Vitamin D Stimulated Calcium Binding Protein from Rat Intestinal Mucosa. Purification and Some Properties*

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ABSTRACT: Rat intestinal calcium binding protein made in response to vitamin D has been purified to homogeneity. Two main purification procedures have been employed, each of which is suitable for preparative purposes. The calcium binding activity from the supernatant fraction of intestinal mucosa has been purified several 100-fold by means of column chromatography with Sephadex G-100 followed by CM-Sephadex. A second procedure involving gel filtration on Bio-Gel P series resins also yields a homogeneous calcium binding protein. Using the latter procedure another protein having a similar charge as the calcium binding protein has been isolated, and is thought to be a precursor of the calcium binding protein. Homogeneity of the calcium binding protein obtained by these methods has been established by column chromatography, urea disc gel electrophoresis, and ultracentrifugation. A molecular weight of between 8000 and 9000 has been estimated by sedimentation equilibrium ultracentrifugation measurements while a molecular weight of 13,000 has been estimated by means of gel filtration and disc gel electrophoresis.

The appearance of a calcium binding protein was first observed in the supernatant fraction from intestines of rachitic chickens given vitamin D (Taylor and Wasserman, 1965; Wasserman and Taylor, 1966). The calcium binding activity was assayed by means of a chelating resin which competes for radioactive calcium with the soluble binding protein. Calcium binding proteins of a similar nature have since been found in the intestinal mucosa of the rat (Kallfelz *et al.*, 1967; Schachter *et al.*, 1967), dog (Taylor *et al.*, 1968), and monkey and calf (Wasserman, 1970). These binding proteins have been implicated by some investigators as the carriers responsible for the transport of calcium in the small intestine in response to vitamin D (Wasserman and Corradino, 1967; Wasserman *et al.*, 1969).

In initial studies (Wasserman and Taylor, 1966; Kallfelz *et al.*, 1967), both chick and rat calcium binding proteins were partially purified by heat fractionation followed by Sephadex G-100 chromatography. Recently, higher degrees of purity have been obtained by preparative disc electrophoresis of partially purified calcium binding fractions (Wasserman *et al.*, 1968; G. Eilon, unpublished communication, 1970). Some physical properties of the purified chick calcium binding protein have been reported; 1 mole of protein binds 1 mole of calcium ion, and the molecular weight by sedimentation equilibrium is about 25,000 (Wasserman *et al.*, 1968). The molecular weight of the vitamin D dependent rat calcium binding protein has been estimated by calibrated gel filtration to be about 13,000 (Schachter, 1970). There is also a calcium binding protein in rat intestinal mucosa which appears at the void volume of a Sephadex G-100 column and which is present in vitamin D deficient rats (Kallfelz *et al.*, 1968). Thus, two calcium binding proteins, of which only the smaller appears in response to vitamin D, have been observed in our and other laboratories (Kallfelz *et al.*, 1968; Schachter, 1970; J. E. Harrison, unpublished communication 1970). Ooizumi *et al.* (1970) have fractionated two calcium binding proteins of estimated molecular weights of 24,000 and 145,000, both of which they report, appear in response to vitamin D. Since their fractionation techniques were quite different from those of other investigators, further work must be done to explain their apparently unique results.

The present study reports the purification, by means of ion exchange and gel filtration chromatography, of the calcium binding protein which appears in rat intestinal mucosa in response to vitamin D.

A preliminary description of some of the properties of the purified material is presented. Also described in this paper is a protein, devoid of calcium binding activity, which is of special interest because of its similarities to the calcium binding protein in size and charge characteristics.

Materials and Methods

Animals. Male 21-day-old albino rats were obtained from the Holtzman Co., Madison, Wis. They were maintained in hanging wire cages and given food and water *ad libitum*. The rats were fed a purified, vitamin D free diet 24 (1.2% calcium–0.12% phosphorus) described by Guroff *et al.* (1963) which induces rickets in the animals. After 5–6 weeks on this diet the animals became severly rachitic and were suitable for use in the preparation of calcium binding protein.

Chemicals. Crystalline vitamin D_3 (choleclaciferol) was obtained from N. V. Philips-Duphar, Weesp, The Netherlands. Calcium-45 used in the binding assays was from International Chemical and Nuclear Corp., City of Industry, Calif. Coomassie brilliant blue stain for disc electrophoretic gels was a product of Colab Laboratories, Inc., Chicago Heights, Ill. Acrylamide, bisacrylamide, and other reagents used for preparing the polyacrylamide gels were Eastman grade, obtained from Distillation Products Industries, Rochester, N. Y. All other chemicals were of analytical reagent grade.

Column Chromatography. The gel filtration resins used were Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and the Bio-Gels P-60 and P-20, 50–150 mesh (Bio-Rad Laboratories, Richmond, Calif.). The ion-exchange resin used was CM-Sephadex C-25. All resins were allowed to

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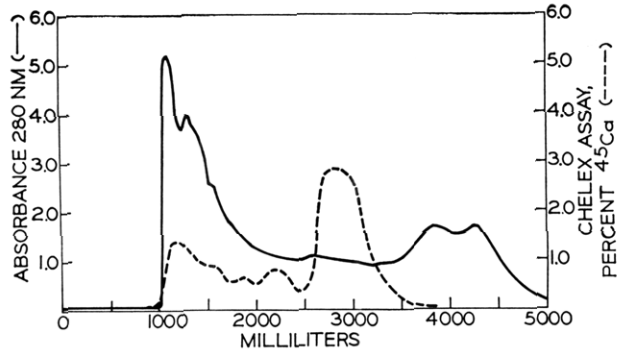

FIGURE 1: Sephadex G-100 chromatogram of the supernatant fraction of intestinal homogenate from rats given vitamin D. Forty rachitic rats were given 250 μg (10,000 IU) vitamin D_3 each 72 hr before sacrifice. The supernatant fraction was prepared and applied to the column as described under Materials and Methods. Column bed dimensions: 7.5 × 70 cm; flow rate: 2 ml/(cm² hr). The column was equilibrated and eluted with 0.08 M NaCl, 0.008% β-mercaptoethanol, and 0.08 M Tris-HCl (pH 7.0). Absorbance at 280 nm (——) or per cent ^{45}Ca by the Chelex assay for calcium binding protein (-----) (see text) is plotted *vs.* milliliters effluent. Vitamin D dependent calcium binding activity is found at about 2.7 void volumes.

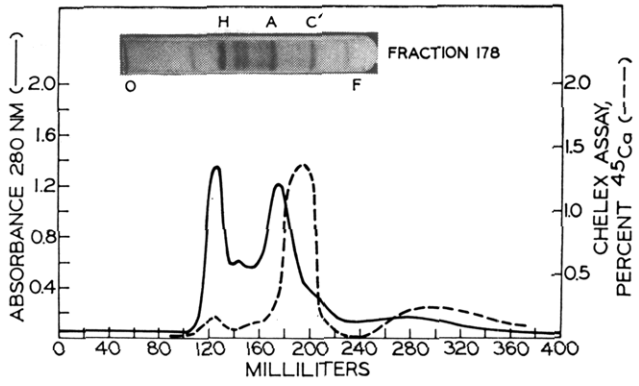

FIGURE 3: Bio-Gel P-60 column chromatogram of proteins associated with calcium binding activity on CM-Sephadex (see Figure 2). Both the peak and shoulder region from the 0.1 M phosphate (pH 7.5) area of a CM-Sephadex chromatogram were combined and concentrated to 5 ml. This was applied to the P-60 column (2 × 97 cm) and eluted with 0.04 M NaCl, 0.008% β-mercaptoethanol, and 0.04 M Tris-HCl (pH 7.0) at a flow rate of 2.3 ml/(cm² hr). The inset is a photo of an electrophoretic gel of the fraction at 178 ml. The origin and front of the gel are marked O and F, respectively.

swell, the fine particles were removed by floatation in deionized water, and were equilibrated with their initial eluent buffer.

Specific dimensions and flow rates of individual columns used in the fractionations are found in the legends to Figures 1–5.

Preparation of Calcium Binding Protein. The vitamin D deficient rats were each given 250 μg of vitamin D as described in Figure 1. Seventy-two hours later they were killed by decapitation and 12 cm of small intestine was excised, slit lengthwise, and immediately rinsed in ice-cold isotonic saline. Washed intestines were quickly frozen to −70° in a beaker submerged in Dry Ice–acetone. A mixture of frozen intestines and an equal amount of Dry Ice was placed in a large mortar, and the tissue was ground to the consistency of fine gravel. The material was homogenized 1.5 min at high speed in a Waring Blendor in a solution of 0.08 M NaCl, 0.008% β-mercaptoethanol, and 0.08 M Tris-HCl (pH 7.0). Approximately 5 ml of solution/intestine was used. The homogenate was centrifuged for 30 min at 40,000*g* in a refrigerated centrifuge and the supernatant retained. The sediment was resuspended and the extraction procedure repeated. The second supernatant was combined with the first, and concentrated in a 400-ml Amicon ultrafiltration cell at 0° using Diaflo UM2 membrane (Amicon Corp., Lexington, Mass.). Final sample volume was 100 ml, which was applied to the Sephadex G-100 column of Figure 1. In all the chromatographic separations the recovery of protein was 90–100%.

Assay for Calcium Binding Protein. The Chelex method for assay of calcium binding protein (Wasserman *et al.*, 1968) was used. The assay depends upon the competition between the chelating resin Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) and the soluble calcium binding protein for added ^{45}Ca (Wasserman *et al.*, 1968). In practice, 1 ml of sample was pipetted into a 3-ml centrifuge tube. A 0.2-ml volume of rapidly stirred, suspended resin (1:1, wet resin–buffer) was pipetted into the tube and immediately swirled several times on a rotary mixer. The buffer was the same as

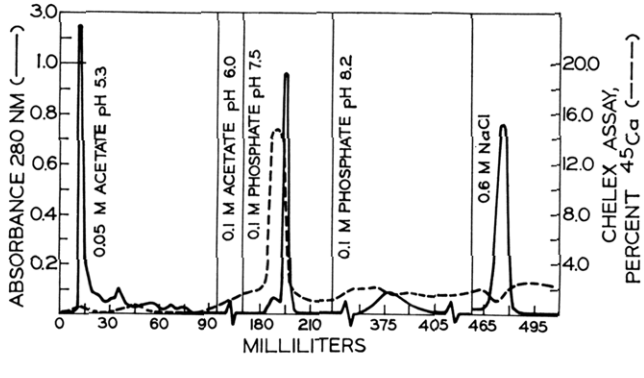

FIGURE 2: Chromatography of crude calcium binding protein from a Sephadex G-100 column on CM-Sephadex. Ordinate and abscissa are marked in the same manner as in Figure 1. Material from the Sephadex G-100 region showing calcium binding activity (Figure 1) was applied to a 1 × 36.5 cm column equilibrated with 0.05 M acetate (pH 5.3). Sample volume was 1 ml and the flow rate was 9 ml/(cm² hr). Stepwise elution was carried out with various buffers as indicated by vertical lines in the figure. All buffers were 0.008% in β-mercaptoethanol. Flow rate was doubled after the activity had been eluted.

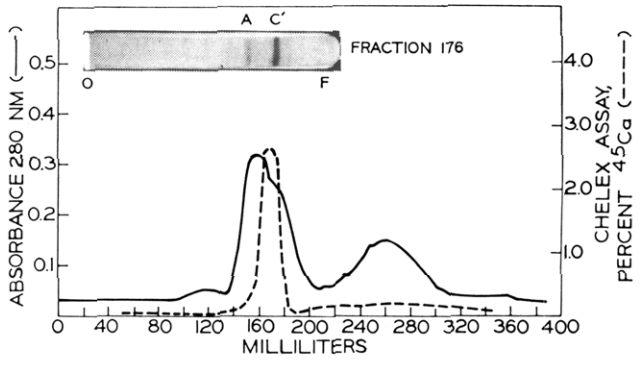

FIGURE 4: Bio-Gel P-60 elution diagram of the region of calcium binding activity from previous P-60 column (Figure 3). The procedure was similar to that described in Figure 3. The sample applied to the disk gel (see inset) was the fraction at 176 ml; bands A and C′ are marked as before. Letters O and F mark the gel origin and front, respectively.

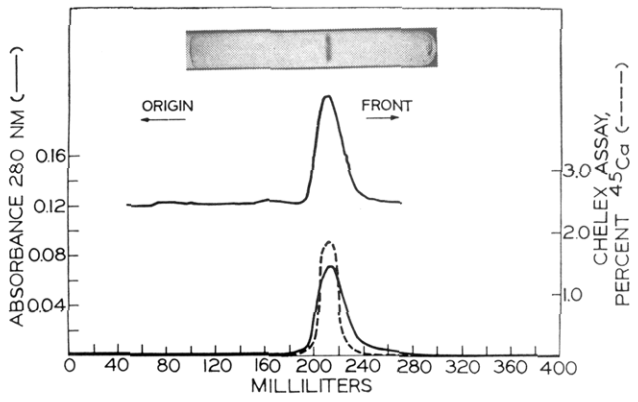

FIGURE 5: Bio-Gel P-20 column chromatography of calcium binding protein from the Bio-Gel P-60 column (Figure 4). A sample of 2 ml volume was applied to the P-20 column (2.2 × 100 cm) and eluted with 0.04 M NaCl, 0.008% β-mercaptoethanol, 0.04 M Tris HCl (pH 7.0) at a flow rate of 1.6 ml/(cm² hr). A photo of the disk gel and densitometric tracing (not in the same scale) of the calcium binding activity peak (band C′, Figure 4) are included. Orientation of the gel is indicated.

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

Fractionation	Sp Act. (%/(mg ml))	Rel Sp Act.
Mucosal supernatant fraction from rats given vitamin D (250 μg)	0.8	1.0
Sephadex G-100 column (Figure 1)	8.8	11.4
First Bio-Gel P-60 column (Figure 3)	11.1	14.4
Second Bio-Gel P-60 column (Figure 4)	31.8	41.2
Bio-Gel P-20 column (Figure 5)	44.1	57.4
Calcium binding fraction from vitamin D deficient rats after Sephadex G-100 fractionation	2.3	
CM-Sephadex columns, initial fractions from calcium binding fraction (Figure 2)	101.3, 795.9	

[a] Fractions were assayed by the Chelex method described in the text. For each chromatogram, the activity in the peak tube was used for calculation of specific activity. Protein concentration was estimated by the expression $A = Ecl$, where A is the absorbance at 280 nm, c is concentration in milligrams per milliliter, and l is the light path length (1 cm). The specific extinction coefficient, E, is set equal to 1.5 mg/(ml cm); that is, the estimate assumes that a protein concentration of 1.0 mg/ml has an absorbance of 1.5 at 280 nm. Specific activity was calculated by dividing the per cent of total ^{45}Ca above background in the supernatant of the Chelex-100 assay tube by the concentration in milligrams per milliliter of protein in the same tube. The mucosal supernatant in the table represents material from rats dosed as described in Figure 1 and prepared as described in the Materials and Methods section. The purification steps involving columns previously illustrated are so designated. Only the calcium binding protein which appears in response to vitamin D is reported in the tabulated results. The supernatant fraction from vitamin D deficient rats reveals a lowered specific activity in the calcium binding protein region of the Sephadex G-100 profile and is included in the table for comparison. The initial fractions of the calcium binding peak from CM-Sephadex yielded low amounts of protein of high specific activity; two representative activity values are listed.

that used for the Sephadex G-100 column (Figure 1). Next, 100 μg of ^{45}Ca solution of approximately 10 μCi/ml was added, and the contents of the tube mixed thoroughly for 60 sec. The resin was sedimented by centrifugation and samples of the supernatant fluid were transferred onto circles of Whatman No. 5203-A filter paper (23 mm diameter). The circles of filter paper were dried in an oven at 80°, placed in counting vials containing a toluene-counting solution, and the ^{45}Ca was determined by means of a Packard Tri-Carb Model 3003 liquid scintillation counter. The counting solution consisted of 0.5% 2,5-diphenyloxazole and 0.025% 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene dissolved in toluene. No quench corrections were found necessary.

Aliquots of supernatant fluid from tubes containing Chelex plus buffer in place of sample gave a low level of radioactivity which represents the ^{45}Ca not bound by the resin (Wasserman *et al.*, 1968). This blank radioactivity equalled the amount found in the case of fractions lacking calcium binding activity. The total ^{45}Ca present was determined by adding 100 μg of ^{45}Ca solution of 1.1 ml of buffer, without Chelex, and counting the resulting solution. Then the calcium binding activity, in units of % supernatant ^{45}Ca, could be calculated from the expression [(S) − (B)]100%/(T) where S, B, and T are the supernatant, blank, and total ^{45}Ca, respectively. Specific activity calculations are discussed in Table I. Protein concentration was determined on the basis of absorbancy at 280 nm with a Beckman DB-G spectrophotometer.

Disc Gel Electrophoresis. The electrophoretic system used to monitor purification was a 7% acrylamide, 9 M urea gel described by Neville (1967). The components other than the acrylamide and bis acrylamide were weighed out into a Pyrex test tube marked at 40 ml. Ten milliliters of a 0.004% aqueous riboflavin solution was added and the mixture was heated to 80° in a water bath with swirling to dissolve the urea. The acrylamide and bisacrylamide were weighed out in a separate test tube and just enough water added to effect solution. The acrylamide solution was quickly poured into the urea–catalyst solution, the volume brought to 40 ml, and dissolved air was removed by means of an aspirator. The gel solution was poured to a height of 90 mm into mounted 8 × 130 mm Pyrex tubes and overlaid with 25 μg of water. The acrylamide was polymerized by means of a fluorescent light. The spacer gel was prepared in a 10-ml volume in a similar manner, and 2.5 ml of 0.004% riboflavin was added. After removing water from the top of the separation gel and rinsing with unpolymerized spacer gel solution, 0.1 ml of the spacer gel was added per tube. The spacer gel was partially polymerized under fluorescent light and no water overlay was used.

Samples for disc electrophoresis were prepared as described by Neville (1967). The apparatus was similar to that of Davis (1964), and electrophoresis was run 6–7 hr under the conditions described by Neville (1967) until the marker dye reached the end of the gel. The gels were removed and exposed for 30 min to 0.05% coomassie blue in 12.5% trichloroacetic acid to stain the proteins as described by Chrambach *et al.* (1967). Removal of the excess coomassie blue from the gels was

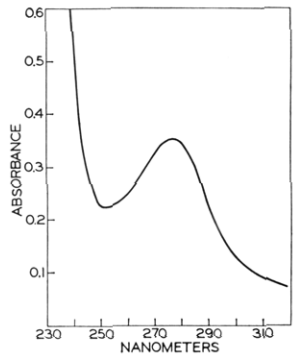

FIGURE 6: Ultraviolet spectrum of purified calcium binding protein. The sample was taken from the active peak eluted from the Bio-Gel P-20 column (Figure 5).

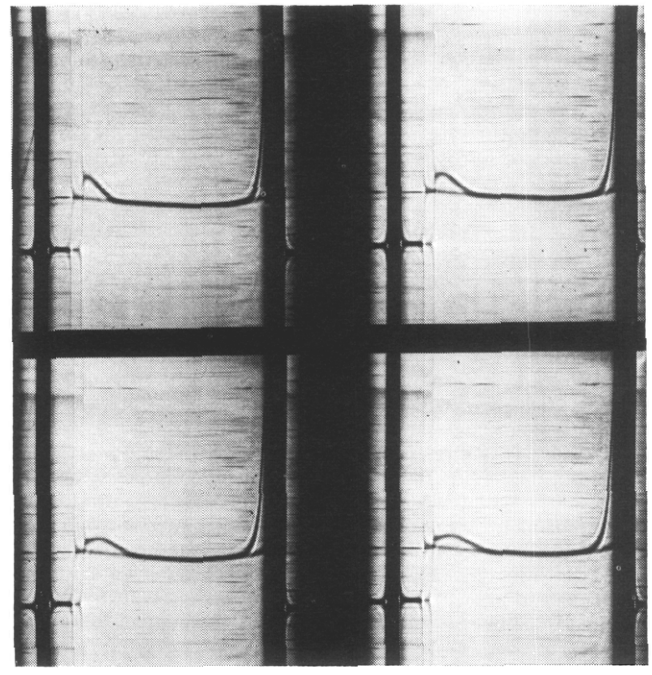

FIGURE 7: Sedimentation of purified calcium binding protein in the analytical ultracentrifuge. The sample used was concentrated material from the Bio-Gel P-20 peak (Figure 5). Exposures of plates (left to right) were taken at 0, 12, 36, and 48 min at 59,780 rpm at 20° in 0.04 M NaCl, 0.008% β-mercaptoethanol, and 0.04 M Tris-HCl (pH 7.0) bar angle 60°.

accomplished with 10% trichloroacetic acid at 45° for 6–12 hr. The gels could be kept in excellent condition for 1 year or more by equilibrating and storing them with an aqueous solution of 7% acetic acid–35% ethylene glycol at −10°. After storage, proteins on the gels can be restained as described above.

Photographs. Gels were positioned in small corked test tubes completely filled with 12.5% trichloroacetic acid, and illuminated from behind with fluorescent light. A Nikomat camera with a Micro-Nikkor Auto 1:3.5 lens, f = 55 m (Nikon, Japan) at a distance of 24 cm and Kodak Plus-X Pan film were used.

Ultraviolet Spectra. Ultraviolet spectra were recorded with a Beckman DB-G recording spectrophotometer.

Analytical Ultracentrifuge Runs. A Spinco Model E analytical ultracentrifuge was used for all measurements. The conditions of the sedimentation velocity experiment are listed in Figure 6 and those of the equilibrium experiment (Yphantis, 1964) in Figure 7. In the determination of molecular weight, fringes were measured with a Gaertner microcomparator.

Results

Preparation of Crude Calcium Binding Protein. The first step in purification of calcium binding protein described by others (Kallfelz *et al.*, 1967; Wasserman *et al.*, 1968) using heat denaturation was avoided because of the possibility of altering the native state of the calcium binding protein. The next step in the purification of calcium binding protein from supernatant was similar to that described by Kallfelz *et al.* (1967) using Sephadex G-100. The 40,000g intestinal supernatant was immediately concentrated and directly applied to the large preparative Sephadex G-100 column (Figure 1). A peak of calcium binding activity as a result of vitamin D supplementation was eluted at approximately 2.7 void volumes, as shown by previous investigators for heat-treated supernatant fraction (Kallfelz *et al.*, 1967; Avioli *et al.*, 1969).

Purification of Calcium Binding Protein with CM-Sephadex. A portion of the calcium binding protein peak from the G-100 Sephadex fraction shown in Figure 1 was applied to a CM-Sephadex column. The elution profile (Figure 2) reveals only one calcium binding peak. A large amount of protein lacking calcium binding activity was eluted at one void volume, which on disc electrophoresis reveals several proteins.

Of the two protein peaks eluted with 0.1 M phosphate (pH 7.5) only the smaller one possesses calcium binding activity. Material from the initial fraction of the smaller peak showed a single protein band (C′) on disc gel electrophoresis (see Figure 3). The last fractions of the smaller peak contained significant amounts of another protein (band A on electrophoresis, see Figure 3). The larger peak contained mostly another protein (band H on electrophoresis, Figure 3). Thus the order of elution of the three proteins on CM-Sephadex was C′, A, and H.

Further development of the CM-Sephadex column (Figure 2) with 0.1 M phosphate (pH 8.2) eluted another peak, probably hemoglobin because of its reddish color. The last peak from the CM-Sephadex column was eluted with 0.6 M NaCl. At this point all of the applied protein had been accounted for.

Separation of Proteins Associated with Calcium Binding Activity by Means of Gel Filtration. Figure 3 shows the chromatogram resulting from the application of the calcium binding fractions from the CM-Sephadex column to a Bio-Gel P-60 column. Calcium binding activity was located in the shoulder region at about 200-ml elution volume (Figure 3). All three of the major proteins detected by disc electrophoresis of the calcium binding region from the CM-Sephadex chromatogram (bands C′, A, and H) again appeared in the P-60 chromatogram (inset Figure 3). The calcium binding activity region of Figure 3 was rechromatographed on another Bio-Gel P-60 column and its elution profile is shown in Figure 4. Only two major staining proteins remained, *i.e.*, electrophoretic bands A and C′. The protein which eluted at 260 ml did not stain with coomassie blue. Calcium binding activity appeared to be associated with the second, lower molecular weight protein, *i.e.*, band C′.

Isolation of a Single Protein with Calcium Binding Activity. Figure 5 shows a further purification of the calcium binding protein on Bio-Gel P-20. Activity was now associated with one chromatographic peak. The peak was shown to be essentially one component on disc gel electrophoresis (band C′). The

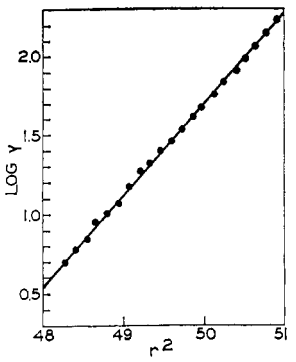

FIGURE 8: High-speed sedimentation equilibrium of purified calcium binding protein. Log y is plotted *vs.* r^2 after the method of Yphantis (1964), where y is the fringe displacement. The sedimentation equilibrium was performed at 12° in a double-sector cell. Equilibrium was achieved after 20 hr at 50,740 rpm. The concentration of protein was 0.5 mg/ml in 0.04 M NaCl, 0.008% β-mercaptoethanol, and 0.05 M Tris-HCl (pH 7.0). Molecular weight was calculated by means of the expression $2RT(2.303)(\text{slope})/(1 - \bar{v}\rho)(\omega^2)$ with the assigned values of $\bar{v} = 0.73$ and $\rho = 1.0$. By this method, the molecular weight of calcium binding protein is found to be 8200.

densitometric tracing (Figure 5) of disc gels in which relatively high concentrations of protein were electrophoresed indicated very small amounts of two higher molecular weight proteins. At lower sample concentrations, these components were not visible.

Ultraviolet Spectrum. Figure 6 shows an ultraviolet absorption spectrum of the purified calcium binding protein of Figure 5. There is a well-defined peak centering at 275 nm, with a slight shoulder at 290 nm.

Sedimentation velocity photographs of the calcium binding protein are shown in Figure 7. Even with the maximum rotor speeds available (59,780 rpm) the separation of the protein boundary from the meniscus failed to occur. However, it appeared that no higher molecular weight proteins were present in the sample.

Molecular Weight by Sedimentation Equilibrium. A sedimentation equilibrium measurement on the binding protein was also performed. A plot of log y *vs.* r^2 (Figure 8) yielded essentially a straight line, indicating the presence of a single component. The molecular weight value obtained from the data was 8200, assuming a partial specific volume of 0.73.

Specific Activity Increase with Purification. Attainment of increased purity as revealed by disc electrophoresis was accompanied by an increase in specific calcium binding activity. Table I summarizes the increase in specific activity of the calcium binding protein accompanying purification. The data concern only the lower molecular weight calcium binding protein which appears following vitamin D administration. Included in the table is the specific activity of the calcium binding region from the G-100 profile for intestinal supernatant taken from vitamin D deficient rats. The specific activity in this case is one-fourth to one-fifth the value for corresponding rats given vitamin D. The purifications illustrated in Figures 1–5 actually represent two routes. A very high degree of purification is achieved by the CM-Sephadex procedure and at least a portion of the isolated protein is homogeneous on disc electrophoresis. However, the yield of protein of this purity is very low. The gel filtration steps with Bio-Gel columns succeed in purifying a larger quantity of material, although the specific activity of the final product is lower, due in part to the limited stability of the calcium binding protein with time.

Discussion

Preparation of Crude Calcium Binding Protein without Ammonium Sulfate or Heat. The Sephadex G-100 chromatographic procedure used in this study as the initial fractionation step appeared superior to the heat or ammonium sulfate fractionation methods used by other investigators because the latter procedures result in an accelerated loss of binding activity during subsequent purification. Such activity loss is a serious problem for the isolation of the protein from rats since the yield in this case is low (perhaps 20–50 μg/rat in these experiments). Also, we have observed that heat and ammonium sulfate treatments result in the artifactual appearance of a new peak at about 1.6 void volumes on Sephadex G-100 chromatograms, apparently the result of disaggregation of large proteins into subunits. Such observations suggest that these fractionation methods may be capable of altering the native state of proteins with calcium binding activity. Accordingly, one possible explanation for the results of Ooizumi *et al.* (1970) may be that they observed a 24,000 molecular weight disaggregation product of the larger (>100,000 molecular weight) calcium binding protein, after heating to 60° for 5 min. Their use of a Diaflo UM-20E membrane for protein concentration indicates that they are studying mainly proteins above 15,000 molecular weight, according to the retention characteristics of the UM-20E membrane (Amicon Publication No. 403, 1970). As mentioned earlier, the work in this paper deals solely with the smaller (13,000 or lower molecular weight) calcium binding protein.

Purification of the Calcium Binding Protein with CM-Sephadex. The CM-Sephadex procedure is very effective in separating calcium binding protein from other contaminating proteins. As can be surmised from Figure 2, the CM-Sephadex method alone can be used for preparing quite pure calcium binding protein if used subsequent to the Sephadex G-100 procedure.

At least five void volumes of each of the acetate buffers must be used for successful operation of the CM-Sephadex column. The use of a lower pH for the first 0.1 M phosphate elution, such as pH 7.0 instead of pH 7.5, results in a more pronounced resolution of the proteins in the calcium binding region. However, it has not been possible to separate the smaller peak completely from the larger, and the calcium binding protein is usually contaminated to some extent with a closely related protein (band A on disc electrophoresis).

Purification by Gel Filtration. The gel filtration procedures using the Bio-Gel columns provide the advantage that proteins similar in size to the calcium binding protein can readily be isolated. This is particularly important since a protein (A on disc gel electrophoresis) believed to be a precursor of the calcium binding protein (Drescher and DeLuca, 1971) can be isolated. That this protein is similar in size and charge to the calcium binding protein is shown by its behavior on CM-Sephadex, Bio-Gel columns, and disc electrophoresis.

The purified calcium binding protein (Figure 5) had only minor impurities which increased on standing at 4°. Minor impurities can readily be detected on the gels since the urea disc electrophoretic system and the staining procedure employed are both extremely sensitive analytical methods (Neville, 1967; Chrambach *et al.*, 1967). The urea used in the gels was passed through a mixed bed ion-exchange resin and recrystallized before use. Purification of the urea in this fashion had no effect on the disc gel electrophoretic results.

Molecular Weight of Calcium Binding Protein. Data from the ultracentrifuge velocity measurement (Figure 7) and equi-

librium measurement (Figure 8) indicate that the molecular weight of the calcium binding protein is surprisingly small. Making the assumptions listed in Figure 8, a value of 8200 is obtained. On the other hand, the estimate of molecular weight by position of activity on calibrated Sephadex G-100 columns and other gel filtration columns, both in ours and other laboratories, has consistently given values around 13,000. A similar value is obtained by means of calibrated urea disc gels in our laboratory. The question arises as to the reason for the discrepancy between molecular weight values obtained by gel filtration *vs.* ultracentrifugation. The striking linearity of the data of Figure 8 argues against a lower molecular weight contaminant in the ultracentrifuge sample. We therefore investigated the possibility of asymmetry of the molecule.

The calcium binding protein is retained by Amicon Diaflo UM-2 membranes, which are rated to retain proteins of at least 2000 molecular weight (Amicon Corp., 1970). However, the protein is not retained by Diaflo PM-10 membranes which are rated to retain molecules the size of cytochrome *c* (12,400 molecular weight, from horse heart) and above. Yet the rat calcium binding protein migrates behind cytochrome *c* on gel filtration and disc gel systems. When cytochrome *c* standard (horse heart, Sigma, St. Louis, Mo.) was run in exactly the same manner as calcium binding protein for determination of molecular weight, a value very close to its true molecular weight was obtained, further supporting the validity of the low molecular weight value for calcium binding protein from ultracentrifuge data.

The results of molecular weight determination for calcium binding protein can be explained in two ways. First, the molecule may have an ellipsoidal shape, as do the postulated bacterial transport carrier proteins (Pardee, 1968). Secondly, the partial specific volume may be higher than the assumed value of 0.73, as might result from bound lipid. At present, the first hypothesis is favored because of analogy to bacterial membrane carriers, but the question has yet to be resolved.

Specific Activity of Purified Calcium Binding Protein. The increase in specific activity of the calcium binding protein with purification by the gel filtration columns is similar to that described for purification of the chick calcium binding protein (Wasserman *et al.*, 1968). The larger apparent increase in specific activity at the CM-Sephadex step may be due to the following causes. First, the sampling of low concentrations of protein can give overestimates of specific activity. Secondly, the shorter time period involved in two *vs.* five column steps allows less opportunity for loss of activity of the calcium binding protein.

It should also be noted that specific activities are compared to the base value of supernatant fraction and not to whole homogenate. This was done because of the great variability experienced in measuring calcium binding activity in whole homogenate using the Chelex assay. There is also present a relatively large amount of a high molecular weight calcium binding protein not related to vitamin D in rat intestinal homogenate, as discussed earlier. Hence the first actual purification step, namely the 40,000*g* centrifugation, is not included in the calculation of relative specific activity increases. Compared to supernatant values, at least a 57-fold purification of calcium binding activity has been achieved by gel filtration, and several 100-fold purification by ion exchange.

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