Isolation and Partial Characterization of a Human Vitamin D-binding Plasma Protein*

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SUMMARY

Vitamin D circulates in human plasma bound to a specific transport protein. This protein differs from the lipoproteins and has a hydrated density greater than 1.21. The purification of the human vitamin D-binding protein was accomplished by use of ammonium sulfate fractionation, DEAE-Sephadex chromatography, sulfoethyl-Sephadex chromatography, and gel chromatography. These procedures resulted in a highly purified preparation of the vitamin D-binding protein which had been purified approximately 15,000-fold. The purified protein appeared homogeneous by Ouchterlony immunodiffusion analyses, immunoelectrophoresis, and by analytical ultracentrifugation. The vitamin D-binding protein separated into two components on electrophoresis, both with $\alpha_1$ mobility. The most anodal component carried vitamin D$_{3}$, whereas the cathodal form of the vitamin D-binding protein was devoid of this form of the vitamin. The molecular weight of the vitamin D-binding protein determined by equilibrium ultracentrifugation and estimated from the sedimentation coefficient and gel chromatography was approximately 53,000. Determinations of the molecular weight of reduced and alkylated vitamin D-binding protein in 6 M guanidine hydrochloride gave the same value as found under physiological conditions, suggesting that this protein is not composed of subunits. The frictional ratio ($f/f_0$) was low for the vitamin D-binding protein, indicating a close to spherical appearance for this protein.

The occurrence of the vitamin D-binding protein in normal serum, normal urine, and normal cerebrospinal fluid was established by Ouchterlony immunodiffusion analyses with use of a specific antiserum against the vitamin D-binding protein. Indirect estimates indicated that the normal concentration of this protein in serum is approximately 5 $\mu$g per ml.

Experimental Procedure

Materials

Plasma—Outdated blood was obtained from the Blood Center, University Hospital, Uppsala. After centrifugation of the blood the plasma was sucked off and used immediately or after storage at -20°C.

Urine—Normal urine was obtained from healthy individuals. Urine collection and sample processing were carried out as described elsewhere (6).

Cerebrospinal Fluid—Samples of cerebrospinal fluid were taken from five individuals in connection with diagnostic myelographies. The cerebrospinal fluids were considered normal, and the subjects were judged to be free from neurological disease. The samples were concentrated as described in a previous publication (7).

Antisera—A polyvalent antiserum against urinary proteins from patients with tubular proteinuria was kindly provided by Dr. I. Berggard. Anti-human serum protein serum and anti-Gc-globulin serum were purchased from Behringwerke AG (Marburg/Lahn, Germany). An antiserum against a highly purified preparation of the vitamin D-binding protein was raised in a rabbit with a previously described technique (8).

Other Materials—Sephadex G-100 and G-200, DEAE-Sephadex A-50, and sulfoethyl-Sephadex C-50, products of Pharmacia Fine Chemicals AB (Uppsala), were treated according to the instructions supplied by the manufacturer. $^{14}$C-Labeled vitamin

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The labeled protein was dialyzed against 6
carrier-free $^{125}I$ (Radiochemical Centre, Amersham) according
standard 12-mm double sector cells with sapphire windows.
tronic speed control device. Determinations were carried out in
equipped with an RTIC temperature control unit and an elec-
gels were handled as described previously (7).

Highly purified vitamin D-binding protein was labeled with
performed in 0.4
roxine-binding globulin, a ribonuclease and carboxypeptidase B
reduced and alkylated in 1
60,000 rpm for sedimentation analyses, and recordings were
accomplished with the photoelectric scanning system set at
280 nm. Calculations were carried out according to the method
of Schachman (10). All values given are corrected to 20° and
water.

Sedimentation equilibrium analyses were performed by the
meniscus-depletion technique of Yphantis (17) as devised by
Teller et al. (18). Recordings were made with the photoelectric
scanning system set at 280 nm. The speed and time required
for establishing equilibrium were estimated as described by van
Holden (19). A final check of the equilibrium conditions was
obtained by taking recordings over a time period of several hours.
It was assumed that when no Redistribution of material could be
detected in recordings taken with a 5-hour interval, equilibrium
had been established.

Calculations of apparent weight average molecular weights
were computed from the following equation (20)

$$M_w = 2RT(d\ln C/dx^2)/(1 - \delta)p$$

where the symbols have their usual meaning. The partial
specific volume was calculated from parallel sedimentation
equilibrium runs in solvents with H2O and D2O, respectively,
according to the equation given by Edelstein and Schachman (21)

$$kM_w = 2RT(d\ln C/dx^2)/(1 - V_{m20}/k)$$

where a value of 1.0155 for $k$ was assumed (21).

**Determination of Stokes Radius—**Analytical gel chromatog-
raphy was performed on a Sephadex G-200 column (100 x
1.0 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, con-
taining 0.15 M NaCl. The details of this procedure have been
outlined elsewhere (9). All experiments were carried out at
$+4^\circ$, fractions of 1.0 ml were collected, and analyses were
performed in duplicate.

Stokes molecular radius, $r_s$, was calculated from the equation
of Laurent and Killander (22)

$$K_{av} = e^{-\lambda L/(r_s + r_c)^2}$$

where $K_{av}$ is the volume available for the analyzed protein in
the gel phase, and $L$ and $r_c$ are constants characteristic of each
gel type. $L$ was determined from the $K_{av}$ of human albumin ($r_c$
= 35.5 A) and $r_s$ was assumed to be 6.5 A (23). The Stokes
radius of the vitamin D-binding protein was used for the calculation of
the molecular weight according to the method of Siegel and
Monty (24)

**Other Methods—**Ouchterlony immunodiffusion analyses (25)
were carried out with a previously described technique (26).
Immunoelectrophoresis was performed according to
Scheidegger (27).

Plasma lipoproteins were separated from non-lipoproteins by
ultracentrifugation in a Spinco L-2 65 preparative ultracentrifuge
with use of rotor 65. Samples of plasma were adjusted to a
density of 1.21 and centrifuged as described by Havel, Edel,
and Bragdon (28).

Protein concentrations in the unpurified fractions containing
the vitamin D-binding protein were estimated by the modified
Folin procedure of Lowry et al. (29) with immunoglobulin G as
the standard. More highly purified fractions were quantita-
tively assayed for protein by measuring the absorbance at 280 nm
assuming an $E_{280}$ of 15.0 based on the relation between the
absorbance at 280 nm and the measured quantity determined by
the Folin method.

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1. Pharmacia Fine Chemicals AB, Uppsala, in preparation.
2. S. F. Nilsson, and P. A. Peterson, manuscript in preparation.
All operations during the isolation procedure were carried out at +4°C.

RESULTS

Distribution of Vitamin D₃ among Human Plasma Proteins

To achieve some information about the number and the size of vitamin D₃-binding proteins in human plasma the following experiment was undertaken.

Plasma, to which small amounts of [³C]-labeled vitamin D₃ had been added, was chromatographed on a column (337 × 2.4 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. It was evident from the chromatogram that [³C]-labeled vitamin D₃ was eluted at two positions. The material emerging at the void volume of the column contained about half of the added radioactivity whereas the residual radioactivity was eluted at a position somewhat later than that of albumin. Fractions in the two positions containing radioactivity were separately pooled, concentrated, and subjected to ultracentrifugation after the density of each sample had been raised to 1.21. All radioactivity of the material recovered in the void fraction was associated with material of density less than 1.21, whereas the [³C]D₃ eluted later than albumin had sedimented and thus was of a density greater than 1.21. A similar distribution of vitamin D₃ has been described for dog plasma after addition of the compound in vivo (3). This pattern is also obtained in plasma from monkeys. In a study on the time course of the distribution of [³C]-labeled vitamin D₃ after in vivo administration to monkeys, it has been shown that the radioactivity first associates with the lipoproteins and later appears in a protein which has similar characteristics as the human counterpart. This protein represents the vitamin D₃ carrier in plasma and was hence isolated.

Purification of Human Vitamin D₃-Binding Protein

The human vitamin D₃-binding protein was isolated from altogether 30 liters of outdated plasma. During the isolation procedure [³C]-labeled vitamin D₃ was added in order to trace the vitamin D-binding protein. In later stages of the purification procedure the vitamin D binding protein was occasionally followed by polyacrylamide gel electrophoresis.

During the first preparations, the starting material consisted of 0.5 to 4 liters of plasma but it was noted that the vitamin D-binding protein was present in amounts too low to obtain quantities of the purified protein sufficient for characterization. The isolation procedure adopted was very reproducible and a large scale purification was achieved. The fractions eluted at the position corresponding to the Kᵥₜ of [³C]D₃ on analytical gel chromatography, elution volume 84 to 100 liters, were pooled and concentrated by ultracentrifugation. Adjacent fractions contained at most negligible quantities of the vitamin D-binding protein. This was shown by addition of [³C]D₃ to these concentrated fractions followed by analytical gel chromatography. For the further purification of the vitamin D-binding protein the concentrated material, 22,700 mg of total protein, was divided into four aliquots which were separately purified according to the procedure described below.

First DEAE-Sephadex Chromatography—The fraction of concentrated vitamin D-binding protein from the gel chromatography step was exhaustively dialyzed against 0.05 M Tris-HCl buffer, pH 7.4, and thereafter subjected to ion exchange chromatography on a column (42 × 6.5 cm) of DEAE-Sephadex, equilibrated with the same buffer. Elution was performed at pH 7.4 with a 5000-mL linear gradient of NaCl from 0 to 0.3 M. Prior to application, [³C]-labeled vitamin D₃ was added to the dialyzed sample. To enhance equilibration between unlabeled endogenous and labeled exogenous vitamin D the sample was incubated at 37°C for 2 hours. A good separation was achieved between the [³C]D₃-labeled vitamin D-binding protein and the bulk of the contaminating proteins. The fractions containing radioactivity were combined and concentrated by ultrafiltration.

Chromatography on Sulfoethyl Sephadex—Various conditions for further purification of the vitamin D-binding protein were tested. The following procedure was finally adopted.

A column (31 × 1.9 cm) of sulfoethyl-Sephadex was equilibrated with 0.02 M sodium acetate buffer, pH 5.05, containing 0.03 M NaCl. The fraction containing the vitamin D-binding protein from the DEAE-Sephadex chromatography step was dialyzed against three changes of the same buffer and then applied to the column. The starting pH is critical since protein in this fraction tends to precipitate at pH 5.0 at the low ionic strength employed. The column was eluted with a linear salt gradient of 600 ml from 0.03 to 0.15 M NaCl in the sodium acetate buffer. Multiple protein peaks were obtained. The [³C]D₃
radioactivity was, however, eluted in a position which did not coincide with any of the protein peaks. Thus a considerable purification of the vitamin D-binding protein was achieved. The radioactive vitamin D₃ fractions were pooled and concentrated.

**Gel Chromatography on Sephadex G-100**—The concentrated fraction containing the vitamin D-binding protein obtained from the sulfoethyl-Sephadex chromatography step was subjected to gel chromatography on a Sephadex G-100 column (130 × 2 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 9.0, containing 0.15 M NaCl. The applied protein consisted of at least three components as indicated by the partly resolved elution peaks. The last eluted material coincided with the distribution of the radioactivity. Eluates corresponding to this position were combined and concentrated by ultrafiltration.

**Second DEAE-Sephadex Chromatography**—The fraction containing the vitamin D-binding protein obtained from the previous purification step was chromatographed on a column of DEAE-Sephadex, equilibrated with 0.05 M Tris HCl buffer, pH 9.0, containing 0.10 M NaCl. The sample was applied after extensive dialysis against the same buffer and the column was run as described in the legend of Fig. 1. The protein was eluted at three positions. ¹⁴C-Labeled vitamin D₃ was present only in the last protein peak. It can be seen from Fig. 1 that the specific activity of [³⁵S]D₃ was constant over the frontal part of this peak. Fractions with the same specific activity were pooled as indicated in the figure, dialyzed exhaustively against distilled, deionized water, and lyophilized. This material constituted purified vitamin D-binding protein.

The tail of the last protein peak which contained the residual amount of the vitamin D-binding protein was rechromatographed under identical conditions. The elution profile was similar to that depicted in Fig. 1, except that the first protein peak of the elution diagram was missing. The protein peak immediately preceding the position of the vitamin D-binding protein was always present on rechromatographs. This material was pooled and concentrated. Evidence will be given below to certify that it represents vitamin D-binding protein. Part of the last eluted protein peak had on rechromatography a constant specific radioactivity. This material was accordingly treated as highly purified vitamin D-binding protein. The total amount of purified vitamin D-binding protein obtained after this purification step was 7.2 mg.

**Homogeneity, Purity, and Immunological Properties of Vitamin D-binding Protein**—The homogeneity of the isolated preparation of the vitamin D-binding protein was tested by gel chromatography on a column of Sephadex G-200. The applied sample emerged as a symmetrical protein peak from the column as did the [¹⁴C]D₃ radioactivity. Thus, the specific radioactivity was constant over the entire protein distribution indicating a high degree of purity and size homogeneity for the tested preparation.

Although the vitamin D-binding protein appeared homogeneous on gel chromatography, polyacrylamide gel electrophoresis revealed charge heterogeneity. Fig. 2 shows that highly purified vitamin D-binding protein gave two protein zones (Fig. 2A), both with α₁ mobility, whereas the tail part of the peak containing the vitamin D-binding protein obtained from the second DEAE-Sephadex chromatography step contained several additional components (Fig. 2B). The two zones of the electrophoretically separated vitamin D-binding protein was eluted from the gel. Analyses of the distribution of [¹⁴C]-labeled vitamin D₃ revealed that only the zone with highest anodal mobility contained radioactivity. To exclude that the electrophoretically slower component was unrelated to the vitamin D-binding protein, Ouchterlony immunodiffusion analysis was performed. It is evident from Fig. 3 that both components reacted with the specific anti-vitamin D-binding protein serum and showed complete immunological identity. This indicated that the vitamin D-binding protein, on complexing with vitamin D₃ had a higher electrophoretic mobility than when free from this form of the vitamin. This was corroborated by the finding that the slower component, after addition of vitamin D₃ changed its electrophoretic mobility to that of the faster [¹⁴C]D₃-containing component. In the same experiment it was shown that the protein peak always emerging somewhat earlier than [¹⁴C]D₃ on the second DEAE-Sephadex chromatography step (cf. Fig. 1) reacted with the specific anti-vitamin D binding protein serum indicating that this peak also constitutes vitamin D-binding protein (Fig. 3).

The purity of the isolated vitamin D-binding protein was tested at various concentrations by immunoelectrophoresis and by Ouchterlony immunodiffusion analysis with polyvalent antisera directed against human serum proteins and against human urinary proteins. It was found that neither antiserum reacted with highly purified vitamin D-binding protein whereas both antisera reacted with the contaminating protein present in the tail part of the [³⁵S]D₃-containing peak obtained by the second DEAE-Sephadex chromatography step (cf. Fig. 1 and Fig. 2B). This contaminant was identified as Gc-globulin by use of a specific anti-serum against this protein. The antiserum raised against the preparation of highly purified vitamin D-binding protein gave a single arc of precipitation with the vitamin D-binding protein when examined on immunoelectrophoresis, giving further evidence for the purity of the vitamin D-binding protein.

**Demonstration of Vitamin D-binding Protein in Normal Biological Fluids**—The occurrence of the vitamin D-binding protein in
Isolation of Vitamin D-binding Protein

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Fig. 2 (left). Polyacrylamide gel electrophoresis in Tris-glycine buffer, pH 8.9, of highly purified vitamin D-binding protein (A) and of the tail part of the peak containing the vitamin D-binding protein obtained from the second DEAE-Sephadex chromatography (B) (cf. Fig. 1).

Fig. 3 (center). Results of Ouchterlony immunodiffusion analysis of different vitamin D-binding protein components. Comparison of the anodal [14C]D-containing component (1) and the cathodic component (2) eluted from a polyacrylamide gel after electrophoretic separation (cf. Fig. 2A), and of the protein peak immediately preceding that containing [14C]D obtained on the second DEAE-Sephadex chromatography (3) (cf. Fig. 1). The center well contained an antiserum against the vitamin D-binding protein.

Fig. 4 (right). Demonstration of the vitamin D-binding protein in normal serum (1), normal urine (2), and normal cerebrospinal fluid (3) by Ouchterlony immunodiffusion analysis. The center well of the plate contained an antiserum against the vitamin D-binding protein.

Fig. 5. Determination of the molecular weight of the vitamin D-binding protein in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. Separate experiments were performed in H2O (A) and D2O (B), with an initial concentration of 0.4 mg per ml. r denotes the distance from the center of rotation, and b the bottom of the cell. The speed for these analyses was 30,000 rpm.

Table I

| Physical properties of human vitamin D-binding protein |
|--------------------------------------------------------|
| Sedimentation constant, $s_{20,w}$ (S) | 3.8 |
| Partial specific volume, $\rho_p$ (ml/g) | 0.73 |
| Stokes molecular radius, $R_m$ (A) | 31 |
| Diffusion constant, $D_{20,1}$ | 6.9 |
| Frictional ratio, $f/f_s$ | 1.22 |
| Molecular weight | |
| Sedimentation equilibrium | 52,800 |
| Sedimentation Stokes radius | 51,000 |
| Gel chromatography | 53,000 |

a Determined by parallel sedimentation equilibrium ultracentrifugations in H2O and D2O.

b Estimated by analytical gel chromatography. The diffusion constant is given as $10^{-6}$ cm$^2$ sec$^{-1}$.

c The speed used for the ultracentrifugations was 30,000 rpm.

d Determined in 6 M guanidine hydrochloride on reduced and alkylated vitamin D-binding protein.

Normal serum, normal cerebrospinal fluid, and normal urine was established by Ouchterlony immunodiffusion analyses. As can be seen in Fig. 4, material reacting like the vitamin D-binding protein was present in these three biological fluids. The vitamin D-binding protein from the different sources gave reactions of complete immunological identity.

Physical Properties of Vitamin D-binding Protein—Some physical properties of the vitamin D-binding protein are summarized in Table I.

Sedimentation velocity analyses were carried out at protein concentrations of 0.02 to 0.1%. The vitamin D-binding protein behaved as a single homogeneous component within this con-
indicates the value obtained for the vitamin D-binding protein. The molecular weight of the vitamin D-binding protein was estimated by sedimentation equilibrium ultracentrifugation. The molecular weight of the vitamin D-binding protein was estimated by sedimentation equilibrium ultracentrifugation.

The two components had apparently the same molecular size and molecular weight. The adopted isolation procedure gave a recovery of approximately 0.5 μg per ml of starting material, indicating a yield of about 10%.

The vitamin D-binding protein isolated from plasma consisted of a mixture of two components, both with α1 mobility, but which separated from each other on polyacrylamide gel electrophoresis. The two components had apparently the same molecular size and weight and exhibited complete immunological identity on Ouchterlony immunodiffusion analysis. The major of these two components contained bound vitamin D3, whereas the other component did not. Since small amounts of protein-bound vitamin D3 were lost during the purification procedure, it is likely that much of the vitamin D-binding protein devoid of vitamin D3 found in the purified preparation arose during the course of the purification. It is, however, also possible that a small amount of vitamin D-free vitamin D-binding protein is normally present circulating in plasma, in analogy to the vitamin A-transporting protein (31). Further work, now in progress, is required for elucidating this question.

Acknowledgments—I wish to thank Dr. S. A. Lidén for introducing me to the large scale gel chromatography method and for assistance during that part of the experiments. I am grateful to Professor T. C. Laurent for placing an ultracentrifuge at my disposal.

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FIG. 6. Determination of the molecular weight of the vitamin D-binding protein by gel chromatography in 6 M guanidine hydrochloride. The Sephadose 6B column (300 x 1.5 cm) was equilibrated with 6 M guanidine hydrochloride titrated to pH 8.0. The column was calibrated with 1, human serum albumin; 2, human thyroxine-binding globulin; 3, heavy chains of human immunoglobulin G; 4, carboxypeptidase B; 5, light chains of human immunoglobulin G; 6, human retin-binding protein; 7, human prealbumin; 8, ribonuclease; 9, β1-microglobulin. The arrow indicates the value obtained for the kav of the vitamin D-binding protein. A protein with a sedimentation coefficient and molecular weight obtained from the ultracentrifugation data. Furthermore, this result indicates that the vitamin D-binding protein has a spherical appearance with a radius of 1.22, indicating a close to spherical appearance assuming "normal" hydration, and consists probably of a single polypeptide chain.

FIG. 5A shows two experiments conducted at 50,000 rpm. Fig. 5A depicts the result obtained in 0.02 M Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl and Fig. 5B shows the result obtained in the same buffer with D2O substituted for H2O. By this procedure the partial specific volume of the vitamin D-binding protein was estimated to 0.73 ml per g. The measured molecular weight was 52,800 and the linear relationship obtained when In C was plotted versus r2 indicated homogeneity for the examined preparation of the vitamin D-binding protein. The molecular weight obtained from the ultracentrifugation data. Furthermore, this result indicates that the vitamin D-binding protein has a "normal" hydration, and consists of a single polypeptide chain.

Gel Chromatography in 6 M Guanidine Hydrochloride—In order to investigate the possibility of a subunit structure for the vitamin D-binding protein it was subjected to gel chromatography on a column of Sepharose 6B equilibrated with 6 M guanidine hydrochloride. The column was calibrated with reference proteins as described in the legend of Fig. 6. The reduced and alkylated vitamin D-binding protein always appeared in the same position as heavy chains of immunoglobulin G (Fig. 6). From these experiments a molecular weight of 53,000 was estimated for the vitamin D-binding protein, a value in good agreement with the ultracentrifugation data. Furthermore, this result indicates that the vitamin D-binding protein probably consists of a single polypeptide chain.

The experiments reported here demonstrate that vitamin D circulates in human plasma bound to a specific plasma protein. This protein has α1 mobility, a molecular weight of 53,000, and apparently differs from all previously identified plasma proteins. The vitamin D-binding protein has a sedimentation constant (kav) of 3.8 S, a frictional ratio (f/f0) of 1.22, indicating a close to spherical appearance assuming "normal" hydration, and consists probably of a single polypeptide chain.

From the level of vitamin D normally circulating in plasma (30), one can estimate the usual level of the vitamin D-binding protein to be approximately 5 μg per ml. This figure, however, assumes that the vitamin D-binding protein carries a single molecule of vitamin D and is saturated with the vitamin. Preliminary studies with use of an immunological method for quantitation of the vitamin D-binding protein has given results in accord with this estimate. The concentration of the vitamin D-binding protein in serum indicates that the final preparation of the vitamin D-binding protein must have been purified about 15,000-fold. The adopted isolation procedure gave a recovery of highly purified vitamin D-binding protein of about 0.5 μg per ml of starting material, indicating a yield of about 10%.

The vitamin D-binding protein isolated from plasma consisted of a mixture of two components, both with α1 mobility, but which separated from each other on polyacrylamide gel electrophoresis. The two components had apparently the same molecular size and weight and exhibited complete immunological identity on Ouchterlony immunodiffusion analysis. The major of these two components contained bound vitamin D3, whereas the other component did not. Since small amounts of protein-bound vitamin D3 were lost during the purification procedure, it is likely that much of the vitamin D-binding protein devoid of vitamin D3 found in the purified preparation arose during the course of the purification. It is, however, also possible that a small amount of vitamin D-free vitamin D-binding protein is normally present circulating in plasma, in analogy to the vitamin A-transporting protein (31). Further work, now in progress, is required for elucidating this question.

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