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Comparison of 25-Hydroxyvitamin D_3 Binding Proteins from Rat Lymph and Plasma[1)]

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The 25-hydroxyvitamin D_3 [25(OH)D_3] binding proteins from rat lymph and plasma were isolated and purified by sequential use of gel filtration, affinity and ion exchange chromatographies. The properties of both proteins were examined by high-performance liquid chromatography [HPLC], electrophoretic and immunological methods.

The results were as follows. (1) On HPLC analysis using a gel permeation column, the proteins showed the same retention times. (2) On disc and sodium dodecyl sulfate disc gel electrophoresis, they showed the same relative mobilities. (3) On chromatofocusing and on isoelectric focusing, they showed the same isoelectric points. (4) On immunoelectrophoresis against rat whole antiserum obtained from rabbit, they showed immunological identity.

These results strongly suggest that the 25(OH)D_3 binding proteins from rat lymph and plasma are the same protein.

Keywords——25-hydroxyvitamin D_3; binding protein; lymph; plasma; HPLC; electrophoresis; chromatofocusing; isoelectric focusing; immunoelectrophoresis

Recently, we reported the existence of a binding protein in rat thoracic duct lymph [*l*-DBP] with a higher affinity to 25-hydroxyvitamin D_3 [25(OH)D_3] than to vitamin D_3.[2)] The purified protein was a single peptide with an apparent molecular weight of 57000 as estimated by high-performance liquid chromatography [HPLC] using a gel permeation column, and was apparently different from lymph albumin in molecular size, charge, and affinity for Blue Sepharose CL-6B. From the results, we thought that *l*-DBP was the same as or very similar to the vitamin D binding protein of rat serum [*s*-DBP] previously reported by Bouillon *et al.*[3)] However, the detailed physicochemical properties of *l*-DBP and its relationship to *s*-DBP are still unknown.

The purpose of this work was to purify *l*-DBP from rat thoracic duct lymph and the binding protein of rat plasma [*p*-DBP], and to investigate the possible identity of the two proteins by polyacrylamide disc and sodium dodecyl sulfate [SDS]–disc gel electrophoreses, HPLC on a gel permeation column, chromatofocusing, isoelectric focusing and immunoelectrophoresis.

Experimental

Chemicals——Commercial grade (Philips-Duphar Co., The Netherlands) 25(OH)D_3 was used as the standard. Organic solvents were purified by the usual methods and distilled before use. Sephadex products, DEAE-Sepharose CL-6B and Blue Sepharose CL-6B were purchased from Pharmacia (Sweden). Other chemicals used were of analytical grade.

Animals——Male Wistar rats weighing about 250 g were used.

Collection of Lymph and Plasma——After peroral administration of cottonseed oil to rats, the thoracic duct was cannulated with polyethylene tubing (size 2, Imamura Co., Ltd.) under light ether anesthesia according to the method of Bollman *et al.*[4)] The lymph was collected in a small flask to which a small amount of sodium azide had been added

as an antimicrobial. The cannula was brought out through the abdominal wound and the rat placed in a restraining cage during the collection of lymph. During several days of lymph collection, rats were fed a commercial chow (Nosanko Co., Osaka) and water containing 0.9% NaCl *ad libitum*. The collection of plasma was performed by heart puncture.

Elimination of Chylomicrons from Lymph——The lymph obtained above was mixed with the same volume of chilled 0.9% NaCl solution in a Polytron homogenizer, and the mixture was centrifuged at 7.8×10^4 g for 60 min at 4 °C to separate chylomicrons. The supernatant chylomicrons were carefully removed with a syringe and the resulting transparent lower liquid layer was designated as chylomicron-free lymph. Seventy-five ml of lymph thus obtained was dialyzed against deionized water at 4 °C for 24 h and then lyophilized. The lyophilized lymph was resolubilized in 40 ml of 1/15 M phosphate buffer (pH 7.4) containing 0.15% NaCl and 0.03% sodium azide.

Incubation of Chylomicron-Free Lymph and Plasma with 25(OH)D_3——The lymph solution obtained above and plasma (40 ml) were incubated with 80 μg of 25(OH)D_3 dissolved in 1 ml of ethanol for 24 h at 4 °C and directly subjected to the following isolation and purification steps.

Purification of *l*-DBP and *p*-DBP——The rat *l*-DBP and *p*-DBP were purified from rat thoracic duct lymph (450 ml; repeated 6 times with 75 ml) and plasma (200 ml; repeated 5 times with 40 ml) by the procedure shown in Chart 1. All columns for gel filtration were equilibrated and eluted with 1/15 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.03% sodium azide. For ion exchange column chromatography, DEAE–Sepharose CL-6B was equilibrated with 0.05 M NaCl and the samples were eluted with a linear gradient of 0.05—0.3 M NaCl in 0.05 M Tris-HCl buffer (pH 7.0). For affinity chromatography, Blue Sepharose CL-6B was equilibrated and eluted with 0.05 M Tris-HCl buffer (pH 7.0) containing 0.1 M KCl. During the purification, both *l*-DBP and *p*-DBP were detected in terms of 25(OH)D_3 measured by HPLC and the absorbance (protein) at 280 nm. Column chromatography was always carried out at about 4 °C. In most instances, the effluent stream was monitored by absorbance measurement at 280 nm with a ultraviolet (UV) monitor (model 111, Gilson Co., Ltd., U.S.A.).

Assay of 25(OH)D_3 by HPLC—Assay of 25(OH)D_3 in the eluates obtained from various column chromatographies was carried out according to our methods reported previously.[2] Exactly 0.5 ml of each eluate was placed in a test tube with a stopper. Three ml of methanol and 1.5 ml of methylene dichloride were added and mixed with a Vortex mixer for 2 min. After standing for 1 h, the mixed solution was centrifuged at 3000 rpm for 5 min. Then, 1 ml of methylene dichloride and 1 ml of water were added. The mixed solution separated into two layers, and the lower layer was taken into a different tube. The upper layer was extracted further with 2 ml of methylene dichloride and the extracted layers were combined. The solvent was evaporated off under reduced pressure, and the residue was dissolved in 0.25 ml of 5.5% isopropanol in *n*-hexane. Then 50 μl of the solution was injected into a Shimadzu LC-3A high-performance liquid chromatograph equipped with an autosampler (WISP Model 710A, Waters Associates, Inc., U.S.A.). Separation of 25(OH)D_3 was carried out in a stainless steel tube (250 × 4.6 mm i.d.) packed with Zorbax SIL (straight-phase type, Dupont Co., U.S.A.) using 5.5% isopropanol in *n*-hexane as a mobile phase. The flow rate was 1.0 ml/min (column pressure: 40 kg/cm^2). Under these conditions, the retention time of 25(OH)D_3 was 13.4 min and 25(OH)D_3 could be easily determined by reading the peak height on the chromatogram.

HPLC Analysis of *l*-DBP and *p*-DBP——HPLC analysis of both *l*-DBP and *p*-DBP was performed on a Shimadzu LC-4A high-performance liquid chromatograph equipped with a Shimadzu SPD-2AS detector (monitored at 280 nm with 0.02 absorbance unit as full scale). Separation of *l*-DBP and *p*-DBP was carried out in a stainless steel tube (600 × 7.5 mm i.d.) packed with hydrophilic, spherical, porous silica gel (TSK G3000SW, Toyo Soda Manufacturing Co., Ltd.), using 1/15 M phosphate buffer (pH 7.0) containing 0.1 M NaCl as a mobile phase. Elution was carried out at a constant flow rate of 0.2 ml/min (column pressure: 9 kg/cm^2) at room temperature.

Determination of Protein——Protein was determined by the method of Lowry *et al.*[5] using human serum albumin as the standard.

Polyacrylamide Disc and SDS–Disc Gel Electrophoreses of *l*-DBP and *p*-DBP——Polyacrylamide disc gel electrophoresis was performed by the method of Davis[6] using a separating gel containing 7.5% (w/v) acrylamide, and with a running buffer of Tris-glycine (pH 8.9). SDS–disc gel electrophoresis was carried out by the method of Weber and Osborn[7] using a separating gel containing 5% (w/v) acrylamide with 0.1% SDS.

Chromatofocusing and Isoelectric Focusing [IEF] of *l*-DBP and *p*-DBP——Chromatofocusing of both *l*-DBP and *p*-DBP was performed under the following conditions. A Mono P HR 5/20 column (Pharmacia, Uppsala, Sweden) was equilibrated with 0.025 M bis. Tris-HCl buffer (pH 6.3). About 100 μg each of *l*-DBP and *p*-DBP was dissolved in 250 μl of the same buffer and then applied to the column. Elution was carried out with 40 ml of Polybuffer 74 (Pharmacia) in the form of a linear pH gradient from pH 6 to 4 at a constant flow rate of 1 ml/min. Isoelectric focusing of both *l*-DBP and *p*-DBP was performed under the following conditions. Agarose–IEF gel (114 × 225 mm) was cast on polyester films (GelBond™, Marine Colloids, U.S.A.) according to the method of Lizana *et al.*[8] The agarose–IEF gel consisted of 0.3 g of Agarose–IEF, 3.6 g of sorbitol, 1.9 ml of pharmalyte and 27 ml of distilled water. This gives a 1% agarose gel 1 mm thick. Furthermore, 3-(*N*-morpholino)propanesulfonic acid [MOPS] was added at a final concentration of 34.48 mg/ml of gel as a chemical apacer. Isoelectric focusing was performed at a constant power (15 W) with maximum set voltage and current of 1500 V and 15 mA, respectively. Cooling water at 10 °C was circulated through the apparatus. The agarose gel was prefocused for 500 V for 30 min. Thereafter, focusing was

continued at up to 1980 V for 90 min. The pH gradient was measured with a surface electrode (Osaka Seimitsu Co., Osaka). After focusing, the gel was fixed, washed, and stained for protein with Coomassie Brilliant Blue.

Immunoelectrophoresis of *l*-DBP and *p*-DBP——Agar–gel immunoelectrophoresis was carried out according to the method of Gravar and Williams.[9] The purified *l*-DBP and *p*-DBP were subjected to electrophoresis at a constant voltage of 10 V/cm for 1.5 h in 1% agar gel with 0.05 M Tris-barbiturate buffer (pH 8.6, $\mu=0.02$) as an electrode buffer. After electrophoresis, immunodiffusion with antiserum was carried out overnight at room temperature. The plate was then immersed in 0.9% NaCl in order to remove any unreacted proteins, dried, and stained for proteins with Coomassie Brilliant Blue.

Results

Purification of *l*-DBP and *p*-DBP

The process of purification of *l*-DBP and *p*-DBP from rat lymph and plasma is outlined in Chart 1. The purification of *l*-DBP and *p*-DBP yielded 21 and 9 mg, respectively, and each of these proteins showed a single, homogeneous peak on Blue Sepharose CL-6B affinity

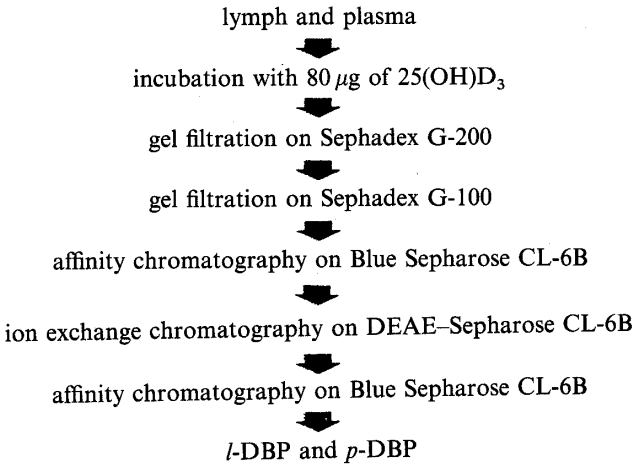

Chart 1. Outline of the Purification of *l*-DBP and *p*-DBP from Rat Lymph and Plasma

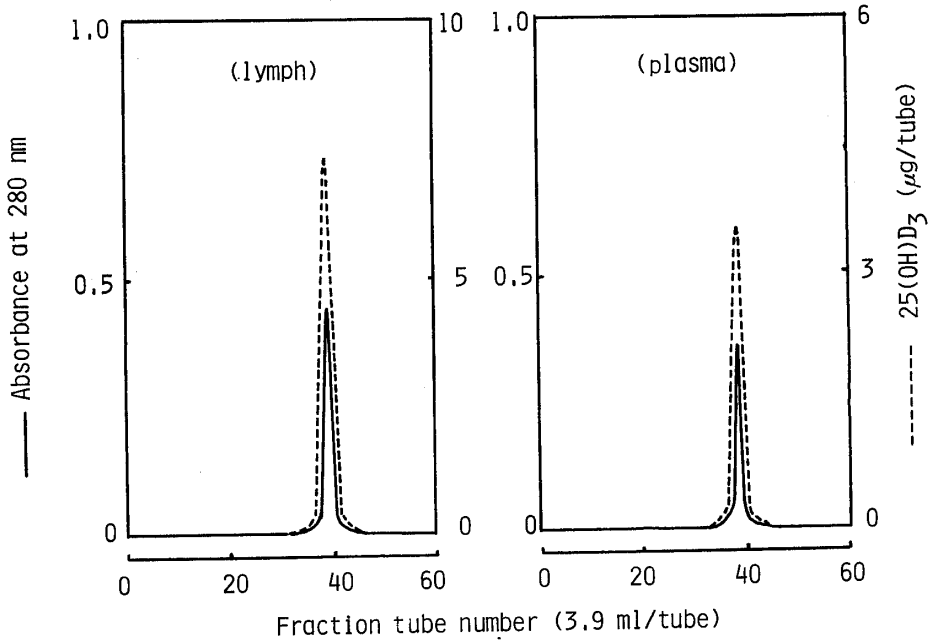

Fig. 1. Affinity Chromatograms of *l*-DBP and *p*-DBP on a Blue Sepharose CL-6B Column

chromatography, used as a final purification step (Fig. 1). Both proteins were purified about 300-fold from the original biological fluids by these procedures. The specific activities [25(OH)D_3 (μg)/protein(mg)] of *l*-DBP and *p*-DBP were 3.9 and 4.3, respectively.

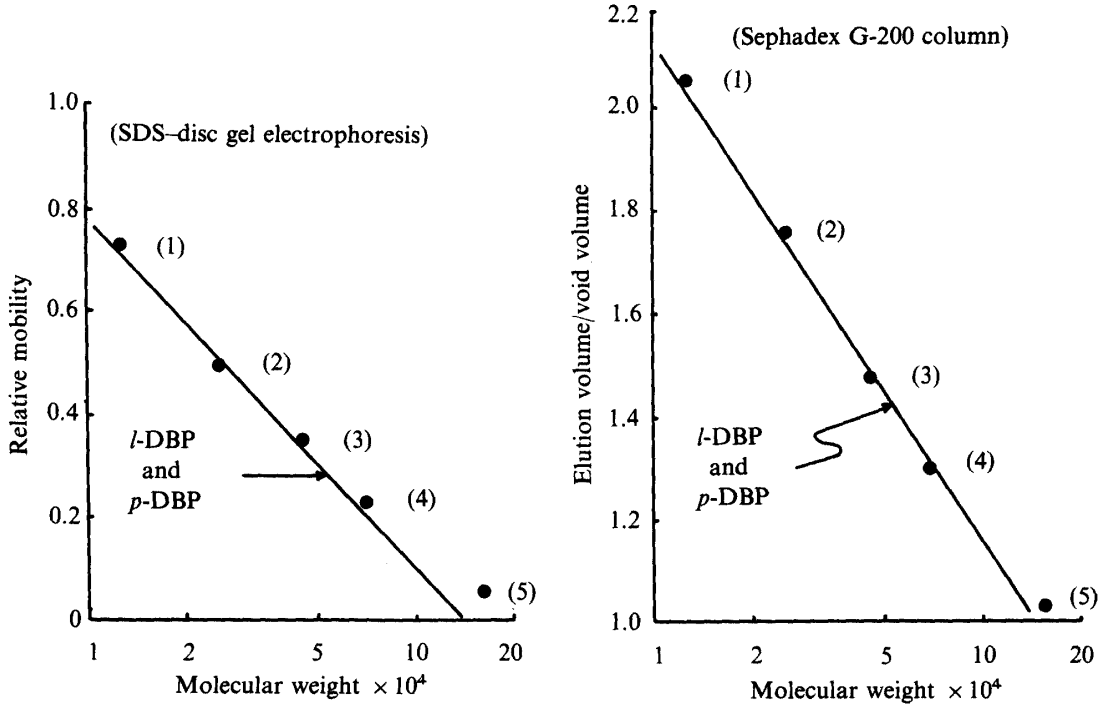

Fig. 2. Molecular Weight Estimation of *l*-DBP and *p*-DBP by SDS–Disc Gel Electrophoresis and Gel Filtration on a Sephadex G-200 Column

(1) cytochrome c [12600]; (2) chymotrypsinogen [25000]; (3) ovalbumin [45000]; (4) bovine serum albumin [69000]; (5) human gamma globulin [160000].

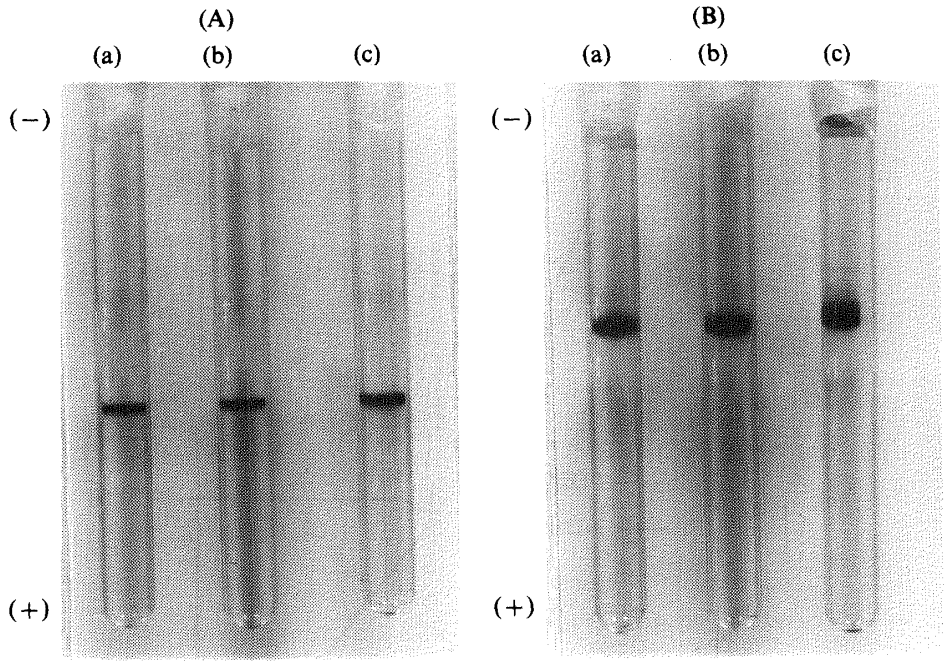

Fig. 3. Disc and SDS–Disc Gel Electrophoreses of *l*-DBP and *p*-DBP

(A): polyacrylamide gel disc electrophoresis.
(B): SDS–polyacrylamide gel disc electrophoresis.
(a) *l*-DBP; (b) *p*-DBP; (c) (a)+(b).

Molecular Weight Determination of *l*-DBP and *p*-DBP

Figure 2 shows a calibration curve for the determination of the molecular weights of *l*-DBP and *p*-DBP on a Sephadex G-200 column. The same value of 5.4×10^4 was obtained for the apparent molecular weight of both proteins. From the relative mobility on SDS–disc gel electrophoresis, the apparent molecular weight of *l*-DBP and *p*-DBP were also both estimated to be 5.4×10^4, respectively (Fig. 3).

Disc and SDS–Disc Gel Electrophoreses of *l*-DBP and *p*-DBP

As shown in Fig. 4, the purified *l*-DBP and *p*-DBP each migrated as a single band of protein on disc and SDS–disc gel electrophoreses, respectively. Furthermore, a mixture of *l*-DBP and *p*-DBP gave a single band of protein on both electrophoreses. These results suggest that *l*-DBP and *p*-DBP have the same molecular size and charge.

HPLC Analysis of *l*-DBP and *p*-DBP

l-DBP and *p*-DBP each gave a single protein peak, and their retention times were the same.

Chromatofocusing of *l*-DBP and *p*-DBP

The isoelectric points of *l*-DBP and *p*-DBP were compared by employing a chromatofocusing method as described in Experimental. As shown in Fig. 4, *l*-DBP and *p*-DBP both gave one major peak which was focused at pH 4.58, although there were a few minor peaks on either side of the major peak. Chromatofocusing is a new method for protein separation developed by Pharmacia Fine Chemicals. The method is based on the effects of differences in protein charge and focusing effects in a pH gradient developed in the column without using an electrophoretic system. Although this method gives very reproducible results and good resolution, the isoelectric point obtained by this method is an apparent value, because the pH of the mobile phase is the pH measured when the buffer emerges from the column. Accordingly, the pH measured by chromatofocusing using an anion exchange column, as here, is lower than the actual pH. Therefore, more accurate determination of the isoelectric points of *l*-DBP and *p*-DBP was performed by employing an isoelectric focusing method.

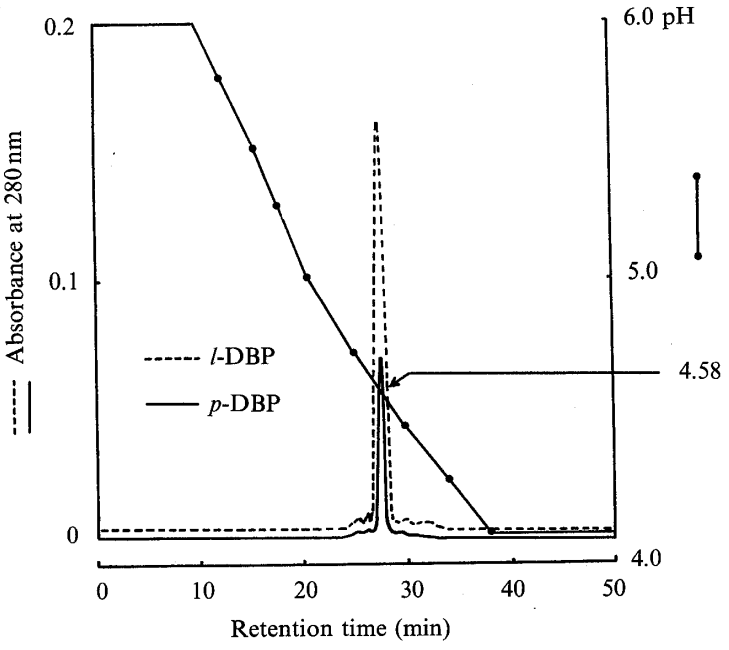

Fig. 4. Chromatofocusing of *l*-DBP and *p*-DBP

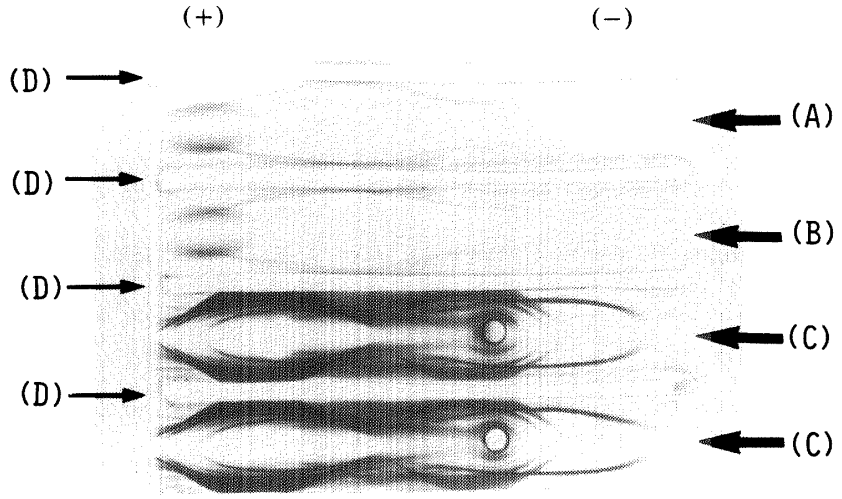

Fig. 5. Immunoelectrophoresis of *l*-DBP and *p*-DBP

(A) *p*-DBP; (B) *l*-DBP; (C) rat plasma; (D) rat whole antiserum (rabbit).

Isoelectric Focusing of *l*-DBP and *p*-DBP

l-DBP and *p*-DBP each gave a single band of protein having the same isoelectric point of 4.93.

Immunoelectrophoresis of *l*-DBP and *p*-DBP

The immunoelectrophoresis patterns of the purified *l*-DBP and *p*-DBP and rat whole plasma are shown in Fig. 5. Both *l*-DBP and *p*-DBP showed identical long precipitation lines developed from the inter α- to α_2-region against rat whole antiserum.

Discussion

It is well known that vitamin D and its hydroxylated metabolites are transported in the blood bound to plasma protein. In human and rat serum, only a single binding protein for vitamin D and all hydroxylated metabolites has been detected, isolated, and characterized.[3,11] The protein is an alpha-globulin with a molecular weight of 52000—58000 containing one sterol binding site per molecule with the highest binding affinity for 25(OH)D_3 and 24,25-dihydroxyvitamin D_3. The protein is synthesized in liver[12] and its only known physiological function is to transfer vitamin D and its metabolites to target tissues *via* the blood stream.

In the present study, we clearly demonstrated that binding protein having high affinity to 25(OH)D_3 also exists in rat thoracic duct lymph, and many physiological properties of the protein were confirmed to be identical with the binding protein from rat plasma. Although identity of amino acid composition and immunological specificity between *l*-DBP and *p*-DBP has not yet been demonstrated, it may be concluded that *l*-DBP and *p*-DBP are the same protein on the basis of our present results and the well-known fact that lymph mainly consists of components filtered from blood. If this is correct, it may be concluded that the binding protein for vitamin D and its metabolites circulates not only in the blood stream but also in the lymph stream. The concentration and physiological significance of *l*-DBP in lymph are still unknown, and are now under investigation.

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References and Notes

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