Isolation and Properties of a Calcium-Binding Protein from Porcine Parathyroid Glands†

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ABSTRACT: A calcium-binding protein (CaBP) has been isolated from normal porcine parathyroid glands. The CaBP was purified by means of gel filtration on Sephadex G-100 and G-50 followed by ion-exchange chromatography on DEAE-cellulose. The molecular weight of the CaBP was approximately 15,000 by calibrated gel filtration and from its amino acid composition. A molecular weight estimate of 11,700 was obtained from sucrose density centrifugation. Preliminary amino acid analysis of the CaBP showed lysine and aspartic acid to be the predominant amino acids. The calcium binding constant was found to be 9.3×10^5 M^{-1} by equilibrium dialysis, and there appear to be two high-affinity binding sites for calcium per molecule of protein. Competitive binding studies showed the affinity of the protein for calcium to be two to four orders of magnitude higher than its affinity for strontium, barium, or magnesium.

A vitamin D dependent calcium-binding protein (CaBP)[1] was first reported to be present in the duodenal mucosa of chicks by Wasserman and Taylor (1966). Similar CaBP have been found in the small intestine of a number of mammals including rat, dog, pig, cow, monkey, and human (Kallfelz *et al.*, 1967; Taylor *et al.*, 1968; Schachter, 1969; Drescher and DeLuca, 1971; Menczel *et al.*, 1971; Wasserman and Taylor, 1971; Hitchman and Harrison, 1972; Fullmer and Wasserman, 1973). Vitamin D dependent CaBP have also been identified in two other tissues across which there is a large flux of calcium, the kidney (Taylor and Wasserman, 1967) and the egg shell gland of laying hens (Corradino *et al.*, 1968). Substantial evidence has been obtained to suggest an intimate involvement of these CaBP in the calcium transport systems of their respective tissues (Wasserman and Taylor, 1968; MacGregor *et al.*, 1970; Sands and Kessler, 1971).

The concentration of calcium in the extracellular fluid is considered to be the principal physiologic factor regulating the rates of biosynthesis and secretion of parathyroid hormone (Raisz, 1963; Sherwood *et al.*, 1966; Hamilton and Cohn, 1969). The possibility that a CaBP may be involved in the transport of calcium in the parathyroid led to the present investigation. We observed that extracts of parathyroid glands from normal pigs exhibited calcium-binding activity. On the basis of soluble protein, the specific calcium-binding activity in the parathyroid extracts was higher than that in extracts of a number of other porcine tissues examined, including muscle, duodenal mucosa, kidney, and brain (Oldham *et al.*, 1971). Further studies showed that this activity was associated with a low molecular weight protein fraction. This paper describes the purification of a CaBP from porcine parathyroid glands and some of its properties, including molecular weight, amino acid composition, and calcium affinity.

Methods

Extraction of Parathyroid Glands. Porcine parathyroid glands were identified and collected as described by Littledike (1967). They were trimmed free of surrounding thymic tissue and were stored frozen at −20°. For each extraction, 10–20 g of parathyroid tissue was thawed, minced to a thick paste with scissors, and homogenized by hand in ground-glass Duall homogenizers in 5 volumes of Tris-saline buffer (0.12 M NaCl–4.74 mM KCl–1 mM β-mercaptoethanol–0.0137 M Tris-HCl (pH 7.4)) (Wasserman *et al.*, 1968). The homogenate was centrifuged for 30 min at 37,000*g* in a Sorvall RC2B refrigerated centrifuge. The resulting supernatant fraction contained essentially all the calcium-binding activity measured in the homogenate. All extraction and subsequent isolation procedures, including assays for calcium-binding activity, were carried out at 4°.

Gel Filtration. The supernatant fraction of the saline homogenate was concentrated to less than 20 ml in 56-mm-diameter dialysis bags placed in dry Sephadex G-100 or G-200. The concentrated material was clarified by centrifugation and was then chromatographed on Sephadex G-100 (Figure 1). Fractions were assayed for calcium-binding activity (see below) and for protein by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. Fractions with a specific calcium-binding activity greater than 50% ^{45}Ca bound/mg of protein were pooled, concentrated as described above, and then chromatographed on Sephadex G-50 (fine grade) (Figure 2).

Ion-Exchange Chromatography. Fractions having specific calcium-binding activity greater than 100% ^{45}Ca bound/mg of protein after gel filtration on Sephadex G-50 were pooled, concentrated by flash evaporation, and desalted by passage through a 2.5 × 80 cm column of Sephadex G-25 equilibrated with 0.01 M Tris-HCl (pH 7.3). The desalted protein fraction was then applied to a column of

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[1] Abbreviations used are: CaBP, calcium-binding protein; [^{131}I]CaBP, ^{131}I-labeled calcium-binding protein.

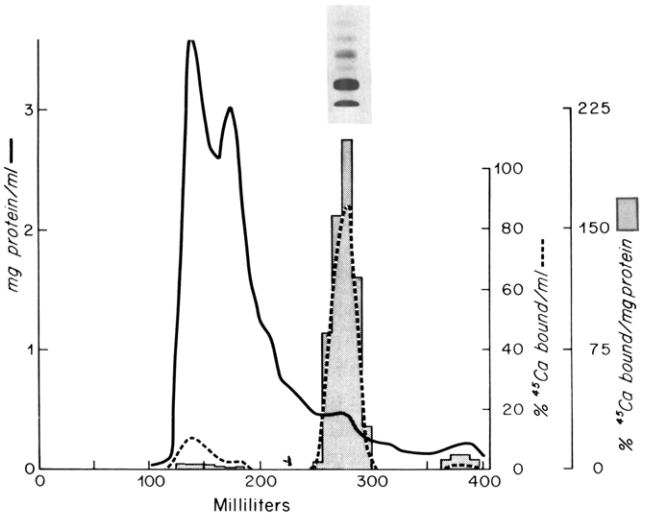

FIGURE 1: Sephadex G-100 chromatogram of concentrated saline-soluble fraction of porcine parathyroid homogenate. Column size, 2.5 × 90 cm; buffer eluate, Tris-saline; flow rate, 0.44 ml/min; fraction size, 4.4 ml. Protein concentration (—), % ^{45}Ca bound/ml as measured in the Chelex-100 assay (- - -) (see Methods), and specific calcium-binding activity, % ^{45}Ca bound/mg of protein (stippled), are plotted against effluent volume. Inset: Photograph of polyacrylamide disc gel on which fraction at 280 ml was electrophoresed (anode is at top).

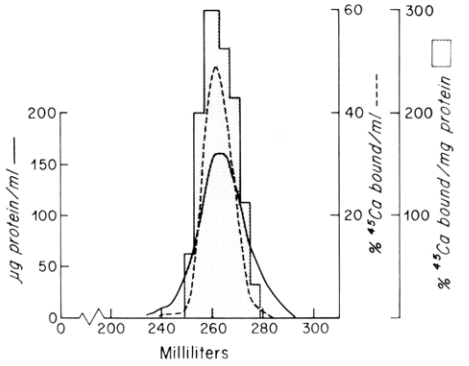

FIGURE 2: Sephadex G-50 fine chromatogram of pooled fractions from Sephadex G-100 chromatography (Figure 1). Column dimensions, buffer eluate, and flow rate as described in Figure 1; ordinate and abscissa are marked as in Figure 1.

DEAE-cellulose and was eluted by stepwise increases in the molarity of Tris buffer (pH 7.3).

Polyacrylamide Disc Gel Electrophoresis. Disc gel electrophoresis was performed according to Davis (1964). The electrophoretic system was as follows: 2.5% acrylamide stacking gels (pH 6.7) (Tris-HCl); 7.5% acrylamide separating gels (pH 7.9) (Tris-HCl); and a Tris-glycine electrode buffer (pH 8.3). Samples were layered on top of the stacking gels in volumes of 20–200 μl as 20% sucrose solutions. Bromophenol Blue was used as a tracking dye. Electrophoresis was carried out at 2 mA/tube for 1 hr and then at 4 mA/tube. Upon completion of the run, the gels were stained in 1% Amido Schwartz in 7% acetic acid and destained in 7% acetic acid or in 0.05% Coomassie Blue (Chrambach *et al.*, 1967). Gels were photographed in 16 × 150 m Pyrex culture tubes.

When gels were to be extracted and assayed for calcium-binding activity, the gels were subjected to preelectrophoresis for 1 hr (2 mA/tube) before application of the samples in order to remove unreacted persulfate and other impurities that could inactivate the CaBP; 12 samples of CaBP fraction were coelectrophoresed as described above. After elecrophoresis, one gel was stained for protein. From this protein pattern, positions for slicing the unstained gels were identified by R_F values such that at least one protein band would be included in each segment. The gel segments ranged from 0.45 to 0.85 cm in length, most being approximately 0.5 cm. The corresponding segments from each gel were pooled and homogenized in 0.5 ml of Tris-saline buffer. The gel particles were removed by centrifugation and extracted a second time as above. Calcium-binding activity was assayed in the combined supernatant fractions.

Calcium-Binding Assay. Assay for calcium-binding activity was based on the competitive-binding Chelex-100 method of Briggs and Fleishman (1965) as modified by Wasserman and Taylor (1966). Samples were added to 13 × 75 mm flint glass test tubes in volumes of 0.5 ml, followed by 0.2 ml of Chelex in suspension (equivalent to 0.04 ml of packed resin). $^{45}CaCl_2$ (0.5 μCi) was added, the contents of the tubes were vigorously mixed for 20 sec, and the tubes were then centrifuged at 600*g* for 10 min in a refrigerated centrifuge. The ^{45}Ca radioactivity in 0.1-ml aliquots of the supernatant was determined by liquid scintillation spectrophotometry (Packard TriCarb Model 3003). The counting solution consisted of Packard Insta-Gel made 0.5% in acetic acid to decrease loss of counting efficiency due to calcium adsorption to the glass vials. Channel ratio values indicated that quench corrections were not required. Control tubes containing 0.5 ml of buffer but no protein were prepared to determine the percentage of ^{45}Ca not bound by the resin in the absence of binding protein and a tube in which the resin was replaced by resin buffer was prepared to determine the total radioactivity added to the assay.

The percentage ^{45}Ca bound (% ^{45}Ca in sample supernatant − % ^{45}Ca in control supernatant) was linearly proportional to the concentration of added CaBP up to approximately 20% binding. In all assays of calcium-binding activity, the amount of protein added was adjusted so that less than 20% of the ^{45}Ca was bound.

Amino Acid Analysis. CaBP purified by DEAE-cellulose chromatography was hydrolyzed with 6 N HCl *in vacuo* for 24 hr at 100° and analyzed with a Beckman 120C amino acid analyzer. Duplicate samples were oxidized with performic acid for 4 hr prior to hydrolysis for determination of cysteine and methionine as cysteic acid and methionine sulfone, respectively. Corrections were made for recovery of norleucine.

Radioiodination. CaBP (1–3 μg) having a specific calcium-binding activity greater than 400% ^{45}Ca bound/mg of protein was labeled with ^{131}I by the method of Hunter and Greenwood (1962). Specific activities of approximately 50 Ci/g were obtained. ^{131}I-labeled protein was separated from remaining free ^{131}I on Sephadex G-25. The iodinated protein was then further purified on a 1.0 × 50-cm column of Sephadex G-50. A single sharp peak of radioactivity eluted at the same V_e/V_o as unlabeled CaBP on the same column. The fraction having maximal radioactivity was used in the molecular weight determinations described below.

Calibrated Gel Filtration. The Stokes radius and the molecular weight of the CaBP were estimated by gel filtration according to the method of Laurent and Killander (1964). The K_{av} of the CaBP and of the standard proteins, ovalbumin, chymotrypsin, and ribonuclease, were determined on a 0.635 by 156.5-cm column of Bio-Rad P-60 in 0.2 M pyridine acetate buffer (pH 4.65).

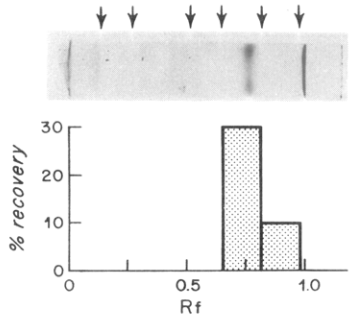

FIGURE 3: Localization of calcium-binding activity on polyacrylamide electrophoretic disc gel. Upper: staining pattern of 50 μg of protein from calcium-binding fraction of Sephadex G-100 chromatography. Equal amounts of same protein fraction were run on 11 additional gels. Unstained gels were cut at positions indicated by arrows, corresponding to R_F values 0.14, 0.27, 0.52, 0.65, 0.82, and 0.98. Lower: recovery of applied calcium-binding activity in extracts of gel segments.

Sucrose Density Centrifugation. The sedimentation coefficient of the CaBP was estimated by centrifugation in a sucrose density gradient according to the method of Martin and Ames (1961) as modified by Ryan *et al.* (1971). A 0.1-ml volume of sample containing 0.3 μCi of ^{131}I-labeled CaBP and 1 mg of standard protein (bovine serum albumin, chymotrypsinogen, or ribonuclease) was overlayed on a 5–27% linear concentration gradient of sucrose in 0.05 M phosphate buffer (pH 7.4). Runs were conducted at 60,000 rpm for 12 hr at 20° in a Spinco Model L2-65 centrifuge with a SW 65 rotor. Upon completion of the run, successive 0.1-ml fractions were collected from the top of the gradient by using an Isco density gradient fractionator and collector. The radioactivity and optical absorbance (225 nm) of each fraction were measured and plotted. The fraction at which a maximum of radioactivity or optical absorbancy occurred was determined by triangulation.

Equilibrium Dialysis. Cation-binding studies were carried out in bags of EDTA-washed 55-mm dialysis tubing, using essentially the procedure described by Ingersoll and Wasserman (1971). For experiments in which 0.5 ml of protein solution was used, the thin-film method of Craig (1967) was used. Samples of the inner and outer solutions were obtained at hourly intervals to determine when equilibrium had been reached. The protein concentration inside the bags at the end of the experiment was determined by the method of Lowry *et al.* (1951). The moles of calcium bound to the protein was calculated from the total radioactivity bound and the specific activity of the ^{45}Ca in the dialysate.

Chemicals. $^{45}CaCl_2$, specific activity 5–25 Ci/g, was obtained from International Chemical and Nuclear Corporation (Irvine, Calif.). Na^{131}I was obtained from Union Carbide. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were obtained from Eastman Chemicals (Rochester, N. Y.). Polyacrylamide P-60 and Chelex-100 (200–400 mesh) were obtained from Bio-Rad Laboratories (Richmond, Calif.). Sephadex G-100, G-50 fine, and G-25 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE-cellulose (Selectacel) was obtained from Brown Paper Company (Vermont). Standard proteins used for molecular weight estimations were obtained from Schwarz/Mann (Orangeburg, N. Y.). All other chemicals were of analytical reagent grade.

Results

Identification and Isolation of Calcium-Binding Protein. The saline-soluble extract of normal porcine parathyroid glands bound ^{45}Ca in the Chelex-100 assay with an average specific binding activity of 5% ^{45}Ca bound/mg of protein. On gel filtration of the extract on Sephadex G-100, the calcium-binding activity eluted at 2.2 void volumes, after the bulk of the total soluble proteins (Figure 1). The specific calcium-binding activity of the peak fractions ranged between 100 and 225% ^{45}Ca bound/mg of protein. In the calcium-binding fraction, a major protein band was found by analytical disc gel electrophoresis. This protein migrated with an R_F of 0.80 relative to Bromophenol Blue. Electrophoresis of other fractions from the column showed that this protein band was present only in those fractions that had high calcium-binding activity.

To identify this protein band as a CaBP, the maximal calcium-binding fraction was subjected to disc gel electrophoresis and calcium-binding activity was measured in extracts of unstained gels (Figure 3). Thirty per cent of the applied calcium-binding activity was recovered in the R_F range of 0.65–0.82; 10% of the calcium-binding activity was recovered in the R_F range of 0.82–0.98. No calcium-binding activity was detected at lower R_F values. The major protein band identified on the stained gels appeared to migrate slower (R_F 0.75) than on gels not subjected to current before application of the sample. However, when the active gel extracts were reelectrophoresed without the prior exposure of the gel to current, only a single protein band migrating with an R_F of 0.80 could be visualized.

A second gel filtration, on Sephadex G-50 fine, of the CaBP fraction obtained by chromatography on Sephadex G-100 resulted in a twofold increase in specific calcium-binding activity (Figure 2). Maximal calcium-binding activity eluted on the leading edge of the protein peak. The most active fractions had a specific calcium-binding activity of approximately 300% ^{45}Ca bound/mg of protein. Electrophoretic examination showed the CaBP fraction still to be heterogeneous.

Chromatography on DEAE-cellulose effectively separated the CaBP from proteins of apparently similar size with which it coeluted during gel filtration. The protein fractions having the highest specific calcium-binding activity obtained from several separate extractions were pooled and applied to a column of DEAE-cellulose in 10 mM Tris-HCl (pH 7.3). Approximately 50% of the total protein came off the column in the void volume. Development of the column with 20 mM Tris-HCl (pH 7.3) failed to elute measurable protein but improved the resolution of the protein peaks that were eluted after an increase to 50 mM (Figure 4). The first peak was comprised of CaBP having a specific calcium-binding activity between 1000 and 1400% ^{45}Ca bound/mg of protein. Conductivity measurements on the immediately preceding fractions indicated that the CaBP was eluting at a Tris-HCl concentration of approximately 40 mM. The second protein peak had no calcium-binding activity.

On disc gel electrophoresis of the protein fraction applied to the DEAE-cellulose column and of the eluted protein peaks, the CaBP appeared to be essentially homogeneous (Figure 5), although small amounts of two slower migrating protein bands could be detected when high concentrations of the CaBP were electrophoresed. The void volume contained exclusively the slower migrating protein bands pres-

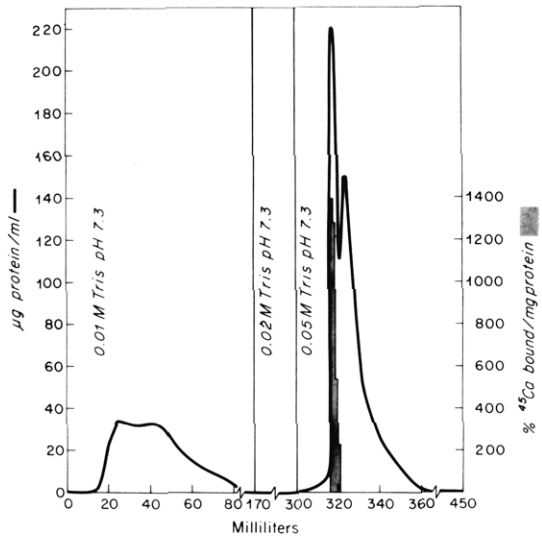

FIGURE 4: DEAE-cellulose chromatogram of CaBP fraction obtained by sequential gel filtration on Sephadex G-100 and Sephadex G-50 fine. Column size, 1.0 × 20 cm; flow rate, 0.10 ml/min; fraction size, 1.5 ml. Sample was applied in a volume of 42 ml. Stepwise elution was carried out with increasing concentrations of Tris-HCl buffer, indicated by vertical lines in figure. Ordinate and abscissa are marked as in Figure 1 except that calcium-binding activity per ml is omitted for simplification. Recovery of added protein was 82%; recovery of calcium-binding activity was 44%.

ent in the applied fraction. The protein peak eluting after the CaBP contained approximately equal amounts of two proteins, one of which migrated identically to the active CaBP (R_F 0.80) and one with an even higher electrophoretic mobility (R_F 0.89). The yield of active CaBP from the DEAE-cellulose step was relatively low and it is possible that the protein that behaved electrophoretically like the CaBP but was devoid of calcium-binding activity could have been CaBP that had been inactivated by this procedure.

The increase in specific calcium-binding activity and recoveries of calcium-binding activity per step achieved during the purification of the CaBP are summarized in Table I. The overall recovery of calcium-binding activity present in the initial soluble extract was approximately 20%. Based on a specific calcium-binding activity of 1,400% ^{45}Ca bound/mg of protein for the purified protein and the total calcium-binding activity in the initial extract, the CaBP appears to

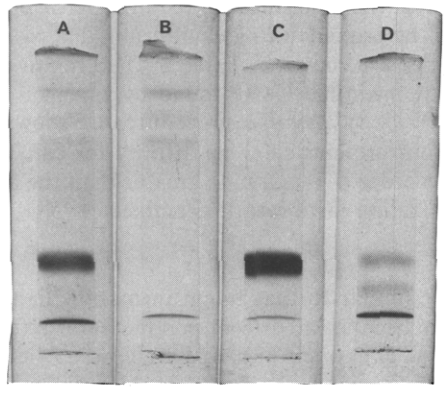

FIGURE 5: Polyacrylamide disc gel electrophoresis of CaBP fraction applied to DEAE-cellulose and of three protein fractions eluted: (A) applied protein fraction, 10 μg; (B) nonretained fraction (40 ml), 7.5 μg; (C) calcium-binding fraction (317 ml), 10.6 μg; (D) nonactive fraction following calcium-binding fraction (324 ml), 7.6 μg. Gels were stained with Coomassie Blue.

TABLE I: Increase in Specific Activity of Calcium-Binding Protein with Fractionation.[a]

Fraction	Specific Activity[b]	Rel Specific Activity	CaB Activity (% recovery per step)
Concentrated supernatant porcine parathyroid homogenate	4.8	1	
Sephadex G-100 (Figure 1)	205	43	78
Sephadex G-50 (Figure 2)	312	65	61
DEAE-cellulose (Figure 4)	1,390	290	44

[a] Fractions were assayed by the Chelex method described in text; for each chromatogram, the activity in the peak tube was used for the calculation of specific activity. The percentage recovery per step is based on each preceding step. [b] As % ^{45}Ca bound/mg of protein.

account for 0.016 ± 0.09% (SD) wet weight of the parathyroid tissue and 0.93 ± 0.68% (SD) of soluble protein. The yield of CaBP was approximately 30 μg of protein/g of wet tissue. Although the individual parathyroid glands varied considerably in size, with an average weight of 35 mg/gland, it is estimated that 1 μg of CaBP was recovered/parathyroid gland.

Amino Acid Analysis. The amino acid composition of the CaBP is shown in Table II. The protein has a relatively high

TABLE II: Amino Acid Composition of Porcine Parathyroid Calcium-Binding Protein.[a]

Amino Acid	Residues/mol of Peptide (combined results)	Mole Integer
Aspartic acid	14.62	15
Threonine	5.62	6
Serine	7.88	8
Glutamic acid	11.30	11
Proline	4.08	4
Glycine	11.35	11
Alanine	10.60	11
Valine	7.00	7
Isoleucine	4.90	5
Leucine	9.52	9–10
Tyrosine	0.14	0
Phenylalanine	6.97	7
Lysine	15.77	16
Histidine	1.99	2
Arginine	2.00	2
Cysteine[b]	0.53	0–1
Methionine[b]	2.78	3
Tryptophan[c]		

[a] Residues/mol of peptide were calculated from mole fraction by best fit based on recovery of all stable residues. The combined results represent the average of duplicate analyses on two separate preparations. [b] Measured as cysteic acid and methionine sulfone. [c] Not determined.

TABLE III: Sucrose Density Centrifugation of Calcium-Binding Protein.

[^{131}I]CaBP plus	Fraction No.[a]		$s_{20,w}$ (S)	
	Standard	[^{131}I]-CaBP	Standard[b]	[^{131}I]CaBP[c]
Bovine serum albumin	32.5	11.8	3.87	1.40
Chymotrypsinogen	21.8	12.5	2.54	1.46
Ribonuclease	16.3	12.8	1.89	1.48
				Mean 1.45 ± 0.04 ±SD

[a] Position of maximal optical density (protein standard) or radioactivity ([^{131}I]CaBP) in 5–27% sucrose linear density gradient after centrifugation at 60,000 rpm for 12 hr at 20°. Maxima were determined by triangulation of plotted values for individual fractions. Fraction 1 would correspond to air–liquid interface. [b] Values experimentally determined (Ryan *et al.*, 1971). [c] Calculated according to Martin and Ames (1961).

content of lysine and aspartic acid and slightly lesser amounts of glutamic acid, glycine, and alanine. The minimum molecular weight calculated from this analysis was 14,500. Due to the small amount of highly purified CaBP obtained, analysis for other constituents has not been possible.

Estimation of Molecular Weight. The molecular weight of the CaBP was estimated by a linear concentration sucrose density centrifugation and by calibrated gel filtration. To facilitate the detection of small amounts of protein, the CaBP was iodinated to low specific activity with ^{131}I. The ^{131}I-labeled CaBP ([^{131}I]CaBP) sedimented as a single iodinated species in the sucrose gradient. The results of a representative sedimentation experiment are shown in Table III. By the procedure of Martin and Ames (1961), an average molecular weight of 11,700 was found for [^{131}I]CaBP.

Both unlabeled and ^{131}I-labeled CaBP were used in the gel filtration experiments. The protein peak of the unlabeled material eluted from the Bio-Rad P-60 column with a K_{av} of 0.293. Two peaks of radioactivity were found when [^{131}I]CaBP was chromatographed on the same column: the first emerged with a K_{av} of 0.293 and the second with a K_{av} of 0.383. The later-emerging radioactive species was attributed to peptide damaged by the iodination procedure or to an impurity in the protein preparation. The K_{av} values obtained for the standard proteins used to calibrate this column and their Stokes radii are listed in Table IV. The Stokes radius for CaBP, 19.7 Å, was obtained from a linear plot of $(-\log K_{av})^{1/2}$ *vs.* the Stokes radius of the standard proteins. From a plot of K_{av} *vs.* log molecular weight of the standard proteins, the molecular weight for the CaBP was estimated as 15,500.

TABLE IV: Stokes Radii and K_{av} of Standard Proteins and Calcium-Binding Protein.

Protein	K_{av}[a]	Stokes Radius
Ovalbumin	0.094	27.3[b]
Chymotrypsin	0.205	22.4[b]
Ribonuclease	0.310	19.2[c]
CaBP	0.293	19.7

[a] Calculated according to Laurent and Killander (1964). [b] From Laurent and Killander (1964). [c] From Ryan (1969).

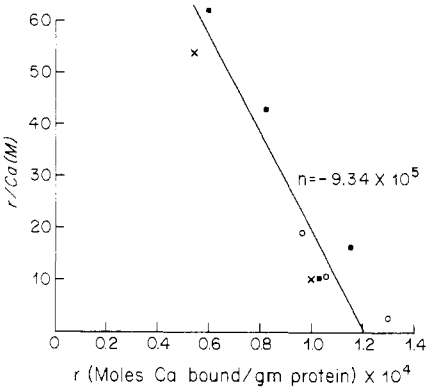

FIGURE 6: Scatchard plot for binding of ^{45}Ca by CaBP. Protein with specific calcium-binding activity of 1140% ^{45}Ca bound/mg of protein was used at a concentration of 75 μg/ml. Dialysis conditions: 0.5 ml of protein solution dialyzed against 225 ml of Tris-saline buffer containing ^{45}Ca (specific activity, 40 μCi/mol of Ca) for 8 hr at 22°. The three different symbols represent data obtained in three separate experiments with the same protein preparation. Line was plotted by linear regression analysis. From the slope of the line, *n*, the association constant (K_f) was estimated to be 9.34×10^5 M^{-1}.

Calcium-Binding Properties. The apparent association constant of the calcium–CaBP complex was determined by equilibrium dialysis. A range of calcium concentrations of 1.0×10^{-6}–5.0×10^{-5} M with ^{45}Ca of constant specific activity (40 Ci/mol) in the dialysate was used. The results of three experiments, plotted according to the method of Scatchard (1949), are shown in Figure 6. The apparent association constant (K_f) estimated from the slope of the line (n) was 9.34×10^5 M^{-1} with a correlation coefficient of 0.936. Extrapolation of the regression line to an infinite calcium concentration indicates a maximal calcium binding of 1.21×10^{-4} mol of Ca/g of protein. From the molecular weight estimated from amino acid analysis, 14,500, there are 1.75 high-affinity binding sites per molecule of protein.

The specificity of the binding site for calcium was examined by determining the concentrations of Ca, Sr, Ba, and Mg, added as the chloride salt, required to displace bound ^{45}Ca from the protein during equilibrium dialysis. The relative affinities are shown in Table V. The relative affinities of the chick intestinal CaBP found by Ingersoll and Wasserman (1971), by a similar procedure, are shown for comparison. Both proteins show an affinity for calcium that is at least one order of magnitude greater than the affinity for the other alkaline earth metals examined.

Discussion

A CaBP similar to that found in mammalian intestinal mucosa has been isolated from porcine parathyroid glands. Gel filtration of extracts of parathyroid tissue yielded a protein fraction in which the CaBP could be identified by its characteristic rapid migration in a basic electrophoretic system. Subsequent chromatography on DEAE-cellulose permitted isolation of the protein in an essentially homogeneous form. The highly purified porcine parathyroid CaBP has a specific calcium-binding activity (calcium bound/mg

of protein) comparable to that of the CaBP isolated from rat intestinal mucosa (Drescher and DeLuca, 1971) and somewhat higher activity than that of the CaBP isolated from chick intestinal mucosa (Wasserman *et al.*, 1968). In contrast to mammalian intestine, which contains a large CaBP in addition to the small vitamin D dependent CaBP, in the parathyroid gland the small CaBP appears to account for 85% of the soluble calcium-binding activity.

The yield of highly purified protein from the DEAE-cellulose step was consistently low, which has hindered efforts toward its complete characterization. The appearance of a protein in the later-eluting fractions of the DEAE-cellulose chromatogram with similar electrophoretic mobility on polyacrylamide gels as the CaBP but no calcium-binding activity suggests that the protein may be partially inactivated by this step. Evidence for an altered form of CaBP was obtained by Hitchman and Harrison (1972) in their isolation of the CaBP from pig intestine and by Fullmer and Wasserman (1973) in their isolation of the CaBP from cow intestine. However, in these cases, the altered species retained calcium-binding activity.

Comparison of the properties of the porcine parathyroid CaBP with those reported for the intestinal CaBP from various animal species suggests that they are closely related molecules. The estimated molecular weight for the parathyroid CaBP (11,700–15,500) is closer to the CaBP isolated from rat, pig, or cow intestine, with molecular weight estimates of 8,000–13,000 (Kallfelz *et al.*, 1967; Drescher and DeLuca, 1971; Hitchman and Harrison, 1972; Fullmer and Wasserman, 1973), than it is to the chick CaBP, with a molecular weight of approximately 28,000 (Bredderman and Wasserman, 1974). The calcium-binding constant of the parathyroid CaBP, $K_f = 9.3 \times 10^5$ M^{-1}, is similar to the calcium-binding constants of the rat, pig, and chick intestinal CaBP, reported to be 1.5×10^6, 1.5×10^6, and 2.0×10^6 M^{-1}, respectively (Schachter, 1969; Hitchman and Harrison, 1972; Bredderman and Wasserman, 1974). The relative affinities of other divalent cations for the parathyroid CaBP, as estimated from their displacement of protein-bound ^{45}Ca, show the same order Ca > Sr > Ba > Mg, as was found for the chick intestinal CaBP (Ingersoll and Wasserman, 1971).

Acidic amino acids comprise approximately one-fourth of the parathyroid CaBP molecule. A prevalence of acidic amino acids appears to be characteristic of the composition of all CaBP. Both the chick and porcine intestinal CaBP are acidic proteins (Wasserman *et al.*, 1968; Dorrington *et al.*, 1974). These amino acids are also prevalent in the CaBP of brain (Moore, 1965; Wolff and Siegel, 1972) and in the high-affinity CaBP associated with the contractile elements in mammalian and fish muscle (Pechère *et al.*, 1971; Schaub *et al.*, 1972; Kretsinger and Nockolds, 1973). The significance of the high dicarboxylic acid content of these proteins can be inferred from the recent work of Kretsinger and Nockolds (1973) who identified four carboxylate groups with no lysine or arginine residue near enough to make formal electrical neutrality in both the calcium-binding sites of carp muscle CaBP.

What possible physiologic role the CaBP may play in the parathyroid gland remains to be investigated. It is of interest to note the low affinity of this protein for magnesium. Magnesium has been shown to influence the rates of secretion of parathyroid hormone in a manner analogous to calcium (Care *et al.*, 1966) but to have no effect, within physiologic limits, on its rate of biosynthesis (Hamilton *et al.*, 1971). We have found that the calcium-binding activity in the parathyroid glands of vitamin D deficient dogs is increased with vitamin D repletion, suggesting that the parathyroid CaBP may be vitamin D induced (Oldham *et al.*, 1974). In these studies, increased calcium-binding activity was associated with decreased parathyroid hormone secretion. It seems likely that the CaBP is involved in calcium transport and, in some way, may mediate the intracellular responses to extracellular calcium concentration. However, until there is direct evidence that parathyroid biosynthetic or secretory activity is influenced by the cellular content of CaBP, this remains conjectural.

TABLE V: Binding Affinities of Porcine Parathyroid and Chick Intestinal Calcium-Binding Proteins with Alkaline Earth Metals.

Cation	Log $(1/I_{50})$[a]	
	Parathyroid[b]	Intestine[c]
Ca^{2+}	5.4	5.3
Sr^{2+}	3.4	4.5
Ba^{2+}	2.2	2.2
Mg^{2+}	1.7	1.4

[a] Binding affinity is shown as log of the inverse of the concentration of chloride salt required to decrease binding of tracer ^{45}Ca to protein by 50%. [b] Determined by equilibrium dialysis. Buffer mixture: 13.7 mM Tris–0.02 M NaCl–4.74 mM KCl–1 mM mercaptoethanol (pH 7.4). [c] From Ingersoll and Wasserman (1971).

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Primary Structure of the C_H2 Homology Region from Guinea Pig IgG2 Antibodies†

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ABSTRACT: The amino acid sequence of a 93-residue cyanogen bromide fragment, C-3, from the Fc region of the heavy chain of strain 13 guinea pig IgG2 has been determined. This fragment spans the region ~N-249 to ~N-341 of the γ_2 chain. It contains one disulfide bond which spans a 61-residue section from ~N-257 to ~N-317 and includes the only known attachment point for oligosaccharides in the γ_2 chain. The fragment appears to have a single sequence which is about 69% homologous with corresponding regions of either rabbit IgG or human IgG1. As this fragment C-3 of the heavy chain corresponds to a large portion of the C_H2 homology region, it may be possible to dimerize it to form a C_H2/C_H2 domain. This domain may be useful in attempts to relate to antibody structure various biologic functions of guinea pig IgG2, notably the initiation of complement activation by the conventional pathway.

A major aim of our determining the primary structure of the heavy chain from normal strain 13 guinea pig IgG2 has been to establish the positions of single residues or groups of residues which had alternative amino acids or amino acid sequences. At this point, all our analyses are consistent with there being a single sequence for the ~327 residues comprising the carboxyl-terminal three-quarters of the γ_2 chain, and we have demonstrated variability at certain residue positions within the amino-terminal quarter, or V_H region, of the chain.

To accomplish a primary structural analysis of the entire γ_2 chain we have isolated eight cyanogen bromide fragments from *normal* heavy γ_2 chain (Birshtein *et al.*, 1971a) which have been formally aligned (Benjamin *et al.*, 1972) and which account for the entire γ_2 chain. The three amino-

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