The Transport of Vitamin D and Its 25-Hydroxy Metabolite in Human Plasma

ISOLATION AND PARTIAL CHARACTERIZATION OF VITAMIN D AND 25-HYDROXYVITAMIN D BINDING PROTEIN

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ABSTRACT This study reports the isolation and partial characterization of vitamin D and 25-hydroxyvitamin D binding protein (DBP), the specific transport protein for vitamin D and its 25-hydroxy metabolite in human plasma. DBP was labeled by the addition of a tracer amount of ^{3}H-labeled 25-OH-D_3 to the original plasma used for protein fractionation. Previous experiments have shown that such 25-OH-D_3 added in vitro binds to the same protein normally responsible for the transport of endogenous 25-OH-D and of vitamin D. The isolation of human DBP was achieved by an extensive sequence of procedures which resulted in a final yield of only approximately 4 mg of purified DBP from a starting volume of 34 liters of plasma. Purified DBP was homogeneous in the analytical ultracentrifuge and showed a single band of protein on analytical polyacrylamide gel electrophoresis. DBP had a sedimentation constant of 3.49*s* and a mol wt of approximately 52,000. The molecular weight was assessed by sedimentation equilibrium analysis and also by sodium dodecyl sulfate-disc-gel electrophoresis and by gel filtration on a standardized column of Sephadex G-150. The amino acid composition of DBP was determined and was generally consistent with the estimated extinction coefficient ($E_{1cm}^{1\%}$ at 280 nm) of about 9.1. The isoelectric point of DBP was estimated as 4.8 from isoelectric focusing experiments. Direct study of the binding capacity of the purified DBP for added 25-OH-D_3 showed that the isolated DBP had a high affinity for 25-OH-D_3, with an apparent maximum binding capacity of one molecule of 25-OH-D_3 per molecule of protein.

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INTRODUCTION

It is now well recognized that vitamin D and its hepatic metabolite 25-hydroxyvitamin D circulate in plasma bound to specific transport proteins. These proteins presumably serve to transport vitamin D to the liver, where it is converted to its 25-hydroxy metabolite, and to then transport this metabolite to the kidney for conversion to 1,25-dihydroxyvitamin D (1–3). 1,25-Dihydroxyvitamin 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 physiologic effects.

Information about the transport of vitamin D and 25-hydroxyvitamin D (25-OH-D)[1] in plasma has been obtained from studies conducted with plasma or serum from humans (4–9), rats (7–10), and chicks (7–9). These studies have shown that chick serum contains two vitamin D-binding proteins, one of which binds mainly cholecalciferol and the other of which binds 25-hydroxycholecalciferol (7, 9). In contrast, rat serum appears to contain a single protein which binds and transports both vitamin D and 25-OH-D (7–9).

We have recently described strong evidence that human serum also contains only a single protein responsible for the normal transport of both vitamin D and 25-OH-D (11). It was shown (11) in addition that tracer 25-OH-D_3 added to human plasma in vitro binds to the

[1] *Abbreviations used in this paper:* DBP, vitamin D and 25-hydroxyvitamin D binding protein; $E_{1cm}^{1\%}$, extinction coefficient at 280 nm; Gc, group-specific component; 25-OH-D, 25-hydroxyvitamin D; RBP, retinol-binding protein; SDS, sodium dodecyl sulfate; V_e, effluent volume; V_o, void volume.

same plasma protein normally responsible for the transport of vitamin D and of 25-OH-D. We now report the isolation and partial characterization of vitamin D and 25-hydroxyvitamin D binding protein (DBP), the specific transport protein for vitamin D and its 25-hydroxy metabolite in human plasma.

METHODS

Plasma. The large scale isolation of DBP was carried out with 34 liters of plasma in acid-citrate-dextrose anticoagulant, obtained from the blood bank of The Presbyterian Hospital, New York. To label the DBP, 1–1.5 liters of plasma was incubated with ^{3}H-labeled 25-OH-D_3, (25-hydroxy-[26(27)-methyl-^{3}H]-cholecalciferol, 2.7 mCi/mg, from Amersham/Searle Corp., Arlington Heights, Ill.) in the amount of 10 μCi added in 2 ml ethanol per liter of plasma. The ^{3}H-labeled 25-OH-D_3 was checked for purity before use by chromatography on a column of Sephadex LH-20 and was found to be greater than 95% pure. After incubation at 4°C for 5 h, the labeled plasma was dialyzed against 25 liters of deionized water three times, centrifuged at 1,600 rpm for 30 min, lyophilized, and stored at −20°. This procedure was repeated a total of 27 times to label the 34 liters of plasma.

Column chromatography. Gel filtration on columns of Sephadex G-200, G-150, or G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and chromatography on columns of DEAE-cellulose (Whatman, Inc., Clifton, N. J.) were carried out as described in previous publications from this laboratory (5, 11–13). Specific details for illustrative examples are indicated in the legends to the figures.

Chromatography on SP-Sephadex C-50 (Pharmacia Fine Chemicals, Inc.) was carried out on a large scale with the Pharmacia Sectional Column KS 370, "the Stack." Samples were applied and elution carried out using 0.02 M Na acetate buffer, pH 5.15, with 0.015 M NaCl and 0.02% Na azide. Details for specific examples of procedures using the sectional column are given in the legends for Figs. 1 and 2.

Chromatography on columns of Blue Sepharose 6B (special experimental samples furnished by Pharmacia Fine Chemicals, Inc.) was carried out using somewhat different conditions at different stages of purification. In the first Blue Sepharose chromatographic run, the sample was applied and elution carried out using 0.025 M Tris-HCl buffer, pH 8.0, with 0.15 M NaCl and 0.03% Na azide. The second Blue Sepharose chromatography employed 0.05 M Tris-HCl buffer, pH 7.0, with 0.1 M KCl (see legend to Fig. 5). The third Blue Sepharose chromatography employed 0.05 M Tris-HCl buffer, pH 7.0, alone.

All column chromatography 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.).

Electrophoresis. Preparative and analytical (disc-) polyacrylamide gel electrophoresis were carried out as reported previously (12–14). The conditions employed (12) included 7% acrylamide gel without a concentrating gel, with a continuous buffer system of Tris-glycine-HCl, pH 8.1. Sodium dodecyl sulfate (SDS)-polyacrylamide disc-gel electrophoresis was performed as described by Weber and Osborn (15).

Isoelectric focusing. Preparative and analytical isoelectric focusing was carried out on polyacrylamide gels, using carrier ampholyte purchased from LKB Instruments, Inc. (LKB Ampholine). Preparative isoelectric focusing was conducted according to the procedure described by Suzuki et al. (16), with some modifications, using apparatus supplied by MRA Corp., Boston, Mass. Preparative gels (2 × 14.5 cm) and indicator (analytical) gels (0.3 × 14.5 cm) were composed of 6% acrylamide, 0.16% bisacrylamide, 2% ampholyte (LKB Ampholine pH 4–6), 5% glycerol, 0.4% TEMED, and 0.03% ammonium persulfate. About 15 mg of protein in 2% carrier ampholyte (pH 3–10) containing 6% sucrose was applied on the preparative gel column. The sample size was about 0.2 mg protein for indicator gels. Electrophoresis was begun at a constant current of 16 mA per preparative column and 1 mA per indicator column. When the voltage reached 400 V, it was maintained at this level for an additional 17–19 h.

The indicator gels and wedge-shaped longitudinal slices cut out from the preparative gels along the long axis were treated with 5% TCA at 90°C for 15 min. By this method protein bands became visible. The positions of protein bands in the original preparative gels were determined from the results observed with the wedge-shaped gel slices. The radioactivity associated with each protein band was determined by slicing and assaying the indicator gels, as described below. Segments of the preparative gel corresponding to each protein band were minced, transferred into vials containing 5 ml water, and left overnight in the refrigerator. About 70% of the protein was recovered in this manner from the gels. The pH of the solution obtained with each gel band was measured. Additional protein was recovered from the minced gel bands by electrophoresis of the gel to cause the protein to migrate out of it. The conditions used were generally the same as those used for analytical polyacrylamide gel electrophoresis, and the procedure was conducted at 400 V at 2°C for 5 h. The protein recovered after isoelectric focusing was dialyzed exhaustively against water and lyophilized.

Analytical ultracentrifugation. Sedimentation velocity and equilibrium analyses were carried out in Dr. P. Feigelson's laboratory in a Spinco model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) equipped with a monochromator and photoelectric scanner. The absorbance of the cell contents at 280 nm was determined at intervals as a function of distance from the center of rotation. Both studies used 0.28 mg of purified DBP per ml in 0.1 M Na phosphate buffer, pH 7.1. Centrifugation was conducted at 4°C at 52,000 rpm for the sedimentation velocity analysis and at 28,000 rpm for 17 h for the equilibrium analysis. The molecular weight was calculated as reported previously (13, 14), with the method of Yphantis (17). These calculations employed the value of 0.73 as an estimate for the term $\bar{V}$, and a value of 1.0094 for ρ.

Molecular weight estimates. The molecular weight of human DBP was also estimated by SDS-disc-gel electrophoresis and by gel filtration on a standardized column of Sephadex G-150. The SDS-disc-gel electrophoresis method was similar to that described previously (13, 15). After electrophoresis the gels were stained for protein and the values of the relative mobility of different proteins were plotted against the logs of their molecular weights. The standard proteins used were: bovine serum albumin, rat serum albumin, ovalbumin, chymotrypsinogen, and trypsin. The gel filtration method was similar to the method described by Whitaker (18), as previously reported from our laboratory (12, 13). The study employed ^{125}I-labeled DBP, labeled by the lactoperoxidase method (19) and prepared in the course of development of a radioimmunoassay

for DBP, to be described in detail in another report.[3] A sample was prepared containing ^{125}I-DBP (approximately 0.82 ng and 0.08 μCi) + 2 mg each of myoglobin and human gamma globulin + a small amount of blue dextran polymer, in 0.6 ml 0.05 M K phosphate buffer, pH 7.4, containing 0.1 M NaCl. The sample was chromatographed on a column of Sephadex G-150, 1.1 × 100 cm in size. Elution with the same buffer was carried out at a flow rate of 7 ml/h; fractions of 0.9 ml each were collected. The absorbance of the effluent stream at 280 nm was monitored continuously. The effluent volume (V_e) of DBP was determined by radioassay of the eluted fractions for ^{125}I. The void volume (V_o) was determined from the effluent volume of the blue dextran polymer. The column was standardized by chromatography on it of small samples (about 2 mg each) of standard proteins, including bovine serum albumin, human prealbumin, ovalbumin, chymotrypsinogen A, and myoglobin.

The proteins of known molecular weight used as standards for these analyses were obtained from Mann Research Labs, Inc., New York, with the following exceptions. Rat serum albumin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio and was purified by preparative gel electrophoresis before use. Human prealbumin was isolated from plasma in this laboratory (14). Linear regression lines for each of the two plots (log molecular weight vs. relative mobility or vs. V_e: V_o) were calculated by the method of least squares with a Wang model 700A electronic calculator (Wang Laboratories, Inc., Tewksbury, Mass.) with programs prepared for that instrument.

Amino acid analyses. Because of the small amount of pure human DBP available, amino acid analyses were carried out on only a very few samples, all subjected to hydrolysis for the single time interval of 24 h. Duplicate samples of 0.1 mg DBP each were hydrolyzed in 6 N HCl in evacuated sealed tubes at 110°C. Samples of human prealbumin were subjected to acid hydrolysis under the same conditions at the same time to serve as standards of known composition (20). Subsequently, for tryptophan determination, one sample of 0.2 mg DBP was hydrolyzed in 4 N methane sulfonic acid containing 0.8% 3-(2-aminoethyl)indole hydrochloride for 20 h at 110°C. Amino acid analysis was performed by the method of Spackman et al. (21) on a JEOL model JLC-6AH amino acid analyzer (JEOL Analytical Instruments, Cranford, N. J.).

Binding capacity. The binding capacity of purified DBP for 25-OH-D_3 was determined by adding increasing amounts of ^{3}H-labeled 25-OH-D_3 to solutions of DBP, followed by gel filtration to determine the amount of radioactivity associated with the DBP in each sample. A series of test solutions, each containing 1.05 μg of DBP and 0.25 mg human prealbumin in 0.5 ml 0.01 M K-phosphate buffer, pH 7.4, 0.15 M NaCl, were incubated with varying amounts of ^{3}H-labeled 25-OH-D_3, added in 20 μl ethanol, at 25°C for 4 h. The prealbumin was added to each solution to provide a sufficient amount of total protein (of molecular weight similar to that of DBP) to prevent the loss of significant portions of the 1 μg of DBP by nonspecific binding (to glassware, etc.). For each test solution, a control solution, containing 0.25 mg prealbumin without DBP in the same buffer solution, with the same amount of added ^{3}H-labeled 25-OH-D_3, was prepared. After incubation, the solution was applied to a standardized column of Sephadex G-100 (0.8 × 63 cm), equilibrated with 0.01 M K-phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.02% Na azide. Elution was carried out with the same buffer at a flow rate of 4.5 ml/h; fractions of 0.9 ml each were collected. 0.5-ml portions of each fraction were assayed for radioactivity.

Other procedures. The extinction coefficient of DBP at 280 nm ($E_{1cm}^{1\%}$) was estimated by measuring the absorbance at 280 nm and the protein concentration by the method of Lowry et al. (22), with human serum albumin as a standard, on the same solution of pure DBP.

Absorbances and absorption spectra were measured with a Gilford model 2400 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) or a Beckman DB spectrophotometer.

Large volumes of column effluents were concentrated by ultrafiltration using the Sartorius Membranfilter Ultrafiltration System (Sartorius Membranfilter, Göttinger, W. Germany).

Radioassay of column effluents or other protein solutions was carried out by adding a measured volume of aqueous sample plus water as needed to provide a total volume of 3.5 ml, to 15 ml of Aquasol (New England Nuclear, Boston, Mass.) in a counting vial, followed by radioassay in a Packard model 3003 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Radioassay of gel slices from indicator gels after isoelectric focusing was carried out by digestion of the minced gel slice with 0.5 ml of H_2O_2 at 50°C overnight in a counting vial, followed by the addition of 10 ml of Aquasol and subsequent direct assay in the scintillation counter.

RESULTS

Purification of DBP. DBP was isolated from human plasma by the following sequence of procedures: chromatography on SP-Sephadex (repeated once); gel filtration on Sephadex G-200; chromatography on DEAE-cellulose, repeated serially for a total of four times; chromatography on Blue Sepharose 6B, repeated a total of three times; gel filtration on Sephadex G-100; preparative isoelectric focusing; and preparative polyacrylamide gel electrophoresis. These procedures resulted in the isolation of a sample of DBP which had been purified at least 13,000-fold from whole plasma.

Chromatography on SP-Sephadex. DBP in plasma was labeled in vitro by the addition of tracer amounts of ^{3}H-labeled 25-OH-D_3, as described under Methods. The lyophilized, labeled plasma (34 liters total) was divided into seven portions, and each portion was chromatographed on a large, sectional column of SP-Sephadex, as described in Fig. 1. After each chromatographic run, the eluted radioactive fractions were combined, concentrated by ultrafiltration, dialyzed against water, lyophilized, and stored at − 20°C.

Second chromatography on SP-Sephadex. The radioactive protein samples obtained after each of the first SP-Sephadex chromatographic runs (seven samples, each obtained as shown in Fig. 1) were combined and dissolved in 3 liters of 0.02 M Na acetate buffer, pH 5.15, with 0.015 M NaCl and 0.02% Na azide. The resulting sample was chromatographed on a sectional column of SP-Sephadex C-50 (37 cm × 30 cm, two sections) equilibrated with the same buffer, with elution

[3] Imawari, M., and DeW. S. Goodman. Manuscript in preparation.

with this buffer then carried out at the rate of 4 liters/h. The eluted radioactive fractions (15 liters) were combined and concentrated by ultrafiltration. The recovery of radioactivity (^3H) during this chromatographic procedure was 78%. The purification of DBP (in terms of radioactivity [dpm] per unit of absorbance at 280 nm) was 8.3-fold, as compared with the original plasma.

Gel filtration on Sephadex G-200. The sample obtained after SP-Sephadex chromatography was subjected to gel filtration as shown in Fig. 2. The recovery of radioactivity was 96%, with an additional 1.84-fold increase in purity (as defined above).

DEAE-cellulose chromatography (four times). DBP was purified further by chromatography on DEAE-cellulose, repeated serially for a total of four times. The results of the first of these chromatographic runs are shown in Fig. 3. The radioactive fractions obtained from this procedure were combined and rechromatographed on a smaller (4 × 45-cm) column of DEAE-cellulose, using conditions generally similar to those described for Fig. 3, but with a flow rate of approximately 100 ml/h. The radioactive fractions from the second DEAE-cellulose chromatographic run were combined and chromatographed on a still smaller (2.5 × 45-cm) column of DEAE-cellulose, followed by a further, fourth chromatography on DEAE-cellulose (column size 2 × 23 cm). The recovery of radioactivity during the series of four DEAE-cellulose chromatographies

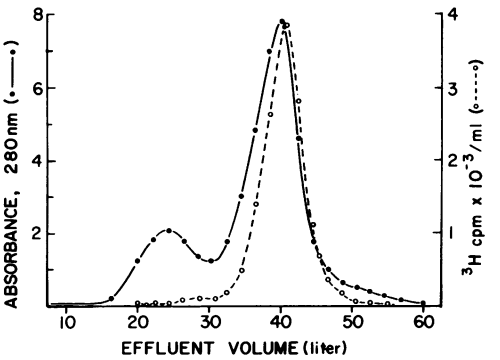

FIGURE 2 Gel filtration on Sephadex G-200. The sample obtained after the second SP-Sephadex chromatography (1,240 ml) was applied to a sectional column (four sections, 37 cm × 60 cm) containing Sephadex G-200 equilibrated with 0.025 M Tris-HCl buffer, pH 8.0, with 0.05 M NaCl and 0.03% Na azide at a flow rate of 3 liters/h. Elution was carried out with the same buffer at a flow rate of 4.5 liters/h, under pressure of 0.11 kg/cm^2. Eluted fractions were assayed for protein (absorbance at 280 nm) and for radioactivity. The fractions containing radioactivity (1,287 ml total) were pooled and used for further purification.

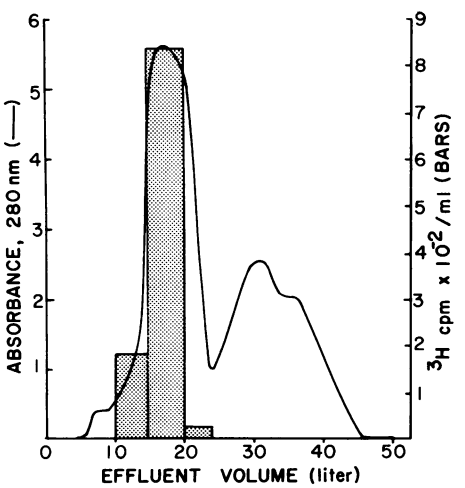

FIGURE 1 Chromatography on SP-Sephadex of human whole plasma labeled in vitro with ^3H-labeled 25-OH-D_3. Lyophilized protein corresponding to 5 liters of plasma was dissolved in 3 liters of 0.02 M Na acetate buffer, pH 5.15, with 0.015 M NaCl and 0.02% Na azide, left overnight at 4°C, and centrifuged at 8,000 rpm for 45 min to clarify. After adding 3 more liters of the same buffer to the supernatant solution, the sample was applied to a column of SP-Sephadex C-50, comprising two sections of "the Stack" (37 cm × 30 cm), previously equilibrated with the same buffer at the rate of 2.5 liters/h. Elution with the same buffer was carried out at a flow rate of 5 liters/h. Eluted fractions were assayed for protein by absorbance at 280 nm, and for radioactivity as described under Methods.

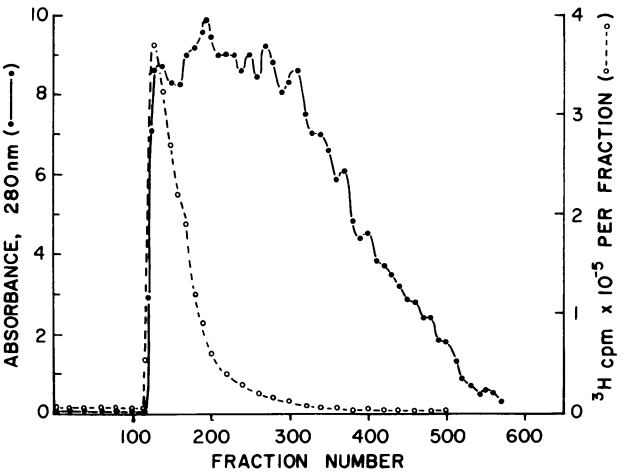

FIGURE 3 First DEAE-cellulose chromatography. The sample obtained after Sephadex G-200 gel filtration (1,287 ml) was applied to a column of DEAE-cellulose (5 × 95 cm) equilibrated with 0.025 M Tris-HCl buffer, pH 8.0, with 0.05 M NaCl and 0.03% Na azide. Elution was carried out with a linear gradient of NaCl, from 0.05 to 0.14 M in 0.025 M Tris-HCl buffer. The collection of fractions, as shown in the Figure, was begun at the time the salt gradient was initiated. Fractions of 18 ml each were collected at a flow rate of 180 ml/h. After assaying eluted fractions for protein (absorbance at 280 nm) and for radioactivity, the fractions showing significant radioactivity (fractions 116–191) were combined to yield a pool of 1,480 ml volume, which was used for repeat chromatography on a smaller column of DEAE-cellulose.

was 26%, with an additional 24-fold increase in purification achieved.

Blue Sepharose 6B chromatography (two times). This procedure was carried out in order to remove plasma albumin, which binds tightly to Blue Sepharose 6B under the appropriate conditions. The radioactive eluate from the fourth DEAE-cellulose chromatography (330 ml) was mixed with 100 ml 0.44 M NaCl (final concentration 0.15 M) and applied to a column of Blue Sepharose 6B equilibrated with 0.025 M Tris-HCl buffer, pH 8.0, with 0.15 M NaCl and 0.03% Na azide. Elution with the same buffer was carried out at a flow rate of 55 ml/h. All the fractions showing absorbance at 280 nm were combined (667 ml total), dialyzed exhaustively against water, and lyophilized. It was estimated that about 75 mg of plasma albumin (which remained bound to the column) was removed from the sample (which comprised a total of approximately 500 mg protein before chromatography). Immunodiffusion of the eluate vs. anti-albumin antiserum showed that there still was present in the preparation about 10 mg of albumin.

Chromatography on Blue Sepharose 6B was repeated under slightly different conditions, as described in Fig.

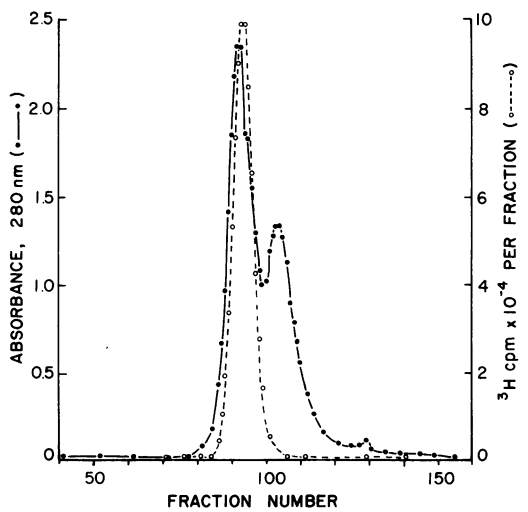

FIGURE 4 Second Blue Sepharose 6B chromatography. The lyophilized protein obtained after the first Blue Sepharose 6B chromatography was divided into three portions. Each portion in turn was dissolved in 4 ml 0.05 M Tris-HCl buffer, pH 7.0, with 0.1 M KCl, and applied to a column of Blue Sepharose 6B (2.5 ×47.5 cm) equilibrated with the same buffer solution. Elution was carried out with this buffer at a flow rate of about 15 ml/h; fractions of 1.8 ml each were collected. After assaying the eluted fractions for protein (absorbance at 280 nm) and for radioactivity, the radioactive fractions (numbers 88 through 98 in this Figure) were pooled, dialyzed against water, and lyophilized. The lyophilized pools from all three chromatographic runs (constituting the entire sample) were subsequently combined for gel filtration on Sephadex G-100.

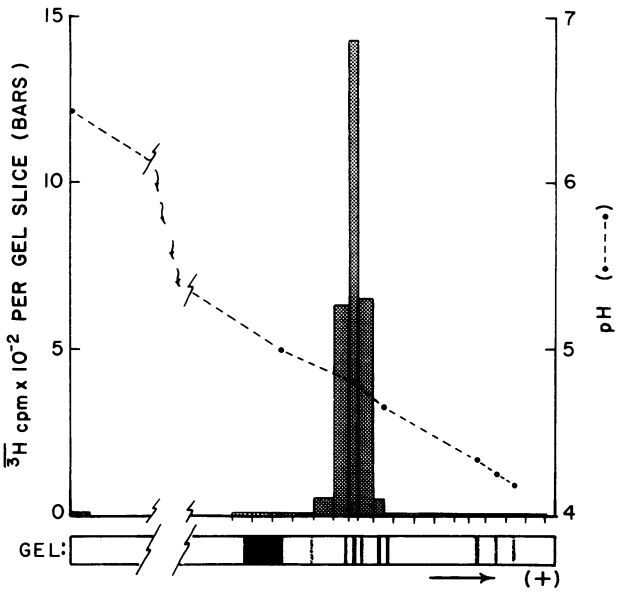

FIGURE 5 Isoelectric focusing on polyacrylamide gel. A sample of approximately 0.2 mg protein was analyzed in an indicator (analytical) gel as described under Methods. The separated proteins at the end of the procedure were visualized as comprising one broad band and nine sharp bands of varying intensity as diagramed in the bottom of this figure (labeled GEL). The protein bands were all found in the anodal half of the gel, representing the pH range 4.0–5.2. Accordingly, in the diagram shown here, one-half of the cathodal half of the gel (which contained no protein bands and no radioactivity) is not shown but is represented by bracketed wavy lines. Gel slices corresponding to each protein band and to areas of gel which lacked protein bands were assayed for radioactivity, and the results are shown above in bar graph form.

4. The recovery of radioactivity was 96%, with a further 2.7-fold increase in purification.

Gel filtration on Sephadex G-100. The sample from the preceding step (see legend to Fig. 4) was dissolved in 5 ml 0.025 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl and 0.03% Na azide, and applied to a column of Sephadex G-100 (2.5 × 144 cm). Elution was carried out with the same buffer. Fractions of 3.2 ml were collected at a flow rate of about 12 ml/h. The radioactive fractions were pooled, dialyzed against water, and lyophilized. The recovery of radioactivity was 88%, with a further 1.8-fold increase in purification.

Third Blue Sepharose 6B chromatography. The sample obtained after gel filtration was applied to a column of Blue Sepharose 6B (2.5 × 47 cm), equilibrated with 0.05 M Tris-HCl buffer, pH 7.0. Elution was carried out with the same buffer. Fractions of 1.4 ml were collected at a flow rate of about 5 ml/h. The radioactive fractions were pooled, dialyzed against water, and lyophilized. Albumin was fully removed by this procedure.

Preparative isoelectric focusing. The sample ob-

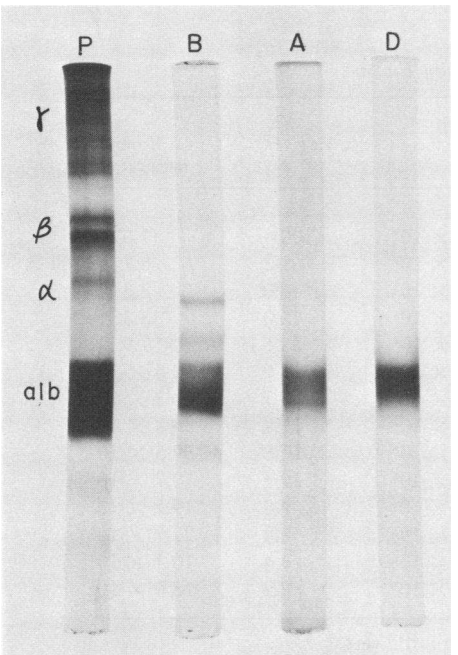

FIGURE 6 Polyacrylamide disc-gel electrophoretic analysis of samples of (from left to right): (a) whole human plasma (P); (b) the partly purified DBP before preparative isoelectric focusing (B); (c) the partly purified DBP after isoelectric focusing (A); and (d) the final purified DBP preparation (D).

tained after the third Blue Sepharose chromatographic step was analyzed by isoelectric focusing and by electrophoresis on polyacrylamide gels (Figs. 5 and 6, respectively). On disc-gel electrophoresis, the sample showed one major and five minor bands of protein (Fig. 6, gel B). On isoelectric focusing (with an analytical, indicator gel) one broad band and nine sharp bands of lesser protein content were revealed (Fig. 5). Radioactivity was associated mainly with the middle band of a group of three sharp bands, as shown in Fig. 5.

Accordingly, further purification of DBP was undertaken by preparative isoelectric focusing, as described under Methods. This procedure resulted in the recovery of 68% of the radioactivity applied to the preparative gel with a further 4.6-fold increase in purification. Disc-gel electrophoresis of the sample obtained after isoelectric focusing showed one major and two minor bands of protein (Fig. 6, gel A).

Polyacrylamide gel electrophoresis. Preparative gel electrophoresis was carried out as described in Fig. 7. Approximately 4 mg of purified DBP was obtained. On analytical (disc-) gel electrophoresis this purified DBP showed only a single band of protein, with mobility similar to that of plasma albumin (Fig. 6, gel D). This DBP was subjected to gel filtration on a small column of Sephadex G-200. The DBP-containing fractions from the Sephadex G-200 column were dialyzed exhaustively against water and lyophilized to dryness. The lyophilized DBP was stored at − 60°C.

Table I summarizes the results obtained throughout the entire sequence of procedures used for the isolation of plasma DBP. The final recovery of radioactivity in the purified DBP was 2.2%. The apparent purification, estimated from the specific radioactivity (expressed as dpm ^{3}H per unit of absorbance at 280 nm), was 12,960. Since it is likely that some radioactive 25-OH-D_3 was removed from its binding protein and lost during the course of the purification sequence, it is probable that the final preparation represented DBP purified more than 13,000-fold.

Analytical ultracentrifuge studies. Sedimentation velocity study showed that purified DBP migrated as a single homogeneous protein with a sedimentation constant ($s_{20,w}$) of 3.49*s*. Fig. 8 shows the results of sedimentation equilibrium analysis. DBP appeared to be a single homogeneous component, as can be judged from the linearity of the data. The mol wt of DBP was estimated to be 51,800.

Spectroscopy. The absorption spectrum of purified DBP in solution showed a peak of absorbance with maximum at 276 nm. The extinction coefficient ($E_{1cm}^{1\%}$) at 280 nm was measured with a solution of DBP showing absorbance at 280 nm of 0.339, and at 276 nm of 0.353. The determination (see Methods) was repeated once, given two values of 9.36 and 8.81. The value of 9.1 was hence taken for the $E_{1cm}^{1\%}$.

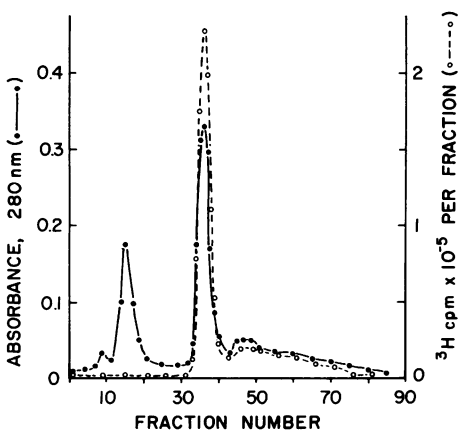

FIGURE 7 Preparative polyacrylamide gel electrophoresis of the DBP preparation obtained after preparative isoelectric focusing. Approximately 9 mg protein was applied to a gel column 7-cm high (about 80 ml volume). The major portion of the electrophoresis was carried out at 300 V with 15 mA current. Fractions of 1.9 ml each were collected at a flow rate of about 16 ml/h. The fractions were assayed for protein (absorbance at 280 nm) and radioactivity. Fractions 33 through 39, comprising the sharp peak of protein and protein-bound radioactivity, were pooled, dialyzed exhaustively against water, and lyophilized.

TABLE I

The Isolation of DBP from Human Plasma

Procedure	Total A_{280}*	Apparent specific radioactivity‡	Recovery of ^3H§	Apparent purification‖
			%	
(Whole plasma)	1.89×10^6	3.14×10^2	(100)	(1.0)
1st SP-Sephadex	1.73×10^5	2.10×10^3	61.3	6.7
2nd SP-Sephadex	1.10×10^5	2.60×10^3	48.0	8.3
Sephadex G-200	5.71×10^4	4.79×10^3	46.0	15.3
1st DEAE-cellulose	1.10×10^4	1.82×10^4	33.5	57.9
2nd DEAE-cellulose	1.73×10^3	7.07×10^4	21.0	22.5×10
3rd DEAE-cellulose	1.04×10^3	9.58×10^4	16.7	30.5×10
4th DEAE-cellulose	6.10×10^2	1.14×10^5	11.7	36.3×10
1st Blue Sepharose	5.17×10^2	1.24×10^5	10.8	39.5×10
2nd Blue Sepharose	1.84×10^2	3.37×10^5	10.4	10.7×10^2
Sephadex G-100	90	6.04×10^5	9.2	19.2×10^2
3rd Blue Sepharose	53.5	7.14×10^5	6.4	22.7×10^2
Isoelectric focusing	8.03	3.25×10^6	4.4	10.4×10^3
Preparative gel electrophoresis	3.24	4.07×10^6	2.2	13.0×10^3

* Calculated as absorbance at 280 nm (A_{280}) × volume in milliliters.

‡ Calculated as total disintegrations per minute of ^3H divided by total absorbance at 280 nm (i.e., dpm per unit of A_{280}).

§ Expressed in cumulative terms (original labeled plasma = 100% of radioactivity).

‖ Calculated from the increase in apparent specific radioactivity and expressed in cumulative terms (original whole plasma = 1.0).

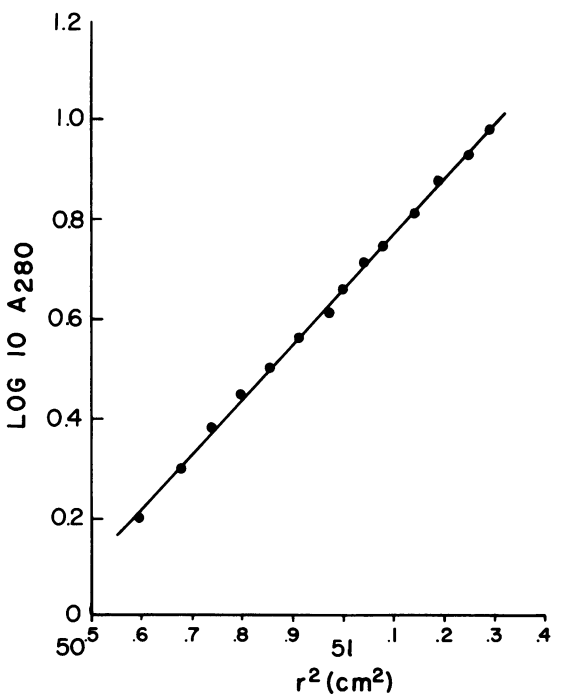

FIGURE 8 Sedimentation equilibrium analysis of purified DBP. The Figure shows the logarithm of 10 times the absorbance at 280 nm vs. the square of the radial distance, within the rotor, from the center of rotation.

Molecular weight. In addition to the sedimentation equilibrium study described above, the molecular weight of purified DBP was also estimated by SDS-disc-gel electrophoresis and by gel filtration on a standardized column of Sephadex G-150. The SDS-disc-gel electrophoresis study was repeated twice, giving the following three values as estimates of the mol wt: 51,500, 52,000, and 51,600. Gel filtration provided a mol wt estimate of 56,000. Thus, the values obtained by the several different methods agreed quite well with each other. Since the sedimentation equilibrium analysis was considered quantitatively the most accurate, the value of 52,000 was taken as an estimate of the mol wt of DBP.

Amino acid composition. The results of the amino acid analyses of purified DBP are presented in Table II. Samples of human prealbumin, subjected to acid hydrolysis and analyzed concurrently, showed results which conformed to the known amino acid composition of prealbumin (20). These assays served to verify the accuracy of the methods employed. The estimated number of residues per DBP molecule, shown in the right-hand column of Table II and calculated from the percentage distribution of observed micromoles, was determined for a protein of mol wt between 51,000 and 52,000. On amino acid analysis of the sample obtained after methane sulfonic acid hydrolysis, no peak was observed before that of tryptophan corresponding to the expected

location for hexosamine (e.g., glucosamine or galactosamine). It was anticipated that such a peak would have been seen if hexosamine comprised more than about 1–2% of the weight of the molecule. No specific assays for carbohydrate were carried out.

Binding capacity of DBP. The binding capacity of purified DBP for ^{3}H-labeled 25-OH-D$_3$ was determined as described under Methods. Increasing amounts of labeled 25-OH-D$_3$ were added to solutions of DBP, followed by gel filtration to determine the amount of ^{3}H (and hence, of 25-OH-D$_3$) associated with DBP in each sample. Solutions of DBP with 25-OH-D$_3$ added in amounts varying from a molar ratio (25-OH-D$_3$: DBP) of 1:10 to one of 20:1 were examined. The results are shown in Fig. 9. When ^{3}H-labeled 25-OH-D$_3$ was added to DBP in a molar ratio of 1:1 or less, radioactivity was eluted as a single peak at the effluent volume expected for DBP. When 25-OH-D$_3$ was, however, added in excess of DBP (molar ratios of 10:1 or 20:1), radioactivity was eluted as two peaks, one with V_e corresponding to DBP, and a later peak with V_e corresponding to low molecular weight compounds. The amount of protein-bound radioactivity eluted with the DBP peak increased as the amount of added ^{3}H-labeled 25-OH-D$_3$ was increased, up to a molar ratio of 1:1 in the sample applied to the column. At higher molar ratios of 25-OH-D$_3$ to DBP, the amount of protein-bound radioactivity represented by specific binding to DBP (see Fig. 9 legend) did not increase significantly beyond that seen at a molar ratio of 1:1. These data suggest that DBP has a high affinity for 25-OH-D$_3$, but with a binding capacity for only one molecule of 25-OH-D$_3$ per molecule of DBP.

To explore further the binding capacity of DBP for 25-OH-D$_3$, the eluted fractions obtained after gel filtration of the solution containing 25-OH-D$_3$ and DBP in a molar ratio of 10:1 (Fig. 9) were analyzed further. The radioactive fractions corresponding to DBP were

TABLE II

Amino Acid Composition of Human DBP

Amino acid	Observed: HCl hydrolysis*	Observed: MSA hydrolysis‡	Distribution of observed micromoles§	Estimated no. of residues per DBP molecule
	μmol × 10^2		%	
Lysine	5.06	5.10	8.88	41
Histidine	0.81	0.85	1.42	6–7
Arginine	1.76	1.43	3.09	14
Aspartic acid	5.40	5.05	9.47	44
Threonine	3.62	3.70	6.49	30
Serine	4.49	4.73	8.30	38
Glutamic acid	6.92	6.33	12.14	56
Proline	3.14	3.28	5.51	25–26
Glycine	2.64	1.85	4.63	21
Alanine	4.08	3.43	7.16	33
Half-cystine	2.57	2.33	4.51	21
Valine	3.02	2.50	5.30	24–25
Methionine	1.02	1.30	1.79	8
Isoleucine	0.57	1.13	1.00	5
Leucine	6.77	5.78	11.88	55
Tyrosine	1.49	1.90	3.33	15–16
Phenylalanine	2.27	1.98	3.98	18
Tryptophan	—	0.65	1.14	5
TOTAL	55.63	53.32	100.0	459–463

* This column represents the mean values observed for each amino acid after hydrolysis of duplicate samples of 0.1 mg DBP each in 6 N HCl. There was good agreement between the results of the duplicate analyses.

‡ This column lists one-half the values observed for each amino acid after hydrolysis of a single sample of 0.2 mg DBP in 4 N methane sulfonic acid (MSA).

§ These values were calculated from the number of micromoles observed for each amino acid, using the values observed after hydrolysis with 4 N methane sulfonic acid for tryptophan, threonine, serine, and tyrosine, and the values observed after hydrolysis with 6 N HCl for the other amino acids.

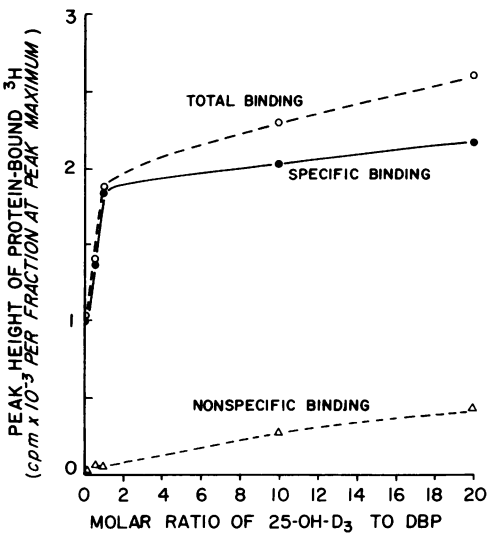

FIGURE 9 The binding capacity of purified DBP for 25-hydroxyvitamin D$_3$. A series of five test solutions was prepared, each containing 1.05 μg of DBP and 0.25 mg prealbumin (see Methods). Each solution was chromatographed on a standardized small column of Sephadex G-100 (see Methods). After each run, the eluted fractions were assayed for protein (absorbance at 280 nm) and for radioactivity. The magnitude of the peak of eluted ^{3}H corresponding to the effluent volume of DBP is shown in this Figure, plotted as the observed peak height of eluted protein-bound ^{3}H (curve labeled "total binding"). Since this observed peak was comprised both of ^{3}H-labeled 25-OH-D$_3$ specifically bound to DBP and of labeled 25-OH-D$_3$ nonspecifically bound to prealbumin, a series of control solutions was prepared containing prealbumin without DBP, but with the same amounts of added ^{3}H-labeled 25-OH-D$_3$. The magnitudes of the small peaks of protein-bound ^{3}H observed with these control solutions are shown in the figure (curve labeled "nonspecific binding"). The amount of ^{3}H-labeled 25-OH-D$_3$ specifically bound to DBP for each molar ratio studied was then calculated by subtracting the value for nonspecific binding from that for total binding, thus giving the data points and curves marked "specific binding" in this Figure.

pooled and assayed for ^3H. The corresponding fractions from the control sample (prealbumin alone, see legend to Fig. 9) were also pooled, and the ^3H content of this sample (representing nonspecific binding) was subtracted from that of the DBP-containing sample, to determine the amount of ^3H-labeled 25-OH-D_3 specifically bound to DBP. From the known specific radioactivity of the added 25-OH-D_3, it was calculated that the concentration of specifically bound 25-OH-D_3 in the sample was 2.00×10^{-4} nmol/ml. The concentration of DBP in the sample was determined by radioimmunoassay,[2] and was estimated as 2.07×10^{-4} nmol/ml (mean of three values from different assays). The molar ratio of 25-OH-D_3 specifically bound to DBP in the sample was hence 1:1. Since the solution originally had a 10-fold molar excess of 25-OH-D_3, it is likely that the DBP was saturated with 25-OH-D_3, and that the molar ratio of 1:1 represents the maximum binding capacity of DBP for 25-OH-D_3.

DISCUSSION

This report describes the isolation and partial characterization of human plasma vitamin D and 25-hydroxyvitamin D binding protein, the transport protein for vitamin D and for 25-OH-D in human blood. This protein, which we have called DBP, has a sedimentation constant of 3.49*s* and a mol wt of approximately 52,000. The molecular weight was assessed by sedimentation equilibrium analysis and also by SDS-disc-gel electrophoresis, and by gel filtration on a standardized column of Sephadex G-150. The isolation of human DBP was achieved by an extensive sequence of procedures which resulted in a final yield of only approximately 4 mg of purified DBP from a starting volume of 34 liters of plasma. The amino acid composition of the purified DBP is presented in Table II, and is generally consistent with the estimated extinction coefficient ($E_{1cm}^{1\%}$) at 280 nm of about 9.1. DBP had mobility similar to that of plasma albumin on polyacrylamide gel electrophoresis under the conditions employed. The isoelectric point of DBP was estimated to be 4.8, from the results of isoelectric focusing.

Plasma DBP was identified in this work by the addition of a tracer amount of ^3H-labeled 25-OH-D_3 to the original plasma used for protein fractionation. Previous experiments have shown that such 25-OH-D_3 added in vitro binds to the same protein normally responsible for the transport of endogenous 25-OH-D_3 and of vitamin D (11). Direct study of the binding capacity of the purified DBP for added 25-OH-D_3 demonstrated that the isolated DBP had a high affinity for 25-OH-D_3, with an apparent maximum binding capacity of one molecule of 25-OH-D_3 per molecule of protein. We presume that DBP would manifest a similar binding capacity for vitamin D_3, although direct studies with vitamin D_3 have not been carried out. Previous studies of the competitive displacement of labeled 25-OH-D_3 from its plasma transport protein, with whole or slightly purified plasma preparations, have demonstrated a higher affinity of the plasma binding protein for 25-OH-D_3 than for vitamin D_3 itself, in both the human (4) and the rat (8).

The isolation and partial characterization of a human vitamin D-binding plasma protein has previously been reported by Peterson (6). This protein was reported to have a mol wt of approximately 53,000, a sedimentation constant of 3.8*s*, and α_1-mobility. Although the properties of this reported protein (6) are in many ways similar to those of DBP reported here, significant differences do exist. Thus, Peterson estimated a value of 15.0 for the $E_{1cm}^{1\%}$ at 280 nm, whereas DBP reported here had a value of approximately 9.1. In addition, major immunologic differences exist between the two preparations, since the preparation reported by Peterson was immunologically different from the group-specific component (Gc) proteins in plasma, whereas the purified DBP reported here has been found to be immunologically identical with the Gc proteins.[2] It should be noted, in addition, that the preparation reported by Peterson was labeled in vitro by the addition of ^{14}C-labeled vitamin D_3 to plasma, and that neither the amino acid composition nor direct data on the binding capacity of the purified preparation were reported.

Human plasma DBP resembles plasma retinol-binding protein (RBP), the specific transport protein for vitamin A, in apparently having one binding site per molecule of protein for one molecule of the appropriate lipid-soluble ligand (12). Both proteins serve the physiologically important role of solubilizing biologically important but water insoluble compounds which require transport to their target tissues or other anatomic sites. The interaction of retinol with RBP has also been shown to protect the unstable retinol molecule against chemical degradation (12, 23). It remains to be determined whether the interaction of 25-OH-D_3 or of vitamin D with DBP serves a similar function. RBP differs from DBP, however, in being a smaller protein (mol wt 21,000), which circulates in plasma in the form of a protein-protein complex with plasma prealbumin. DBP and RBP also appear to differ significantly with regard to their metabolic regulation. It is known that RBP is secreted from the liver and circulates in plasma almost entirely as the retinol-RBP complex (i.e., the holoprotein) (23–24). In contrast, most of plasma DBP appears to be unsaturated with either 25-OH-D or with vitamin D. Thus, it has been reported that rat serum contains a relatively high binding capacity for 25-OH-D_3, representing approximately 20 times the nor-

mal concentration of circulating 25-OH-D_3 (25). In addition, recent experiments in our laboratory using a radioimmunoassay procedure developed for DBP [2] have demonstrated that immunoreactive DBP is present in normal human plasma in considerable molar excess as compared to either 25-OH-D or to vitamin D itself. Studies of this and related questions are under continuing investigation.

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