Filter Assay for 1α,25-Dihydroxyvitamin D_3. Utilization of the Hormone's Target Tissue Chromatin Receptor†

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ABSTRACT: A radioreceptor assay for 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3) has been developed by utilizing competitive binding to the receptor system from chick intestinal mucosa. This target tissue binding system consists of a high affinity cytosol receptor protein and acceptor sites on nuclear chromatin. Reconstituted cytosol-chromatin is incubated with radioactive 1α,25-$(OH)_2$-D_3, and the bound and free sterol are separated by trapping the hormone-receptor-chromatin complex on glass fiber filters. Washing away of free or nonspecifically bound sterol is carried out with 1% Triton X-100. The amount of specifically bound sterol is linearly related to the quantity of cytosol-chromatin in the incubation and the receptor system is saturated at 5 nM 1α,25-$(OH)_2$-[^{3}H]D_3. An isotope-dilution standard curve obtained with increasing amounts of authentic nonradioactive 1α,25-$(OH)_2$-D_3 indicates that 17 pg of hormone can be detected by this assay. After extraction and extensive purification of the suspected 1α,25-$(OH)_2$-D_3 fraction from normal chick and human plasma, the circulating levels of the hormone were determined to be 8.0 and 4.0 ng/100 ml, respectively. These values were verified by demonstrating that the assayable material elutes with tracer 1α,25-$(OH)_2$-[^{3}H]D_3 on Celite liquid-liquid partition columns. By similar analysis, rachitic chick plasma contained <0.05 ng of 1α,25-$(OH)_2$-D_3/100 ml. The lower limit of sensitivity of the assay for human plasma samples (20 ml) is 1.0 ng/100 ml of 1α,25-$(OH)_2$-D_3 and the coefficient of variation is 12%. Patients with chronic renal failure and anephric subjects have undetectable circulating levels of the kidney-produced 1α,25-$(OH)_2$-D_3 hormone as determined by the assay. These data implicate a lack of 1α,25-$(OH)_2$-D_3 in the etiology of renal osteodystrophy and point to the kidney as the unique site of synthesis of 1α,25-$(OH)_2$-D_3 in humans. This assay should not only be of great utility in diagnosing other defects in vitamin D metabolism, but the concept of employing the two-step receptor binding system for the sterol may find application in the assay of steroid hormones.

Apparently, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3)[1] is the metabolite of vitamin D_3 that mediates the biochemical events that result in increased calcium absorption from the intestine (Haussler *et al.*, 1971; Holick *et al.*, 1971) and bone calcium resorption (Holick *et al.*, 1972; Wong *et al.*, 1972) in experimental animals. This metabolite is produced by hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) in the kidney and is considered to be the hormonal form of the vitamin (Fraser and Kodicek, 1970; Lawson *et al.*, 1971; Gray *et al.*, 1971). In order to study further the metabolism of vitamin D_3 to 1α,25-$(OH)_2$-D_3 as well as investigate the role of this hormone in calcium metabolism in humans, we have developed a sensitive, competitive protein binding assay for 1α,25-$(OH)_2$-D_3 utilizing the hormone's chromatin receptor in the chick intestine.

Assays of steroid hormones employing their respective cytoplasmic receptors have been reported (Korenman, 1968; Ballard and Baxter, 1974). These intracellular proteins are ideally suited for measurement of hormone binding activity due to their great specificity and affinity (Jensen *et al.*, 1971). However, the observed thermolability of the chick intestinal cytoplasmic receptor for 1α,25-$(OH)_2$-D_3 precluded our development of an assay for the hormone using this protein alone. Recent experiments (Brumbaugh and Haussler, 1973, 1974a,b) also indicate that the chromatin receptor for 1α,25-$(OH)_2$-D_3 has an affinity and specificity for this hormone similar to that of the cytosol receptor. In addition, once the 1α,25-$(OH)_2$-D_3-receptor complex associates with the chromatin, binding is markedly thermostable (P.F. Brumbaugh and M.R. Haussler, unpublished experiments). Thus, a radioreceptor assay for 1α,25-$(OH)_2$-D_3 employing the chromatin of the hormone's target tissue was developed.

Separation of macromolecular bound from unbound steroid in competitive protein binding assays for steroid hormones is generally made by gel filtration chromatography or adsorption of uncomplexed steroid on activated charcoal. These techniques are not applicable to chromatin; therefore, a filter assay, which depends on the ability of a filter to trap chromatin under wash conditions where unbound sterol may be removed, was suited for quantitation of receptor-bound sterol. Unfortunately, free steroids cannot easily be washed from nitrocellulose filters used for assays of this nature (Yarus and Berg, 1970; Santi *et al.*, 1973). However, it has been recently shown that several steroid hormones, including [^{3}H]dexamethasone (Santi *et al.*, 1973) and 1α,25-$(OH)_2$-[^{3}H]D_3 (Brumbaugh and Haussler, 1974b) can be washed from DEAE-cellulose filters at low ionic strength. In addition, the nonionic detergent, Triton X-100, has been shown to be highly effective in removing nonspecifically bound sterol from chromatin (Haussler *et al.*, 1968). Therefore, this detergent was used to remove free 1α,25-$(OH)_2$-D_3 from chromatin trapped on filters, providing a rapid method for separation of bound from free 1α,25-$(OH)_2$-D_3.

In previous reports (Brumbaugh *et al.*, 1974a,b) we showed that a radioreceptor assay based on the chromatin receptor for 1α,25-$(OH)_2$-D_3 in chick intestine was capable of detecting this hormone in human plasma. We report here experimental results demonstrating the validity of this filtration technique for the separation of bound from free hormone as well as data pertinent to the precision, accuracy, sensitivity, and specificity of this assay for 1α,25-$(OH)_2$-D_3.

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

Materials and Methods

Materials. Animals used to prepare the chick intestinal receptor system were White Leghorn cockerels (kindly donated by Demler Farms, Anaheim, Calif.) that were raised for 3–4 weeks on a vitamin D deficient diet (McNutt and Haussler, 1973). 25-Hydroxy[26(27)-*methyl*-^{3}H]vitamin D_3 (6.0 Ci/mmol) was obtained from Amersham–Searle. Crystalline 25-hydroxyvitamin D_3 was a gift from Dr. John C. Babcock of the Upjohn Co. 25-Hydroxycholesterol was purchased from Steraloids. Crystalline 1α-hydroxyvitamin D_3 was a gift from Dr. Maurice M. Pechet, Research Institute for Medicine and Chemistry, Cambridge, Mass. Filters (Versapor, 0.9 μ; Metricel, 0.45 μ; and Glass Fiber type A) were obtained from Gelman. Whatman DE81 and No. 2 filters were purchased from Reeve-Angel. Filters had diameters of 24 mm to 1 in. for use in a 30-place Millipore Sampling Manifold. All solvents used in chromatography procedures were reagent grade and were glass distilled before use.

Preparation of 1α,25-Dihydroxyvitamin D_3. 1α,25-Dihydroxy[26(27)-*methyl*-^{3}H]vitamin D_3 was produced, *in vitro*, by a modification of the method of Lawson *et al.* (1971) and purified by column chromatography on silicic acid, Sephadex LH-20, and Celite as previously described (Brumbaugh and Haussler, 1974a). The final radiochemical purity of the 1α,25-(OH)$_2$-[^{3}H]D_3 as determined by rechromatography on Celite was greater than 98%. Nonradioactive 1α,25-(OH)$_2$-D_3 was prepared and purified by similar procedures (Haussler, 1972); the sterol was virtually free of contaminating materials as judged by mass spectrometry and ultraviolet absorption spectrophotometry. 1α,25-(OH)$_2$-D_3 was quantitated by ultraviolet absorption spectrophotometry at 265 nm and was stored in redistilled ethanol at −20°.

Preparation of Subcellular Fractions. Preparation of purified chromatin and cytosol fractions from 20% homogenates of small intestines of rachitic chicks in 0.25 M sucrose in 0.05 M Tris-HCl (pH 7.4), 0.025 M KCl, and 0.005 M $MgCl_2$ (sucrose–buffer A) was carried out as previously described (Haussler *et al.*, 1968; Brumbaugh and Haussler, 1974a). Chromatin was washed once with 1% Triton X-100 in 0.01 M Tris-HCl (pH 7.5) (Triton X-100–buffer B) and once with buffer B prior to use in the assay. The chromatin and cytosol fractions were kept at 0–4° and reconstituted immediately before use.

Binding Assay. To each assay tube containing 1α,25-(OH)$_2$-[^{3}H]D_3 and unlabeled sterol was added a 200-μl portion of the reconstituted cytosol–chromatin receptor system. The final concentration of 1α,25-(OH)$_2$-[^{3}H]D_3 was 4.3 nM and the amount of DNA added to each tube was *ca.* 150 μg. After incubation of mixtures at 25° for 20 min, the quantity of labeled sterol bound to the chromatin was determined by filtration (described below). To determine nonspecifically bound sterol, parallel reactions were performed in which a 100-fold excess of unlabeled 1α,25-(OH)$_2$-D_3 was included in incubation mixtures as a competitor for binding to the 1α,25-(OH)$_2$-D_3 chromatin receptor. Sterol specifically bound to the 1α,25-(OH)$_2$-D_3 receptor is calculated by subtracting the nonspecifically bound sterol from that bound in the absence of excess nonradioactive sterol.

Filtration assays were performed as follows: filters were soaked in distilled water prior to use, placed on a 30-place filter manifold (Millipore), and washed with ice-cold 1% Triton X-100–buffer B during application of a low vacuum. The 200-μl samples were uniformly applied to each of the filters. After 2–4 min the vacuum was increased to achieve uniform flow rates of *ca.* 1 ml/min and each of the filters was washed with 8 ml of 1% Triton X-100. Following filtration, the filters were placed in 10 ml of MeOH–$CHCl_3$ (2:1, v/v) in a scintillation vial for 1 hr to extract 1α,25-(OH)$_2$-[^{3}H]D_3 from the filters. The filters were then removed from the vial and contents dried under a stream of air. Sterols were then solubilized in 5 ml of toluene counting solution consisting of 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene. Samples were counted in a Beckman LS-233 liquid scintillation spectrometer. Efficiency for tritium was 39% and was identical for all counted samples.

DNA Assay. DNA was determined by the diphenylamine method (Dische, 1930).

Purification of 1α,25-Dihydroxyvitamin D_3 from Plasma. To each plasma sample (~20 ml) a small 1α,25-(OH)$_2$-[^{3}H]D_3 tracer (2000 cpm) was added to quantitate losses of 1α,25-(OH)$_2$-D_3 in the ensuing purification procedure. Plasma samples were extracted with 4 vol of methanol–chloroform (2:1 v/v) by the procedure of Bligh and Dyer (1959). The chloroform phase obtained was flash evaporated and the sterols solubilized in diethyl ether. 1α,25-(OH)$_2$-D_3 was purified by silicic acid, Sephadex LH-20, and Celite column chromatography as described below.

Silicic Acid. Chromatography on silicic acid was carried out as described by Haussler and Rasmussen (1972) except that columns (1 × 40 cm; 18 g; Figures 5 and 7, Table II) were eluted with 220 ml of diethyl ether (fraction 1) and 75 ml of acetone (fraction 2) (containing 1α,25-(OH)$_2$-D_3). In the initial purification of 1α,25-(OH)$_2$-D_3 from plasma for identification (Figure 6), a modified silicic acid chromatography system (2 × 18 cm; 25-g column) was employed. Elution was carried out with 5% acetone in ether; 1α,25-(OH)$_2$-[^{3}H]D_3 eluted in 160–300 ml.

Sephadex LH-20. Chromatography on Sephadex LH-20 was by the procedure of Holick and DeLuca (1971). Columns (1 × 15 cm; 5 g) were eluted with 65% chloroform in hexane. Fractions (45 ml) were collected. 1α,25-(OH)$_2$-D_3 eluted in fraction 2.

Celite. Liquid–liquid partition chromatography on Celite (Figure 6) was by the method of Haussler and Rasmussen (1972). The solvent scheme was 10% ethyl acetate in hexane (mobile) and 45% water in ethanol (stationary). Fractions (5 ml) were collected. "Micro-Celite" columns containing 0.8 g of Celite and the same solvent system as above were used for rapid and efficient purification of 1α,25-(OH)$_2$-D_3 from plasma samples. Fraction 1 was 4 ml, and fraction 2 was 8 ml. 1α,25-(OH)$_2$-D_3 eluted in fraction 2.

Periodate Oxidation. Plasma extracts were treated for 10 min with 10 ml of periodic acid (1 mg/ml) in a mixture of water and ethanol (67:33, v/v). This procedure converts 24,25-dihydroxyvitamin D_3 and 25,26-dihydroxyvitamin D_3 to the corresponding 24-aldehyde and 25-ketone derivatives (M. R. Haussler, manuscript in preparation).

Results

1α,25-Dihydroxyvitamin D_3 Receptors. The development of a specific radioreceptor assay for 1α,25-(OH)$_2$-D_3 arose from recent elucidation (Brumbaugh and Haussler, 1974a,b) of the early events in the interaction of 1α,25-(OH)$_2$-D_3 with its target tissue. 1α,25-(OH)$_2$-D_3 enters the intestinal mucosa cell and binds to a cytoplasmic receptor. This hormone receptor complex then migrates into the nucleus where it associates with the chromatin. The movement of the 1α,25-(OH)$_2$-D_3–receptor complex into the nucleus is temperature dependent, occurring more rapidly at 25–37° than at 0°. The competitive protein binding assay depends on the specificity of the cytoplasmic receptor for 1α,25-(OH)$_2$-D_3 and the migration of the hor-

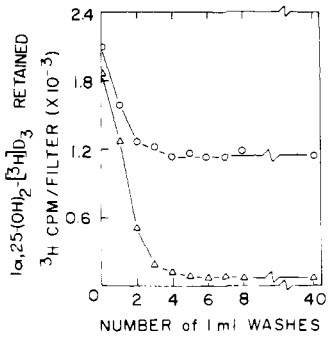

FIGURE 1: 1α,25-Dihydroxy[^{3}H]vitamin D_3 retained on glass fiber filters after incubation of reconstituted cytosol-chromatin with 4.3×10^{-9} M 1α,25-$(OH)_2$-[^{3}H]D_3 (6.0 Ci/mmol) (○) and 4.3×10^{-7} M 1α,25-$(OH)_2$-[^{3}H]D_3 (60 mCi/mmol) (△). Incubation mixtures were applied to glass fiber filters and washed with 1% Triton X-100-buffer B as described under Materials and Methods.

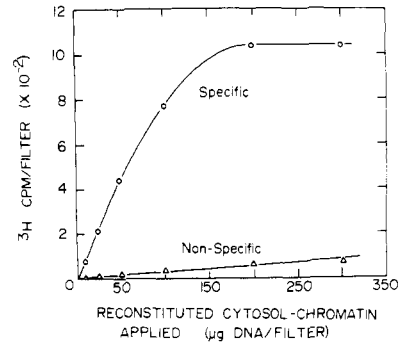

FIGURE 2: 1α,25-Dihydroxy[^{3}H]vitamin D_3 retained on glass fiber filters at increasing amounts of reconstituted cytosol-chromatin (DNA). Various amounts of reconstituted cytosol-chromatin (cytosol protein, 10 mg/ml; DNA, 500 μg/ml) were incubated with 4.3×10^{-9} M 1α,25-$(OH)_2$-[^{3}H]D_3 (6.0 Ci/mmol) or 4.3×10^{-7} M 1α,25-$(OH)_2$-[^{3}H]D_3 (60 mCi/mmol) (△). Incubations were filtered and washed as described under Materials and Methods. Specifically bound 1α,25-$(OH)_2$-[^{3}H]D_3 (○) was determined by subtracting nonspecifically bound sterol (△) from the total retained (not shown).

mone-receptor complex to its chromatin binding sites in the nucleus.

Binding Assay. When 1α,25-$(OH)_2$-[^{3}H]D_3 is applied to glass fiber filter disks directly or preincubated with intestinal mucosa cytosol prior to application, washing with 3 ml of Triton X-100-buffer B removes almost all radioactivity from the filter disk (data not shown). However, when reconstituted cytosol-chromatin from intestinal mucosa is incubated with 1α,25-$(OH)_2$-[^{3}H]D_3 prior to application, substantial radioactivity is retained on the filters even when they are washed extensively with Triton X-100-buffer B (Figure 1). The amount of 1α,25-$(OH)_2$-[^{3}H]D_3 retained by the chromatin which is trapped on the filter remains essentially constant after 4 to 40 ml of wash. However, the radioactivity retained is significantly reduced when incubation of 1α,25-$(OH)_2$-[^{3}H]D_3 with reconstituted cytosol-chromatin is carried out with a 100-fold excess of nonradioactive 1α,25-$(OH)_2$-D_3 as a competitor for specific hormone binding to the chromatin (Figure 1). The difference between the filter-retained sterol in the presence and in the absence of excess unlabeled 1α,25-$(OH)_2$-D_3 is a measure of the receptor-hormone complex retained on the filter.

The amount of specifically bound sterol retained on the filters is linearly related to the amount of reconstituted cytosol-chromatin in the incubation (as measured by the quantity of DNA). This linearity is observed (Figure 2) up to about 200 μg of DNA per incubation at which point hormone binding to the chromatin is apparently limited by sterol concentration. Thus, the number of chromatin binding sites for 1α,25-$(OH)_2$-D_3 limits the hormone retention on the filters under standard binding assay conditions (4.3×10^{-9} M 1α,25-$(OH)_2$-[^{3}H]D_3 (4300 cpm), 150 μg of DNA, cytosol containing about 2 mg of protein in 0.25 M sucrose-buffer A, final volume 0.20 ml).

The titration of a constant amount of intestinal mucosa reconstituted cytosol-chromatin (120 μg of DNA) at increasing 1α,25-$(OH)_2$-[^{3}H]D_3 concentration is shown in Figure 3. Saturation occurs at 5 nM added hormone. The amount of 1α,25-$(OH)_2$-[^{3}H]D_3 retained on the filters at saturation is 23 pmol/15 mg of DNA (chick intestinal equivalent), a value which correlates well with the previously reported saturation value for this receptor (Brumbaugh and Haussler, 1974a). However, determination of the saturation value for this chromatin receptor by a filter assay depends on the efficiency of the filtration procedure. The efficiency of a filter assay may be defined as the probability that a protein-ligand complex will survive the filtration and washing procedure and be detected when the filter is assayed (Yarus and Berg, 1970). This parameter was determined by harvesting chromatin independently on filters and by centrifugation at 30,000*g* for 30 min following parallel incubations of reconstituted cytosol-chromatin under standard binding conditions. By determining the ratio of 1α,25-$(OH)_2$-[^{3}H]D_3 retained on the filter to that extracted by methanol-chloroform (2:1) from the chromatin pellets, filter trapping efficiencies were maximized to reproducible values of >95%. Optimal binding to glass fiber filters was found to occur if the incubation mixtures were permitted to adsorb to the filters 2–4 min prior to wash and if uniform filtration rates of *ca.* 1 ml/min were maintained.

The isotope dilution standard curve for the 1α,25-$(OH)_2$-D_3 assay using glass fiber filters is shown in Figure 4. The assay is sensitive enough to detect 17 pg of added hormone. Without optimizing filtration efficiencies, standard curves virtually identical with that pictured in Figure 4 were obtained employing several other types of filters, including DEAE-cellulose, Whatman No. 2, and 0.45- or 0.9-μ membrane filters. Apparently this filtration procedure relies on the filter's ability to trap pieces of sheared chromatin in small pores rather than any specific binding of the chromatin to the filter. Thus, a wide range of filter varieties is suitable for this type of assay.

Sample Extraction and Chromatography. The binding assay was used to determine the concentration of 1α,25-$(OH)_2$-D_3 purified from a human plasma sample by various procedures (Figure 5). A methanol-chloroform extract was purified by chromatography on a silicic acid column and two Sephadex LH-20 columns. The extract was then divided into

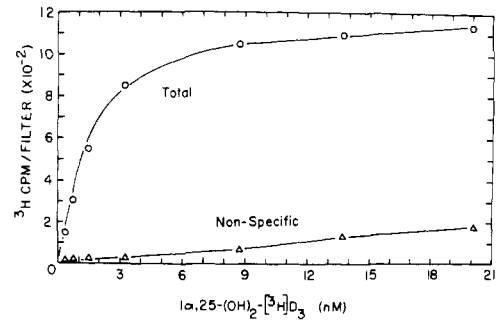

FIGURE 3: Titration of chromatin receptor with 1α,25-dihydroxy-[^{3}H]vitamin D_3. Indicated amounts of 1α,25-$(OH)_2$-[^{3}H]D_3 (6.0 Ci/mmol) in the absence (total, ○) or presence (nonspecific, △) of a 100-fold excess of unlabeled 1α,25-$(OH)_2$-D_3 were incubated with reconstituted cytosol-chromatin (120 μg of DNA) and assayed as described under Materials and Methods.

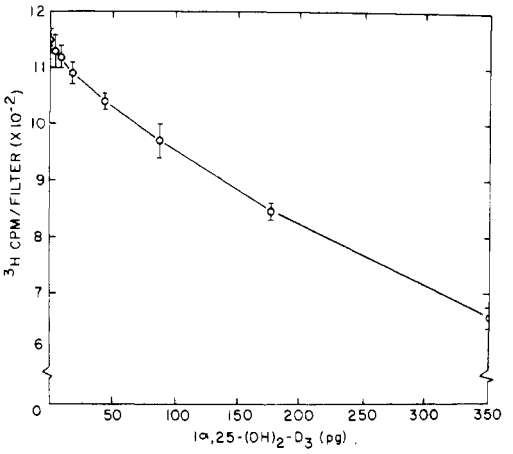

FIGURE 4: Isotope-dilution standard curve for 1α,25-dihydroxyvitamin D_3 assay. Reconstituted cytosol-chromatin was incubated with 1α,25-(OH)$_2$-[^{3}H]D_3 (360 pg, 6.0 Ci/mmol) and the indicated amount of unlabeled 1α,25-(OH)$_2$-D_3. Filtration was through glass fiber filters as described under Materials and Methods. Each point represents the average of triplicate assays ± standard error of the mean.

three fractions which were: (1) assayed, (2) purified by periodate oxidation followed by Sephadex LH-20 chromatography, or (3) purified by a "micro-Celite" column. These additional purification procedures remove 25,26-dihydroxyvitamin D_3, a circulating metabolite of vitamin D which migrates with 1α,25-(OH)$_2$-D_3 on silicic acid and Sephadex LH-20 columns (Haussler and Rasmussen, 1972). The assay results (Figure 5) indicate that an interfering substance or substances in the assay are removed from the extract by the "micro-Celite" column but not by periodate oxidation of the extract. Thus, this substance is probably not 25,26-dihydroxyvitamin D_3. Recovery of 1α,25-(OH)$_2$-D_3 after plasma extraction and chromatography was determined by initial addition of 1α,25-(OH)$_2$-[^{3}H]D_3 to each plasma sample, and the assay results were corrected for losses of tritium. Recoveries ranged from 51 to 64%.

Identification of the Assayable Compound as 1α,25-Dihydroxyvitamin D_3. The compound that competes with 1α,25-(OH)$_2$-[^{3}H]D_3 for binding to the chromatin receptor in the assay was identified as 1α,25-(OH)$_2$-D_3 by its migration with authentic labeled hormone on a large Celite liquid-liquid partition column (1 × 35 cm). Pooled plasma samples from rachitic and normal chicks containing 1α,25-(OH)$_2$-[^{3}H]D_3 tracer were extracted with methanol-chloroform (2:1) and purified by silicic acid and Sephadex LH-20 chromatography as outlined in Figure 5. The extracts were divided into two aliquots which were further purified by "micro-Celite" chromatography or by chromatography on a large Celite column. Individual fractions from the large Celite column were assayed for 1α,25-(OH)$_2$-D_3 binding activity. As shown in Figure 6A, the binding activity in the plasma extract from the normal chick migrated exactly with the added 1α,25-(OH)$_2$-[^{3}H]D_3 tracer. The total assayable 1α,25-(OH)$_2$-D_3 in the plasma sample corresponds to 8.0 ng/100 ml of plasma (2×10^{-10} M). Triplicate assays on the normal chick plasma sample purified by "micro-Celite" chromatography indicated a 1α,25-(OH)$_2$-D_3 concentration of 7.7 ± 0.5 ng/100 ml of plasma. Therefore, the 1α,25-(OH)$_2$-D_3 binding activity in normal chick plasma corresponds to 1α,25-(OH)$_2$-D_3 as identified by migration with authentic labeled hormone on a Celite liquid-liquid partition column, the best known chromatography system for the resolution and purification of dihydroxyvitamin D_3 metabolites (Haussler and Rasmussen, 1972). The pooled plasma sample from rachitic chicks contained no assayable 1α,25-(OH)$_2$-D_3 when purified by a large Celite column (Figure 6B) or when

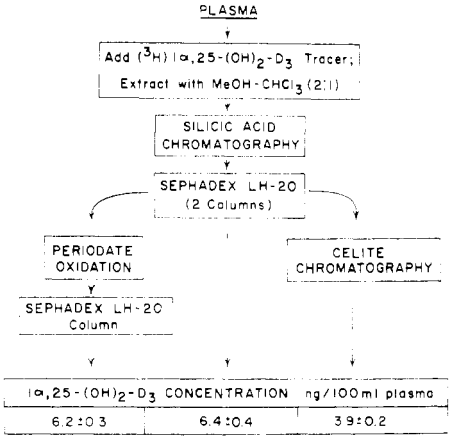

FIGURE 5: Purification of 1α,25-dihydroxyvitamin D_3 from human plasma. 1α,25-(OH)$_2$-[^{3}H]D_3 (8800 cpm, 6.0 Ci/mmol) was added to human plasma (90 ml). A methanol-chloroform extract of the plasma was purified by chromatography on a silicic acid and two Sephadex LH-20 columns (recovery of ^{3}H = 64%). The extract was divided into three fractions and further purified by periodate oxidation and Sephadex LH-20 chromatography (final recovery of ^{3}H = 59%) or by chromatography on a "micro-Celite" column (final recovery of ^{3}H = 51%). 1α,25-(OH)$_2$-D_3 assays and purification procedures are described under Materials and Methods. 1α,25-(OH)$_2$-D_3 measured are the result of triplicate assays ± standard error of the mean.

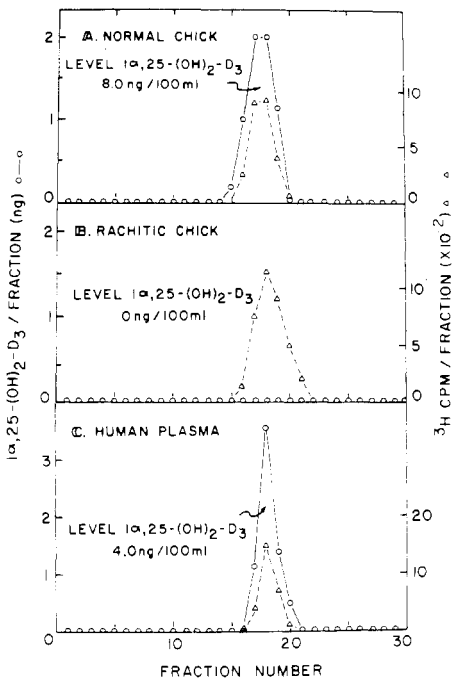

FIGURE 6: Identification of assayable material in normal chick and human plasma as 1α,25-dihydroxyvitamin D_3 by Celite chromatography. 1α,25-(OH)$_2$-[^{3}H]D_3 (5600 cpm; 6.0 Ci/mmol) was added to a pooled plasma sample from rachitic chicks (188 ml), normal chicks (78 ml), and five normal humans (165 ml). 1α,25-(OH)$_2$-D_3 was purified from methanol-chloroform extracts of the plasma by chromatography on silicic acid, Sephadex LH-20 columns, and Celite columns. Purification methods were as outlined under Materials and Methods (recovery of ^{3}H from normal chick extract = 47%; from rachitic chick extract = 65%; and from human extract = 47%). Assayable 1α,25-(OH)$_2$-D_3 in each 5-ml fraction from the final Celite column (○) from normal chick extract (panel A), from rachitic chick extract (panel B), and from human extract (panel C) was determined as described under Materials and Methods. Migration of 1α,25-(OH)$_2$-[^{3}H]D_3 marker on the Celite columns (△) was determined by counting an aliquot of each fraction.

TABLE I: Competition of 1α,25-Dihydroxy[^{3}H]vitamin D_3 and Various Steroids and Vitamin D Analogs for Chromatin Receptor.[a]

Sterol or Steroid	Concn (M)	Binding of 1α,25-$(OH)_2$-[^{3}H]D_3 to Filter (%)
None		100
1α,25-$(OH)_2$-D_3	2.5×10^{-9}	68
25-OH-D_3	2.5×10^{-6}	50
1α-Hydroxyvitamin D_3	2.5×10^{-6}	56
Vitamin D_3	2.5×10^{-6}	100
Vitamin D_2	2.5×10^{-6}	100
Dihydrotachysterol$_2$	2.5×10^{-6}	100
25-Hydroxycholesterol	2.5×10^{-6}	100
Cholesterol	2.5×10^{-6}	100
Cortisol	2.5×10^{-6}	100
17β-Estradiol	2.5×10^{-6}	100

[a] Reconstituted cytosol–chromatin was equilibrated with 4.3×10^{-9} M 1α,25-$(OH)_2$-[^{3}H]D_3 and unlabeled compounds as described under Materials and Methods. The binding of labeled hormone to the chromatin, as measured by the filter assay, was determined.

purified by a "micro-Celite" column. Based on the sensitivity of the assay of 17 pg (Figure 4) the circulating concentration of 1α,25-$(OH)_2$-D_3 in these vitamin D deficient chicks is less than 0.05 ng/100 ml of plasma, a value which is 1/160th the concentration of this hormone in the normal chick.

In order to show that the assayable material in human plasma is 1α,25-$(OH)_2$-D_3, a similar experiment was carried out using a pooled plasma sample from five normal humans. After preliminary purification on silicic acid and Sephadex LH-20 columns, the 1α,25-$(OH)_2$-[^{3}H]D_3 tracer and 1α,25-$(OH)_2$-D_3 binding activity comigrated on a large Celite column (1 × 35 cm) (Figure 6C). The total hormone binding activity indicates that the average 1α,25-$(OH)_2$-D_3 concentration in these individuals is 4.0 ng/100 ml of plasma (1×10^{-10} M). Triplicate assays on this plasma sample purified by a "micro-Celite" column indicated an observed 1α,25-$(OH)_2$-D_3 concentration of 4.2 ± 0.4 ng/100 ml of plasma. The results in Figure 6 show that the 1α,25-$(OH)_2$-[^{3}H]D_3 competitive binding activity in chick and human plasma results only from the presence of the hormone in these plasma samples. Furthermore, binding activity that migrates with 1α,25-$(OH)_2$-[^{3}H]D_3 on a large Celite column is the same as that activity determined when the plasma sample is purified on a "micro-Celite" column. Therefore, purification of plasma extracts by silicic acid, Sephadex LH-20 (two columns), and "micro-Celite" chromatography appears to be the most valid method for preparation of plasma in this assay.

TABLE II: Concentration of 1α,25-$(OH)_2$-D_3 in Plasma of Normal Individuals and in Patients with Chronic Renal Disease.[a]

Group (No.)	Range 1α,25-$(OH)_2$-D_3 (ng/100 ml)	Av 1α,25-$(OH)_2$-D_3 (ng/100 ml ● SD)
Normal (6)	3.2–5.1	3.9 ± 0.8
Untreated renal failure (3)	0.8–1.7	1.3 ± 0.5[b]
Hemodialysis (5)	Undetectable[c]	<0.5[b]
Anephric (2)	Undetectable[d]	<1.0[b]

[a] Unless otherwise designated, plasma samples were approximately 20 ml. Purification of 1α,25-$(OH)_2$-D_3 from plasma extracts was carried out as described under Materials and Methods. [b] Significantly different from normal, $P < 0.005$. [c] All samples contained 1α,25-$(OH)_2$-D_3 concentrations below the sensitivity of the assay (0.5 ng/100 ml in 30–40-ml plasma samples). [d] All samples contained 1α,25-$(OH)_2$-D_3 concentrations below the sensitivity of the assay (1.0 ng/100 ml in 20-ml plasma samples).

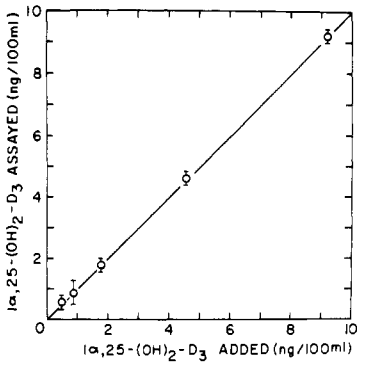

FIGURE 7: Accuracy of 1α,25-dihydroxyvitamin D_3 assay. 1α,25-$(OH)_2$-[^{3}H]D_3 (1900 cpm; 6.0 Ci/mmol) was added to aliquots of a human plasma sample (19 ml each). Varying amounts of nonradioactive 1α,25-$(CH)_2$-D_3 (up to 9.2 ng/100 ml) were added to each plasma sample. Purification of 1α,25-$(OH)_2$-D_3 from the plasma samples and assay was carried out as described under Materials and Methods. Each point represents the average of triplicate assays ± standard error of the mean. The endogenous concentration of 1α,25-$(OH)_2$-D_3 in the plasma sample (2.8 ng/100 ml), determined by addition of no 1α,25-$(OH)_2$-D_3, has been substracted from each assay point.

Specificity of the 1α,25-Dihydroxyvitamin D_3 Assay. Various steroids and vitamin D analogs listed in Table I were added to the assay incubations. Only 1α,25-$(OH)_2$-D_3, 25-OH-D_3, and 1α-hydroxyvitamin D_3 competed with 1α,25-$(OH)_2$-[^{3}H]D_3 for the chromatin receptor at the concentrations tested. In order to confirm that the monohydroxyvitamin D_3 analogs are eliminated from the plasma extracts in the purification scheme, these sterols were added to plasma from rachitic chicks in amounts of 10 μg/100 ml of plasma. Of those sterols added only 1α,25-$(OH)_2$-D_3 results in measurable 1α,25-$(OH)_2$-D_3 binding activity. The other known circulating metabolites of vitamin D_3, 24,25-dihydroxyvitamin D_3 and 25,26-dihydroxyvitamin D_3, are resolved from 1α,25-$(OH)_2$-D_3 in the purification sequence (Haussler and Rasmussen, 1972) and are not measured in the assay since no 1α,25-$(OH)_2$-D_3 binding activity occurs in the elution position of these sterols on Celite columns (Figure 6).

Method Blank. Three 50-ml samples of distilled water were processed through the purification scheme in order to determine the method blank. No 1α,25-$(OH)_2$-D_3 binding activity was observed in the assay of these samples.

Sensitivity and Accuracy of Assay. Only that range of the standard curve from 20 to 350 pg is used, and 25% of the plasma extract is assayed. Based on 40% minimum recovery to the point of assay from a 20-ml plasma extract, the routine method is applicalbe at 1α,25-$(OH)_2$-D_3 levels of 1 ng/100 ml or higher. Larger plasma samples containing less than 1 ng/100 ml of plasma can be assayed (Table II).

To determine accuracy of the assay, various amounts of 1α,25-$(OH)_2$-D_3 were added to aliquots of a human plasma sample and triplicate assays were performed. Average recov-

eries of 100–110% (following correction for procedural losses) were obtained over the range of 0.5–9.2 ng/100 ml of added hormone (Figure 7). The endogenous level of 1α,25-$(OH)_2$-D_3, determined at no addition of hormone, was subtracted from each assay value.

Precision of Assay. A pooled plasma sample was divided into six aliquots which were processed for assay. The mean 1α,25-$(OH)_2$-D_3 concentration was 4.4 ng/100 ml, and the coefficient of variation was 12%. The mean difference of duplicate determinations of 26 plasma samples was 15.9 ± 11.7 (SD) %. Thus the specificity, sensitivity, accuracy, and precision of the assay indicate that it is a reliable method for quantitation of 1α,25-$(OH)_2$-D_3 in human plasma.

1α,25-Dihydroxyvitamin D_3 Assays in Chronic Renal Failure. In order to further verify the validity and usefulness of this assay as a diagnostic tool in measuring 1α,25-$(OH)_2$-D_3 levels in human disease states, we determined the circulating level of 1α,25-$(OH)_2$-D_3 in patients with renal disease (Table II). All renal patients had strikingly lower concentrations of hormone, whether they were subjected to hemodialysis or not. The fact that patients with advanced renal failure and anephric individuals have undetectable levels of hormone (Table II) suggests that synthesis of 1α,25-$(OH)_2$-D_3 is impaired in kidney disease and that the kidney is the major site of production of 1α,25-$(OH)_2$-D_3 in humans.

Discussion

In this report a filtration assay is described for 1α,25-$(OH)_2$-D_3 employing the chromatin receptor for the hormone in the target tissue. In the past, filtration assays for steroid hormones have been largely unsuccessful because of significant and unreproducible binding of free steroid to filters. In the present assay, conditions have been defined under which free steroid can be removed from chromatin and filters by the nonionic detergent Triton X-100. A reconstituted cytosol–chromatin mixture is incubated with a known amount of 1α,25-$(OH)_2$-[^{3}H]D_3 and a plasma extract containing unlabeled hormone. Hormone–receptor complexes are formed in the cytosol which then associate with the chromatin. The chromatin is then adsorbed on various filters, and the filter is washed free of unbound sterol.

The standard procedure for purification of 1α,25-$(OH)_2$-D_3 from plasma extracts entails serial purification using a silicic acid column, two Sephadex LH-20 columns, and a final "micro-Celite" column. Our initial attempts to devise a reliable chromatography system for purification of 1α,25-$(OH)_2$-D_3 from human plasma were only partially successful (Brumbaugh *et al.*, 1974a,b). After purification on silicic acid and Sephadex LH-20 (two columns), we routinely obtained a circulating level of 6 ng/100 ml for 1α,25-$(OH)_2$-D_3 in normal humans. Yet plasma samples which were expected not to contain appreciable hormone, such as those from vitamin D deficient chicks and anephric humans, yielded values of approximately 2 ng/100 ml by this method. These findings suggested that a significant blank value of 2 ng/100 ml existed in the assay performed on plasma purified in this fashion. Experiments with large plasma volumes such as the one pictured in Figure 6, in which individual fractions eluted from a long Celite column are assayed for both hormone and radioactive marker, indicate that the level of 1α,25-$(OH)_2$-D_3 in man is 4 ng/100 ml (10^{-10} M). We also report here the development of a "micro-Celite" chromatography system for purification of 1α,25-$(OH)_2$-D_3 from individual 20-ml plasma extracts. This procedure reduces our blank to near 0 and assays of plasma samples from 45 normal individuals using the "micro-Celite" purification scheme confirm that the previously reported average value of 6.4 ng/100 ml for circulating 1α,25-$(OH)_2$-D_3 (Brumbaugh *et al.*, 1974a,b) should be revised downward to 4 ng/100 ml.

The principle of radioreceptor assay based on the chromatin receptor for the 1α,25-$(OH)_2$-D_3 should be applicable to steroid hormones. Since this assay employs the final physiologic binding site of the hormone in the nucleus, it should be possible to develop an assay for total plasma hormone activity which measures any compound that binds to the hormone's cytoplasmic receptor and migrates to the chromatin. Because it utilizes the molecular receptor site for the forms of the hormone, such an assay can be the most meaningful assessment of biologically active hormone. A total plasma cortisol activity assay has been reported using hepatoma cell glucocorticoid cytosol receptor (Ballard and Baxter, 1974). In addition, the techniques in this report are applicable to the assay of steroid hormone–chromatin receptors and investigation of the interaction of steroid hormone–receptor complexes with experimentally altered forms of chromatin.

In summary, an accurate assay has been developed for the newly discovered sterol hormone, 1α,25-$(OH)_2$-D_3. The assay employs a high affinity, saturable receptor system from the hormone's target tissue to generate a standard curve with a useful range of 20–350 pg. Extensive chromatographic purification of plasma from humans reduces blank values virtually to zero and permits valid measurement of the hormone in normal individuals and patients with defects in vitamin D metabolism. It is evident that this assay will be of value not only in studying vitamin D metabolism in a basic sense, but also as a diagnostic tool in elucidating the possible involvement of 1α,25-$(OH)_2$-D_3 in disorders of mineral metabolism.

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References

Ballard, P. L., and Baxter, J. D. (1974), *Clin. Res. 22,* 212A.

Bligh, E. C., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37,* 911.

Brumbaugh, P. F., and Haussler, M. R., (1973), *Biochem. Biophys. Res. Commun. 51,* 74.

Brumbaugh, P. F., and Haussler, M. R. (1974a), *J. Biol. Chem. 249,* 1251.

Brumbaugh, P. F., and Haussler, M. R. (1974b), *J. Biol. Chem. 249,* 1258.

Brumbaugh, P. F., Haussler, D. H., Bressler, R., and Haussler, M. R. (1974a), *Science 183,* 1089.

Brumbaugh, P. F., Haussler, D. H., Haussler, M. R., and Ogden, D. A. (1974b), *Clin. Res. 22,* 205A.

Dische, F. (1930), *Mikrochemie 8,* 42.

Fraser, D. R., and Kodicek, E. (1970), *Nature (London) 228,* 764.

Gray, R., Boyle, I., and DeLuca, H. F. (1971), *Science 172,* 1232.

Haussler, M. R. (1972), *Steroids 20,* 639.

Haussler, M. R., Boyce, D. W., Littledike, E. T., and Rasmussen, H. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 177.

Haussler, M. R., Myrtle, J. F., and Norman, A. W. (1968), *J. Biol. Chem. 243,* 4055.

Haussler, M. R., and Rasmussen, H. (1972), *J. Biol. Chem. 247,* 2328.

Holick, M. F., and DeLuca, H. F. (1971), *J. Lipid Res. 12,* 460.
Holick, M. F., Garabedian, M., and DeLuca, H. F. (1972), *Science 176,* 1146.
Holick, M. F., Schnoes, H. K., DeLuca, H. F., Suda, T., and Cousins, R. J. (1971), *Biochemistry 10,* 2799.
Jensen, E. V., Numata, M., Brecher, P. I., and DeSombre, E. R. (1971), *in* The Biochemistry of Steroid Hormone Action, Smellie, R. M. S., Ed., London, Academic Press, p 133.
Korenman, S. G. (1968), *J. Clin. Endocrinol. Metab. 28,* 127.
Lawson, D. E. M., Fraser, D. R., Kodicek, E., Morris, H. R., and Williams, D. H. (1971), *Nature (London) 230,* 228.
McNutt, K. W., and Haussler, M. R. (1973), *J. Nutr. 103,* 681.
Santi, D. V., Sibley, C. H., Perriard, E. R., Tomkins, G. M., and Baxter, J. D. (1973), *Biochemistry 12,* 2412.
Wong, R. G., Myrtle, J. F., Tsai, H. C., and Norman, A. W. (1972), *J. Biol. Chem. 247,* 5728.
Yarus, M., and Berg, P. (1970), *Anal. Biochem. 35,* 450.

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.