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Purification of Human Renal Calcium Binding Protein from Necropsy Specimens[1] (37878)

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(Introduced by J. P. Hannon)

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A calcium binding protein (CaBP) has been reported in the small intestine, colon, and kidney of vitamin D_3-treated rachitic chicks but was not detectable by the same methods in the liver, muscle, pancreas, or bone (1, 2). A similar protein has been reported in rat intestine (3), dog intestine (4), human intestine (5–8), and human kidney (5). The concentration of the protein in intestinal mucosa is correlated with the rate of calcium transport across the intestine in vitamin D-repleted rachitic chicks (9), dietary calcium deficiency or excess, and dietary phosphorus deficiency or excess (10). A vitamin D dependency has been demonstrated in the chick (9) and rat (3). However, the vitamin D dependency and functional importance of CaBP in humans is not established. The purpose of this study was to purify human CaBP for use in development of a radioimmunoassay for the protein, after which the functional importance of CaBP in various nutritional and disease states can be studied in humans.

Methods. Tissue preparation. Kidneys were obtained 6–24 hr postmortem from cadavers which had been refrigerated soon after death. The specimens were frozen for later preparation, at which time they were thawed, cut into small pieces, suspended 1:4 in pH-7.4 Tris buffer,[2] homogenized with a blender, centrifuged at 30,000*g* for 20 min, heated to 65° for 15 min, cooled in an ice bath for at least 1 hr, and recentrifuged at 30,000*g* for 30 min. The supernate was then dialyzed against distilled water and lyophilized.

DEAE-cellulose separation. One-hundred-and-ten grams of Whatman Cellulose DE52 (W. and R. Balson, Maidstone, Kent, England) was suspended in 1 liter of 0.05 *N* HCl for approximately 1 hr, degassed under vacuum, allowed to sediment, and the supernate decanted. The granules were resuspended in previously degassed pH-7.0 Tris buffer.[3] The pH of the suspension was then adjusted to 7.0 with 1.0 *M* Tris and washed 3 times with pH-7.0 Tris buffer. The suspension was again degassed immediately prior to pouring into a 2.6 × 40 cm column. The column was packed, equilibrated, and run at a flow rate of 75 ml/hr. Approximately 3 liters of buffer were required for equilibration, at which time the pH of the buffer leaving the column was equal to that of the entering buffer. The lyophilized crude homogenate was dissolved in pH-7.0 Tris buffer (30 mg protein/ml buffer), centrifuged at 30,000*g* for 30 min to remove traces of insoluble precipitate, degassed, and applied to the equilibrated column. Up

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

[2] The buffer contained 0.01 *M* Tris, 0.12 *M* sodium chloride, 0.004 *M* potassium chloride, and 0.02% sodium azide.

[3] The buffer contained .05 *M* Tris adjusted to pH 7.0 with HCl.

to 16 g of lyophilized crude protein could be applied to 110 g of DE52 without column saturation. After the sample was applied, the column was washed with pH-7.0 Tris buffer until the eluate was free of protein, at which time the adsorbed protein was eluted with a linear gradient of 0–1.0 *M* sodium chloride in pH-7.0 buffer (total volume = 500 ml).

Gel filtration separation. A 5 × 110 cm column of Sephadex G75 superfine gel (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) was prepared and equilibrated with pH-7.4 buffer. The DE52 eluate fractions which contained calcium binding activity were pooled and concentrated by suction ultrafiltration to 30–80 mg/ml. Twenty-five to one-hundred milliliters of the concentrate was applied to the column and the protein eluted with pH-7.4 buffer.

Acrylamide gel electrophoresis separation. The low-molecular-weight calcium-binding-activity fractions were pooled, and approximately 100 mg of protein was concentrated by suction ultrafiltration to 0.5–1.0 ml. The concentrate was further purified by preparative acrylamide gel electrophoresis using an E-C vertical gel slab apparatus (E-C Apparatus Corp., Philadelphia, PA) and a continuous alkaline buffer system. The acrylamide gel (Cyanogum 41 from E-C Apparatus Corp.) concentration was 7% and the buffer was 89 m*M* Tris, 88.5 m*M* boric acid, and 2.5 m*M* disodium EDTA (pH 8.3). The protein migration pattern was determined by cutting off a portion of the preparative gel slab, staining with amido black, and destaining electrically. Separate bands were cut out and placed in 8 mm i.d. disc gel electrophoresis destaining tubes (Shannon Southern Inst., Inc., Sewickley, PA) which were previously plugged with gel. A piece of dialysis tubing was mounted on the destaining tubes, and the protein was electrophoretically eluted into the dialysis bags. The buffer used for elution was 89 m*M* Tris and 88.5 m*M* boric acid. The eluted protein was dialyzed against distilled water and concentrated by suction ultrafiltration to approximately 4 ml. The extent of isolation was tested by analytical disc gel electrophoresis using the same gel and buffer system as that employed in the gel slab separation.

Protein analysis. The protein concentration of individual column fractions was determined turbimetrically (11) by adding 1 ml of 3% trichloracetic acid to 0.2 ml of sample and allowing to stand for 10 min. The precipitated protein was compared to barium sulfate standards which had been standardized against human albumin concentrations using the same procedure for protein precipitation. The method of Lowry *et al.* (12) was employed for protein concentration determinations, which were to be used as a basis for specific calcium-binding-activity determinations and purified CaBP quantitation.

Calcium-binding-activity assays. The calcium binding activity was determined by a modification of the method reported earlier by Wasserman *et al.* (13). The test reagent volumes were modified to 0.2 ml of sample, 0.1 ml of ^{45}Ca solution (approximately 10,000 cpm/0.1 ml in pH-7.4 buffer), and 0.1 ml of Chelex 100 (200–400 mesh) resin (Bio-Rad Laboratories, Richmond, CA) suspension (1 part resin:1 part pH-7.4 buffer). The calcium binding activity was expressed as

$$\text{S/R net} = \frac{\%\ ^{45}\text{Ca in test supernate}}{\%\ ^{45}\text{Ca in test resin}} - \frac{\%\ ^{45}\text{Ca in blank supernate}}{\%\ ^{45}\text{Ca in blank resin}}.$$

The rationale for expressing the calcium binding activity as S/R net has been reported earlier (13). Radioactivity was measured by liquid scintillation counting.

Results and Discussion. Figure 1 illustrates a typical DEAE-cellulose chromatography run. It is apparent from the calcium-binding-activity curve that at least two calcium-binding proteins are retained on the DE52 column from the heat-treated crude homogenate, but the two are not well-resolved by this method. DE52 separation was used as the initial isolation step because it provides an efficient means of eliminating a fairly large amount of undesired protein while retaining the proteins of interest,

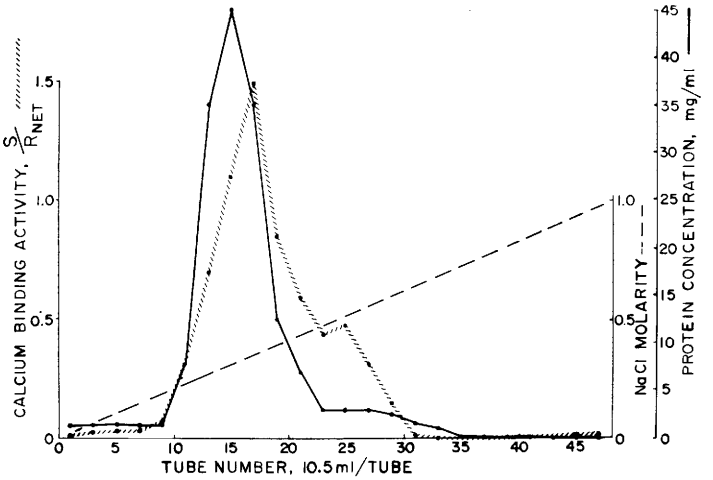

FIG. 1. DEAE cellulose chromatography. Eight grams of heat-treated supernate from renal tissue was applied to 110 g of DE52 in a 2.6 × 40 cm column. The adsorbed protein was eluted from the column with a 0–1.0 *M* linear sodium chloride gradient.

thus reducing the number of gel-filtration column runs required. One of the calcium-binding proteins was eluted by 0.2–0.3 *M* sodium chloride, as could be expected from the results of Piazolo *et al.* (5), and the other calcium binding protein is eluted in the 0.4–0.5 *M* sodium chloride range. The fractions containing calcium binding activity eluted from the DE52 column by the sodium-chloride gradient were pooled for gel filtration.

The gel-filtration step is illustrated in Fig. 2. The calcium-binding-activity curve illustrates that the two calcium-binding proteins are well-resolved by this method, but the larger-molecular-weight one (Peak A) was still heavily contaminated with other proteins while the low-molecular weight one (Peak B) is markedly increased in specific activity by this treatment. Peak A was not

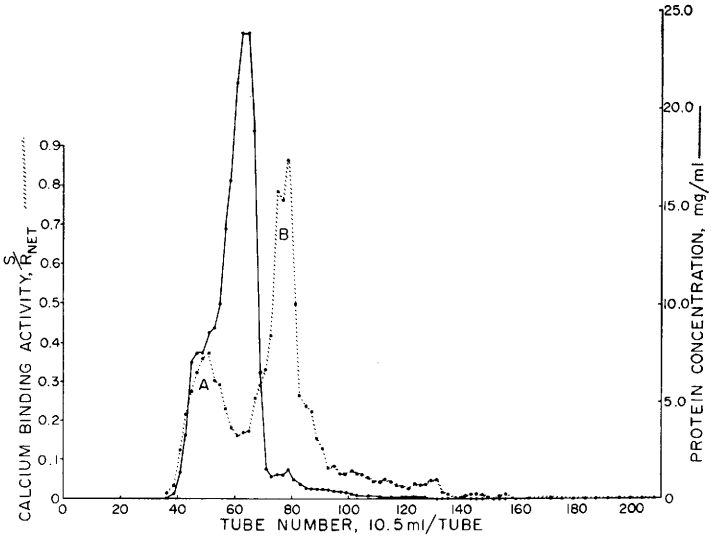

FIG. 2. Gel filtration chromatography. Approximately 2 g of DE52 purified CaBP from renal tissue was applied to a 5 × 110 cm column of Sephadex G75 superfine gel. The sample was applied in 25 ml of pH-7.4 Tris buffer, and the protein was eluted with the same buffer using a pressure head of 100 cm.

mentioned by Piazolo *et al.* (5), but is not as apparent when Sephadex G75 is utilized as the initial separation step instead of DEAE-cellulose, because peak A elutes in the void volume with a number of other proteins, resulting in a relatively low specific calcium binding activity. A 145,000 MW vitamin D-dependent calcium-binding protein has been reported in rat intestinal mucosa (14), but the authors are not aware of any reports of its presence in kidney from any species. Alpers *et al.* (6) reported two calcium-binding proteins in human intestine, but both were approximately 21,000 MW. However, the human intestinal CaBP reported by Hitchman and Harrison (8) was only 12,000–13,000 MW. Peak A is eluted in the void volume of Sephadex G75, indicating that it has a molecular weight of 70,000 or larger. Thus, it could be either albumin (69,000 MW) or another calcium-binding protein of MW < 70,000. Peak A has been pooled and retained for later isolation and characterization, but Peak B (CaBP) was further subjected to acrylamide gel electrophoresis as described above. The first passage through acrylamide gel slab resulted in elimination of all non-CaBP protein except for a trace of the band migrating just behind the CaBP. A second run through the acrylamide gel slab procedure resulted in protein which migrated as a single band on gel electrophoresis (Fig. 3) and had a very high specific calcium binding activity (Table I). Segments of acrylamide gel adjacent to the CaBP band did not yield any calcium binding activity when treated in the same manner as the CaBP-containing gel segment, indicating that the electrophoretic elution procedure and dialysis had effectively removed the EDTA from the buffer used in the initial electrophoretic separation.

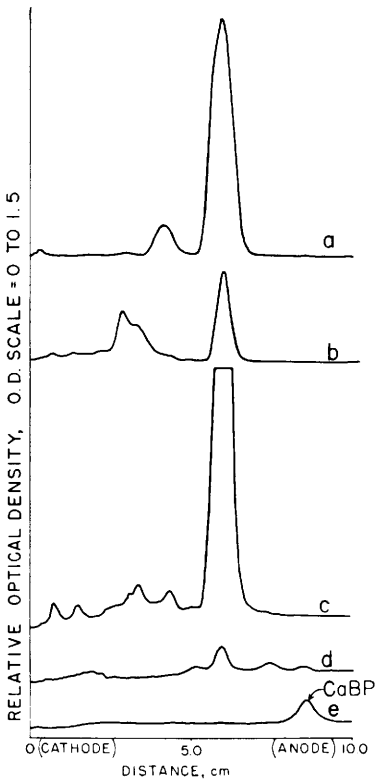

FIG. 3. Acrylamide gel electrophoresis of material from stages of purification: (a) 300 μg of human albumin fraction V; (b) 250 μg of heat-treated supernate; (c) 500 μg of DEAE purified material; (d) 125 μg of gel-filtration-purified material; (e) 78 μg of acrylamide-gel-electrophoresis-purified material. Samples were placed in slots at the top of the gel slab and migrated toward the anode (bottom) with 200 V applied for 3 hr. The gel slab was 3 mm thick and was 7% acrylamide in pH-8.3 buffer. Optical density was recorded with a recording densitometer (Model 345 Electrophoresis Densitometer, Clifford Instruments, Inc., Natick, MA).

TABLE I. Specific Calcium Binding Activity.

Protein	S/R_{net}/(mg protein/ml supernate)
Human albumin fraction V	0.017
Heat-treated supernate	0.018
DEAE-cellulose purified	0.054
Gel filtration purified	0.744
Acrylamide gel purified	3.140

The effect of the various isolation steps on the specific calcium binding activity is indicated in Table I, and the electrophoretic patterns for the same samples are illustrated in Fig. 3. The specific calcium binding activity of human albumin fraction V (Sigma Chemical Co., St. Louis, MO) is also included, using the same methodology as that employed for the other measurements. The method described herein results in a 174-fold increase in specific calcium binding

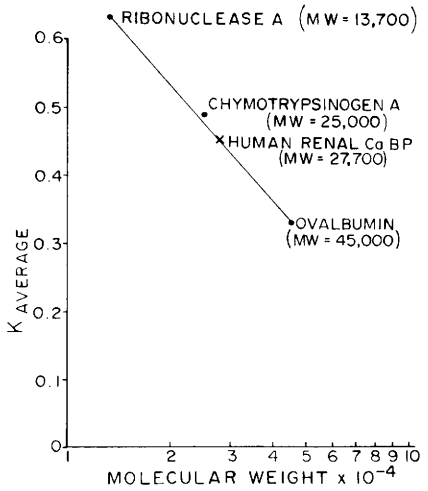

FIG. 4. The molecular weight of human renal CaBP was estimated by gel filtration with a 2.6 × 38.2 cm column of Sephadex G150 superfine gel. $K_{av} = V_e - V_0/V_t - V_0$, where V_e = elution volume for the protein, V_0 = elution volume for Blue Dextran 2000, and V_t = total bed volume.

activity compared to the heat-treated crude homogenate and a 184-fold greater specific calcium binding activity than that observed for human albumin fraction V. Piazolo *et al.* (5) reported a 36.4-fold increase in specific calcium binding activity during isolation and an eight-fold greater specific calcium binding activity than very pure human serum albumin (Behring Werke, Germany). A direct comparison of the two procedures is not possible because of the difference in the albumin and tissue sources. However, the presently reported method yields a highly purified CaBP and offers the advantage of readily concentrating CaBP from a relatively dilute initial source such as necropsy tissues.

Approximately 8 mg of purified CaBP (Peak B) has been prepared by the above method. The molecular weight of human renal CaBP was approximated by gel filtration. Figure 4 illustrates the results. The 27,700 MW of human renal CaBP is quite close to the 28,000 MW CaBP reported in the chick (11), and approximately two times the molecular weight of the human intestinal CaBP reported by Hitchman and Harrison (8).

Summary and Conclusions. A method has been developed for the purification of a 27,000 MW CaBP from human renal necropsy specimens. The purification was attained by application of DEAE-cellulose chromatography, gel filtration chromatography, and preparative acrylamide gel electrophoresis. The method yields a protein which migrates as a single band on acrylamide gel electrophoresis and has a specific calcium binding activity 184-fold greater than that of human albumin fraction V.

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