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STUDIES OF THE CHICK RENAL MITOCHONDRIAL 25-HYDROXYVITAMIN D-3 24-HYDROXYLASE

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Summary

The vitamin D metabolite, 24,25-dihydroxyvitamin D-3, is made from 25-hydroxyvitamin D-3 by an enzyme (25-hydroxyvitamin D-3 24-hydroxylase) located in the renal mitochondria of vitamin D and calcium-replete chicks. The apparent K_m for 25-hydroxyvitamin D-3 for this reaction was found to be $3 \cdot 10^{-7}$ M, although simple Michaelis-Menten kinetics were not observed at the higher substrate concentrations. Enzymatic activity was reduced by the mitochondrial metabolic inhibitors, antimycin A and dinitrophenol, as well as the inhibitors of mixed function oxidases, 2,4-dichloro-6-phenylphenoxyethylamine, 2-diethylaminoethyl-2,2-diphenylvalerate and metyrapone. Increasing the calcium concentration from 0 to $1 \cdot 10^{-5}$ M or the potassium concentration from 0 to 50 mM stimulated enzymatic activity 4- to 8-fold. Increasing the phosphate or acetate concentration from 0 to 50 mM stimulated enzymatic activity 6-fold. Raising the CO_2/HCO_3^- content from 0/0 to 10%/10 mM stimulated enzymatic activity 5-fold. The effects of the cations were additive to

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Abbreviations: 25OHD-3, 25-hydroxyvitamin D-3; $1,25(OH)_2$D-3, 1,25-dihydroxyvitamin D-3; $24,25(OH)_2$D-3, 24,25-dihydroxyvitamin D-3; $25,26(OH)_2$D-3, 25,26-dihydroxyvitamin D-3; $1,24,25-(OH)_3$D-3, 1,24,25-trihydroxyvitamin D-3; DPEA, 2,4-dichloro-6-phenylphenoxyethylamine; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences — National Research Council.

those of the anions except in the presence of 50 mM phosphate or acetate. The effect of ions on 24,25-dihydroxyvitamin D-3 production by renal mitochondria from vitamin D-replete chicks resembles the effect of these ions on production of 1,25-dihydroxyvitamin D-3 by renal mitochondria from vitamin D-deficient chicks, suggesting that the same mechanisms may be utilized.

Introduction

Mitochondria from the kidney metabolize the major circulating form of vitamin D-3, 25-hydroxyvitamin D-3 (25OHD-3), either to 1,25-dihydroxyvitamin D-3 ($1,25(OH)_2$D-3) or to 24,25-dihydroxyvitamin D-3 ($24,25(OH)_2$D-3) [1]. Animals fed diets deficient in vitamin D or calcium produce $1,25(OH)_2$D-3 [2], but within hours after the administration of $1,25(OH)_2$D-3 an animal raised on a high-calcium diet will start producing $24,25(OH)_2$D-3 instead of $1,25(OH)_2$D-3 [3—6]. These results have been confirmed in vitro [7,8]. This changeover from $1,25(OH)_2$D-3 production to $24,25(OH)_2$D-3 production requires protein synthesis [4,5,7]. Thus, it appears that changes in enzyme synthesis underlie the ability of $1,25(OH)_2$D-3 and calcium, over a period of hours, to increase $24,25(OH)_2$D-3 production while decreasing $1,25(OH)_2$D-3 production. However, this may not be the only mechanism by which vitamin D metabolism is regulated.

We [9—12] and others [13—16] have emphasized that $1,25(OH)_2$D-3 production may be under acute control by factors which do not require changes in enzyme synthesis and which exert their effects in vitro within minutes. The similarities between the enzyme producing $1,25(OH)_2$D-3 (25OHD-3 1-α-hydroxylase) and the enzyme producing $24,25(OH)_2$D-3 (25OHD-3 24-hydroxylase) suggested to Ghazarian [17] that these 'two' enzymes might share at least some of the same components. We [12] proposed a model postulating a particular spatial arrangement of the components (NADPH dehydrogenase, non-heme iron, cytochrome *P*-450) of the 25OHD-3 1-α-hydroxylase within the inner membrane of the mitochondrion. The spatial arrangement of these components within the mitochondrial membrane is such that oxidation of NADPH dehydrogenase by the non-heme iron occurs at the outer surface of the membrane, whereas the cytochrome *P*-450-mediated conversion of 25OHD-3 and O_2 to $1,25(OH)_2$D-3 and H_2O occurs at the inner surface. As such, the protons released upon oxidation of the dehydrogenase are separated from the protons required by the cytochrome *P*-450-mediated reaction by a proton-impermeable membrane. Cations such as Ca^{2+} and K^+, which stimulate proton efflux from mitochondria [12], stimulate the 1-α-hydroxylase by facilitating the release of protons at the point at which NADPH dehydrogenase (a flavoprotein) is oxidized by the non-heme iron. Anions such as phosphate and acetate, which donate protons to the more alkaline mitochondrial matrix [18], stimulate the 1-α-hydroxylase [9—12] by providing protons to the cytochrome *P*-450-mediated conversion of 25OHD-3 and O_2 to $1,25(OH)_2$D-3 and H_2O. If this model were correct and if the components of the 25OHD-3 24-hydroxylase were similarly arranged in the inner mitochondrial membrane, then one would expect that the effect of ions on $24,25(OH)_2$D-3 production

would be similar to the effect of these ions on $1,25(OH)_2D$-3 production. The data presented in this report indicate that this expectation is fulfilled.

Methods

Chicks were raised from hatching on a diet containing 3% Ca, 0.65% P, and 20 μg vitamin D-3 per kg diet (Teklad Diet 75007, modified by the addition of the appropriate amount of calcium, phosphate and vitamin D-3). At 14 to 17 days the chicks were killed, and renal mitochondria were prepared in EGTA-containing buffers as previously described [9]. The basic incubation medium of 1 ml contained 4—6 mg mitochondrial protein, 10 mM sodium malate, 2 mM $MgSO_4$, 90 mM sucrose, 16 mM Tris-HCl, 10 mM EGTA with sufficient $CaCl_2$ added to give the desired free Ca^{2+} concentration at pH 6.8 [19]. Incubations in which different concentrations of K^+ were included were maintained at equivalent ionic strength by adding the K^+ in a solution balanced with Na^+ each as the chloride salt, such that the total final added cation concentration was 50 mM. Likewise, addition of phosphate or acetate (sodium salts) were balanced with NaCl such that the total final added anion concentration was 50 mM. In general, the incubations were performed at 37° C under 1 atm O_2. After a 5-min preincubation, 25 pmol of [26,27-^{3}H]25OHD-3 (spec. act. 2 Ci/mmol) were added in 50-μl ethanol to begin the incubation. After 30 min the reaction was terminated with 3 ml $CHCl_3$/MeOH (1 : 2, v/v) containing 0.017% butylated hydroxytoluene, and the $CHCl_3$ extracts were prepared for Whatman SG81 paper chromatography as previously described [20]. The chromatography system employed in these experiments was modified to improve the resolution of $24,25(OH)_2D$-3 by changing the solvent system to $CHCl_3$/ethylacetate/benzene (40 : 40 : 20) and shortening development time to 12—14 h. The chromatograms were scanned by a Packard radiochromatogram scanner, and the radioactive peaks were cut out directly or 1 cm slices of the entire chromatogram (50 cm) were prepared, each of which was analyzed for ^{3}H by standard liquid scintillation spectroscopy techniques. The amount of radioactivity in each peak was used to determine metabolite production. In all experiments $CHCl_3$/MeOH was added to one of the incubation vessels prior to the addition of the 25OHD-3. This sample served as background for the calculations.

Periodate cleavage was performed as previously described [20]. Protein was determined by using the method of Lowry et al. [21]. Sephadex LH20 column chromatography was performed with 0.8 × 48 cm columns by using the method of Holick and DeLuca [22].

High-pressure liquid chromatography (HPLC), using a Waters Assoc. Model 440 HPLC apparatus, was performed by using the method of Eisman et al. [23] with a 10 μm silicic acid column (Waters Assoc., Inc.) and a hexane/isopropanol (9 : 1) solvent system.

Radioactively labelled metabolites of vitamin D used as chromatography standards were biosynthesized from [23,24-^{3}H]25OHD-3 (spec. act. 80 Ci/mmol) using renal mitochondrial preparations [9] from chicks raised on a 0.43% Ca, 0.3% P vitamin D-deficient diet (for the synthesis of $1,25(OH)_2D$-3) or from chicks raised on a 3% Ca, 0.65% P vitamin D-supplemented diet (for the synthesis of $24,25(OH)_2D$-3, $25,26(OH)_2D$-3, and $1,24,25(OH)_3D$-3). The

metabolites were purified by SG81 paper and Sephadex LH20 column chromatography and characterized by periodate cleavage. The identity and purity of $1,25(OH)_2D$-3 and $24(R),25(OH)_2D$-3 were verified by co-chromatography on a high-pressure liquid chromatographic system with their respective chemically synthesized non-radioactive compounds.

Non-radioactively labelled $24(R),25(OH_2D$-3 and $1,25(OH)_2D$-3 were gifts from M.R. Uskokovic, Hoffmann-La Roche, non-radioactively labelled 25OHD-3 was a gift from John Babcock, Upjohn, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) was a gift from R.E. McMahon, Lilly, and 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) was a gift from Smith, Kline and French. All other chemicals were of reagent grade and commercially supplied.

Estimates of V and K_m for the 25OHD-3 24-hydroxylase were obtained by fitting the velocity and substrate data to the Lineweaver-Burk equation using a least-squares non-linear regression computer program [24] implemented by a Hewlett-Packard 9830-A calculator.

Results

Identification of metabolites

The SG81 paper chromatographic system used in these experiments separates $24,25(OH)_2D$-3 and $1,24,25(OH)_3D$-3 from the other major metabolites of vitamin D-3 (Fig. 1). In Fig. 2 and Table I(A) are recorded the results of periodate cleavage of the metabolites biosynthesized from [23,24-^{3}H]-

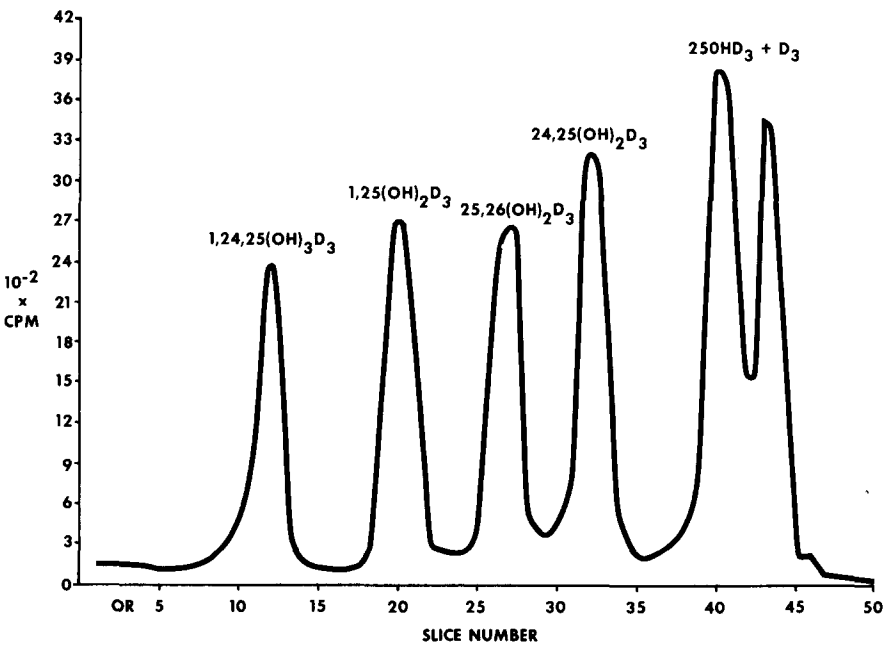
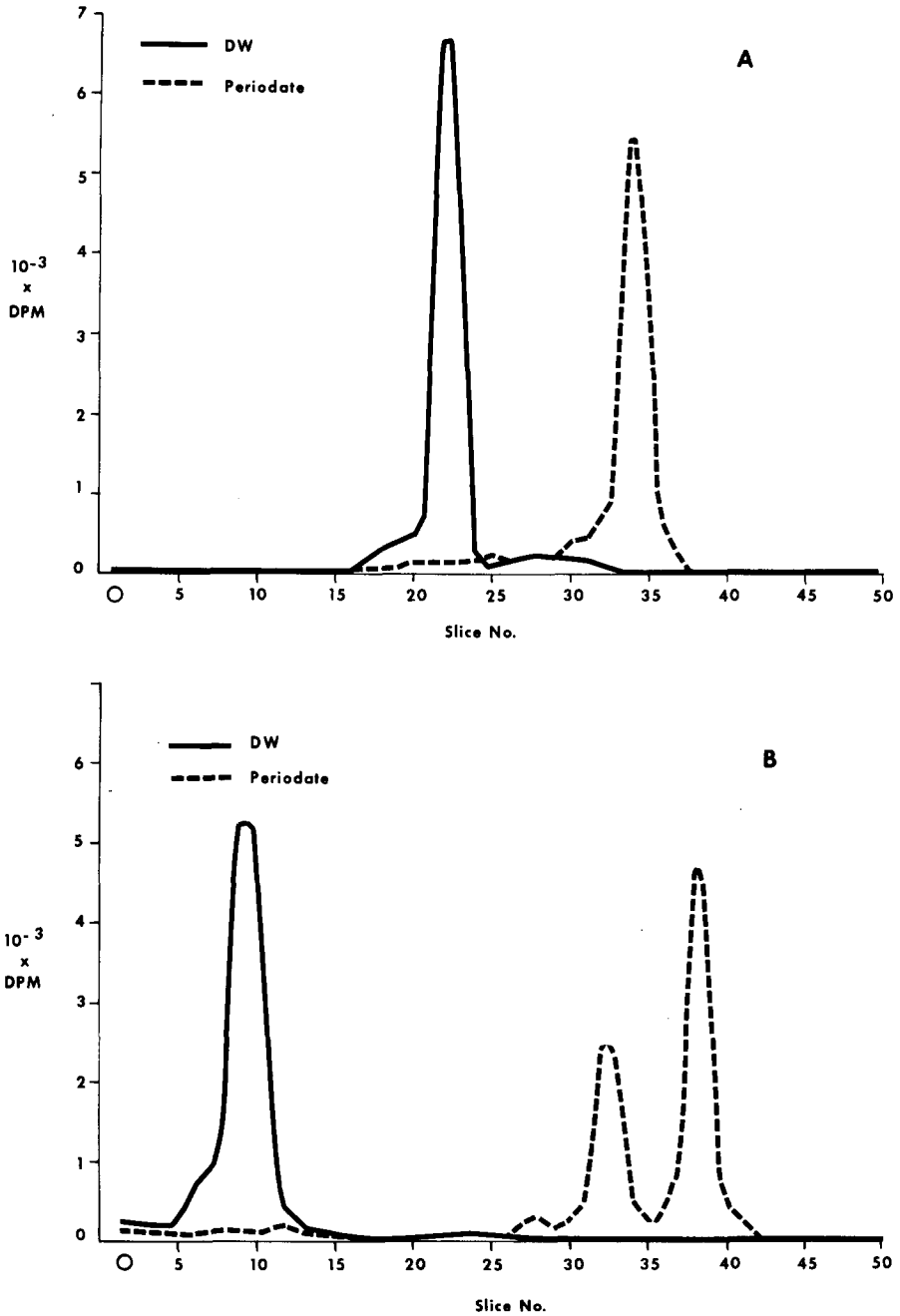


Fig. 1. The separation of purified metabolites by chromatography on SG-81 paper using $CHCl_3$/ethylacetate/benzene (40 : 40 : 20).



25OHD-3 and used as standards in the chromatogram of Fig. 1. These results are compared to periodate cleavage of the material eluted from the 1,24,25$(OH)_3$D-3 and 24,25$(OH)_2$D-3 region of the SG81 chromatogram of a $CHCl_3$ extract of renal mitochondria from vitamin D- and calcium-supplemented chicks incubated with [26,27-^{3}H]25OHD-3 under comparable condi-

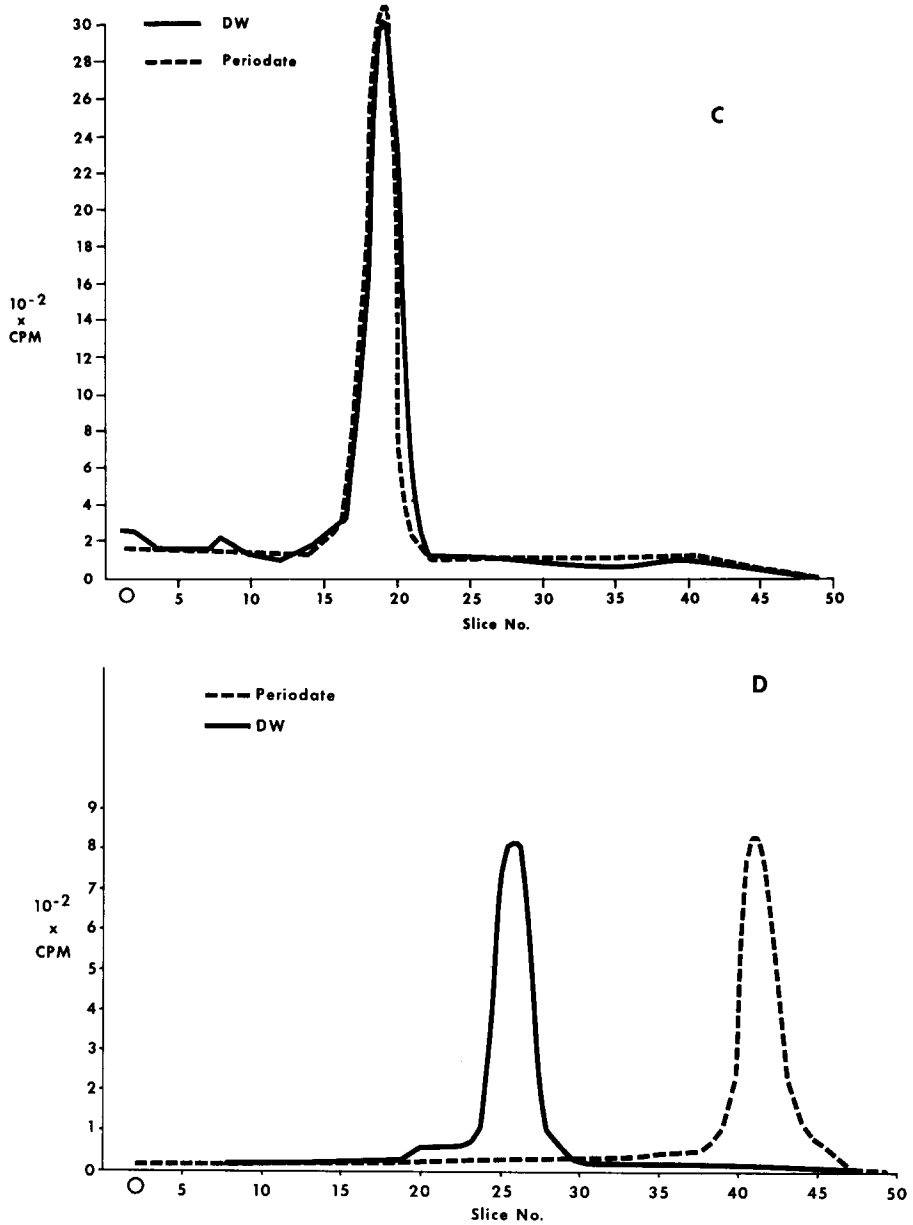


Fig. 2. Periodate treatment of the following metabolites biosynthesized from [23,24-^{3}H]25OHD-3. (A) 24,25$(OH)_2$D-3, (B) 1,24,25$(OH)_3$D-3, (C) 1,25$(OH)_2$D-3, (D), 25,26$(OH)_2$D-3. Equal aliquots of each metabolite were incubated for 15 min with or without periodate, extracted, and chromatographed on SG-81 paper. The chromatograms for the two preparations of each metabolite are shown. The total dpm for each peak on the chromatograms is recorded in Table I(A). The chromatograms of 24,25$(OH)_2$D-3 and its periodate cleavage products were developed for 8 h, whereas the others were developed for 14 h. DW, distilled water.

tions to those used in this report (Table I(B)). The purpose of studying both (23,24-^{3}H)-labelled and (26,27-^{3}H)-labelled metabolites was to confirm the location of the periodate-sensitive glycol linkage. In particular, 24,25-dihydroxy compounds would lose all ^{3}H in the 26,27 positions but none of the ^{3}H

TABLE I

PERIODATE STUDIES

The effect of periodate on various metabolites of vitamin D biosynthesized by chick renal mitochondria and separated using SG-81 paper chromatography. (A) The metabolites labelled in the 23,24 positions were purified by a combination of SG-81 paper chromatography and Sephadex LH20 and were used for the studies shown in Figs. 1 and 2. (B) These metabolites were taken directly from a chromatogram of the $CHCl_3$ extract of chick renal mitochondria incubated in the presence of [26,27-^{3}H]25OHD-3.

Presumptive metabolite	dpm in peak		
	Control	Periodate	
		Original	New *
(A) (23,24-^{3}H) label			
24,25$(OH)_2$D-3	13 346	959	12 704
1,24,25$(OH)_3$D-3	15 652	325	6126, 9640
1,25$(OH)_2$D-3	22 317	21 135	—
25,26$(OH)_2$D-3	6 568	256	6 562
(B) (26,27-^{3}H) label			
24,25$(OH)_2$D-3	643 925	44 959	— **
1,24,25$(OH)_3$D-3	52 232	97	— **

* New peaks appearing after periodate treatment on the SG-81 chromatogram.

** No new peak was observed on the chromatogram but substantial radiolabel appeared in the aqueous phase, presumably as acetone.

in the 23,24 positions. By the criterion of periodate sensitivity, at least 93% of the 24,25$(OH)_2$D-3 peak and 99.8% of the 1,24,25$(OH)_3$D-3 peak on SG81 paper chromatograms of such a mitochondrial extract are comprised of metabolites with a 24,25 glycol linkage. The periodate sensitivity of these peaks (Table I(B)) is comparable to that of the purified standards (Table I(A)). No other metabolite could be resolved from the 1,24,25$(OH)_3$D-3 peak by Sephadex LH20 chromatography. However, a metabolite accounting for a small fraction of the radioactivity contained in the total SG81 paper chromatogram 24,25$(OH)_2$D-3 peak could be separated from 24,25$(OH)_2$D-3 by Sephadex LH20 column chromatography. This 'contaminant' comigrated with 25OHD-3 on Sephadex LH20 columns. No other contaminant was observed on HPLC which showed a comigration of the biosynthesized 24,25$(OH)_2$D-3 with chemically synthesized 24(R),25$(OH)_2$D-3 (data not shown). The extent of contamination of the 24,25$(OH)_2$D-3 peak was reduced by using low substrate concentrations (25 pmol 25OHD-3) which yield greater percent conversion, and by correcting the amount of radioactivity in the 24,25$(OH)_2$D-3 peak for background (see Methods). The location of the periodate-sensitive glycol linkage in the 24,25 positions is indicated by the complete loss of radioactivity from the $CHCl_3$-extractable metabolites when the radioactive label was in the 26,27 positions (Table I(B)), but full recovery of radioactivity in a 'new' metabolite when the radioactive label was in the 23,24 positions (Fig. 2A and B and Table I(A)). Two new metabolites appeared after periodate treatment of [23,24-^{3}H]1,24,25$(OH)_3$D-3 (Fig. 2B), but only one was detected after periodate treatment of [23,24-^{3}H]24,25$(OH)_2$D-3 (Fig. 2A).

Assay kinetics

The production of $24,25(OH)_2D$-3 was linear with respect to time for 30 min (Fig. 3) such that over 40% of the 25OHD-3 was converted to $24,25(OH)_2D$-3 under optimal conditions. Under such conditions $1,24,25(OH)_3D$-3 also was produced (Fig. 3), although the amount of $1,24,25(OH)_3D$-3 produced did not exceed 11% of the amount of $24,25(OH)_2D$-3 produced in 30 min. At shorter incubation times, the ratio of $1,24,25(OH)_3D$-3 to $24,25(OH)_2D$-3 was even smaller. We were unable to detect $1,25(OH)_2D$-3 in these experiments. The amount of $25,26(OH)_2D$-3 produced was inconsistent but always less than 5% of the total substrate added. The recovery of radioactivity in the sum total of metabolite peaks on the chromatogram was consistently greater than 85% of the amount added to the incubation.

The apparent K_m and V values of the 25OHD-3 24-hydroxylase for 25OHD-3 at substrate concentrations less than $1 \cdot 10^{-6}$ M were found to be $3.07 \pm .09 \cdot 10^{-7}$ M and 20.7 ± 5 pmol/30 min per mg protein, respectively. However, saturation of the enzyme by higher substrate concentrations could not be achieved. A Lineweaver-Burk plot of the data (Fig. 4) was not linear at the higher substrate concentrations. Accordingly, the calculation of these kinetic parameters is suspect. At all substrate concentrations $24,25(OH)_2D$-3 was the major metabolite produced, accounting for approx. 70% of the sum of the metabolites produced from 25OHD-3 throughout the entire range of substrate concentrations.

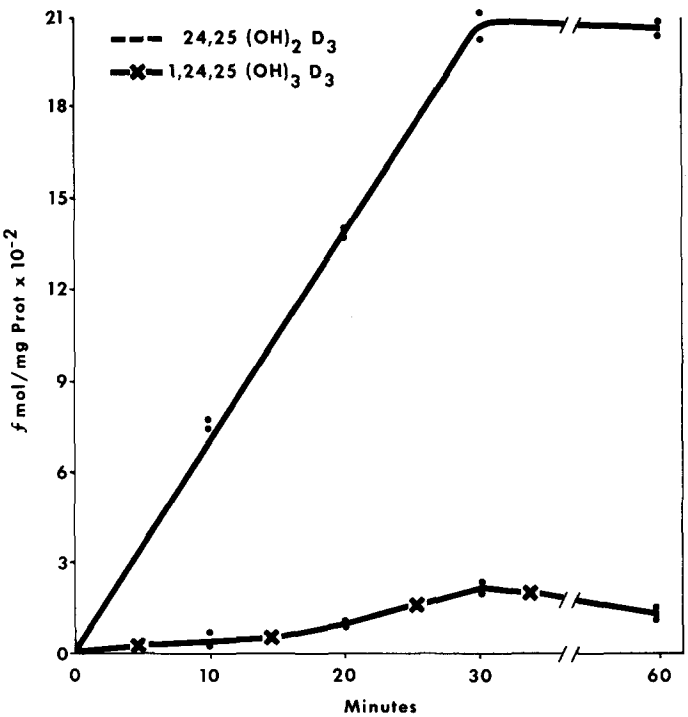


Fig. 3. The rate at which $24,25(OH)_2D$-3 and $1,24,25(OH)_3D$-3 are produced by mitochondria incubated in the presence of 50 mM K^+, 10 mM phosphate and less than $1 \cdot 10^{-9}$ M Ca^{2+} (10 mM EGTA).

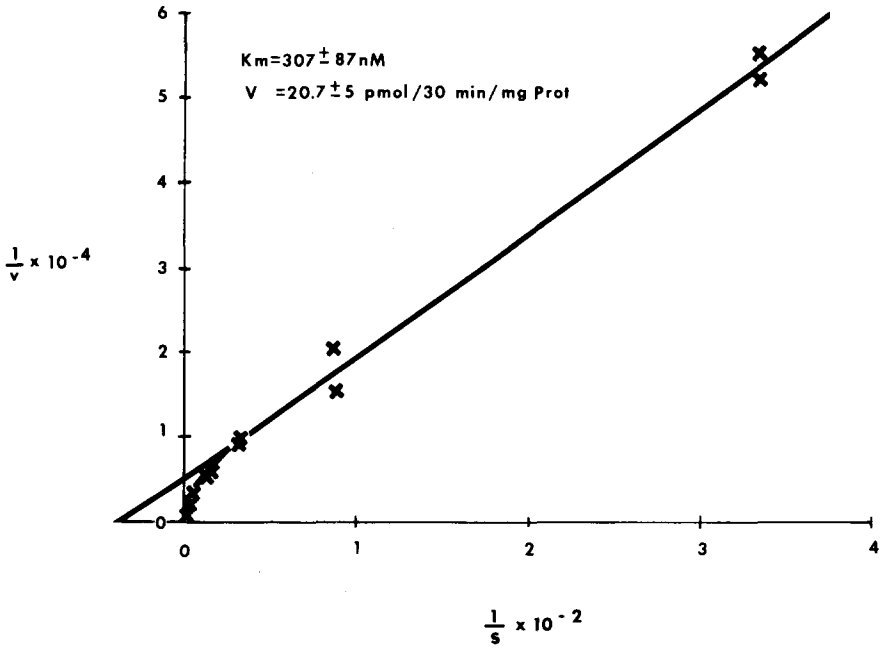


Fig. 4. Lineweaver-Burk plot of 24,25(OH)$_2$D-3 production at different concentrations of 25OHD-3 by mitochondria incubated in the presence of 50 mM K^+, 10 mM phosphate and 10 mM EGTA. The computer-generated fit of the data for the three lowest substrate concentrations is depicted with the resulting calculations for K_m and V ± S.E. $1/S$ = 1/nmol, $1/v$ = 1/(fmol/30 min per mg protein).

Effect of inhibitors

The production 24,25(OH)$_2$D-3 and 1,24,25(OH)$_3$D-3 was reduced by the inhibitors of mitochondrial metabolism, antimycin A and dinitrophenol (Table II). Such inhibitors blocked 1,24,25(OH)$_3$D-3 production more effectively than 24,25(OH)$_2$D-3 production (75% compared to 60% inhibition, respectively). Likewise, the inhibitors of cytochrome *P*-450 mixed function oxidases, DPEA, SKF-525A and metyrapone, blocked both 24,25(OH)$_2$D-3 and

TABLE II

METABOLIC INHIBITORS

DNP, dinitrophenol; DPEA, 2.4-dichloro-6-phenylphenoxyethylamine (HBr salt); SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate (HCl salt). The mitochondria were incubated in the presence of 50 mM K^+ and 10 mM phosphate in the absence of Ca^{2+} (10 mM EGTA). The inhibitors were added in 10 µl ethanol (antimycin A, DNP, metyrapone) or water (DPEA, SKF-525A).

Inhibitor	fmol/mg protein		% inhibition	
	24,25(OH)$_2$D-3	1,24,25(OH)$_3$D-3	24,25(OH)$_2$D-3	1,24,25(OH)$_3$D-3
Control	2080	223	0	0
Antimycin (0.83 µg/ml)	877	57	58	75
DNP ($2.5 \cdot 10^{-5}$ M)	835	57	60	75
DPEA (18.3 µg/ml)	663	30	68	87
SKF-525A (25 µg/ml)	1218	87	42	61
Metyrapone ($1 \cdot 10^{-5}$ M)	1308	68	37	70

$1,24,25(OH)_3D$-3 production. Again, $1,24,25(OH)_3D$-3 production was more sensitive to these inhibitors than $24,25(OH)_2D$-3 production.

Effect of ions

Both Ca^{2+} and K^+, in the absence of stimulatory anions, stimulated $24,25(OH)_2D$-3 production (Fig. 5). Na^+ was ineffective (data not shown). A Ca^{2+} concentration of $1 \cdot 10^{-5}$ M was optimal, although stimulation was observed at $1 \cdot 10^{-6}$ M. At concentrations greater than $1 \cdot 10^{-5}$ M, Ca^{2+} inhibited the reaction. At $1 \cdot 10^{-5}$ M Ca^{2+} the production of $24,25(OH)_2D$-3 was increased 4-fold above the basal rate. K^+ stimulated $24,25(OH)_2D$-3 production more effectively than Ca^{2+} such that an 8- to 10-fold stimulation was observed by increasing the K^+ concentration from 0 to 50 mM. Na^+ did not stimulate the reaction (data not shown).

Raising the phosphate or acetate concentrations from 0 to 50 mM in the absence of Ca^{2+} and K^+ stimulated $24,25(OH)_2D$-3 production 6-fold (Fig. 6). The presence of 10 mM phosphate or acetate was nearly as effective as at 50 mM. Likewise, raising the CO_2/HCO_3^- content from 0%/0 mM to 10%/10 mM, maintaining the pH at 6.8, stimulated $24,25(OH)_2D$-3 production 4-fold (Fig. 6). Cl^- did not exert a stimulatory effect (data not shown).

The stimulation by the cations was additive to that by the anions (Figs. 7 and 8) except when $1 \cdot 10^{-5}$ M Ca^{2+} and 50 mM phosphate or acetate were combined (Fig. 7). Such combinations disrupt the metabolic function of the mitochondria [11].

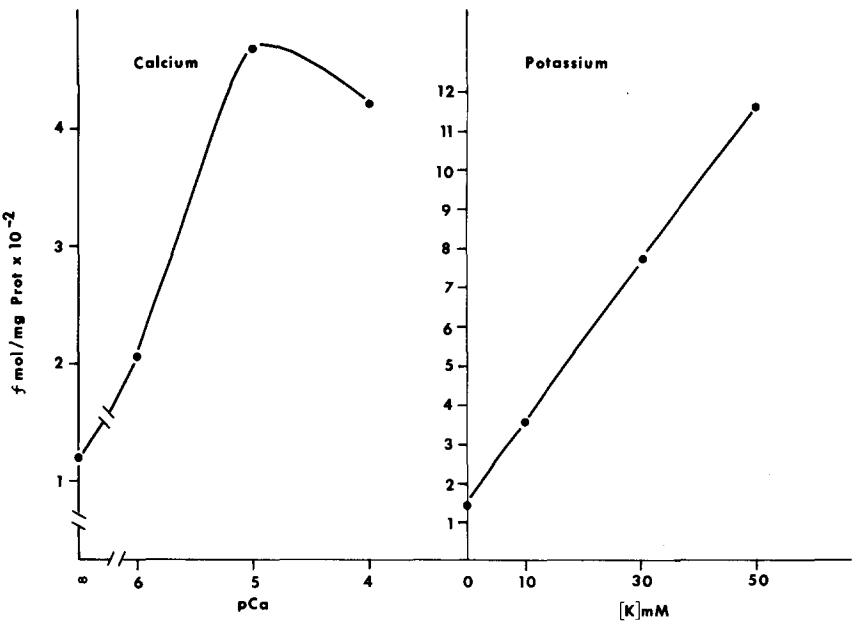


Fig. 5. Stimulation of $24,25(OH)_2D$-3 production by Ca^{2+} or K^+. The free Ca^{2+} concentrations were buffered by EGTA. No phosphate or acetate was included in the incubation mixture. $pCa = -\log [Ca]_{free}$.

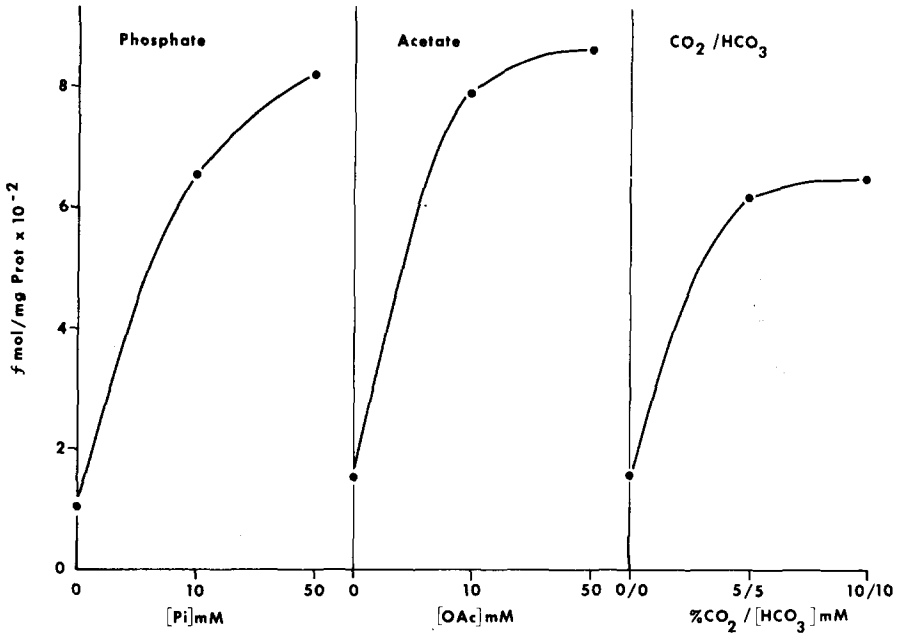


Fig. 6. Stimulation of $24,25(OH)_2$D-3 production by phosphate, acetate (OAc), or CO_2/HCO_3^-. No Ca^{2+} (10 mM EGTA) or K^+ was included in the incubation mixture.

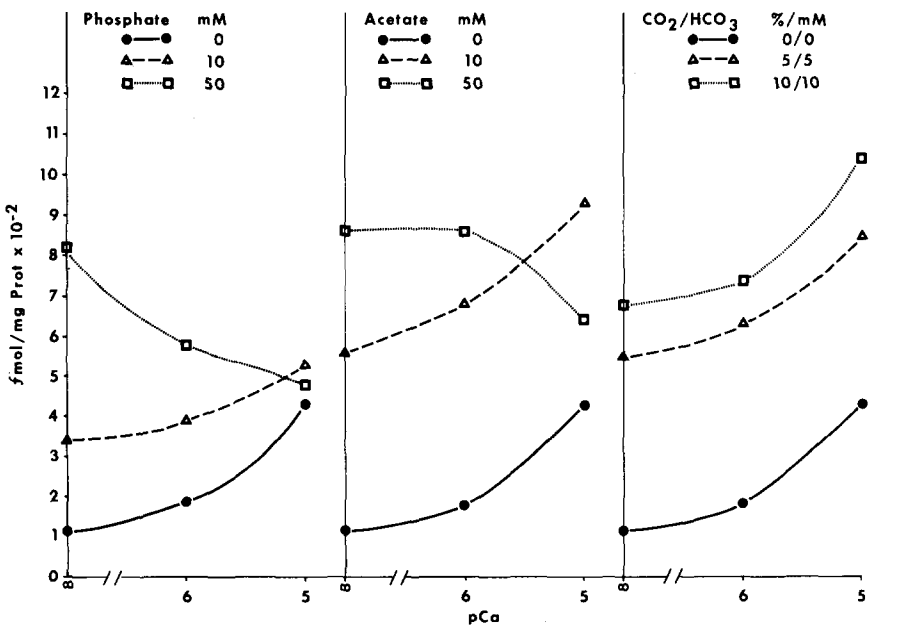


Fig. 7. The effects of phosphate, acetate and CO_2/HCO_3^- on the ability of Ca^{2+} to stimulate $24,25(OH)_2$D-3 production.

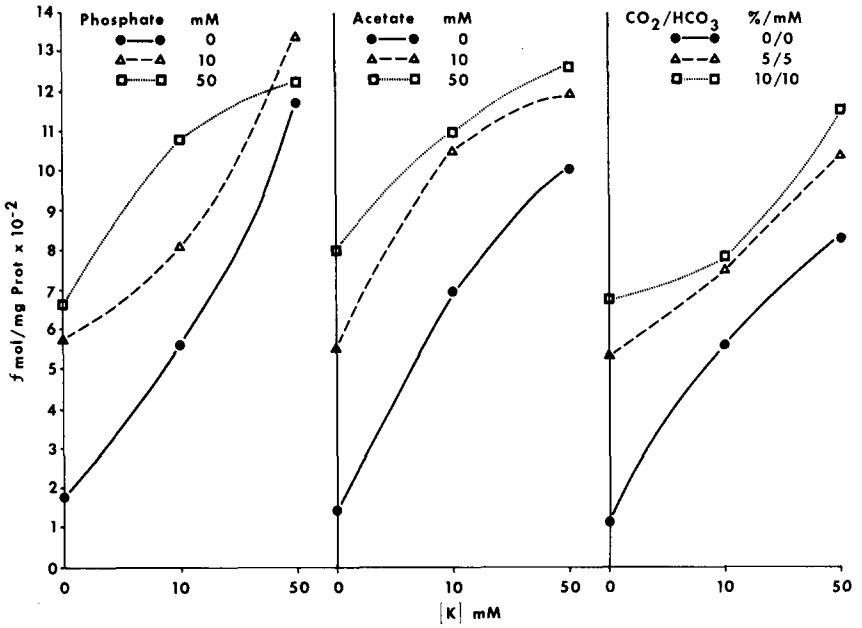


Fig. 8. The effects of phosphate, acetate and CO_2/HCO_3^- on the ability of K^+ to stimulate 24,25$(OH)_2$D-3 production.

Discussion

The control of 24,25$(OH)_2$D-3 production, like that of 1,25$(OH)_2$D-3 production, may be exerted both on synthesis of the enzyme responsible (25OHD-3 24-hydroxylase and 25OHD-3 1-α-hydroxylase, respectively) and on the activity of the existing enzyme. On the basis of inhibitor studies, Tanaka et al. [3] calculated that the 24-hydroxylase and its mRNA have half-lives of approx. 1 and 6 h, respectively, similar to their calculations for the 1-α-hydroxylase [25]. The ability of 1,25$(OH)_2$D-3 and Ca^{2+} to stimulate 24,25$(OH)_2$D-3 production [1—8] while reducing 1,25$(OH)_2$D-3 production most likely is due to new protein synthesis since the effect takes hours and can be blocked by protein-synthesis inhibitors [4,5,7,26]. Similarly, parathyroid hormone, estrogens, cortisol and prolactin are capable of stimulating the 1-α-hydroxylase over a period of hours [27—33] while reducing 24-hydroxylase activity, although as yet the effects of protein-synthesis inhibitors on this process have not been reported. In general, it appears that physiological factors which lead to increased 24-hydroxylase synthesis result in the disappearance of 1-α-hydroxylase and vice versa. This reciprocal control of enzyme synthesis does not mean, however, that the activities of the enzymes need show a similar reciprocity with regard to a possible acute control. In fact, the number of similarities between the two enzymes suggest that if they are subject to an acute control by ions, the mechanism might be similar or identical. Both enzymes appear to be composed of three components, the flavoprotein NADPH dehydrogenase, a non-heme iron ferredoxin-like protein and

cytochrome *P*-450 [17,34—36]. The enzymes are located in the mitochondria of kidney [37,38], probably in the inner membrane, although other tissues contain the 24-hydroxylase [39,40]. Both enzymes incorporate elemental oxygen (O) into 25OHD-3 from molecular oxygen (O_2) [41,42]. Ghazarian [17] recently proposed that only the cytochrome *P*-450 may differ in the two enzymes.

We studied the 24-hydroxylase with an approach similar to our studies of the 1-α-hydroxylase [9—12]. First we modified the chromatography system somewhat to improve the resolution of 24,25$(OH)_2$D-3 (Fig. 1) and verified the nature of the metabolites in the various peaks on the chromatogram (Fig. 2, Table I). We noted that our mitochondrial preparations from vitamin D-supplemented high-calcium diet chicks synthesized 1,24,25$(OH)_3$D-3 and 25,26$(OH)_2$D-3 in addition to 24,25$(OH)_2$D-3, but did not produce detectable amounts of 1,25$(OH)_2$D-3. The production of 1,24,25$(OH)_3$D-3 but not 1,25$(OH)_2$D-3 indicates that some 1-α-hydroxylase activity is present in the mitochondria. However, it may be more exposed to or prefer 24,25$(OH)_2$D-3 relative to 25OHD-3, and/or any 1,25$(OH)_2$D-3 produced may be immediately 24-hydroxylated. The amount of 25,26$(OH)_2$D-3 produced relative to 24,25$(OH)_2$D-3 varied from experiment to experiment and usually was inisignificant, although 1,24,25$(OH)_3$D-3 production during the 30-min incubation consistently was about 10% of the amount of 24,25$(OH)_2$D-3 produced. The lag in 1,24,25$(OH)_3$D-3 production with respect to time (Fig. 3) compared to 24,25$(OH)_2$D-3 production and the inability to detect 1,25$(OH)_2$D-3 suggest that 25OHD-3 is first 24-hydroxylated before being 1-hydroxylated by these mitochondria. The apparent K_m value of the 24-hydroxylase for 25OHD-3 was found to be $3 \cdot 10^{-7}$ M, a value comparable to that determined by a number of groups for the 1-α-hydroxylase [14,34,37,43] but lower than that found by Knutson and DeLuca [38]. However, the double-reciprocal plot of the data presented by Knutson and DeLuca [38], as well as our own, is not linear. We used a wider range of substrate concentrations than Knutson and DeLuca [38] and used the data from the lowest concentrations to determine the K_m value. If one uses the data from the lowest substrate concentrations reported by Knutson and DeLuca [38] and redetermines the apparent K_m value, a value closer to ours is found. The reason for the curvilinear double-reciprocal plot of the data for 24-hydroxylase is obscure and contrasts with that for the 1-α-hydroxylase [43]. Similar kinetics have been observed for fumarase and interpreted as substrate activation [44]. However, the data may also indicate the presence of two or more 24-hydroxylases with different apparent K_m values.

Inhibitors of mitochondrial metabolism, antimycin A and dinitrophenol, blocked 24,25$(OH)_2$D-3 and 1,24,25$(OH)_3$D-3 production (Table II) but not to the extent that they blocked 1,25$(OH)_2$D-3 production by mitochondria from vitamin D-deficient chicks [9]. One explanation is that the 1-α-hydroxylase experiments were performed with stimulating concentrations of Ca^{2+} in the medium, whereas the 24-hydroxylase experiments were performed with stimulating concentrations of K^+. The Ca^{2+} gradient across the mitochondria was much larger than the K^+ gradient under the conditions of these experiments so that Ca^{2+} transport into mitochondria is likely to be more sensitive than K^+ transport to mitochondrial metabolic inhibitors. As such, Ca^{2+}-

stimulated enzymatic activity would be inhibited more readily. This hypothesis remains to be tested. At any rate, the extent of inhibition by antimycin in these experiments is comparable to, and that of dinitrophenol greater than, the results reported by Knutson and DeLuca [38]. That antimycin A and dinitrophenol are partial inhibitors of the 24-hydroxylase indicates a requirement for maintenance of an electrochemical gradient across the mitochondrial membrane for optimal 24-hydroxylase activity. The ability of the specific mixed function oxidase inhibitors, DPEA [45], SKF-525A [46] and metyrapone [47], to inhibit the 24-hydroxylase (as well as the 1-α-hydroxylase) (Table II) supports the concept that this enzyme is a mixed function oxidase [38].

After characterizing the 24-hydroxylase in the above manner we evaluated the effects of cations and anions. Like the 1-α-hydroxylase, the 24-hydroxylase was stimulated by physiological concentrations of Ca^{2+} and K^+ (Fig. 5 and Refs. 9, 10 and 12), cations which induce proton efflux from mitochondria at comparable concentrations [12]. Similarly, the permeant anions, phosphate and acetate, as well as CO_2/HCO_3^-, stimulated both 24-hydroxylase and 1-α-hydroxylase (Fig. 6 and Refs. 11 and 12). These anions, as well as CO_2, appear to cross the mitochondrial membrane in acid form and donate protons to the alkaline matrix [18]. The effects of the cations and anions are additive for the stimulation of both the 24-hydroxylase (Figs. 7 and 8) and the 1-α-hydroxylase [9,10,12].

The numerous similarities between the renal mitochondrial 24-hydroxylase and 1-α-hydroxylase support the hypothesis [17] that they are two variants of the same enzyme complex, differing only in the cytochrome *P*-450 component, the component which determines whether a 24-OH or 1-α-OH group is inserted into 250HD-3. Presumably, changing the cytochrome *P*-450 from one type to the other requires time and protein synthesis, but regulation of the electron and proton flow from NADPH to cytochrome *P*-450 does not. Since the effect of ions on the 24-hydroxylase resembles that of the 1-α-hydroxylase, the same mechanisms may serve both hydroxylases. We [12] have proposed a model to explain the effect of ions on the 1-α-hydroxylase which can readily be extended to the 24-hydroxylase. The model requires further testing. However, the concept that mitochondrial steroid hydroxylases share similar properties and even components with each other and may be controlled in a fashion analogous to the electron transport system of oxidative phosphorylation [48,49] is intriguing.

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