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DIHYDROTACHYSTEROL INDUCTION OF INTESTINAL CALCIUM-BINDING ACTIVITY IN THE CHICK*

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Abstract—Appearance of the calcium-binding protein (CaBP) in intestinal mucosal preparations has previously been regarded as a vitamin D-dependent process. However, dihydrotachysterol, a compound stereo-chemically related to vitamin D, was found to be capable of inducing CaBP activity in vitamin D-deficient chicks. Dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3) were less active than vitamin D_3 at lower dosage levels (i.e. up to 325 nmoles); while a high dose (i.e. 3·25 μmoles) of the sterols demonstrated activities equivalent to or surpassing that for vitamin D_3. At the high dosage level, DHT_2 appeared more active than either DHT_3 or vitamin D_3.

Stimulation of calcium absorption was detectable at 5 hr and CaBP activity at 10 hr following DHT_2 repletion (325 nmoles), giving approximately the same time course of events as an equivalent dose of vitamin D_3. Induction of CaBP activity was actinomycin sensitive, although the action of DHT_2 was not completely blocked by the antibiotic. Such action for DHT in the intestine was discussed in view of possible hydroxylation *in vivo* at C-25, similar to vitamin D.

IT HAS been known for over 30 years that vitamin D exerts a major biological effect on intestinal calcium absorption.[1] More recent advances have provided a deeper insight concerning the mechanism of this vitamin D action. Studies using ^{3}H-vitamin D_3 have revealed the rapid appearance of several metabolites in vitamin D-repleted animals.[2] The main circulating metabolite in blood has been identified as 25-hydroxy vitamin D_3,[3] while the tissue active form in the intestine appears to be 1,25-dihydroxy vitamin D_3.[4]

Another important achievement has been the isolation of a vitamin D-dependent intestinal calcium-binding protein (CaBP).[5] Appearance of the CaBP seems to correlate closely with the increase in calcium absorption; however, its specific role in the calcium absorption process is still uncertain.

Due to the recent identification of several vitamin D metabolites,[3,6] it has become possible to discuss these compounds with regard to a tissue specific action.[7] It therefore seems apparent that the use of vitamin D_3-related compounds (e.g. vitamin D_2, DHT_2 and DHT_3) can provide greater detail concerning the mechanism of action for vitamin D and its metabolites. However, prior to extracting meaningful data from such experimentation, the biological activity of each compound should be known for the various vitamin D-dependent systems (e.g. calcium transport and CaBP activity).

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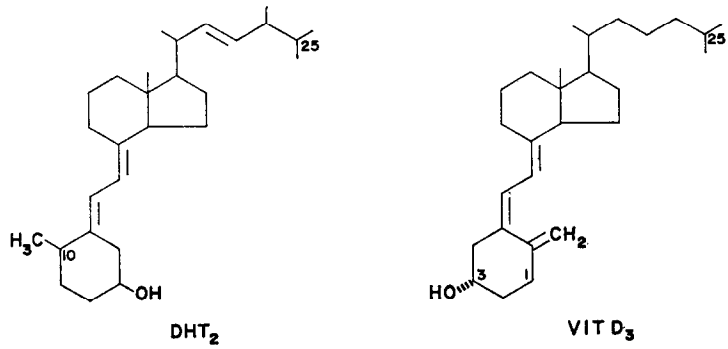

FIG. 1. Structural diagram showing similarity of DHT_2 and Vit D_3.

Dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3),* active hypercalcemic agents,[8–10] have been reported to be functional in stimulation of intestinal calcium absorption, although to a lesser degree than vitamin D.[11,12] Whether these compounds act in a similar fashion regarding the calcium transport system, namely CaBP, prompted the current study.

MATERIALS AND METHODS

One-day-old White Leghorn cockerels (DeKalb) were kept in a room devoid of ultraviolet light and given a rachitogenic diet.[13] The chicks were maintained on this diet for approximately 21 days, at which time the characteristic symptoms of rickets were evident. Subsequent vitamin D_3 dosage was effective in elevating the plasma calcium concentration and intestinal calcium absorption in these chicks.

The vitamin D and DHT† to be used in these studies were checked for purity by: (1) thin-layer chromatography (plates coated with a mixture of 4 parts cellulose to 7·5 parts Silica gel and developed using 15% acetone in *N*-heptane); (2) ultraviolet absorption spectrum; and (3) gas–liquid chromatography.‡

Calcium absorption studies were performed *in situ* using the duodenal loop. Chicks were fasted for 15 hr and then anesthetized with chloral hydrate (35 mg/100 g body wt., given i.m.). The duodenum was exposed, ligated and washed free of lumenal contents using 5 ml of a phosphate-free bicarbonate solution (120 mM NaCl, 4·9 mM KCl, 9·2 mM $NaHCO_3$; pH = 7·0).

Through use of a syringe and 18 gauge needle, 0·2 ml of [^{45}Ca]phosphate-free bicarbonate solution (0·5 $\mu c^{45}CaCl_2$/ml, 6 mM $CaCl_2$) was placed within the duodenal segment. The loop was replaced in the peritoneal cavity and the animal was killed 20

* Tachysterol, one of the irradiation by-products leading to the synthesis of vitamin D, can be reduced to form dihydrotachysterol. DHT differs from vitamin D in that the C-19 methylene group on the A ring is reduced, giving a conjugated diene, and the A ring is rotated 180°, resulting in the 3-hydroxy function occupying the C-1 position. The remainder of the molecule appears identical to vitamin D. The term dihydrotachysterol (i.e. DHT) will be used to denote both dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3), much the same as the term vitamin D encompasses both vitamin D_2 and vitamin D_3 (Fig. 1).

† Vitamin D_3 and DHT_2 were purchased from Mann Research Laboratories. Vitamin D_2 was a product of Philips-Roxane, while DHT_3 was a gracious gift of Philips-Duphar.

‡ Hewlett Packard Mod. 402. Column was 4 ft long, packed with Gas-ChromZ and maintained at 250°. Flow rate was 80 ml/min.

min later. The duodenal segment was excised and dry ashed at 600° for 48 hr. Per cent ^{45}Ca absorption was calculated using the following formula:

$$\text{Per cent } {}^{45}Ca_{abs} = \left[1 - \frac{{}^{45}Ca_R}{{}^{45}Ca_A}\right] 100$$

where $^{45}Ca_R$ refers to the ^{45}Ca activity remaining in the duodenal loop and $^{45}Ca_A$ denotes the ^{45}Ca added to each loop.

A modification of the method described by Wasserman and Taylor[14] was used to prepare supernatant fractions from mucosal homogenates. Chicks were killed by cervical dislocation, the duodenum was removed and placed in cold tris-NaCl solution (80 mM tris-HCl, 80 mM NaCl, 10 mM-β-mercaptoethanol; pH 7·4). The duodenal loop was everted, rinsed free of lumenal contents with cold 150 mM NaCl solution and blotted free of excess moisture. Mucosal tissue was obtained by gentle scraping with the edge of a glass slide. A 20 per cent (w/v) mucosal homogenate was prepared using the Tris-NaCl solution and then centrifuged at 38,000 *g* (4°) for 20 min. The supernatant solution was heat-treated (60°) for 8 min, cooled and the precipitated protein removed by centrifugation (38,000 *g*, 5 min). A nearly constant calcium concentration between individual supernatant preparations was obtained by washing each sample with a cation exchange resin, Chelex-100* (2·5 ml supernatant to 0·25 ml resin).

Calcium-binding activity in the final supernatant was determined using a modification of the previously developed Chelex assay.[14] The assay consisted of the uniform and rapid mixing of 1 ml supernatant, 0·1 ml Chelex resin and 0·1 μc ^{45}Ca for 20 sec. Near equilibrium in the partitioning of ^{45}Ca was obtained between the soluble calcium-binding molecule and Chelex resin during the mixing procedure. The resin was rapidly and extensively sedimented by centrifugation (i.e. 3000 *g*, 30 sec) with an aliquot of the supernatant solution counted for estimation of ^{45}Ca partitioning. Prior normalization of the endogenous calcium concentration was deemed necessary in an effort to assure that the only assay variable was the CaBP concentration.† Accordingly, a greater percentage of the ^{45}Ca remained in the supernatant with increasing CaBP concentrations. CaBP activity was then expressed as per cent ^{45}Ca pool or per cent ^{45}Ca pool/mg protein as exemplified in the following equation:

$$\text{Per cent } {}^{45}Ca \text{ pool} = \left[\frac{{}^{45}Ca_S}{{}^{45}Ca_A}\right] (100) = \text{CaBP activity}$$

where $^{45}Ca_S$ is the supernatant radioactivity and $^{45}Ca_A$ is the dose of radioactivity added (i.e. ^{45}Ca pool) to the assay mixture. Background measurements using buffer alone gave a value of 1% ^{45}Ca pool. Protein concentrations of 3–6 mg/ml gave a correspondingly linear assay response. Radioactive ^{45}Ca was detected using a low-background planchet system (Beckman Instrument Co.), and protein was determined

* 200–400 mesh (Bio-Rad Laboratories, Richmond, California).

† The binding of calcium by a CaBP is depicted in the following equation:

$$^{40}Ca + {}^{45}Ca + CaBP \leftrightarrow CaBP \times {}^{40}Ca + CaBP \times {}^{45}Ca$$

An increase in [^{40}Ca] will tend to compete with [^{45}Ca] for the CaBP molecules. Part of the ^{45}Ca which is displaced would then be sequestered by the Chelex resin, in effect lowering the detectable supernatant radioactivity. Since the amount of supernatant radioactivity is taken as an estimate of CaBP activity, a fallaciously low value would be obtained for samples containing high levels of endogenous ^{40}Ca.

by the method of Lowry *et al.*[15] Calcium was estimated by using atomic absorption spectrophotometry (Instrumentation Laboratories).

RESULTS

Preliminary studies indicated that pharmacological doses of DHT_2 (1 mg/chick) induced calcium-binding activity in vitamin D-deficient chicks. Such binding activity appeared dependent on the presence of a proteinaceous molecule(s), since incubation with trypsin (1 mg/ml, 30° for 5 hr) degraded the activity. Furthermore, the calcium-binding activity in the supernatant from DHT_2-repleted chicks was retained following extensive dialysis and could be fractionated on a Sephadex G-100 column. The calcium-binding activity appeared to be confined to a single peak. Similar results have been obtained for the supernatant from vitamin D_3-repleted chicks, wherein it was demonstrated by disc gel electrophoresis that one protein was responsible for the calcium-binding activity.[14,16] This protein was appropriately named the calcium-binding protein (CaBP). Accordingly, the calcium-binding activity induced by either vitamin D or dihydrotachysterol will be designated as CaBP activity throughout this paper.

Realizing that high dosages of DHT_2 could induce CaBP activity, it became of interest to see how active the sterol was with respect to vitamin D. A dose–response curve (Fig. 2) revealed that vitamin D_3 induced CaBP activity at a lower dosage than

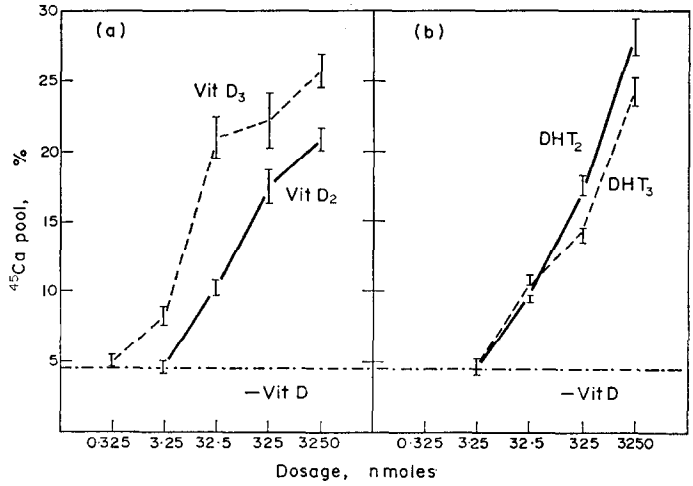

FIG. 2. The appropriate amount of (A) vitamin D or (B) DHT was dissolved in corn oil (0·2 ml) and given to vitamin D-deficient chicks 40 hr prior to assaying for calcium-binding activity in heat-treated supernatant solutions. Control chicks received the vehicle alone. Each point represents the average of seven or more observations. The vertical lines represent the standard error of the mean.

either vitamin D_2 or dihydrotachysterol. CaBP activity was detected following a vitamin D_3 dose of 3·25 nmoles (i.e. 50 I.U.), while a similar phenomenon for vitamin D_2 required a 32·5 nmole dose (i.e. 500 I.U.). This difference in activity remained nearly constant to 3250 nmoles, indicating that vitamin D_2 was about one-tenth as active as vitamin D_3. Similarly, vitamin D_3 exceeded the activity of DHT_2 and DHT_3

at both 32·5 and 325 nmole dosages. However, at the level of 3250 nmoles, DHT appeared equal in activity to vitamin D. DHT_2 was the more active compound at the high dose level, exceeding DHT_3 in stimulation of CaBP activity. Thus, vitamin D_3 gave half the maximal response of vitamin D_2, DHT_2 or DHT_3 at one-tenth the dose level.

The possibility that a nuclear process was involved in the DHT-induced CaBP activity was investigated using actinomycin D* (17) prior to DHT_2 treatment (Table 1). In both experiments the antibiotic effected a significant decrease ($P < 0·01$) in CaBP

TABLE 1. EFFECT OF ACTINOMYCIN D UPON DHT_2-INDUCED CALCIUM-BINDING ACTIVITY*

Experiment	Treatment		% ^{45}Ca ^{45}Ca/mg protein
	Act. D	DHT_2	
A†	+	−	0·86 ± 0·04 (6)
	+	+	1·35 ± 0·07‡ (6)
	−	−	0·90 ± 0·03 (6)
	−	+	1·98 ± 0·08 (6)
B§	+	−	1·06 ± 0·08 (4)
	+	+	2·51 ± 0·10‡ (4)
	−	−	0·98 ± 0·01 (4)
	−	+	3·19 ± 0·10 (4)

* Chicks were sacrificed 20 hr following DHT_2 dosage (325 nmoles/chick). Mucosal supernatant solutions were prepared from individual chicks as described in the text. Values in parentheses denote the number of animals. Results are presented as mean ± S.E.M.

† In experiment A, deficient chicks were injected with Act. D (50 μg/100 g, i.p.) 2 hr prior to oral DHT_2 dosage. Control chicks received dosage of corn oil.

‡ Significantly different ($P < 0·01$) from (−) Act. D. (+) DHT_2.

§ In experiment B, deficient chicks were injected with Act. D (60 μg/100 g, i.v.) 3 hr prior to i.v. injection of DHT_2. Control chicks received i.v. injections of 95% ethanol.

activity. However, complete blockage of CaBP activity was not accomplished in either experiment. Increasing the actinomycin dosage (1 μg/g) did not enhance the blockage.

Taylor and Wasserman[18] using fluorescent antibody techniques have reported localization of the CaBP in the goblet cell and surface coat-microvillar region of intestinal epithelial cells. Several theories of action for the CaBP were postulated by the authors involving concentration of calcium at the brush border or facilitated movement of the cation across the plasma membrane. Physiological support for such a theory would seemingly be provided by complementary time-course profiles for calcium absorption and CaBP activity. Subsequent data (Fig. 3) supported that idea whereby the two parameters were found to be related ($r = 0·88$; $P < 0·05$; $N = 5$)[19] following DHT_2 repletion.

* Courtesy of Merck, Sharpe & Dohme.

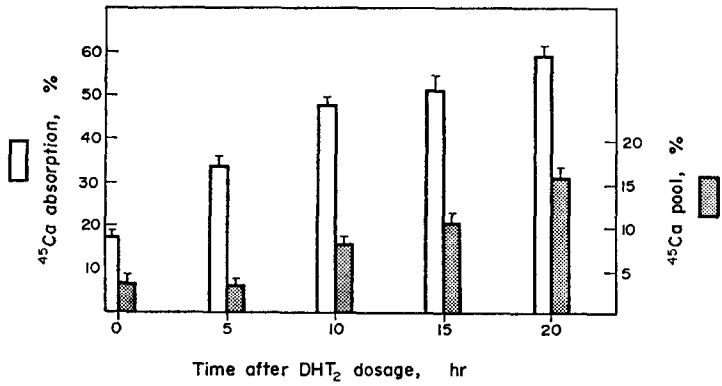

FIG. 3. The DHT_2 (325 nmoles/chick) was given i.v. (in 95% ethanol) at the appropriate time periods. Methods for determination of calcium absorption and calcium-binding activity are explained in the text. Results are the average of four to eight chicks, with the standard error of the mean given by the vertical lines.

Calcium absorption, which was significantly increased at 5 hr ($P < 0{\cdot}01$), continued to exhibit increased values to 20 hr post DHT_2 administration (Fig. 3). The CaBP was not detected until 10 hr had elapsed but, similar to calcium absorption values, it continued to show increased levels at 20 hr. Other data (not tabulated) showed that the CaBP continued to increase in activity up to 44 hr post DHT_2 dosage, differing from calcium absorption which plateaued at approximately 20 hr.

DHT_2-repleted chicks also showed an elevated plasma calcium (Table 2) in addition to stimulation of calcium transport and induction of CaBP activity. Elevation of plasma calcium can be attributed to the sterol's influence on bone resorption and

TABLE 2. INDUCTION OF CALCIUM-BINDING ACTIVITY AND ELEVATION OF PLASMA CALCIUM AFTER DHT_2 DOSAGE*

Compound	Dosage (nmoles)	Calcium-binding act. (% ^{45}Ca pool/mg protein)	Plasma Ca (mg/100 ml)
DHT_2	32·5	1·37 ± 0·09†	6·11 ± 0·43
DHT_2	325	2·60 ± 0·18†	6·65 ± 0·35‡
DHT_2	3250	4·40 ± 0·25†	7·51 ± 0·21†
Vit. D_3	1·1 (daily)	1·30 ± 0·11†	10·04 ± 0·35†
Corn oil		0·73 ± 0·03	5·57 ± 0·39

* DHT_2 was given orally, 44 hr before kill. Values given as mean ± S.E. from three to six observations.

† Significantly different from oil control group ($P < 0{\cdot}01$).

‡ Significantly different from oil control group ($P < 0{\cdot}05$).

intestinal calcium transport, since both tissues respond to DHT_2[11,12] and no attempt was made to separate these effects. Plasma calcium and CaBP activity increased with the log dose of DHT_2. Chicks maintained on vitamin D revealed normal plasma calcium values and CaBP activity approximating that for 32·5 nmole-repleted group.

DISCUSSION

Induction of CaBP activity, generally considered vitamin D dependent, was effected by dihydrotachysterol. Equivalent activities for both DHT_2 and DHT_3 were observed at all dosages tested up to 3·25 μmoles, where DHT_2 demonstrated a greater activity ($P < 0{\cdot}02$). A previous study by Hibberd and Norman,[11] comparing the activity of DHT_2 and DHT_3 in promoting intestinal calcium absorption, indicated that DHT_3 was the more active compound. These investigators proposed that the side-chain of DHT_2 and DHT_3 was indicative of the compounds' physiological loci of action. It was suggested that DHT_2 and vitamin D_2 act preferentially in bone resorption, while DHT_3 and vitamin D_3 act more specifically in calcium absorption. Such conclusions differ from the earlier work of Bosmann and Chen[20] where DHT_2 and DHT_3 demonstrated nearly equivalent activities in the maintenance of blood calcium and bone ash. In addition, the present study (Fig. 2) revealed a slightly greater activity for DHT_2 toward induction of CaBP activity, a result which would not have been predicted by the previously mentioned theory of Hibberd and Norman.[11]

In chicks, vitamin D_3 is more active than vitamin D_2 by a factor of about 10:1.[20] In this work vitamin D_3 exhibited a greater activity than either vitamin D_2 or dihydrotachysterol. However, DHT_3 was not more active than DHT_2 toward induction of CaBP activity as might have been predicted from the differing activities for vitamin D_3 and vitamin D_2 in the chick. Apparently, alteration of the side-chain affects the activity of vitamin D_3 only when the stereo-chemical integrity of ring A is maintained. Vitamin D_2 differs from vitamin D_3 by a modification of the side-chain,* while DHT_2 and DHT_3 mimic the parent vitamins, except for a modification of the A ring.

The metabolically active form of vitamin D in the intestine is a metabolite which contains additional hydroxyl groupings on the side-chain (C-25) and A ring (C-1).[4,21]

This dihydroxy metabolite of vitamin D_3 (i.e. 1,25-dihydroxy vitamin D) is quite specific for the calcium transport system,[22] although a large pulse of 25-hydroxy vitamin D_3 (i.e. 25-HCC) can mimic its action.[23] In a similar manner, high dosages of DHT gave a CaBP response equivalent or surpassing that of vitamin D_3 (Fig. 2). Recently, Suda *et al.*[24] synthesized 25-hydroxy DHT_3 and showed that it possessed increased bone mobilization activity. Thus, it seems probable that DHT is hydroxylated *in vivo* at the C-25 position,† similar to vitamin D.[6] Such a reaction would, in effect, give DHT hydroxyl functions at carbons 1 and 25, thereby fulfilling an important criterion for the active form of vitamin D in the intestine. The intestinal transport activity for DHT would seemingly be most pronounced at high dosage levels where mass action of the compound could overcome its structural deficiencies (e.g. loss of triene system, C-3 hydroxyl and C-10 methylene groupings). Data from this report are consistent with such a hypothesis, yet further studies are required before the mechanism of action can be fully understood.

Detection of CaBP activity appears to lag behind the observable stimulation of calcium absorption (Fig. 3). However, appearance of the CaBP has been shown to coincide very closely with the increase in calcium transport by use of the rabbit antibody to CaBP and immuno diffusion techniques.[25] The Chelex assay used in this study was incapable of detecting small amounts of CaBP, which may account somewhat for the lag time.

* The side-chain of vitamin D_2 is characteristic of ergosterol and differs from vitamin D_3 which is a product of 7-dehydrocholesterol.

† R. B. Hallick and H. F. DeLuca, *J. biol. Chem.*, in press.

Following vitamin D repletion, a time period of several hours precedes detection of a physiological response.[26] This lag time can be shortened by the use of high vitamin dosages. Ebel *et al.*[25] reported that deficient chicks dosed with 6·5 or 325 nmoles of vitamin D_3 showed increased calcium absorption at 14 and 4 hr, respectively, and CaBP activity (Chelex assay) at 18 and 14 hr. Under similar conditions a 325-μmole DHT_2 dosage induced calcium absorption and CaBP activity at 5 and 10 hr, respectively, equating the time response for a similar dose of vitamin D_3. In contrast, vitamin D_3 appears to be the more active agent at lower doses.

To explain the above mentioned lag period, it has been proposed that vitamin D functions at the nuclear level to effect assembly of the calcium transport system.[27,28] A similar lag time was evidenced following DHT dosage (Fig. 3). The induction of CaBP by DHT was actinomycin D sensitive, also indicating the possibility of a nuclear involvement. However, actinomycin treatment did not completely block the action of DHT, similar to previous results using vitamin D.[29] Thus, experiments using agents which block translational activity are required before a definite statement can be made with regard to DHT's site of action.

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