Chemical Composition, Affinity for Calcium, and Some Related Properties of the Vitamin D Dependent Calcium-Binding Protein†

Paul J. Bredderman‡ and Robert H. Wasserman*

ABSTRACT: The concentration of a vitamin D dependent intestinal high-affinity Ca-binding protein (CaBP) is known to be highly correlated with the vitamin D dependent enhancement of intestinal Ca absorption. Purified CaBP from chick duodenal mucosa was analyzed for lipids, glycoprotein carbohydrate components, amino acid composition, and Ca-binding properties. It was free of lipid, carbohydrate, phosphorus, and other ash-producing substances. The molecular weight from amino acid composition and sodium dodecyl sulfate polyacrylamide gel electrophoresis was near 28,000. CaBP contains only three half-cystine residues. Several spectrophotometric methods, including a new three-wavelength method, indicated the presence of two tryptophan residues. Polar residues make up 53% of the 242 residues and 61 residues contain side-chain carboxyl groups. The calculated isoelectric point is 4.2 and the average charge per residue, 0.384. The computed partial specific volume and molecular volume were 0.734 g cm^{-3} and ~34,000 $Å^3$, respectively. $\epsilon^{H_2O}_{1\%,\,1\text{ cm}}$ (pH 6.5) at 280 nm was 9.03. A study of the thermal stability of CaBP indicated that its immunoreactivity, high-affinity binding of Ca and electrophoretic mobility were unchanged after a heat treatment of up to ~80°, but declined precipitously between 80 and 90°. Equilibrium dialysis studies revealed that Ca was bound exchangeably at four strong Ca-binding sites with apparent intrinsic association constant, k_i, of 2×10^6 M^{-1} in 0.15 M KCl (pH 6.8). Based on published competitive binding data (Ingersoll, R. J., and Wasserman, R. H. (1971), *J. Biol. Chem. 246*, 2808), the log k_i for several divalent cations were calculated to be: Ca, 6.30; Cd, 5.10; Sr, 4.39–4.58; Mn, 4.37; Zn, 3.71; Ba, 3.18–3.24; Co, 2.84; Mg, 2.44. Binding affinity appears to be related to the crystal ionic radius of these various cations. Additional Ca binding appeared abruptly when the concentration of free Ca^{2+} reached 3×10^{-3} M.

Vitamin D, a sterol which undergoes metabolic conversion to more active forms in the liver and kidney, is required by several species for the optimal intestinal absorption of Ca and the vitamin influences Ca metabolism in kidney, bone, and the avian shell gland. Its metabolism and its metabolic effects have been recently summarized (Wasserman and Corradino, 1971; Wasserman and Taylor, 1972). The direct correspondence between the magnitude of absorption of Ca and the concentration of CaBP[1] in chick intestinal mucosa under a variety of physiological and nutritional situations (Wasserman *et al.*, 1974) indicates CaBP to be intimately related to the vitamin D dependent translocation of calcium. Although intestinal Ca-binding proteins have been identified in a number of other species, including the human (Wasserman *et al.*, 1974), the function of these proteins is still undetermined.

This paper presents the results of detailed analyses of the lipid, carbohydrate, and amino acid composition of chick CaBP which expand upon, and provide refinements and modifications to, previously published preliminary results (Wasserman *et al.*, 1968). Also presented are the results of equilibrium dialysis studies of the protein's Ca-binding properties along with estimates of its binding affinity for several other divalent cations, based on their competitive inhibition of Ca binding as published by Ingersoll and Wasserman (1971).

Materials and Methods

Inorganic, organic, and biochemicals were, when available, of reagent grade or better. Guanidine hydrochloride was a spectroscopic grade from Mann Research.

The regenerated cellulose dialysis membrane was pretreated as recommended by Craig (1967), including treatment with 10^{-6} M EDTA.

The purification of CaBP was outlined by Wasserman *et al.* (1968), incorporating several minor modifications; among them was the use of sodium azide (0.02%) and mercaptoethanol (1 mM) in all solutions.

Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard and using the experimentally determined factor of 1.04 to obtain CaBP concentration. Nitrogen was determined by the sealed-tube method of Jacobs (1964). For the lipid analyses, CaBP was extracted with 20 volumes (v/v) of 3:1 ethanol–ether (Boyd, 1936). Qualitative analysis of the extracts for neutral lipids and phospholipids was by thin-layer chromatography (tlc). Cholesterol and cholesterol esters were estimated by the method of Zak *et al.* (1954). Free fatty acids were

† From the Department of Physical Biology, New York State Veterinary College, Cornell University, Ithaca, New York 14850. *Received August 20, 1973.* Supported by U. S. Public Health Service Grant AM-4652, U. S. Atomic Energy Commission Contract AT(11-1)-3167, and the National Institutes of Health Training Grant 5-T01-DE-009. The sodium dodecyl sulfate electrophoresis studies were done at the University of Rochester by Paul J. Bredderman supported by the National Institutes of Health Training Grant 5-T01-DE00175 and under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project and has been assigned Report No. UR-3490-360.

‡ Present address: Department of Radiation Biology and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y. 14642.

[1] Abbreviations used are: CaBP, vitamin D dependent calcium-binding protein; NANA, *N*-acetylneuraminic acid; PAS, periodic acid–Schiff reagent; Pipes, piperazine-*N*,*N*-bis(2-ethanesulfonic acid); T_m, temperature of maximum melting.

determined by gas–liquid chromatography. Lipid phosphorus analysis was based on the methods of Bartlett (1959) and Morrison (1964). For all analyses, glassware was prerinsed with 2:1 chloroform–methanol and the results were expressed as the difference between the amount of lipid in the CaBP extract and in the blank extract. Total carbohydrate was measured by the method of Dubois *et al.* (1956) on CaBP passed through a 10 μ Millipore filter. The method of Gibbons (1955) was employed for methylpentose analysis. Total hexosamine was estimated according to Cessi and Piliego (1960) on samples hydrolyzed for 4 hr at 100° in 4 N HCl under nitrogen at a CaBP concentration of 0.1%. The method of Warren (1959) was employed to estimate free sialic acid in samples hydrolyzed in 0.1 N H_2SO_4 at 80° for 1 hr using synthetic NANA as standard. Sialic acid was also estimated by the method of Svennerholm (1957), without prior hydrolysis. Each of these carbohydrate analyses was performed with the proper internal controls to correct for carbohydrate destruction, protein interference, and nonspecific absorption, all of which proved to be minor.

For the analysis of tryptophan and tyrosine, bovine serum albumin, α-chymotrypsinogin A, and lysozyme were used as controls and several spectrophotometric methods were employed (Goodwin and Morton, 1946; Bencze and Schmid, 1957; Edelhoch, 1967), including a new three-wavelength method to be described in detail elsewhere (P. J. Bredderman, in preparation).

Total half-cystine and methionine were determined according to Moore (1963) on performic acid oxidized CaBP hydrolyzed for 20, 24, 48, and 72 hr. Amide nitrogen was estimated as outlined by Wilcox (1967) and also from linear extrapolation to zero time of hydrolysis, based on the ammonia content of CaBP hydrolysates prepared for amino acid analysis. This latter method was not very satisfactory because the variability in the ammonia values among hydrolysates made extrapolation difficult. A third method for amide nitrogen came from the observation that the fluctuations in the concentrations of four amino acids (serine, threonine, methionine, and tyrosine) accounted quantitatively for the wide fluctuations in the ammonia content of the various CaBP hydrolysates, the sum of these five components being essentially constant for all hydrolysates. This suggested that amide nitrogen could be estimated more precisely as the total ammonia in each hydrolysate minus that ammonia arising from non-protein sources and from the destruction of these four amino acids.

Amino acids other than tryptophan, tyrosine, methionine, and half-cystine were determined by ion-exchange chromatography (Moore and Stein, 1963) on a Beckman Model 120C analyzer. Two batches of CaBP were analyzed; for each batch, two samples were hydrolyzed for each of four hydrolysis times (12, 24, 48, and 72 hr) in sealed degassed tubes at 110 ± 1° in 6 N HCl at a final protein concentration of 0.1%. Hydrolysis blanks were used to correct for non-protein ammonia. Hydrochloric acid was removed from the hydrolysis tubes by rotary evaporation at 40° and 5 mm. From plots of amino acid concentration *vs.* the hours of hydrolysis, serine and threonine, which showed gradual destruction during hydrolysis, were determined as the linearly extrapolated zero-time values, whereas all other acids were determined from the plateau values.

The molecular weight of CaBP was estimated from the amino acid composition by the method of Nyman and Lindskog (1964), and the results were utilized to calculate the amino acid residue nearest integer values. The molecular weight was also estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, using 16 standard proteins ranging in molecular weight from 11,700 to 67,000 and employing the sodium dodecyl sulfate polyacrylamide gel electrophoresis system of Fairbanks *et al.* (1971) (T = 11.1%, C = 0.9%).[2]

To assess the thermal stability of CaBP, homogenates of duodenal mucosa of vitamin D repleted chicks were prepared in 1:4 (w/v) dilution with Tris-buffered saline (pH 7.4). Aliquots of supernatant from a 30 min, 78,000g centrifugation were treated for 10 min at temperatures ranging from 25 to 100° and recentrifuged. As indications of the amount and quality of the CaBP in the resulting supernatants, the CaBP band on analytical polyacrylamide gel electrophoresis was scanned densitometrically, the high-affinity Ca-binding activity was measured using equilibrium dialysis and tracer ^{45}Ca, and the amount of immunoreactive CaBP was assessed by radial immunoassay, kindly performed by Dr. A. N. Taylor, using specific rabbit antibody. As part of the study of the Ca-binding properties of CaBP, liquid scintillation methodology was used to measure ^{45}Ca in Bray's solution (Bray, 1960). Quench corrections were not required. Total Ca was measured by atomic absorption spectrometry with standards prepared from a certified Ca standard (Fisher Scientific).

To measure the exchangeability of the bound Ca, purified CaBP (0.700 mg/ml) was first preequilibrated at 4° with 4×10^{-5} M $CaCl_2$ in 0.15 M KCl and 10^{-3} M Pipes (pH 6.8). Then, starting with equal concentrations of ^{45}Ca in retentate and dialysate, the specific radioactivity of both solutions and the protein concentration of the retentate were monitored at 12-hr intervals during a 180-hr dialysis at 4°. Exchangeability was assessed from the ratio of the specific activities of calcium in each compartment.

In a second study the affinity and binding capacity of CaBP for Ca were determined by equilibrium dialysis, employing plastic dialysis cells with 1-ml chambers and a range of equilibrium ionic Ca concentrations, $(Ca)_f$, from 2×10^{-7} to 1×10^{-2} M in 0.15 M KCl–10^{-3} M Pipes (pH 6.8) with a CaBP concentration of 0.700 mg/ml. Dialysis was for 48 hr at 4°, about twice the time required for equilibration. The Ca contamination in the KCl–Pipes buffer was estimated by the "method of additions" (Dickson and Johnson, 1966) to be about 2.5×10^{-7} M, in close agreement with the results of others (Nanninga and Kempen, 1971), and constituted a negligible fraction of the total Ca in almost all cells. The data were plotted as described by Scatchard (1949).

The k_i and n obtained above for the high-affinity Ca-binding sites of CaBP were used in conjunction with previously published data (Ingersoll and Wasserman, 1971) to estimate the affinity of other divalent cations for these sites. The experimental data of Ingersoll and Wasserman (1971) indicated the degree to which various cations (Sr^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}) at 10^{-3} M inhibited Ca binding when $(Ca)_f$ was 5×10^{-6} M, using an equilibrium dialysis procedure. The k_i for each of these cations was calculated from the following expression

$$k_i^X = \frac{\alpha}{1-\alpha} k_i^{Ca} (Ca)_f / (X)_f^{\frac{n}{\bar{\nu}}} \quad (1)$$

[2] The gels are described by the notation of Hjerten (1962), where T = percentage (w/v) of acrylamide monomers and C = percentage (w/w) of N,N'-methylenebisacrylamide expressed as per cent of total weight of monomers.

where k_i^{Ca} and k_i^X are the apparent intrinsic association constants for Ca and the competing divalent cation, X, respectively; α is the fractional inhibition of Ca binding at $(Ca)_f$ and $(X)_f$; and $\bar{\nu}$ is the average number of Ca ions bound to the n high-affinity sites of CaBP at $(Ca)_f$ in the *absence* of the competing divalent cation. The derivation of the above equation is straightforward and omitted here because of limitation of space. However, it should be pointed out that the assumptions underlying the derivation are, as follows: (1) the competing ion has no allosteric effects on the protein; (2) the competition is of equal intensity at all n sites; (3) the values of $(Ca)_f$ remain the same whether or not the competing ion is present; and (4) all of the unbound competing ion is present in solution as the free divalent cation. The validity of assumption (3) was assured by the experimental design which used a large volume ratio of dialysate to retentate. About 25% of the Cd^{2+} probably was present as the $CdOH^+$ and $CdCl^+$ complexes (Sillén and Martell, 1971), but this has only a minor effect on the degree of competitive inhibition by Cd^{2+} and on its computed association constant.

Results

Lipid Analysis. Both 90-mg batches of CaBP yielded indistinguishable neutral lipid patterns on tlc. However, quantitative analysis indicated that the CaBP contained less than 0.1% lipid, much less than a 1:1 lipid:protein molecular ratio. The absence of phospholipid was also indicated by direct elemental analysis of CaBP which showed that less than 0.02% P was present.

Carbohydrate Analysis. The amounts of CaBP tested in each of the several analyses would have allowed a 1:1 carbohydrate:CaBP molecular ratio to be easily detected. In each case, however, the absorption observed was close to the limits of sensitivity of the assay and only about twice that produced by the ultrafiltrate of the CaBP solution, thereby indicating the absence of carbohydrate as an integral part of the molecule. We also observed that the CaBP band on polyacrylamide gel electrophoresis of freshly prepared 70° heat-treated mucosal homogenates reacted negatively to PAS staining (Fairbanks *et al.*, 1971), even when very large amounts of CaBP were present.

Tryptophan and Tyrosine Analysis. The method of Goodwin and Morton (1946) and that of Bencze and Schmid (1957) gave unreliable estimates of tryptophan and tyrosine in the three control proteins analyzed. The simultaneous determination of these two amino acids in 6 M guanidine hydrochloride (Edelhoch, 1967) overestimated tryptophan and slightly underestimated tyrosine in bovine serum albumin, possibly as a result of the need to correct for "irrelevant absorption" in the albumin. When tyrosine was estimated independently prior to the determination of tryptophan (Edelhoch, 1967), correct estimates were obtained for all three control proteins. However, the three-wavelength method for tryptophan gave the most accurate values. It was concluded that CaBP contains two residues of tryptophan and eight residues of tyrosine per molecule.

Half-cystine and Methionine Analysis. After correcting the cysteic acid content of performic acid oxidized CaBP for the 94.7% conversion of cystine to cysteic acid determined experimentally, the average plateau values of the ratios between the amounts of cysteic acid and each of six stable amino acids resulted in an estimate of 2.58 half-cystine residues/molecule of CaBP. In a similar manner, it was determined that CaBP contains eight methionine residues per mol wt 28,000.

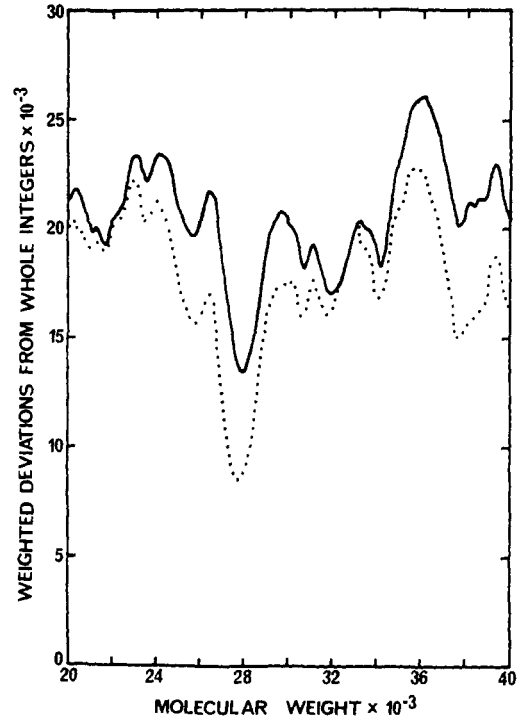

FIGURE 1: Estimation of molecular weight of calcium-binding protein from amino acid composition by the method of Nyman and Lindskog (1964). The upper curve includes weighted deviations of half-cystine residues and the lower curve does not. Both yield, as best estimate, a mol wt of slightly less than 28,000.

Amide Nitrogen Analysis. The method described by Wilcox (1967) indicated 18.36 amide groups/mol wt 28,000. By extrapolation, the ammonia in the two sets of CaBP hydrolysates yielded values of 22.7 and 21.4 residues. These were rather uncertain, however, and the results obtained in this manner are generally considered maximum amide values. By the third method of amide analysis (see Materials and Methods), values of 17.1 and 17.2 residues were obtained for the two sets of hydrolysates. These latter values, because they too would appear to be maximum values, were accepted as the best estimates.

Overall Amino Acid Analysis. Table I is a summary of the amino acid analysis. Of the 108 ± 2.2 μg (SE) of protein nitrogen present in the samples, 107 μg is accounted for by the composition shown. The composition shown also accounts for 101.3% of the protein determined by the method of Lowry *et al.* (1951).

Molecular Weight Determinations. Both the estimate of the molecular weight from the amino acid composition (Figure 1) by the procedure of Nyman and Lindskog (1964) and the estimate by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 2) indicate a value of close to 28,000 ± about 1000. This value is in accord with the value of 28,000 obtained previously by molecular exclusion chromatography (Wasserman *et al.*, 1968). However, when the computed partial specific volume (Table I) was used to recalculate the three sedimentation equilibrium molecular weight estimates (Wasserman *et al.*, 1968), only one of these values (24,972 ± 1,518 (±SE); 25,247 ± 1,671; 27,462 ± 1,339) is in reasonable agreement with the other independent estimates.

Isoelectric Point. The point of zero net charge of CaBP was computed from the amino acid composition using average

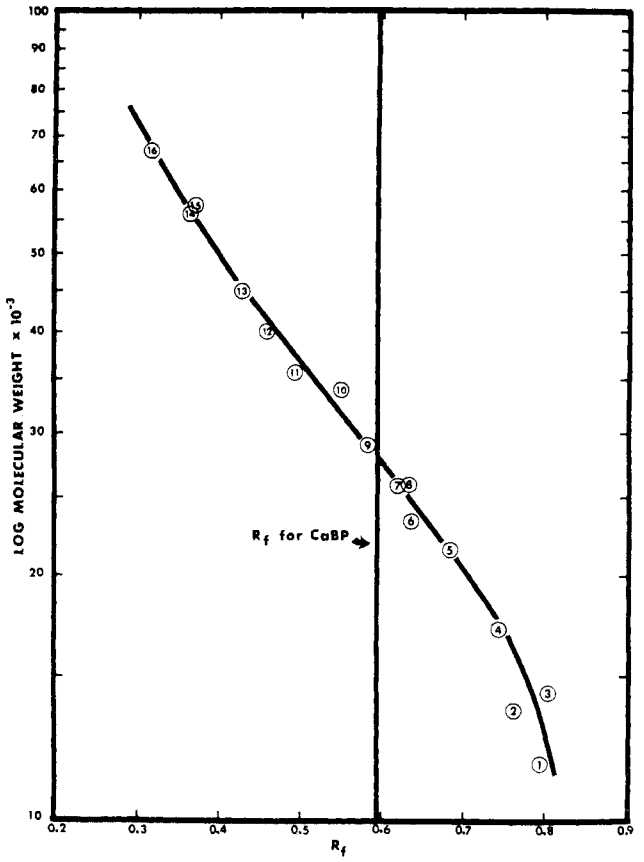

FIGURE 2: Molecular weight determination of calcium-binding protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The reference proteins and their molecular weights are, as follows: 1, cytochrome *c* (equine, 11,700); 2, ribonuclease A (bovine, 13,700); 3, lysozyme (egg white, 14,300); 4, myoglobin (whale, 17,200); 5, soybean trypsin inhibitor (Kunitz, 21,500); 6, papain, 23,400; 7, α-chymotrypsinogen A (bovine, 25,700); 8, elastase (porcine, 25,900); 9, carbonic anhydrase (bovine erythrocyte, 30,000); 10, carboxypeptidase A (bovine, 34,400); 11, glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 35,700); 12, aldolase (rabbit muscle, 40,000); 13, ovalbumin (45,000); 14, L-glutamic acid dehydrogenase (bovine, 56,100); 15, catalase (bovine erythrocyte, 57,500); 16, bovine serum albumin (67,000). R_F values are mobilities relative to the tracking dye, Pyronin-Y.

pK_a values for aspartic (3.85) and glutamic (4.4) side chains and the end-group carboxyl (3.1) (Cohn and Edsall, 1943) after arbitrarily assigning the amide groups to an equal percentage of the two types of side-chain carboxyls. The estimate, 4.2, was the same as the experimentally determined isoelectric point reported by Ingersoll and Wasserman (1971).

Extinction Coefficient. The percentage nitrogen (15.4), calculated from the nearest integer residue values, along with total nitrogen and 280-nm absorption values of CaBP in water at pH 6.5, were used to calculate an $\epsilon_{1\%,\,1\text{ cm}}$ value of 9.03.

Thermal Stability of CaBP. Figure 3 shows that a large fraction of the total protein in mucosal homogenates started precipitating at about 40°, with only about 33% remaining soluble at 60° and only about 15% soluble at 100°. However, CaBP appeared to be unaffected up to almost 80°, based on electrophoretic mobility, immunoreactivity, and high-affinity Ca binding. (The low value for the electrophoresis of the control resulted from the protein overload on the gel.) Above 80°, however, the values of all three of these parameters declined precipitously and then plateaued at 5–20% of the initial values. The temperature of maximum melting, T_m, would appear to be about 85°.

Exchangeability of CaBP-Bound Calcium. After equilibrium was attained at 40 hr, the mean specific activity ratio (retentate:dialysate) for the subsequent 140-hr dialysis was 1.039 ± 0.019 (SE), indicative of the complete exchangeability of bound Ca. It was also calculated from atomic absorption measurements of the Ca in the dialysate and retentate and from protein determinations on the retentate (0.700 mg/ml) that CaBP binds four Ca^{2+} per molecule at 4.12×10^{-5} M $(Ca)_f$, in complete accord with the findings described below. It is noteworthy that direct elemental analysis of CaBP, indicating less than 0.01% ash, is consistent with the presence of nonexchangeable metal ions in no more than a 1:10 metal:CaBP molecular ratio.

Calcium-Binding Capacity and Affinity of CaBP. Under the experimental conditions Donnan effects were insignificant over the entire $(Ca)_f$ range (2×10^{-7} to 1×10^{-2} M). The ionic strength was essentially constant at 0.16 for $(Ca)_f \leq 1 \times 10^{-3}$ M. Above this range, the increase in ionic strength due to an increase in $CaCl_2$ up to 1×10^{-2} M would have depressed the activity coefficient of Ca^{2+} by no more than about 2.2%. Ingersoll and Wasserman (1971) found that the degree of depression in Ca binding caused by increasing the ionic strength over the range of 0.02–0.15 was the same regardless of whether KCl or NaCl was used, suggesting that there is no specific binding of the K^+ of the buffer to the Ca-binding sites. Figure 4 is a Scatchard plot of the results (Scatchard, 1949). The plotted values were obtained from 24 dialysis cells of two 12-cell experiments and cover 14 different Ca concentrations. The ordinate in Figure 4b, where $(Ca)_f$ for the plotted values ranges from 2.1×10^{-7} to 1.4×10^{-4} M, covers 250 times the range covered in Figure 4a, where $(Ca)_f$ for the plotted values ranges from 1.6×10^{-4} to 1.0×10^{-2} M. From the slope in Figure 4b, the apparent intrinsic association constant, k_i, for the high-affinity sites was estimated as 2×10^6 M^{-1} (range 1.6×10^6 to 2.5×10^6 M^{-1}). The curve in the subfigure intercepts the abscissa to yield a value of 4 for the number of high-affinity sites (n) per mol wt 28,000. The slight downward curvature of the experimental points may indicate slight cooperativity among the four sites, but this is uncertain because of the size of the standard error estimates.

Figure 4a shows that no more calcium becomes associated with the protein until a $(Ca)_f$ of 3×10^{-3} M is reached; above this concentration there is a sharp upturn in bound calcium. By subtracting the contribution of the four high-affinity sites, one should be left with a linear plot reflecting the relationship

$$\bar{\nu}/(Ca)_f = k_i(n - \bar{\nu}) \qquad (2)$$

for binding to a second set of preexisting identical, independent sites. Instead, however, a curvilinear plot (not shown) was obtained that passed through the origin at 3×10^{-3} M $(Ca)_f$ as would occur if n for these sites went to zero at 3×10^{-3} M $(Ca)_f$ as $\bar{\nu}$ went to zero. From extrapolation of the linear portion of this latter curve, the rough estimates of $k_i \simeq 30$ M^{-1} and $n \simeq 32$ were obtained for these low-affinity sites.

Apparent Intrinsic Association Constants for Other Divalent Cations. The competitive inhibition data used to calculate these association constants (Ingersoll and Wasserman, 1971) are plotted in Figure 5 as a function of crystal ionic radius (Pauling, 1960). The log of the association constants obtained as described in Materials and Methods are: Ca, 6.30; Cd, 5.10; Sr, 4.39–4.58; Mn 4.37; Zn, 3.71; Ba, 3.18–3.24; Co, 2.84; Mg, 2.44. The range in the values for Ba and Sr represent the results obtained at two concentrations of these ions.

TABLE I: Summary of the Amino Acid Analysis.

Residue	Concentration		Min Mol Wt	Residues/ Molecule	Nearest Integer Residues	Wt %[a]	Partial Sp Vol	
	μmol/ml	μg/ml					cm^3/g^b of Residue	cm^3/g of Protein
Ala	0.4105	29.174	1,696	16.54	17	4.32	0.74	0.0320
Arg	0.125	19.522	5,569	5.04	5	2.79	0.70	0.0195
Asp	0.839	96.552	830	33.81	34	13.99	0.60	0.0839
$^1/_2$-Cys	0.064	6.537	10,878	2.58	3	1.10	0.63	0.0069
Glu	1.093	141.053	637	44.02	44	20.31	0.66 0.67	0.1358[b]
Gly	0.327	18.662	2,129	13.18	13	2.65	0.64	0.0170
His	0.081	11.108	8,595	3.26	3	1.47	0.67	0.0098
Ile	0.277	31.348	2,513	11.16	11	4.45	0.90	0.0400
Leu	0.774	87.594	899	31.19	31	12.54	0.90	0.1129
Lys	0.592	75.877	1,176	23.85	24	11.00	0.82	0.0902
Met	0.199	26.107	3,498	8.02	8	3.75	0.75	0.0281
Phe	0.326	48.058	2,132	13.16	13	6.84	0.77	0.0527
Pro	0.072	6.992	9,669	2.90	3	1.04	0.76	0.0079
Ser	0.226	19.678	3,080	9.11	9	2.80	0.63	0.0176
Thr	0.222	22.444	3,136	8.94	9	3.25	0.70	0.0228
Trp	0.049	9.124	14,207	1.97	2	1.33	0.74	0.0098
Tyr	0.205	33.448	3,396	8.26	8	4.66	0.71	0.0331
Val	0.130	12.890	5,355	5.24	5	1.77	0.86	0.0152
Amide				(17.1)	(17)			
Totals		696.164			242	100		0.7342

[a] Based on a molecular weight of 27,900 determined from the amino acid composition as described in the text. [b] These values are from Cohn and Edsall (1943); all the amide groups have been arbitrarily assigned to glutamic acid.

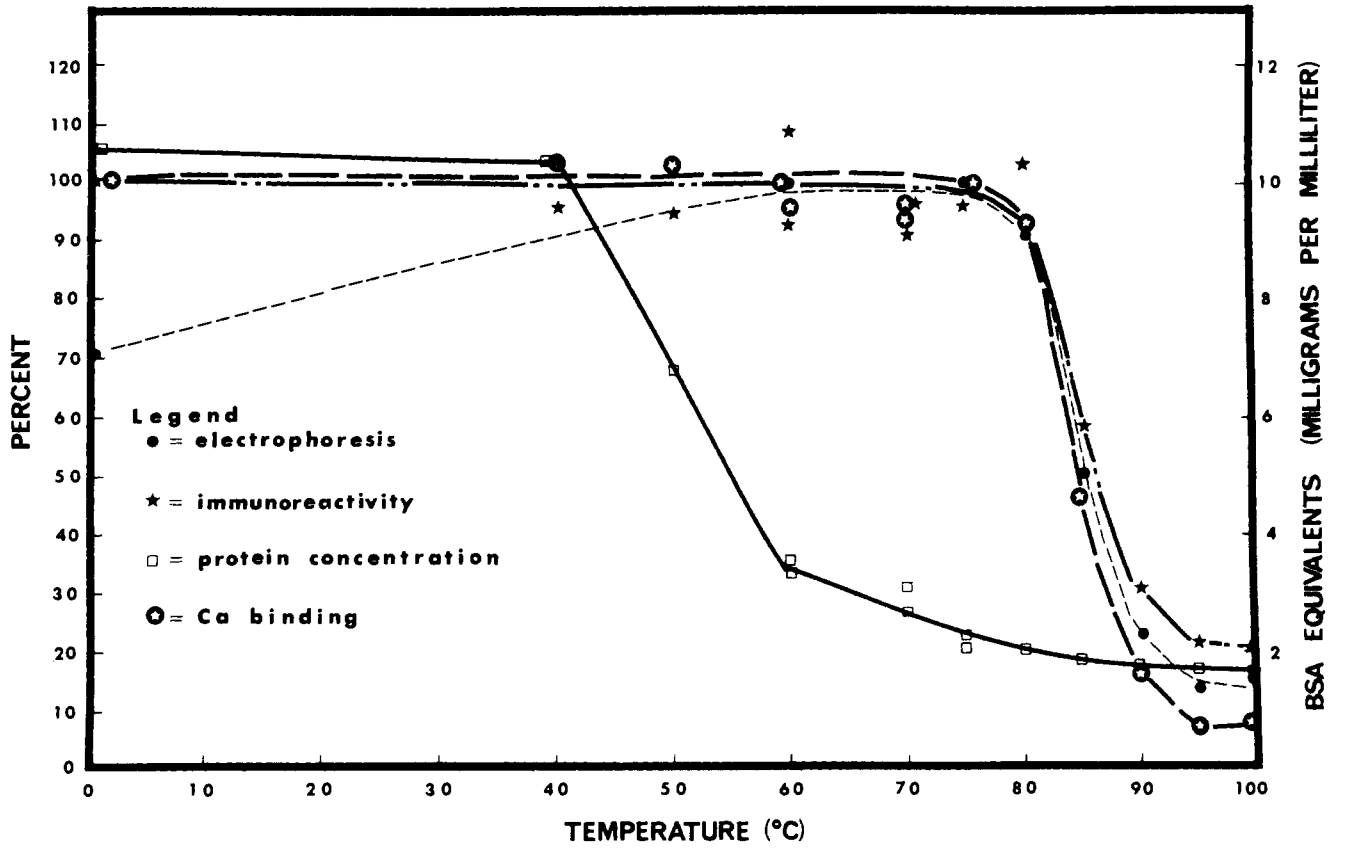

FIGURE 3: Effect of a prior heat treatment on the Ca-binding activity, immunoreactivity, and electrophoretic mobility of CaBP. The values, except for protein content and mobility, are percentages of the non-heat-treated preparation. Mobility uses the 60° treatment as 100%. Protein is expressed in bovine serum albumin equivalents.

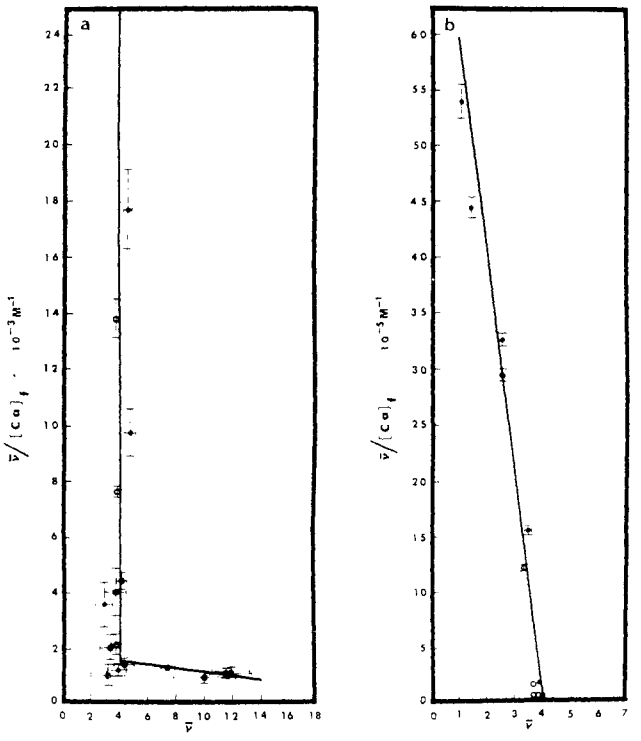

FIGURE 4: Scatchard plot of equilibrium dialysis data. The left graph (4a) covers the range of free calcium concentrations, $(Ca)_f$, from 1.6×10^{-4} to 1×10^{-2} M. The plotted values in part b are for the $(Ca)_f$ range from 2.1×10^{-7} to 1.4×10^{-4} M. The standard error estimates are indicated where their size exceeds that of the symbols used. Open circles refer to values from the second set of dialysis cells.

Discussion

This report confirms the preliminary findings of Wasserman *et al.* (1968) that CaBP isolated in this manner is essentially free of lipid, carbohydrate, and phosphorus; it also contains no nonexchangeable calcium or other ash-producing components. It can possess, at most, one disulfide bond.

The high proportion of dicarboxylic amino acids (32% in CaBP) is also found in hydrolysates of troponin A (33%) and other high-affinity Ca-binding proteins (Sottacassa *et al.*, 1972; Nockolds *et al.*, 1972; Ebashi, *et al.*, 1971; Fullmer and Wasserman, 1973). Sixty-one of the 78 aspartic and glutamic acid residues, or more than 25% of the 242 residues of CaBP, possess side-chain carboxyl groups, and a comparable percentage is present in troponin A, based on preliminary amide nitrogen values (Hartshorne and Pyun, 1971). Thirty-two of the 242 residues (13.2%) of the CaBP are capable of being positively charged compared to 13.8% for carp myogen and 12.4% for troponin A. The polar residues (Asp, Asn, Glu, Gln, His, Arg, Lys, Ser, and Thr) comprise 53% of the residues of both CaBP and troponin A but only 46% of myogen, which is 18.5% alanine.

The nonpolar residues of CaBP contain more tyrosine and tryptophan and less glycine and alanine than either troponin A or myogen, giving an average residue hydrophobicity ($H\Phi_{av}$) (Bigelow, 1967) for CaBP of 1059 cal/mol.

These three Ca-binding proteins not only have similar isoelectric points, but also exhibit an unusually large average number of charges per residue (CaBP, 0.384; troponin A, 0.37–0.38; carp myogen 0.362.)

The 28,000 molecular weight of CaBP, as estimated from the amino acid composition, from sodium dodecyl sulfate polyacrylamide gel electrophoresis, and by molecular exclusion

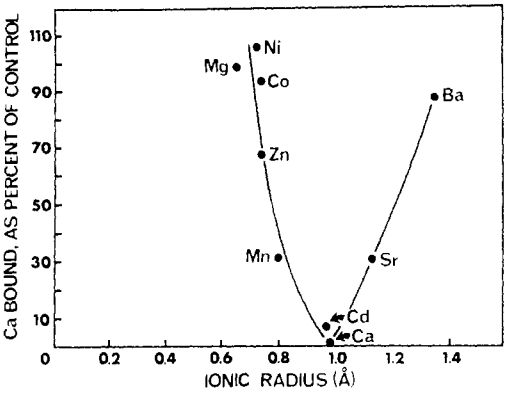

FIGURE 5: Plot of inhibition of the binding of tracer ^{45}Ca to CaBP by various divalent cations as a function of ionic radius.

chromatography (Wasserman *et al.*, 1968), is larger than that of troponin A (18,000–20,000), carp myogen (11,500), and intestinal Ca-binding proteins of the rat (8000–13,000; Drescher and DeLuca, 1971), bovine (11,000; Fullmer and Wasserman, 1973), pig (12,000–13,000, Hitchman and Harrison, 1972), and human (12,000–21,000; Hitchman and Harrison, 1972; Alpers *et al.*, 1972). Its molecular displacement volume, based on a partial specific volume of 0.734 cm^3 g^{-1}, is 34,100 $Å^3$, equal to that of a sphere of 20.1-Å radius.

The apparent intrinsic association constant for the four high-affinity Ca-binding sites of CaBP ($k_i = 2 \times 10^6$ M^{-1}) is about an order of magnitude larger than that reported earlier by Wasserman *et al.* (1968). It is, however, comparable to that of the sites of troponin A (Fuchs, 1971; Hartshorne and Pyun, 1971), myogen (Benzonanna *et al.*, 1972) and to that of Ca-binding proteins of sarcoplasmic reticulum (Yamada and Tonomura, 1972), mitochondria (Sottocassa *et al.*, 1972), and those from intestinal mucosa of some other species (Hitchman and Harrison, 1972). This association constant corresponds to an apparent standard free energy of binding ($\Delta G°$) of 8000 cal/mol at 4° so that Ca, by a cross-linking effect on the native conformation of CaBP, could contribute to the observed thermal stability. High thermal stability (60° or higher) is a feature of the intestinal CaBP of all species thus far examined (Hitchman and Harrison, 1972; Wasserman and Taylor, 1966). Calcium apparently also adds stability to thermolysin and subtilisin (Stauffer and Treptow, 1973), bacterial neutral proteases (Feder *et al.*, 1971), trypsin and its zymogen (Abita *et al.*, 1969; Sipos and Merkel, 1970), troponin A (Nagy *et al.*, 1973), carp myogen (Kretsinger and Nockolds, 1973), staphylococcal nuclease (Taniuchi *et al.*, 1967), and to the Ca-binding protein that activates the cyclic adenosine 3′:5′-monophosphate phosphodiesterase of beef heart (Teo and Wang, 1973).

On the other hand, Bull and Breese (1973) found a correlation coefficient of +0.960 between T_m (melting point) and average residue volume in a study of 14 proteins. Using their least-squares equation and the average residue volume of CaBP (84.87 cm^3 mol^{-1}), the predicted T_m for CaBP is 86°, essentially identical with the value estimated from Figure 3.

Both the n and k_i for Ca binding to the high-affinity sites of CaBP differ from the values previously reported by Wasserman *et al.* (1968) obtained from ion-exchange data using the equation and method of Schubert *et al.* (1950). A reexamination of the Schubert equation revealed that it is not applicable to Ca binding to a set of identical, independent sites on a protein. The correct equation is

$$\log [(K_d^0/K_d) - 1] = \log [nk_i(1 - \nu)] + \log [Pr]_T \quad (3)$$

where k_i is the apparent intrinsic association constant, $[Pr]_T$ is the total protein concentration, ν is the fractional occupancy of the sites under the experimental conditions, n is the number of binding sites, and K_d and K_d^0 refer to the fraction of resin-bound Ca to solution Ca in the presence and absence of protein, respectively. Note that when eq 3 is used to interpret a plot of $\log [K_d^0/K_d) - 1]$ *vs.* $\log [Pr]_T$, the slope is 1.0 and not equal to n as Wasserman *et al.* (1968) and, more recently, Hitchman and Harrison (1972) interpreted it to be. Although such a plot does not provide an estimate of either n or k_i, the ordinate intercept values reported by Hitchman and Harrison (1972) suggest that k_i for pig CaBP is comparable to that of chick and rat CaBP.

Figure 5, which relates binding affinity to crystal cationic radius, suggests that ionic size is one factor that determines binding affinity, and that Ca has the optimum radius. The similarity between this plot and results for troponin A (Fuchs, 1971) is striking. The selectivity series for the alkaline earths (Ca > Sr > Ba > Mg) does not, however, correspond to any of the seven series predicted by Sherry (Diamond and Wright, 1969) on the basis of rigid anionic site charge density and the hydration energies of the cations. It may be that the binding sites lie within coil regions of CaBP as in carp myogen (Kretsinger and Nockolds, 1973); this would provide a certain amount of steric flexibility for binding while nearby helical regions provide the required conformational stability. CaBP is only 30–40% α helix and undergoes only a small change in helical content when Ca is bound (Ingersoll and Wasserman, 1971).

The fact that high-affinity Ca binding by CaBP is destroyed by chemical denaturants (Ingersoll and Wasserman, 1971) and by heat suggests that it is bound in a polydentate chelate complex. The magnitude of the association constant compared with the stability constants for Ca binding to model compounds (*e.g.*, see Baker and Saroff, 1965) also suggests that 3 or more of the 62 carboxyl groups of CaBP are coordinated with each Ca ion; the precipitous loss of binding activity below pH 5 (Ingersoll and Wasserman, 1971) is no doubt caused by protonation of these ligands. Chemical modification of carboxyl groups results in the loss of Ca binding by collagen (Davis and Walker, 1972), erythrocyte membrane protein (Forstner and Manery, 1971), and to troponin A (McCubbin and Kay, 1973). X-Ray crystallographic structural analysis has shown that carboxyl groups, along with carbonyl oxygens, coordinate in approximate octahedral geometry with Ca in carp myogen (Kretsinger and Nockolds, 1973), thermolysin (Mathews *et al.*, 1972), and to staphylococcal nuclease (Cotton *et al.*, 1971).

The pH dependency of Ca binding to CaBP (Ingersoll and Wasserman, 1971) suggests at least the indirect importance of some of the 32 positively charged residues. In thermolysin a lysine residue neutralizes the excess charge in a "buried" site (Mathews *et al.*, 1972).

The onset of additional calcium binding above 3×10^{-3} M $(Ca)_f$ (Figure 4) is too abrupt to be explained as the filling of a second set of preexisting sites of low affinity but possibly reflects a binding-induced conformational change in the protein (Steinhardt and Reynolds, 1969).

Speculation about the physiological significance of these properties of CaBP is unwarranted until other aspects of the problem are resolved, particularly the exact cellular and subcellular localization of the protein in intestine, kidney, and shell gland.

Acknowledgments

The authors thank and acknowledge the assistance of Mr. Francis Davis with the purification of CaBP, and Dr. D. B. Zilversmit for his laboratory facilities to conduct the lipid analyses and his guidance concerning this aspect of this investigation. The authors also thank Dr. Alex J. Winter for his generous scheduling of time on his amino acid analyzer.

References

Abita, J. P., Delaage, M., Lazdunski, M., and Savrda, J. (1969), *Eur. J. Biochem. 8*, 314.
Alpers, D. H., Lee, S. W., and Avioli, L. V. (1972), *Gastroenterology 62*, 559.
Baker, H. P., and Saroff, H. A. (1965), *Biochemistry 4*, 1670.
Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Benzonanna, G., Capony, J-P., and Pechere, J-F. (1972), *Biochim. Biophys. Acta 278*, 110.
Bigelow, C. C. (1967), *J. Theoret. Biol. 16*, 187.
Boyd, E. M. (1936), *J. Biol. Chem. 114*, 223.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Bull, H. B., and Breese, K. (1973), *Arch. Biochem. Biophys. 158*, 681.
Cessi, C., and Piliego, F. (1960), *Biochem. J. 77*, 508.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, New York, N. Y., Reinhold.
Cotton, F. A., Bier, C. J., Day, V. W., Hazen, E. E., Jr. and Larsen, S. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 243.
Craig, L. C. (1967), *Methods Enzymol. 11*, 870.
Davis, N. R., and Walker, T. E. (1972), *Biochem. Biophys. Res. Commun. 48*, 1656.
Diamond, J. M., and Wright, E. M. (1969), *Annu. Rev. Physiol. 31*, 581.
Dickson, R. E., and Johnson, C. M. (1966), *Appl. Spectrosc. 20*, 214.
Drescher, D., and DeLuca, H. F. (1971), *Biochemistry 10*, 2302.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, E. (1956), *Anal. Chem. 28*, 350.
Ebashi, S., Wakabayashi, T., and Ebashi, F. (1971), *J. Biochem. (Tokyo) 69*, 441.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10*, 2606.
Feder, J., Garrett, L. R., and Wildi, B. S. (1971), *Biochemistry 10*, 4552.
Forstner, J., and Manery, J. F. (1971), *Biochem. J. 125*, 343.
Fuchs, F. (1971), *Biochim. Biophys. Acta 245*, 221.
Fullmer, C. S., and Wasserman, R. H. (1973), *Biochim. Biophys. Acta 317*, 172.
Gibbons, M. N. (1955), *Analyst 80*, 268.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Hartshorne, D. J., and Pyun, H. Y. (1971), *Biochim. Biophys. Acta 229*, 698.
Hitchman, A. J. W., and Harrison, J. E. (1972), *Can. J. Biochem. Physiol. 50*, 758.
Hjerten, S. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 147.
Ingersoll, R. J., and Wasserman, R. H. (1971), *J. Biol. Chem. 246*, 2808.
Jacobs, S. (1964), *Analyst 89*, 489.
Kretsinger, R. H., and Nockolds, C. E. (1973), *J. Biol. Chem.*

248, 3313.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mathews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., and Neurath, H. (1972), *Nature (London), New Biol. 238*, 41.
McCubbin, W. D., and Kay, C. M. (1973), *Biochemistry 12*, 4228.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Morrison, W. R. (1964), *Anal. Biochem. 7*, 218.
Nagy, B., Greaser, M. L., and Gergely, J. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 570 Abstr.
Nanninga, L. B., and Kempen, R. (1971), *Biochemistry 10*, 2449.
Nockolds, C. E., Kretsinger, R. H., Coffee, C. J., and Bradshaw, R. A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 581.
Nyman, P-O., and Lindskog, S. (1964), *Biochim. Biophys. Acta 85*, 141.
Pauling, L. (1960), The Nature of the Chemical Bond and the Structure of Molecules and Crystals, Ithaca, N. Y., Cornell University Press, p 514.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schubert, J. S., Russell, E. R., and Myers, L. S., Jr. (1950), *J. Biol. Chem. 185*, 387.
Sillén, L. G., and Martell, A. E. (1971), *Chem. Soc. Spec. Publ. No. 25*, 14.
Sipos, T., and Merkel, J. R. (1970), *Biochemistry 9*, 2766.
Sottacassa, G., Sandri, G., Panfili, E., de Bernard, B., Gazzotti, P., Vasington, F. D., and Carofoli, E. (1972), *Biochem. Biophys. Res. Commun. 47*, 808.
Stauffer, C. E., and Treptow, R. S. (1973), *Biochim. Biophys. Acta 295*, 457.
Steinhardt, J. and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N. Y., Academic, p 26.
Svennerholm, L. (1957), *Biochim. Biophys. Acta 24*, 604.
Taniuchi, H., Anfinsen, C. B., and Sodja, A. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1235.
Teo, T. S., and Wang, J. H. (1973), *J. Biol. Chem. 248*, 5950.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Wasserman, R. H., and Corradino, R. A. (1971), *Annu. Rev. Biochem. 40*, 501.
Wasserman, R. H., Corradino, R. A., and Taylor, A. N. (1968), *J. Biol. Chem. 243*, 3978.
Wasserman, R. H., and Taylor, A. N. (1966), *Science 152*, 791.
Wasserman, R. H., and Taylor, A. N. (1972), *Annu. Rev. Biochem. 41*, 179.
Wasserman, R. H., Taylor, A. N., and Fullmer, C. S. (1974), *Biochem. Soc. Spec. Publ. No. 3* (in press).
Wilcox, P. E. (1967), *Methods Enzymol. 11*, 63.
Yamada, S., and Tonomura, Y. (1972), *J. Biochem. (Tokyo) 72*, 417.
Zak, B., Moss, N., Boyle, A. J., and Zlatkis, A. (1954), *Anal. Chem. 26*, 776.

Practical Aspects of Calculating Protein Secondary Structure from Circular Dichroism Spectra†

Thomas D. Barela‡ and Dennis W. Darnall*,§

ABSTRACT: Protein secondary structure fractions were calculated from analysis of circular dichroism (CD) spectra assuming that the protein CD spectrum between 190 and 240 nm could be described by a linear combination of model secondary structure spectra (the α helix, β structure, and random coil of poly-L-lysine). This method is identical with that reported by Greenfield and Fasman (*Biochemistry 8*, 4108 (1969)). Wavelength dependence of calculated secondary structure fractions was determined by subdividing the 191–240-nm wavelength region into smaller subregions and calculating structure fractions based on the smaller subregions. Standard deviations for calculated structure fractions were generally less than 4%. Between 190 and 240 nm a particular secondary structure fraction could be varied as much as 39% by altering the wavelengths chosen for analysis. As judged by higher standard deviations about the calculated structure fractions, as well as calculation of negative structure fraction values, the 220–240-nm segment was the region of greatest error. We concluded that the greatest uncertainty associated with the use of this method was the choice of the experimental wavelengths used in the analysis, and further, that no choice of wavelengths could be made which would be useful for all proteins.

Optical rotatory properties of the protein polypeptide backbone have been extremely valuable for examining protein secondary structures in solution. Greenfield *et al.* (1967) and Magar (1968) quantitated secondary structures of proteins from optical rotatory dispersion (ORD) spectra by a least-squares regression analysis of a protein ORD spectrum assuming a linear relationship between the protein spectrum and spectra of the α helix, β structure, and random coil of poly-L-lysine. Technical advantages of circular dichroism (CD) over optical rotatory dispersion led to an analogous

† From the Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003. *Received November 12, 1973.* This work was supported in part by Grant AM-13665 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service. Presented in part at the 166th Annual Meeting of the American Chemical Society, Chicago, Ill., Aug 1973. This work constitutes a portion of the Ph.D. Thesis of T. D. B., New Mexico State University.

‡ Present address: The Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Okla. 73401.
§ Recipient of U. S. Public Health Service Research Career Development Award, GM-32014.