therefore appeared highly desirable to study the release process apart from calcium uptake. In earlier experiments dealing with the release of calcium, a high-ATP, phosphate-deficient medium was used (Engstrom and DeLuca, 1962) with the subsequent addition of hexokinase-glucose at 10 or 15 minutes to eliminate the excess ATP. More recently only enough ATP was added to induce complete calcium binding, which eliminated the need for hexokinase and glucose. In both instances the uptake and release phases were studied in the same medium with the possibility that products of uptake remained to influence the process of calcium release. Furthermore, it was not possible to examine the requirements for the release process independent of uptake. Therefore a new procedure was attempted in which the mitochondria were first incubated in the complete medium for 5–10 minutes at 30° to allow the mitochondria to bind the calcium. Then they were centrifuged, the supernatant fluid was decanted, and the pellet was resuspended in a fresh medium. The incubation was then continued at 10° (method 2). By omitting single components from the medium, the requirements for a demonstration of the vitamin D–stimulated release of calcium were determined (Table I). The retention of calcium by the organelles required the presence of an oxidizable substrate and magnesium ions. It was only in the presence of substrate that a vitamin D–dependent release of calcium was demonstrated. In other experiments a requirement for sucrose (osmotic considerations) and buffer as well as substrate was found (Table I). The composition of the medium was simplified as much as possible in these experiments, and it was found that

TABLE II
EFFECT OF METABOLIC INHIBITORS ON CALCIUM RELEASE FROM RAT KIDNEY MITOCHONDRIA[a]

Inhibitor	Inhibitor Concn	Mitochondrial Ca −D (μmoles)	Mitochondrial Ca +D (μmoles)
None (water)	—	1.80	1.60
Dinitrophenol	2×10^{-5} M	0.23	0.17
Cyanide	10^{-3} M	0.92	0.90
Azide	10^{-3} M	0.29	0.34
Gramicidin	10^{-7} M	0.27	0.18
Antimycin A	10^{-7} M	0.25	0.25
Malonate	10^{-2} M	0.40	0.42
Oligomycin B	10^{-6} M	0.79	0.43
Amytal	10^{-3} M	1.57	0.85

[a] The mitochondria from 200 mg of kidney from +D or −D rats were incubated with 14 mM succinate, 0.125 mM ATP, 0.025 mM cytochrome c, 3 mM $MgCl_2$, 12.5 mM KCl, 15.6 mM imidazole buffer, pH 7.0, 115 mM sucrose, and 0.3 mM Ca^{40}-Ca^{45} in 6.4 ml for 10 minutes at 30°, at which time all samples had bound 1.8 μmoles of calcium. They were then removed by centrifugation, resuspended in 6.4 ml of medium containing 16.4 mM sucrose, 15.6 mM imidazole buffer, pH 7.3, and 14 mM succinate, and incubated at 10° for 20 minutes (method 2).

the best medium for demonstrating the effect of vitamin D was one containing imidazole buffer, sucrose, and substrate. In all cases the omission of substrate resulted in a rapid and vitamin D–independent release of calcium.

Further support for the substrate requirement in the retention of calcium by mitochondria was obtained from inhibitor studies. The data shown in Table II indicate that inhibitors of respiration and uncouplers of oxidative phosphorylation induce a rapid release of calcium from kidney mitochondria independent of vitamin D. Only with oligomycin or amytal was it possible to demonstrate the effect of vitamin D and even then it occurred earlier than in the uninhibited control.

It should be mentioned that these experiments have been reproduced many times without failure. However, the amount of calcium released in a given time may vary from experiment to experiment but the effects demonstrated in the tables are completely reproducible. For example, the effect of vitamin D is shown in Tables I and II, but the amount of calcium released in the case of mitochondria from rats fed vitamin D is 1.44 μmoles for 20 minutes in Table I and 0.2 μmole for 20 minutes in Table II, although the media used were identical. Many factors may contribute to this variation because of the involved manipulations which are necessary to perform these experiments.

The effect of different substrates on the vitamin D–stimulated release process was investigated using method 1. The results shown in Figure 1 clearly indicate that with glutamate the vitamin D effect occurred earlier, revealing a more dramatic effect than with succinate or any other substrate tried. Therefore it seemed advisable to use glutamate and to re-examine the effect of vitamin D on the release of calcium and phosphate. The curves for calcium release are essentially the same as those obtained previously with succinate. An important observation was that phosphate was released simultaneously with the calcium (Fig. 2). This might be predicted from the fact that phosphate is deposited with the calcium in mitochondria (Brierley *et al.*, 1963; Lehninger *et al.*, 1963). In our experiments the phosphate must have been bound at the same time as the calcium and had to originate from the added ATP or endogenous phosphate, since no

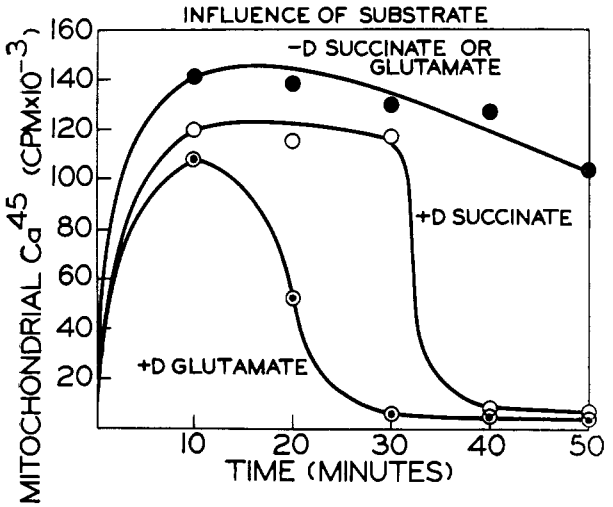

FIG. 1.—Influence of substrate on vitamin D–stimulated release of calcium from rat kidney mitochondria. The mitochondria from 200 mg of tissue from −D or +D rats were incubated with 9.4 mM glutamate or 14 mM succinate, 0.125 mM ATP, 3 mM $MgCl_2$, 15.6 mM imidazole buffer, pH 7.4, 0.3 mM $CaCl_2$ (labeled with Ca^{45}), 0.025 mM cytochrome c, 12.5 mM KCl, and 115 mM sucrose at 30° in a volume of 6.4 ml (method 1). 1.5×10^5 cpm represents 1.90 μmoles calcium.

inorganic phosphate was added. Similar results with regard to the simultaneous translocation of calcium and phosphate have also been obtained with succinate as the substrate. In all cases examined by the authors phosphate has always been transported together with calcium. This is also the case with the parathyroid hormone–stimulated release of calcium (DeLuca *et al.*, 1962).

In a previous communication the high degree of specificity of vitamin D and parathryoid hormone added *in vitro* in stimulating the release of calcium from the mitochondria was demonstrated (DeLuca *et al.*, 1962). The question of the degree of specificity with regard to the divalent cation transported was of obvious interest. Mitochondria bound strontium and barium ions under the same conditions used for inducing calcium binding, while zinc, cadmium, and magnesium were poorly bound (G. W. Engstrom and H. F. DeLuca, in preparation). As shown in Figures 3 and 4, vitamin D administration to the animals had the greatest effect on the release of calcium with much smaller effects in the case of strontium and barium. No effect of vitamin D was found on the release of cadmium, magnesium, or zinc.

Recently, A. W. Norman and H. F. DeLuca (in preparation), using tritium-labeled vitamin D of very high specific activity, were able to demonstrate a considerable accumulation of vitamin D in the kidneys, intestine, liver, and skeleton, with smaller accumulations in other organs such as heart and brain. It was then of interest to determine if a correlation could be found between these findings and the effect of vitamin D on the release of calcium from the mitochondria. Clearly, a striking *in vivo* effect of vitamin D was found with mitochondria from liver (Fig. 5) but not from brain or heart (not shown). Snellgrove and DeLuca (unpublished data) have demonstrated a similar effect with mitochondria prepared from intestine. It should be mentioned that, in experiments not shown here to conserve space, the *in vitro* addition of vitamin D to heart mitochondria did stimulate the release of calcium, which suggests that these particles will respond if sufficient vitamin D is present.

It appeared important to determine the calcium

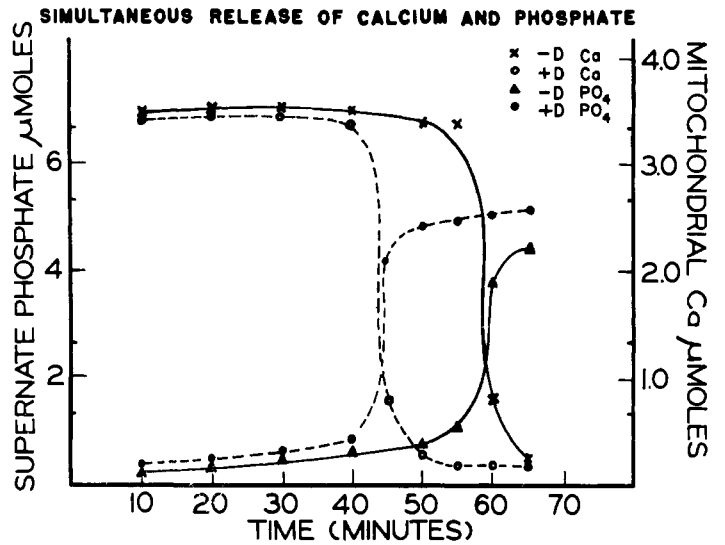

FIG. 2.—Simultaneous release of calcium and phosphate from rat kidney mitochondria. Mitochondria from 400 mg tissue were incubated at 30° with 9.4 mM glutamate, 3 mM $MgCl_2$, 0.125 mM ATP, 15.6 mM imidazole buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with Ca^{45}), 12.5 mM KCl, 115 mM sucrose, and 0.025 mM cytochrome c in 12.8 ml. No inorganic phosphate was added.

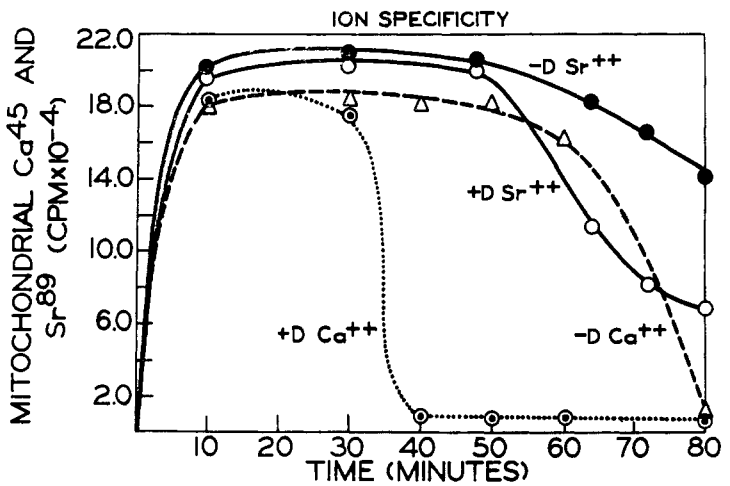

FIG. 3.—The effect of vitamin D on the release of calcium and strontium from rat kidney mitochondria. The reaction mixture contained 9.4 mM glutamate, 3 mM $MgCl_2$, 0.125 mM ATP, 15.6 mM imidazole buffer, *p*H 7.4, 0.3 mM $CaCl_2$ (labeled with Ca^{45}) or 0.3 mM $SrCl_2$ (labeled with Sr^{89}), mitochondria from 200 mg tissue, 12.5 mM KCl, 115 mM sucrose, and 0.025 mM cytochrome c in 6.4 ml. 2.0×10^5 cpm represents 1.90 μmoles calcium; 2.3×10^5 cpm represents 1.90 μmoles strontium.

concentration range within which an effect of vitamin D could be demonstrated. The results shown in Table III demonstrate that an effect of vitamin D is observable from 0.05 to 0.3 mM calcium in a medium with 0.7 mg of mitochondrial protein/ml of incubation mixture. At concentrations above 0.3 mM the release of calcium became increasingly independent of vitamin D.

The results in Table IV show that the effect of vitamin D on the release of calcium from mitochondria could be demonstrated regardless of the previous calcium and phosphate nutritional status of the animals from which the mitochondria were prepared. It was necessary to increase the calcium concentration of the medium in the case of mitochondria from rats fed the low-calcium diets before a vitamin D effect was demonstrated.

DISCUSSION

The mechanism whereby calcium ions are bound by isolated mitochondria is becoming increasingly clear through the efforts of many investigators (DeLuca and Engstrom, 1961; Vasington and Murphy, 1962; Brierley *et al.*, 1963; Lehninger *et al.*, 1963; Rasmussen and DeLuca, 1963). This process is believed to be an active one on the basis of its energy requirements. It is now evident that either ATP or an oxidizable substrate can supply energy for this process and that inorganic phosphate is bound with the calcium ions. Vitamin D and parathryoid hormone are not required for calcium uptake but both stimulate the subsequent release of calcium from these particles. Of these two agents vitamin D is of primary importance since the hormonal stimulation of calcium release requires the presence of vitamin D, while the converse is not true (DeLuca *et al.*, 1962).

The release of calcium from the particles, as might be expected, is accompanied by a simultaneous release of phosphate (Fig. 2), and in all cases studied thus far the calcium and phosphate are simultaneously translocated perhaps as an ion pair. This is important in view of the fact that vitamin D improves phosphate absorption from the small intestine secondarily to calcium (Nicolaysen and Eeg-Larsen, 1953).

TABLE III
EFFECT OF CALCIUM CONCENTRATION ON SENSITIVITY OF KIDNEY MITOCHONDRIA TO DIETARY VITAMIN D[a]

Calcium Added (mM)	Amount Bound Initially (μmoles)	Final Mitochondrial Ca: No Vitamin D (μmoles)	Final Mitochondrial Ca: Plus Vitamin D[b] (μmoles)
0.05	0.30	0.18	0.05
0.1	0.61	0.61	0.12
0.3	1.80	1.80	0.30
0.6	2.04	0.90	0.57
1.0	2.52	0.83	0.55
3.0	6.25	1.94	1.86
4.5	5.60	0.95	0.95

[a] These experiments were done according to method 1 described in the text. Samples were taken initially and after 20 minutes of incubation in all cases except with 0.05 and 0.1 mM calcium (40 minutes). The reaction mixture contained mitochondria from 200 mg kidney (4.4 mg protein), 14 mM succinate, 0.125 mM ATP, 0.025 mM cytochrome c, 3 mM $MgCl_2$, 12.5 mM KCl, 15.6 mM imidazole buffer, pH 7.2, 115 mM sucrose, and the indicated calcium concentration in 6.4 ml. The ATP was absent in the case of the 0.05 and 0.1 mM calcium concentrations. [b] Each rat in this group received 75 IU of vitamin D_2 every 3 days.

The mechanism of calcium and phosphate release from mitochondria in response to vitamin D is under study at the present time. It does not appear to be merely due to a disruption of the mitochondria, since the addition of more ATP after the calcium has been released as a result of vitamin D will bring about the binding of calcium once again. Further, previous work had revealed that the mitochondria isolated from vitamin D–deficient rats were structurally damaged relative to those from vitamin D–fed rats (DeLuca *et al.*, 1960) but they retained their calcium much longer.

It now appears that the retention of calcium by mitochondria depends upon at least a portion of the oxidative phosphorylation machinery, mitochondrial integrity (sucrose), and pH. Inhibitors of electron transport or uncouplers of oxidative phosphorylation cause an immediate release of calcium independent of vitamin D. It is possible that the generation of high-energy phosphate compounds serves only to preserve the integrity of the particles. On the other hand they may be necessary for the synthesis of compound(s) which may function in controlling calcium passage through the membrane. In any case, in all results obtained by the authors the effect of vitamin D on the release of calcium was found only in the presence of oxidizable substrate and in the absence of external ATP.

The effect of vitamin D on the release of calcium is quite specific in a number of respects. (a) A high degree of specificity is found for the vitamin D molecule added *in vitro* (DeLuca *et al.*, 1962). (b) The effect of vitamin D administration is found regardless of the calcium and phosphate content of the diet. (c) It is found with a wide range of calcium concentration in the incubation medium. (d) It shows a degree of specificity with regard to the cation being translocated. (e) The parathyroid hormone stimulates the release of calcium from mitochondria obtained from vitamin D–fed rats but not from vitamin D–deficient rats (DeLuca *et al.*, 1962).

With regard to point (d), the vitamin is known to improve the intestinal absorption of calcium, strontium, barium, and magnesium in that order (Worker and Migicovsky, 1961a, b). This correlates well with the mitochondrial system except in the case of magnesium. The major problem here is that magnesium ions are bound to only a small degree by mitochondria under the conditions used in the present experiments. An effect of vitamin D on magnesium transfer therefore may not have been fairly tested. Similar questions might well be raised in the cases of cadmium and zinc

The effect of vitamin D on the release of calcium is not confined to kidney mitochondria. Liver (Fig. 5) as well as intestinal mitochondria (Snellgrove and DeLuca, unpublished data) also reveal a dramatic effect of vitamin D while none was observed in mitochondria from heart or brain. This correlates well with the fact that physiological doses of tritium-labeled vitamin D accumulate in sizable amounts in liver, kidney, and intestine, while it appears only in small quantities in heart and brain tissue. When vitamin D is added *in vitro* to heart mitochondria, a stimulation of calcium release occurs, suggesting that the limiting feature in these preparations is the lack of sufficient vitamin D.

The importance of calcium-ion concentration in experiments of this type merits emphasis in view of the great tendency to use large and inhibitory levels of calcium in the study of mitochondrial binding of that cation (Vasington and Murphy, 1962; Brierley *et al.*, 1963; Lehninger *et al.*, 1963). The present data show that the effect of vitamin D with kidney mitochondria

TABLE IV
THE INFLUENCE OF DIETARY CALCIUM AND PHOSPHORUS CONTENT AND VITAMIN D ON THE RELEASE OF CALCIUM FROM RAT KIDNEY MITOCHONDRIA[a]

Diet: % Ca	Diet: % P	Vitamin D[b]	Mitochondrial Calcium: 25 Minutes (μmoles)	Mitochondrial Calcium: 35 Minutes (μmoles)	Mitochondrial Calcium: 45 Minutes (μmoles)
0.45	0.3^1	−	1.21	1.16	1.08
		+	1.16	0.94	0.06
0.45	0.02^1	−	1.10	1.08	0.71
		+	1.09	0.10	0.05
0.02	0.03^2	−	1.80	0.85	0.14
		+	0.57	0.09	0.09
0.02	0.02^2	−	1.71	1.08	0.10
		+	1.35	0.30	0.09

[a] The medium contained 0.2^1 mM or 0.3^2 mM Ca^{40} (labeled with carrier-free Ca^{45}), 0.125 mM ATP, mitochondria from 200 mg of kidney, 9.4 mM glutamate, 0.025 mM cytochrome c, 3 mM $MgCl_2$, 12.5 mM KCl, 15.6 mM imidazole buffer, pH 7.3, 115 mM sucrose, and 0.3 mM Ca^{40}-Ca^{45} in a volume of 6.4 ml. Initially (after 5 minutes' incubation) the mitochondria in all cases bound 1.2^1 and 1.8^2 μmoles of calcium, respectively. [b] 75 IU of vitamin D was given to each rate very 3 days where indicated.

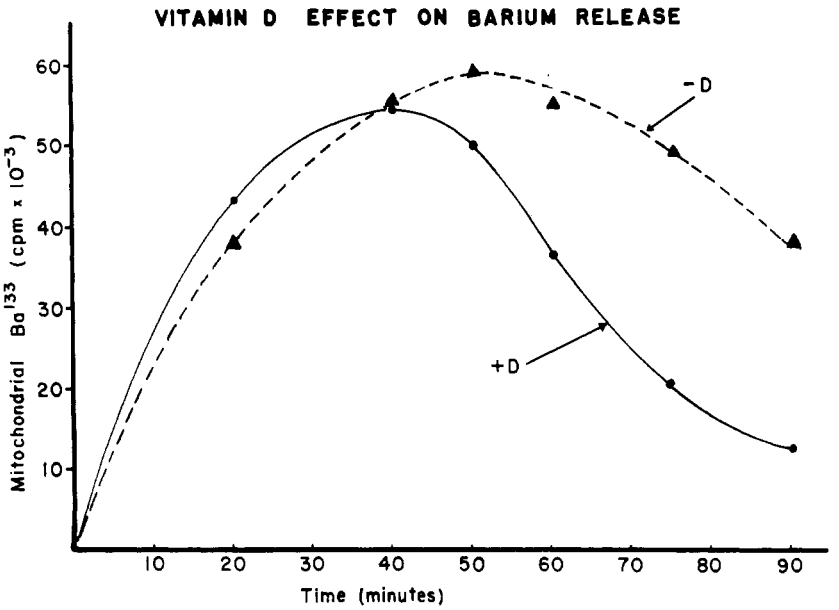

FIG. 4.—Vitamin D-stimulated release of barium from rat kidney mitochondria. The reaction mixture contained 9.4 mM glutamate, 0.125 mM ATP, 15.6 mM imidazole buffer, *p*H 7.4, 0.3 mM $BaCl_2$ (labeled with Ba^{133}), 3 mM $MgCl_2$, mitochondria from 200 mg tissue, 12.5 mM KCl, 115 mM sucrose, and 0.025 mM cytochrome c in 6.4 ml. 9.8×10^4 cpm represents 1.9 μmoles barium.

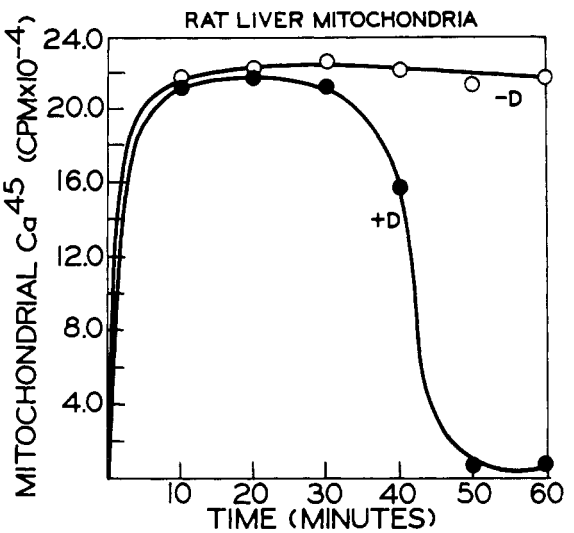

FIG. 5.—Release of calcium from liver mitochondria. Mitochondria from 200 mg of liver from −D and +D rats were incubated with 14 mM succinate, 0.08 mM ATP, 0.2 mM $CaCl_2$ (labeled with Ca^{45}), 15.6 mM imidazole buffer, *p*H 7.4, 12.5 mM KCl, 3 mM $MgCl_2$, 115 mM sucrose, and 0.025 mM cytochrome c at 30° in a total volume of 6.4 ml. 2.45×10^5 cpm represents 1.2 μmoles calcium.

is not demonstrable with calcium concentrations above 1.0 mM. This is not surprising since others have shown that calcium at these levels uncouples oxidative phosphorylation and inhibits respiration (Vasington and Murphy, 1962; Slater and Cleland, 1953; Siekevitz and Potter, 1953; Potter *et al.*, 1953; Lindberg and Ernster, 1954; Hunter and Ford, 1955), and, as pointed out previously, uncouplers of phosphorylation mask the effect of vitamin D. It is important to note that an effect of vitamin D is found at low calcium concentrations (0.05 mM), which probably approaches more closely the calcium concentrations to which these membranes are exposed under normal conditions.

The physiological significance of the vitamin D–stimulated release of calcium is not known at the present time. It may be that vitamin D plays a role in the regulation of calcium exchange in subcellular particles as they exist in the cell, and that this is in some manner linked to the transcellular transport of calcium. However, adequate experimental techniques to test this possibility are not available at the present time. On the other hand it appears likely that the calcium system of mitochondria is only one of a number of systems found in subcellular membranes which may function in the transport of calcium. If this is the case the mitochondrial membrane may well serve as a model in the elucidation of the mechanism of action of vitamin D. It is interesting to note that kidney mitochondria contain a large proportion of the vitamin D found in that organ (Norman and DeLuca, in preparation).

ACKNOWLEDGMENT

We are grateful to Merck and Co. for its supply of vitamins. We wish to acknowledge the excellent technical assistance of Helen Frank and Marguerite Sykes. We are grateful to Howard Rasmussen for many helpful discussions.

REFERENCES

Brierley, G. P., Murer, E., and Green, D. E. (1963), *Science 140*, 60.
Carlsson, A. (1952), *Acta Physiol. Scand. 26*, 212.
Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.
DeLuca, H. F., and Engstrom, G. W. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1744.
DeLuca, H. F., Engstrom, G. W., and Rasmussen, H. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1604.
DeLuca, H. F., Gran, F., Steenbock, H., and Reiser, S. (1957), *J. Biol. Chem. 228*, 469.
DeLuca, H. F., Reiser, S., Steenbock, H., and Kaesberg, P. (1960), *Biochim. Biophys. Acta 40*, 526.
Dowdle, E. B., Schachter, D., and Schenker, H. (1960), *Am. J. Physiol. 198*, 269.
Engstrom, G. W., and DeLuca, H. F. (1962), *J. Biol. Chem. 237*, PC974.
Engstrom, G. W., and DeLuca, H. F. (1964), *Biochemistry 3*, in press.
Gran, F. C. (1960), *Acta Physiol. Scand. 50*, 132.
Harrison, H. E., and Harrison, H. C. (1941), *J. Clin. Invest. 20*, 47.

Harrison, H. E., and Harrison, H. C. (1955), *J. Lab. Clin. Med. 46*, 662.
Harrison, H. E., and Harrison, H. C. (1960), *Am. J. Physiol. 199*, 265.
Harrison, H. E., and Harrison, H. C. (1961), *Am. J. Physiol. 201*, 1007.
Hunter, F. E., Jr., and Ford, L. (1955), *J. Biol. Chem. 216*, 357.
Johnson, M. J. (1941), *J. Biol. Chem. 137*, 575.
Lehninger, A. L., Rossi, C. S., and Greenawalt, J. W. (1963), *Biochem. Biophys. Res. Commun. 10*, 444.
Lindberg, O., and Ernster, L. (1954), *Nature 173*, 1038.
Nicolaysen, R., and Eeg-Larsen, N. (1953), *Vitamins Hormones 11*, 29
Norman, A. W., and DeLuca, H. F. (1963), *Biochemistry 2*, 1160.
Potter, V. R., Siekevitz, P., and Simonson, H. C. (1953), *J. Biol. Chem. 205*, 893.
Rasmussen, H., and DeLuca, H. F. (1963), *Ergeb. Physiol., Biol. Chem. Exptl. Pharmakol. 53*, 1.
Schachter, D., and Rosen, S. M. (1959), *Am. J. Physiol 196*, 357.
Siekevitz, P., and Potter, V. R. (1953), *J. Biol. Chem. 201*, 1.
Slater, E. C., and Cleland, K. W. (1953), *Biochem. J. 55*, 566
Steenbock, H., and Herting, D. C. (1955), *J. Nutr. 57*, 449.
Vasington, F. D., and Murphy, J. Y. (1962), *J. Biol. Chem. 237*, 2670.
Worker, N. A., and Migicovsky, B. B. (1961a), *J. Nutr. 74*, 490.
Worker, N. A., and Migicovsky, B. B. (1961b), *J. Nutr. 75*, 222.

Amino Acid Incorporation into Protein by Cell-free Preparations from Rat Skeletal Muscle. I. Properties of the Muscle Microsomal System

JAMES R. FLORINI

From the Biochemistry Research Department, Biochemical Research Section, Lederle Laboratories, American Cyanamid Company, Pearl River, New York

Received August 12, 1963

Rat skeletal muscle contains the enzymes, cofactors, and particulate fractions required for amino acid incorporation into protein by the classical pathway involving amino acid adenylates and amino acyl s-RNA (soluble RNA) as intermediates. The muscle system requires a monovalent cation (NH_4^+, K^+, or Rb^+), and Mg^{++}, an ATP-generating system, GTP, and both microsomes and soluble fraction or *p*H 5 precipitate. Incorporation of phenylalanine is stimulated by the addition of polyuridylic acid. The muscle system is much less active than similar preparations from normal rat liver; this difference could be explained only in part by the large differences in RNA content of the microsome fractions of these tissues, and is not attributable to the presence of inhibitors in the muscle fractions.

The incorporation of labeled amino acids into submitochondrial preparations from bacteria, plants, and various mammalian tissues has been studied extensively in recent years (Simpson, 1962), but thus far no detailed report on incorporation by similar systems from mammalian skeletal muscle has been published.[1] This paper presents a description of the requirements for amino acid incorporation into protein by microsome–*p*H 5 precipitate preparations from rat skeletal muscle and the inhibition or stimulation of this system by various additions to the medium. The muscle system is similar in many respects to systems in which labeling of specific proteins has been demonstrated (Allen and Schweet, 1962; Campbell and Kernot, 1962). However, comparison of the muscle system with similar liver preparations indicates that muscle microsomes are very much less active than liver microsomes even when allowance is made for the rather large differences in RNA[2] content of these fractions. This difference in activity is not attributable to the presence of an inhibitor in muscle microsomes.

[1] A preliminary report of this work was presented at the 145th annual meeting of the American Chemical Society, New York, September, 1963, and a report on the incorporation of labeled amino acids into crude muscle preparations has been published (Florini, 1962).

[2] Abbreviations used in this work: RNA, ribonucleic acid; s-RNA, soluble RNA; ATP, adenosine triphosphate; GTP, guanosine triphosphate; poly-U, polyuridylic acid; Tris, tris(hydroxymethyl)aminomethane; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl.

EXPERIMENTAL

Materials.—L-Leucine-4,5-H^3 was prepared by the catalytic reduction of Δ^4-L-leucine in the presence of carrier-free H_2^3 gas by a procedure similar to that of du Vigneaud *et al.* (1962).[3] Exchangeable tritium was removed by repeated lyophilization from dilute acid, base, and aqueous solution; the leucine-H^3 was then separated from unreduced starting material by paper chromatography in butanol–acetic acid–water (4:1:5). The purity of the radioactive material eluted from the paper strip was established by paper chromatography and extensive countercurrent distribution. The specific activity of the final product was 16 c/mmole. To minimize radiation decomposition, the purified L-leucine-4,5-H^3 was dissolved in a small quantity of water and diluted by ethanol followed by benzene to a final solvent ratio of 1:20:80; the concentration of tritium was 0.5–1.0 mc/ml. This solution was divided into convenient aliquots and stored at −10°. Fresh preparations were made from the original reaction mixture (similarly frozen in water-ethanol-benzene) every 4–6 months.

Coenzymes and other biochemicals were purchased from the following suppliers: L-phenylalanine-U-C^{14} (142 mc/mmole) and GTP, Schwarz BioResearch; ATP, Pabst Laboratories; creatine phosphate (Na salt) and creatine phosphokinase, California Corp. for Biochemical Research or Mann Laboratories;

[3] Δ^4-L-Leucine was generously provided by Dr. R. Purdy, Boston City Hospital.