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Existence of Vitamin D_3 and 25-Hydroxyvitamin D_3 in Rat Lymph[1)]

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The transport forms of vitamin D_3 and 25-hydroxyvitamin D_3 [25(OH)D_3] in lymph were studied in rats with thoracic duct cannulas. Under *in vitro* and *in vivo* conditions, the majority of vitamin D_3 recovered from lymph was found in the chylomicrons and other lipoproteins fractions. On the other hand, the major fraction containing 25(OH)D_3 corresponded to the albumin and α-globulin fractions. In order to confirm the presence of binding protein having affinity to 25(OH)D_3 in lymph, the binding protein was isolated and purified by procedures of gel filtration, affinity and ion exchange chromatography from the incubated mixture of thoracic duct lymph with 25(OH)D_3. The purified binding protein showed a single band of protein on disc and sodium dodecyl sulfate-disc gel electrophoreses. Furthermore, the protein also gave a single peak on high-performance liquid chromatography (HPLC) using a gel permeation column. The molecular weight of the protein was calculated from the results of HPLC as 57000, a value which is apparently different from that of lymph albumin.

These results suggest that vitamin D_3 and 25(OH)D_3 are mainly transported from the intestine to the liver *via* the lymph duct by different carrier mediators as lipoprotein complex and protein-bound forms, respectively, in rats.

Keywords——vitamin D_3; 25-hydroxyvitamin D_3; lymph; transport form; binding protein; isolation; identification

It has been considered that vitamin D, as a fat-soluble vitamin, is absorbed from the intestine by a mechanism similar to that of dietary triglyceride, requiring bille salts for incorporation into micelles and subsequently being released into lymph chylomicrons.[2)] The majority of vitamin D_3 in lymph is found as an association complex with chylomicrons.[3)] 25-hydroxyvitamin D_3 [25(OH)D_3] is a metabolite of vitamin D_3 which is formed by hydroxylation at the 25 position in the liver. The metabolite is still fat-soluble, but the solubility is less than that of the parent vitamin. Therefore, its transfer into blood is mainly carried out *via* lymph.[3)] Both vitamin D and 25(OH)D_3 exist in natural foodstuffs and they are frequently used for oral treatment of metabolic bone disease and hypocalcemia. It was reported that 25(OH)D_3 is perhaps more active than vitamin D_3 on the calcium mobilizing.[4)] Therefore, their efficient absorption from the intestine and effective transport into lymph are required to maintain the calcium homeostasis. There have been a few reports on the efficiency and mechanism of intestinal absorption of vitamin D and 25(OH)D.[2,3,5)] However, in most of them, the intestinal absorption of vitamin D and 25(OH)D was mainly elucidated only from clinical and phenomenal aspects. Little information is available on the mechanism of transport of vitamin D and 25(OH)D in rat lymph. Detailed results on the transport mechanism are presented in this paper.

Experimental

Chemicals——Commercial grades (Philips-Duphar Co., The Netherlands) of vitamin D_3 and 25(OH)D_3 were

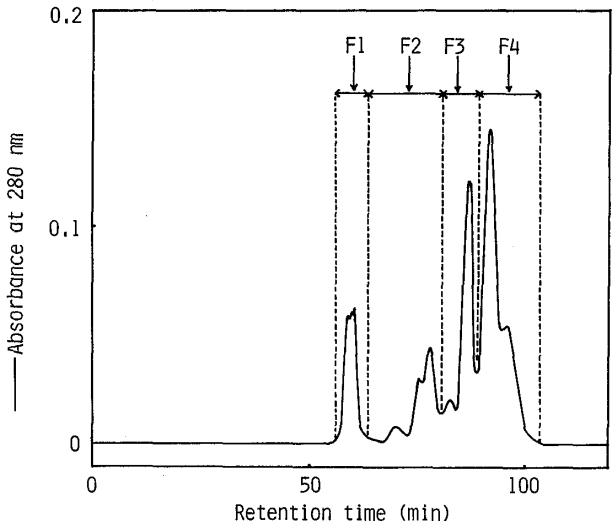

Fig. 1. HPLC Profile of Lymph on a Gel Permeation Column

Fifty μl of lymph prepared according to the procedure described in "Experimental" was subjected to HPLC on a single column of gel (TSK-Gel G3000SW, 600 × 7.5 mm i.d.) using 1/15 M phosphate buffer (pH 7.0) as a mobile phase. The flow rate was 0.2 ml/min (column pressure: 9 kg/cm^2).

used as the respective standard compounds. Organic solvents were purified by the usual methods and distilled before use. Sephadex products, Blue Sepharose CL-6B and DEAE-Sepharose CL-6B were purchased from Pharmacia Fine Chemicals Co. (Sweden). Other chemicals were of analytical grade.

Animal——Male Wistar rats weighing 250—300 g were used.

Collection of Lymph——After peroral administration of small amounts of cottonseed oil, rats were given light ether anesthesis, and a thoracic duct cannula consisting of polyethylene tubing (Size 2, Imamura Co., Ltd.) was positioned according to the method of Bollman *et al.*[6] The lymph was collected in a small flask to which a small amount of sodium azide had been added. The cannula was brought out through the abdominal wound and the rat was placed in a restraining cage during the collection of lymph.

Binding of Vitamin D_3 and 25(OH)D_3 to the Protein Components of Lymph——The binding of vitamin D_3 and 25(OH)D_3 to the protein components of lymph was examined by means of a simple high-performance liquid chromatography (HPLC) method using a gel permeation column under *in vitro* and *in vivo* conditions.

(A) *In Vivo* Experiment: At 24 h after thoracic duct cannulation, animals were perorally given 5 μg of vitamin D_3 or 25(OH)D_3 in 50 μl of ethanol and the lymph was collected at every hour until 7 h after administration. Exactly 50 μl of lymph was subjected to HPLC analysis. The conditions of HPLC analysis are described later.

The characteristic HPLC profile of lymph is shown in Fig. 1. The separation of each fraction (F1—F4 in the figure) was performed on the basis of the retention time. F1 (57.6—64.0 min), F2 (64.0—80.5 min), F3 (80.5—89.5) and F4 (89.5—104.0 min) mainly contain α,β-lipoproteins, macroglobulin (IgM), immunoglobulins (IgG, IgA, IgE), and albumin and α-globulin, respectively.[7] The assay of vitamin D_3 and 25(OH)D_3 in each fraction was carried out as described later.

(B) *In Vitro* Experiment: One ml of lymph was incubated with 2.4 μg each of vitamin D_3 and 25(OH)D_3 in 10 μl of ethanol in a mini-vial at 4 °C for 7 h. Aliquots of exactly 50 μl of the mixture were periodically taken from the vial and subjected to HPLC analysis. The assay of vitamin D_3 and 25(OH)D_3 in each fraction was carried out as described later.

Preparation of Chylomicron-Free Lymph——The lymph was mixed with the same volume of chilled 0.9% NaCl solution in a Polytron homogenizer, and the mixture was centrifuged at $7.8 \times 10^4 g$ 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.

Incubation of Lymph with 25(OH)D_3——Seventy-five ml of lymph obtained above 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, with 0.15 M NaCl and 0.03% sodium azide and incubated with 120 μg of 25(OH)D_3 dissolved in 1 ml of ethanol, then directly subjected to the following isolation and purification steps.

HPLC Analysis of Proteins——HPLC analysis of proteins was performed on a Shimadzu LC-4A high-performance liquid chromatograph with a Shimadzu SPD-2AS detector (monitored at 280 nm with 0.02 absorbance unit as full scale). Separation of proteins was carried out in a stainless steel tube (600 × 7.5 mm i.d.) packed with hydrophilic, spherical, porous silica gel (TSK-Gel G3000SW, Toyo Soda Manufacturing Co., Ltd., Japan) 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.

Assay of Vitamin D_3 and 25(OH)D_3 by HPLC——Assay of vitamin D_3 and 25(OH)D_3 in the eluates obtained from various column chromatographies and the HPLC was carried out according to our methods as previously

reported.[8] Exactly 0.5 ml of 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. The mixture was allowed to stand for 1 h, then centrifuged at 3000 rpm for 5 min, and 1 ml of methylene dichloride and 1 ml of water were added to it. The mixed solution was separated into two layers and the lower methylene dichloride layer was taken into a different tube. The upper water layer was extracted further with 2 ml of methylene dichloride and the extracts were combined. The solvent was evaporated off under reduced pressure, and the resulting residue was dissolved in 0.25 ml of 5.5% isopropanol in *n*-hexane. Then, 50 μl of the solution was applied to a Shimadzu LC-3A high-performance liquid chromatograph equipped with an auto-sampler (WISP model 710A, Waters Associates, Inc., U.S.A.). Separation of vitamin D_3 and 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 times of vitamin D_3 and 25-(OH)D_3 were 6.0 and 13.4 min, respectively and both compounds were easily determined by reading the appropriate peak heights on chromatograms.

Determination of Protein——Protein was determined by the method of Lowry *et al.*[9] using human serum albumin as a standard protein.

Disc Gel Electrophoresis——Electrophoresis was performed with 7.5% polyacrylamide gel (pH 8.9) at a constant current (3 mA/tube) for 45 min at room temperature. The protein in the gel was stained with amido black 10 B.

SDS–Disc Gel Electrophoresis——Electrophoresis was carried out with 5% polyacrylamide gel containing 0.1% SDS according to the method of Weber and Osborn.[10]

Column Chromatography——Gel filtration on columns of Sephadex G-200 or G-100, ion exchange column chromatography on DEAE-Sepharose CL-6B and affinity column chromatography on Blue Sepharose CL-6B were carried out according to the isolation procedures described for human plasma vitamin D-binding protein by Imawari *et al.*[11] The detailed conditions are indicated in each figure caption. Column chromatography was always carried out at about 4 °C. In most instances, the effluent stream was monitored continuously at 280 nm with a UV detector (model 111, Gilson Co., Ltd., U.S.A.).

Determination of Molecular Weight——The molecular weight of the purified 25(OH)D_3 binding protein obtained from lymph was estimated by the HPLC method using γ-globulin, albumin, ovalbumin, chymotrypsinogen, myoglobin, cytochrome c and bacitracin as standard proteins. The separation of the seven standard proteins and the binding protein was carried out under the same conditions as described above.

Results and Discussion

Binding of Vitamin D_3 and 25(OH)D_3 to the Protein Components of Lymph

It was considered that carrier mediators bound preferentially to vitamin D_3 or 25(OH)D_3 might be present in lymph and that they might be transported *via* lymph ducts to the blood stream. In order to confirm these assumptions, a simple *in vitro* binding test as mentioned in Experimental was first performed by HPLC using a gel permeation column. As shown in Fig.

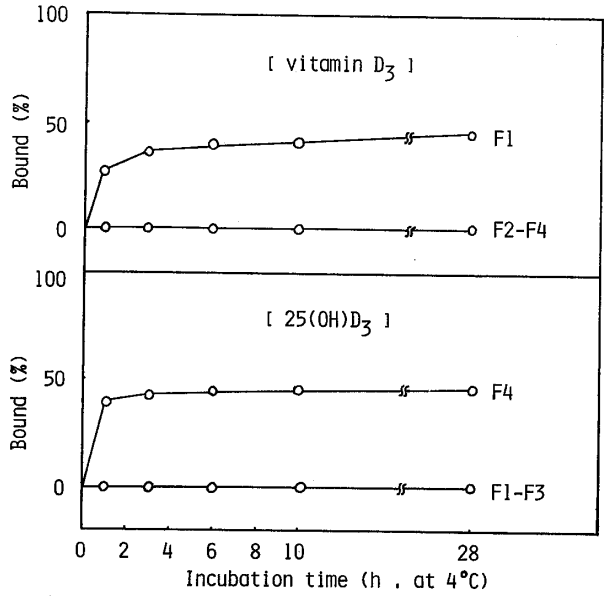

Fig. 2. Binding Ratios of Vitamin D_3 and 25(OH)D_3 to the F1—F4 Fractions in the *in Vitro* Experiment

1, good separation of various lymphatic proteins was obtained within two hours. When a preliminary experiment was performed by using human serum albumin as a marker protein, the recovery of the protein was satisfactory. As shown in Fig. 2, the majority of vitamin D_3 was recovered from the F1 fraction (mainly containing high molecular lipoproteins), while the vitamin was not detected in the other fractions. This result suggests that vitamin D_3 is predominantly bound to lipoproteins to form an association complex. On the other hand, most of the 25(OH)D_3 was recovered from the F4 fraction (mainly containing albumin and α-globulin), while the compound was not detected in the other fractions, including F1. In the *in vivo* experiment, similar results were observed, as shown in Fig. 3. Since plasma albumin is

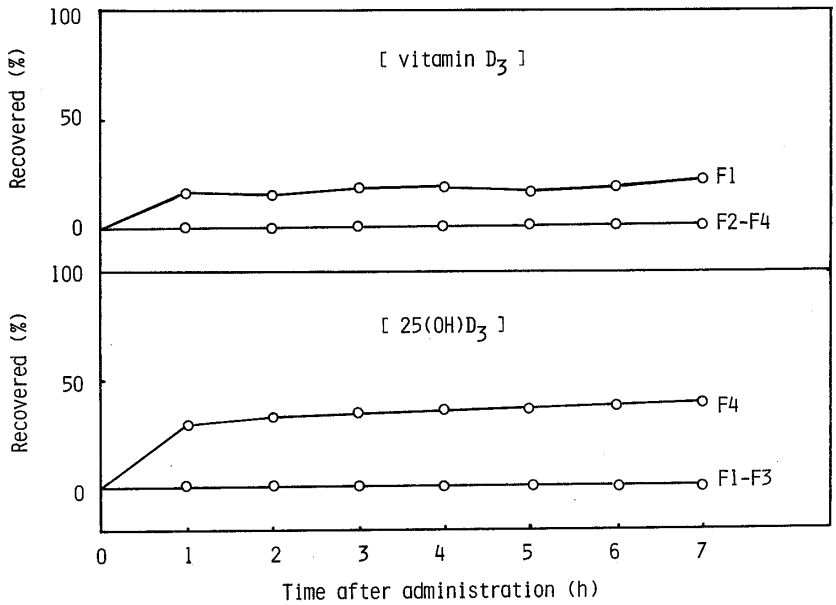

Fig. 3. Binding Ratios of Vitamin D_3 and 25(OH)D_3 to the F1—F4 Fractions in the *in Vitro* Experiment

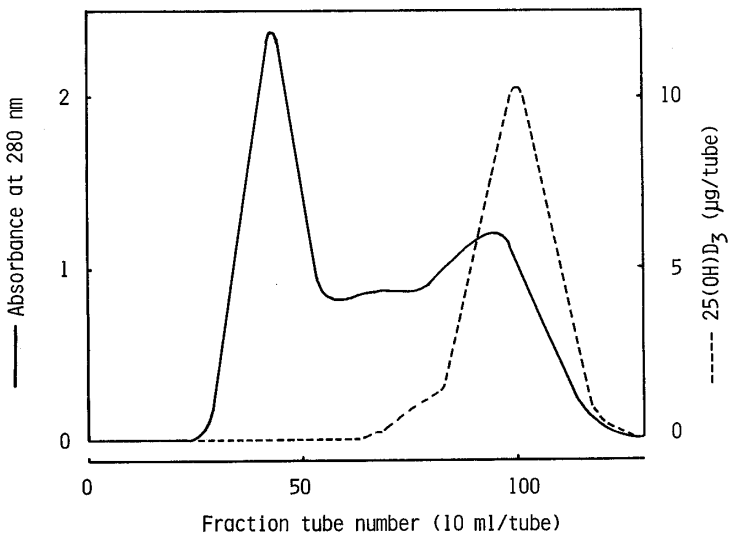

Fig. 4. Gel Filtration on Sephadex G-200 of Lymph

The sample was applied to a column (5 × 65 cm) of Sephadex G-200 equilibrated with 1/15 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.03% sodium azide at a flow rate of 40 ml/h. Elution was carried out with the same buffer solution and at the same flow rate. Fractions of 10 ml were collected. The absorbance at 280 nm and the concentration of 25(OH)D_3 were measured. Fractions 63—111 were combined for further purification.

known to bind non-specifically to various kinds of drugs,[12] lymph albumin may be involved in the binding of 25(OH)D_3. However, α-globulin is also a candidate 25(OH)D_3-binding protein because plasma α-globulin usually contains significant amounts of Gc-protein, which is known to be a specific binding protein for vitamin D_3 and its metabolites.[13] In order to clarify the binding specificity of the protein with 25(OH)D_3 and to determine whether the lymph albumin and/or α-globulin are involved in the binding, the following isolation and purification

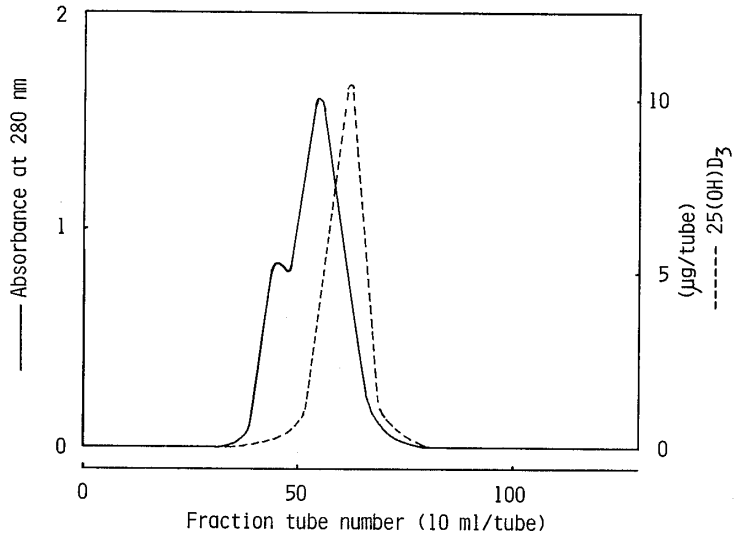

Fig. 5. Gel Filtration on Sephadex G-100 of Lymph 25(OH)D_3-Binding Protein

The sample was applied to a column (5 × 65 cm) equilibrated with 1/15 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.03% sodium azide at a flow rate of 70 ml/h. Elution was carried out with the same buffer solution and at the same flow rate. Fractions of 10 ml were collected. The absorbance at 280 nm and the concentration of 25(OH)D_3 were measured. Fractions 55—65 were combined for further purification.

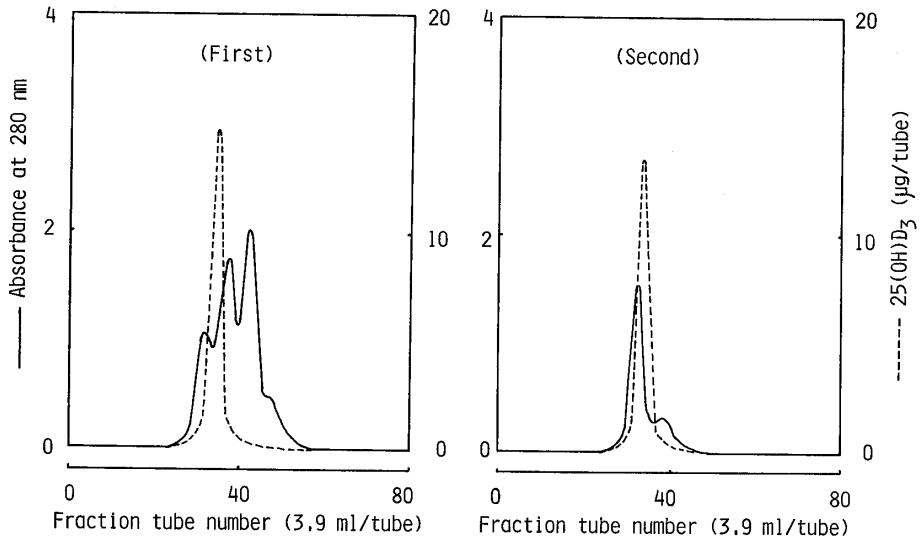

Fig. 6. Affinity Chromatogram on Blue Sepharose CL-6B of Lymph 25(OH)D_3-Binding Protein

The fraction obtained by Sephadex G-100 chromatography was applied to a column (2 × 89 cm) of Blue Sepharose CL-6B equilibrated with 0.05 M Tris·HCl buffer (pH 7.0) containing 0.1 M KCl and 0.03% sodium azide at a flow rate of 20 ml/h. Elution was carried out with the same buffer and at a flow rate of 17 ml/h. Fractions of 3.9 ml were collected. The absorbance at 280 nm and the concentration of 25(OH)D_3 were measured. Fractions 27—40 were combined and applied again to the same column. In the second chromatography, fractions 28—34 were combined for further purification.

procedures were performed.

Purification of 25(OH)D_3-Binding Protein in Lymph

The 25(OH)D_3-binding protein from rat lymph was purified by gel filtration on Sephadex G-200 and G-100, chromatography on Blue Sepharose CL-6B (twice), chromatography on DEAE-Sepharose CL-6B and rechromatography on Blue Sepharose CL-6B as described below. The crude 25(OH)D_3-binding protein isolated from lymph was purified at least 307-fold.

1) Gel Filtration on Sephadex G-200——Lymph which had been incubated with 25-(OH)D_3 as mentioned in Experimental was gel-filtered on Sephadex G-200. As shown in Fig. 4, the protein fraction binding 25(OH)D_3 appeared as a single peak in the fraction of low molecular weight proteins containing albumin and α-globulin.[14] The eluates containing 25(OH)D_3 (fractions 63—111) were combined, dialyzed against deionized water, and lyophilized. The product was gel-filtered on Sephadex G-100.

2) Gel Filtration on Sephadex G-100——The 25(OH)D_3-binding protein fraction obtained above was applied to a Sephadex G-100 column. As shown in Fig. 5, the chromatogram showed one major peak and one minor peak. However, since these peaks were not exactly coincident with the peak of 25(OH)D_3, the fractions containing 25(OH)D_3 (fractions 55—65) were combined, then dialyzed and lyophilized.

3) Blue Sepharose CL-6B Chromatography (Twice)——This procedure was carried out in order to remove lymph albumin, which binds tightly to Blue Sepharose CL-6B under appropriate conditions. The lyophilized protein fraction obtained above was applied to a Blue Sepharose CL-6B column. The chromatograms are shown in Fig. 6. As shown on the left side of Fig. 6, three major peaks and one minor peak appeared in terms of absorbance at 280 nm. The fractions containing 25(OH)D_3 (fractions 27—40) were combined and then applied again to the same column. As shown on the right side of Fig. 6, further purification was achieved by

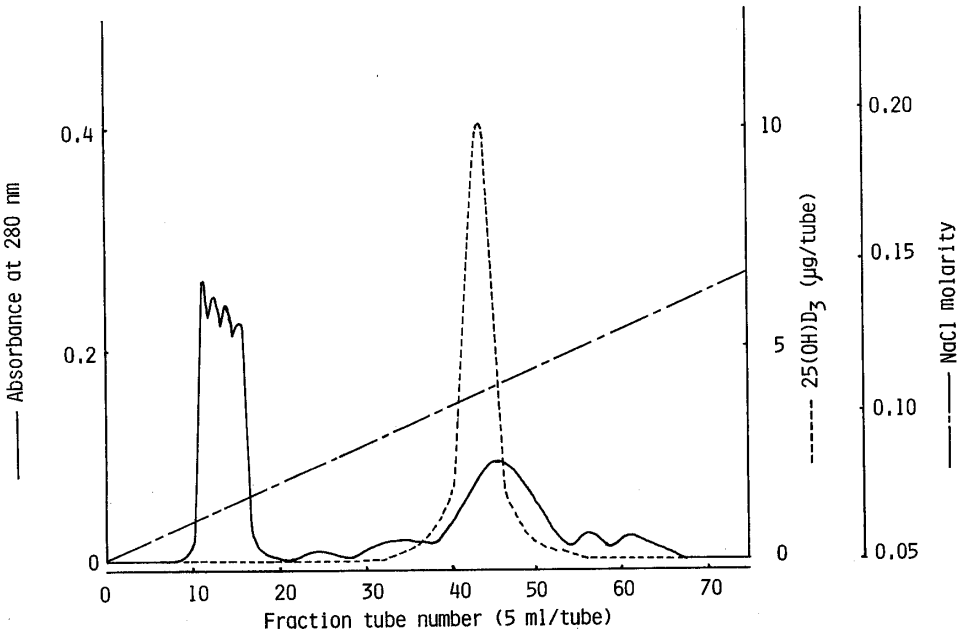

Fig. 7. Ion Exchange Chromatogram on DEAE-Sepharose CL-6B of Lymph 25(OH)D_3-Binding Protein

The sample obtained by the Blue Sepharose CL-6B chromatography was applied to a column (2.5 × 18 cm) of DEAE-Sepharose CL-6B equilibrated with 0.05 M Tris·HCl buffer (pH 7.0). Elution was carried out with a linear gradient from 0.05—0.3 M NaCl in the same buffer. Fractions of 5 ml were collected. The absorbance at 280 nm and the concentration of 25(OH)D_3 were measured. Fractions 38—49 were combined for further purification.

the second chromatography. Since the recovery of 25(OH)D_3 over both chromatographies was better than 90%, it is concluded that 25(OH)D_3 did not bind with lymph albumin, but with α-globulin or other unknown proteins. For further purification, the fractions containing large amounts of 25(OH)D_3 in the second chromatography (fractions 28—34) were combined, dialyzed against deionized water and lyophilized.

4) DEAE-Sepharose CL-6B Chromatography——The 25(OH)D_3-binding protein fraction obtained above was further purified by ion exchange chromatography on DEAE-Sepharose CL-6B. The chromatogram is shown in Fig. 7. The major protein peaks were in the neighborhood of the void volume of column, while five minor peaks were eluted by further development. Since the peak of 25(OH)D_3 is the 4th on the chromatogram, the fractions corresponding to this peak (fractions 38—49) were combined and further purified by affinity chromatography on Blue Sepharose CL-6B.

5) Blue Sepharose CL-6B Chromatography——The 25(OH)D_3-binding protein fraction obtained above was again subjected to chromatography on Blue Sepharose CL-6B as described above. As shown in Fig. 8, two major and three minor peaks were observed in the chromatogram and the second peak clearly coincided with the peak of 25(OH)D_3. The fractions containing 25(OH)D_3 (fractions 38—41) were collected and subjected to electrophoretic and HPLC analyses to confirm the homogeneity of the 25(OH)D_3-binding protein thus purified.

Disc and SDS-Disc Gel Electrophoreses

The homogeneity and electrophoretical properties of the purified 25(OH)D_3-binding protein obtained above were examined by means of disc and SDS-disc gel electrophoreses. As shown in Fig. 9, the purified protein showed only a single band in both electrophoreses, and its mobility was apparently different from that of lymph albumin. Furthermore, the result of SDS-disc gel electrophoresis suggested that the purified protein might consist of a single peptide chain because the protein was not cleaved into subunits by reduction with 2-mercaptoethanol.

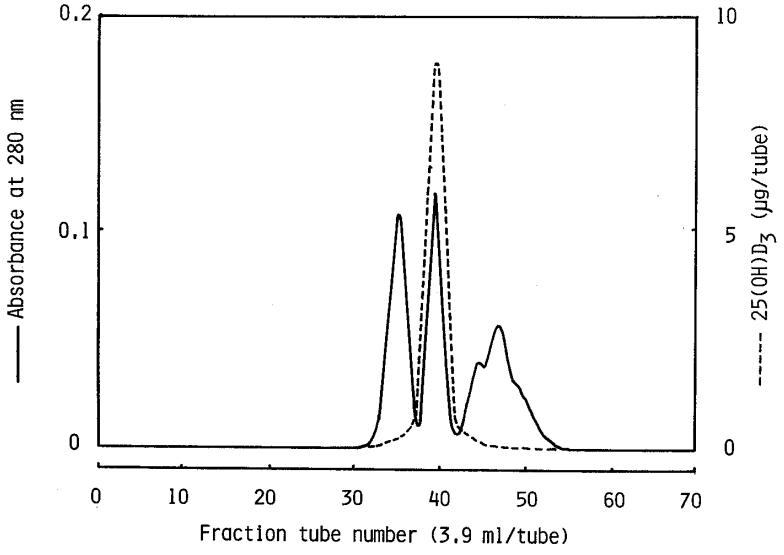

Fig. 8. Final Affinity Chromatogram on Blue Sepharose CL-6B of Lymph 25(OH)D_3-Binding Protein

The sample obtained after the ion exchange chromatography was applied to a column (2 × 89 cm) of Blue Sepharose CL-6B. Elution and fractionation were carried out under the same conditions as described in Fig. 6. Fractions 33—37 were combined and subjected to HPLC and electrophoresis as described in Fig. 9.

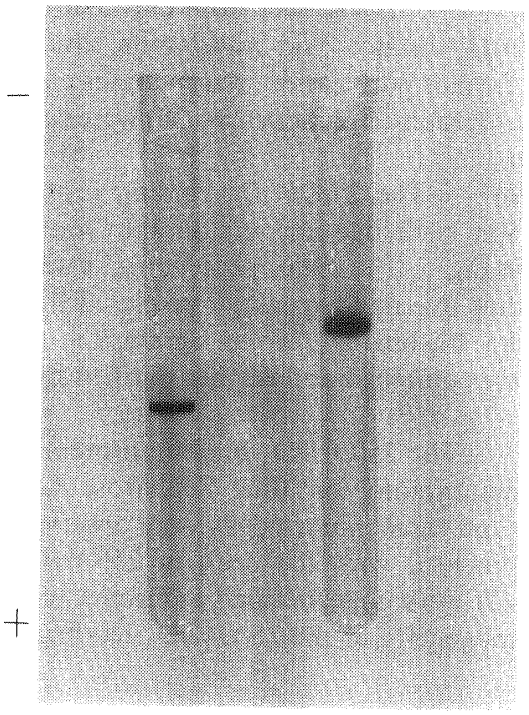

Fig. 9. Disc and SDS-Disc Gel Electrophoreses of the Purified Lymph 25(OH)D_3-Binding Protein

Left column: Disc gel electrophoresis. Right column: SDS-disc gel electrophoresis. The conditions are described in "Experimental."

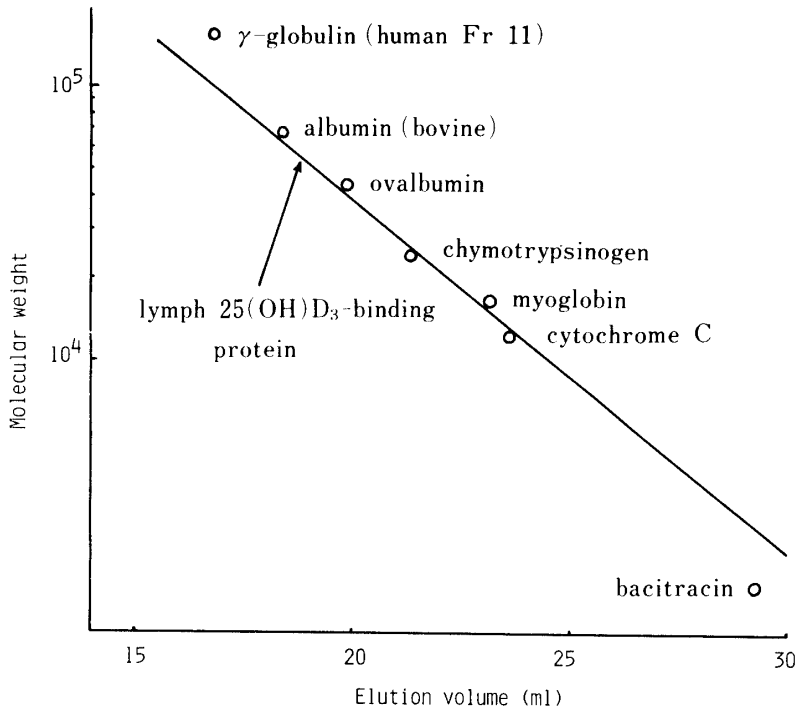

Fig. 10. Determination of Molecular Weight of the Purified Lymph 25(OH)D_3-Binding Protein by HPLC Using a Gel Permeation Column

The conditions are described in "Experimental".

Determination of Molecular Weight by HPLC

The molecular weight of the purified protein was estimated by HPLC using a gel permeation column. Since the affinity of this column (packed with hydrophilic, spherical, porous silica gel) is unaffected by the charge of proteins, the order of elution from the column directly reflects the apparent molecular weights of proteins, as is the case with gel filtration on

Sephadex and SDS-disc gel electrophoresis. When the retention time of the purified 25(OH)D_3-binding protein was compared with the calibration curve prepared with seven standard proteins, the apparent molecular weight was calculated as 5.7×10^4. This value is very similar to that of the rat plasma vitamin D-binding protein.[15]

We are now investigating whether or not the protein purified from rat lymph is identical with the rat plasma vitamin-D-binding protein.

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