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Effect of Calcium on the Monomer-Dimer Association of Calcium-Binding Protein in the Serum of Laying Hens (40687)

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The laying hen provides a good model for studying calcium in a biological system because of the marked changes in calcium metabolism associated with reproduction (1). A 2-kg hen, containing 25 mg of calcium in a total of 100 ml of plasma (2), can deposit a shell containing 1.5–2.0 g of calcium in approximately 15 hr (1). Laying hens have long been known to have a higher level of blood calcium than males or nonlayers (3). Virtually all of this increase is due to an increase in bound, nondiffusible calcium (4). The nature of the molecules responsible for the binding has been open to question. Evidence for and against colloidal calcium (5, 6), albumin (7, 8), phosvitin (9–12), and high molecular weight phosphoproteins (13), has been presented. The molecule appears to bind a large amount of calcium but with an average dissociation constant of 10^{-1} to 10^{-3} (14, 15). The increased calcium present in the serum of estrogenized roosters, was found to be associated with two proteins, X_1 (a phosphoprotein) and X_2 (a lipoprotein) which could be separated by ultracentrifugation (15). A fast-moving electrophoretic component was found by gel filtration to have a molecular weight of 183,000 (28). The electrophoretic mobility of this component decreased as the calcium ionconcentration increased (29). This was attributed to a decrease in net charge on the molecule. The molecule appeared to bind a large amount of calcium. Kuan and Clegg (30) found 110 calcium-binding sites with an association constant of 1.66×10^6. Deeley *et al.* (16) found a single phosphoprotein, vitellogenin, in the serum of estrogenized roosters. The native molecule, of 480,000 M_r, was a dimer composed of two 240,000 M_r subunits as determined by gel electrophoresis. Vitellogenin is apparently broken down by proteolysis in the yolk into phosvitin and α- and β-lipovitellin. Bergink and Wallace (17) have also identified a 200–250,000 M_r phosphoprotein in estrogen-treated roosters. It seems possible that the calcium-binding proteins X_1 and X_2 observed by Urist (15) were the dimer and monomer forms of vitellogenin, and that the electrophoretically separated component I (29, 30) was the monomer form of vitellogenin.

In the related *Xenopus* system (18) estrogenized males contain a similar plasma phosphoprotein vitellogenin of about 400,000 M_r, which is also a dimer of approximately 200,000 M_r subunits.

The present investigation (19) was undertaken to determine the relation between monomer–dimer association and calcium binding of the calcium-binding protein in the blood of hens and estrogenized roosters.

Materials and methods. Animals and blood collection. Regularly laying, 1- to 2-year-old, single-comb, white Leghorn hens, weighing about 2 kg were used. They were fed a standard laying ration and water *ad libitum* until the night before blood collection when food was removed to keep blood lipid content low. Roosters were injected intramuscularly with 20 mg/kg body wt of estradiol valerate (E. R. Squibb & Sons, Inc.).

For blood collection, the birds were anesthetized by intramuscular injection of chloral hydrate (1.4 ml of a 35% solution). Blood was withdrawn from the carotid artery. Plasma was separated from the blood cells by centrifugation at 2000 rpm in a refrigerated Sorvall RC-2 centrifuge for 30 min.

Lipid flotation and preparation of Fraction 4. Lipid flotation was performed on plasma using a modification of the method of DelGatto *et al.* (20). Plasma (40 ml) was added slowly with gentle mixing to 80 ml of an ice-cold solution containing 3.94 *M* NaBr, 0.045 *M* NaCl, and 3.2 m*M* $CaCl_2$. The solution was then centrifuged in a Spinco Type

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50 rotor at 43,500 rpm at 18° for 24 hr. The contents of the tube were divided into four equal fractions. The contents of the bottom quarter of each tube were dialyzed against the standard buffer (0.10 *M* NaCl, 0.01 *M* Tris hydroxymethylaminomethane, 0.01 *M* $CaCl_2$, and 0.02% NaN_3, adjusted to pH 7.5 with HCl) and labeled as Fraction 4.

In a preliminary experiment, Fraction 4 was shown to contain approximately 80% of all calcium-binding activity, as determined by equilibrium dialysis. Therefore, all further work was performed on this fraction.

Gel chromatography. Fraction 4 was purified on Sephadex G-200 (Pharmacia) and Sepharose gels 6B and 2B (Pharmacia) using 2.5 × 30 and 2.5 × 80 cm chromatographic columns (Glenco). Elution was by upward flow at rates not exceeding 0.5 ml/min. Ultraviolet absorption of the eluate was monitored at 254 nm with an Isco monitor and the absorption of the fractions after elution measured at 280 and 260 nm with a Beckman DU-2 spectrophotometer.

Protein concentrations were determined by the method of Lowry *et al.* (21) as modified by Oyama and Eagle (22). Carbohydrate was detected by the orcinol–sulfuric acid method of Francois *et al.* (23).

Equilibrium dialysis. Protein in standard buffer was placed on one side of the membrane of an equilibrium dialysis cell (Chemical Rubber Co.). Buffer plus $^{45}CaCl_2$ (New England Nuclear, carrier free) was placed on the other side. The solutions were maintained at 4° on a rotary shaker. The 25-μl samples were removed periodically (in triplicate) and counted on a Nuclear Chicago Model 1105, spectro-shield, low-background, gas-flow, planchet counter.

Analytical ultracentrifugation. Analytical ultracentrifugation was performed on the Beckman Model E analytical ultracentrifuge with schlieren and ultraviolet optics. Solution densities were determined by pycnometry. Viscosities were determined in Cannon viscometers. Boundary runs were performed in 12-mm Kel-F centerpieces at 44,770 rpm (24). Meniscus depletion runs were performed in double-sector cells (24) at 9945 rpm for 24 hr after an initial overspeed using schlieren optics.

Sucrose density gradient centrifugation. Linear 5–20% sucrose gradients containing 0.01 *M* Tris buffer were prepared at 4° and centrifuged for 27.5 hr in an SW 25 rotor in a Beckman L2-65B centrifuge at 23,000 rpm.

Results. Analytical sedimentation and chromatography of Fraction 4. Analytical ultracentrifugation of Fraction 4 using schlieren optics showed three components with sedimentation coefficients of 3.4–4.4, 5.9–8.0, and 11.6–15.6 S. Representative uncorrected sedimentation coefficients are given in Table I. Density and viscosity correction factors were less than 1%.

Sephadex G-200 chromatography of Fraction 4 gave two UV-absorbing peaks (Fig. 1), the first peak eluting in the void volume. Rechromatography of the first-peak material

TABLE I. REPRESENTATIVE PLASMA AND RED BLOOD CELL (RBC) VOLUMES, AND SEDIMENTATION COEFFICIENTS OF FRACTION 4 ENTITIES

Bird	Plasma volume (ml)	RBC volume (ml)	Plasma: RBC ratio	Sedimentation coefficients[a] (Svedberg units, S)		
3	40	ND[b]	—	4.0	7.0	14.3
4	45	25	1.8	4.4	8.0	13.5
6	26	ND	—	3.4	6.0	11.8
9[c]	90	80	3.0	3.5	7.9	11.6
16	52	ND	ND	4.0	7.1	15.6

[a] Uncorrected.
[b] Not determined.
[c] This was a long-term estrogenized bird.

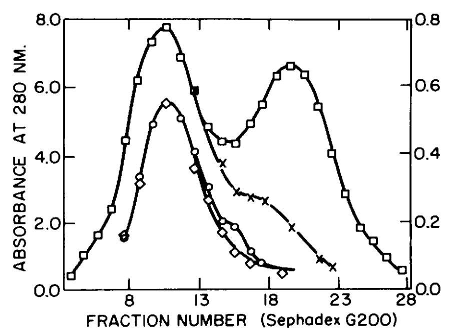

FIG. 1. Sephadex G-200 elutions of Fraction 4 (bird 9). UV absorption and carbohydrate profiles. The material from tubes 8–13 was combined, concentrated, and reeluted on Sephadex G-200. Carbohydrate (540 nm) was detected by the orcinol–sulfuric acid test. (□) Original Sephadex G-200 elution at 280 nm; (○) Sephadex G-200 reelution of material from tubes 8–13 at 280 nm; (×) original Sephadex G-200 elution at 540 nm; (◇) Sephadex G-200 elution of material from tubes 8–13 at 540 nm.

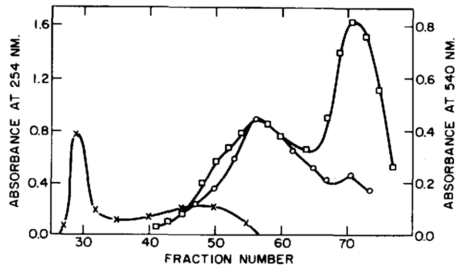

FIG. 2. Sepharose 6B elution of Fraction 4 (bird 16). UV and carbohydrate profiles. The sharp dextran 2000 peak at tube 29 denotes the void volume. Carbohydrate (540 nm) was determined by the orcinol-sulfuric acid test. $S_{20,w}$ values from representative fractions: Tube 50: 7.8, 16.2 S; tube 56; 7.9, 16.4 S; tube 61: 7.8, 16.3 S; tube 70: 3.7 S. (×) Dextran 2000 at 254 nm; (□) absorbance at 254 nm; (○) absorbance at 540 nm.

successfully removed trailing second-peak material (Fig. 1). The elution profiles on Sephadex G-200 were similar for Fraction 4 from plasma or serum, normal laying or estrogenized birds. However, long-term estrogenized birds had a larger proportion of the first-peak material. A bimodal elution profile also resulted when Fraction 4 was chromatographed on Sepharose 6B (Fig. 2). The dextran 2000 elution pattern indicates that all Fraction 4 components eluted well after the void volume. When material from the first peak eluted on Sephadex G-200 (Fig. 1, tubes 8–13) was chromatographed on Sepharose 2B only one absorption peak was detected (data not shown).

When the peak materials from these columns were tested by analytical sedimentation, the first peak on both columns (Fig. 1, Fraction 11, and Fig. 2, Fraction 56) contained the two fast-sedimenting components (8 and 16 S); the second-peak material (Fig. 1, Fraction 20, and Fig. 2, Fraction 70) contained the slow-sedimenting component (4 S).

The carbohydrate content of Fraction 4 material eluted on Sephadex G-200 and Sepharose 6B is shown in Figs. 1–2. In each case the first peak eluted, which contains the calcium-binding material of Fraction 4, contained about 5% carbohydrate.

Localization of calcium-binding activity. Eighty percent of the soluble calcium-binding activity in serum was found in the bottom quarter (Fraction 4) of the tube following flotation. Calcium binding was further localized by adding ^{45}Ca to Fraction 4 prior to sucrose density centrifugation. The UV absorption and ^{45}Ca profiles obtained following sucrose density gradient centrifugation of Fraction 4 indicated a peak of ^{45}Ca associated with the fast-sedimenting proteins (Fig. 3). The contents of tubes 9–11 (Fig. 3) were incubated with additional ^{45}Ca and eluted on Sephadex G-200; the protein eluted in the void volume and had ^{45}Ca associated with it. Materials from tubes 22–24 (Fig. 3), similarly treated, eluted later on Sephadex G-200 with no associated ^{45}Ca. Equilibrium dialysis confirmed that the fast-sedimenting, G-200 void volume material contained the calcium binder: This material bound 23% of the total calcium when the free calcium concentration was 1 m*M* and the protein concentration was 1.5 mg/ml.

Molecular weight determination. Resolution of the two fast-sedimenting entities by gel chromatography or sucrose gradient sedimentation was unsuccessful. The relative amounts of these two fast-sedimenting entities (8 and 16 S) were variable, and on several occasions only the fast-sedimenting entity (16

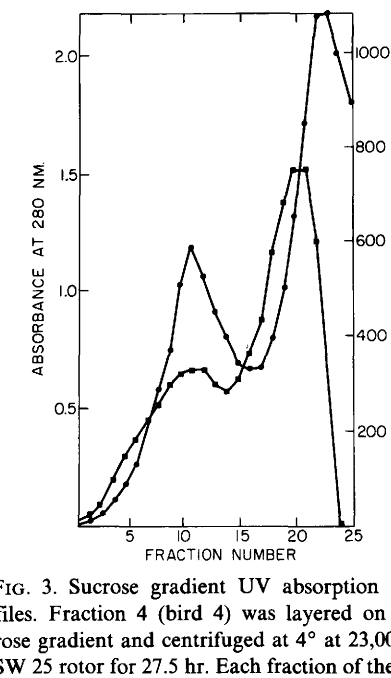

FIG. 3. Sucrose gradient UV absorption and ^{45}Ca profiles. Fraction 4 (bird 4) was layered on a 5–20% sucrose gradient and centrifuged at 4° at 23,000 rpm in an SW 25 rotor for 27.5 hr. Each fraction of the gradient was monitored for absorption at 280 nm and for ^{45}Ca. (In the absence of protein, ^{45}Ca decreased exponentially from the top of the tube). (■) Absorbance at 280 nm; (●) ^{45}Ca cpm.

S) was observed. When equilbrium sedimentation (meniscus depletion) was performed on material eluted first from a Sepharose 2B column a $5.4 \pm 0.1 \times 10^5$ M_r was obtained (assuming $\bar{v} = 0.72$) with no evidence of heterogeneity. After dialysis against 0.10 *M* citrate, 0.01 *M* Tris (pH 7.5), a 2.7×10^5 M_r was calculated. When material containing both peaks (16, 8 S) was subjected to equilibrium sedimentation, heterogeneity in molecular weight was apparent. However, when such fractions were first dialyzed against citrate, and then subjected to equilibrium sedimentation, molecular weights were similar to those obtained when the 16 S entity had been citrate treated. Analytical sedimentation confirmed that dialysis against citrate or EDTA converted a mixture of 8 and 16 S components to 8 S, and, furthermore, showed that subsequent dialysis of the 8 S component against calcium ions again produced a mixture of 8 and 16 S (Fig. 4).

Determination of frictional factor and axial ratio. The molecular weight and $S_{20,w}$ values obtained for the calcium-binding material were used to determine the molecular frictional coefficients and axial ratios, assuming a hydration of 0.2 g H_2O/g protein for the calcium-binding material (25). For the 5.4×10^5 M_r material a value of f/f_{min} of 1.6 and an axial ratio of 8.7 were determined. For the 2.7×10^5 M_r material, f/f_{min} was 2.0 and the axial ratio 16.0, assuming a prolate ellipsoid model.

Discussion. We have identified the major calcium-binding protein in chicken serum as an asymmetric ($f/f_m = 1.6$) 5.4×10^5 M_r dimer which dissociates in the absence of calcium into 2.7×10^5 M_r monomers which are also highly asymmetric ($f/f_m = 2.0$). The

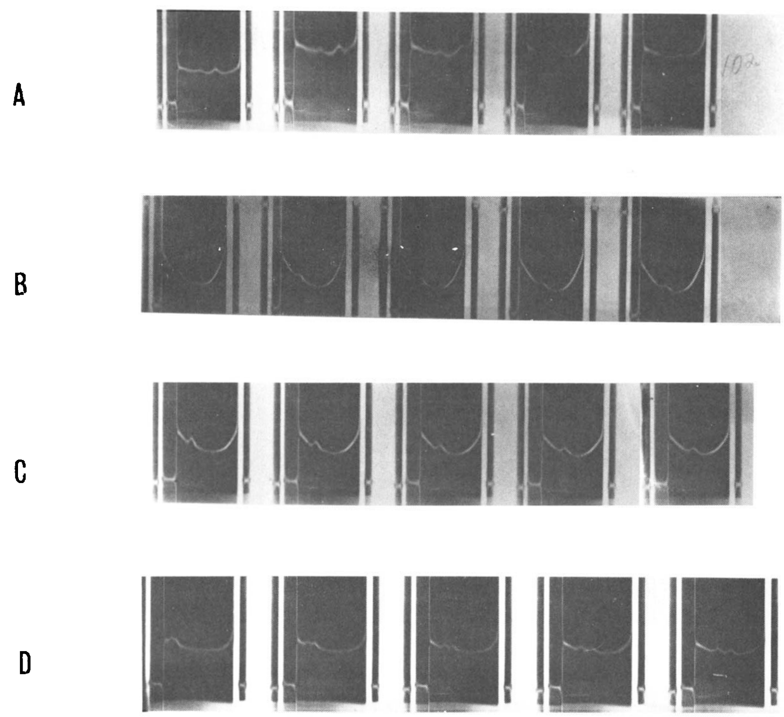

FIG. 4. Analytical boundary sedimentation of "fast" calcium-binding material. The fast calcium-binding component isolated from a sucrose gradient (Fig. 3, Fractions 8–12) was treated as follows below and then spun at 44,770 rpm in an analytical centrifuge using schlieren optics. Frames are shown at 8-min intervals. (A) Sucrose gradient fractions 8–12 were pooled and dialyzed against Sephadex buffer containing 1 m*M* $CaCl_2$. The two sedimenting peaks have values of 17.4 and 10.4 S. (B) The pooled fractions (A) were dialyzed against 0.1 *M* EDTA, pH 7.5. This gave a value of 7.0 S. (C) The pooled fractions were dialyzed against 0.1 *M* sodium citrate, 0.01*M* Tris, pH. 7.5. This gave a value of 7.3 S. (D) The citrate was dialyzed out against sephadex buffer. Peaks of 16.6 and 9.6 S resulted.

monomers partially reassociate to the dimer in the presence of calcium ions.

The phosphoprotein complex vitellogenin characterized by Deeley (16) in etrogenized roosters is also a dimer of approximate 480,000 M_r consisting of lipovitellin and phosvitin. The 450,000 M_r vitellogenin in the plasma of estrogenized male *Xenopus* (18) is also an asymmetric (f/f_m = 1.5; calculated from the data of Wallace (26)) dimer. The simplest conclusion from these comparisons is that the 5.4×10^5 M_r calcium-binding protein in chicken serum is vitellogenin.

The three sedimenting entities observed in analytical centrifugation of the high-density plasma fraction may be identified as the dimer and monomer vitellogenin and chicken serum albumin. The two peaks eluting from gel columns are vitellogenin and serum albumin. The lack of resolution of the monomer and dimer forms by gel chromatography can be explained by the extreme asymmetry of these molecules. Recently Guyer *et al.*[2] have shown by gel chromatography the presence in hen serum of two calcium-binding proteins which they have identified immunologically as vitellogenin and chicken serum albumin. They find an estimated molecular weight for the calcium-binding protein related to vitellogenin of 6×10^5 M_r.

The calcium-binding property of the vitellogenin can be attributed to both the phosvitin components which contain a high proportion of phosphoserine and the phosphate groups present in the lipovitellin components (27). We find by equilibrium dialysis that at a concentration of 1.5 mg/ml protein and 1.3 mM calcium, 23% of the calcium is bound, indicating a minimum of 100 calcium-binding sites on the 5.4×10^5 M_r complex. Kuan and Clegg (30) found 110 calcium-binding sites per molecule for their component I.

The reversible association–dissociation of the vitellogenin dimer–monomer system depends on the presence of calcium, and the simplest explanation is that calcium bridges form between two monomers. Dimerization lowers the asymmetry slightly, suggesting side-to-side association of rod-shaped molecules. The arrangement of phosvitin and lipovitellin in the dimer must be such that the phosphate groups are not available for further intermolecular association since larger complexes are not found. Attempts to observe the molecules directly by electron microscopy are in progress.

[2] We are grateful to Guyer *et al.* for a preprint of their manuscript submitted to Proc. Soc. Exp. Biol. Med.

Summary. A major calcium-binding protein of laying hen plasma was fractionated and partially characterized. After lipid flotation, approximately 80% of the total binding activity was localized in a high-density plasma fraction. Chromatography on Sephadex G-200 or Sepharose 6B gave a bimodal elution pattern. Sucrose gradient centrifugation yielded a similar bimodal pattern; the fast- and slow-sedimenting ultraviolet (UV) absorption peaks appeared identical to the first and second chromatographic elution peaks, respectively. Equilibrium dialysis localized the calcium-binding activity to the faster-sedimenting material. An orcinol–sulfuric acid test indicated a total carbohydrate content of 5% for the fast peak.

Analytical velocity ultracentrifugation measurements of this calcium-binding component yielded varying amounts of two entities with sedimentation coefficients ($S_{20,w}$) of approximately 16 and 8 S. The 16 S material was shown by equilbrium centrifugation (meniscus depletion) to have a 5.4×10^5 M_r. Dialysis of the 16 S moiety against citrate or ethylene diamine tetraacetic acid (EDTA) buffer produced 8S material having a 2.7×10^5 M_r and this monomer–dimer association was partially reversible by subsequent dialysis against calcium ions. The calcium-binding molecule appears to be vitellogenin. Calcium-binding protein was found at elevated levels in plasma from long-term estrogenized birds but not from nonlaying birds.

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