The Regulation of Rat Liver Calciferol-25-hydroxylase*

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SUMMARY

Rats treated with vitamin D₃ show decreased levels of liver calciferol-25-hydroxylase activity. The extent of the depression and its persistence are related to the dose of vitamin D₃. The decrease in enzyme activity measured in vitro cannot be accounted for by dilution of the labeled substrate by unlabeled vitamin D₃ remaining in the liver. The in vitro decrease is reflected in vivo by a decrease in the rate and extent of appearance of [³H]-labeled 25-hydroxyvitamin D₃ in the blood and liver following a dose of [³H]-labeled vitamin D₃ administered to rats pretreated with unlabeled vitamin D₃. These data show the existence of a mechanism which regulates the activity of the rat liver calciferol-25-hydroxylase.

In recent years it has become clear that the metabolism of vitamin D to more polar biologically active metabolites is an important aspect of the vitamin's function. In 1966 Lund and DeLuca (1) demonstrated the existence of biologically active polar metabolites of vitamin D₂ (D₂), one of which proved to be the major metabolite circulating in blood. Blunt et al. (2) identified this metabolite as 25-hydroxyvitamin D₁ (25-OH-D₁). Subsequent work (3) established not only its potent antirachitic activity but also its ability to stimulate bone calcium mobilization and intestinal calcium transport more rapidly than vitamin D₁. The difference in time of response to the two compounds was considered to be the time needed for conversion of vitamin D₁ to 25-OH-D₁.

In an effort to determine the location of the calciferol-25-hydroxylase, Ponchon et al. (4) studied the metabolism of [¹²⁵I]-labeled vitamin D₃ (D₃), a major, if not the only, site for the 25-hydroxylation of vitamin D₃ in the rat.

In this report evidence for the existence of a mechanism responsible for regulating the activity of rat liver calciferol-25-hydroxylase will be presented.

MATERIALS AND METHODS

Animals—Male albino rats (Holtzman Co., Madison, Wis.) were maintained in hanging wire cages and fed ad libitum a low vitamin D diet of Steenbock (7), modified by using skim milk powder and butter instead of whole milk. Rats weighing 200 to 250 g were used for each experiment.

Vitamin D₃ Compounds—The [¹²⁵I]-labeled vitamin D₃ used (spec. act. 1500 or 1262 dpm per pmole) was prepared in our laboratory according to the method of Neville and DeLuca (8). Crystalline vitamin D₃ and 25-OH-D₃ were gifts of the Philips-Duphar Co. of Weesp, The Netherlands.

Rat Liver Homogenate Incubations—The rat livers were removed and chilled immediately in ice-cold 0.25 M sucrose. A 25% homogenate in 0.25 M sucrose was used.

The homogenate was homogenized in a Potter-Elvehjem homogenizer. To a 5-ml aliquot of this homogenate (200 to 250 mg of liver), 5 ml of buffer-cofactor solution (0.1 M potassium phosphate, 0.4 mM TPN, 160 mM nicotinamide, 20 mM ATP, 22.4 mM glucose adjusted to pH 7.4) was added, and then with 2.5 ml of salt solution (5 mM MgCl₂, 0.1 M KCl) to give a final incubation volume of 10 ml. Incubations were carried out for 2 hours at 37° with 120 oscillations per min at which time the reactions were terminated by addition of 25 ml of MeOH plus 12.5 ml of CHCl₃.

Extraction Procedure—Extractions were carried out essentially according to the method of Bligh and Dyer (9). After the reactions were terminated, the resulting single phase was allowed to extract overnight at 4°. The extracts were filtered into separatory funnels to remove precipitated protein. The incubation flasks and precipitated protein were rinsed with 2.5 ml of buffer-cofactor solution (0.1 M K₂HPO₄, 0.4 mM TPN, 160 mM nicotinamide, 20 mM ATP, 22.4 mM glucose adjusted to pH 7.4), and then with 2.5 ml of salt solution (5 mM MgCl₂, 0.1 M KCl) to give a final incubation volume of 10 ml. Incubations were carried out for 2 hours at 37° with 120 oscillations per min at which time the reactions were terminated by addition of 25 ml of MeOH plus 12.5 ml of CHCl₃.

The abbreviations used are: D₁, vitamin D₁; 25-OH-D₁, 25-hydroxyvitamin D₁; D₂, vitamin D₂; 25-OH-D₂, 25-hydroxyvitamin D₂; D₃, vitamin D₃; 25-OH-D₃, 25-hydroxyvitamin D₃.
diluted to 50 ml with 100% EtOH and aliquots were removed for determination of total radioactivity. The remainder was evaporated to dryness with a flash evaporator and the residue dissolved in 1 ml of 50% CHCl₃ in Skellysolve B for application to columns. Liver and serum samples collected from in vivo studies with [³H]-vitamin D₃ were extracted in an analogous manner. Twenty-five per cent liver homogenates were prepared with 0.25 M sucrose and then extracted. Serum samples were brought to 10 ml with distilled water and extracted. In this case, samples were not filtered and 10 ml of saturated NaCl were used instead of water to prevent emulsification of serum extracts.

Column Chromatography—All lipid extracts were applied to columns (2 x 30 cm) containing 20 g of Sephadex LH-20 equilibrated with 50% CHCl₃ in Skellysolve B (10). The samples were eluted with the same solvent. A maximum of six such columns were run simultaneously on a time-operated fraction collector. The flow rate for each column was adjusted to deliver exactly 2.5- to 3.0-ml fractions every 2 min. Forty such fractions were collected directly in 5-ml counting vials. The solvents were evaporated under a stream of air, and the resulting residues were redissolved in 4 ml of toluene counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazoly)]benzene per liter of toluene). The [³H] radioactivity in the vials was determined with a Packard Tri-Carb model 3375 liquid scintillation counter. After each group of determinations the columns were stripped with 200 ml of 65% CHCl₃ in Skellysolve B. They were regenerated by washing with 50 ml of 50% CHCl₃ in Skellysolve B before applying a new sample.

Silicic acid column chromatography was carried out as described by Ponchon and DeLuca (11) while liquid-liquid partition chromatography followed the procedure of Blunt et al. (2).

Calciferol-25-hydroxylase Assay—The basic properties of the rat liver homogenate calciferol-25-hydroxylase system were studied in order to set up an assay for the enzyme. That the product produced is 25-OH-D₃ was established by co-chromatography of the peak of radioactivity produced on incubation of vitamin D₃ with rat liver homogenates together with synthetic 25-OH-D₃, in three different chromatographic systems (Fig. 1). Two of these chromatographic procedures were used to isolate 1.3 mg of 25-OH-D₃ in pure form (2), while the relatively new Sephadex LH-20 method has potent resolving powers (10).

Maximum 25-hydroxylation was shown to require the presence of added TPN and ATP (Fig. 2). The tissue concentration used for routine work (1.25 g of liver per 10 ml of reaction mixture) was chosen since it is within the range which shows a linear relationship between enzyme activity and tissue concentration. The pH maximum was determined to be 6.9, using 50 mM phosphate buffer to maintain the pH. Since the reaction using 25 mM phosphate buffer as already described (pHₘₐₓ = 7.2, pHₘₐₓ = 6.8) proceeds slightly better than the one with 50 mM phosphate buffer, pH 8.8, 25 mM phosphate buffer was used routinely. The substrate concentration chosen gives a measure of initial reaction velocity at 2 hours even in the most active preparations (5).

RESULTS

In vitro Calciferol-25-hydroxylase Activity Decrease in Response to Vitamin D₃ Administration—Rats were injected with 0.65 nmole (10 i.u.) of unlabeled vitamin D₃ intrajugularly in 50 µl of 95% EtOH while control rats received the EtOH vehicle. Ten hours later the animals were killed and their livers assayed for 25-hydroxylase activity. The 10-hour period was chosen as a time when the unlabeled vitamin D₃ remaining in the liver was sufficiently low so as not to dilute the added labeled substrate.
injected with 0.65 nmole of unlabeled vitamin D₃ as described in the text. Ten hours later they were killed and their livers were assayed for calciferol-25-hydroxylase activity. The column profile on the left is from a control rat. The profile on the right is from a vitamin D-treated rat.

The rats were injected intrajugularly with 0.33, 0.65, or 1.3 nmoles (5, 10, or 20 i.u.) of unlabeled vitamin D₃ in 50 μl of 95% EtOH and killed at various times after dosing. Calciferol-25-hydroxylase activities were determined in homogenates of their livers as described in the text. Averages of the activities from two rats at each time point are reported, with the ends of the bars indicating the two actual values obtained: (---) 0.33-nmole dose; (-----) 0.65-nmole dose; (------) 1.3-nmole dose.

As shown in Fig. 3, animals which had received a prior dose of vitamin D₃ had very little in vitro 25-hydroxylase activity.

The change in enzyme activity as a function of time following three dose levels of vitamin D₃ is shown in Fig. 4. The 25-hydroxylase activity at each dose level fell rapidly from 15 min to 1 hour after administration of the vitamin and then remained low. The extent of the decrease depended upon the amount of vitamin D₃ administered, and was greatest for the highest dose level. Looking at the recovery phase, animals which had received 1.3 nmoles (20 i.u.) of vitamin D₃ took several days longer to return to control enzyme levels than did animals which had received only 0.65 nmole (10 i.u.).

In a parallel experiment, vitamin D₃ and 25-OH-D₃ levels were determined in the livers of the animals whose in vitro 25-hydroxylase activities had been previously assayed. To do this rats were injected with 0.65 nmole of [{}^{3}H]D₃ intrajugularly in 50 μl of 95% EtOH and were killed at various times after dosing. Lipid extracts of their livers were chromatographed on Sephadex LH-20 columns. From the data in Table I, it is clear that simple dilution of the labeled vitamin D₃ added to the assay mixture by the unlabeled vitamin D₃ present in the liver tissue could not account for the enzyme activity decrease observed in response to a 0.65-nmole dose of vitamin D₃. At the 15-min time point, only 0.045 nmole of unlabeled vitamin D₃ would be present in 1.25 g of liver (the amount of tissue in each incubation vessel), while 0.26 nmole of [{}^{3}H]D₃ substrate was added to each assay flask. Furthermore, 15 min after dosing there was much more vitamin D₃ in the liver tissue than at 10 hours. Yet there was more in vitro 25-hydroxylase activity at 15 min than at 10 hours.

In one further experiment performed to rule out dilution of substrate radioactivity as an explanation for the in vitro 25-hydroxylase activity decrease, rats were injected with 0.65 nmole of [{}^{3}H]D₃ rather than unlabeled vitamin D₃. Twelve hours later, 25% homogenates of their livers were prepared. Five milliliters of homogenate were extracted immediately to determine initial [{}^{3}H]D₃ and 25-OH-[{}^{3}H]D₃ levels. Five milliliters of homogenate were then incubated with the same specific activity as the [{}^{3}H]D₃ predose. Control liver homogenate incubations were prepared from untreated animals and from animals treated with 0.65 nmole of unlabeled vitamin D₃ 12 hours prior to killing. As shown in Table II, liver homogenates from [{}^{3}H]D₃-treated rats showed the same decrease in 25-hydroxylase activity as did the liver homogenates of animals treated with unlabeled vitamin D₃.

**Table I**

| Time after dosing | [{}^{3}H]D₃ | 25-OH-[{}^{3}H]D₃ |
|-------------------|------------|-----------------|
| 15 min            | 41.1 (35.8) | 0.72 (0.70)     |
| 1 hr              | 28.7 (29.6) | 1.1 (1.2)       |
| 3 hrs             | 18.7 (18.6) | 1.1 (1.2)       |
| 5 hrs             | 10.3 (10.2) | 0.79 (0.77)     |
| 10 hrs            | 6.7 (7.5)   | 0.44 (0.46)     |

Decrease in 25-OH-D₃ Production in Vivo in Response to Prior Vitamin D₃ Administration—Since in vitro calciferol-25-hydroxylase activity decreases in response to a dose of vitamin D₃, an effort was made to determine whether a corresponding decrease in calciferol-25-hydroxylation could be observed in vivo. Rats were injected with 0.65 nmole of unlabeled vitamin D₃ intrajugularly. Ten hours later when both in vitro enzyme activity and in vivo pool sizes of unlabeled vitamin D₃ were low (see Fig. 4 and Table I), the rats were injected with a second 0.65-nmole dose of [{}^{3}H]D₃. Control animals received only the [{}^{3}H]D₃ dose. The animals were killed at various times following the [{}^{3}H]-labeled vitamin D₃ dose. Lipid extracts of the sera and livers were chromatographed on Sephadex LH-20 columns to determine metabolite levels. Indeed, the rate and extent of appearance of 25-OH-[{}^{3}H]D₃ in the blood and liver of animals
pretreated with 0.65 nmole of vitamin D₃ were considerably lower than in the case of the untreated animals (Fig. 5). Yet from Fig. 6 it is apparent that the predosed animals still showed a rapid uptake of [³H]D₃ into their livers.

By adding together the amounts of vitamin D₃ and 25-OH-D₃ known to be present in the liver due to the 10-hour predose and the amounts of [³H]-metabolites present in the liver 15 min after the dose of [³H]D₃, the dilution of [³H]D₃ in the predosed animals was estimated. As shown in Fig. 7, dilution of the label was small and the metabolite levels alone compared with those of the control animals (no vitamin D₃ predose) could not explain the reduced rate of 25-OH-D₃ production observed in the pretreated animals.

### TABLE II

**Effect of [³H]D₃ administration on in vitro calciferol-25-hydroxylase activity**

| Pretreatment | Before incubation (A) | After incubation with added substrate (B) | 25-OH [³H]D₃ (B-A) |
|--------------|------------------------|------------------------------------------|-------------------|
| [³H]-Vitamin D₃ | 0.60 (0.68) | 2.02 (1.57) | 1.42 (0.90) |
| Unlabeled D₃ | 0.55 | 1.12 | 0.57 |
| EtOH vehicle only | 0.63 (1.13) | 1.63 (1.13) | 0.63 |

*No significant 25-hydroxylation occurred during incubation without added substrate.*

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**DISCUSSION**

This report shows the existence of a mechanism which regulates the level of liver calciferol-25-hydroxylase activity according to the amount of vitamin D₃ presented to the rat. After a rat receives a dose of vitamin D₃, the calciferol-25-hydroxylase activity as measured in vitro quickly falls (Fig. 4). The enzyme activity slowly returns to initially observed levels, with the amount of time required for return to control levels increasing as the dose of vitamin D₃ increases. Since the extent to which the 25-hydroxylase activity falls also increases as the dose increases, possibly this mechanism operates in vivo to maintain a low level of 25-OH-D₃ in the blood even when large amounts of vitamin D are being ingested. Certainly the daily requirement for vitamin D₃ is many times less than the amount required to produce toxicity symptoms.

The decrease in calciferol-25-hydroxylase activity observed in vitro cannot be explained by dilution of labeled substrate used in the assay system by unlabeled vitamin D₃ present in the rat liver tissue, since that amount is too small to significantly dilute the [³H]D₃ added to the assay mixture (Table I).

Also, when dilution of substrate radioactivity is eliminated by predosing animals with [³H]D₃ instead of unlabeled vitamin D₃, the same decrease in calciferol-25-hydroxylase activity is observed (Table II).

A decrease in the rate and extent of appearance of 25-OH-D₃...
in the liver and blood of vitamin D-treated rats compared to control rats (Fig. 5) indicates that the in vitro decrease in calciferol-25-hydroxylase activity has significance in vivo. The liver vitamin D3 and 25-OH-D3 levels of the treated and control rats 15 min after injection of 0.65 nmole of [3H]D3 are very comparable (Fig. 7), and yet the treated animals do not put 25-OH-D3 into the blood at the rate of control animals.

Recent attempts to bring about a decrease in in vivo 25-OH-D3 production and in vitro 25-hydroxylase activity by pretreatment of rats with unlabeled 25-OH-D3 have shown that calciferol-25-hydroxylase activity is not sensitive to 25-OH-D3 injected into the blood stream of the rat. These data indicate that metabolites of 25-OH-D3 are not responsible for the decrease in the enzyme activity.

It is interesting to follow the 25-OH-D3 levels in the liver (Table I) and blood (Fig. 5) of rats which have received a single intrajugular injection of 0.65 nmole of [3H]D3. Upon injection, vitamin D3 is very quickly taken up from the blood by the liver. The 25-OH-D3 formed is immediately released into the blood; it does not accumulate in the liver, but remains at very low concentrations there, while the 25 O H D3 concentration in the blood increases to a plateau level. Thus at 15 min after dosing, the 25-OH-D3 level in the liver is if anything higher than the 25-OH-D3 level measured 10 hours after dosing (Table I). Yet the calciferol-25-hydroxylase activity of the liver 10 hours after dosing is much less than that measured 15 min after dosing. These data imply that product inhibition cannot explain the decrease in enzyme activity observed in response to a dose of vitamin D3.

Attempts are currently being made to determine the mechanism whereby pretreatment of rats with vitamin D3 decreases the calciferol-25-hydroxylase activity. Meanwhile, this phenomenon must be taken into account by investigators attempting to measure the vitamin D 25-hydroxylase of the liver.

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