Kinetics of Liver Microsomal Cholecalciferol 25-Hydroxylase in Vitamin D-Depleted and -Repleted Rats

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(Received 31 August 1977)

Kinetics of vitamin D-depleted and -repleted rat liver microsomal cholecalciferol 25-hydroxylase were studied. Anaerobiosis, CO, omission of a NADPH-generating system and addition of detergents all decreased the activities, showing that the hydroxylase behaves like a cytochrome P-450-dependent enzyme. An apparent K_m of $0.18 \mu\text{M}$ and V_{max} of 32 pmol/min per g of tissue were found for vitamin D-deficient animals. Although both apparent K_m and V_{max} were significantly altered in vitamin D-repleted animals no inhibition of the enzyme was elicited. These latter results show that at normal vitamin D intake, rat liver cholecalciferol 25-hydroxylase is not feedback-inhibited.

The predominant role of vertebrate liver in the conversion, *in vivo* and *in vitro*, of cholecalciferol (vitamin D₃) into 25-hydroxycholecalciferol has been well established (Ponchon & DeLuca, 1969; Ponchon *et al.*, 1969; Horsting & DeLuca, 1969; Tucker *et al.*, 1973). Although chick kidney and intestine were also shown to convert cholecalciferol into 25-hydroxycholecalciferol *in vitro* (Tucker *et al.*, 1973), such hydroxylating systems could not be elicited under normal intake of vitamin D₃ (DeLuca & Schnoes, 1976). Cholecalciferol 25-hydroxylase, initially thought to be a mitochondrial enzyme (DeLuca, 1971a), was later mainly located in the microsomal fraction (Bhattacharyya & DeLuca, 1974a). Like the kidney mitochondrial 25-hydroxycholecalciferol 1 α -hydroxylase (Colston *et al.*, 1973) and the liver cholic acid hydroxylase (Shefer *et al.*, 1976), it requires the cytosolic fraction to show full catalytic activity (Bhattacharyya & DeLuca, 1974a). It shares with the mixed-function oxidases the requirements for an NADPH-generating system and molecular O₂ (Bhattacharyya & DeLuca, 1974b). However, unlike these, it is not inhibited by CO, nor induced by anti-convulsants (DeLuca & Schnoes, 1976) and is claimed to be subject to feedback inhibition by products (Horsting & DeLuca, 1970; Bhattacharyya & DeLuca, 1973, 1974b).

To characterize this enzyme and thus to help to show whether or not it is a mixed-function oxidase, we have, by using rat liver as the source of enzyme, studied some of its kinetic characteristics.

Materials and Methods

Reagents

NADPH (sodium salt), glucose 6-phosphate (sodium salt), glucose 6-phosphate dehydrogenase

(EC 1.1.1.49; type VII), cholecalciferol, Triton X-100 and Tween 80 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Brij-35 was obtained from Hyland Laboratories (Costa Mesa, CA, U.S.A.). [$1\alpha,2\alpha(n)^3\text{H}$]Cholecalciferol (sp. radioactivity 12 Ci/mmol) and 25-hydroxy[26,27- $\text{Me-}^3\text{H}_2$]cholecalciferol (sp. radioactivity 12 Ci/mmol), purchased from Amersham-Searle (Oakville, Ont., Canada), were periodically tested for their purity by high-pressure liquid chromatography by the method developed by Jones & DeLuca (1975). *NN'*-Diphenyl-*p*-phenylenediamine, a lipid-peroxidation inhibitor (Hochstein & Ernster, 1963), was from Eastman Kodak Co. (Rochester, NY, U.S.A.). Silicic acid, obtained from Bio-Rad Laboratories (Mississauga, Ont., Canada), was activated at 110°C for at least 1 h before use. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals (Montreal, Que., Canada). All solvents used for chromatography were of spectral grade. All other reagents were obtained from Fisher Scientific (Montreal, Que., Canada).

Enzyme preparation

Male weanling Holtzman rats (Canadian Breeding Farms, St. Constant, Que., Canada) were fed on either a normal (Ralston-Purina Canada, Woodstock, Ont., Canada) or a vitamin D-deficient diet (no. 170640; Teklad Test Diets, Madison, WI, U.S.A.) for 4 weeks. They were killed by decapitation after starvation overnight. The livers of the vitamin D-deficient animals were removed at once, blotted, weighed and homogenized in a Potter-Elvehjem homogenizer fitted with a Teflon pestle with ice-cold 0.25 M-sucrose containing 1 mM- NaH_2PO_4 buffer adjusted to pH 7.4 with 1 M-NaOH. The livers of the control animals were perfused with the cold buffered sucrose described above before the homogenization.

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The nuclei and cell debris were sedimented at an integrated field-time of 8300g-min at r_{av} 7.3 cm in the SS-34 rotor of a Sorvall RC-2B centrifuge equilibrated at 4°C. The supernatant (10ml/g of liver) was centrifuged at an integrated field-time of 196200g-min in the apparatus described above and the supernatant, consisting of the microsomal and cytosol fractions, was retained for the cholecalciferol 25-hydroxylase assays.

Enzyme assay

The cholecalciferol 25-hydroxylase assay was modified from that of Bhattacharyya & DeLuca (1974b). Unless otherwise specified, a 2ml sample of the 10-fold diluted supernatant was added to a 25ml Erlenmeyer flask containing 2ml of a cofactor solution consisting of: 0.025M-KH₂PO₄ adjusted to pH 7.4 with 1M-NaOH, 0.25mM-NADPH, 1.25mM-MgCl₂, 0.025M-KCl, 6.0mM-glucose 6-phosphate, 6 units of glucose 6-phosphate dehydrogenase (where 1 unit of activity is defined as the amount of enzyme required to convert 1 μ mol of substrate/min) and 0.01mM-*NN'*-diphenyl-*p*-phenylenediamine in 10 μ l of ethanol. The flasks were equilibrated at 37°C under a constant stream of O₂ for 5 min. The reaction was initiated by the addition of cholecalciferol (0.02–0.5 μ M) and [1 α ,2 α (n)-³H]cholecalciferol (10⁵ d.p.m.) dissolved in 20 μ l of propylene glycol. The flasks were oxygenated for the entire period of incubation. The reaction was terminated by quantitatively transferring the content of the flasks to screw-cap tubes immersed in an ice bath and containing 5ml of diethyl ether. Three extractions, each with 5ml of diethyl ether, were performed. The combined extracts were evaporated under a stream of N₂.

The separation of cholecalciferol, 25-hydroxycholecalciferol and more polar metabolites was performed on silicic acid columns as follows. Activated silicic acid, thoroughly equilibrated with 60% (v/v) diethyl ether in hexane, was poured to a height of 4cm in transfer pipettes plugged with cottonwool. The extracts in 60% (v/v) diethyl ether in hexane (1ml) were applied to the column and cholecalciferol was eluted with 4ml of the same solvent; 25-hydroxycholecalciferol was eluted with 6ml of diethyl ether and more-polar metabolites were eluted from the columns with methanol. The fractions were evaporated under N₂, redissolved in ethanol and counted for radioactivity in a Packard Tri-Carb 3385 liquid-scintillation spectrometer with Aquasol [New England Nuclear (Canada), Montreal, Que., Canada] as scintillation fluid.

The units of activity reported are, unless otherwise specified, pmol of 25-hydroxycholecalciferol/min per g wet wt. of tissue. The supernatant from the same individual liver provided the enzyme for each set of experiments.

Results

The elution profile obtained from the lipid extracts of the incubation mixture with the chromatographic procedure described in the Materials and Methods section is illustrated in Fig. 1. A clear separation of 25-hydroxycholecalciferol from cholecalciferol and from more-polar metabolites was obtained. The identity of the radioactivity peak, attributed to 25-hydroxycholecalciferol, was confirmed by comparing its migration characteristics with that of crystalline reference 25-hydroxycholecalciferol on Sephadex LH-20 by the method of Holick & DeLuca (1971) and by high-pressure liquid chromatography on silicic acid columns (10 μ m/particle) essentially by the method reported by Jones & DeLuca (1975).

Under the conditions described, the production of 25-hydroxycholecalciferol was directly proportional to the time up to 90 min at both 0.02 μ M- (Fig. 3) and 0.5 μ M-cholecalciferol, whereas no activity could be detected when a sample of boiled-enzyme preparation was incubated for the same length of time. The pH-

Fig. 1. Elution profile obtained from chromatography of lipid extracts of the incubation mixture

Chromatography of part of the extract from an incubation procedure described (continuous line) in the Materials and Methods section (concentration of cholecalciferol, 0.02 μ M) was carried out on a silicic acid column (0.5 cm × 4 cm). The broken line represents the elution position of reference [1 α ,2 α (n)-³H]cholecalciferol (A) and 25-hydroxy [26,27-Me-³H₂]cholecalciferol (B). The composition of eluent was: 1, 60% ether/hexane; 2, ether; 3, methanol. Fractions of 1ml volume each were collected.

Fig. 2. pH-activity curve of rat liver microsomal cholecalciferol 25-hydroxylase

The buffer consisted of 0.025M-KH₂PO₄ adjusted at different pH with 1M-NaOH. Incubations were carried out as described in the Materials and Methods section with 0.02 μM-cholecalciferol. Activity is expressed as c.p.m. of 25-hydroxycholecalciferol (25-OHD₃).

Fig. 3. Cholecalciferol 25-hydroxylase activity as a function of time

Incubations were carried out as described in the Materials and Methods section with 0.02 μM-cholecalciferol. Boiled enzyme (1 min at 100°C) was incubated for the same time period to serve as a control 'blank'. Results are expressed as pmol of 25-hydroxycholecalciferol produced per assay.

activity curve revealed a wide optimum between pH 7.0 and 7.5 (Fig. 2) with no pH change occurring during the incubation. Therefore we chose pH 7.4 for the assay, as did Tucker *et al.* (1973). Although Bhattacharyya & DeLuca (1973) reported a slightly

Fig. 4. Cholecalciferol 25-hydroxylase activity as a function of supernatant concentration

The incubations were carried out as described in the Materials and Methods section. The supernatant, originally 10% (w/v) (see the text), was used concentrated or was diluted with buffered 0.25M-sucrose to give a concentration in the incubation medium of 5.0, 2.5, 1.25, 0.75, 0.50 and 0.25%. Activity is expressed as pmol of 25-hydroxycholecalciferol produced in 90 min at the different contents of enzyme.

more acidic pH optimum for the reaction, they subsequently measured their activity at a pH similar to ours (Bhattacharyya & DeLuca, 1974a). The production of 25-hydroxycholecalciferol was directly proportional to the concentration of enzyme up to 0.5% (w/v) final supernatant concentration but decreased appreciably thereafter (Fig. 4). Tucker *et al.* (1973) also observed, although to a lesser extent, the same phenomenon for chick liver cholecalciferol 25-hydroxylase activity. Since a coupled enzyme system is used for the assay, our results could be interpreted as an inadequate regeneration of NADPH at high enzyme concentration, thereby lowering the active cofactor concentration and hence the hydroxylase activity.

Interference with the cytochrome reductase pathway by omission of NADPH or of a NADPH-generating system, or by anaerobiosis, inhibits the enzyme activity considerably (Table 1). Furthermore, at variance with the claims of DeLuca & Schnoes (1976), CO, known to combine with cytochrome P-450 (Conney *et al.*, 1957), also inhibits the activity by nearly 80% (Table 1). Triton N-101 and X-100 have been shown to convert cytochrome P-450 into inactive cytochrome P-420 and to decrease drug hydroxylation in direct proportion to the amount of cytochrome P-420 produced (Gillette, 1966; Silverman & Talalay, 1967). Addition of such surfactants as Triton X-100, Brij-35 or Tween 80 to the incubation medium strongly inhibited cholecalciferol 25-hydroxylase activity in proportion to their concentration (Table 2). Finally, treatment of the micro-

Table 1. *Effect of N₂, CO and omission of cofactors on cholecalciferol 25-hydroxylase activity*

Values for hydroxylase activity are means of duplicate experiments.

Incubation condition	Hydroxylase activity (pmol/min per g of tissue)	Hydroxylase activity (% of control)
Control	31.3	100
+N ₂ *	7.8	25
+CO*	5.9	19
-NADPH	21.6	69
-Glucose 6-phosphate,	4.0	13
-glucose 6-phosphate dehydrogenase		
-NADPH, -glucose	2.8	9
6-phosphate, -glucose 6-phosphate dehydrogenase		

* A stream of CO or N₂ was bubbled for 1 min on the surface of the reaction mixture described in the Materials and Methods section. The Erlenmeyer flasks were then capped during the incubation period. The concentration of cholecalciferol was 0.02 μ M.

Table 2. *Effects of surfactants on microsomal cholecalciferol 25-hydroxylase activity*

These experiments were done essentially as described in the Materials and Methods section except that cholecalciferol was dissolved in the different surfactants. Owing to its different solubility in each of them, concentration of substrate varied. In the control experiment, the substrate concentration was adjusted accordingly. The cholecalciferol concentrations for each detergent were: Brij-35, 0.01 μ M; Triton X-100, 0.02 μ M; Tween 80, 0.05 μ M. Values for hydroxylase activity are means of duplicate experiments.

Detergent	Final detergent concentration (% w/v)	Hydroxylase activity (% of control)
Brij-35	0.15	1.5
	0.015	9.0
Triton X-100	0.15	0.5
	0.015	5.0
Tween 80	0.15	5.0

somal fraction with 0.2 μ M-*p*-cumenyl hydroperoxide, a scavenger of cytochromes similar to linoleoyl hydroperoxide (Jeffrey *et al.*, 1977), completely abolished the activity (unpublished results). The data thus suggest the association of the hydroxylase with a microsomal membrane-bound system and possibly with the cytochromes.

The double-reciprocal plot (Lineweaver & Burk, 1934) reveals an apparent Michaelis constant (K_m) of 0.18 μ M (Fig. 5) and a maximum velocity (V_{max}) of 32 pmol/min per g of tissue for the hydroxylase from the vitamin D-depleted animals. We observed

Fig. 5. *Double-reciprocal plot of activity as a function of substrate concentration for the vitamin D-depleted rats*

The incubations were carried out as described in the text with the cholecalciferol concentration varying from 0.02 μ M to 0.5 μ M. The amount of enzyme used was equivalent to 20 mg of liver. The regression line was obtained from the pooled data of seven animals, each assay at every substrate concentration being carried out in triplicate. The vertical bars represent $\pm$ S.D. from the mean.

monophasic kinetics and hence our results differ from those of Suda *et al.* (1977) who by liver perfusion obtained biphasic kinetic data with K_m values lying on either side of our value by approximately one order of magnitude.

The apparent K_m and V_{max} from vitamin D-depleted and -repleted animals are compared in Table 3. These results show a significant modification of those constants in the latter group. To avoid the possibility of having vitamin D or 25-hydroxycalciferol present in the liver homogenates, the livers of the vitamin D-repleted rats were thoroughly perfused with cold buffered sucrose. Hence the risks of isotopic dilution of the substrate or of interference by 25-hydroxycalciferol were diminished. In our experimental conditions, the size of the substrate pool *in vivo*, expressed as the serum concentration of 25-hydroxycalciferol (Table 3), affected the kinetics, without, however, showing a feedback inhibition of the hydroxylase as advocated by Horsting & DeLuca (1970) and Bhattacharyya & DeLuca (1973, 1974b).

Discussion

The data in the present paper show that the rat liver microsomal fraction hydroxylates cholecalciferol to 25-hydroxycalciferol, thus corroborating the results of Bhattacharyya & DeLuca (1974a). The requirements of NADPH, molecular O₂ and

Table 3. *Michaelis constant (K_m), maximum velocity (V_{max}) and serum 25-hydroxycholecalciferol concentrations in vitamin D-depleted (D^-) and -repleted (D^+) rats*

The incubations were carried out as described in the Methods section. The K_m and V_{max} were derived from the regression line of the double-reciprocal plot (Lineweaver & Burk, 1934) for each separate experiment. The means ($\pm$ S.D.) for the K_m and V_{max} were then calculated from each value obtained. 25-Hydroxycalciferol was measured by the method of Haddad & Chyu (1971). 25-Hydroxycalciferol refers to the sum of 25-hydroxycholecalciferol and 25-hydroxyergocalciferol concentrations since both have the same binding constants for the binding protein. The significance levels for the difference in means (P values) were calculated by Student's t test for independent observations with 9 degrees of freedom. N.D., not detected.

	D^-	D^+	P
K_m (μM)	0.18 ± 0.07 ($n = 7$)	0.44 ± 0.09 ($n = 4$)	< 0.05
V_{max} . (pmol of 25-hydroxycholecalciferol/min per g of tissue)	32 ± 12 ($n = 7$)	103 ± 6 ($n = 4$)	< 0.01
25-Hydroxycalciferol (pmol/ml)	N.D.	52 ± 5	—

integrity of the microsomal membranes and of cytochrome *P*-450 suggest that the enzyme is a hydroxylase. The clear inhibition by CO, reported here, is in contradiction with the data published by DeLuca (1971b). It should be noted, however, that a mitochondrial fraction was used in the latter study, whereas we worked solely with the microsomal and cytosolic fractions.

Whether cholecalciferol 25-hydroxylase is a mixed-function oxidase remains unresolved. Delvin *et al.* (1977) have shown that rat liver hydroxylase is not induced by short-term treatment with phenobarbital. Moreover, Sulimovici & Roginsky (1977) have shown cholecalciferol 25-hydroxylase inhibition by phenobarbital and diphenylhydantoin, when administered for longer periods of time. On the other hand, Hahn *et al.* (1972) have suggested that the low concentration of circulating 25-hydroxycalciferol in patients treated with anti-convulsant drugs is the consequence of hepatic enzyme induction diverting the vitamin D metabolism towards the production of more-polar biologically inactive metabolites.

Our data showing classical monophasic Michaelis-Menten kinetics are at variance with those of Suda *et al.* (1977) who proposed two modes of cholecalciferol hydroxylation in liver. Their protocol, however, differed markedly from ours since they were working with a dynamic system (isolated perfused liver) in which such variables as the rate of substrate cellular uptake and product efflux have not been considered, prohibiting close comparison of the data. Our results also show that vitamin D repletion decreases the affinity of the enzyme for cholecalciferol (higher K_m). This finding may explain the apparent product feedback inhibition observed in vitamin D-repleted rats (Bhattacharyya & DeLuca, 1973) and chicks (Bhattacharyya & DeLuca, 1974b). The latter workers measured cholecalciferol 25-hydroxylase at a single substrate concentration (approximately 30 nM) well below the apparent Michaelis constant of the enzyme. In these conditions and from our results

it would be expected that cholecalciferol would be less readily transformed into 25-hydroxycholecalciferol in the vitamin D-repleted animals, with no need to invoke a feedback inhibition. This hypothesis is supported by the report of Tucker *et al.* (1973), who did not elicit inhibition of the hydroxylase in vitamin D-repleted cockerels with 10-fold higher substrate concentration.

Further characterization of the enzyme complex is needed to show whether or not cholecalciferol 25-hydroxylase is a mixed-function oxidase and to further understand cholecalciferol hepatic hydroxylation mechanisms.

E. E. D. is a recipient of an Establishment Grant of the Conseil de la Recherche en Santé du Québec. F. H. G. is a scientist of the Queen Elizabeth II Research Fund to aid in research on the diseases of children. This work was supported by the Shriners of North America. We are grateful to Mrs. D. Bissonnette for her expert secretarial assistance with the manuscript.

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