Human Serum Binding Protein for Vitamin D and Its Metabolites

I. PHYSICOCHEMICAL AND IMMUNOLOGICAL IDENTIFICATION IN HUMAN TISSUES*

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The physicochemical and immunological characteristics of the human tissue binding protein for 25-hydroxycholecalciferol (25-OH-D₃) were studied. The serum 25-OH-D₃ binding protein sedimentation in 5 to 20% linear sucrose gradients was 3 to 4 S whether detected by 25-OH-D₃ binding, the radiodination of the purified protein, or by specific radioimmunoassay. All 25-OH-D₃ binding by serum was attributable to immunoassayable material in the 3 to 4 S region. In contrast, all 25-OH-D₃ binding and immunoassayable material in high speed supernatants of tissue extracts migrated in the 5 to 6 S region. The serum and tissue binding proteins were revealed to be indistinguishable by immunoassay and double immunodiffusion studies. In contrast to serum binding protein (58,000), the tissue binding protein was estimated to have a molecular weight of 95,000 by calibrated gel filtration analyses and was not altered by treatment with reducing agents, 0.5 M KCl and Triton X-100. ¹²⁵I-labeled serum binding protein, when incubated with extracts of cultured human skin fibroblasts at physiological pH, formed a ¹²⁵I-labeled complex which was indistinguishable from the tissue binding protein labeled with 25-OH-D₃. The ¹²⁵I-labeled complex was dissociated by 6 μM guanidine HCl or sodium dodecyl sulfate (SDS) treatment, resulting in ¹²⁵I sucrose gradient sedimentation and SDS-polyacrylamide gel electrophoresis migration characteristic of the serum binding protein. Collectively, these data provide clear evidence that the tissue binding protein represents an interionic association between an unidentified tissue factor and the serum binding protein.

Specific binding of vitamin D sterol is recognized to involve a serum DBP for vitamin D and its metabolites and two tissue binding proteins. Human serum DBP has been isolated and partially characterized in several laboratories (1-3). It is a 3.5 S, M, = 58,000 single polypeptide chain which has a binding preference for 25-OH-D₃ over vitamin D₃ and 1,25-(OH)₂D₃ (1) and which circulates in the serum at 6 to 8 x 10⁻⁶ M, predominantly in the apo form (4-6). A soluble 3.7 S tissue binding protein, which preferentially binds 25-OH-D₃, has been demonstrated in several target tissues such as avian intestine (7), human and avian parathyroid (8, 9), and rat intestine (10) and bone (11). Although it has not been isolated (12), the latter protein appears to be a "receptor" (13), analogous to those proposed for steroid hormone action (14).

The nature and role of the other tissue binding protein are less well defined. A 5 to 6 S, soluble binding protein, which preferentially binds 25-OH-D₃ has been demonstrated in all nucleated tissues examined in man (8), rat (15-19), and chicken (20, 21). The 5 to 6 S tissue binding protein does not dissociate into a serum DBP form during 0.5 M KCl or dithioerythritol treatment (15). However, serum DBP appeared when the 105,000 x g supernatant (cytosol) from tissue extracts, containing the 5 to 6 S binding protein, was heated to 60°C, and the 5 to 6 S moiety was formed when dilutions of serum were mixed with tissue cytosol. The cytosolic protein which binds to serum DBP was reported not to have sterol binding ability and to be heat labile (17). These authors raised the possibility that the 5 to 6 S binding protein reflected plasma contamination of tissues during cytosol preparation, and others have interpreted its widespread occurrence and lack of species specificity as evidence for its artifactual nature (22).

The relationship between serum DBP and the 5 to 6 S binding protein (tissue DBP) has been an area of considerable interest since the tissue form apparently displays a higher binding affinity for 25-OH-D₃ (15, 16). All previous work has depended on ligand binding to monitor the presence of and experimental changes in these binding proteins. We have employed immunological techniques and radiolabeled, purified human DBP as well as ligand binding in studies designed to examine rigorously the nature of the 5 to 6 S tissue DBP. Our studies demonstrate an interionic binding between DBP and a heat-labile cytosol protein. In the accompanying paper (23), data are presented which indicate that the binding of DBP to the cytosol protein is of high affinity and specificity.

EXPERIMENTAL PROCEDURES

Materials

All chemicals used were reagent grade or best grade available. Sephadex G-25, DEAE-Sephadex, DEAR-cellulose, and Sephadex LH-20 were purchased from Pharmacia. Ovalbumin, grade III ("egg albumin"), lactoperoxidase, trypsin, agarose, type I, bovine serum albumin (Fraction IV powder), trypsin (bovine pancreas, type III), ribonuclease, and bovine γ-globulin were obtained from Sigma Chemical Co. Proteins of known molecular weight (human γ-globulin, human serum albumin, ovalbumin, and pancreatic chymotrypsino-
gen) were purchased as a "molecular marker" kit from Schwarz/ Mann. Soybean trypsin inhibitor was from Worthington. Hanks' balanced salt solution, trypsin-EDTA, and fetal calf serum were purchased from Grand Island Biological Co. Enriched minimal essential media (MEM-a) was provided by the Cancer Biology Center.
Washington University. Crystalline reference 25-OH-D₃ was a generous gift from Dr. John Babcock of the Upjohn Co.

Carrier-free sodium-¹²⁵I was purchased from Amersham. 25-Hydroxy-26,27-7-Hdrocalceiferol (specific activity 11.3, 11.7, 80, or 94 C/mmol) was obtained from Amersham and purified on a column (1 × 50 cm) of LH-20 slurred in CHCl₃-N-hexane (65:35, v/v) before use (24). It was stored in a sterile grade benzene under N₂ at -4°C. Aqueous acetylation counting was performed in Hydromix (Yorktown) or Formula 963 (New England Nuclear).

**Human Serum and Tissues—**Serum samples were obtained from six healthy adult volunteers and human tissues were obtained from surgical specimens that remained after pathological examination: jejunum, parathyroid, and skeletal muscle. All other tissues (lung, testis, and liver) were obtained within 2 h of death. Both surgical and autopsy specimens were kept frozen or on ice until cytosols were prepared. Mucosal scrapings from the small intestine of chicks were prepared immediately prior to use.

**Cultured Human Fibroblasts—**A primary fibroblast cell line was established from a punch biopsy of normal adult human skin. The cells were grown as monolayers in enriched minimal essential media supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ humidified atmosphere. At confluency, the cells were split one to four. The cell suspensions in a balanced salt solution containing 0.001 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% soybean trypsin inhibitor. After a 10-min incubation at 4°C, the cell suspension was pelleted as before and resuspended three times in 30 ml of the NaCl/PO₄ buffer. The final cell pellet was resuspended in 3 ml of 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl (Buffer A) and homogenized in a glass tube using a motor-driven Teflon pestle. The homogenate was centrifuged in a Beckman L 62-B ultracentrifuge in an SW 50.1 rotor for 45 min at 105,000 × g. Supernatants, excluding floating lipid, were used in all studies and called “cytosols.”

The surgical and autopsy tissues were minced and washed three or four times with the NaCl/PO₄ buffer. The minced tissues were homogenized in this buffer in a Virtis “23” homogenizer or with a Teflon pestle and then centrifuged at 300 × g for 20 min at 4°C. The supernatants were centrifuged at 105,000 × g as described above.

Protein determinations were made on all of the cytosol preparations by the method of Lowry (25) using bovine serum albumin as standard. The cytosols were stored frozen at -4°C.

**Preparation of Cytosol—**Confluent adult human skin fibroblasts (about 30 million cells/150-cm² culture flask) were washed as monolayers in enriched minimal essential media supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ humidified atmosphere. At confluency, the cells were split one to four. The average doubling time for these cells was 2 days. The cells were harvested by 0.025% trypsin and 0.002% EDTA and centrifuged for 5 min at 1,500 rpm. The supernatant was gently aspirated and the cells were resuspended in 30 to 50 ml of Hanks’ balanced salt solution containing 0.001 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% soybean trypsin inhibitor. After a 10-min incubation at 4°C, the cell suspension was pelleted as before and resuspended three times in 30 ml of the NaCl/PO₄ buffer. The final cell pellet was resuspended in 3 ml of 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl (Buffer A) and homogenized in a glass tube using a motor-driven Teflon pestle. The homogenate was centrifuged in a Beckman L 62-B ultracentrifuge in an SW 50.1 rotor for 45 min at 105,000 × g. Supernatants, excluding floating lipid, were used in all studies and called “cytosols.”

**Preparation of Cytosol—**Confluent adult human skin fibroblasts (about 30 million cells/150-cm² culture flask) were washed as monolayers in enriched minimal essential media supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ humidified atmosphere. At confluency, the cells were split one to four. The average doubling time for these cells was 2 days. The cells were harvested by 0.025% trypsin and 0.002% EDTA and centrifuged for 5 min at 1,500 rpm. The supernatant was gently aspirated and the cells were resuspended in 30 to 50 ml of Hanks’ balanced salt solution containing 0.001 M CaCl₂ and 0.317 mg/ml of soybean trypsin inhibitor. After a 10-min incubation at 4°C, the cell suspension was pelleted as before and resuspended three times in 30 ml of the NaCl/PO₄ buffer. The final cell pellet was resuspended in 3 ml of 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl (Buffer A) and homogenized in a glass tube using a motor-driven Teflon pestle. The homogenate was centrifuged in a Beckman L 62-B ultracentrifuge in an SW 50.1 rotor for 45 min at 105,000 × g. Supernatants, excluding floating lipid, were used in all studies and called “cytosols.”

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**Identification of Human Serum DBP in Tissues—**DBP was isolated from Cohn IV fractions of human plasma by DEAE-cellulose and DEAE-Sephadex chromatography, gel filtration on Sephadex G-200, and preparative polyacrylamide gel electrophoresis. The purified preparation was homogeneous as judged by analytical polyacrylamide gel electrophoresis, analytical ultracentrifugation, immunoelectrophoresis against rabbit anti-human serum, and its ability to raise monospecific anti-DBP antiserum in rabbits. Details of these procedures are described elsewhere (1).

DBP was iodinated using the lactoperoxidase technique (26). Three to five micrograms of purified, lyophilized DBP was dissolved in 20 µl of 0.05 M sodium phosphate buffer, pH 7.4, and transferred to a small glass tube. The dispersion was made viscous with 2 µl of the same buffer and 1 or 2 µl of carrier-free sodium-¹²⁵I. In 25 µl of buffer were added to the reaction vessel. After 1 µl of 0.088 M hydrogen peroxide was added (1 to 2 s), 1.0 ml of the phosphate buffer was added and the entire iodination mixture was immediately applied to a column (1 × 17 cm) of Sephadex G-25 that had been equilibrated at room temperature in 0.01 M Tris-HCl, pH 7.5, containing 0.025% sodium azide and 0.025% egg albumin (Buffer B). The filtration was carried out at a rate of 1.0 ml/min and the DBP iodination was carried out by descending flow. The first 60 ml were discarded, and the last 5/6 of the outer volume (3.0 ml) was retained for further purification. One milliliter aliquots were layered onto a column (1 × 110 cm) of Sephadex G-200 equilibrated in the same buffer at 4°C, and ascending flow was carried out at 13 ml/h. The iodinated material which eluted at 68 to 72 ml was bound by anti-DBP antiserum and had the same sedimentation velocity as DBP-25-OH-¹²⁵I, and reference DBP. It was, therefore, used in all experiments and estimates of its specific activity ranged from 50 to 100 µCi/pg.
Physicochemical Treatments of DBP and Cytosol

All treatments were carried out in buffer A with the conditions as listed. Dithioerythritol (0.002 M) was incubated for 30 min at 4°C, with 0.002 M dithioerythritol present in the sucrose gradient. 0.005 M p-hydroxymercuribenzoate was incubated for 1 h at 4°C. Iodoacetamide (0.05 M) was incubated for 10 min at 23°C and then for 60 min at 4°C with cytosol; but 0.05 M iodoacetamide was incubated with 0.002 M dithioerythritol for 1 h at 37°C with DBP, 0.5 M KCl for 30 min at 4°C, also present in the sucrose gradient. Six molar guanidine HCl was incubated for 2 h at 45°C under nitrogen alone and with the addition of 0.1 M dithioerythritol and 0.3 M iodoacetamide for an additional 30 min. Sodium dodecyl sulfate (1%) was present overnight and in the sucrose gradient. Trypsin (1 mg/ml) or 0.5 mg/ml of RNase was incubated for 1 h at 23°C. Heat treatment consisted of 60°C for 60 min. In the pH treatment, 125I-DBP was dialyzed overnight at 4°C in buffer pH 4.0, 6.0, 7.4, 9.0, or 11.0 and then analyzed on gradients made with the corresponding pH buffer. The p-hydroxymercuribenzoate, iodoacetamide, and 6 M guanidine HCl incubations were dialyzed against Buffer A prior to analysis. The method and quantity analyzed are indicated in Table I. Each experiment contained control gradients with samples of serum DBP and cytosol DBP that were not treated, as well as a bovine serum albumin marker.

RESULTS

Immunological Similarity of Serum and Tissue DBP—The open circles in Fig. 1A and B, indicate the sedimentation positions of 25-OH-3HJD3 bound to human small intestinal mucosal cytosol and human serum after sucrose gradient ultracentrifugation. A aliquots of each fraction from both sucrose gradients were examined in a DBP radioimmunoassay. Although the immunologically related material described a broader fraction, it was very similar to that indicated by the bound ligand in both experiments.

On sucrose gradients (Fig. 2), iodinated DBP co-sedimented with 25-OH-3HJD3 bound to serum, and both were less dense than the bovine serum albumin marker (Mr = 68,000). As reported previously for rat muscle (15), the 25-OH-3HJD3 bound by human skeletal muscle cytosol sedimented in the 5 to 6 S region, faster than bovine serum albumin.

To avoid serum contamination during cytosol preparation, human fibroblasts grown in monolayers in tissue culture were trypsin-treated and washed extensively prior to use. Aliquots of washed fibroblast cytosol demonstrated ligand binding in the 5 to 6 S region on sucrose gradient analysis, but only when exposed to very high specific activity 25-OH-3HJD3 (78 Ci/mM) (23). 125I-DBP incubated with human fibroblast cytosol co-sedimented on sucrose gradient analysis with the binding protein in skeletal muscle (tissue DBP) as shown in Fig. 2. Human serum incubated with fibroblast cytosol and then with

Fig. 1 (left). Sucrose gradient ultracentrifugation of human small intestinal cytosol and human serum both incubated with 25-OH-3HJD3. Gut cytosol protein (1.9 mg) (A) and 0.88 mg of human serum protein (B) in Buffer A were incubated at 4°C for 30 min with 1.1 and 1.9 ng of 25-OH-3HJD3, respectively. A 200-µl aliquot of each incubation mixture was layered onto a sucrose gradient and collected as described in the legend to Fig. 1. The sedimentation of bovine serum albumin (BSA) is indicated by the arrow.

Fig. 2 (center). Sucrose gradient ultracentrifugation of human serum and muscle cytosol incubated with 25-OH-3HJD3 and of 125I-DBP alone and incubated with fibroblast cytosol. Serum protein (0.56 mg) (□) and 1.42 mg of muscle cytosol (○) were incubated with 27 and 57 pg, respectively, of 25-OH-3HJD3 for 30 min at 4°C. Two hundred picograms of 125I-DBP alone (■) and 300 pg of 125I-DBP with 0.22 mg of fibroblast cytosol (●) were incubated under the same conditions. A 200-µl aliquot of each incubation mixture was layered onto a sucrose gradient and collected as described in the legend to Fig. 1. The sedimentation of bovine serum albumin is indicated by the arrow.

Fig. 3 (right). Radioimmunoassay of human serum DBP in serial dilutions of cytosol from various human tissues. The curved line connecting the closed circles (●) demonstrates the displacement of 125I-DBP from anti-DBP antibody by serial dilutions of human serum. Each serum concentration represents the mean ± S.E. of identical serum dilutions from six healthy adult volunteers. The tissue cytosol samples are: testis (□), lung (■), small intestine (△), liver (□), muscle (◊), and fibroblast cytosol incubated with dilutions of serum for 30 min at 4°C prior to assay (○). Tissue cytosol protein content is indicated on the lower abscissa. The upper abscissa indicates the volume of serum alone and serum diluted with cytosol.
25-OH-[3H]D3 also co-sedimented with these 5 to 6 S peaks (not shown).

Immunological similarity was shown between serum DBP and the tissue extracts of human testis, lung, liver, and small intestine, as well as fibroblast cytosol incubated with human serum. The DBP radioimmunoassay (Fig. 3) revealed that all of these tissues contained material that was immunologically related.

Ouchterlony double immunodiffusion studies of the same tissue cytosols, serum, and purified DBP against monospecific DBP antiserum (Fig. 4) confirm the radioimmunoassay data. After staining of the immunoprecipitin lines, only chevrons of immunological identity were present between the tissue extracts and serum DBP. These observations provide strong evidence for the close immunological similarity, if not identity, of tissue and serum DBP.

Characterization of Tissue and Serum DBP by Gel Filtration—As shown in Fig. 5, 25-OH-[3H]D3 bound by skeletal muscle cytosol eluted from a Sephadex G-200 column at a position that correlated to that of a globular protein with a molecular weight of 98,000. Serum DBP, similarly marked with radioactive ligand, eluted later. Under low ionic buffer conditions, dissociation of tissue DBP into serum DBP was not observed.

The Effect of Denaturing Conditions on Tissue DBP—When filtered on a column of Sephadex G-200 equilibrated in 6 M guanidine HCl, the elution of 125I-DBP was similar to that of human serum DBP detected by radioimmunoassay (Fig. 6). A small amount of immunoreactive material eluted earlier, but this material was not further characterized or studied. Replicate analyses on the same column provided minor variations in results, which were likely due to the variability of slow flow rate experienced. Human skeletal muscle cytosol was filtered over the same column, but the ligand binding ability and immunoreactivity in the dialyzed eluates did not provide any sharp elution peaks. This failure may have resulted from the necessity to filter the larger muscle protein sample at room temperature and to the slower column flow rate. However, 125I-DBP incubated with fibroblast cytosol (which co-sedimented with muscle tissue DBP) (Fig. 2) was recovered in fractions similar to those of DBP and 125I-DBP under these denaturing conditions (Fig. 6).

Fig. 7 (inset) demonstrates that 125I-DBP incubated with...
Identification of Human Serum DBP in Tissues

**Fig. 7 (left).** Electrophoresis of $^{125}$I-DBP alone and $^{125}$I-DBP incubated with fibroblast cytosol on 5% polyacrylamide, 0.1% SDS gels. One hundred fifty picograms of $^{125}$I-DBP alone ($\Delta$) or 150 pg of $^{125}$I-DBP incubated for 30 min at 4°C with 27.5 pg of fibroblast cytosol protein (●) were incubated overnight in Buffer A containing 1% SDS. Fifty microliters of each sample was layered on a gel and electrophoresed at room temperature at 8 mA/gel. The $^{125}$I content of each 3-mm gel segment is plotted against the relative front (RF) using the migration of bromophenol blue as unity. Inset, Sucrose gradient ultracentrifugation of $^{125}$I-DBP alone and $^{125}$I-DBP incubated with fibroblast cytosol with or without SDS treatment before centrifugation. Five hundred ninety-two picograms of $^{125}$I-DBP alone (●) or 1.2 ng of $^{125}$I-DBP incubated with fibroblast cytosol for 30 min at 4°C (○) were analyzed. An aliquot of the $^{125}$I-DBP-cytosol incubation was incubated in 1% SDS overnight (A) and then analyzed. After centrifugation for 20 hr at 42,000 rpm, fractions were analyzed for radioactivity. The sedimentation of bovine serum albumin is shown by the arrow.

**Fig. 8 (right).** Sucrose gradient ultracentrifugation of parathyroid adenoma cytosol; analysis by 25-OH-[$^3$H]D$_3$ binding and DBP radioimmunoassay in the presence and absence of various concentrations of Triton X-100. Top panel, each of five samples containing 1.43 mg of parathyroid adenoma cytosol in 0.01 M Tris-HCl, pH 8.3, was incubated with 1.4 ng of 25-OH-[$^3$H]D$_3$ for 30 min at 4°C. Aliquots (250 µl) were then layered onto 5 to 20% sucrose gradients in the same buffer containing no Triton X-100 (○), 0.035% Triton X-100, 0.05% Triton X-100 (●), 0.1% Triton X-100 (□), 0.1% Triton X-100 (△), or 1.0% Triton X-100. Fractions were collected by bottom puncture and aliquots were assayed for tritium. Only three of the five curves are displayed for clarity. Bottom panel, 50 µl of each fraction from the gradient with no Triton X-100 (○) and the gradient with 0.1% Triton X-100 (△) were diluted 1:1 with Buffer B. A separate radioimmunoassay standard curve was carried out in 0.05% Triton X-100, and the results of each analysis are displayed. In each panel, the arrow indicates the sedimentation of bovine serum albumin (BSA).

Effect of Physicochemical Treatments on $^{125}$I-DBP-Cytosol Complex Formation—Table I lists the effects of a variety of physicochemical treatments of DBP or fibroblast cytosol, or both, on their ability to form tissue DBP in vitro. Reducing agents such as dithioerythritol, p-hydroxymercuribenzoate, and iodoacetamide did not prevent complex formation, nor did incubation and ultracentrifugation in 0.5 M KCl. Six molar guanidine HCl denaturation of DBP, followed by extensive dialysis in 0.01 M Tris-HCl buffer, did not prevent complex formation. Heating the cytosol to 60°C for 1 h also prevented complex formation. SDS treatment prevented complex formation as documented in Fig. 7. The complex formed at physiological pH 7.4 and pH 9.0, but not any other pH in the spectrum tested. Finally, the complex was not formed if the cytosol was preincubated with trypsin but was formed in the presence of RNase.

The 5 to 6 S tissue complex was stable in the nonionic detergent Triton X-100. Fig. 8 demonstrates the faster sedimenting complex from a human parathyroid adenoma extract in the absence and presence of Triton X-100. The quantity of immunoreactive material in the tissue peak, as detected by radioimmunoassay, did not decrease in the presence of Triton X-100. However, ligand binding did progressively decrease in proportion to the concentration of Triton X-100 employed.

**Discussion**

In contrast to serum DBP and the tissue 1,25-(OH)$_2$D$_3$ binding protein, the 5 to 6 S tissue DBP had been defined less clearly. Its presence in all nucleated tissue extracts has been recognized by many investigators, often as the dominant bind-
ing moiety of antiricketic sterols (8, 11, 15–21). A relationship between serum and tissue DBP was suggested by their similar ligand binding preferences. However, 0.5 M KCl and dithioerythritol treatment of the tissue DBP failed to produce serum DBP, and initial attempts to produce tissue DBP from serum DBP mixed with tissue extracts were unsuccessful (15). Subsequently, the in vitro complexing of 25-OH-[3H]DBP to a tissue cytosol constituent was demonstrated to produce tissue DBP sedimentation (5 to 6 S) of tritium when serum was diluted 1:20 beforehand. Since heating DBP to 60°C produced serum DBP sedimentation (3 to 4 S), the complex of cytosol and serum DBP was considered to be noncovalent (17). The time course of these studies, however, did not exclude an enzymatic cleavage of the tissue DBP.

The present data clearly indicate the immunological similarity, if not identity, of serum and tissue DBP. The demonstration of the 5 to 6 S binding protein in intestinal extracts by ligand binding and radiolmmunoassay is the first report of this binding moiety in a human vitamin D target tissue. Since DBP antiserum recognized the complexed form of DBP in tissue extracts, the association of DBP with the cytosol-complexing factor does not conceal the antigenic determinants of DBP. Until tissue DBP is isolated for precise quantitation in the radiommmunoassay, some minor degree of immunological dissimilarity is not excluded. Our immunological studies, however, directly and clearly indicate that the 5 to 6 S tissue DBP is not explained by a shift of 25-OH-[3H]DBP binding, but rather by the association of serum DBP with cytosol factor(s). The failure to detect immunoassayable DBP in its 3 to 4 S form in cytosol preparations provides evidence for the presence of a considerable excess of the cytosol-complexing factor in all tissues.

The loss of the cytosol-complexing activity after trypsin treatment indicates that the cytosol factor is a protein. The gel filtration experiments indicate that tissue DBP represents an approximate increase of M. = 40,000 over serum DBP. This finding and its recognized 5 to 6 S sedimentation are consistent, suggesting that the cytosol-complexing protein is probably not a lipoprotein. Furthermore, since Triton X-100 treatment removed the lipid ligand from DBP, but did not dissociate serum DBP from tissue DBP, the DBP-tissue interaction is less likely to be of a protein-lipid nature.

The development and application of immunological methods and radiolabeled DBP provided the means for a rigorous examination of the nature of the association between DBP and tissue extracts. The production of serum DBP from tissue DBP denatured with 6 M guanidine or SDS indicates that the 5 to 6 S tissue DBP represents an interionic, noncovalent bond of serum DBP with a tissue cytosol protein. A disulfide linkage between DBP and cytosol-complexing protein was excluded by the failure of reducing agents and sulfhydryl blocking agents to prevent tissue DBP formation or affect a dissociation of the complex. Since the denaturation, reduction, and carboxymethylation of DBP prevented association with tissue extracts, its tertiary structure is essential for this interaction to occur. The interaction was apparently optimal in the physiological pH range, and the heat lability or denaturation of the cytosol-complexing factor was verified.

The apparent excess of cytosol-complexing factor in tissues, the demonstrated formation of tissue DBP in vitro, and the unavoidable serum contamination of cytosol preparations have suggested that this complex may be artifactual. However, several lines of evidence indicate that this complex may also occur in vivo and have physiological significance. Tissue DBP is found in physiological fluids such as milk (17), which derive from normal apocrine function. In easily washed tissues, such as human blood mononuclear cells and cultured human fibroblasts, extracts reveal a higher ratio of DBP to an extracellular marker, human serum albumin, than the ratio of DBP to human serum albumin in serum (23). Tissue DBP exhibits a higher affinity binding of 25-OH-D₃ than serum DBP (15, 16). The 5 to 6 S complex has been reported to be capable of transferring vitamin D metabolites to nuclei (29). The complex is found in well perfused tissues and relatively nonvascular tissue, such as cartilage (15). The absence of the 5 to 6 component in cultured cells grown without serum (17) does not argue against a physiological role, since serum DBP cellular entry may be a necessary occurrence. Other serum carriers of lipid ligands are recognized to have surface receptors (30, 31) and gain entry into cells (30). The dissociation of the complex requires severe denaturing conditions such as heating and high concentrations of salt, suggesting a high affinity interaction between DBP and the tissue component. Investigations of the affinity and specificity of the binding of serum DBP to tissue are reported in the accompanying paper (23).

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