Studies on the transport of vitamin D and of 25-hydroxyvitamin D in human plasma

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Abstract A study was conducted to investigate whether human plasma contains one or more than one protein for the transport of vitamin D and of 25-hydroxyvitamin D (25-OH-D). Serum was labeled in vivo with a mixture of radioactive vitamin D_3 (derived from orally administered tracer vitamin D_3) and of endogenously synthesized labeled 25-OH-D_3. Samples of such serum were subjected to several different protein fractionation procedures. Only a single peak of protein-bound radioactivity was observed after each of these procedures. The fractions comprising the ascending and the descending limbs of the single peak of protein-bound radioactivity (after each procedure) were separately pooled. In each instance the ratio of radioactive 25-OH-D_3 to radioactive vitamin D_3 was found to be almost identical in both the ascending and the descending limbs. Taken together, these findings provide strong evidence that human serum contains only a single binding protein responsible for the normal transport of both vitamin D and 25-OH-D.

Plasma labeled in vitro with added ^{3}H-labeled 25-OH-D_3 was subjected to gel filtration on Sephadex G-200 and to chromatography on columns of DEAE-cellulose and of SP-Sephadex. After each of these procedures a single peak of protein-bound radioactivity was observed, with elution profiles of protein and of radioactivity that were identical with those observed with in vivo labeled serum. These data indicate that tracer 25-OH-D_3 added to plasma in vitro binds to the same plasma protein normally responsible for the transport of vitamin D and of 25-OH-D.

Supplementary key words transport protein · binding in vivo · binding in vitro

It is now well established that the biologic expression of vitamin D activity requires the prior metabolism in the liver of the vitamin to its 25-hydroxy derivative, which in turn is converted to 1,25-dihydroxy-vitamin D in the kidney (1–3). 1,25-Dihydroxy-vitamin D, the hormonal form of the vitamin, is then secreted by the kidney and transported to target tissues, such as the intestinal mucosa, to exert its biologic effects.

Since both D and 25-OH-D are insoluble in water, the mechanisms whereby D is transported to the liver, and 25-OH-D from the liver to the kidney, represent important components of the overall metabolism of vitamin D. Recent studies have demonstrated clearly that specific plasma proteins exist for the transport of both of these compounds in plasma (4–9). Some information about the characteristics of these plasma transport proteins has been obtained from studies carried out with plasma or serum obtained from humans (4–9), rats (7–9), and chicks (7–9). These studies have shown that chick serum contains two cholecalciferol-binding proteins, one of which binds mainly cholecalciferol, and the other of which binds 25-hydroxy-cholecalciferol (7, 9). In contrast, rat serum appears to contain a single protein that binds and transports both D and 25-OH-D. Although the limited evidence available generally supports the conclusion that only one transport protein, for both compounds, is present in human plasma (7, 9), data have been reported suggesting that two transport proteins with differing specificities for D and 25-OH-D may be present in human plasma (8).

The studies reported here were designed to explore further the question of whether human plasma contains one or more than one protein for the transport of D and of 25-OH-D. In addition, data were obtained as to whether 25-OH-D added to plasma in vitro binds to the same plasma protein as does endogenous 25-OH-D.

METHODS AND MATERIALS

In vivo labeling

Two healthy volunteers (Subject R, a 21-year-old male, and Subject H, a 23-year-old female) served as experimental subjects. Each subject was given orally 2.0 ml of a monoolein–oleic acid–taurocholate emulsion containing 4 μCi of ^{3}H-labeled vitamin D_3 and 2 μCi of ^{14}C-labeled vitamin D_3.

Abbreviations: D, vitamin D; 25-OH-D, 25-hydroxyvitamin D.

The emulsion was prepared by dissolving the appropriate amount of radioactive vitamin D_3 in 0.10 ml acetone, adding to this 0.25 ml of a mixture of monoolein–oleic acid (1:2, w/w), followed by 4.65 ml of 12 mM Na taurocholate solution in 10 mM Na phosphate buffer pH 7.4 with 125 mM NaCl. A stable emulsion was produced by vigorous mixing on a Vortex mixer. Samples of blood (200–250 ml each) were collected after 2, 5, and 12 days (labeled samples R-1, R-2, R-3 and H-1, H-2, and H-3, respectively, for the two subjects). After allowing the blood to clot, serum was collected and stored at −20°C for further study. Small portions of each sample were extracted with $CHCl_3$–CH_3OH (see below), followed by chromatography on Sephadex LH-20 to determine the sample's content of radioactive D_3 and 25-OH-D_3. Larger portions of serum were subsequently subjected to gel filtration, chromatography, and/or electrophoresis as described below.

Protein fractionation procedures

Gel filtration on columns of Sephadex G-200 (Pharmacia) and chromatography on columns of DEAE-cellulose (Whatman Inc., Clifton, N.J.) were carried out as described in previous publications from this laboratory (5, 10, 11). (The procedures used with DEAE-cellulose were similar to those previously described with DEAE-Sephadex.) Specific details for illustrative examples are indicated in the legends to the figures. Chromatography on SP-Sephadex (C-50) was carried out with a column equilibrated with 0.02 M Na acetate buffer, pH 5.15, 0.02 M NaCl; elution was carried out with a linear gradient of NaCl, from 0.02 M to 0.15 M, in 0.02 M Na acetate buffer, pH 5.15. All column chromatography of serum or protein samples was carried out in a cold room at about 5°C. In most instances the effluent stream was monitored continuously for absorption of light at 280 nm with a Uvicord II Absorptiometer (LKB Instruments, Inc., Rockville, Md.).

Preparative polyacrylamide gel electrophoresis was performed with a Polyprep 100 apparatus (Buchler Instruments, Inc., Fort Lee, N.J.), as described previously (5, 10).

Extraction and chromatography of vitamin D compounds

Samples of serum, or of pooled fractions of serum proteins after chromatography or electrophoresis, were extracted with 20 volumes of $CHCl_3$–CH_3OH, 2:1 (v/v) as described previously (5). The extracting solvents contained butylated hydroxytoluene, 1 mg/100 ml, as an antioxidant, and 0.1–0.2 mg of unlabeled carrier cholecalciferol. Portions of each total lipid extract were chromatographed on columns of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), as described by Holick and DeLuca (12), using hexane–chloroform 35:65 (v/v) as eluting solvent. This procedure resulted in the complete separation of vitamin D from 25-OH-D; the chromatographic procedure was standardized and validated with pure, unlabeled vitamin D_3 and 25-OH-D_3. Fractions eluted from the Sephadex LH-20 columns were assayed for radioactivity, and the content of radioactive D_3 and 25-OH-D_3 in each serum sample was then calculated. The recovery of radioactivity after Sephadex LH-20 chromatography was usually close to 100%.

The ratio of radioactive 25-OH-D_3 to radioactive D_3 in a given sample of serum, or of fractionated serum proteins, was determined by extraction of the sample with $CHCl_3$–CH_3OH, followed by chromatography of the lipid extract on Sephadex LH-20. The amounts of radioactivity present in the pooled fractions representing D_3 and 25-OH-D_3 were measured, and the ratio of radioactive 25-OH-D_3 to D_3 present was calculated from these measured values.

In vitro labeling

200 ml of "out-dated" plasma, obtained from the blood bank of The Presbyterian Hospital was incubated with 2 μCi of ^{3}H-labeled 25-hydroxyvitamin D_3 for three hours at 4°C under N_2 in the dark. The labeled 25-OH-D_3 was added slowly, with stirring, to the serum in 1.5 ml ethanol. The labeled plasma was divided into several portions and stored at −20°C. Portions of the labeled plasma were subsequently subjected to chromatography on columns of Sephadex G-200, DEAE-cellulose, or SP-Sephadex, and the elution profiles for protein and for radioactivity were compared to those obtained with serum containing "in vivo labeled" vitamin D_3 and 25-OH-D_3.

Materials, other procedures

Radioassay of lipid extracts or of fractions from Sephadex LH-20 columns was carried out by dissolving samples in 15 ml of 0.5% diphenyloxazole in toluene, followed by assay with a Packard liquid scintillation counter (Packard Instrument Co., Dowers Grove, Ill.). Aqueous samples (e.g., effluent fractions from chromatography or electrophoresis) were assayed for radioactivity using 15 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) plus 3.5 ml of aqueous sample (or water plus aqueous sample) as scintillation mixture. Where appropriate,

TABLE 1. Serum concentrations of radioactive Vitamin D_3 and 25-Hydroxy-vitamin D_3 after oral radioactive Vitamin D_3

Sample Number	Day[a]	^{14}C Concentration in Serum	% Distribution of ^{14}C[b]	
			D_3	25-OH-D_3
		dpm/5 ml		
R-1	2	889	31	69
R-2	5	654	6	94
R-3	12	503	6	94
H-1	2	1274	61	39
H-2	5	804	22	79
H-3	12	644	16	84

[a] After oral administration of the labeled vitamin D_3.
[b] Determined by measuring the distribution of ^{14}C in D_3 and 25-OH-D_3 after Sephadex LH-20 chromatography of a total lipid extract of serum.

quenching was corrected for by means of an automatic external standardization system.

Elution of nonradioactive D and/or 25-OH-D from Sephadex LH-20 columns was monitored by measuring the absorbance of eluted fractions at 264 nm.

Radioactive vitamin D_3 ([1α,2α-^{3}H]vitamin D_3, 21 mCi/mg; [4-^{14}C]vitamin D_3, 83.8 μCi/mg) and 25-hydroxy-vitamin D_3 ([26,27-methyl-^{3}H]25-hydroxyvitamin D_3, 2.7 mCi/mg) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. The radioactive compounds were checked for purity by thin-layer chromatography and by chromatography on columns of Sephadex LH-20 before use, and all were found to be more than 95% pure. Nonradioactive vitamin D_3 was obtained from Schwartz/Mann (Division of Becton, Dickinson and Co., Orangeberg, N.Y.). Nonradioactive 25-hydroxy-vitamin D_3 was the generous gift of Dr. John Babcock, The Upjohn Co., Kalamazoo, Michigan.

RESULTS

In vivo labeling

Serum labeled in vivo with a mixture of radioactive vitamin D_3 and 25-OH-D_3 was subjected to several different protein fractionation procedures. If only one peak of protein-bound radioactivity was observed after a given procedure, then the experimental plan called for further separate analysis of each of the two limbs of the peak in order to determine the ratio of radioactive 25-OH-D_3 to radioactive D_3 in each limb.

The subjects in this study were each given a mixture of 4-^{14}C-D_3 and of 1α,2α-^{3}H-D_3, in the hope that a small peak of radioactivity enriched in ^{14}C relative to ^3H, and hence possibly representing 1,25-dihydroxy-D_3, might be observed after Sephadex LH-20 chromatography. Such a peak of radioactivity was, however, not observed in any of the samples. Radioactive compounds chromatographically identical with authentic D_3 and with 25-OH-D_3 were found in all serum samples. Virtually identical results were obtained with each of the two isotopes; accordingly, only the data for ^{14}C are presented.

Table 1 shows the distribution of radioactivity between D_3 and 25-OH-D_3 in each of the three serum samples from each subject. Subject R metabolized the administered labeled to 25-OH-D_3 more rapidly than did Subject H.

Gel filtration

A portion of serum sample R-1, containing a mixture of radioactive D_3 and 25-OH-D_3 (see Table 1), was chromatographed on a column of Sephadex G-200. The results are shown in **Fig. 1.** A single peak of radioactivity was eluted from the Sephadex column with an elution volume slightly greater than that of serum albumin. The fractions comprising the ascending and the descending limbs of the peak of radioactivity were separately pooled (see legend to Fig. 1), extracted, and the lipid extracts chromatographed on Sephadex LH-20 as described under Methods. The ratio of radioactive 25-OH-D_3 to radioactive D_3 (dpm:dpm) was found to be 2.94 in the pooled fractions comprising the ascending limb, and 3.14 in the pooled fractions comprising the descend-

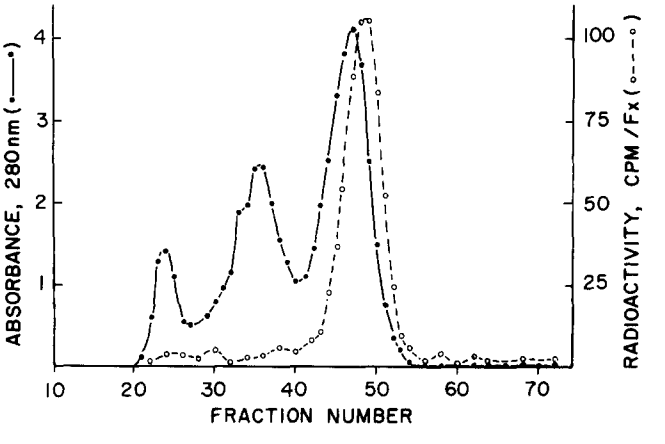

Fig. 1. Gel filtration of human serum containing radioactive vitamin D_3 and 25-hydroxy-vitamin D_3. Eight ml of serum sample R-1 was dialyzed extensively against a large volume of 0.05 M Na phosphate buffer, pH 7.4, containing 0.1 M NaCl. The sample was applied to a column (2.5 × 80 cm) of Sephadex G-200 equilibrated with the same buffer. Fractions of 100 drops each were collected by elution with the same buffer at a flow rate of 15 ml/hr. The fractions were assayed for protein by absorbance at 280 nm, and for radioactivity as described under Methods. Fractions 44–47, comprising the ascending limb of the peak of radioactivity, and fractions 50–52, comprising the descending limb, were subsequently pooled and analyzed as described in the text.

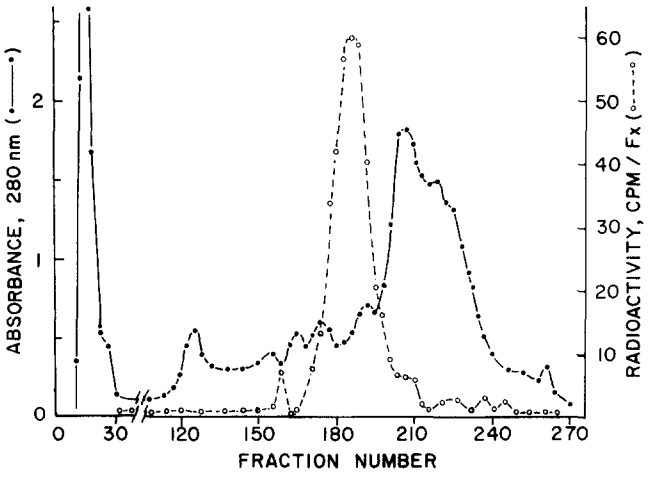

Fig. 2. Chromatography on DEAE-cellulose of human serum containing radioactive vitamin D_3 and 25-hydroxy-vitamin D_3. 10 ml of serum (5 ml each of samples H-1 and H-2) was dialyzed three times, each time against 1 l of 0.025 M Tris-HCl buffer, pH 8.0, and then applied to a column (2.5 × 26 cm) of DEAE-cellulose (DE-52) equilibrated with the same buffer. Elution was carried out with a linear gradient of NaCl from 0 to 0.15 M. Fractions of 4 ml each were collected at a flow rate of 60 ml/hr. After assaying the fractions for protein and for radioactivity, fractions 172–182, comprising the ascending limb of the peak of radioactivity, and fractions 190–200, comprising the descending limb, were pooled and analyzed as described in the text.

ing limb. The slight difference between these two values is within experimental error, and the two values are considered to be essentially identical.

DEAE-Cellulose chromatography

A sample of serum consisting of 5 ml each of samples H-1 and H-2 was chromatographed on a column of DEAE-cellulose. The results are shown in **Fig. 2.** A single peak of radioactivity was eluted from the column, appearing just before the major peak of protein (representing serum albumin). As with the gel filtration experiment (see above), the fractions comprising the ascending and descending limbs of the peak of radioactivity were separately pooled and extracted, and the lipid extracts were each chromatographed on Sephadex LH-20. The ratio of radioactive 25-OH-D_3 to radioactive D_3 was found to be 1.89 in the pooled fractions comprising the ascending limb, and 1.74 in the pooled fractions comprising the descending limb. Again, the slight difference between these two values is within experimental error, and the two values are considered to be essentially identical.

Polyacrylamide gel electrophoresis

50 ml of serum (25 ml of sample H-1 and 25 ml of sample H-2) was chromatographed on a column of DEAE-cellulose. An elution pattern similar to that shown in Fig. 2 was obtained. The fractions comprising the single peak of protein-bound radioactivity were pooled, dialyzed exhaustively against distilled water, and lyophilized. The resulting material was dissolved in 5 ml of 0.025 M Tris-HCl buffer, pH 8.0, containing 0.04 M NaCl, and then chromatographed on a column of Sephadex G-200. The fractions comprising the single peak of protein-bound radioactivity (cf. Fig. 1) were pooled and applied to a smaller DEAE-cellulose column equilibrated with 0.025 M Tris-HCl buffer, pH 8.0, 0.04 M NaCl. A single peak of protein-bound radioactivity was eluted from this column by means of a linear gradient of NaCl, from 0.04 M to 0.15 M. The radioactive fractions comprising this peak were pooled, dialyzed against water, and lyophilized.

The resulting sample was dissolved in 5 ml of 5% sucrose solution and subjected to preparative polyacrylamide gel electrophoresis. The results are shown in **Fig. 3.** Only a single peak of protein-bound radioactivity was eluted from the gel column, with mobility similar to the leading edge of the peak of serum albumin. The fractions comprising the ascending and descending limbs of the peak of radioactivity (see legend to Fig. 3) were separately pooled, extracted, and the lipid extracts were each chro-

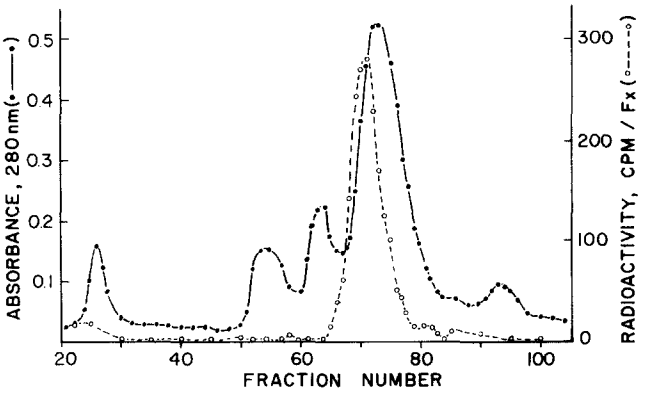

Fig. 3. Preparative polyacrylamide gel electrophoresis. Serum containing radioactive vitamin D_3 and 25-OH-D_3 (equal portions of samples H-1 and H-2) was chromatographed on a column of DEAE-cellulose, followed by gel filtration on Sephadex G-200 and repeat DEAE-cellulose chromatography as described in the text. The sample (5 ml) of partially purified serum protein containing protein-bound radioactivity was applied to a gel column 7.5 cm high. Electrophoresis was conducted at 300 V with 15 mA current. Fractions of 30 drops (1.5 ml) each were collected at a flow rate of 20–22 ml/hr. After assaying the fractions for protein and for radioactivity, fractions 65–69, comprising the ascending limb of the peak of radioactivity, and fractions 72–76, comprising the descending limb, were pooled and analyzed as described in the text. The major peak of eluted protein (absorbance peak at fractions 70–75) coming just after the peak of radioactivity was immunologically identified as human serum albumin.

matographed on Sephadex LH-20. The ratio of radioactive 25-OH-D_3 to radioactive D_3 was found to be 2.66 in the pooled fractions comprising the ascending limb, and 2.63 in the pooled fractions comprising the descending limb. The specific radioactivity of the radioactive sample obtained after gel electrophoresis (dpm per mg protein) was 116-times that of the original serum used in the sequence of procedures, representing a purification of the D/25-OH-D transport protein of at least 116-fold.

In vitro labeling

A sample of plasma (5 ml) labeled in vitro with ^{3}H-labeled 25-OH-D_3 was subjected to gel filtration on a column of Sephadex G-200. The observed elution profiles of protein and of protein-bound radioactivity were identical with those shown in Fig. 1 (for serum labeled in vivo). Thus, protein-bound radioactivity was eluted as a single peak with an elution volume slightly greater than that of serum albumin.

A second portion of the in vitro labeled plasma (10 ml) was subjected to chromatography on a column of DEAE-cellulose, using the conditions as described in the legend to Fig. 2. The observed elution patterns of protein and of protein-bound radioactivity were identical with those shown in Fig. 2. Thus, protein-bound radioactivity was eluted as a single peak that appeared just before the elution of the major peak of protein (representing serum albumin).

A third portion of the in vitro labeled plasma (10 ml) was subjected to chromatography on a column of SP-Sephadex. Protein-bound radioactivity was eluted as a single peak that appeared just after the void volume of the column and before the start of the linear gradient of NaCl (see Methods). In order to compare this result with the result that would be obtained with in vivo labeled serum, 10 ml of sample R-2, containing radioactivity almost entirely as 25-OH-D_3 (see Table 1), was chromatographed under the same conditions on a second column of SP-Sephadex. The observed elution profiles (of protein and of protein-bound radioactivity) were identical with those observed with the in vitro labeled plasma.

DISCUSSION

These experiments provide strong evidence that human serum contains only a single protein responsible for the normal transport of both vitamin D and 25-OH-D. The experiments employed serum that was labeled in vivo with a mixture of radioactive D_3, derived from orally administered tracer D_3, and of endogenously synthesized 25-OH-D_3. Samples of such serum were subjected to several different protein fractionation procedures in order to determine whether one peak or more than one peak of radioactivity would be observed. It was anticipated that if more than one protein were involved in the transport of D and of 25-OH-D, then separation of these proteins might be observed after one or another fractionation procedure. Only a single peak of protein-bound radioactivity was, however, observed after each of the three kinds of procedures carried out, viz., gel filtration on Sephadex G-200, ion-exchange chromatography on DEAE-cellulose, and electrophoresis on polyacrylamide gel.

The possibility was then considered that the single peak of protein-bound radioactivity observed after each of these procedures might really represent two closely overlapping but nonidentical peaks of radioactivity. In other words, the possibility was considered that two transport proteins with very similar properties might be present, but with different relative specificities for the binding of vitamin D and of 25-OH-D. If this possibility were true, and if the two postulated proteins separated slightly from each other during fractionation, then it would be anticipated that the ascending and the descending limbs of the peak of protein-bound radioactivity would contain different ratios of the two overlapping but nonidentical proteins.

In order to test this possibility, after each procedure the fractions comprising the ascending and the descending limbs of the single peak of protein-bound radioactivity were separately pooled, and each pool was analyzed to determine the ratio of radioactive 25-OH-D_3 to radioactive D_3 present. In each one of the three instances studied, the ratio of radioactive 25-OH-D_3 to radioactive D_3 was found to be almost identical in both the ascending and the descending limbs of the single peak of radioactivity. These findings thus strongly support the conclusion that a single transport protein is involved in the transport of both D and 25-OH-D in human plasma.

Experiments were also conducted to determine whether tracer 25-OH-D_3 added to plasma in vitro binds to the same plasma protein as does endogenously synthesized 25-OH-D_3. Information about this question was required before we could undertake the large-scale isolation of the plasma transport protein for vitamin D. Plasma labeled in vitro with added ^{3}H-labeled 25-OH-D_3 was subjected to gel filtration on Sephadex G-200 and to chromatography on columns of DEAE-cellulose and SP-Sephadex.

The elution profiles of protein and of radioactivity obtained after each of these procedures were compared with the corresponding profiles obtained after carrying out the same procedure with in vivo-labeled serum. After each of the three procedures carried out with in vitro-labeled plasma, a single peak of protein-bound radioactivity was observed. In each instance, the observed elution profiles of protein and of protein-bound radioactivity were identical with those observed with in vivo-labeled serum. These data indicate that tracer 25-OH-D_3 added to plasma in vitro binds to the same plasma protein normally responsible for the transport of vitamin D and of 25-OH-D. The addition of labeled 25-OH-D to plasma in vitro would hence be an effective way to label the vitamin D transport protein in studies aimed at its isolation and characterization.

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