Radioligand Receptor Assay for 25-Hydroxyvitamin D_2/D_3 and 1α,25-Dihydroxyvitamin D_2/D_3

APPLICATION TO HYPERVITAMINOSIS D

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ABSTRACT A competitive protein binding assay for measurement of the plasma concentration of 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] has been extended to include the immediate precursor of this hormone, 25-hydroxyvitamin D_3 (25-OHD_3). In addition, the assay system is capable of measuring the two metabolic products of ergocalciferol, namely, 25-hydroxyvitamin D_2 (25-OHD_2) and 1α,25-dihydroxyvitamin D_2 [1α,25-$(OH)_2D_2$]. The target tissue assay system consists of a high affinity cytosol receptor protein that binds the vitamin D metabolites and a limited number of acceptor sites on the nuclear chromatin. By utilizing a series of chromatographic purification steps, a single plasma sample can be assayed for any of the four vitamin D metabolites either individually or combined. Therefore, the assay procedure allows for both the quantitative and qualitative assessment of the total active vitamin D level in a given plasma sample.

To show that the binding assay was capable of measuring 1α,25-$(OH)_2D_2$ as well as 1α,25$(OH)_2D_3$, two groups of rats were raised. One group, supplemented with vitamin D_3, produced assayable material that represented 1α,25-$(OH)_2D_3$. The other group, fed only vitamin D_2 in the diet, yielded plasma containing only 1α,25-$(OH)_2D_2$ as the hormonal form of the vitamin. The circulating concentrations of the two active sterols were nearly identical (15 ng/100 ml) in both groups, indicating that the competitive binding assay can be used to measure both hormonal forms in plasma.

In a separate experiment, 1α,25-$(OH)_2D_2$ was generated in an in vitro kidney homogenate system using 25-OHD_2 as substrate. Comparison of this sterol with 1α,25-$(OH)_2D_3$ in the assay system showed very similar binding curves; the D_2 form was slightly less efficient (77%). Comparison of the respective 25-hydroxy forms (25-OHD_2 vs. 25-OHD_3) at concentrations 500-fold that of 1α,25-$(OH)_2D_3$, again suggested that the binding of the D_2 metabolite was slightly less efficient (71%).

Finally, the assay was employed to measure the total active vitamin D metabolite pools in the plasma of normal subjects and patients with varying degrees of hypervitaminosis D. The normal plasma levels of 25-OHD and 1α,25-$(OH)_2D$ measured in Tucson adults were 25–40 ng/ml and 2.1–4.5 ng/100 ml, respectively. Both sterols were predominately (> 90%) in the form of vitamin D_3 metabolites in this environment. Typical cases of hypervitaminosis D exhibited approximately a 15-fold increase in the plasma 25-OHD concentration, and a dramatic changeover to virtually all metabolites existing in the form of D_2 vitamins. In contrast, the circulating concentration of 1α,25-$(OH)_2D$ was not substantially enhanced in vitamin D-intoxicated patients. We therefore conclude that hypervitaminosis D is not a result of abnormal plasma levels of 1α,25-$(OH)_2D$ but may be caused by an excessive circulating concentration of 25-OHD.

INTRODUCTION

It is now well established that 25-hydroxyvitamin D_3 (25-OHD_3)[1] and 1α,25-dihydroxyvitamin D_3 [1α,25-

[1] *Abbreviations used in this paper:* 25-OHD_2, 25-hydroxyvitamin D_2 or 25-hydroxyergocalciferol; 25-OHD_3, 25-hydroxyvitamin D_3 or 25-hydroxycholecalciferol; 1α,25-$(OH)_2D_2$, 1α,25-dihydroxyvitamin D_2; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3. When no subscript is present after the D, both D_2 and D_3 are implied.

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$(OH)_2D_3$] are two metabolites of the native vitamin involved in regulating calcium and phosphorus metabolism (1). Formation of 25-OHD_3 occurs in the liver, intestine, and kidney (2), and subsequent activation of the sterol to 1α,25-$(OH)_2D_3$ occurs exclusively in the kidney (3), producing what is considered to be the hormonal form of the vitamin. 1α,25-$(OH)_2D_3$ has been shown to be the most active metabolite of vitamin D in stimulating intestinal calcium transport (4) and bone calcium mobilization (5). Although the exact sequence of events that mediate the effect of 1α,25-$(OH)_2D_3$ on its target tissue is not yet completely understood, an early step has been shown to be the interaction between the hormone and a specific macromolcular component in the target tissue cytoplasm (6). The hormone-receptor complex subsequently translocates to the nuclear compartment where it attaches to specific acceptor sites (6–8). Our laboratory has utilized this series of events to develop a sensitive competitive binding assay for the hormonal form of vitamin D (9). The method utilizes a saturable, reconstituted receptor system consisting of intestinal mucosa cytosol and nuclear chromatin. An isotope-dilution curve is obtained by incubating reconstituted cytosol-chromatin and [^{3}H] 1α,25-$(OH)_2D_3$ with increasing amounts of nonradioactive 1α,25-$(OH)_2D_3$ and then separating the free and bound sterol by trapping the sterol-receptor-chromatin supercomplex on glass fiber filters. This technique has been shown to be a reliable and valid method for quantitation of hormone concentrations as low as 1.0 ng/100 ml plasma (10).

The present study was undertaken for the following reasons: (*a*) to determine if the presently available chromatography methods could separate vitamin D_3 from vitamin D_2 metabolites, (*b*) to ascertain if the discrimination against ergocalciferol metabolites by the chick is at the level of the sterol's target tissue receptor, (*c*) to extend the 1α,25-$(OH)_2D_3$ assay (9, 10) to incorporate 25-hydroxyvitamin D_2 (25-OHD_2), 25-OHD_3, and 1α,25-dihydroxyvitamin D_2 [1α,25-$(OH)_2D_2$], thus developing a single method for the qualitative and quantitative assessment of these biologically active forms of vitamin D and, (*d*) to measure these metabolites in hypervitaminosis D patients, possibly allowing a more complete delineation of the disorder.

METHODS

Animals. Chicks used were White Leghorn cockerels (donated by Demler Farms, Anaheim, Calif.) that were raised on a vitamin D-deficient diet (11) for 3–4 wk. Male weanling Holtzman rats were used in the experiment generating 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_2$ in vivo.

Sterols. Crystalline 25-OHD_2 and 25-OHD_3 were a generous gift from Dr. Jack Hinman and Dr. John Babcock of the Upjohn Company, Kalamazoo, Mich. [26,27-*methyl*-^{3}H]-25-OHD_3 (6.5 Ci/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. [26,27-*methyl*-^{3}H]1α,25-$(OH)_2D_3$ (6.5 Ci/mmol) was produced in vitro, by a modification (12) of the method of Lawson et al. (13) and purified by column chromatography as previously described (7, 14). Nonradioactive 1α,25-$(OH)_2D_3$ was either prepared and purified via similar procedures (12) or obtained as the crystalline, synthetic compound from Dr. M. Uskokovic of Hoffman-LaRoche Inc., Nutley, N. J. Biologically generated and synthetic nonradioactive 1α,25-$(OH)_2D_3$ yielded identical quantitative competition in the binding assay, verifying the equivalency of sterol from these two sources. Nonradioactive 1α,25-$(OH)_2D_2$ was generated in vitro in a similar fashion (12). Sterols were quantitated by ultraviolet absorption spectrophotometry and stored in distilled ethanol at -20°C.

Chromatography. After addition of 2,000 cpm each of tritiated 25-OHD_3 and 1α,25-$(OH)_2D_3$ (6.5 Ci/mmol) to the plasma and extraction of sterols as described elsewhere (10), chromatographic procedures were carried out as follows. (See Fig. 1 for schematic diagram.) Sephadex LH-20 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) column chromatography was performed as described previously (10). One 1×15-cm (5 g) column resulted in the separation of 25-OHD from 1α,25-$(OH)_2$D; 25-OHD emerged between 0 and 40 ml elution volume, whereas 1α,25-$(OH)_2$D eluted between 40 and 95 ml. Throughout the remainder of the purification scheme the sterols were treated separately. 25-OHD was purified on silicic acid (Bio-Rad Laboratories, Richmond, Calif., minus 325 mesh; activated by heating at 120°C for 24 h in a vacuum oven) by eluting first with 20 ml hexane-diethyl ether (1:1) and then with 20 ml diethyl ether; 25-OHD emerged in the second fraction with the ether. 1α,25-$(OH)_2$D was purified similarly on silicic acid by elution with diethyl ether (10 ml) and then acetone (8 ml); the hormone emerged in the second fraction with the acetone. The entire procedure was miniaturized to 0.8×6.4-cm columns to provide a very rapid and simple means of removing contaminating lipid. Final purification of the sterols was accomplished by Celite (Johns-Manville, New York) liquid-liquid partition chromatography (14). The solvent system (System I) used for 25-OHD was 100% hexane (mobile) and 15% water in methanol (stationary); the solvent system (System II) for the purification of 1α,25-$(OH)_2$D was 10% ethyl acetate in hexane (mobile) and 45% water in ethanol (stationary). Celite chromatography was miniaturized to a "micro" scale (0.8×8.0 cm), when rapid purification without separating D_3 and D_2 forms was desired (Fig. 1B, D). 10-ml glass pipettes, fitted with glass wool plugs, serve as columns. Elution was carried out with mobile phase, and each sterol emerged between 7 and 22 ml on its respective column. When 12 samples were processed simultaneously, the total time from the initial drawing of the blood to a purified assayable sample is approximately 2 h/sample per technician using the micro-silicic acid and micro-Celite chromatography method (Fig. 1B, D). If it is necessary to separate 25-OHD_2 from 25-OHD_3 and 1α,25-$(OH)_2D_2$ from 1α,25-$(OH)_2D_3$, a longer (1×35-cm) column (with 5-ml fractions) was required (Fig. 1A, C). A slow flow rate (15 min/fraction) produced defined peaks with minimal overlap. When sample purification on a successive series of Sephadex LH-20, silicic acid, and Celite chromatography was utilized, any possibility of cross-contamination between the mono- and dihydroxy-metabolites was avoided (14). All samples for sterol assay were evaporated under nitrogen and solubilized in 400 μl ethanol. After determination of yield by counting an aliquot of each sample for tritium (yields range from 40 to 80%), radioreceptor assays were performed on 100-μl portions. Triplicate assays

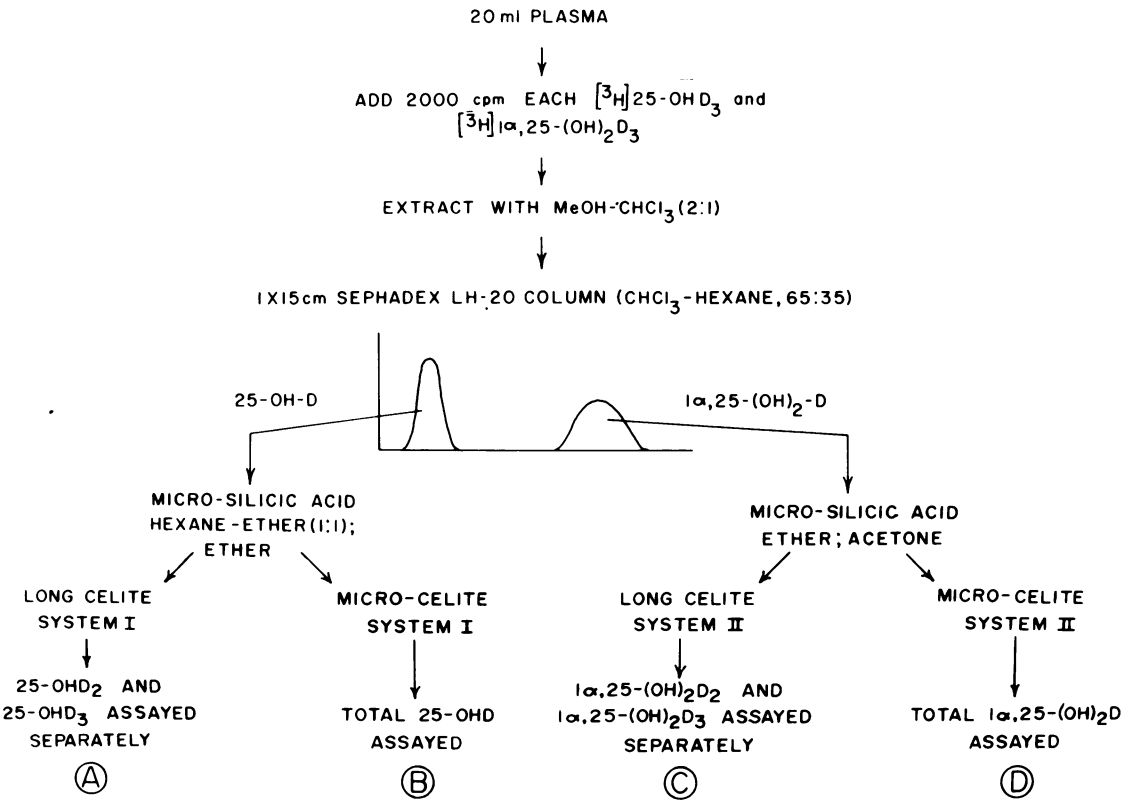

FIGURE 1 Purification scheme for 25-OHD$_2$ and(or) 25-OHD$_3$ (A, B), and 1α,25-(OH)$_2$D$_2$ and(or) 1α,25-(OH)$_2$D$_3$ (C, D). For a complete vitamin D metabolite profile of the plasma, pathways A and C are used. When distinctions between D$_2$ and D$_3$ metabolites are not required, the micro-Celite is employed as a final purification step (pathways B and D) and results are expressed as the "total" D-metabolite concentration. All assays are run in triplicate. Extraction of plasma is carried out as described elsewhere (10). Celite solvent systems I and II and other details are described under Methods.

were carried out on all samples in the present study, and interassay variation was 10–15%. All solvent used in chromatographic procedures and sterol storage were reagent grade and glass distilled before use.

Isolation of subcellular fractions. Intestinal mucosa (6 g) from two rachitic chicks was homogenized in 25 ml of 0.25 M sucrose in 0.05 M Tris-HCl (pH 7.4), 0.025 M KCl, and 0.005 M $MgCl_2$. Cytosol fraction was obtained by centrifugation at 100,000 *g* for 1 h. Chromatin was prepared from crude nuclei (isolated from original homogenate by centrifugation at 1,000 *g* for 10 min) by homogenizing successively in one 25-ml portion of 0.8 mM EDTA, 25 mM NaCl, pH 8; one 25-ml portion of 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), 0.01 M Tris-HCl, pH 7.5; and one 25-ml portion of 0.01 M Tris-HCl, pH 7.5. The chromatin was harvested by sedimentation at 30,000 *g* for 10 min after each wash. The entire chromatin pellet from 6 g of mucosa was reconstituted with the cytosol fraction by homogenization to create a cytosol-chromatin receptor system for the competitive binding assay. The reconstituted mixture was prepared fresh daily and immediately before use. All operations were performed at 0–4°C.

Binding assay. To each assay tube containing [^{3}H]1α,25-(OH)$_2$D$_3$ and unlabeled sterol (dried together with a stream of nitrogen) were added 20 μl of distilled ethanol and 200 μl of reconstituted cytosol-chromatin system. The ethanol that was added just before the receptor system aids in solubilizing the sterols to achieve a higher binding efficiency and reduces nonspecific binding; this represents a modification of our earlier procedures (9, 10). The final concentration of [^{3}H]1α,25-(OH)$_2$D$_3$ was 4.3 nM. After incubation, for 20 min at 25°C, the quantity of labeled sterol bound to chromatin was determined by filtration (Millipore Corp., Bedford, Mass., 30-place manifold). 1 ml of cold 1% Triton X-100 in 0.01 M Tris (pH 7.5) was added to each assay tube, and the entire mixture was applied to a Type A or A/E glass fiber filter (Gelman Instrument Co., Ann Arbor, Mich.) at very low vacuum. After 2–4 min the vacuum was increased to achieve uniform flow rates of approximately 1 ml/min, and each of the filters was washed with 2 ml of 1% Triton X-100, 0.01 M Tris, pH 7.5. After filtration, the filters were placed in liquid scintillation vials with 5 ml of methanol-chloroform (2:1, vol/vol). After 20 min the methanol-chloroform was evaporated on a hot plate under a stream of air. Without removal of the glass filter, sterols were solubilized in 5 ml of toluene counting solution consisting of 4 g of PPO and 50 mg of POPOP per liter of toluene. Samples were counted in a refrigerated Beckman LS-233 liquid scintillation spectrometer (Beckman Instruments, Inc., Scientific Instruments Div., Irvine,

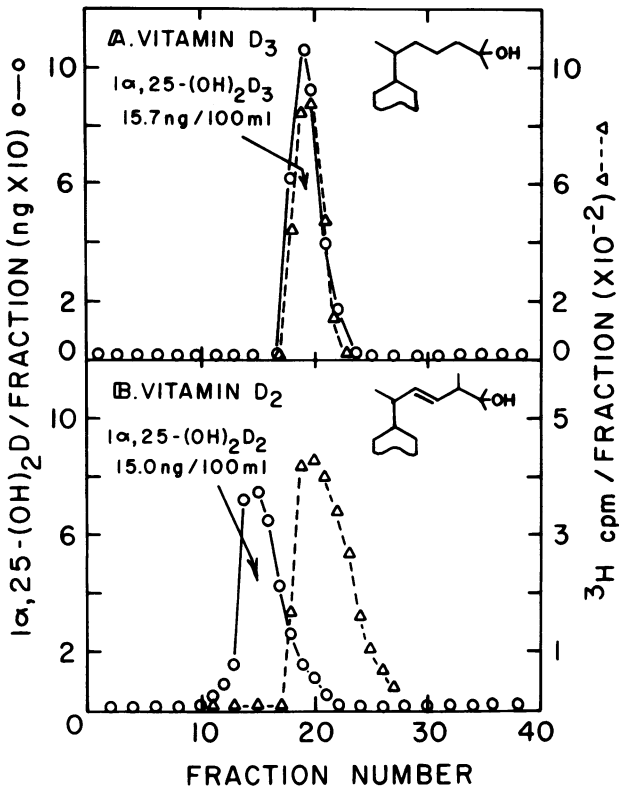

FIGURE 2 Celite chromatography (1 × 35 cm; System II) of purified plasma from rats fed. (A) Vitamin D_3, or (B) vitamin D_2. [^{3}H]1α,25-$(OH)_2D_3$ (4,200 cpm; 6.5 Ci/mmol) was added to each pooled plasma sample from 12–15 rats (32 ml) and purified for 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ as outlined under Methods and in Fig. 1, pathway C. Recovery of tritium after extraction and chromatography was 63% for D_3-fed animals and 68% for D_2-fed animals. The radioreceptor assay was used to quantitate 1α,25-$(OH)_2D$ in each 5-ml fraction the final Celite column (○). Migration of [^{3}H]1α,25-$(OH)_2D_3$ marker (△) was determined by counting an aliquot of each fraction.

Calif). Efficiency for tritium was 48% and was identical for all counted samples. Routinely, 2,000–3,000 cpm of the approximately 6,000 cpm of [^{3}H]1α,25-$(OH)_2D_3$ present in the incubation are recovered in the chromatin in the absence of competing nonradioactive sterol (33–50% binding efficiency). Other aspects of the validity, sensitivity, specificity, and accuracy of this assay are described elsewhere (10, 15).

Patients. For routine assays, blood was drawn from healthy volunteers (ages 20–50 yr) in Tucson, Ariz. In addition, blood was obtained from three patients receiving high doses of vitamin D_2. The first patient (A) was a 59-yr-old man with osteomalacia due to unexplained vitamin D resistance. He had been receiving as much as 500,000 IU vitamin D_2/day, but at the time of this study he was receiving 250,000 IU D_2/day. The serum calcium was 14.0 mg/100 ml (N = 9.5–10.5 mg/100 ml), serum phosphorus was 4.6 mg/100 ml (N = 3.0–4.5 mg/100 ml), alkaline phosphatase was 165 U (N = 5-13 U), and serum magnesium was normal. Bone biopsy revealed osteomalacia (unknown etiology) with increased osteoid seams. The second patient (B) was a 47-yr-old woman with postsurgical hypoparathyroidism receiving 150,000 IU D_2/day. Her serum calcium was 11.1 mg/100 ml and phosphorus was 3.2 mg/100 ml. The third patient (C) was a 60-yr-old man with postsurgical hypoparathyroidism. This subject was receiving 100,000 IU D_2/day but did not exhibit vitamin D intoxication since his serum calcium was 9.6 mg/100 ml and serum phosphorus was 3.2 mg/100 ml. All patients receiving vitamin D_2 had normal creatinine clearance within 1 mo of blood drawing.

RESULTS

Rat in vivo generation of 1α,25-$(OH)_2D$. To determine if the 1α,25-$(OH)_2D_3$ assay of Brumbaugh et al. (10) also measures 1α,25-$(OH)_2D_2$, both sterols were generated in weanling rats in vivo. Rats were chosen for the study because it is thought that these animals do not discriminate between cholecalciferol and ergocalciferol as does the chick (16). Two groups of animals were raised for 3 wk on a normal calcium (0.6%) and phosphorus (0.6%) diet. Both groups were treated identically except that the diet of one group was supplemented with vitamin D_3 (2 IU/g diet), whereas, the second group received only vitamin D_2 (2 IU/g) in the diet. Blood was obtained by cardiac puncture, pooled within each group, and subjected to chromatographic purification. Tracer [^{3}H] 1α,25-$(OH)_2D_3$ (4,200 cpm) was added to the samples to quantitate losses and serve as a column marker for authentic hormone. After lipid extraction with methanol-chloroform (2:1) and Sephadex LH-20 chromatography (see Methods), the extracts were further purified on a 35-cm Celite column, and individual fractions were assayed for 1α,25-$(OH)_2D$ binding activity (Fig. 2). As shown in Fig. 2A, the binding activity of the plasma extract from vitamin D_3-fed animals migrated exactly with the added [^{3}H] 1α,25-$(OH)_2D_3$ tracer. The total assayable 1α,25-$(OH)_2D_3$ in the plasma sample corresponds to 15.7 ng/100 ml of plasma (0.4 nM), and this value correlates well with previously reported (17) concentrations of the hormone in normal rat plasma. Fig. 2B depicts the Celite profile of the pooled plasma sample from vitamin D_2-fed rats. The assayable material does not migrate with authentic 1α,25-$(OH)_2D_3$, and the value of 15.0 ng/100 ml correlates well with 15.7 ng/100 ml obtained in Fig. 2A. Taken in combination with the fact that vitamin D_3 and vitamin D_2 are believed to be metabolized equally in the rat, the data suggest that the assayable metabolite (Fig. 2B) is 1α,25-$(OH)_2D_2$. This preliminary identification will be supported (below) by the fact that the elution position of this metabolite corresponds with the elution position of 1α,25-$(OH)_2D_2$ that was generated in vitro from authentic, crystalline 25-OHD_2. In addition, the data of Fig. 2 which illustrate the near equivalency of levels of the two generated sterols as assayed by the chick intestinal receptor system, indicate that the binding pro-

tein does not discriminate between them but rather binds 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_2$ with approximately the same affinity.

In vitro generation of 1α,25-$(OH)_2D_2$. In the next experiment, a more direct approach was undertaken to generate 1α,25-$(OH)_2D_2$. Since the kidney is the sole location of the 1α-hydroxylase enzyme that produces the hormonal form of the vitamin, renal tissue from 4-wk-old, severely rachitic chicks (12 animals, plasma $Ca^{++} \leq 5.5$ mg%) was removed. Kidney homogenates were incubated in a phosphate buffer and NADPH generating system as previously described (2, 12). The reaction was initiated with 4,500 IU (292.5 nmol) crystalline 25-OHD_2 and was allowed to proceed under air at 37°C for 2 h; the generated dihydroxy-sterol was purified on silicic acid, Sephadex LH-20, and Celite and finally quantitated by ultraviolet absorption spectrophotometry. These identical conditions are routinely employed for the production of 1α,25-$(OH)_2D_3$ from 25-OHD_3; it is reasonable that the substrate 25-OHD_2 will produce 1α,25-$(OH)_2D_2$ in this system. Moreover, the sterol displayed a uv spectrum with a minimum at 228 nm and a maximum at 265 nm which is characteristic of 1α,25-$(OH)_2D$. In addition, the dihydroxy-metabolite migrated on Celite to the same position (fraction 14) as the in vivo-produced sterol of the previous experiment.

Competitive binding of 1α,25-$(OH)_2D_2$ and 25-OHD_2. To verify that the target tissue receptor system efficiently binds 1α,25-$(OH)_2D_2$, the in vitro-generated sterol was added to the reconstituted cytosol-chromatin system and allowed to compete with [^{3}H] 1α,25-$(OH)_2D_3$ for the receptor. Fig. 3A illustrates the competitive binding curves obtained when either 1α,25-$(OH)_2D_3$ or 1α,25-$(OH)_2D_2$ is the competing sterol. Results indicate that 1α,25-$(OH)_2D_3$ is approximately 1.3 times more efficient than the dihydroxyergocalciferol metabolite. Nevertheless, the assay receptor system can bind and therefore detect both the D_2 and D_3 forms of the hormone.

In the next experiment 25-OHD binding to the receptor was explored. Nonradioactive 25-OHD_3 is approximately 500 times less effective than nonradioactive 1α,25-$(OH)_2D_3$ in the ability to displace [^{3}H] 1α,25-$(OH)_2D_3$ from its intestinal receptor (18). Therefore, if cytosol-chromatin is incubated with the standard amount (360 pg) of [^{3}H] 1α,25-$(OH)_2D_3$, a 500-fold excess (180 ng) of 25-OHD_3 will result in 50% competition at the receptor (Fig. 3B). Since the dihydroxy-D_2 metabolite was capable of binding (Fig. 3A), the monohydroxy-D_2 metabolite (25-OHD_2) was also tested for its competitive ability. Fig. 3B illustrates that nanogram quantities of both 25-OHD_3 and 25-OHD_2 are capable of competing with picogram amounts of [^{3}H] 1α,25-$(OH)_2D_3$ but that 25-OHD_3 is approximately 1.4 times more efficient. This is consistent with the results of Fig. 3A, indicating that

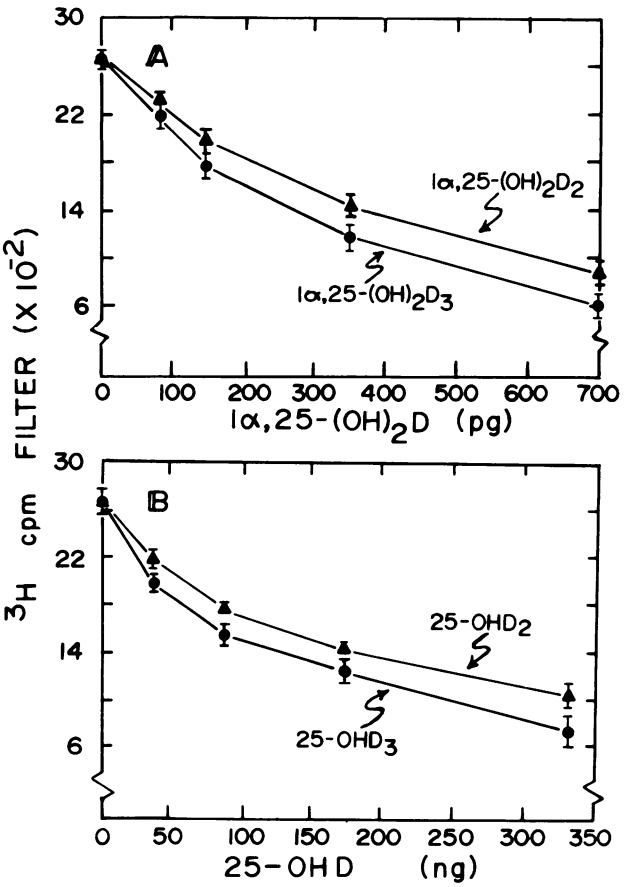

FIGURE 3 Competitive binding standard curves for 1α,25-$(OH)_2D_3$ or 1α,25-$(OH)_2D_2$ (A), and 25-OHD_3 or 25-OHD_2 (B). A sample of [^{3}H]1α,25-$(OH)_2D_3$ (360 pg; 6.5 Ci/mmol) was incubated with increasing amounts of nonradioactive sterol in the reconstituted cytosol-receptor system. The amount of bound tritiated compound (as determined by filtration) is plotted as a function of the amount of nonradioactive sterol in the incubation mixture. Each point represents the average of quadruplicate assays±SEM.

cholecalciferol metabolites bind slightly better than ergocalciferol metabolites. Therefore, standard competitive binding curves can be obtained for all four of the sterols of interest, and this allows for the measurement of these metabolites in plasma.

Isolation and measurement of plasma vitamin D metabolites. Before the circulating concentration of these metabolites can be measured by the competitive binding method, the plasma sample must first be purified to separate the sterols of interest and remove contaminating lipid. Fig. 1 is a schematic diagram depicting the purification process and the chromatographic options that are employed in the isolation of these biologically active vitamin D sterols from plasma. A single 20-ml plasma sample (with added radioactive tracers to quantitate purification yields) is processed in such a way that the

25-hydroxymetabolites are separated from the 1α,25-dihydroxy metabolites on Sephadex LH-20 (Fig. 1). Each of the two groups are then purified on micro silicic acid columns using elution systems appropriate for the particular metabolite (see Methods). Final resolution of 25-OHD$_3$ from 25-OHD$_2$ and 1α,25-(OH)$_2$D$_3$ from 1α,25-(OH)$_2$D$_2$ is achieved by Celite liquid-liquid partition chromatography on 1 × 35-cm columns (Fig. 1, pathways A and C). Subsequent radioreceptor assay of the separated sterols results in a complete metabolite profile of the four most biologically active forms of vitamin D in the circulation.

In many instances (see Discussion) it is not necessary to distinguish between the D$_2$ and D$_3$ metabolite concentrations. Consequently, a simplified version of the purification scheme has been developed that utilizes a "micro" (0.8 × 8.0-cm) Celite column that does not resolve D$_2$ from D$_3$ metabolites (Fig. 1, pathways B and D). To demonstrate the inability of the micro-Celite system to separate the two hormonal sterols, rats, which apparently metabolize D$_2$ and D$_3$ equivalently, were raised on either vitamin D$_2$ or D$_3$ in a similar manner to the experiment illustrated in Fig. 2. After purification (as outlined in Fig. 1D) the average 1α,25-(OH)$_2$D in the D$_2$- and D$_3$-raised rats was 18.8±2.0 ng/100 ml (SD) ($n = 3$; 30 total rats) and 17.1±3.0 ng/100 ml (SD) ($n = 6$; 60 total rats), respectively. Thus, the micro-Celite chromatography system is necessary to remove contaminating

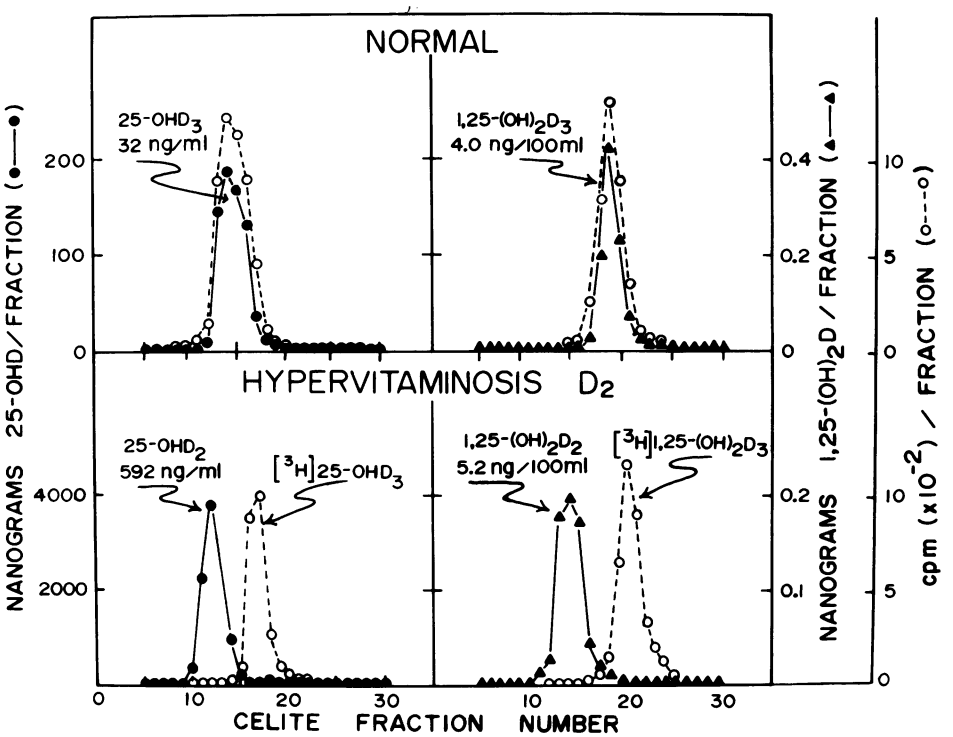

FIGURE 4 Celite resolution and radioligand receptor assay of D metabolites in normal subjects and in patient A with hypervitaminosis D. All Celite columns were 1 × 35 cm and 5-ml fractions were collected. The plasma from normals and a hypervitaminosis D patient was purified as outlined in Fig 1A and C and as described under Methods. The plasma from the normal subjects (four donors; 40 ml) yielded assayable 25-OHD$_3$ (●), which migrates with authentic [^{3}H]25-OHD$_3$ (○) in system I solvents (top left), and 1α,25-(OH)$_2$D$_3$, (▲) which migrates with [^{3}H]1α,25-(OH)$_2$D$_3$ (○) in system II solvents (top right). The plasma (23 ml) from the hypervitaminosis D patient (patient A; see Methods) yielded assayable material that does not migrate with authentic [^{3}H]25-OHD$_3$ in system I solvents (lower left), or [^{3}H]-1α,25-(OH)$_2$D$_3$ in system II solvents (lower right). These assayable peaks (fractions 12 and 14, respectively) migrate to the exact positions of crystalline 25-OHD$_2$ and in vitro generated 1α,25-(OH)$_2$D$_2$ (see Results). Purification yields were as follows: pooled normal subjects = 49% 25-OHD$_3$ and 56% 1α,25-(OH)$_2$D$_3$; hypervitaminosis D$_2$ subject = 76% 25-OHD$_3$ and 60% 1α,25-(OH)$_2$D$_3$. In calculating the 25-OHD$_2$ and 1α,25-(OH)$_2$D$_2$ plasma concentrations the factors 1.4 and 1.3, respectively, are included since the assay standard curves are obtained using the respective D$_3$ metabolites (see text). Yields are assumed to be identical for D$_2$ and D$_3$ forms.

lipid from 1α,25-$(OH)_2D$ and permit valid radioreceptor assay (10), but the column yields a mixture of 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$. This column greatly simplifies the final purification step before assay and allows for the routine processing of numerous samples to yield "total" 25-OHD and 1α,25-$(OH)_2D$ concentrations in plasma.

With the use of this technique, the biologically active metabolites of vitamin D have been measured in humans. Normal plasma 1α,25-$(OH)_2D$ in humans (78 subjects) is 3.3±0.6 ng/100 ml (SD) or approximately 0.1 nM. Utilizing 2 SD in either direction from the average, we obtain a normal range of 2.1–4.5 ng/100 ml for 95% of all normals assayed. The concentration of 25-OHD in normal human plasma as measured by this assay is 25–40 ng/ml and this value correlates well with data obtained by other assay techniques (19–21).

Determination of D_2 vs. D_3 forms in the human and application of assay to hypervitaminosis D. The vitamin D metabolite assay, coupled with long Celite columns (Fig. 1, pathways A and C), was next used to monitor the plasma concentrations of 25-OHD_2, 25-OHD_3, 1α,25-$(OH)_2D_2$, and 1α,25-$(OH)_2D_3$ in healthy human subjects and patients with varying degrees of vitamin D intoxication (Figs. 4 and 5). All of the assayable 25-OHD in normal human plasma migrates with authentic [^{3}H] 25-OHD_3 at a concentration of 32 ng/ml (Fig. 4, top left); 25-OHD_2 was undetectable.[2] When the 1α,25-$(OH)_2D$ concentration was examined (Fig. 4, top right) all of the assayable material migrated with [^{3}H] 1α,25-$(OH)_2D_3$; 1α,25-$(OH)_2D_2$ was undetectable[2] in these normal volunteers. A circulating level of 4.0 ng/100 ml was obtained when the column peak fractions were assayed. Therefore, Celite preparative chromatography and the radioreceptor assay has allowed for the measurement of both 25-OHD and 1α,25-$(OH)_2D$ in human plasma and demonstrates that greater than 90%[2] of the circulating concentration in the normal subject is in the form of cholecalciferol metabolites.

In contrast to these normal values, the assayed plasma from a hypervitaminosis D patient (Methods, subject A) contained 592 ng/ml 25-OHD (Fig. 4, lower left), and all of the assayable material migrated on the Celite column (fractions 10–15) as 25-OHD_2. Similarly, the only measurable hormone concentration in the plasma of this hypervitaminosis D patient was also the ergocalciferol metabolite; the 1α,25-$(OH)_2D_2$ concentration was determined to be 5.2 ng/100 ml of plasma and 1α,25-$(OH)_2D_3$ was undetectable (Fig. 4, lower right). This value is only slightly above the normal human range of 2.1–4.5 ng/100 ml, whereas the assay value for 25-OHD of 592 ng/ml represents more than a 15-fold increase over the normal human subjects. The plasma from two additional patients with postsurgical hypoparathyroidism, who were receiving large doses of vitamin D_2 (patients B and C) was also studied (Fig. 5). The circulating concentration of 25-OHD was elevated 10- to 16-fold above normal values (Fig. 5, left panels) while the 1α,25-$(OH)_2D$ concentration only slightly increased (Fig. 5, right panels). It is possible that the mass action effect of the elevated 25-OHD substrate on the renal 25-hydroxy-1α-hydroxylase may account for the slight increase seen in the hormone's concentration. In any case, the results in Figs. 4 and 5 indicate that vitamin D-intoxicated patients with intact kidneys maintain near normal hormone levels in the form of 1α,25-$(OH)_2D_2$, but display highly elevated circulating concentrations of 25-OHD_2.

DISCUSSION

Elucidation of the chick intestinal cytoplasmic and nuclear receptor system for 1α,25-$(OH)_2D_3$ by Brumbaugh and Haussler (6–8) has allowed for the development of a competitive binding radioreceptor assay for this hormone (9, 10). The value obtained by this assay for the circulating concentration of 1α,25-$(OH)_2D$ in normal humans (0.1 nM; see reference 10) has recently been confirmed by Hill et al. (23) using a bioassay. However, several questions remained regarding the applicability of the radioreceptor assay to the measurement of active vitamin D metabolites in animals and patients. Most notable of these was the uncertainty as to whether 1α,25-$(OH)_2D_2$ was also detected by the radioreceptor assay. Haddad and Hahn (22) have shown that patients treated with therapeutic doses of vitamin D_2 have a significant plasma concentration of 25-OHD_2. Since many human disorders such as hypoparathyroidism, renal osteodystrophy, and vitamin D-resistant rickets are routinely treated with ergocalciferol, the measurement of 1α,25-$(OH)_2D_2$ is critical to the assessment of the vitamin D status in these cases. Because the binding system utilized for the radioreceptor assay is derived from the chick intestine and the chick is known to discriminate against the D_2 vitamins (16), it was essential to determine directly the effectiveness of 1α,25-$(OH)_2D_2$ binding to the intestinal receptor. Furthermore, in the purification scheme of Brumbaugh et al. (10) it was not known whether 1α,25-$(OH)_2D_2$ was resolved from 1α,25-$(OH)_2D_3$. Thus, 1α,25-$(OH)_2D_2$ could have been either excluded completely from measurement by the chromatography or quantitated in combination with the 1α,25-$(OH)_2D_3$.

[2] The sensitivity of the dual assay is 17 pg of 1α,25-$(OH)_2D$ hormone (10) and 8.5 ng of precursor 25-OHD. With these values, if a 20-ml plasma sample is purified to a 50% sterol yield, it can be calculated that > 90% of the circulating vitamin D metabolites in a normal (Tucson) human will be in the D_3 form. This value correlates well with the results of Haddad and Hahn (22) who measured 25-OHD in normal (St. Louis, Mo.) subjects.

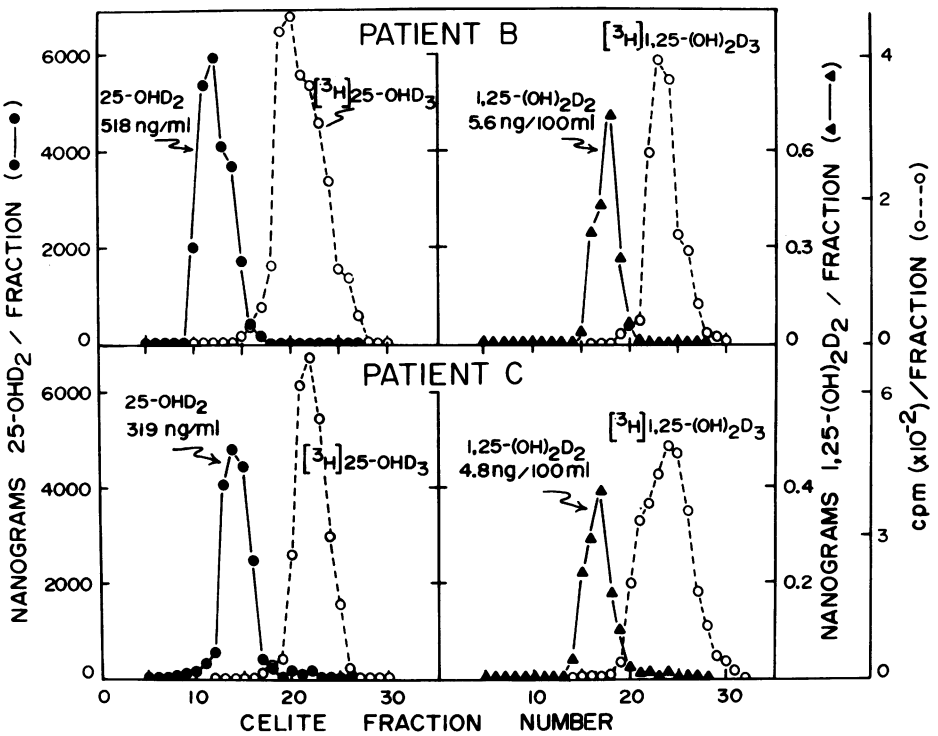

FIGURE 5 Celite resolution and radioligand receptor assay of D metabolites in two patients with postsurgical hypoparathyroidism treated with large doses of vitamin D_2. The assayable material (●) from an additional D_2-intoxicated subject (patient B) as well as the material from a patient with normal serum calcium on a high dose of vitamin D_2 (patient C; see Methods) does not migrate with authentic [^{3}H]25-OHD_3 in system I solvents (left panels), or with [^{3}H]1α,25-$(OH)_2D_3$ in system II solvents (right panels). The plasma was processed according to Fig. 1 (pathways A and C) and concentrations calculated as described in the legend to Fig. 4. Celite columns were 1 × 35 cm and 5-ml fractions were collected. Plasma volume and purification yields were as follows: patient B (top panels), 22 ml, 49.5% 25-OHD_3, and 68% 1α,25-$(OH)_2D_3$; patient C (lower panels), 22 ml, 78.5% 25-OHD_3, and 43.5% 1α,25-$(OH)_2D_3$.

The present results (Figs. 2 and 3) clearly demonstrate that the chick receptor system binds 1α,25-$(OH)_2D_2$ with an efficiency approximating that of 1α,25-$(OH)_2D_3$ and sufficient to allow quantitation of this ergocalciferol metabolite in rats and man (Figs. 2, 4, and 5). Moreover, 1 × 35-cm Celite liquid-liquid partition columns are capable of separating 1α,25-$(OH)_2D_2$ from 1α,25-$(OH)_2D_3$. This represents the first reported chromatographic resolution of these two hormonal sterols.[3] Therefore, to determine separately 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ in a given plasma sample, a 1 × 35-cm Celite column must be used in the final chromatographic step before the radioligand receptor assay. Since Brumbaugh et al. (10) employed micro-Celite columns similar to those used in some of the current experiments (Fig. 1B and D) and these small columns do not resolve 1α,25-$(OH)_2D_2$ from 1α,25-$(OH)_2D_3$, the original radioreceptor assay for 1α,25-$(OH)_2D_3$ actually measures total 1α,25-$(OH)_2D$.

Supportive information concerning the possible mechanism (24) of discrimination by the chick against the D_2 vitamins is also obtained in the current study. Both 25-OHD_2 and 1α,25-$(OH)_2D_2$ bind to the intestinal receptor with slightly lower affinity than their D_3 counterparts (Fig. 3). While this is consistent with a lower biologic potency of vitamin D_2 in the chick, the magnitude of the binding difference (1.3- to 1.4-fold) is markedly less than the 10-fold difference in biologic activities between vitamin D_2 and D_3 in the chick (16). Also, Belsey et al. (24) have observed that the chick plasma binding protein for 25-OHD_3 favors this sterol over 25-OHD_2 by approximately a factor of 10. This discrimination by the plasma binding protein can explain the relative ineffectiveness of vitamin D_2 in the chick, although other factors, such as an altered rate of vitamin D_2 metabolism, may play a part. Clearly, only a small portion of the

[3] Since the original submission of this manuscript, G. Jones and H. F. DeLuca reported (*J. Lipid Res.* **16**: 448–453, 1975) the resolution of 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_2$ via high-speed liquid chromatography.

discrimination against D_2 can be accounted for by the slightly lower affinity of D_2 metabolites for the intestinal receptor system.

Another extension of the radioreceptor assay for 1α,25-$(OH)_2D_3$ which is reported here is the application of this binding system to the measurement of circulating 25-OHD_3 and 25-OHD_2. As reported previously (18), this receptor will bind 25-OHD_3 but only when this sterol is present in concentrations in 500-fold excess of that required for hormone binding. Although the competitive binding system is 500-fold less sensitive for 25-OHD_3 compared to 1α,25-$(OH)_2D_3$ (Fig. 3), this assay becomes possible because 25-OHD_3 circulates in approximately 1,000-fold greater concentrations than 1α,25-$(OH)_2D_3$ in man. Other workers have successfully quantitated 25-OHD by competitive binding methods utilizing several proteins. Belsey et al. (19) have reported a sensitive, competitive binding assay for measuring both vitamin D and 25-OHD which uses a specific protein in rat serum. More recently, this group has developed a rapid system (without chromatography) for measuring 25-OHD in plasma (21). Haddad and Chyu (20) have published a sensitive competitive binding assay for 25-OHD using a specific protein from rat kidney cytosol. Both of the latter assays are approximately 40-fold more sensitive than the radioreceptor assay for 25-OHD reported here, and they require considerably less sample volume and preparation. However, the use of intestinal receptor to measure 25-OHD has certain distinct advantages. For instance, 25-OHD_2 as well as 25-OHD_3 (after chromatographic resolution) can be quantitated separately with this binding protein since both sterols bind with similar affinities (Fig. 3B). Yet the most important consideration regarding this assay lies not in its insensitivity in measuring 25-OHD, but in its ability to quantitate the four major circulating cholecalciferol and ergocalciferol metabolites with a single competitive binding system.

The ability to monitor these biologically active metabolites in plasma may prove important in the understanding of many vitamin D-related diseases. Of particular interest in this regard is hypervitaminosis D or vitamin D intoxication. Brumbaugh and Haussler (25), based upon the fact that 25-OHD_3 (in higher concentrations) will bind to the 1α,25-$(OH)_2D_3$ intestinal receptor, originally hypothesized that 25-OHD may be the toxic agent in hypervitaminosis D. Recently, it has been reported by Counts et al. (26) that vitamin D intoxication and hypercalcemia can occur in the anephric human; the serum concentration of 25-OHD in their patient was nearly 28 times larger than the normal circulating concentration of this metabolite. Since 1α,25-$(OH)_2D_3$ is produced exclusively in renal tissue (3), and since anephric human subjects have undetectable 1α,25-$(OH)_2D$ in the plasma (27), it is apparent that the hypercalcemia in this patient was probably due to the markedly elevated 25-OHD concentrations in the plasma. In the present study (Figs. 4 and 5), we measured the 25-OHD in two hypervitaminosis D patients with intact kidneys and in one patient (normal calcium) being treated with a large dose of vitamin D_2. Our results confirm the finding that 25-OHD is considerably increased in D intoxication. In addition, we found that the 1α,25-$(OH)_2D$ concentration is near normal in these patients (Figs. 4 and 5) when the kidneys are present. Although 1α,25-$(OH)_2D_3$ is the physiologic hormone regulating calcium translocation, a 100- to 500-fold greater concentration of precursor 25-OHD_3 will mediate (in vitro) bone calcium resorption (5) and intestinal receptor binding (25). Therefore, the causative agent in hypervitaminosis D is probably the highly elevated 25-OHD, rather than the slightly elevated 1α,25-$(OH)_2D$ hormone. This conjecture is supported by the findings of Shen et al. (28) that patients with idiopathic hypercalciuria have similar (slightly elevated) 1α,25-$(OH)_2D$ levels as we presently report for D-intoxicated patients, but are able to maintain a normal serum calcium concentration. It should be mentioned that these data do not rule out the possibility that some other vitamin D metabolite such as 1,24,25-trihydroxyvitamin D (29) might be involved in this disorder. Nevertheless, it seems reasonable to conclude that the hypercalcemia seen in D intoxication is not a result of abnormal plasma levels of 1α,25-$(OH)_2D$, but is caused by an excessive circulating concentration of 25-OHD.

It is of interest that there are undetectable ergocalciferol metabolites in normal Tucson Caucasian adults who undoubtedly ingest vitamin D_2 in their diet. Haddad and Hahn (22), using a more sensitive assay for measuring 25-OHD in serum, have reported that St. Louis adults exhibit approximately 90% of their circulating 25-OHD as the cholecalciferol metabolite. The present report also confirms the finding (22) that 25-OHD_2 is the predominant form when a patient is receiving large doses of ergocalciferol, and it extends this work to include the measurement of 1α,25-$(OH)_2D$. It is possible that preferential metabolism of the two forms of vitamin D occur under different physiological conditions, however further assessment of the metabolic disposition of natural and synthetic sources of vitamin D is required.

Vitamin D_2 and D_3 metabolites are not equivalent in this receptor system, the D_3 forms being 1.3–1.4 times more potent. Therefore, when rapid micro-Celite purification is used, equivalent displacement of radioactive 1α,25-$(OH)_2D_3$ by a typical sample might represent one part vitamin D_3 metabolite, 1.3–1.4 parts vitamin D_2 metabolite, or a variable combination of the two sterols. Nevertheless, meaningful clinical information has been obtained utilizing this technique for total 1α,25-$(OH)_2D$ measurement. Abnormal circulating levels of 1α,25-

$(OH)_2D$ have been found in numerous human disorders including untreated hypoparathyroidism (15), pseudohypoparathyroidism (15), primary hyperparathyroidism (30), idiopathic hypercalciuria (28), and patients with chronic renal failure (9). Further clinical insights await refinement and simplification of the radioligand receptor assay, especially the advent of higher specific activity [^{3}H] 1α,25-$(OH)_2D_3$ to allow for reduced size of plasma samples and less arduous chromatographic purification requirements.

Note added in proof. We have recently confirmed the structure of the in vitro produced 1α,25-$(OH)_2D_2$ used in this study (see Results) by direct-probe mass spectrometry.

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REFERENCES

1. DeLuca, H. F. 1974. Vitamin D: The vitamin and the hormone. *Fed. Proc.* **33**: 2211–2219.
2. Tucker, G., III, R. E. Gagnon, and M. R. Haussler. 1973. Vitamin D_3-25-hydroxylase: Tissue occurrence and apparent lack of regulation. *Arch. Biochem. Biophys.* **155**: 47–57.
3. Fraser, D. R., and E. Kodicek. 1970. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature (Lond.)*. **228**: 764–766.
4. Haussler, M. R., D. W. Boyce, E. T. Littledike, and H. Rasmussen. 1971. A rapidly acting metabolite of vitamin D_3. *Proc. Natl. Acad. Sci.* **68**: 177–181.
5. Raisz, L. G., C. C. Trummel, M. F. Holick, and H. F. DeLuca. 1972. 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. *Science (Wash. D. C.)*. **175**: 768–769.
6. Brumbaugh, P. F., and M. R. Haussler. 1974. 1α,25-dihydroxycholecalciferol receptors in intestine. Temperature-dependent transfer of the hormone to chromatin via a specific cytosol receptor. *J. Biol. Chem.* **249**: 1258–1262.
7. Brumbaugh, P. F., and M. R. Haussler. 1974. 1α,25-dihydroxycholecalciferol receptors in intestine. Association of 1α,25-dihydroxycholecalciferol with intestinal mucosa chromatin. *J. Biol. Chem.* **249**: 1251–1257.
8. Brumbaugh, P. F., and M. R. Haussler. 1975. Specific binding of 1α, 25-dihydroxycholecalciferol to nuclear components of the chick intestine. *J. Biol. Chem.* **250**: 1588–1594.
9. Brumbaugh, P. F., D. H. Haussler, R. Bressler, and M. R. Haussler. 1974. Radioreceptor assay for 1α,25-dihydroxyvitamin D_3. *Science (Wash. D. C.)*. **183**: 1089–1091.
10. Brumbaugh, P. F., D. H. Haussler, K. M. Bursac, and M. R. Haussler. 1974. Filter assay for 1α,25-dihydroxyvitamin D_3. Utilization of the hormone's target tissue chromatin receptor. *Biochemistry*. **13**: 4091–4097.
11. McNutt, K. W., and M. R. Haussler. 1973. Nutritional effectiveness of 1α,25-dihydroxycholecalciferol in preventing rickets in chicks. *J. Nutr.* **103**: 681–689.
12. Haussler, M. R. 1972. Characterization of the metabolites of vitamin D_3 in the chick. *Steroids.* **20**: 639–650.
13. Lawson, D. E. M., D. R. Fraser, E. Kodicek, H. R. Morris, and D. H. Williams. 1971. Identification of 1α, 25-dihydroxycholecalciferol. A new kidney hormone controlling calcium metabolism. *Nature (Lond.)*. **230**: 228–230.
14. Haussler, M. R., and H. Rasmussen. 1972. The metabolism of vitamin D_3 in the chick. *J. Biol. Chem.* **247**: 2328–2335.
15. Haussler, M. R., D. J. Baylink, M. R. Hughes, P. F. Brumbaugh, J. E. Wergedal, F. H. Shen, R. L. Nielsen, S. J. Counts, K. M. Bursac, and T. A. McCain. 1976. The assay of 1α,25-dihydroxyvitamin D_3: Physiologic and pathologic modulation of circulating hormone levels. *Clin. Endocrinol.* **5**: 151s–165s.
16. Norman, A. W. 1968. The mode of action of vitamin D. *Biol. Rev. (Camb.)*. **43**: 97–137.
17. Hughes, M. R., P. F. Brumbaugh, M. R. Haussler, J. E. Wegedal, and D. J. Baylink. 1975. Regulation of plasma 1α,25-dihydroxyvitamin D_3 by calcium and phosphate in the rat. *Science (Wash. D. C.)*. **190**: 578–580.
18. Hughes, M. R., P. F. Brumbaugh, and M. R. Haussler. 1975. A dual assay for measuring the plasma concentration of 1α,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3. *Fed. Proc.* **34**: 893. (Abstr.)
19. Belsey, R. E., H. F. DeLuca, and J. T. Potts, Jr. 1971. Competitive binding assay for vitamin D and 25-OH vitamin D. *J. Clin. Endocrinol. Metab.* **33**: 554–557.
20. Haddad, J. G., and K. J. Chyu. 1971. Competitive protein-binding radioassay for 25-hydroxycholecalciferol. *J. Clin. Endocrinol. Metab.* **33**: 992–995.
21. Belsey, R. E., H. F. DeLuca, and J. T. Potts, Jr. 1974. A rapid assay for 25-OH-vitamin D_3 without preparative chromatography. *J. Clin. Endocrinol. Metab.* **38**: 1046–1051.
22. Haddad, J. G., Jr., and T. J. Hahn. 1973. Natural and synthetic sources of circulating 25-hydroxyvitamin D in man. *Nature (Lond.)*. **244**: 515–517.
23. Hill, L. F., E. B. Mawer, and C. M. Taylor. 1975. Determination of plasma levels of 1,25-dihydroxy-cholecalciferol in man. *In* Vitamin D and Problems Related to Uremic Bone Disease. A. W. Norman, K. Schaefer, H. G. Grigoleit, D. V. Herrath, and E. Ritz, editors. Walter De Gruyter & Co., Berlin. 755–762.
24. Belsey, R. E., H. F. DeLuca, and J. T. Potts, Jr. 1974. Selective binding properties of vitamin D transport protein in chick plasma *in vitro*. *Nature (Lond.)*. **247**: 208–209.
25. Brumbaugh, P. F., and M. R. Haussler. 1973. 1α,25-dihydroxyvitamin D_3 receptor: Competitive binding of vitamin D analogs. *Life Sci.* **13**: 1737–1746.
26. Counts, S. J., D. J. Baylink, F-H. Shen, D. J. Sherrard, and R. O. Hickman. 1975. Vitamin D intoxication in an anephric child. *Ann. Intern. Med.* **82**: 196–200.
27. Haussler, M. R. 1974. Vitamin D: Mode of action and biomedical applications. *Nutr. Rev.* **32**: 257–266.
28. Shen, F., D. Baylink, R. Nielsen, D. Sherrard, and M. Haussler. 1976. A study of the pathogenesis of idiopathic hypercalciuria. *Clin. Res.* **24**: 157a. (Abstr.)
29. Holick, M. F., A. Kleiner-Bossaller, H. K. Schnoes, P. M. Kasten, I. T. Boyle, and H. F. DeLuca. 1973. 1,24-25-Trihydroxyvitamin D_3. A metabolite of vitamin D_3 effective on intestine. *J. Biol. Chem.* **248**: 6691–6696.
30. Haussler, M. R., K. M. Bursac, H. Bone, and C. Y. C. Pak. 1975. Increased circulating 1α,25-dihydroxyvitamin D_3 in patients with primary hyperparathyroidism. *Clin. Res.* **23**: 322a. (Abstr.)