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1α-Hydroxyvitamin D_3. An Analog of Vitamin D Which Apparently Acts by Metabolism to 1α,25-Dihydroxyvitamin D_3†

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ABSTRACT: 1α-Hydroxyvitamin D_3 (1α-OH-D_3) is a synthetic sterol with biological characteristics similar to those of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3), the apparent hormonal form of vitamin D. The synthetic sterol is virtually equipotent to the natural hormone, *in vivo,* and has been utilized recently to treat patients with defects in vitamin D metabolism. However, no information is presently available on its biochemical mode of action. In order to determine if 1α-OH-D_3 functions by binding directly to target tissue receptors for 1α,25-$(OH)_2$-D_3 or is first metabolized to 1α,25-$(OH)_2$-D_3, we have carried out a detailed examination of the comparative biologic effects as well as the receptor binding properties of the two sterols. After a single oral dose of 162.5 pmol (2.5 IU) of either 1α-OH-D_3 or 1α,25-$(OH)_2$-D_3, rachitic chicks display equivalent increases in calcium absorption from the intestine; following a 2–3-hr latent period, both sterols elicit maximal effects at 9 hr and the responses undergo parallel decay between 24 and 72 hr. Comparison of the relative amounts of 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 required to stimulate calcium absorption at 9 hr indicates that the sterols are equipotent in the dose range of 19.5–650 pmol (0.3–10 IU). In chronic administration studies, the synthetic sterol was slightly more antirachitic than 1α,25-$(OH)_2$-D_3, with both sterols being 2–6 times more active than native vitamin D_3. By contrast, *in vitro* studies utilizing competitive binding for the 1α,25-$(OH)_2$-D_3 cytosol-chromatin receptor system from intestine demonstrate that 1α-OH-D_3 binds to the receptor two–three orders of magnitude less avidly than 1α,25-$(OH)_2$-D_3. Since the equipotency of these two sterols was not reflected at the molecular level, we performed the following experiment to show that 1α-OH-D_3 is converted to 1α,25-$(OH)_2$-D_3, *in vivo.* Rachitic chicks were given 1α-OH-D_3 and 3 hr later their intestinal chromatin was extracted. The suspected 1α,25-$(OH)_2$-D_3 fraction was isolated by Celite chromatography and a saturating amount (26 pmol) of 1α,25-$(OH)_2$-D_3 was detected in the chromatin by competitive protein binding assay. Since 1α,25-$(OH)_2$-D_3 was present in the chromatin receptor prior to the stimulation of calcium absorption by 1α-OH-D_3, we conclude that 1α-OH-D_3 is rapidly metabolized to 1α,25-$(OH)_2$-D_3 and probably functions by conversion to the hormone. The conversion of 1α-OH-D_3 to 1α,25-$(OH)_2$-D_3 was also observed in intestinal mucosa homogenates, *in vitro,* further verifying the occurrence of this enzymatic reaction.

It is well documented that 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3)[1] is the hormonal form of vitamin D_3 (Holick *et al.,* 1971; Haussler *et al.,* 1971; Lawson *et al.,* 1971; Brumbaugh *et al.,* 1974a). This active form is produced from the parent vitamin D_3 (D_3) by conversion to 25-hydroxyvitamin D_3 (25-OH-D_3) in several tissues (Ponchon and DeLuca, 1969; Tucker *et al.,* 1973) and then 25-OH-D_3 is subsequently metabolized to 1α,25-$(OH)_2$-D_3 exclusively in the kidney (Fraser and Kodicek, 1970).

Recently, a chemically synthesized sterol, 1α-hydroxyvitamin D_3 (1α-OH-D_3), has been reported to have biological activity comparable to that of the natural hormone (Barton *et al.,* 1973; Holick *et al.,* 1973; Haussler *et al.,* 1973). 1α-OH-D_3 was found to be as efficacious as 1α,25-$(OH)_2$-D_3 both in stimulating intestinal calcium absorption in rats and chicks and in promoting bone calcium mobilization *in vivo.* Since 1α-OH-D_3 is considerably easier and less expensive to synthesize than 1α,25-$(OH)_2$-D_3, synthetic 1α-OH-D_3 has found recent clinical application in the treatment of patients with possible defects in the 1α hydroxylation of 25-OH-D_3 (Chalmers *et al.,*

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[1] Abbreviations used are: D_3, vitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1α,25-$(OH)_2$-D_3, 1α,25-dihydroxyvitamin D_3.

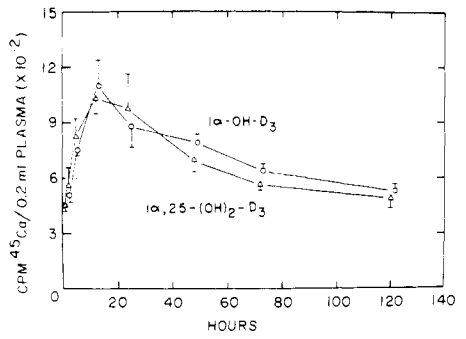

FIGURE 1: Time course of action of 1α-OH-D$_3$ and 1α,25-(OH)$_2$-D$_3$ in stimulating intestinal calcium absorption; 162.5 pmol of either 1α-OH-D$_3$ (○) or 1α,25-(OH)$_2$-D$_3$ (△) was administered orally and calcium transport from intestine measured, *in vivo*, at the indicated time after dosing. Absorption of calcium was monitored by counting plasma samples 45 min after an oral supplement of ^{45}Ca as described under Materials and Methods. Each point is the average of five rachitic chicks ± standard error of the mean.

1973; Pechet and Hesse, 1974). It is possible that 1α-OH-D$_3$ will become the sterol of choice in the therapeutic management of such metabolic bone diseases as renal osteodystrophy (Brickman *et al.*, 1972) and hypoparathyroidism (DeLuca, 1972). It is therefore of critical importance to understand the action of 1α-OH-D$_3$ at the biochemical level. Furthermore, it is evident that this potent analog will be of value in elucidating the metabolism and molecular mode of action of vitamin D in affecting calcium metabolism.

The question of whether 1α-OH-D$_3$ is further metabolized to 1α,25-(OH)$_2$-D$_3$ or acts directly upon the hormone receptor sites in intestine and bone has recently been raised (Holick *et al.*, 1973; Haussler *et al.*, 1973). Based on the similar time courses of action of the two sterols reported in a preliminary communication (Haussler *et al.*, 1973) and verified in detail in the present report, it appears that unless the 25-hydroxylation of 1α-OH-D$_3$ is exceedingly rapid, the synthetic sterol might function as an ideal analog of the hormone at the tissue receptor sites. On the other hand, the recently discovered occurrence of vitamin D$_3$ 25-hydroxylase in a number of tissues including the intestine (Tucker *et al.*, 1973), plus the fact that an analog similar in structure to 1α-OH-D$_3$, namely dihydrotachysterol$_3$, is rapidly hydroxylated at position 25 *in vivo* (Hallick and DeLuca, 1972), suggests that significant metabolism of 1α-OH-D$_3$ to 1α,25-(OH)$_2$-D$_3$ is not unlikely.

The present report presents a comparison of 1α-OH-D$_3$ and 1α,25-(OH)$_2$-D$_3$ in biological activity experiments, *in vivo*, and receptor binding studies *in vitro*. A striking disparity is found between the relative effectiveness of 1α-OH-D$_3$ *in vivo* and *in vitro*, and this disparity is accounted for by demonstrating that 1α-OH-D$_3$ is metabolized to 1α,25-(OH)$_2$-D$_3$ *in vivo*. Moreover, this conversion occurs rapidly enough to saturate the physiologic receptors in intestine with 1α,25-(OH)$_2$-D$_3$ prior to eliciting an increase in calcium absorption.

Materials and Methods

Animals and Materials. Animals used in all experiments were White Leghorn cockerels that were raised for 3–4 weeks on a rachitogenic diet (McNutt and Haussler, 1973). Vitamin D$_3$ was purchased from Calbiochem. 1α,25-(OH)$_2$-D$_3$ was produced enzymatically from crystalline 25-OH-D$_3$ (courtesy of Dr. John C. Babcock, Upjohn) by a modification (Haussler, 1972) of a published procedure (Lawson *et al.*, 1971). Crystalline 1α-OH-D$_3$ (courtesy of Dr. Maurice M. Pechet, Research Institute for Medicine and Chemistry, Cambridge, Mass.) was synthesized as described elsewhere (Barton *et al.*, 1973). All sterols were virtually free of contaminating materials as judged by mass spectrometry (Haussler *et al.*, 1973) and ultraviolet absorption spectrophotometry (Cary, Model 15). Absorbance at 265 nm was used to quantitate solutions of 1α-OH-D$_3$ and 1α,25-(OH)$_2$-D$_3$. 1α,25-(OH)$_2$-[26(27)-*methyl*-^{3}H]D$_3$ (9.8 Ci/mmol) was generated from 25-OH-[26(27)-*methyl*-^{3}H]D$_3$ (Amersham–Searle) as described previously (Brumbaugh and Haussler, 1974a) and was employed in competitive binding studies and the radioreceptor assay experiment.

Bioassays. Stimulation of calcium transport was quantitated as previously described (McNutt and Haussler, 1973). Antirachitic activity of the sterols in question was carried out as described elsewhere (Cork *et al.*, 1974).

Competitive Binding Assay for 1α,25-(OH)$_2$-D$_3$. The radioreceptor assay for 1α,25-(OH)$_2$-D$_3$ has been described in an earlier communication (Brumbaugh *et al.*, 1974a) and is extensively detailed in the preceding paper (Brumbaugh *et al.*, 1974b). The assay can be utilized either to measure minute quantities of nonradioactive 1α,25-(OH)$_2$-D$_3$ (sensitivity = 20 pg) or to probe the efficacy of vitamin D analogs in competing with 1α,25-(OH)$_2$-[^{3}H]D$_3$ for the chromatin receptor as previously reported (Brumbaugh and Haussler, 1973a).

Isolation of 1α,25-(OH)$_2$-D$_3$ from Chick Intestinal Chromatin. A single dose of 650 pmol of 1α-OH-D$_3$ in 0.2 ml of 1,2-propanediol was orally administered to each of 20 rachitic chicks which were sacrificed 3 hr later. The 20 control chicks received only 0.2 ml of 1,2-propanediol. Subsequently, both groups were treated identically. The intestines were excised and placed in 0.25 M sucrose at 0–2°, immediately after the chicks were killed. Triton X-100 washed intestinal chromatin was then prepared as previously described (Brumbaugh and Haussler, 1974a). Sterols were extracted from the two chromatin preparations (control and 1α-OH-D$_3$ treated chicks) with methanol–chloroform (2:1). After flash evaporation of the chloroform layer (Bligh and Dyer, 1959) the sterols were dissolved in ether. At this point, tracer amounts (17,000 dpm) of 1α,25-(OH)$_2$-[26(27)-*methyl*-^{3}H]D$_3$ were added to each extract to facilitate quantitation of losses and location of 1α,25-(OH)$_2$-D$_3$ during the purification procedure. Each extract was applied to a 2 × 18 cm (25 g) silicic acid column, which was eluted with 5% acetone in diethyl ether (v/v). Fractions (40 ml) were collected and 10% aliquots were counted to locate the 1α,25-(OH)$_2$-[^{3}H]D$_3$ peak. In each case the entire 1α,25-(OH)$_2$-D$_3$ peak was then rechromatographed on a Celite liquid–liquid partition column as previously described (Haussler and Rasmussen, 1972). 1α,25-(OH)$_2$-D$_3$ peaks of the Celite columns were dried under nitrogen and dissolved in 2.0 ml of distilled ethanol for 1α,25-(OH)$_2$-D$_3$ radioreceptor assay.

Results

Acute Activity of 1α-OH-D$_3$ Compared to 1α,25-(OH)$_2$-D$_3$. The time course of calcium absorption in rachitic chicks pursuant to an oral dose of 162.5 pmol (2.5 IU)[2] of either 1α-OH-D$_3$ or 1α,25-(OH)$_2$-D$_3$ is shown in Figure 1. At this dose of 2.5 IU, which produces approximately 50% of the maximal possible response at 9 hr, the time courses of stimulation of calcium transport with the two sterols are practically identical. 1α,25-(OH)$_2$-D$_3$ may function slightly more rapidly than 1α-OH-D$_3$, which has a slightly more sustained action, but at no time are the data statistically significantly different for the two sterols (Figure 1). As has been previously reported (Haussler *et*

[2] One international unit (IU) of all vitamin D sterols is defined as 65 pmol (Norman, 1972).

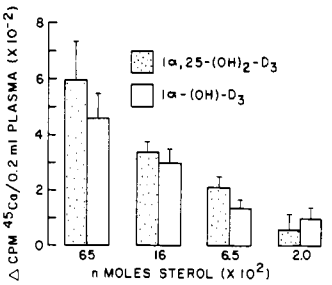

FIGURE 2: Calcium absorption response of rachitic chicks to various doses of 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3. Doses of 0.02–0.65 nmol of 1α-OH-D_3 or 1α,25-$(OH)_2$-D_3 were administered to chicks and calcium absorption was measured 9 hr later. Calcium absorption is expressed as *increment* (Δ cpm of ^{45}Ca) above the transport in untreated controls. Each bar represents the average of six animals ± standard error of the mean.

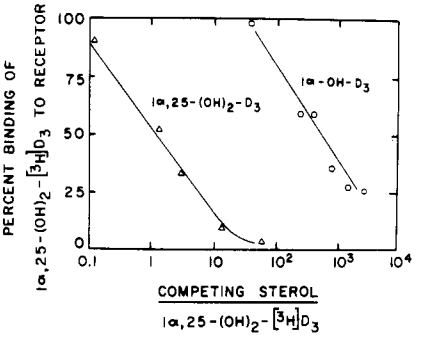

FIGURE 3: Relative ability of 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 to compete with 1α,25-$(OH)_2$-D_3 for intestinal chromatin receptor sites, *in vitro*. Varying amounts of 1α-OH-D_3 (○) or 1α,25-(OH)-D_3 (△) were added to a reconstituted cytosol–chromatin receptor system from chick intestinal mucosa and incubated in the presence of 5 nM 1α,25-$(OH)_2$-[^{3}H]D_3 (Brumbaugh *et al.*, 1974b). The amount of tritium associated with the chromatin (trapped on filters) in the absence of nonradioactive competitor is taken as 100% and the displacement data with nonradioactive sterols expressed as the per cent of this control binding. The concentration of nonradioactive competing sterol to labeled 1α,25-$(OH)_2$-D_3 is depicted on the abscissa. The possibility that nonradioactive 1α-OH-D_3 is converted to 1α,25-$(OH)_2$-D_3 by the intestinal 25-hydroxylase enzyme is eliminated by the fact that the 25-hydroxylase resides in the microsomal fraction and only cytosol and Triton X-100 washed chromatin fractions are present in the incubation.

al., 1973; Holick *et al.*, 1973), the hormone and its synthetic analog rapidly stimulate calcium absorption, following a 2–3 hr lag period, to yield a maximal effect 9 hr after administration of sterol. The decay in the response is less dramatic than has been previously demonstrated by others (Myrtle and Norman, 1971; Omdahl *et al.*, 1971), but is consistent with our earlier report of a 50% decay by 40 hr (Haussler *et al.*, 1971).

We next quantitated the dose–response relation at 9 hr after administration of 1α-OH-D_3 or 1α,25-$(OH)_2$-D_3 at various doses (19.5–650 pmol). As can be seen in Figure 2, virtually identical activity was obtained at all doses tested. Thus, regardless of the sterol dose or the time of assay, 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 are virtually indistinguishable in these *in vivo* experiments. The data in Figures 1 and 2 are consistent with the concept that these two sterols function by identical mechanisms, but do not eliminate the possibility that the conversion of 1α-OH-D_3 to 1α,25-$(OH)_2$-D_3 is sufficiently rapid to precede the slower induction of calcium absorption.

Chronic Activity of 1α-OH-D_3 Compared to 1α,25-$(OH)_2$-D_3. When administered daily for a 3-week period to chicks raised on a rachitogenic diet, 1α-OH-D_3 is slightly more potent than 1α,25-$(OH)_2$-D_3 in preventing rickets (Table I). The results expressed in Table I illustrate that, as in single dose experiments, the biological activity of 1α-OH-D_3 is equivalent to or greater than that of 1α,25-$(OH)_2$-D_3. Of the four antirachitic parameters tested, 1α-OH-D_3 was more potent than the natural hormone in three cases: growth promotion, maintenance of plasma calcium, and increase of per cent bone ash. Again these results provide evidence for the equivalence of the synthetic sterol and the natural hormone *in vivo*. However, the greater efficacy of chronically administered 1α-OH-D_3 is also suggestive of its serving as a precursor for 1α,25-$(OH)_2$-D_3 with physiologic amounts of 1α,25-$(OH)_2$-D_3 being continually produced from a reservoir of stored 1α-OH-D_3.

Relative Effectiveness of 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 in Binding to Receptors in Intestinal Mucosa. Previous investigations of the initial site of action of 1α,25-$(OH)_2$-D_3 in the intestine (Haussler *et al.*, 1968; Tsai *et al.*, 1972; Brumbaugh and Haussler, 1973b, 1974a,b) have led to the concept that the hormone binds initially to a cytosol receptor and is then transported to its apparent ultimate site of action in the nuclear chromatin. The evidence correlating this receptor system with the biological response to the vitamin has been discussed previously (Tsai *et al.*, 1972; Brumbaugh and Haussler, 1973a, 1974a,b). By assessing the ability of various vitamin D sterols to compete with radioactively labeled 1α,25-$(OH)_2$-D_3 for this intestinal receptor, we utilize this receptor system as an assay for vitamin D activity at the molecular level. As depicted in Figure 3, when 1α-OH-D_3 is tested in such a competitive bind-

TABLE I: Antirachitic Activity of Chronically Administered 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 in Chicks.

Sterol Tested[a]	Relative Activity[b]			
	Promotion of Growth	Maintenance of Plasma Ca	Increase in % Bone Ash	Alleviation of Radiol. Signs of Rickets
D_3	1.0	1.0	1.0	1.0
1α,25-$(OH)_2$-D_3	4.8	2.6	2.4	2–6
1α-OH-D_3	5.1	3.4	3.7	2–6

[a] All sterols were administered daily for 3 weeks (orally). [b] Relative activity is computed by determining the daily dose of sterol required to produce a 50% curative effect and then comparing it to the level of vitamin D_3 (D_3) necessary to elicit an equivalent response. Vitamin D_3 potency is set at 1.0 for all parameters tested. The details of these determinations are published elsewhere (Cork *et al.*, 1974).

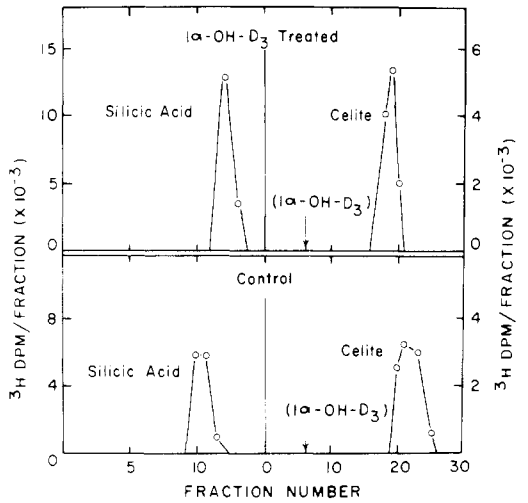

FIGURE 4: Chromatographic purification of suspected $1\alpha,25\text{-}(OH)_2\text{-}D_3$ from intestinal chromatin of rachitic chicks given 1α-OH-D_3. Twenty rachitic chicks each received 10 IU of nonradioactive 1α-OH-D_3 and were killed 3 hr later. Preparation of chromatin and extraction of sterols was carried out as described under Materials and Methods. After addition of marker $1\alpha,25\text{-}(OH)_2\text{-}D_3$ (17,000 dpm), purification of this extract ("1α-OH-D_3 treated") was performed on successive silicic acid and Celite columns as depicted in the top panels. In separate experiments, crystalline 1α-OH-D_3 (monitored by absorbance at 265 nm) was found to migrate approximately to fraction 5 on identical silicic acid columns and to fractions 6–7 on identical Celite columns. Thus, 1α-OH-D_3 is completely resolved from $1\alpha,25\text{-}(OH)_2\text{-}[^3H]D_3$ on these columns. Details of the chromatographic procedures are presented under Materials and Methods. The bottom two panels illustrate the purification of a "control" extract prepared from 20 chicks that received dosing vehicle alone. Final yields of tracer $1\alpha,25\text{-}(OH)_2\text{-}[^3H]D_3$ were 60% in both 1α-OH-D_3 treated and control extracts.

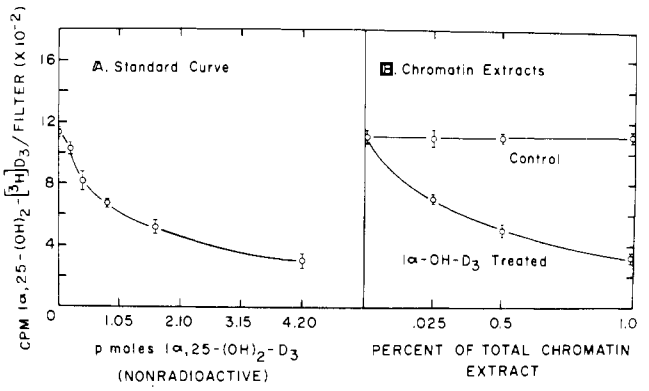

FIGURE 5: Radioreceptor assay of suspected $1\alpha,25\text{-}(OH)_2\text{-}D_3$ isolated from chicks treated with 1α-OH-D_3. (A) Standard curve obtained with authentic nonradioactive $1\alpha,25\text{-}(OH)_2\text{-}D_3$. The details of this assay are presented in the previous paper (Brumbaugh *et al.*, 1974b). (B) Assay of purified chromatin extracts (see Figure 4) from control and 1α-OH-D_3 treated chicks; 5-, 10-, and 20-μl aliquots (in triplicate) of the suspected $1\alpha,25\text{-}(OH)_2\text{-}D_3$ peaks (in 2.0 ml of ethanol) were assayed simultaneously with standard amounts of sterol as depicted in panel A. Each assay was carried out in triplicate and the results are ± standard error of the mean.

ing assay, it competes with the radioactive hormone two–three orders of magnitude less efficiently than the nonradioactive $1\alpha,25\text{-}(OH)_2\text{-}D_3$. Thus, although 1α-OH-D_3 is equipotent to $1\alpha,25\text{-}(OH)_2\text{-}D_3$ in all *in vivo* assays, it is strikingly less active at the molecular level. The data pictured in Figure 3 suggest that 1α-OH-D_3 must be transformed to $1\alpha,25\text{-}(OH)_2\text{-}D_3$ before acting upon the target intestine and eliminate the possibility that the synthetic sterol represents an analog of the hormone with similar binding characteristics.

Proof That 1α-OH-D_3 Is Metabolized to $1\alpha,25\text{-}(OH)_2\text{-}D_3$, in Vivo. In order to show that *in vivo* metabolism of 1α-OH-D_3 occurs as a crucial step in its action to stimulate calcium absorption, it was deemed necessary to demonstrate this metabolic conversion in the chick directly. Since radioactively labeled 1α-OH-D_3 is not currently available, the experiment was carried out with nonradioactive 1α-OH-D_3 and the suspected $1\alpha,25\text{-}(OH)_2\text{-}D_3$ was assayed *via* radioreceptor assay (Brumbaugh *et al.*, 1974a,b). Furthermore, the experiment was set up to measure only that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ which was associated with the intestinal chromatin receptor site and had originated from 1α-OH-D_3 during the 3-hr latent period of action of 1α-OH-D_3. Putative $1\alpha,25\text{-}(OH)_2\text{-}D_3$ fractions were isolated by silicic acid and Celite chromatography from chromatin extracts of control chicks and chicks treated for 3 hr with 1α-OH-D_3. Assessment of loss of nonradioactive $1\alpha,25\text{-}(OH)_2\text{-}D_3$ during the purifications was done by monitoring tracer $1\alpha,25\text{-}(OH)_2\text{-}[^3H]D_3$ added at the beginning of the isolation. Figure 4 pictures the results of purifications on the control and 1α-OH-D_3 treated chromatin extracts. Final recovery of tracer $1\alpha,25\text{-}(OH)_2\text{-}[^3H]D_3$ was 60% in the control extract and 60% in the extract from 1α-OH-D_3 treated chicks. Radioreceptor assay of the Celite purified $1\alpha,25\text{-}(OH)_2\text{-}D_3$ fractions from both chromatin extracts is shown in Figure 5. Figure 5A is the isotope dilution standard curve for authentic nonradioactive $1\alpha,25\text{-}(OH)_2\text{-}D_3$. Figure 5B indicates that the $1\alpha,25\text{-}(OH)\text{-}D_3$ fraction from control chicks produces no deflection in the assay, while the $1\alpha,25\text{-}(OH)_2\text{-}D_3$ fraction from 1α-OH-D_3-treated chicks yields a significant competition for receptor

TABLE II: Production of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ from 1α-OH-D_3 by Intestinal Mucosa *in Vitro*.[a]

1α-OH-D_3 Added[b] (pmol)	Homogenate Pretreatment	Time of Incubation (min)	$1\alpha,25\text{-}(OH)_2\text{-}D_3$ Produced[c] (pmol)	% Conversion
1300	None	0	0	0
1300	None	30	46	3.5
1300	100° for 20 min	30	0	0

[a] Intestinal mucosa from rachitic chicks was homogenized (10%) as previously described (Tucker *et al.*, 1973). Homogenate (0.5 ml/flask) was diluted to 10 ml with phosphate buffer (pH 7.4) and an NADPH generating system and incubations were carried out as described elsewhere (Tucker *et al.*, 1973). [b] Sterol was added in 50 μl of ethanol. [c] Production of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ was determined as previously described (Brumbaugh *et al.*, 1974b). Methanol–chloroform (2:1) extracts of homogenates were purified to isolate suspected $1\alpha,25\text{-}OH_2\text{-}D_3$ by silicic acid, Sephadex LH-20, and Celite chromatography and competitive protein binding assay was used to quantitate the $1\alpha,25\text{-}(OH)_2\text{-}D_3$ product.

binding sites. When the level of 1α,25-$(OH)_2$-D_3 is calculated from the results of Figure 5B (taking into account the purification losses), each chick intestinal chromatin (15 mg of DNA equivalent) contains 26 pmol of 1α,25-$(OH)_2$-D_3 3 hr subsequent to a physiologic dose of 1α-OH-D_3. The saturation value for 1α,25-$(OH)_2$-D_3 in intestinal chromatin following direct administration of labeled hormone has been previously determined to be 25 pmol (Brumbaugh and Haussler, 1974a). Therefore, sufficient 1α,25-$(OH)_2$-D_3 is produced from 1α-OH-D_3 to saturate the physiologic receptors in the intestine. Since this phenomenon occurs prior to the initiation of the calcium transport increases by 1α-OH-D_3, these data strongly suggest that 1α-OH-D_3 functions by metabolism to the natural hormone.

Production of 1α,25-(OH)$_2$-D$_3$ from 1α-OH-D$_3$ in Vitro. In order to further demonstrate the ability of 1α-OH-D_3 to be metabolized to 1α,25-$(OH)_2$-D_3, intestinal mucosa homogenates were prepared and 1300 pmol of 1α-OH-D_3 was added to the mixture (Tucker *et al.*, 1973). The data depicted in Table II illustrate that after 30 min of incubation at 37°, 1α-OH-D_3 is converted to significant amounts of 1α,25-$(OH)_2$-D_3. Moreover, if the homogenate is boiled for 20 min prior to the addition of 1α-OH-D_3, no production of 1α,25-$(OH)_2$-D_3 occurs during the subsequent 30-min incubation. These data provide independent evidence that intestinal vitamin D_3-25-hydroxylase enzyme is capable of catalyzing the conversion of 1α-OH-D_3 to the natural 1α,25-$(OH)_2$-D_3 hormone. The boiled enzyme experiment (Table II) also excludes the possibility that in the *in vivo* experiment described above (Figures 4 and 5) 1α-OH-D_3 is nonenzymatically oxidized to 1α,25-$(OH)_2$-D_3 during extraction and chromatographic isolation of suspected 1α,25-$(OH)_2$-D_3 fractions for radioreceptor assay.

Discussion

The present results confirm and extend earlier investigations of 1α-OH-D_3. This synthetic analog is found to be equivalent to the natural 1α,25-$(OH)_2$-D_3 hormone in experiments, *in vivo*. Since strong evidence is presented that 1α-OH-D_3 acts by metabolism to 1α,25-$(OH)_2$-D_3 (Figures 4 and 5), it is somewhat surprising that 1α-OH-D_3 can function as rapidly as 1α,25-$(OH)_2$-D_3 (Figure 1) and especially puzzling that even at doses as low as 19.5 pmol (0.3 IU), both sterols yield equal biologic responses (Figure 2). Although differential absorption of the sterols might influence the observed temporal relationships and the dose–response relationships and cannot be ruled out at this time, the 25-hydroxylation of 1α-OH-D_3 is apparently rapid as shown by *in vitro* intestinal mucosa studies (Table II). Confirmation of the enzymatic conversion of 1α-OH-D_3 to the natural hormone should also occur when radioactive 1α-OH-D_3 becomes available for study.

Our conclusion that 1α-OH-D_3 functions by metabolism to 1α,25-$(OH)_2$-D_3 is based upon the reliability of certain facets of our experimental protocol and is also dependent upon the biologic significance of the cytosol–chromatin receptor system for 1α,25-$(OH)_2$-D_3 in intestine. Previous reports have documented that Celite liquid–liquid chromatography is the only system capable of unequivocally resolving all known dihydroxyvitamin D metabolites (Haussler and Rasmussen, 1972) and this chromatographic procedure also removes all lipids which might interfere with the radioreceptor assay for 1α,25-$(OH)_2$-D_3 (Brumbaugh *et al.*, 1974b). This fact is further documented in the current data (Figure 5B) where no deflection is obtained in the 1α,25-$(OH)_2$-D_3 assay with suspected 1α,25-$(OH)_2$-D_3 fractions from rachitic chicks not receiving 1α-OH-D_3. The high specificity and selectivity of the radioreceptor assay (Brumbaugh *et al.*, 1974b) add further credibility to our conclusion that the Celite purified material is actually 1α,25-$(OH)_2$-D_3. Taken in combination, the coelution of assayable material with authentic 1α,25-$(OH)_2$-[^{3}H]D_3 from Celite columns and its significant competition in the specific radioreceptor assay indicate that 1α,25-$(OH)_2$-D_3 arises from 1α-OH-D_3 *in vivo*. Absolute proof that this metabolite is 1α,25-$(OH)_2$-D_3 will require physical characterization *via* mass spectrometry.

Moreover, the conclusion that 1α-OH-D_3 must be metabolized to 1α,25-$(OH)_2$-D_3 in order to induce intestinal calcium absorption is dependent upon the validity of the cytosol–chromatin receptor system as a molecular manifestation of the initial events in the action of 1α,25-$(OH)_2$-D_3 in the intestinal cell. The physiologic relevance of this receptor system has been extensively tested in three independent laboratories (Norman *et al.*, 1969; Kodicek, 1974; Brumbaugh and Haussler, 1974a,b) and, to date, no convincing evidence has been published which suggests that this system does not represent the true site of action of the hormone. We therefore suggest that the fact that 1α-OH-D_3 is converted, *in vivo*, to sufficient 1α,25-$(OH)_2$-D_3 to saturate the intestinal chromatin receptors for the hormone within the lag period of the sterol, demonstrates that a mandatory step in the action of 1α-OH-D_3 is its metabolism to the natural hormone. This conclusion, of course, applies only to "physiologic doses" of 1α-OH-D_3 and pharmacologic doses can simulate 1α,25-$(OH)_2$-D_3 by acting directly when present in about 500-fold excess (Figure 3).

From these studies it is possible to predict the activity of 1α-OH-D_3 in several *in vitro* systems for calcium translocation. Assuming that the 1α,25-$(OH)_2$-D_3 receptors in bone have a similar discriminatory affinity between 1α-OH-D_3 and the natural hormone as intestinal receptors, the activity of 1α-OH-D_3 should be about 1/500th that of 1α,25-$(OH)_2$-D_3 in mobilizing calcium from bone tissue culture (Raisz *et al.*, 1972). Although the intestinal organ culture system (Corradino, 1973) might be expected to also display a similar relative responsiveness to the two sterols, the presence of an intestinal vitamin D_3-25-hydroxylase (Table II) will undoubtedly affect the results. Thus, by incubating 1α-OH-D_3 or 1α,25-$(OH)_2$-D_3 with intestines in culture for 48 hr, sufficient 1α,25-$(OH)_2$-D_3 could be produced from 1α-OH-D_3 to cause the sterols to have an apparent equal activity, *in vitro*. This problem of 25-hydroxylation of 1α-OH-D_3 may not occur, however, in shorter duration, perfused intestine experiments (Olson and DeLuca, 1969). Therefore, certain isolated bone and intestinal systems offer a testing ground for the concept that the 1α-OH-D_3 may be approximately two–three orders of magnitude less active than the natural hormone, *in vitro*.

Thus, the present results increase our understanding of the biochemical action of 1α-OH-D_3. Further study of this sterol will require an understanding of its enzymatic conversion to 1α,25-$(OH)_2$-D_3 and ultimately a complete comprehension of the mechanism by which 1α,25-$(OH)_2$-D_3 in turn regulates calcium transport. Knowing that 1α-OH-D_3 acts by conversion to the hormone should assist clinicians in judging the therapeutic regimen of this sterol. Because it requires transformation to 1α,25-$(OH)_2$-D_3, the synthetic sterol may offer a certain protective capacity to patients with renal osteodystrophy and hypoparathyroidism, in that it may not possess the potentially toxic qualities of the direct doses of 1α,25-$(OH)_2$-D_3. Thus, larger doses of the synthetic sterol may be required to regulate calcium balance, but by providing a protective reservoir of hormone precursor, 1α-OH-D_3 could be a superior supplement. Also, since 1α,25-$(OH)_2$-D_3 can now be assayed in patients (Brumbaugh *et al.*, 1974a), assessment can be made of circu-

lating levels of 1α,25-$(OH)_2$-D_3 following treatment with 1α-OH-D_3.

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Primary Structure of the Mcg λ Chain†

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ABSTRACT: The complete primary structure of the Mcg L chain has been determined. Three new amino acid substitutions which occur in the constant region at positions 116, 118, and 167 have been detected. In addition, a substitution of threonine for glycine at position 103 in the variable region in this λ-type chain protein is noted for the first time. The primary sequence of this L chain will be of great value in relating the tertiary structure of the crystalline dimer to its ligand binding activity.

The products of a number of structural genes have been described for the C regions of immunoglobulin L chains (Hilschmann and Craig, 1965; Ein and Fahey, 1967; Milstein *et al.*, 1967; Ein, 1968; Ponstingl and Hilschmann, 1969; Hess *et al.*, 1971; Terry *et al.*, 1969; Gally and Edelman, 1972). Possible evidence for others has been indicated but not fully explored (Milstein, 1967; Milstein *et al.*, 1967). An increase in the number of variations in this region of the L chain will markedly increase the possible numbers of antibody molecules unless some linkage between these substitutions in the constant region exists or unless constant region variations will relate in some manner to variable region sequences. Different amino acid substitutions in the constant region of the L chain may also play a role in their interactions with the C_1 region of the H chain and correlate with different substitutions in this part of

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