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The Purification of Calcium-Binding Protein from the Uterus of the Laying Hen (39369)

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Intestinal calcium-binding proteins from several species, including the chick (1–3), rat (4), and monkey (5) have been shown to be dependent upon vitamin D for their formation, and a large body of evidence presently exists suggesting a role for these proteins in the vitamin D-mediated intestinal transport of calcium. Proteins similar or identical to the chick intestinal calcium-binding protein have also been detected in the chick kidney (2) and brain (6) and in the uterus of the laying hen (7). The hen uterine calcium-binding protein has been shown to be dependent upon vitamin D_3 for its formation (7) and to increase in concentration in the tissue with the onset of egg-laying and decrease in concentration with its cessation (8), and has been localized in the tubular gland cells of the shell gland (9). Unlike the intestinal calcium-binding protein, the uterine protein does not appear to be influenced by long-term dietary calcium-restriction (8).

A recent report (10) suggested that the uterine calcium-binding protein might be different from the intestinal protein, although earlier studies, by another group (7), indicated that the two had similar electrophoretic mobilities. In the present study, uterine calcium-binding protein was isolated and some of its properties compared to purified intestinal calcium-binding protein, including immunological and electrophoretic properties and amino acid composition. The similarities of these characteristics indicate that the two proteins are identical.

Methods and materials. Hen uterus was obtained from normal mature White Leghorn hens in lay. Animals were sacrificed by cervical dislocation, the uterus was excised, and the latter homogenized in Tris buffer mixture, pH 7.4 (1:2, w/v), containing 0.1% β-mercaptoethanol. Homogenization and all successive steps were conducted at 4°. The homogenates were then centrifuged at 23,000 *g* (max.) for 45 min and the supernatant solutions retained.

The original supernatant solution was subjected to $(NH_4)_2SO_4$ fractionation by the addition of 48 g of solid $(NH_4)_2SO_4$ per 100 ml supernatant solution with vigorous stirring. Mixing was continued for 2 hr following the last addition, and the resulting suspension centrifuged as described before. The $(NH_4)_2SO_4$-soluble supernate (700 ml) was dialyzed exhaustively against Tris buffer and concentrated to 23 ml by membrane ultrafiltration, using an Amicon Model 2000 Diafiltration unit equipped with a UM-10 Diaflow membrane (Amicon Corp., Lexington, Mass.).

Ten milliliters each of the original mucosal supernatant solution and concentrated $(NH_4)_2SO_4$-soluble supernate were applied separately to a 2.5 × 87-cm calibrated Sephadex G-100 column and eluted with Tris buffer mixture. Columns were run in the upward direction by gravity flow with a buffer flow rate of 0.5 ml/min. Three-milliliter (60 drop) fractions were collected. The column calibration procedure has been described previously (11). Fractions from gel filtration column chromatography of the $(NH_4)_2SO_4$-soluble supernate that contained calcium-binding activity and exhibited only several bands on analytical acrylamide gel electrophoresis were pooled and concentrated to 1.0 ml by membrane ultrafiltration and subjected to preparative gel electrophoresis. The procedures for analytical and preparative acrylamide disk gel electrophoresis have been described in detail previously (11).

Column fractions were assayed for total protein concentration by the procedure of Lowry *et al.* (12) and all values expressed on the basis of bovine serum albumin standard solutions.

Calcium-binding activity was assayed by

the Chelex-100 ion-exchange procedure of Briggs and Fleischman (13) as modified by Wasserman *et al.* (3).

Double immunodiffusion analyses were conducted in a humidified chamber at room temperature on micro Ouchterlony plates prepared with 1.5% agar (special Agar-Noble, Difco Laboratories, Detroit, Mich.) in barbital buffer (pH 8.6; ionic strength, 0.075). Preparation and demonstration of the specificity of the antiserum have been described previously (14). Twenty-microliter aliquots of both sample and specific antiserum were applied to the agar wells and diffusion allowed to continue for 24 hr.

Immunoelectrophoresis was performed by first subjecting samples to analytical discontinuous gel electrophoresis in 10% acrylamide. At the completion of electrophoresis, gels were slit longitudinally and one-half of each gel embedded in 1.5% agar on a microscope slide. A slot was then cut in the agar parallel to the gel and specific antiserum added. Diffusion was allowed to continue for 24 hr at room temperature in a humidified compartment.

The radial immunodiffusion procedure of Mancini *et al.* (15) as modified by López and Golder (16) was employed for quantitative immunoanalysis of calcium-binding protein in some instances. Specific antiserum was dissolved in 1.5% agar (in barbital buffer, pH 8.6) at a concentration of 4% and 0.5 ml of the mixture delivered to cylindrical wells (4-mm deep × 14-mm diameter) and allowed to harden. Three-millimeter holes were cut in the center of each well and a 20-μl sample placed therein. Diffusion was allowed to proceed for 24 hr, after which the diameter of the precipitin rings were measured. Concentration of calcium-binding protein was determined from a semi-log plot of standard protein solutions.

Amino acid analyses were conducted essentially according to the method of Moore and Stein (17) with the aid of a Beckman model 120 C amino acid analyzer. Approximately 0.5 mg of lyophilized calcium-binding protein was hydrolyzed in 6 *N* HCl, under vacuum, at 110° for 22 hr. HCl was removed by rotary evaporation at 40° and samples were brought to 1.1 ml with pH 2.2 sample-diluting buffer. One-half-milliliter aliquots were applied in duplicate to both the long and short columns for analysis.

Results and Discussion. The results of gel filtration column chromatography on the original mucosal supernatant and the $(NH_4)_2SO_4$-soluble supernatant solutions from hen uterus are shown in Fig. 1. Included for comparison is a chromatographic elution profile of the original supernatant solution from chick intestinal mucosa obtained under identical conditions. On the basis of previous column calibration runs, a V_e/V_0 ratio of 2.0 was obtained for each of the sample runs in Fig. 1, corresponding to a molecular weight of about 28,000. As indicated above, the calcium-binding profile for the original chick intestinal supernate and the concentrated uterine $(NH_4)_2SO_4$-soluble supernate were easily obtained by means of the Chelex-100 ion exchange procedure. However, the method lacked sufficient sensitivity when applied to the original uterine supernatant solution due to the low concentrations of protein therein, as verified by quantitative measurements of calcium-binding protein concentration by radial immunodiffusion. Therefore, the elution profile of original uterine supernate was determined by the use of the sensitive radial immunoassay procedure. With this procedure, it was clearly shown that the column fractions containing uterine calcium-binding protein eluted in essentially the same volume as the calcium-binding activity peak from chick intestinal supernate and the concentrated $(NH_4)_2SO_4$-soluble preparation from the hen uterus.

Bar and Hurwitz (10) have recently reported a slightly greater molecular size for the uterine protein as compared to the intestinal calcium-binding protein on the basis of SDS acrylamide gel electrophoresis. In the present study, estimation of molecular size by gel filtration column chromatography showed essentially no difference in molecular size for the two proteins. Both were estimated to be approximately 28,000 daltons, a value which has been confirmed previously for the chick intestinal calcium-binding protein by gel filtration (3, 18), SDS acrylamide gel electrophoresis (19), and amino acid compositional studies (18, 19). Furthermore, on SDS acrylamide gel elec-

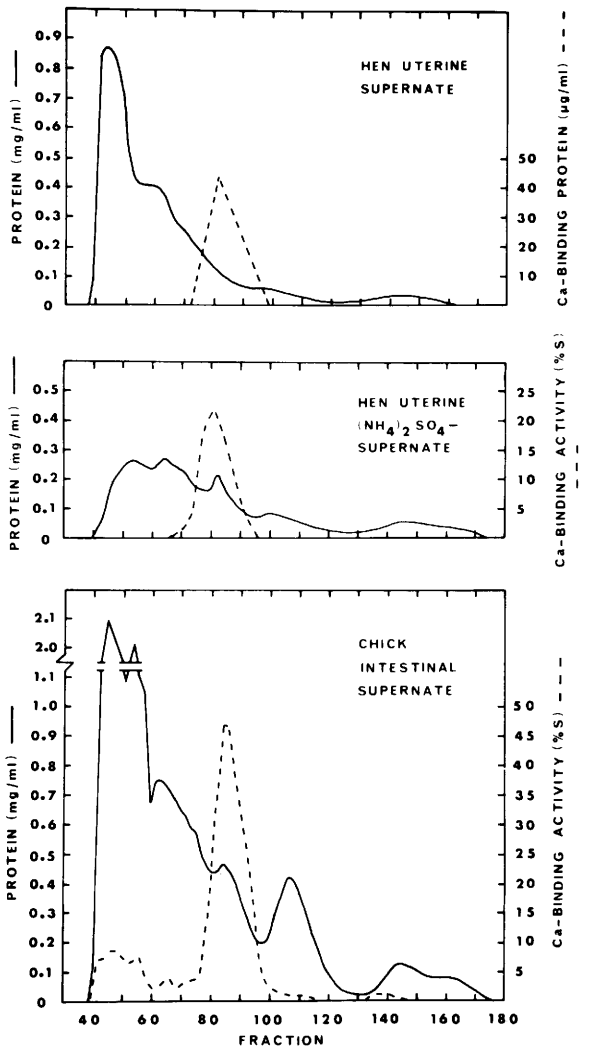

FIG. 1. Separation of original and $(NH_4)_2SO_4$ soluble proteins from hen uterus by gel filtration on Sephadex G-100. Included for comparison is a similar elution profile run on original supernatant solution from chick intestinal mucosa. (●——●), Total protein; (O- - -O), Ca-binding activity.

trophoresis, the hen uterine and chick intestinal calcium-binding protein migrated coincidentally on a single gel, again suggesting identity.

Double immunodiffusion analyses of purified hen uterine calcium-binding protein and intestinal calcium-binding protein are shown in Fig. 2. Immunological identity of the two proteins is verified by total coalescence of the precipitin lines resulting from each. From the immunoelectrophoresis studies (Fig. 3), the location of the precipitin arc in the embedded gels, and the protein bands in the stained gels showed that

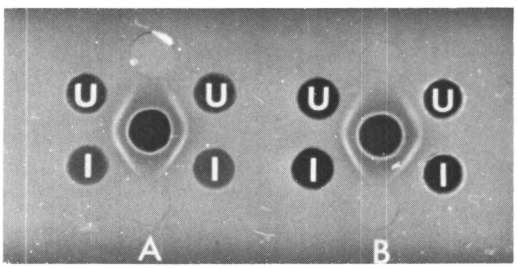

FIG. 2. Double immunodiffusion analysis performed on the original supernatant solutions (A) and purified calcium-binding proteins (B) from hen uterus (U) and intestine (I). Diffusion against antiserum specific for chick intestinal calcium-binding protein (center wells).

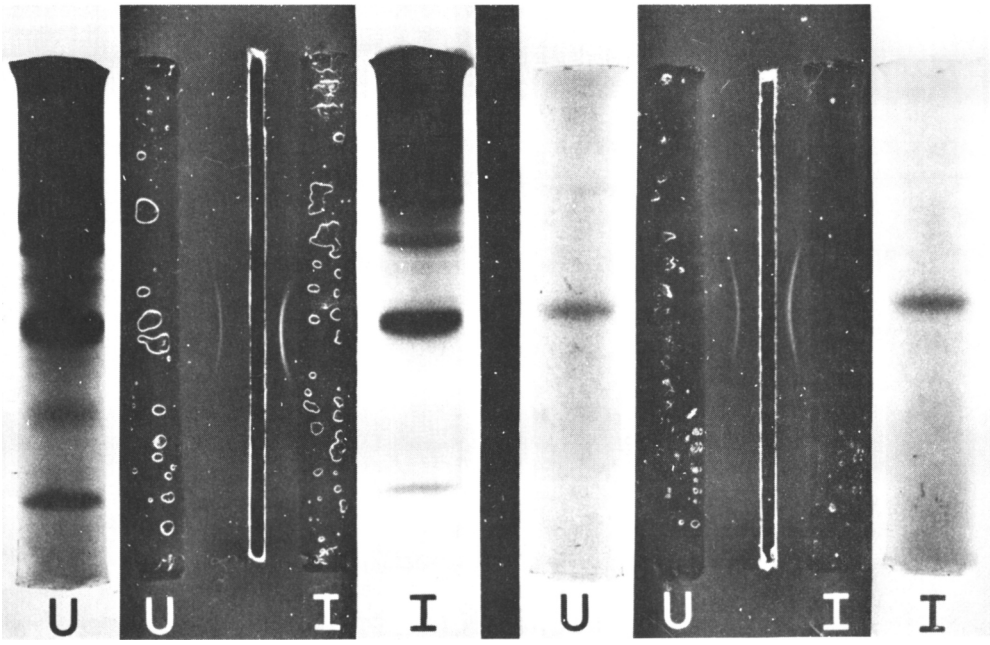

FIG. 3. Immunoelectrophoresis performed on supernatant solutions (left) and purified calcium-binding proteins (right) from hen uterus (U) and intestine (I). Amounts applied to stained gels were, as follows: uterine supernate, 200 μl; intestinal supernate, 20 μl; purified uterine calcium-binding protein, 20 μg; purified intestinal calcium-binding protein, 20 μg. Amounts applied to gels embedded for immunoelectrophoresis were: uterine supernatant, 50 μl; intestinal supernatant, 20 μl; purified uterine calcium-binding protein, 10μg; purified intestinal calcium-binding protein, 10 μg. Electrophoresis was toward the anode (bottom).

the chick intestinal and hen uterine calcium-binding proteins were electrophoretically identical.

The results of amino acid analyses performed on 22-hr acid hydrolysates of purified chick intestinal and hen uterine calcium-binding proteins are shown in Table I. Essentially no significant differences in composition exist between calcium-binding proteins isolated from these sources.

The evidence presented in this study suggests that calcium-binding proteins isolated from laying hen uterus and chick intestinal mucosa are identical with respect to electrophoretic mobility, immunological reactivity, molecular size, and amino acid composition.

Summary. Calcium-binding proteins from the chick intestine and uterus of the laying hen (*Gallus domesticus*) were isolated and purified by identical methods. Proteins from each source were shown to exhibit identical electrophoretic mobility and were immunologically identical. Estimation of molecular size by gel filtration indicated a value of approximately 28,000 daltons for both the chick intestinal and hen uterine calcium-binding proteins. The amino acid compositions of chick intestinal and hen uterine cal-

TABLE I. AMINO ACID COMPOSITION OF CHICK INTESTINAL AND HEN UTERINE CALCIUM-BINDING PROTEINS.

	Residues/molecule	
	Chick intestine	Hen uterus
Lysine	23.3	23.3
Histidine	4.3	4.3
Arginine	5.8	5.6
Aspartic acid	29.5	28.7
Threonine	8.9	8.7
Serine	9.2	9.2
Glutamic acid	34.6	35.7
Proline	2.8	3.0
Glycine	13.3	13.1
Alanine	16.6	16.6
½-Cystine	ND*	ND*
Valine	5.0	5.0
Methionine	6.9	6.1
Isoleucine	10.6	10.8
Leucine	27.6	27.2
Tyrosine	6.5	6.2
Phenylalanine	13.4	13.3

* ND is not determined.

cium-binding proteins were essentially identical.

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