Purification of calciferol-binding proteins from kidney: physicochemical and immunological properties

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Abstract The calciferol-binding system of rat kidney cytosol has been purified and is shown to consist of two proteins, each capable of binding either 25-hydroxyvitamin D_3 (25-OH-D_3) or 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). The two proteins, designated A and B, have similar sedimentation coefficients ($S_{20,w}$) of 5.2 S. Component A binds 25-OH-D_3 with a dissociation constant (K_d) of 10^{-7} M while component B binds 1,25-$(OH)_2D_3$ with a K_d of 1.6×10^{-8} M. The estimated molecular weights (M_r) of the two proteins are 105,000 for component A and 250,000 for component B. Amino acid analyses revealed that glutamic acid is the most abundant residue in both proteins, comprising 12% of the total number of amino acid residues. Immunodiffusion tests using commercial anti-human serum group-specific protein antiserum gave a precipitin reaction when purified rat serum calciferol-binding protein was used as an antigen, but no reactions could be detected with proteins A and B. This result significantly eliminated the possibility of the presence of the rat serum binding protein in either of the purified kidney proteins. In contrast, anti-rat serum calciferol-binding protein antiserum prepared in rabbits interacted with the rat serum and kidney proteins. This result suggests that the antigenic determinants recognized by the antiserum against the rat serum calciferol-binding protein appear to be similar to those recognized in the kidney proteins A and B. Immunoelectrophoresis of the three rat proteins demonstrated dissimilar electrophoretic mobilities with the serum protein showing the least mobility, a property consistent with its higher lysine content relative to proteins A and B.

Supplementary key words cytosol · receptor · hormone · vitamin D_3

Vitamin D_3 undergoes sequential hydroxylation reactions, first in liver microsomes (1) and then in kidney mitochondria (2), resulting in the formation of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 derivatives, respectively. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is currently regarded as the physiologically active hormone associated with the movement of calcium and phosphate ions in the intestine and skeleton (3, 4).

The mode of action of vitamin D_3 is considered analogous to that of a prehormone, which ultimately yields the active secosteroid[2] hormone 1,25-$(OH)_2D_3$ (5). Therefore, efforts have been made to establish whether specific binding proteins (receptors) exist for the intercellular and intracellular translocation of vitamin D_3 and its metabolites. As a result, binding proteins have been found for vitamin D_3 and its metabolites in blood (6–9) and in the cytosol fraction of several tissues (10–12). For example, in most mammalian species, including man, the plasma-circulating vitamin D_3 and 25-OH-D_3 occur in protein complexes that have α-globulin mobility (13, 14) and molecular weights ranging from 40,000 to 60,000 (6, 8, 14). Their sedimentation coefficients have been reported to range from 3.1 S to 4 S (8, 10). Further studies (7, 8, 15) have indicated that plasma vitamin D_3 and its metabolites are transported by the same plasma protein with dissociation constants ranging from 0.064 μM to 0.43 μM. It has been suggested by Daiger, Schanfield, and Cavilli-Sforza (16) that the human plasma group-specific (G_c) proteins, for which a worldwide polymorphism exists, and the plasma vitamin D-binding proteins are in fact the same. This suggestion has received much support from the finding that the purified human plasma calciferol-binding protein and human sera produce lines of identity with commercial anti-human G_c antisera in radial immunodiffusion experiments (7, 15).

The binding proteins of vitamin D_3 and its metab-

Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; DTT, 1,4-dithiothreitol.

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[2] Calciferol is a generic name for vitamin D which does not distinguish the side chain isomers. Cholecalciferol is vitamin D_3. In keeping with the nomenclature recommended by the International Union of Pure and Applied Chemistry Commission, cholecalciferol may be considered a steroid. The chemical name is 9,10-secocholesta-5,7,10(19)-trien-3β-ol.

olites in tissues other than blood generally appear in crude cytosolic preparations as molecules sedimenting in the range of 5 S to 6 S (10, 12). The presence in kidney of a high-affinity binding protein for 25-OH-D_3 is consistent with the functional role of the mitochondria of this tissue (17, 18) in the production of the physiologically active hormone 1,25-$(OH)_2D_3$. However, the discovery of similar high-affinity binding proteins for 25-OH-D_3 in other tissues (muscle, skin, brain, lung, intestine, etc.) (10, 11, 19, 20) not known to be involved in the activation of 25-OH-D_3 requires the assignment of alternate cellular functions for such proteins.

In view of the hormonal nature of 1,25-$(OH)_2D_3$ it was expected that specific receptor proteins for this metabolite would be found in its target cells. Tsai and Norman (21) have described the in vitro binding of 1,25-$(OH)_2D_3$ to an intestinal mucosal cytoplasmic receptor protein of 65,000–150,000 daltons and have found that the binding of 1,25-$(OH)_2D_3$ to this receptor protein was an obligatory step for the eventual appearance of 1,25-$(OH)_2D_3$ in the chromatin fraction of the mucosal cell. Furthermore, the in vitro studies of Brumbaugh and Haussler (22) have demonstrated that the 1,25-$(OH)_2D_3$ metabolite selectively associates with the chromatin–receptor complex of the intestine. It is very likely that this interaction of 1,25-$(OH)_2D_3$ is associated with the response of the intestine to vitamin D. A recent study by Kream et al. (23) has shown that cytosols prepared from fetal chick and rat bone homogenates contain a high-affinity binding protein for 1,25-$(OH)_2D_3$ similar to that observed in chick intestinal cytosol. This finding has led to the suggestion that the action of 1,25-$(OH)_2D_3$ in bone may be mediated by an analogous receptor protein mechanism.

Recently, the purification of the rat serum calciferol-binding protein was reported (24). Antibodies against this protein cross-reacted with the kidney cytosolic 25-OH-D_3-binding protein, indicating identity of the antigenic determinants of the two tissue proteins (25). This observation gave us the impetus to explore further the relationships between the serum and kidney binding proteins in the hope of elucidating the mechanisms of intercellular translocation of vitamin D_3 and its metabolites. We now report purification of rat kidney calciferol-binding protein and its resolution into two components. Examination of the binding properties of the two components showed that the relative ligand preferences are sufficiently different to distinguish them either as 25-OH-D_3 or 1,25-$(OH)_2D_3$ specific. The physicochemical and immunological properties of the two components revealed that, although there are immunochemical similarities between the proteins A and B, these antigens may be structurally dissimilar.

EXPERIMENTAL PROCEDURE

Materials

All reagents and chemicals were of analytical grade. Deionized glass-distilled water was used throughout the experiments. Crystalline 25-OH-D_3 was a gift from Dr. John Babcock of the Upjohn Co., Kalamazoo, MI. Crystalline 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2$-[26(27)-methyl-^{3}H]-vitamin D_3 (9.3 Ci/mmol) were the kind gifts of Drs. Jacob Lemann and Richard W. Gray. Crystalline vitamin D_3 was purchased from Nutritional Biochemicals Co., Cleveland, OH, and stock 25-OH-[26(27)-methyl-^{3}H]-vitamin D_3 (7–10 Ci/mmol) from Amersham-Searle Corp., Arlington Heights, IL. Beef liver catalase, horse heart cytochrome *c*, and crystalline cholesterol were purchased from Sigma Chemicals, St. Louis, MO. Fresh hemoglobin and monomeric bovine serum albumin were prepared according to published procedures (26, 27). Aldolase, ovalbumin, chymotrypsinogen A, Sephadex G-200, DEAE–Sephadex A-25 and A-50 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Human sera for typing the group-specific protein phenotypes G_c1-1, G_c2-1, and G_c2-2, and anti-human G_c antiserum were purchased from Behring Diagnostics, American Hoechst Corp., Somerville, NJ. The stock 25-OH-[26(27)-methyl-^{3}H]-vitamin D_3 solution in benzene was evaporated to dryness under a stream of nitrogen. The steroid was redissolved in the original volume of absolute ethanol. An aliquot was tested for radiochemical purity on micro-Celite (17, 28).

Methods

Amino acid analysis. These experiments were performed in the laboratories of Dr. Beatrice Kassell, under her guidance. The proteins were hydrolyzed in 4 M methane sulfonic acid containing 0.2% tryptamine (Pierce Co., Rockford, IL) in sealed, evacuated glass tubes at 115°C for 24 hr (29). The hydrolysates were analyzed on a modified Beckman 120 B amino acid analyzer as previously described (30).

Analytical ultracentrifugation. Sedimentation velocity analyses by observing the moving boundary were performed in a Spinco model E ultracentrifuge equipped with schlieren optics. Protein solutions of either 2.3 mg/ml or 1.4 mg/ml in 15 mM Tris–acetate, 1 mM DTT, and 0.1 mM EDTA at pH 7.4 were employed. The ultracentrifugation was conducted at 5°C at 60,000 rpm. Photographs were taken at a

phase plate angle of 60°. The sedimentation coefficients ($S_{20,w}$) were calculated using a partial specific volume value of 0.75 (31).

Competitive binding assays. Binding activities were measured with a fixed amount of protein in a final volume of 0.25 ml of 15 mM Tris–acetate buffer, 1 mM DTT, and 0.1 mM EDTA at pH 7.4. To each tube a constant amount of the appropriate radiolabeled metabolite was added as a mixture with a competing nonradiolabeled metabolite in a small volume of absolute ethanol. The ethanol was evaporated under a stream of nitrogen. The protein in the Tris–acetate buffer was then added, mixed thoroughly, and allowed to stand on ice for 1 hr. The free metabolites were separated from the bound by adding 0.25 ml of a suspension of Dextran 20-coated charcoal (1.25 mg) prepared as previously described (32). After centrifugation at 6000 rpm for 20 min at 4°C, duplicate 0.2-ml supernatant aliquots were counted for bound radioactivity. Dissociation constants were calculated as described by Munck (33).

Electrophoresis. Analytical and preparative acrylamide electrophoreses were performed in 7% acrylamide gels polymerized in Canalco instruments according to published procedures (34, 35). In the preparative procedures a separating gel column of 4.25 cm high with a 0.5 cm stacking gel was utilized. The gel was prerun overnight at 4 mA and all the buffers were then discarded. The chambers were refilled with fresh buffers prior to sample application. Electrophoresis was performed at 6 mA and, after 4 hr, elution was started with 15 mM Tris–acetate, 1 mM DTT, 0.1 mM EDTA pH 7.4 buffer at a flow rate of 40 ml/hr. Fraction collection was initiated 6 hr after elution was started.

Plasma-binding protein. Normal rat serum 25-OH-D_3-binding protein was purified as previously reported (24).

Preparation of antisera. One milligram of the purified serum 25-OH-D_3-binding protein in 1 ml of 0.05 M Tris–acetate buffer pH 7.4 was mixed with an equal volume of Freund's complete adjuvant. The mixture was injected intradermally at multiple sites both in the nuchal and inguinal regions of New Zealand White rabbits (5–7 lb). The rabbits were boosted at 28 days by intramuscular injection of 1 mg of the antigen without adjuvant and bled from the ear vein 7–10 days after the booster immunization. Antibody titer was determined by the Ouchterlony double-diffusion procedure (36). The double-diffusion was performed in gels prepared in Hemagglutination Buffer (Difco Labs, Detroit, MI) containing 0.7% agarose (Bio-Rad Laboratories, Richmond, CA), 3% polyethylene glycol 6000 (Union Carbide Corp., New York, NY) and 0.01% Thimerosal (ethylmercurithiosalicylate, Sigma Chemical Co.). Reactions were allowed to proceed in a humid chamber at 4°C for 24–48 hr.

Immunoelectrophoresis (IEP). IEP was performed on microscope slides covered with 3 ml of 1% Ionagar No. 2 (Colab Laboratories, Chicago Heights, IL) in barbital buffer pH 8.5, ionic strength 0.05 (37). Proteins were electrophoresed at a constant voltage of 212 V for 1 hr at room temperature. Subsequently, parallel troughs were made in the gels and were filled with 0.1 ml of anti-25-OH-D_3-binding protein antiserum. Reactions were allowed to proceed as above.

Other methods. Protein was determined by the method of Lowry et al. (38). Molecular weights were estimated by comparison with protein standards on a Sephadex G-200 gel filtration column (1 × 50 cm) (39).

Kidney binding proteins. Normal male Sprague-Dawley rats (175–200 g) were the generous gift of Dr. John J. Lech. The animals were killed by decapitation and their kidneys were immediately removed and perfused via the renal arteries with physiologic saline solution containing 1% potassium oxalate until the kidneys were completely blanched. The kidneys were then decapsulated and chilled in ice-cold buffer (0.25 M sucrose–15 mM Tris–acetate, 1 mM DTT, 0.1 mM EDTA pH 7.4). After all the connective tissues were removed, the kidneys (281 g) were homogenized in three portions in 4 volumes of the sucrose–Tris–acetate buffer at 0°C in a Waring blender operated for 15 sec at full-speed. The homogenate was centrifuged at 15,000 rpm for 1 hr (IEC model B-35 refrigerated preparative ultracentrifuge equipped with A-57 rotor). The supernatant was filtered through cheesecloth and stored overnight at 0°C. The pellets were discarded without washing. The stored premicrosomal preparation was then centrifuged at 35,000 rpm for 1 hr. The pellets were discarded. To the supernatant powdered enzyme-grade ammonium sulfate (Schwarz/Mann, Orangeburg, NY) was added with constant stirring at 0°C to 30% saturation. The mixture was allowed to stand for 15 min and then was centrifuged at 19,000 rpm for 1 hr. The pellets were discarded. The supernatant was filtered through glass wool and brought to 60% saturation with ammonium sulfate as above. The mixture was then centrifuged at 19,000 rpm for 1 hr. The supernatant fractions were discarded and the pellets were dissolved in 180 ml of the sucrose–Tris–acetate buffer and dialyzed for 12 hr at 4°C against 2 × 2-liters of the buffer. The dialysis was then continued for 3 additional hr against deionized, distilled

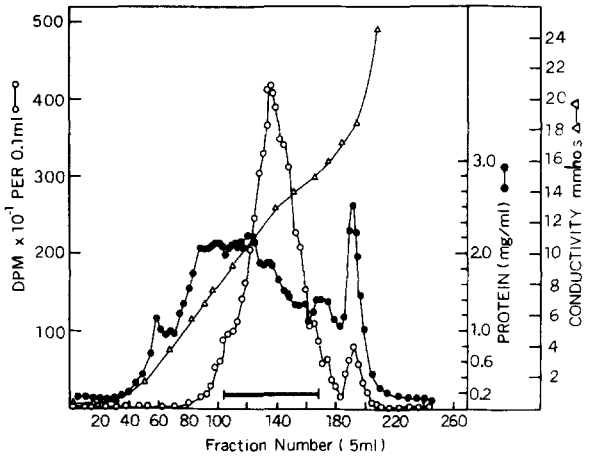

Fig. 1. DEAE–Sephadex A-50 chromatography of the 30–60% ammonium sulfate fraction of normal rat kidney cytosol labeled with ^{3}H-25-OH-D$_3$. The experimental conditions are given under Methods.

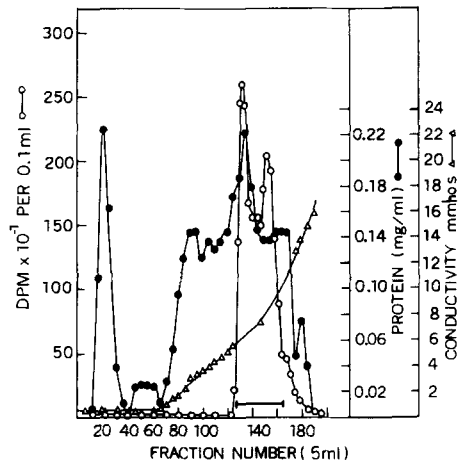

Fig. 3. DEAE–Sephadex A-25 chromatography of the pooled fractions containing the tritium from the Sephadex G-200 gel filtration step. See text for details.

water and finally for 6 more hr against the sucrose–Tris–acetate buffer adjusted to pH 8.3.

To one-third of the dialyzed preparation (85 ml, total protein 2.59 g) 250 μl of the stock 25-OH-[26(27)-methyl-^{3}H]-vitamin D$_3$ in ethanol was added drop-wise with constant stirring at 4°C and then allowed to stand in the cold overnight. Radioactivity determinations were made in a total volume of 3 ml containing preblended 3a70B scintillation fluid (Research Products International Corp., Elk Grove Village, IL) and 100 μl of the sample to be counted. A Packard 3324 spectrometer was used with a tritium counting efficiency of 10%.

The radiolabeled preparation was applied to a DEAE–Sephadex A-50 ion-exchange column (2.5 × 29 cm) equilibrated with 15 mM Tris–acetate, 1 mM DTT, and 0.1 mM EDTA pH 8.3. The column was washed overnight with the same buffer until a total effluent volume of 520 ml was collected at a flow rate of 35 ml/hr. Subsequently, the column was eluted with a linear gradient from 0 to 0.5 M KCl in the Tris–acetate buffer at pH 8.3 (**Fig. 1**). Five-ml fractions were collected. The fractions containing the radioactivity (no. 105–169) were combined and concentrated to a volume of 12 ml in an Amicon cell fitted with a PM-30 membrane. The total protein recovered was 430 mg. The concentrated sample was applied to a Sephadex G-200 column (2.5 × 84 cm) equilibrated and eluted with the above Tris–acetate buffer but at pH 7.4 (**Fig. 2**). Again, 5-ml fractions were collected at a flow rate of 12.5 ml/hr by gravity. The combined volume of the fractions containing the radioactivity (no. 40–55) was 72.6 ml and contained 152.5 mg of protein. This was applied to a second ion-exchange column (2.5 × 27 cm) packed with DEAE–Sephadex A-25 equilibrated with the pH 7.4 buffer. The sample was eluted with a linear gradient from 0 to 0.5 M KCl in buffer (**Fig. 3**). Fractions, 5 ml each, were collected from the start of sample application at a flow rate of 50 ml/hr by gravity. The fractions containing the radioactivity (no. 125–166) were pooled and concentrated as above to a volume of 16.1 ml (36.4 mg total protein). This preparation was concentrated further to 2 ml by vacuum dialysis using conical collodion membrane with a molecular weight cut-off of 25,000 (Schleicher and Schuell, Inc., Keene, NH) and stored in the cold overnight to permit the precipitation of flocculent extraneous proteins (this was experienced during pilot experiments). The precipitate was removed by centrifugation at 4,000 rpm for 15 min at 4°C (Sorvall

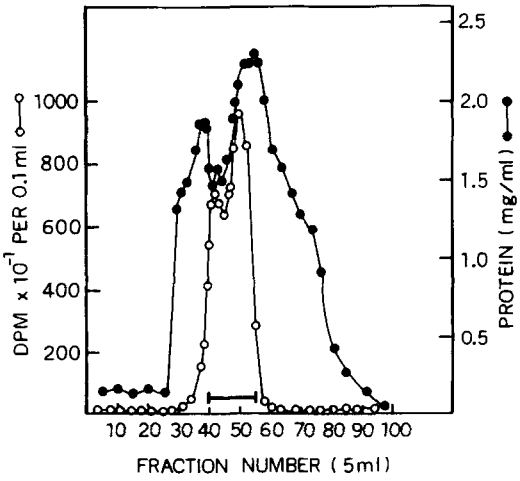

Fig. 2. Sephadex G-200 chromatography of the pooled fractions containing the tritium from the DEAE–Sephadex A-50 step. See text for details. Void volume of column is 130 ml.

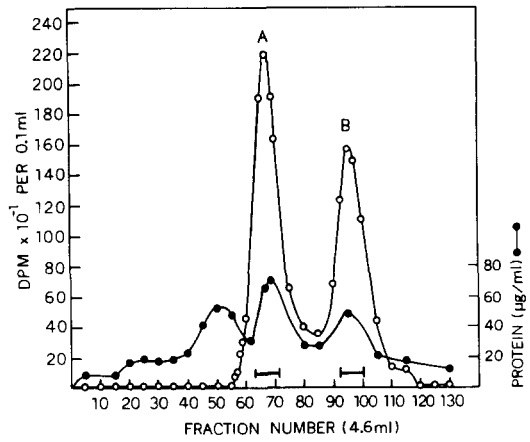

Fig. 4. Preparative acrylamide gel electrophoresis of the partially purified kidney cytosol calciferol-binding protein. Approximately 16 mg of the tritiated protein was applied to a 7% acrylamide gel column 4.75 cm high (0.5 cm stacking gel, 4.25 cm separating gel, 320 mm² cross-sectional area). Electrophoresis was performed at 6 mA.

RC-2 with SS-34 rotor). The pellet was washed three times with Tris–acetate buffer by centrifugation as above. The combined supernatants (7.5 ml) contained 15.7 mg of protein and could be stored overnight unfrozen without precipitation.

The preparative acrylamide electrophoresis unit was prepared and prerun as described earlier. Just prior to sample application, the protein sample was concentrated by vacuum dialysis to a volume of 1.0 ml. The collodion membrane was rinsed with 0.5 ml of 50% sucrose and the combined volumes (1.5 ml) were subjected to electrophoresis as described. Fractions, 4.6 ml each, were collected. Fractions 63–71, designated peak A (**Fig. 4**), were combined and concentrated to 1 ml in an Amicon cell fitted with an XM-50 membrane. This peak contained 2.45 mg of protein and appeared to prefer the binding of 25-OH-D_3. Fractions 92–100, designated peak B, were also combined and concentrated likewise to 0.8 ml. The protein content of peak B was 1.14 mg and represented the high-affinity binding component of kidney cytosol for 1,25-$(OH)_2D_3$.

RESULTS

Table 1 summarizes the purification of components A and B. That the isolated kidney proteins were specific for calciferol metabolites was ascertained when chloroform-extractable radioactivity, either from peak A or B, was shown to comigrate with authentic ^{3}H-25-OH-D_3 on micro-Celite columns (28). Furthermore, neither A nor B has enzymatic activity capable of metabolizing 25-OH-D_3 in vitro.

TABLE 1. Purification of calciferol-binding components of rat kidney

Purification Step	Total Protein	Specific Activity	Yield	Purification Factor[a]
	mg	*dpm × 10⁻³/mg*	%	
Ammonium sulfate	2,590	6	100	2
DEAE A-50	430	15	44	5
Sephadex G-200	152.5	34	34	11
DEAE A-25	36.4	89	21	30
Precipitation	15.7	203	21	68
Electrophoresis				
Peak A	2.45	305	5	102
Peak B	1.14	409	3	136

[a] Relative to unfractionated cytosol as determined by the binding assay described under Methods but in the absence of unlabeled substrate. The purification factors indicated in this table are minimal because quantitative binding assays could not be performed with crude whole kidney homogenates that contain large amounts of lipids.

Labeled A or B (approximately 10 μg) incubated for 30 min at 37°C with 0.5 mM NADH or NADPH failed to convert 25-OH-D_3 to metabolites that could be detected on micro-Celite columns (17, 28).

Molecular weights and sedimentation coefficients

Components A and B have molecular weights of 105,000 and 250,000, respectively (**Fig. 5**), as estimated by filtration on Sephadex G-200. When A and B were examined by SDS–acrylamide gel electro-

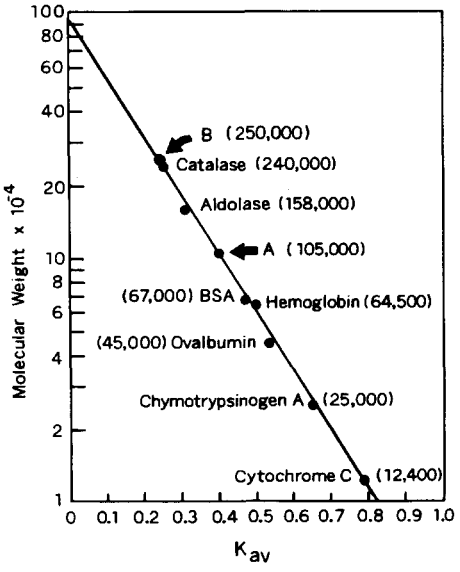

Fig. 5. Estimation of molecular weights of the purified components A and B by gel filtration. Samples of A or B approximately 20,000 dpm were applied to a column of Sephadex G-200 (1 × 50 cm) equilibrated with 15 mM Tris–acetate, 1 mM DTT, 0.1 mM EDTA pH 7.4 buffer. Elution was performed with the same buffer at a flow rate of 30 ml/hr. Fractions of 0.1 ml in volume were collected and counted for radioactivity after the addition of preblended 3a70B scintillant.

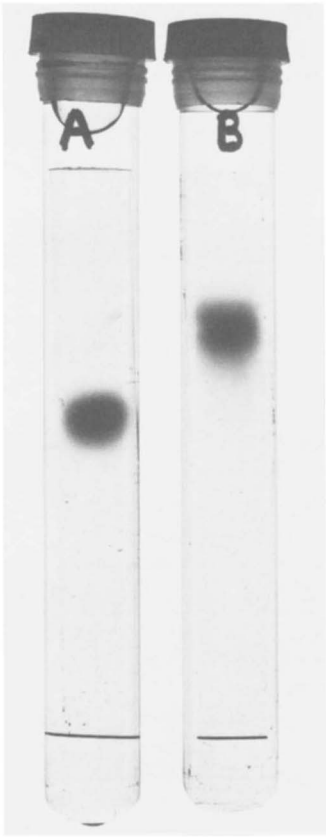

Fig. 6. Analytical gel electrophoresis of components A and B. The amount of protein applied was 32 μg of A and 24 μg of B. Electrophoresis was performed at 4°C at a current of 3 mA per column. The gels were 7% acrylamide. Coomassie Blue was used for protein staining.

phoresis, the results indicated that these proteins are oligomeric in nature. An analytical gel electrophoresis of components A and B is shown in **Fig. 6.** No contaminating proteins appear to be present in either of the two gel columns. The relative mobilities are consistent with that seen in the preparative electrophoresis step of purification.

Although it is premature to delineate the physical nature of the proteins A and B, we cannot exclude a possible monomer–dimer relationship between A and B. In support of this relationship are the amino acid compositions of A and B (see **Table 2**) which suggest a similarity between these proteins. However, when component A or B is subjected to repeated gel-filtration chromatography or electrophoresis, there is no apparent dissociation or aggregation of either A or B. This is clear evidence that both A and B represent discrete entities at least in the present investigation. Whether they exist as such in the intact kidney cell is another matter. Further insight into the likely structural relationships that may exist between the proteins A and B is given in the immunologic studies described below.

Sedimentation velocity studies showed that the purified components A and B migrated as single homogenous proteins (**Fig. 7A** and **B**) each with a sedimentation coefficient ($S_{20,w}$) of 5.2 S. Although this value is reasonable for a protein with a molecular weight of 105,000 as in component A, it appears unusual for a protein with a molecular weight of 250,000 as in component B. No conclusion could be

TABLE 2. Amino acid composition of rat calciferol-binding proteins

	Serum		Kidney Cytosol			
			A		B	
Residue	Residues/ 1000 Residues	Estimated Number of Residues/ Molecule	Residues/ 1000 Residues	Estimated Number of Residues/ Molecule	Residues/ 1000 Residues	Estimated Number of Residues/ Molecule
Tryptophan	15	7	15	14	14	32
Lysine	106	49	52	49	61	135
Histidine	17	8	26	24	19	42
Arginine	39	18	49	46	48	106
Aspartic acid	76	35	91	85	101	226
Threonine	63	29	56	52	56	125
Serine	71	33	60	56	59	131
Glutamic acid	119	55	123	115	133	296
Proline	52	24	64	60	56	126
Glycine	65	30	76	71	72	161
Alanine	80	37	79	74	80	179
½ Cystine	32	15	9	8	6	14
Valine	50	23	64	60	63	141
Methionine	15	7	20	19	22	48
Isoleucine	28	13	41	38	45	101
Leucine	93	43	104	97	97	217
Tyrosine	30	14	34	32	29	64
Phenylalanine	50	23	38	36	39	87
Total		463		936		2,231
Molecular weight	51,842		104,333		249,768	

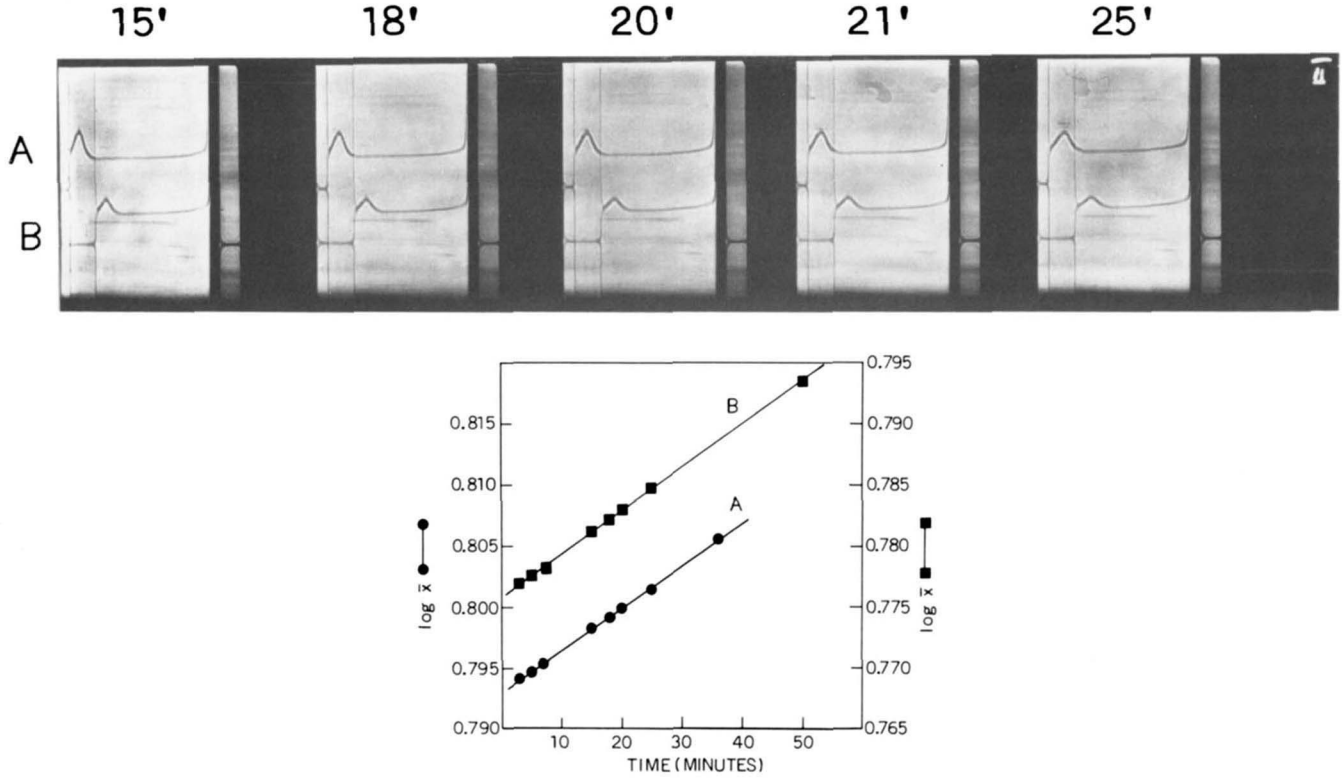

Fig. 7. *A*. Sedimentation patterns of components A and B at a concentration of 2.3 mg/ml for A and 1.4 mg/ml for B in 15 mM Tris–acetate, 1 mM DTT, 0.1 mM EDTA pH 7.4 buffer. The numbers represent the time course of sedimentation in minutes after the rotor had reached the maximum speed of 60,000 rpm at 5°C. *B*. Sedimentation velocity analyses of components A and B by moving boundary technique. See text for details.

drawn on the physical nature of component B in the absence of further data. Suffice it to say that the calculated sedimentation coefficient of 5.2 S is distinctly different from the reported 3.4 ($S_{20,w}$) for mammalian serum binding protein (7, 8), but is in good agreement with the values reported for crude kidney cytosol preparations (10, 40, 41).

Amino acid composition

The results of amino acid analyses of the two binding proteins are shown in Table 2. Because of the limited availability of the components A and B, the analyses represent uncorrected values based on a single hydrolysate for each protein. The two proteins, in general, appear to be similar in their amino acid composition. Glutamic acid is by far the most predominant residue of the two proteins, comprising 12–13% of the total number of amino acids calculated for each of the two proteins. It is important to note that the serum binding protein also contains about 12% glutamic acid residues. In contrast to the cytosol proteins, the serum protein in addition contains a large number of lysine residues (110 per 1000 residues). However, the similarities between the amino acid compositions of the rat serum binding protein reported here and that reported for human serum are striking (7, 8). The human serum binding protein likewise contains a disproportionate number of glutamic acid and lysine residues relative to the remaining amino acids.

Binding specificity of the purified components

In view of the ability of A or B to bind ^{3}H-25-OH-D_3, it was of interest to examine by competitive binding assays whether the two proteins could be distinguished on the basis of their relative preference to bind vitamin D_3, 25-OH-D_3 or 1,25-$(OH)_2D_3$. In competitive binding studies, a quantity of radiolabeled ligand is usually used as a mixture with a competing substance. The amount of radioactivity bound in the absence of a competing ligand is given an arbitrary value of 100%. The efficacy of a competing unlabeled ligand when present is evaluated by measuring percent of unbound radioactivity. The results indicated that component A selectively binds 25-OH-D_3 while component B demonstrates a preference for 1,25-$(OH)_2D_3$. This is seen in **Fig. 8A** where the results of the competition between ^{3}H-25-OH-D_3 and unlabeled 1,25-$(OH)_2D_3$ to bind component A (open triangles) show that 1,25-$(OH)_2D_3$ was able to compete with ^{3}H-25-OH-D_3 only when its concentration approached 100-fold mole excess. Yet, under identical

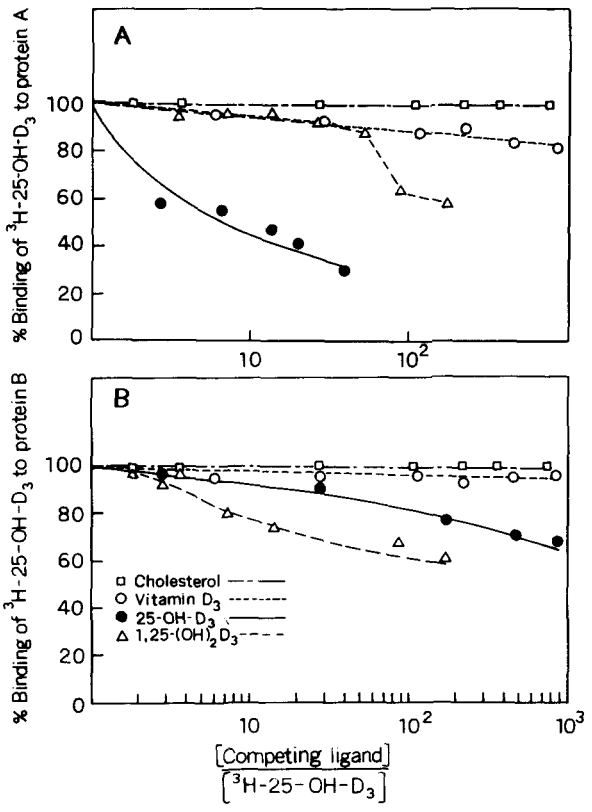

Fig. 8. Competitive binding of ligands to the components A and B. The amounts of ^{3}H-25-OH-D$_3$ (calculated from specific radioactivity) in the competition assays employing 24 μg of component A (229 pmol) were as follows: 0.49 ng (1.2 pmol) with vitamin D$_3$ or cholesterol;[3] 0.74 ng (1.9 pmol) with 25-OH-D$_3$; and 0.61 ng (1.5 pmol) with 1,25-(OH)$_2$D$_3$ as the competing unlabeled ligands (panel *A*). The amount of ^{3}H-25-OH-D$_3$ in the competition assays employing 26 μg of component B (104 pmol) was 0.94 ng (2.2 pmol) with all the unlabeled ligands used (panel *B*). Following incubation of the mixtures on ice for 1 hr, the bound ligand was separated from the unbound by the Dextran 20-coated charcoal procedure. See text for details.

conditions, unlabeled 25-OH-D$_3$ (solid circles) at 50-fold mole excess allowed only 30% binding of ^{3}H-25-OH-D$_3$. While 1,25-(OH)$_2$D$_3$ was unable to compete with ^{3}H-25-OH-D$_3$ for the binding sites of component A, it appeared to be the preferred ligand for the binding sites on component B (**Fig. 8B,** open triangles). This conclusion is partly based on the data shown for the competition between unlabeled 25-OH-D$_3$ and ^{3}H-25-OH-D$_3$ for protein B (Fig. 8B, solid circles). Although the competition by 1,25-(OH)$_2$D$_3$ appears to level off at about 60% binding, a significant competition by this metabolite resulted even when it was present at 8-fold mole excess, a ratio where no competition could be measured when 25-OH-D$_3$ was used. The results shown in Fig. 8A or 8B are indicative of a complex system of reactions which could not be unequivocally explained. The affinities shown in Fig. 8 represent only qualitative avidities of A and B for 25-OH-D$_3$ or 1,25-(OH)$_2$D$_3$. It should be noted that the kidneys, from which proteins A and B were purified, were obtained from normal rats, hence the purified proteins would not be devoid of endogenously bound vitamin D metabolites. Therefore, endogenous bound ligands and secondary low affinity binding sites are likely to interfere with stoichiometric measurements. Nevertheless, even though proteins A and B bind either 25-OH-D$_3$ or 1,25-(OH)$_2$D$_3$, their relative ligand preferences are sufficiently different to associate them with the selective binding of either 25-OH-D$_3$ or 1,25-(OH)$_2$D$_3$. There was little indication that vitamin D$_3$ or cholesterol is a ligand for the binding proteins A or B.

[3] The concentrations of the unlabeled vitamin D$_3$, 25-OH-D$_3$ and 1,25-(OH)$_2$D$_3$ were calculated from the UV spectrum of stock solutions in 95% ethanol using a value for $\epsilon_{264\,nm}$ of 18,000 M^{-1} cm^{-1}. Cholesterol concentration was calculated from dilutions of a 0.1% solution in absolute ethanol.

Dissociation constants

The results obtained from the competitive binding assays were used to approximate the dissociation constants as described under Methods. The decay curves shown in **Fig. 9A** and **B** represent dissociation rather than binding site denaturation because preparations that contained the nonsaturating radiolabeled ligands showed no loss of bound radioactivity when kept on ice for several weeks.

Although a dissociation constant of 10^{-7} M for the apparent avidity of component A for 25-OH-D$_3$ is at variance with some reported values (perhaps due to the uncertainty in the amount of endogenous bound ligands) (10, 11), it is nevertheless of the same order of magnitude of a more recent value reported for the purified human serum protein (7). It is apparent from Fig. 8A that a K_d value describing the avidity of component A for 1,25-(OH)$_2$D$_3$ would be very large and insignificant in terms of hormone–receptor interactions. On the other hand, data obtained using component B show a K_d value of 1.6×10^{-8} M for 1,25-(OH)$_2$D$_3$. This number describes a preferential affinity for the 1,25(OH)$_2$D$_3$ metabolite over 25-OH-D$_3$. The avidity of component B for 25-OH-D$_3$ described by a K_d value of 7.5×10^{-8} M is not significantly different from 10^{-7} shown for A.

Immnochemical relationships between serum and kidney calciferol-binding proteins

The results shown in **Fig. 10** show that commercial anti-human G$_c$ antiserum reacts with the phenotypes of its natural human antigens G$_c$1-1, G$_c$2-1, and G$_c$2-2 (rosette on left side) as well as with the rat serum 25-OH-D$_3$-binding protein (lower left

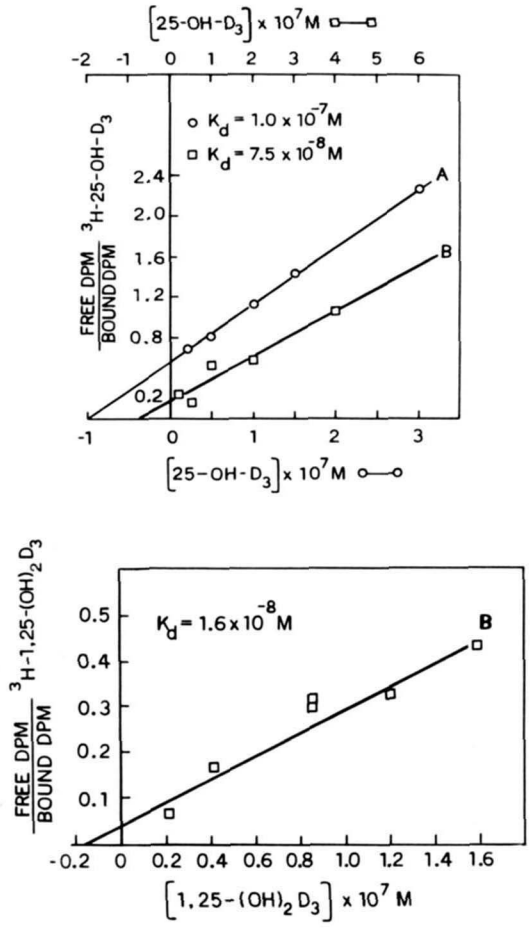

Fig. 9. *A*. Determination of dissociation constants. 24 μg of component A, or 26 μg of component B, was added to mixtures containing 0.74 ng (1.9 pmol) ^{3}H-25-OH-D$_3$ and increasing amounts of unlabeled 25-OH-D$_3$. After incubation of the mixtures on ice for 1 hr, the unbound ligand was adsorbed to Dextran 20-coated charcoal. After centrifugation, supernatant aliquots were counted for bound radioactivity. *B*. 26 μg of component B was added to mixtures containing 0.94 ng (2.2 pmol) ^{3}H-1,25-(OH)$_2$D$_3$ and increasing amounts of unlabeled 1,25-(OH)$_2$D$_3$. The mixtures were analyzed as described in *A* after incubation on ice for 1 hr.

well of rosette on right side). This observation is consistent with earlier reports of the immunologic identity of the serum calciferol-binding protein with serum group-specific components (G$_c$) (7, 15, 16). However, it should be noted that the percipitin arcs shown in Fig. 10 do not reveal complete immunologic identity between the human and rat antigens. The results suggest, therefore, that the rat and human sera 25-OH-D$_3$-binding proteins share immunologic determinants and that there are apparent species differences among these proteins in spite of their in vivo functional similarities. In contrast to the above findings, the commercial antibodies clearly failed to react with the kidney cytosol proteins A and B (upper and lower right wells of rosette on right side). This result significantly eliminates the possibility of the

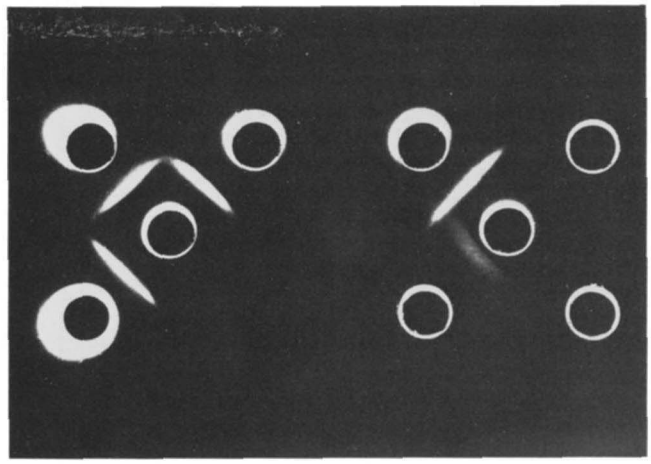

Fig. 10. Ouchterlony immunodiffusion profile. The center well of each gel diffusion pattern contained 10 μl of commercial anti-human G$_c$ antiserum. Titer = 0.3 mg protein/ml. (Gel diffusion pattern on left side) upper left, 5-μl control human serum for typing G$_c$1-1; lower left, 5-μl control human serum for typing G$_c$2-2; upper right, 5-μl control human serum for typing G$_c$2-1. (Gel diffusion pattern on right side) upper left, 5-μl control human serum for typing G$_c$1-1; lower left, 5.9-μg purified rat serum calciferol-binding protein; lower right, 4.4 μg of protein A; upper right, 4.5 μg of protein B.

presence of the rat serum binding protein (as a complex or otherwise) in either of the purified cytosol proteins A and B.

While the commercial anti-human G$_c$ antiserum did not react with the proteins A and B, anti-rat serum 25-OH-D$_3$-binding protein antiserum prepared in rabbits in this laboratory reacted with both proteins. However, as can be seen in **Fig. 11,** a much weaker

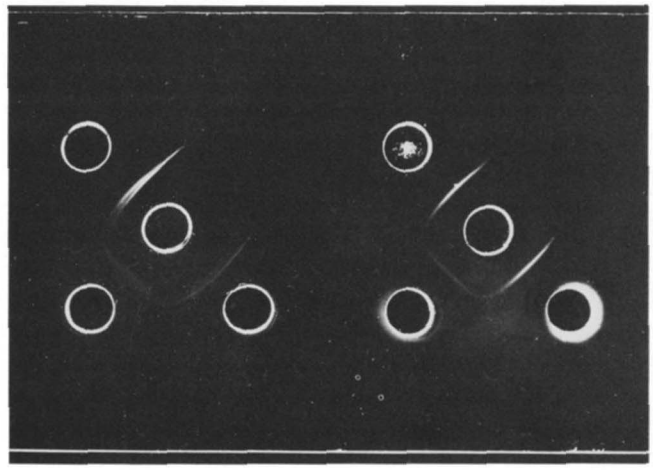

Fig. 11. Ouchterlony immunodiffusion profile. The center well of each gel diffusion pattern contained 10 μl of anti-rat calciferol-binding protein antiserum (270 μg protein). (Diffusion pattern on left side) upper left, 2.7 μg of purified rat serum calciferol-binding protein; lower left, 8.7 μg of protein A; lower right, 8.9 μg of protein B. (Diffusion pattern on right side) upper left, 2.7 μg of purified rat serum calciferol-binding protein; lower left, 21.8 μg of protein A; lower right, 22.2 μg of protein B.

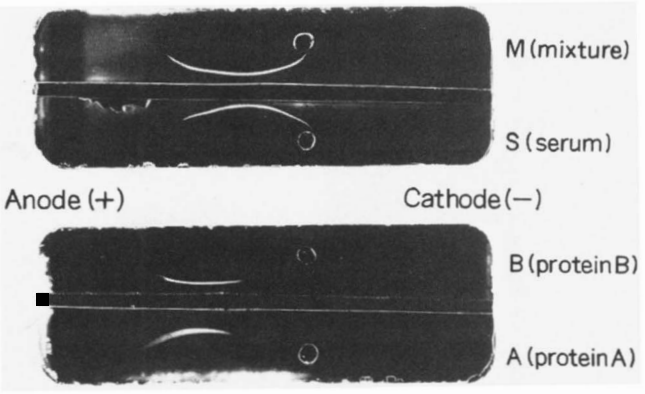

Fig. 12. Co-immunoelectrophoresis of the rat kidney cytosol and rat serum calciferol-binding proteins. Well *A* contained 32.7 μg of protein A; well *B* contained 33.3 μg of protein B; well *S* contained 8.1 μg of purified rat serum calciferol-binding protein; well *M* contained a mixture of the three proteins equivalent to 10.9 μg of A, 11.1 μg of B, and 2.7 μg of the serum protein. After electrophoresis, 100 μl of anti-rat serum calciferol-binding protein antiserum (2.7 mg protein) was placed in the trough and allowed to stand in a humid chamber at 4°C for 48 hr.

immunologic reaction was obtained with protein A than with protein B at similar protein concentrations. The results shown in Figs. 10 and 11 collectively suggest that *a*) although there are immunochemical similarities between the proteins A and B, nevertheless these antigens may be structurally dissimilar, *b*) the antigenic determinants in the rat serum binding protein recognized by the anti-human G_c antiserum appear to be different from those recognized by the anti-rat calciferol-binding protein antiserum, and *c*) the determinants recognized by the antiserum against the rat serum calciferol-binding protein appear to be similar to those recognized in rat cytosol proteins A and B.

To further elucidate the relationships between the three rat proteins, immunoelectrophoresis was performed as described and the results are shown in **Fig. 12.** Clearly, the three proteins migrate with dissimilar electrophoretic mobilities with the serum protein showing the least mobility, a property consistent with its higher lysine content relative to proteins A and B as shown in Table 2. This result also suggests that the serum protein is absent from either A or B. The behavior of the faster moving protein A is also consistent with its electrophoretic behavior on the polyacrylamide gels described earlier.

DISCUSSION

We have isolated two 5.2 S components of the calciferol-binding system of rat kidney cytosol. The two components show a distinct ligand preference and appear to be high-affinity binding proteins for the active metabolites of vitamin D_3. They show the same binding activity as the crude kidney cytosol preparations. The reported findings that a single calciferol-binding protein is detectable in partially purified kidney cytosol fractions (11, 41) suggest that the binding proteins are usually extracted as a complex of components A and B. This complex is completely resolved upon electrophoresis.

The similarity in the sedimentation coefficients of A and B suggests that in competition experiments using ^{3}H-25-OH-D_3 and unlabeled 1,25-$(OH)_2D_3$ analyzed by sucrose gradients, no discernible differences in peak areas should be detectable with increasing concentrations of 1,25-$(OH)_2D_3$. Although exchange of bound metabolites may occur on the appropriate binding protein, the sedimentation profile would show a persistent peak at 5.2 S. However, a diminishing peak area of the 5.2 S region would eventually result with large excesses of the competing metabolite 1,25-$(OH)_2D_3$. This would lead to the erroneous conclusion that a single binding protein is present in kidney cytosol (and other tissues as well) with a higher affinity for 25-OH-D_3 (10, 11). Only in the intestine, a target tissue for 1,25-$(OH)_2D_3$, has there been a clear demonstration of a mucosal receptor specific for 1,25-$(OH)_2D_3$ that functions in the mediation of 1,25-$(OH)_2D_3$ translocation into the intestinal nucleus (3, 23). Hence, results from the gradient experiments have prompted the search for discrete physiologic roles for 25-OH-D_3 in tissues other than the intestine.

The amino acid compositions of A and B show that the two proteins are basically similar. When compared with the composition of the serum binding protein, the variations seen in the lysine contents could explain the reported chromatographic differences shown to exist between the serum and cytosol proteins (41). Such properties could also explain the dissimilar immunoelectrophoretic mobilities demonstrated for the three proteins with the serum protein showing the least mobility.

Previous work (7, 15, 16) has shown immunochemical identity between human sera calciferol-binding protein and group-specific components, a class of α-globulins whose three common phenotypes (G_c1-1, G_c2-1, G_c2-2) are determined by a pair of autosomal alleles (*G_c1* and *2*). Our results support this identity, but in addition, they show that there are apparent species differences among these proteins in spite of their in vivo functional similarities. Although commercial anti-human G_c antiserum reacted with the rat serum calciferol-binding protein, it clearly failed to recognize the rat kidney proteins A and B

as functionally appropriate antigens. The results we have presented in this paper, therefore, support the conclusion that both A and B represent discrete protein entities. However, the possibility that the different properties of A and B may be reflections of different functional behavior of a single protein that may exist in more than one form cannot be definitely excluded at the present time. It is clear that further experiments will be required for the thorough examination of the physical properties of the separated proteins A and B. The description of a functional role for A and B, for example in the bilateral translocation of vitamin D_3 metabolites across cellular membranes, must await detailed investigations.

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