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THE *IN VITRO* EFFECTS OF VITAMIN D ON OXIDATIVE PHOSPHORYLATION AND ADENOSINE TRIPHOSPHATASE ACTIVITY BY RAT LIVER MITOCHONDRIA*

PRAKORN CHUDAPONGSE and SIRISILN LOWCHAREONKUL
Department of Pharmacology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 4, Thailand

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Abstract—Ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) were found to depress ATP synthesis, state 3 and 2,4-dinitrophenol–stimulated respiration of rat liver mitochondria. This action of the D vitamins is not antagonized by the addition of excess dithiothreitol. The degree of inhibition on mitochondrial oxidative phosphorylation is greater when the mitochondria are respiring in the presence of NAD-linked substrates than with succinate. Vitamin D does not inhibit the mitochondrial ATPase (ATP phosphohydrolase, EC 3.6.1.4) activity induced by 2,4-dinitrophenol; however, in the absence of the uncoupler, vitamin D was found to significantly stimulate this mitochondrial enzyme. The vitamin D-stimulated ATPase activity is inhibited by oligomycin. Dihydrotachysterol$_2$ has little or no effect on mitochondrial oxidative phosphorylation and ATPase activity. The sites of action of vitamin D on the energy transfer pathway of rat liver mitochondria and the possible implications of these observations with regard to the biological actions of vitamin D are discussed.

The role of vitamin D in calcium metabolism is now well-documented. Vitamin D is generally accepted to have three main physiological functions, namely stimulation of intestinal calcium and phosphate absorption, promotion of bone calcification and mobilization of bone minerals[1]. Current evidence indicates that vitamin D enhances intestinal calcium transport by inducing the synthesis of calcium-binding protein[2] which has been isolated, purified and the biochemical properties studied[3]. However it is not certain whether vitamin D promotes bone calcification by the above mechanism, and whether the induction of calcium-binding protein synthesis is the sole mechanism underlying the vitamin D-induced calcium transport at small intestine. There is strong evidence supporting the role of mitochondrial calcium transport in regulating cytoplasmic calcium activity and transcellular calcium movement in many tissues[4]. The possible involvement of calcium transport by mitochondria in the molecular biology of calcification has received considerable experimental support[5, 6]. More than 10 years ago, DeLuca and collaborators demonstrated that vitamin D inhibited the uptake and stimulated the release of calcium from kidney mitochondria incubated *in vitro* [7, 8]. Kimberg and Goldstein[9] have shown that liver mitochondria from vitamin D-repleted rats accumulated and lost calcium at a more rapid rate than did mitochondria from vitamin D-depleted animals. These findings suggest the participation of mitochondria in the biological actions of vitamin D. The possible role of mitochondria in the vitamin D-induced intestinal calcium transport has been discussed in a recent review[4]. Since calcium accumulation and retention by mitochondria is the process which is dependent on electron transport and oxidative phosphorylation[10], it is of interest to investigate whether the D vitamins have any effect on these mitochondrial reactions. In this paper we present results of experiments on the action of vitamin D and related compound on energy-linked reactions by mitochondria from rat liver which indicate that vitamin D inhibits oxidative phosphorylation at the point above the site of action of 2,4-dinitrophenol in the energy-conservation pathway and stimulates latent ATPase of the mitochondria.

*Abbreviations: DHT_2, dihydrotachysterol$_2$; DNP, 2,4-dinitrophenol; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; vitamin D_2, ergocalciferol; vitamin D_3, cholecalciferol.

MATERIALS AND METHODS

Rat liver mitochondria in 0·25 M sucrose were prepared by the method of Hogeboom[11] as described by Myers and Slater[12]. The protein concentrations were determined by the method of Lowry *et al.*[13] as modified by Miller[14] using bovine serum albumin as standard. Measurements of oxygen uptake were carried out in the chamber of a Gilson oxygraph with a Clark oxygen electrode. The rates of oxygen consumption were expressed as μatom O/ml per min. Inorganic phosphate was determined, after millipore filtration of the reaction mixtures, by the method of Fiske and Subbarow[15]. The temperature was 26° in all experiments.

Vitamin D_2, vitamin D_3, dihydrotachysterol$_2$, dithiothreitol and oligomycin were obtained from Sigma Chemical Co. and HEPES from Calbiochem. Vitamin D_2, vitamin D_3, dihydrotachysterol$_2$ and oligomycin were added to the reaction mixtures in the form of ethanolic solution.

RESULTS AND DISCUSSION

Effect of vitamin D on oxidative phosphorylation. Figure 1 shows the effect of vitamin D_2 at different concentrations on the rate of respiratory response of rat liver mitochondria to ADP and DNP.

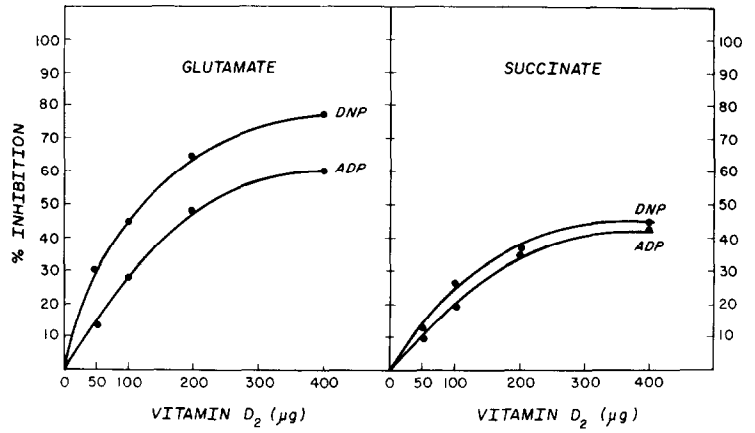

Fig. 1. The effect of vitamin D_2 at different concentrations on state 3 and DNP-stimulated respiration by rat liver mitochondria with glutamate or succinate as substrate. Composition of reaction system: 30 mM HEPES buffer pH 7·4, 7·3 mM $MgCl_2$, 15·7 mM potassium glutamate or succinate (pH 7·4), 2·61 mM potassium phosphate, KCl to 250 mosM, 0·26 mM ADP, 0·05 mM DNP and vitamin D_2 as indicated. Mitochondria were preincubated with and without vitamin D_2 for 5 min before the addition of ADP. DNP added 2 min after ADP. 1·57 mg mitochondrial protein per ml. Total vol 1·91 ml.

Significant inhibition of both ADP- and DNP-stimulated respiration was observed with 50 μg vitamin D_2 and the degree of inhibition reached maximum at about 400 μg. The doses of vitamin D required to depress oxidative phosphorylation were comparable to those used by DeLuca *et al.*[7] to demonstrate the effect on calcium transport by kidney mitochondria. It should be noted that much of the vitamin comes out of solution when it is added in ethanol to the reaction mixtures. It therefore appeared likely that much less amounts of the vitamin are actually required to depress state 3 and DNP-stimulated respiration. When mitochondria were respiring in the presence of NAD-linked substrates such as glutamate and malate plus pyruvate, the degree of inhibition was found to be greater than with succinate. Thus the process of energy conservation associated with the early part of the electron transport chain appeared to be more sensitive to the inhibitory action of vitamin D. It was also observed that with NAD-linked substrates the DNP-stimulated respiration was more depressed by vitamin D than was state 3 respiration.

Experiments were also performed to compare the effects of D vitamins and related compounds on mitochondrial respiration. The results of these experiments are recorded in Fig. 2. Curve A shows the control response of rat liver mitochondria, respiring in the presence of glutamate as substrate, to the additions of ADP + P_i and DNP. Note that there was a sharp cut-off (transition from state 3 to

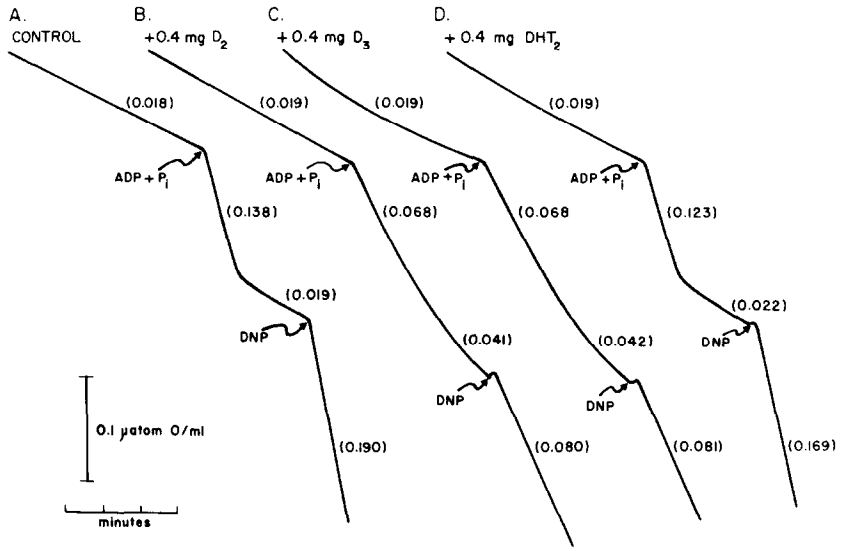

Fig. 2. The effects of vitamin D and related drug on state 3 and DNP-stimulated respiration by rat liver mitochondria. Composition of reaction system: 30 mM HEPES buffer pH 7·4, 7·3 mM $MgCl_2$, 15·7 mM potassium glutamate (pH 7·4), 2·61 mM potassium phosphate, KCl to 250 mosM, 0·26 mM ADP, 0·05 mM DNP, vitamin D_2, vitamin D_3 and dihydrotachysterol$_2$ (DHT_2) as indicated. 1·57 mg mitochondrial protein per ml. Total vol 1·91 ml. The figures in parentheses represent rate of oxygen consumption in μatom O/ml per min.

state 4 respiration) when added ADP had been phosphorylated to ATP. Preincubation of the mitochondria with vitamins D_2 or D_3 for 5 min depressed the stimulation of respiration caused by both ADP + P_i and DNP (Curves B and C). Moreover in both cases, there were no obvious cut-offs and state 4 respiratory rates were significantly elevated as compared to the control. The degree of inhibition produced by vitamin D_2 was found to be about equal to that caused by vitamin D_3. In contrast dihydrotachysterol$_2$, a reduction product of vitamin D_2, at the same concentration had only slight inhibitory effect (Curve D). Note that there was a sharp cut-off and state 4 respiratory rate was only slightly increased. The inhibitory action of vitamin D on state 3 respiration indicated the impairment of mitochondrial ATP synthesis. This conclusion was confirmed by the finding in other experiments not reported here that vitamin D indeed inhibited ATP production by the mitochondria whereas dihydrotachysterol$_2$ was much less effective. The degree of inhibition on ATP synthesis produced by vitamin D was somewhat greater than that of state 3 respiration.

It is known that certain compounds inhibit oxidative phosphorylation by a mechanism involving combination with the mitochondrial sulfhydryl groups[16] and this inhibition is reversed by dithiothreitol (DTT), a reagent known to protect sulfhydryl groups[16, 17]. As reported in Fig. 3, excess concentration of dithiothreitol did not antagonize the action of vitamin D_2 on state 3 and DNP-stimulated respiration. In fact, the degree of inhibition caused by vitamin D_2 was significantly greater in the presence than in the absence of dithiothreitol. Similar results were also obtained with vitamin D_3. Thus it seems unlikely that the D vitamins inhibit oxidative phosphorylation by combining, through their triene structures, with the sulfhydryl groups present in mitochondrial membrane.

Since vitamin D was found to inhibit the respiratory response of mitochondria to both ADP + P_i and DNP, the vitamin appears to interfere with oxidative phosphorylation at the point above the site of action of DNP in the energy-conservation pathway although the possibility that vitamin D also inhibits phosphorylation mechanism cannot be excluded. To test this possibility, the action of vitamin D on DNP-induced ATPase activity by rat liver mitochondria was studied. As reported in Table 1, vitamins D_2 and D_3 at the doses which markedly depressed oxidative phosphorylation did not inhibit the DNP-activated ATPase. In fact, these compounds were found to further enhance the enzyme activity. Dihydrotachysterol$_2$ was also inactive in this respect. In other experiments not reported here vitamin D_2 (100 μg) was found to enhance significantly the ATPase activity induced by concentrations of DNP ranging from 5 to 200 μM. Because the ATPase activity induced by uncouplers of the dinitrophenol-type represents a reversal of phosphorylation reactions[18], these

Table 1. The effects of vitamin D_2, vitamin D_3 and dihydrotachysterol$_2$ on 2,4-dinitrophenol-stimulated ATPase activity of rat liver mitochondria

Experiment	Inorganic phosphate liberated (μmoles/10 min)
Control	2·29
0·4 mg vitamin D_2	2·54
0·4 mg vitamin D_3	2·54
0·4 mg dihydrotachysterol$_2$	2·27

Composition of reaction system: 34 mM HEPES buffer pH 7·4, 8·5 mM $MgCl_2$, KCl to 250 mosM, 5 mM ATP, 0·1 mM DNP, vitamin D_2, vitamin D_3 and dihydrotachysterol$_2$ as indicated. DNP was added after 5 min preincubation of mitochondria with the drugs. ATP added 1 min after DNP. 2·06 mg mitochondrial protein per ml. Total vol 2·01 ml.

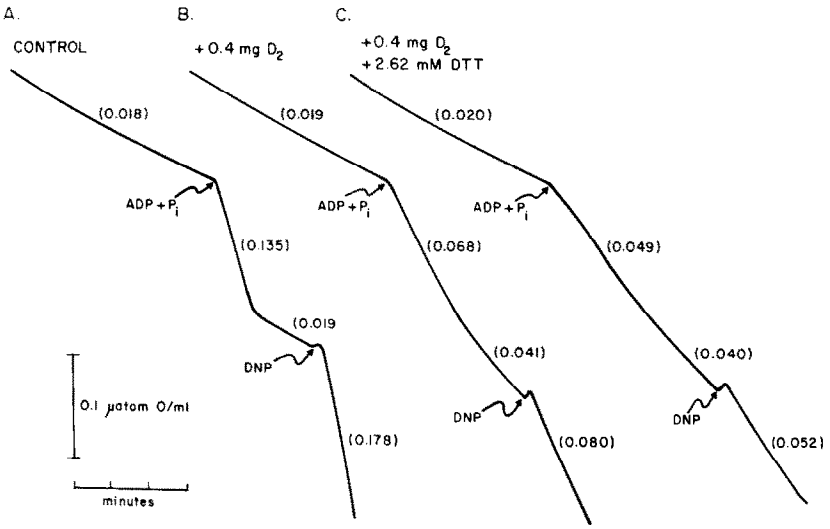

Fig. 3. The effect of dithiothreitol on the action of vitamin D_2 on state 3 and DNP-stimulated respiration by rat liver mitochondria. Composition of reaction system: 30 mM HEPES buffer pH 7·4, 7·3 mM $MgCl_2$, 15·7 mM potassium glutamate (pH 7·4), 2·61 mM potassium phosphate, KCl to 250 mosM, 0·26 mM ADP, 0·05 mM DNP, vitamin D_2 and dithiothreitol (DTT) as indicated. 1·57 mg mitochondrial protein per ml. Total vol 1·91 ml. The figures in parentheses represent rate of oxygen consumption in μatom O/ml per min.

observations suggested that D vitamins did not retard the phosphorylation of ADP *per se* and the vitamin most probably inhibited oxidative phosphorylation at the early stage of energy-conserving reactions or at the level of respiratory chain itself. Preliminary studies on the effects of vitamin D on calcium-stimulated respiration by rat liver mitochondria also support this conclusion. Both the rate of respiratory jump and extra oxygen uptake caused by the addition of calcium chloride to respiring mitochondria were found to be depressed by vitamins D_2 and D_3 but not by dihydrotachysterol$_2$. These findings strongly suggested that the D vitamins produced the effects on mitochondrial metabolism by interfering with the formation of the so-called 'primary energy pressure' by electron transport chain which is necessary not only for the synthesis of ATP but also for the respiration-linked cation accumulation by the mitochondria[5].

Effect of vitamin D on ATPase. The ability of vitamin D to further enhance the DNP-activated ATPase of rat liver mitochondria led us to investigate the action of this compound on mitochondrial latent ATPase in the absence of the uncoupler. The effect of vitamin D_2 at different concentrations on ATPase activity of rat liver mitochondria is recorded in Fig. 4. Significant stimulation of the ATPase was observed when the mitochondria were preincubated with 25 μg vitamin D_2 and the degree of stimulation increased as the dose of the vitamin was raised. The ATPase activity increased rather rapidly with the first 100 μg of vitamin D_2 and leveled off with higher doses of the drug. This is in contrast to the DNP-induced ATPase in which the enzyme activity declines with high concentrations of the uncoupler[19]. It is clear from this experiment that the latent ATPase of mitochondria can be unmasked by vitamin D as well as by uncouplers of the dinitrophenol-type[20]. However, unlike dinitrophenol, vitamin D does not appear to uncouple mitochondrial respiration since addition of vitamin D, even at high dose, to rat liver mitochondria respiring in the presence of glutamate as substrate produces only a slight increase in the rate of respiration (Fig. 1). The ability of vitamin D to activate mitochondrial latent ATPase clearly provides an explanation as to why, in the presence of the sterol, 'state 4' respiratory rate following 'state 3' respiration is elevated as compared to the control response in the absence of vitamin D.

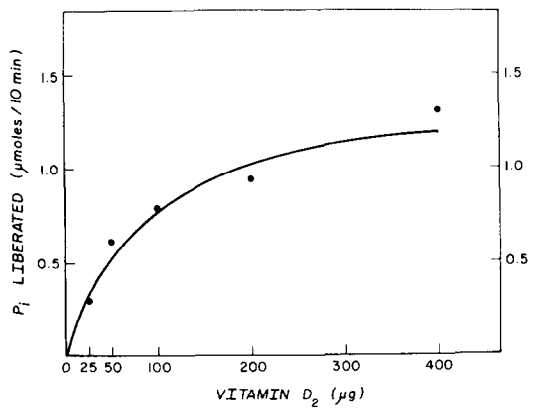

Fig. 4. Stimulation of the latent ATPase activity of rat liver mitochondria by different concentrations of vitamin D_2. Composition of reaction system: 35·8 mM HEPES buffer pH 7·4, 9 mM $MgCl_2$, KCl to 250 mosM, 5 mM ATP and vitamin D_2 as indicated. ATP was added after 5 min preincubation of the mitochondria with or without vitamin D_2. 2·62 mg mitochondrial protein per ml. Total vol 2·01 ml.

Results of experiments on the effects of the D vitamins and dihydrotachysterol$_2$ on ATPase activity of rat liver mitochondria are reported in Table 2.

Table 2. The effects of vitamin D_2, vitamin D_3 and dihydrotachysterol$_2$ on ATPase activity of rat liver mitochondria

Experiment	Inorganic phosphate liberated (μmoles/10 min)
Control	0·09
0·4 mg vitamin D_2	1·39
0·4 mg vitamin D_3	1·19
0·4 mg dihydrotachysterol$_2$	0·04

Composition of reaction system: 36 mM HEPES buffer pH 7·4, 9 mM $MgCl_2$, KCl to 250 mosM, 5 mM ATP, vitamin D_2, vitamin D_3 and dihydrotachysterol$_2$ as indicated. ATP was added after 5 min preincubation of the mitochondria with or without the drugs. 2·78 mg mitochondrial protein per ml. Total vol 2·01 ml.

It is seen that dihydrotachysterol$_2$, at the same dose as vitamins D_2 and D_3, did not stimulate mitochondrial ATPase. In fact, this compound seemed to produce a slight inhibition on the enzyme. Vitamin D_3 appeared to be a little less active than vitamin D_2 in stimulating ATPase activity. It should be noted in this connection that dihydrotachysterol$_2$ has been reported to have a very weak antirachitic activity[21] and did not influence calcium transport by rat kidney mitochondria[7]. Our results also indicate that dihydrotachysterol$_2$ is much less effective than the D vitamins in inhibiting oxidative phosphorylation and stimulating latent ATPase activity by rat liver mitochondria.

The vitamin D-stimulated ATPase activity was studied further in the presence of oligomycin, the antibiotic known to be a powerful inhibitor of latent ATPase and DNP-stimulated ATPase of the mitochondria[22, 23]. As reported in Table 3, the mitochondrial ATPase induced by the D vitamins was inhibited by oligomycin. Thus vitamin D, like dinitrophenol, appears to act beyond the site of action of oligomycin in the ATPase reaction but the exact location and mechanism of action are unknown at present. The extremely weak uncoupling action of vitamin D excludes this mechanism as being responsible for the activation of ATPase activity by the sterol. One of the possible mechanisms is that vitamin D may act directly on the ATPase complex in mitochondrial inner membrane. Experiments are in progress to determine whether the D vitamins can stimulate the activity of purified ATPase system from rat liver mitochondria.

Whether the effects of vitamin D on mitochondrial reactions have any implication in the biological actions of this compound is at present a matter of speculation. Relatively little studies have been

Table 3. Inhibition of vitamin D-stimulated ATPase activity of rat liver mitochondria by oligomycin

Experiment	Inorganic phosphate liberated (μmoles/10 min)
Control	0·36
0·4 mg vitamin D_2	2·08
0·4 mg vitamin D_2 + 10 μg oligomycin	0·44
0·4 mg vitamin D_3	1·89
0·4 mg vitamin D_3 + 10 μg oligomycin	0·58

Composition of reaction system: 36 mM HEPES buffer pH 7·4, 9 mM $MgCl_2$, KCl to 250 mosM, 5 mM ATP, vitamin D_2, vitamin D_3 and oligomycin as indicated. ATP was added after 5 min preincubation of the mitochondria in the presence or absence of the D vitamins. Oligomycin, when present, was added at the same time as vitamin D. 2·69 mg mitochondrial protein per ml. Total vol 2·02 ml.

done on the action of vitamin D on mitochondrial metabolism despite the growing body of evidence that mitochondria play a significant role in the processes of transcellular calcium transport [4] and calcification [5, 6]. Very recent experiments carried out in organ cultures [24] and whole animals [25] clearly demonstrate that vitamin D itself, when used in high concentrations, is active without having to be hydroxylated and may substitute for 1,25-dihydroxy vitamin D, the activated metabolite [26]. These observations strongly indicate common mechanism of action among the D vitamins and their active metabolites and permit the use of parent sterols to study their biochemical mechanism of action. In the present studies liver mitochondria were employed as the model to demonstrate the *in vitro* effects of vitamin D on mitochondrial functions. Although the amounts of vitamin D used in this work are relatively large, the actual concentrations required to influence mitochondrial reactions are most probably only the small fractions of the added vitamin since much of the vitamin comes out of solution when it is added to the reaction mixtures. Moreover, the relative insensitivity of liver mitochondria to the action of small quantities of vitamin D may be related to the very low efficacy of this compound as compared to the hydroxylated metabolites [24–26] and to the fact that intestinal and skeletal tissues, rather than hepatic tissue, are the target organs of vitamin D [1, 2]. Considering these factors, it appears likely that intestinal and bone mitochondria would be more responsive to vitamin D than liver mitochondria and the degree of sensitivity would be even greater with the hydroxylated vitamins. Since data concerning the sensitivity of mitochondria from target organs is lacking, the observed effects of vitamin D on mitochondrial metabolism described here may not be related to the physiological mechanism of action of this compound but represent the pharmacological and/or toxic effects resulting from repeated administration of large doses of the sterol.

There is evidence indicating the participation of mitochondria in the vitamin D-induced intestinal calcium transport. Administration of vitamin D to vitamin D-deficient animals has been shown to cause an increase in the number and density of calcium granules in the intestinal mitochondria [27]. This is associated with a rise in calcium efflux from mitochondria and intestinal cells [4]. The vitamin D-dependent calcium-binding protein has been reported to stimulate the release of calcium from mitochondria of intestinal mucosal cells incubated *in vitro* [28]. However, it is doubtful whether this reaction can occur in the *in vivo* condition since calcium-binding protein appears to localize at the intestinal brush border surface rather than in the cytosol [29]. It is well-established that accumulation and retention of calcium by mitochondria is dependent on energy derived from electron transport or ATP hydrolysis [5, 10]. Because vitamin D was found to inhibit oxidative phosphorylation at the early stage of energy-conserving reactions and stimulate latent ATPase of the mitochondria, a reduction in the intracellular ATP level is conceivable. These conditions would tend to limit calcium accumulation and facilitate calcium release by the mitochondria [5, 10]. This view is also supported by the observation that following the *in vitro* ATP depletion produced by the addition of glucose and hexokinase to the medium, liver mitochondria from vitamin D-repleted rats lost the accumulated calcium at a more rapid rate than did mitochondria from vitamin D-depleted animals [9]. The vitamin D-stimulated calcium release from kidney mitochondria has been shown by DeLuca *et al.* [7, 8] to occur only when the reaction mixtures contained small concentrations of ATP. High level of ATP will inhibit calcium efflux [7, 8]. Thus the consequence of these effects would be a rise in cytoplasmic calcium activity, particularly after the calcium-binding protein-induced calcium influx at the intestinal brush border surface [27, 29], and hence the increase in calcium efflux and transcellular calcium movement [4]. In this hypothesis vitamin D stimulates intestinal calcium transport by a mechanism involving both the induction of calcium-binding protein synthesis and direct action on mitochondrial metabolism. Much further work is needed to determine whether the mitochondrial actions of vitamin D could play a role in the intestinal calcium transport and other biological actions of this compound, especially the demonstration that the hydroxylated metabolites at low physiological doses can indeed modify the energy-linked reactions of isolated intact mitochondria from intestinal and skeletal tissues. It is hoped that this report will serve to stimulate further investigations along this line.

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