25-Hydroxyvitamin D_3-24-Hydroxylase. Subcellular Location and Properties†

Joyce C. Knutson and Hector F. DeLuca*

ABSTRACT: The 25-hydroxyvitamin D_3-24-hydroxylase activity is shown to be localized in renal mitochondria of chickens raised on a high calcium (3%), vitamin D_3-supplemented diet. The product, 24,25-dihydroxyvitamin D_3, was identified through periodate oxidation and Sephadex LH-20, silicic acid, and Celite liquid–liquid partition cochromatography. This reaction requires NADPH and oxygen. When NADPH is supplied through the oxidation of succinate and malate, the electron transport inhibitors, cyanide, antimycin, and carbon monoxide, inhibit the reaction; however, when the NADPH is supplied directly, these inhibitors have no effect on the 24-hydroxylase activity. Neither added phosphate nor magnesium is required for 24-hydroxylase activity, but each will stimulate the activity. The 24-hydroxylase is inhibited by its product, 24,25-dihydroxyvitamin D_3, and by another metabolite of 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, but is not affected by vitamin D_3, vitamin D_2, dihydrotachysterol$_3$, or 25-hydroxydihydrotachysterol$_3$ when present in the incubation mixture at a 2:1 molar ratio with the substrate.

The metabolism of 25-hydroxyvitamin D_3 (25-OH-D_3)[1] was first demonstrated to be influenced by dietary factors by Boyle *et al.* (1971). These investigators showed that the major metabolite of 25-OH-D_3 in the blood of young, vitamin D-supplemented rats fed a low calcium diet is 1,25-$(OH)_2D_3$, while a less polar metabolite of 25-OH-D_3 predominates in the blood of rats fed a high calcium diet. This less polar metabolite, initially thought to be 21,25-$(OH)_2D_3$ (Suda *et al.*, 1970), has been identified as 24,25-$(OH)_2D_3$ (Holick *et al.*, 1972), a structure which has been confirmed by synthesis (Lam *et al.*, 1973). Further study on the regulation of 25-OH-D_3 metabolism *in vivo* has shown that the plasma concentration of 24,25-$(OH)_2D_3$ increases in rats which have little, if any, circulating parathyroid hormone (Garabedian *et al.*, 1972) or which have high plasma and kidney cortex inorganic phosphorus levels (Tanaka and DeLuca, 1973).

Since the discoveries that 24,25-$(OH)_2D_3$ is the major metabolite of 25-OH-D_3 in the "normal" rat (Boyle *et al.*, 1971), normal chicken (Knutson, 1973), and man (Gray *et al.*, 1973) as well as in animals with rachitic lesions induced by feeding a strontium diet (Omdahl and DeLuca, 1972), attention has focused on the enzymatic production of this metabolite.

The kidney has been shown to be the major, if not the only, site of production of 24,25-$(OH)_2D_3$ since this metabolite was not detected in the blood of nephrectomized rats injected with physiologic doses of 25-OH-[^{3}H]D_3 (Omdahl *et al.*, 1972). It is the purpose of this communication to report the subcellular location of the 25-OH-D_3-24-hydroxylase in chicken kidney and to describe some of the *in vitro* properties of this system.

Materials and Methods

Chickens. One-day old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, Wis.) were housed in group cages maintained at 30° for 1 week. They were fed *ad libitum* a purified diet composed of the following in per cent: soy protein, 25.6; sucrose, 57.9; DL-methionine, 0.6; glycine, 0.4; cellulose, 0.2; cottonseed oil, 4.0; $CaHPO_4$, 1.2; KH_2PO_4, 1.6; $CaCO_3$, 6.6; NaCl, 0.8; with trace salts and vitamins added as previously reported (Imrie *et al.*, 1967) except that the fat-soluble vitamins were added as crystalline compounds or as a gelatin-coated powder. The chickens received a daily oral administration of 12.5 IU of vitamin D_3 dissolved in 0.1 ml of cottonseed oil.

Radioactive 25-OH-D_3. 25-OH-[26,27-^{3}H]D_3 (1.2 Ci/mmol) was synthesized in this laboratory according to the method of Suda *et al.* (1971). Periodically the tritiated 25-OH-D_3 was purified by chromatography on a Sephadex LH-20 column developed with chloroform–Skellysolve B (petroleum ether, bp 67–68°) 50:50 (Holick and DeLuca, 1971).

Nonradioactive 25-OH-D_3 was a gift from the Philips-Duphar Company of Weesp, The Netherlands.

Preparation of Mitochondria. The chickens were killed by decapitation; the kidneys were removed and placed in ice-cold 0.25 M sucrose. The tissue was dissected free of mesentery, minced, and weighed. A 20% (w/v) homogenate was prepared in 0.25 M sucrose. For experiments in which the NADPH generating system was used, the 20% homogenate was prepared in 0.25 M sucrose containing 15 mM Tris-acetate (pH 7.4), 1 mM dithiothreitol, and 0.1 mM EDTA. The tissue was homogenized using three strokes with a motor-driven glass–Teflon Potter-Elvehjem homogenizer (A. H. Thomas, Philadelphia, Pa.). The homogenate was centrifuged at 270*g* for 10 min; the pellet was washed once. The combined supernatants were centrifuged at 3300*g* for 10 min. The crude mitochondrial pellet was washed once, and resuspended in the appropriate medium at approximately 12–16 mg/ml of protein.

Incubation Conditions. In a 25-ml erlenmeyer flask, 0.5 ml of mitochondrial suspension was mixed with 1.0 ml of buffer–cofactor solution A or B. Cofactor solution A, which was used for the succinate-supported reaction, contained 22.5 mM Tris-acetate (pH 7.4), 7.5 mM succinate, 3 mM $MgCl_2$ and 0.25 M sucrose; cofactor solution B, which was used for the NADPH generating system supported reaction, contained 15 mM Tris-acetate (pH 7.4), 0.75 mM $NADP^+$, 7.5 mM nicotin-

† From the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received October 24, 1973.* Supported by grants from the National Institutes of Health (AM-14881 and AM-15512) and a Training Grant (GM00236-BCH) from the National Institute of General Medical Sciences.

[1] Abbreviations used are: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 21,25-$(OH)_2D_3$, 21,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3.

amide, 7.5 mM glucose 6-phosphate, 7.5 mM $Mg(OAc)_2$, 15 mM $Ca(OAc)_2$, 0.25 M sucrose, 1 mM dithiothreitol, and 0.1 mM EDTA. The incubation flask was flushed for 1 min with a stream of 100% oxygen. The substrate, 1.25 nmol of 25-OH-[^{3}H]D_3 (1.2 Ci/mol) in 0.01 ml of 95% ethanol, was then added to the contents of the flask. The cork-stoppered flask was then incubated at 37° for 15 min with continuous agitation of 80 oscillations/min with a 1-in. stroke length. For experiments in which the NADPH generating system was used, 1.5 units of glucose 6-phosphate dehydrogenase in 0.01 ml of 3.2 M ammonium sulfate was added to the incubation mixture just prior to the addition of substrate. For the experiment in which the NADH generating system was used, 0.75 mM NAD^+ replaced the $NADP^+$, nicotinamide, and glucose 6-phosphate in cofactor solution B. Then 0.05 ml of 95% ethanol and 1.5 units of alcohol dehydrogenase were added to the incubation mixture just prior to the 25-OH-[^{3}H]D_3. All incubations were performed in duplicate. The reaction was terminated by addition of 6 ml of 1:0.8 chloroform–methanol.

Extraction Procedure. The contents of the incubation flask and a 4-ml methanol rinse of the flask were poured into a separatory funnel. The resulting single phase remained at room temperature for 1 hr. The solution was separated into two phases with the addition of 2.5 ml of water and 5 ml of chloroform and was kept at 4° overnight. The chloroform phase was then removed, and the aqueous phase was re-extracted with 5 ml of chloroform. An aliquot of the combined chloroform phases was removed for determination of total radioactivity. The remaining solution plus a few milliliters of absolute ethanol, added to remove any residual water, was evaporated to dryness with a rotary evaporator and the residue was redissolved in 1 ml of 65:35 chloroform–Skellysolve B for chromatographic analysis.

Chromatography. The lipid extract from each incubation mixture was chromatographed on a 16-g Sephadex LH-20 column (2 × 19.5 cm) equilibrated with 65:35 chloroform–Skellysolve B (Holick and DeLuca, 1971). The column was developed with the same solvent at a flow rate of 1 ml/min. Thirty-seven fractions (5 ml) were collected into 1-dram vials. The solvents were evaporated under a stream of air and the residues were redissolved in 4 ml of toluene scintillation solution (Bhattacharyya and DeLuca, 1973). The 1-dram vials were placed in 20-ml scintillation counting vials, and the radioactivity was determined using either a Packard Tri-Carb liquid scintillation spectrometer Model 3375 or a Nuclear Chicago Model 6868 Isocap liquid scintillation spectrometer. The average counting efficiency for tritium in these samples was 40 and 45%, respectively. The total radioactivity recovered usually ranged from 90 to 100% of the incubated substrate radioactivity.

Subcellular Fractionation. The subcellular fractionation procedure followed was essentially that of DeDuve *et al.* (1955) except that the initial centrifugation was 270*g* for 10 min; the final centrifugation, 105,000*g* for 60 min. The assay mixture for the 24-hydroxylase contained 6–7 mg of protein from each fraction/ml.

The isolation of "pure" nuclei was performed by a modified procedure of Lawson *et al.* (1969). Only three strokes were used to homogenize the tissue; the homogenate was not filtered through cloth; and the initial centrifugation was 270*g* for 10 min. The nuclei appeared free of contaminants under a light microscope.

Procedures for the Identification of the Product. The Celite liquid–liquid partition and silicic acid chromatographies were performed as described by Suda *et al.* (1970). The Sephadex LH-20 chromatography was performed as described by Holick and DeLuca (1971).

Periodate Reaction. Each sample was dissolved in 0.02 ml of methanol and treated with 0.01 ml of 5% $NaIO_4$ (aqueous) at room temperature for 20 hr. The solution was extracted three times with 0.2 ml of chloroform. The combined chloroform extracts were placed in scintillation vials and evaporated. The residues were redissolved in scintillation counting solution.

Alteration of the Incubation Atmosphere. All components of the incubation mixture except the mitochondria, the 25-OH-[^{3}H]D_3, and the glucose 6-phosphate dehydrogenase were placed in the incubation flask and sealed with a rubber serum stopper. The flasks were attached to a vacuum line by needles inserted through the stopper, evacuated, flushed with nitrogen, and then reevacuated. The flasks were then removed from the vacuum line and filled with the appropriate gas or gas mixture by means of a gas-filled syringe. The remaining incubation components were introduced into the flask through syringes.

Analytical Procedures. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. DNA was determined by the method of Schneider (1957) using calf thymus DNA as a standard.

Results

The subcellular distribution of the 25-OH-D_3-24-hydroxylase activity in the kidneys of chickens raised on a high calcium diet is shown in Table I. Almost all of the recovered enzyme activity was located in the crude nuclear-debris and heavy mitochondrial fractions. The greatest specific activity, however, was located in the heavy mitochondrial fraction. Nuclei were purified by sedimentation through 2.4 M sucrose to determine whether the nuclei possessed 24-hydroxylase activity or the activity associated with this fraction was a result of contamination from whole cells and mitochondria.

While the heavy mitochondria and crude nuclei produced 11.8 and 3.3 pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}, respectively, an assay of the 2.4 M sucrose nuclei containing a similar protein concentration showed no ability to convert 25-OH-D_3 to 24,25-$(OH)_2D_3$.

TABLE I: Subcellular Distribution of 25-OH-D_3-24-Hydroxylase Activity of Chicken Kidney.[a]

Fraction	Specific Activity	Total Enzyme Activity[b]	Total Protein (mg)	Total DNA (mg)
Homogenate	0.80	1220	1530	44
Crude nuclear-debris	0.68	426	631	41
Heavy mitochondria	2.14	451	211	1
Light mitochondria	0.13	8	60	ND[c]
Microsomes	0.01	1	115	ND
Cytoplasm	0	0	360	ND
% recovered		73	90	95

[a] A subcellular fractionation of chicken kidney was performed according to a modification of the method of DeDuve *et al.* (1955). Each fraction (6–7 mg of protein/ml) was assayed for 24-hydroxylase activity using the conditions described in the text for the succinate-supported reaction. [b] pmol of 24,25-$(OH)_2D_3$ min^{-1} fraction^{-1}. [c] Not determined.

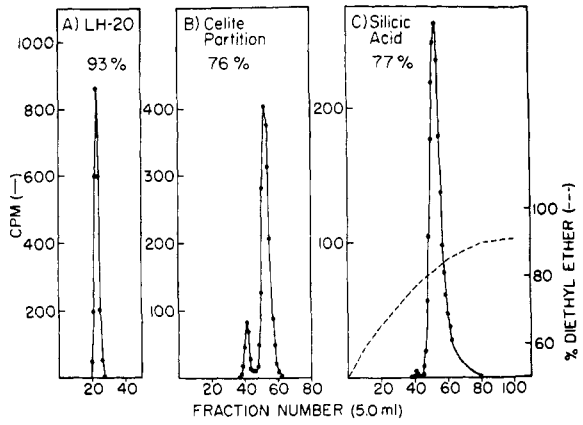

FIGURE 1: Cochromatography of the product derived from the incubation of chicken kidney mitochondria with 25-OH-[^{3}H]D_3 and standard 24,25-$(OH)_2D_3$. The *in vitro* product (2100 cpm) produced as described in the text and eluted from the Sephadex LH-20 column used in the assay procedure was mixed with standard 24,25-$(OH)_2$-D_3 (970 cpm) for cochromatography in (a) Sephadex LH-20 (Holick and DeLuca, 1971), (b) Celite liquid–liquid partition (Suda *et al.*, 1970), and (c) silicic acid (Suda *et al.*, 1970). The percentages represent the per cent recovery of the applied radioactivity. (——) Radioactivity was determined using a Packard Model 3375 liquid scintillation counter.

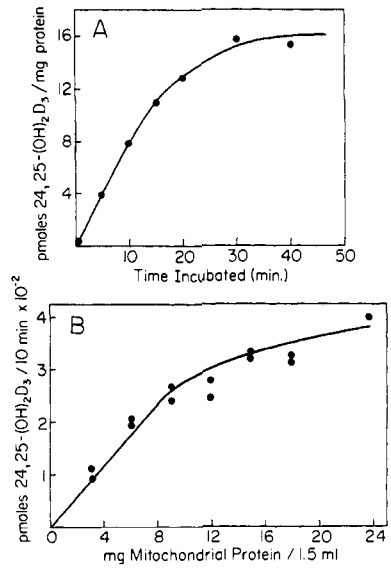

FIGURE 2: The production of 24,25-$(OH)_2D_3$ by chicken kidney mitochondria supported by succinate as a function of time (A) and protein concentration (B). The incubation medium and conditions are described in the text.

Cochromatography of the product of the mitochondrial reaction with standard 24,25-$(OH)_2D_3$ identified by Holick *et al.* (1972) in three systems, Sephadex LH-20, silicic acid, and Celite liquid–liquid partition, is shown in Figure 1. All three of these chromatographic systems are capable of resolving structurally similar metabolites. The silicic acid and Celite liquid–liquid partition chromatography was used to isolate the *in vivo* metabolite in pure form (Suda *et al.*, 1970), while the Sephadex LH-20 chromatography has been shown to be capable of resolving vitamin D_3 metabolites, particularly those more polar than 25-OH-D_3 (Holick and DeLuca, 1971). With each chromatographic system 2100 cpm of the product and 970 cpm of the standard 24,25-$(OH)_2D_3$ were applied to the column. The recoveries observed are within the expected range for each chromatographic system (Holick and DeLuca, 1971). The small peak observed with liquid–liquid partition chromatography appeared in the chromatography of the standard 24,25-$(OH)_2D_3$ and also appeared with this chromatographic procedure in the initial isolation of this compound by Suda *et al.* (1970). Perhaps this compound is 24,25-$(OH)_2$ previtamin D_3, which is in thermal equilibrium with the vitamin.

Since reaction with periodate will cleave the bond between carbon atoms bonded to vicinal hydroxyl functions, reaction of 24,25-$(OH)_2$-[26,27-^{3}H]D_3 with periodate should release the radioactivity as tritiated acetone. The [^{3}H]acetone could then be removed through evaporation. The per cent radioactivity remaining in the nonvolatile fraction following treatment with periodate of the standard 24,25-$(OH)_2D_3$, the *in vitro* product, and 25-OH-D_3 was 11, 12, and 80%, respectively. The similar loss in radioactivity from the standard 24,25-$(OH)_2D_3$ and from the product generated *in vitro* and the cochromatography in three systems indicate that 24,25-$(OH)_2D_3$ is the product of this *in vitro* mitochondrial reaction.

The production of 24,25-$(OH)_2D_3$ by chicken kidney mitochondria as a function of time from 5 to 40 min and as a function of protein concentration from 3 to 23 mg/flask are shown in Figure 2. The production of 24,25-$(OH)_2D_3$ appears linear up to 15 min and with a protein concentration up to 8 mg/flask. The incubation conditions selected for assay were 15 min in length and 6–8 mg of mitochondrial protein/flask.

A Lineweaver–Burk plot of the dependence of the 24-hydroxylation of 25-OH-D_3 on the concentration of substrate revealed that the apparent K_m for 25-OH-D_3 for this mitochondrial reaction is approximately 10^{-6} M (Figure 3).

Succinate, malate, and isocitrate supported the enzyme activity to a similar extent, producing 15.7, 16.1, and 15.3 pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}, respectively. In the absence of any other source of reducing equivalents, one of the tricarboxylic acid cycle intermediates was required to observe enzyme activity.

Since intact mitochondria are impermeable to externally generated reduced pyridine nucleotides (Lehninger, 1951), to determine if the enzyme activity required these reducing equivalents, the mitochondria were swollen with 10 mM calcium to allow them to enter (Guerra *et al.*, 1966). As shown in Figure 4, in mitochondria swollen with calcium, NADPH but not NADH was able to support the production of 24,25-$(OH)_2D_3$. Externally generated NADPH could not support

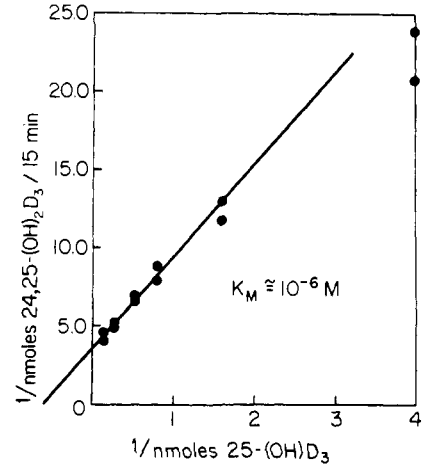

FIGURE 3: A Lineweaver–Burk plot for the 25-OH-D_3-24-hydroxylase. The apparent K_m for 25-OH-D_3 was calculated to be 10^{-6} M.

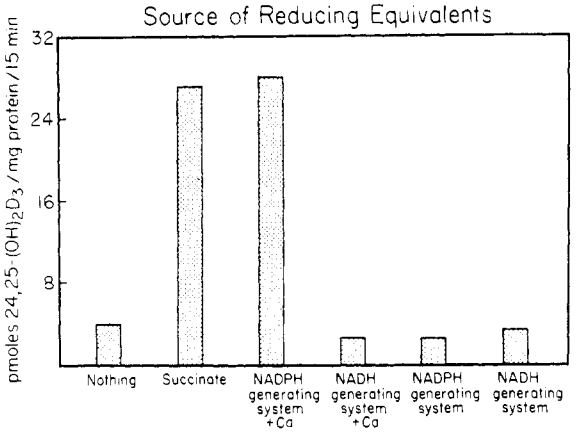

FIGURE 4: The ability of different sources of reducing equivalents to support 25-OH-D_3-24-hydroxylase activity in chicken kidney mitochondria. For the assay labeled "nothing," the incubation medium was the standard one minus succinate. The respective NADPH and NADH generating systems replaced succinate in the medium in the indicated reactions. For the assays labeled "+ Ca," 10 mM calcium acetate was added to swell the mitochondria to allow entry of the reduced pyridine nucleotides.

the reaction in intact mitochondria, indicating a degree of mitochondrial integrity.

The data shown in Table II indicate that molecular oxygen is required for the conversion of 25-OH-D_3 to 24,25-$(OH)_2D_3$. The experiments with the different sources of reducing equivalents were performed at different times with different mitochondrial preparations so the absolute production of 24,25-$(OH)_2D_3$ cannot be compared.

The concentration of oxygen in air (21%) appeared limiting with the succinate-supported reaction. The usual method of supplying oxygen, flushing the flask with a stream of oxygen for 1 min, provided a nonlimiting supply of oxygen. After 15 min under nitrogen there is still sufficient enzyme activity remaining to indicate that the lack of production of 24,25-$(OH)_2D_3$ in a 100% nitrogen atmosphere was a result of lack of oxygen rather than destruction of the enzyme activity by nitrogen.

The production of 24,25-$(OH)_2D_3$ was studied in the presence of cyanide, antimycin, and dinitrophenol. The electron transport inhibitors, cyanide (10^{-3} M) and antimycin (10^{-5} M), inhibited the succinate-supported reaction to a greater extent than the malate-supported reaction (Table III). These concentrations of inhibitors are sufficient to inhibit respiration (Slater, 1967). The formation of NADPH from succinate or malate is apparently blocked by these inhibitors since the inhibition could be bypassed by supplying the NADPH directly. The uncoupler, dinitrophenol (10^{-5} M), had little effect on the reaction.

The effect of carbon monoxide (50 and 75%) on the 24-hydroxylation of 25-OH-D_3 on the succinate and NADPH generating system supported reactions is shown in Table IV. At these concentrations of carbon monoxide, the NADPH-supported enzyme activity was apparently unaffected. The succinate-supported reaction was inhibited by 32%, probably the result of cytochrome oxidase inhibition.

The effect of phosphate on the succinate-supported 24-hydroxylation of 25-OH-D_3 is shown in Figure 5. Added phosphate is not required for the production of 24,25-$(OH)_2D_3$, but adding phosphate increased the rate of production of this metabolite. Controls were performed with potassium chloride to test whether the addition of the potassium ion or the change in ionic strength was responsible for the increased rate of 24,25-$(OH)_2D_3$ production. Figure 5 shows that the phosphate ion is responsible for the increase in rate.

The effect of magnesium and calcium ions on the 24-hydroxylase is shown in Table V. Added magnesium, like phosphate, was not required for enzyme activity, but stimulated the activity. Calcium, however, inhibited the succinate-supported production of 24,25-$(OH)_2D_3$ by 60% at both 1 and 2.5 mM.

TABLE II: Dependence on Molecular Oxygen of the 24-Hydroxylation of 25-OH-D_3.

Expt[a]	Source of Reducing Equivalents	Atmosphere[b]	pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}
1	Succinate	100% O_2	23.2
	Succinate	100% N_2	0
	Succinate	Air	17.3
	Succinate	Flushed with O_2[c]	23.6
	Succinate	100% N_2, then O_2[d]	17.4
2	NADPH	100% O_2	12.7
	NADPH	100% N_2	0
	NADPH	Air	12.9
	NADPH	100% N_2, then O_2[d]	6.5

[a] The experiments with succinate and NADPH generating system were performed with different mitochondrial preparations. [b] The incubation flask was first evacuated; then the appropriate gas was introduced using a syringe. [c] A stream of oxygen was blown on the surface of the reaction mixture for 1 min. [d] The reaction mixture was incubated under nitrogen for 15 min, then opened, flushed for 1 min with oxygen, and reincubated for 15 min.

TABLE III: Effect of Cyanide, Antimycin, and Dinitrophenol on the 25-OH-D_3-24-Hydroxylase.

Expt[a]	Source of Reducing Equivalents	Compd[b]	Concn (M)	pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}	% Inhibition
1	Succinate			19.1	
	Succinate	KCN	10^{-3}	4.2	78
	Succinate	Antimycin	10^{-5}	3.8	80
	Succinate	Dinitrophenol	10^{-5}	19.3	
	Malate			19.1	
	Malate	KCN	10^{-3}	9.1	52
	Malate	Antimycin	10^{-5}	8.3	57
	Malate	Dinitrophenol	10^{-5}	17.1	10
2	NADPH			12.0	
	NADPH	KCN	10^{-3}	12.1	
	NADPH	Antimycin	10^{-5}	12.2	

[a] Experiments 1 and 2 were performed with different mitochondrial preparations. [b] Antimycin and dinitrophenol were added in 0.01 ml of 95% ethanol.

TABLE IV: Effect of Carbon Monoxide on the Conversion of 25-OH-D_3 to 24,25-$(OH)_2D_3$.

Expt[a]	Source of Reducing Equivalents	Atmosphere[b]	pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}
1	Succinate	50% CO	15[c]
	Succinate	50% N_2	22[c]
2	NADPH	100% O_2	16
	NADPH	50% CO	15
	NADPH	75% CO	16
	NADPH	75% N_2	16

[a] Different mitochondrial preparations were used for experiments 1 and 2. [b] The portion of the atmosphere not designated is oxygen. The incubation flask was first evacuated and the appropriate gasses were then introduced using a syringe. [c] Significantly different, $p \leq 0.01$.

If vitamin D_3, vitamin D_2, dihydrotachysterol$_3$, or 25-hydroxydihydrotachysterol$_3$ are present in the incubation mixture at a 1:1 or 2:1 molar ratio with the substrate, 25-OH-D_3, the enzymatic conversion to 24,25-$(OH)_2D_3$ was not significantly inhibited (Table VI). This would indicate a degree of specificity of the enzyme for 25-OH-D_3. However, the two metabolites of 25-OH-D_3, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, inhibited the 24-hydroxylase. The inhibition by 1,25-$(OH)_2D_3$ was dramatic, even at the lower concentration, while the inhibition by 24,25-$(OH)_2D_3$ was only apparent at the 2:1 molar ratio with the substrate.

Discussion

The data presented demonstrate that heavy mitochondria from chickens fed a high-calcium, vitamin D-supplemented diet are capable of converting 25-OH-D_3 to 24,25-$(OH)_2D_3$. Although the crude nuclear fraction showed 24-hydroxylase activity, highly purified nuclei sedimented through 2.4 M sucrose showed no activity. Because NADPH can support the reaction only in the presence of high levels of calcium, it seems likely that heavy mitochondria are the major subcellular site of 24-hydroxylase activity. The requirements of NADPH and molecular oxygen for the enzymatic production of 24,25-$(OH)_2D_3$ suggest that the enzyme is an hydroxylase, and possibly a mixed function oxidase.

TABLE V: Effect of Magnesium and Calcium on the 25-OH-D_3-24-Hydroxylase.[a]

Ion	Concn (mM)	pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}	% control
$MgCl_2$		22.2	69
$MgCl_2$	1.5	32.0[b]	99
$MgCl_2$	2.0	32.2[b]	100
$MgCl_2$	3.0	29.1[b]	91
$CaCl_2$[a]	1	12.5	39
$CaCl_2$[a]	2.5	12.7	40

[a] The incubation medium and conditions are described in the text. The calcium chloride was added in addition to 2.0 mM magnesium chloride. [b] Significantly different from reaction without added $MgCl_2$ at $p \leq 0.05$.

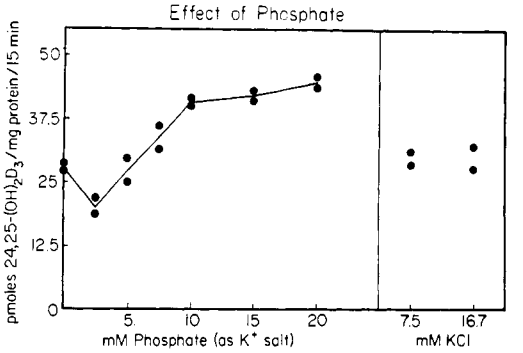

FIGURE 5: The effect of added phosphate on chicken kidney mitochondria 25-OH-D_3-24-hydroxylase. A phosphate buffer was added to the standard incubation medium for the succinate-supported enzyme assay. Osmolarity was maintained by appropriately decreasing the sucrose concentration. The controls with KCl tested the effect of the potassium ion and a change in ionic strength on the 24-hydroxylase.

The tricarboxylic acid cycle intermediates apparently support the hydroxylation by providing a source of NADPH. The mechanism by which this occurs is open to speculation. Two major sources of NADPH have been postulated for mitochondrial hydroxylations of steroids in the adrenal. They are: (a) the malic enzyme which converts malate to pyruvate with the reduction of $NADP^+$ (Simpson and Estabrook, 1969), and (b) the reversal of the electron transport chain followed by transhydrogenation of NADH to NADPH (Hall, 1972). The data obtained for the 25-OH-D_3-24-hydroxylase from the studies with cyanide and antimycin resemble that obtained for the 11β-hydroxylation of 11-deoxycorticosterone, for which the malic enzyme has been postulated as the source of NADPH (Guerra *et al.*, 1966; Simpson and Estabrook, 1969).

TABLE VI: Effect of Structural Analogs of 25-OH-D_3 on the 25-OH-D_3-24-Hydroxylase Activity.

Expt[a]	Analog	nmol of Analog–nmol of 25-OH-D_3	pmol of 24,25-$(OH)_2D_3$ mg of protein^{-1} 15 min^{-1}
1	None		14.4
	Vitamin D_3	1:1	14.0
		2:1	15.5
	Dihydrotachysterol$_3$	1:1	13.3
		2:1	13.7
	25-Hydroxydihydro-tachysterol$_3$	1:1	13.8
		2:1	13.7
2	None[b]		20.4
	Vitamin D_2	1:1	20.9
		2:1	21.0
	1,25-$(OH)_2D_3$	1:1	4.4
		2:1	2.1
	24,25-$(OH)_2D_3$	1:1	20.4
		2:1	10.7

[a] Experiments 1 and 2 were performed with different mitochondrial preparations. [b] Each incubation contained 1.25 nmol of 25-OH-[^{3}H]D_3 and the appropriate amount of the analog added in a maximum of 0.01 ml of 95% ethanol.

The stimulation of the 24-hydroxylase activity by phosphate *in vitro* appears to support the suggestion that inorganic phosphate has a role in the regulation of 25-OH-D_3 metabolism (Tanaka and DeLuca, 1973). It is also possible, however, that the phosphate is not influencing the enzyme directly, but rather mitochondrial structure or function which in turn influences enzyme activity. The inhibition of the succinate-supported reaction by calcium is probably a result of the inhibition of the generation of reducing equivalents since an external NADPH generating system allows activity in the presence of calcium. Whether or not calcium inhibits the enzyme itself cannot be determined from the present results.

It is interesting to note that of the structural analogs of vitamin D_3 examined, only 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ significantly inhibited the 24-hydroxylase (Table VI). To determine whether these inhibitions operate in the regulation of 25-OH-D_3 metabolism *in vivo* requires further investigation.

A comparison of the properties of the 25-OH-D_3-1-hydroxylase (Gray *et al.*, 1972; Fraser and Kodicek, 1970, 1973; Ghazarian and DeLuca, 1974) and the 25-OH-D_3-24-hydroxylase reveals both similarities and differences. In addition to the mitochondrial location and requirement for NADPH and molecular oxygen, the *in vitro* rate of metabolism of 25-OH-D_3 as a function of time and protein concentration and the K_m for 25-OH-D_3 for the two enzyme systems are similar. However, the 24-hydroxylase differs from the 1-hydroxylase in that (a) a carbon monoxide to oxygen ratio of 3:1 does not inhibit the 24-hydroxylase, (b) added magnesium is not required for activity, and (c) 25-hydroxydihydrotachysterol$_3$, when present at a 1:1 ratio with 25-OH-D_3, does not inhibit the reaction.

Although the function of 24,25-$(OH)_2D_3$ is not yet known, the regulation of its enzymatic production by dietary and hormonal changes (Boyle *et al.*, 1971; Garabedian *et al.*, 1972; Tanaka and DeLuca, 1973; Omdahl *et al.*, 1972) suggests an important physiologic role for this metabolite in calcium and/or phosphorus homeostasis. A possible role of 24,25-$(OH)_2D_3$ in intestinal calcium transport through conversion to a more polar metabolite has been suggested by Boyle *et al.* (1973). It might also function at its site of production in the renal handling of calcium or phosphorus. However, only continued investigation can reveal what, if any, its role is.

Acknowledgment

We gratefully acknowledge the technical assistance of Mary Jo Graber.

References

Bhattacharyya, M. H., and DeLuca, H. F. (1973), *J. Biol. Chem. 248*, 2969.

Boyle, I. T., Gray, R. W., and DeLuca, H. F. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2131.

Boyle, I. T., Omdahl, J. L., Gray, R. W., and DeLuca, H. F. (1973), *J. Biol. Chem. 248*, 4174.

DeDuve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Applemans, F. (1955), *Biochem. J. 60*, 604.

Fraser, D. R., and Kodicek, E. (1970), *Nature (London), 228*, 764.

Fraser, D. R., and Kodicek, E. (1973), *Nature (London), New Biol. 241*, 163.

Garabedian, M., Holick, M. F., DeLuca, H. F., and Boyle, I. T. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1673.

Ghazarian, J. G., and DeLuca, H. F. (1974), *Arch. Biochem. Biophys. 160*, 63.

Gray, R. W., Omdahl, J. L., Ghazarian, J. G., and DeLuca, H. F. (1972), *J. Biol. Chem. 247*, 7528.

Gray, R. W., Weber, H. P., and Lemann, J. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 917.

Guerra, F., Péron, F. C., and McCarthy, J. L. (1966), *Biochim. Biophys. Acta 117*, 433.

Hall, P. F. (1972), *Biochemistry 11*, 2891.

Holick, M. F., and DeLuca, H. F. (1971), *J. Lipid Res. 12*, 460.

Holick, M. F., Schnoes, H. K., DeLuca, H. F., Gray, R. W., Boyle, I. T., and Suda, T. (1972), *Biochemistry 11*, 4251.

Imrie, M. H., Neville, P. F., Snellgrove, A. W., and DeLuca, H. F. (1967), *Arch. Biochem. Biophys. 120*, 525.

Knutson, J. C. (1973), Ph.D. Thesis, University of Wisconsin, Madison.

Lam, H. Y., Schnoes, H. K., DeLuca, H. F., and Chen, T. C. (1973), *Biochemistry 12*, 4851.

Lawson, D. E. M., Wilson, P. W., Barker, D. C., and Kodicek, E. (1969), *Biochem. J. 115*, 263.

Lehninger, A. L. (1951), *J. Biol. Chem. 190*, 345.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Omdahl, J. L., and DeLuca, H. F. (1972), *J. Biol. Chem. 247*, 5520.

Omdahl, J. L., Gray, R. W., Boyle, I. T., Knutson, J., and DeLuca, H. F. (1972), *Nature (London), New Biol. 237*, 63.

Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.

Simpson, E. R., and Estabrook, R. W. (1969), *Arch. Biochem. Biophys. 129*, 384.

Slater, E. C. (1967), *Methods Enzymol. 10*, 48.

Suda, T., DeLuca, H. F., and Hallick, R. B. (1971), *Anal. Biochem. 43*, 139.

Suda, T., DeLuca, H. F., Schnoes, H. K., Ponchon, G., Tanaka, Y., and Holick, M. F. (1970), *Biochemistry 9*, 2917.

Tanaka, Y., and DeLuca, H. F. (1973), *Arch. Biochem. Biophys. 154*, 566.