THE REGULATION OF CALCIFEROL-25-HYDROXYLASE IN THE CHICK[+]

by

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The administration of 1.3 (20 IU) and 3.25 (50 IU) nmoles but not 0.65 nmoles of ^{3}H-vitamin D_3 intravenously results in a marked diminution of hepatic vitamin D_3-25-hydroxylase activity in vitamin D-deficient chicks within 24 hours. A similar reduction in *in vivo* production of 25-hydroxyvitamin D_3 was observed following the 1.3 and 3.25 nmole but not the 0.65 nmole dose of vitamin D_3. These changes in *in vivo* and *in vitro* vitamin D_3 hydroxylation could not be due to dilution of the ^{3}H-vitamin D_3 substrate and thus represents regulation of the hepatic vitamin D_3-25-hydroxylase of the chick as has been observed in the rat. Regulation in the chick requires a somewhat higher dose of vitamin D_3 than is the case in the rat, however.

Vitamin D_3-25-hydroxylase in the rat is regulated in such a way that rats administered vitamin D_3 show a dramatic decrease in liver vitamin D_3-25-hydroxylase activity as early as 15 minutes following vitamin D_3 administration (1). The 25-hydroxylase activity then remains low for a period of time which increases as the dose of vitamin D_3 increases (1). Following the administration of 10 IU (0.65 nmoles) of vitamin D_3, *in vitro* vitamin D_3-25-hydroxylase activity remains low for 24 hours and begins to approach original deficiency levels by 48 hours.

Stimulus for the present investigation came from the observations of Tucker *et al.* (2) which demonstrated that liver from chicks given 10 IU (0.65 nmoles) of vitamin D_3 per day for 14 days showed the same *in vitro* vitamin D_3-25-hydroxylase activity as did those from vitamin D-deficient chicks.

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These results seemed to contradict our previous results in the rat. The aim of the present ivestigation was to determine whether vitamin D_3-25-hydroxylation activity of chick liver is reduced following vitamin D_3 administration. The results demonstrate that the chick system is also sensitive to vitamin D_3 administration but larger amounts are required.

MATERIALS AND METHODS

Animals. White Leghorn cockeral chickens were obtained from Northern Hatcheries of Beaver Dam, Wisconsin. They were maintained in cages at 38°, shielded from ultraviolet light, and fed ad libitum a vitamin D-deficient diet described previously (3). At four weeks of age, chicks averaging 250 grams in weight and ranging from 230-280 grams were used for experimentation.

Vitamin D_3 compounds. The [1,2-^{3}H]-vitamin D_3 used (specific activity 782 DPM/pmole) was prepared according to the method of Neville and DeLuca (4). Crystalline vitamin D_3 was a gift of the Philips-Duphar Company of Weesp, The Netherlands.

Measurement of vitamin D_3-25-hydroxylase activity. Chick livers were removed and chilled immediately in ice cold 0.25 M sucrose. A 10% homogenate in 0.25 M sucrose was prepared using a Potter-Elvehjem homogenizer. A 5 ml aliquot of the homogenate was added to a 125 ml Erlenmeyer flask containing 2.5 ml of buffer cofactor solution (0.1 M K_2HPO_4, 0.4 mM TPN, 160 mM nicotinamide, 20 mM ATP, 22.4 mM glucose, pH 7.2), and 2.5 ml of salt solution (5 mM $MgCl_2$, 0.1 M KCl). To this incubation mixture was added 25 µl of 95% EtOH containing 394 pmole (6 IU) of [^{3}H]-vitamin D_3. Incubations were carried out for 2 hours at 37° C at 120 oscillations per minute, at which time the reactions were terminated, the mixture extracted, and the extract chromatographed on 20 gram Sephadex LH-20 columns as already described (1).

RESULTS

In vitro liver vitamin D_3-25-hydroxylase activity in vitamin D-deficient and vitamin D_3-repleted chicks. Rachitic chicks, prepared as described in Materials and Methods, were administered 0.65, 1.30, or 3.25 nmole (10, 20, or 50 IU) of [^{3}H]-vitamin D_3 in 50 µl 95% EtOH by wing vein injection. Control chicks received the EtOH vehicle only. Twenty-four hours after the injections, the chicks were killed and their livers assayed for *in vitro* vitamin D_3-25-hydroxylase activity as described in Materials and Methods. The [^{3}H]-vitamin D_3 injected *in vivo* was of the same specific activity as that added to the liver homogenate *in vitro*, thus eliminating any dilution of the [^{3}H]-vitamin D_3 added *in vitro* by that remaining in the liver from the *in vivo* dose.

In the case of the chicks treated with [^{3}H]-vitamin D_3 *in vivo*, 10 ml aliquots of their liver homogenates were extracted directly without incubation to determine initial liver concentrations of [^{3}H]-vitamin D_3 and

Table 1: Effect of [^{3}H]-vitamin D_3 Administration on In Vitro Calciferol-25-Hydroxylase Activity*

Pre-treatment (nmoles [^{3}H]-vitamin D_3	[^{3}H]-25-OH-D_3 Before incubation (A)	[^{3}H]-25-OH-D_3 After incubation with added substrate (B)	[^{3}H]-25-OH-D_3 produced (B - A)
	pmoles/incubation flask		
Control vehicle only	-	11.9	11.9
	-	13.3(13.2)	13.3(13.2)
	-	18.1	18.1
	-	10.6	10.6
0.65 (10 IU)	1.4	9.9	8.5(11.3)
	0.7	14.7	14.0
1.30 (20 IU)	0.7	4.8	4.1(4.9)
	0.7	6.3	5.6
3.25 (50 IU)	1.5	3.2	1.7(2.3)
	1.4	4.3	2.9

*Chicks were injected with [^{3}H]-vitamin D_3 or the vehicle (EtOH). Twenty-four hours later they were killed and their livers assayed for calciferol-25-hydroxylase activity as described in the text. The values for individual chicks are presented with their averages in parentheses.

[^{3}H]-25-hydroxyvitamin D_3[1] as a consequences of the in vivo dose.

As shown in Table 1, no significant decrease in 25-hydroxylase activity was observed 24 hours following a 0.65 nmole (10 IU) dose of vitamin D_3 in agreement with the results of Tucker et al. (2). When the dose was increased to 1.30 and 3.25 nmole, striking decreases in enzyme activity were observed which were more pronounced at the higher level. The chick liver vitamin D_3-25-hydroxylase enzyme thus appears to be regulated as is the rat liver enzyme, but requires a higher dose of vitamin D_3 in order to bring about a reduction in enzyme activity.

In vivo vitamin D_3-25-hydroxylation in rachitic and vitamin D_3-treated chicks. Rachitic chicks were administered 0.65 or 3.25 nmole (10 or 50 IU) of unlabeled vitamin D_3 in 50 µl 95% EtOH by wing vein injection. Control chicks received the EtOH vehicle only. Twenty-four hours later, when in vivo pools of vitamin D_3 were small, all chicks were administered 0.65 nmole (10 IU) [^{3}H]-vitamin D_3 by wing vein injection and the rates of appearance of [^{3}H]-25-OH-D_3 in the blood were

[1] [^{3}H]-25-OH-D_3

measured as described in Materials and Methods. The livers of the control and the pre-treated chicks with 3.25 nmole of vitamin D_3 were analyzed for [^{3}H]-vitamin D_3 concentrations at the 1 hour time point.

As shown in Figure 1, the rate of appearance of [^{3}H]-25-OH-D_3 in the blood

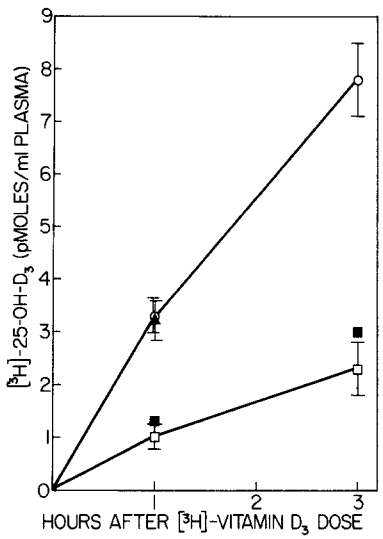

Figure 1: Plasma concentrations of [^{3}H]-25-OH-D_3 following [^{3}H]-vitamin D_3 administration to rachitic and vitamin D_3 treated chicks. Rachitic chicks were injected with 0.65 nmole or 3.25 nmoles of unlabeled vitamin D_3 or the ethanol vehicle. Twenty-four hours later they were all injected with 0.65 nmoles of [^{3}H]-vitamin D_3. At the indicated times the chicks were killed and the concentrations of [^{3}H]-25-OH-D_3 in the plasma were determined. Each point represents the average of values from three chicks. The brackets show the standard errors of the mean. O, rachitic chicks; Δ ,chicks pre-treated with 0.65 nmoles vitamin D_3; □ , chicks pre-treated with 3.25 nmoles vitamin D_3; ■ , 3.25 nmoles pre-treatment values corrected for approximate dilution of [^{3}H]-vitamin D_3 (refer to text and Table 2)

of chicks pre-treated with 3.25 nmole (50 IU) of unlabeled vitamin D_3 was considerably lower than that of the controls. This happened in spite of the fact that the livers of the control and pre-treated chicks took up similar amounts of [^{3}H]-vitamin D_3 (27.7 ± 1.0 and 33.2 ± 1.0 pmole/gm liver at 1 hour for control and pre-treated chicks, respectively). In contrast, the 1 hour [^{3}H]-25-OH-D_3 concentration in the blood of chicks pre-treated with 0.65 nmole (10 IU) vitamin D_3 was the same as the concentration in the control chicks. These

in vivo data support the measurements of *in vitro* vitamin D_3-25-hydroxylase activity already presented (Table 1).

Calculation of [^{3}H]-vitamin D_3 dilution by unlabeled vitamin D_3 remaining in the liver 24 hours after pre-treatment. In the *in vivo* experiments just described, the [^{3}H]-vitamin D_3 taken up by the liver following the injection of 0.65 nmole [^{3}H]-vitamin D_3 could be diluted by unlabeled vitamin D_3 remaining in the liver from the pre-treatment 24 hours earlier. An estimate of this dilution must be made to determine whether dilution of labeled substrate ([^{3}H]-vitamin D_3) could account for the decreased rate of appearance of blood [^{3}H]-25-OH-D_3 observed in the chicks pre-treated with 3.25 nmole (50 IU) of unlabeled vitamin D_3.

To do this, liver [^{3}H]-vitamin D_3 concentrations were determined 1 hour following the administration of 0.65 nmole [^{3}H]-vitamin D_3 to chicks pre-treated with 3.25 nmole unlabeled vitamin D_3. The amount of vitamin D_3 remaining in the liver 24 hours after adminstration of 3.25 nmole vitamin D_3 was obtained from the analyses made in the *in vitro* experiments presented in Table 1. The approximate dilution of liver [^{3}H]-vitamin D_3 by unlabeled vitamin D_3 remaining in the liver was calculated as shown in Table 2. The value is approximate because the amount of [^{3}H]-vitamin D_3 initially taken up by the liver has been estimated by the amount present 1 hour after [^{3}H]-vitamin D_3 administration. In the rat, the amount of liver vitamin D_3 is greater at 15 minutes than 1 hour after vitamin D_3 administration (1). Thus the 1.28 fold dilution calculated here is most likely greater than the actual dilution of label which took place. In any case, as shown by the solid squares plotted in Figure 1, the reduction in rate of appearance of [^{3}H]-25-OH-D_3 in the blood of chicks pre-treated with 3.25 nmole of vitamin D_3 cannot be accounted for by dilution of the liver [^{3}H]-vitamin D_3 by unlabeled vitamin D_3 present due to prior vitamin D_3 adminstration.

DISCUSSION

The rate of 25-hydroxylation of vitamin D_3 as measured either *in vitro* or *in vivo* is sharply and rapidly decreased following the administration of

Table 2: Calculation of Approximate Dilution of Liver [^{3}H]-vitamin D_3 by Unlabeled Vitamin D_3 Remaining in the Liver*

Vitamin D_3 remaining in the liver 24 hr after administration of 3.25 nmoles vitamin D_3	[^{3}H]-vitamin D_3 in liver 1 hr after administration of 0.65 nmoles [^{3}H]-vitamin D_3 to chicks pre-treated with 3.25 nmoles unlabeled vitamin D_3	Approximate dilution of [^{3}H]-vitamin D_3 by unlabeled vitamin D_3
(pmoles/gm liver)	(pmoles/gm liver)	
10.1	33.4	
(9.5)		
8.8	31.5 (33.2)	$\frac{33.2 + 9.5}{33.2}$ = 1.28 fold dilution
	34.8	

*Liver vitamin D_3 concentrations were determined as described in the text. The values for individual chicks are presented with their averages in parentheses.

physiological doses of vitamin D_3 to the rat (1). Subsequent doubts were raised about whether such a mechanism exists in a species other than the rat, especially the chick (2, 5). The present report demonstrates that hepatic vitamin D_3-25-hydroxylation in the chick is also decreased following physiological doses of vitamin D_3. Twenty-four hours following the administration of 1.30 nmole (20 IU) of vitamin D_3, *in vitro* liver vitamin D_3-25-hydroxylase activity was measured to be 37% of the control value. Following 3.25 nmole (50 IU), enzyme activity was 17% of control. No significant reduction in *in vitro* vitamin D_3-25-hydroxylase activity was observed 24 hours following the administration of 0.65 nmole (10 IU) of vitamin D_3. These results agree with the observations of Tucker *et al.* (2) that the administration of 10 IU vitamin D_3 per day to chicks did not reduce *in vitro* measured liver vitamin D_3-25-hydroxylase activity. (Presumably their *in vitro* measurement was made 24 hours after the final daily dose of vitamin D_3). Thus the chick liver vitamin D_3-25-hydroxylase is subject to the same type of regulation as is the rat enzyme. For both enzymes the amount of reduction increases as the dose of vitamin D_3 increases. Interestingly enough, the chick requires a higher level of vitamin D_3 to bring about a measureable

decrease in enzyme activity. This may be related to a higher requirement for vitamin D by the chick.

Measurements of *in vivo* chick vitamin D_3-25-hydroxylation were also made. As shown in Figure 1, a reduction in the rate of appearance of [^{3}H]-25-OH-D_3 in the blood was observed in chicks pre-treated with 3.25 nmole (50 IU) of unlabeled vitamin D_3. Such a reduction was not observed in chicks pre-treated with 0.65 nmole (10 IU) of vitamin D_3. Thus the *in vivo* measurements agree well with the *in vitro* measurements of vitamin D_3-25-hydroxylase activity in the liver.

This observation raises questions about the role of organs other than the liver in vitamin D_3-25-hydroxylation. Tucker *et al.* (2) have presented *in vitro* data which suggest that chicken kidneys and intestines might also 25-hydroxylate vitamin D_3. The fact is, however, that the *in vivo* rate of appearance of [^{3}H]-25-OH-D_3 in the blood of chicks is considerably reduced by prior vitamin D_3 administration. Thus, if chick kidneys and intestine also 25-hydroxylate vitamin D_3, either those organs do not contribute a major portion of the [^{3}H]-25-OH-D_3 appearing in the blood, or the vitamin D_3-25-hydroxylation in those organs is also regulated as is the case for the liver. Certainly the possibility that organs other than the liver do carry out vitamin D_3-25-hydroxylation should be investigated thoroughly with very careful identification of products formed *in vitro* and an attempt at determining *in vivo* significance of the *in vitro* findings.

The mechanism whereby the hepatic vitamin D_3-25-hydroxylase activity is reduced following vitamin D_3 adminstration remains unknown. Several possibilities are being considered ranging from simple product inhibition to increased enzyme degradation and allosteric changes in the 25-hydroxylation enzyme. It is significant that as much as 13 nmoles of exogenously administered 25-OH-D_3 does not reduce hepatic hydroxylation. However, very little exogenous 25-OH-D_3 enters the liver (6) which precludes conclusions regarding possible role of hepatic levels of 25-OH-D_3 in this regulation.

The physiological significance of the regulation of hepatic vitamin D_3-

25-hydroxylase is also unsettled at the present time. This system may serve to provide some protection against vitamin D toxicity, or it may be a mechanism to conserve vitamin D_3. Only additional investigation can provide solutions to these questions. However, the present report demonstrates that this phenomenon is not limited to one species as might have been surmised from previously published results.

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