Yang, J. T., Chen, G. C., & Jirgensons, B. (1976) *Handbook of Biochemistry and Molecular Biology* (Fasman, G. D., Ed.) 3rd ed., Vol. III, pp 3–13, CRC Press, Cleveland, OH.

Yazawa, M., & Yagi, K. (1977) *J. Biochem.* (*Tokyo*) *82*, 287–289.

Yazawa, M., Sakuma, M., & Yagi, K. (1980) *J. Biochem.* (*Tokyo*) *87*, 1313–1320.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Bovine Dentin Phosphophoryn: Composition and Molecular Weight[†]

W. G. Stetler-Stevenson and A. Veis*

ABSTRACT: The molecular weight of phosphophoryn, an acidic phosphoprotein unique to dentin matrix, has been difficult to determine because of a combination of neutral protease activities in this tissue and the intrinsic high charge density of the molecule. In this study, bovine dentin phosphophoryn (BDPP) was isolated by a procedure designed to prevent proteolysis. Bovine unerupted third molar powder was demineralized by ethylenediaminetetraacetic acid (EDTA). The EDTA-soluble phosphophoryn fraction was isolated and purified by sequential calcium chloride precipitation, gel filtration in sodium dodecyl sulfate ($NaDodSO_4$) containing buffer, anion-exchange chromatography, and finally gel filtration in 4 M guanidine hydrochloride (4 M Gdn·HCl) buffer. Sedimentation equilibrium, sedimentation velocity, and diffusion coefficient data, viscosity studies in a high ionic strength buffer, and $NaDodSO_4$ gradient gel electrophoresis data gave consistent results for the molecular weight of BDPP, all being in the range of 151 000–167 000. This range is much higher than any previously reported value. An anomalous behavior was observed in nongradient $NaDodSO_4$ gel electrophoresis. Dissociative analytical gel filtration chromatography in 4 M Gdn·HCl gave a molecular weight value of 100 000. This discrepancy was resolved by studying the viscosity of BDPP in 4 M Gdn·HCl which showed BDPP does not assume a true random-chain conformation in this solvent.

Phosphophoryns are major protein constituents of dentin extracellular matrix, accounting for as much as 50% of the noncollagenous proteins in some species. They are extremely rich in aspartyl, seryl, and phosphoseryl residues. Phosphophoryns bind calcium ions with high affinity (Lee et al., 1977; Zanetti et al., 1981) and can be preferentially precipitated by relatively high concentrations of calcium ion (Kuboki et al., 1979; Butler et al., 1981). They have been shown to markedly affect the in vitro crystallization of hydroxyapatite (Nawrot et al., 1976; DeSteno et al., 1975; Termine & Conn, 1976; Termine et al., 1980c). Autoradiographic (Weinstock & Leblond, 1973), biosynthetic (Dimuzio & Veis, 1978b; Maier et al., 1983), and analytical studies (Carmichael & Dodd, 1973) support the hypothesis that phosphophoryns are rapidly synthesized and secreted directly at the mineralization front. On the basis of these varied data, phosphophoryns have been postulated to play a number of important roles in normal dentinogenesis including nucleation and initial localization of calcium phosphate crystals within the hole regions of the collagen matrix (Butler, 1972; Veis, 1978; Dimuzio & Veis, 1978b; Glimcher, 1981; Veis et al., 1981).

However, discrepancies still exist in the literature concerning the molecular sizes and amino acid compositions of the phosphophoryns. Values for the molecular weight of bovine dentin phosphophoryn determined in earlier studies are on the order of 35 000–39 000 (Veis et al., 1972; Lee et al., 1977). Typically, these earlier studies failed to prevent proteolysis during demineralization and isolation procedures. Recent studies have shown that the strict prevention of proteolysis during isolation yields fetal bovine dentin phosphophoryn with a molecular weight on the order of 100 000 as determined by gel filtration in 4 M guanidine hydrochloride (Gdn·HCl) and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis (Termine et al., 1980b).

Recent studies of rat incisor dentin have shown that the rat incisor has at least two highly phosphorylated phosphoproteins (Dimuzio & Veis, 1978a,b; Linde et al., 1980; Butler et al., 1981). Different methods have given widely varying results for the molecular weights of these rat incisor phosphophoryns. For example, gel filtration chromatography in 3 M Gdn·HCl gave values of 65 000–71 000 for the molecular weights (Dimuzio & Veis, 1978a) whereas high-speed sedimentation equilibrium analysis performed in 0.1 M phosphate buffers containing 1 mM ethylenediaminetetraacetic acid (EDTA) at neutral pH showed no apparent concentration-dependent behavior and gave a molecular weight estimate of 30 000 (Jontell & Linde, 1977; Jontell et al., 1980). $NaDodSO_4$ gel electrophoresis has also been used to estimate the molecular weight of rat incisor phosphophoryns. When enzymatically dephosphorylated rat incisor phosphophoryns were run on $NaDodSO_4$ gel electrophoresis (7.5% acrylamide), a value of 72 000 was obtained for the molecular weight (Linde et al., 1980). When corrected for the addition of the phosphate, the estimate of the molecular weight became about 100 000. More recently, values of about 90 000 for the phosphorylated phosphophoryns have been obtained by using an acrylamide gradient $NaDodSO_4$ electrophoresis system (Butler et al., 1981). However, Jontell et al. (1982) have recently shown that rat incisor phosphophoryns behave anomalously in $NaDodSO_4$ gel electrophoresis systems as well as gel filtration chromatography in 6 M Gdn·HCl. Using sedimentation equilibrium analysis, these investigators also found nonideal behavior of the phosphophoryns from rat incisors in 1 M NaCl solutions. However, after dephosphorylation, these proteins showed ideal behavior in the analytical ultracentrifuge. The molecular weight of the dephosphorylated phosphophoryn was determined by this method to be 28 000. When corrected for

---

[†] From the Departments of Oral Biology and Molecular Biology (Biochemistry Program), Northwestern University Medical and Dental Schools, Chicago, Illinois 60611. *Received December 20, 1982.* This research has been supported by a grant from the National Institute for Dental Research (DE-01734).

* Address correspondence to this author at the Department of Oral Biology, Northwestern University Dental School.

0006-2960/83/0422-4326$01.50/0

the addition of the phosphate, this value increased to 38 000. These divergent values for the M_r of the rat incisor phosphophoryns have not been adequately explained and remain a major point of confusion.

The use of sulfonated polystyrene columns for the purification of phosphophoryns results in the removal of a nonpolar amino acid fraction (Munksgaard et al., 1977; Linde et al., 1980). This has led these investigators to question the purity of other phosphophoryn preparations. It has been suggested that phosphophoryns purified by Dowex resins represent degradation products enriched in aspartyl, seryl, and phosphoseryl residues by removal of intervening sequences containing nonpolar amino acid residues (Veis et al., 1981).

Kuboki et al. (1979), using calcium chloride to precipitate the phosphophoryns from bovine dentin prior to further purification by anion-exchange chromatography, also found few nonpolar amino acids in their purified phosphophoryns. However, these investigators did not add protease inhibitors during the demineralization and subsequent purification.

In this report, we describe the isolation, purification, and initial hydrodynamic characterization of an undegraded bovine dentin phosphophoryn (BDPP). Extraction was carried out by using EDTA demineralization at pH 7.5 in the presence of protease inhibitors. Subsequent purification was done in the presence of either protease inhibitors or 0.1% NaDodSO$_4$. The molecular weight of BDPP characterized by several techniques is significantly higher than that for any preparation previously reported. The discrepancy between values reported here for the molecular weight of BDPP and values from analytical gel filtration in 4 M Gdn·HCl is resolved by viscosity studies of BDPP in 4 M Gdn·HCl.

Materials and Methods

Tissue Preparation. Mandibles from cattle approximately 2 years old were collected and stored on ice immediately after slaughter. All subsequent operations were performed at 4 °C. Unerupted third molars were extracted; the periodontal sac, pulp, and other adherent soft tissues were removed mechanically. The teeth were rinsed with cold tap H_2O before being soaked overnight in 15% (w/v) NaCl containing the following protease inhibitors: 50 mM ϵ-amino-*n*-caproic acid, 2.5 mM benzamidine hydrochloride, 0.5 mM *N*-ethylmaleimide (NEM), and 0.3 mM phenylmethanesulfonyl fluoride (PMSF). The teeth were rinsed in distilled H_2O, frozen in liquid nitrogen, lyophilized, and stored at −20 °C.

Dried teeth were immersed in liquid nitrogen prior to being splintered with a hammer. The teeth shards were milled to a coarse powder in a prechilled mill (CRC, 4 °C) by using repeated short power bursts. The powder was sieved by using a 2-mm mesh (Newark Wire Cloth Co.), and then it was suspended in cold 15% (w/v) NaCl containing protease inhibitors as before. The resulting suspension was allowed to settle overnight. The salt solution was decanted and discarded. The residue was washed 3 times with cold distilled H_2O before it was frozen, lyophilized, and stored at −20 °C.

Demineralization of Bovine Molar Powder. Bovine molar powder was demineralized in 0.5 M EDTA, 50 mM ϵ-amino-*n*-caproic acid, 2.5 mM benzamidine hydrochloride, 0.5 mM NEM, and 0.3 mM PMSF, pH 7.5 (EDTA + PI), by using Spectra Por 6 dialysis tubing with a molecular weight cutoff (MWCO) of 3500 (70 mL of EDTA + PI per g of bovine molar powder). Demineralization was carried out at 4 °C. The outer dialysate solution was changed for fresh EDTA + PI every fourth day. The demineralization was discontinued after 14 days. The dialysis bags containing the EDTA-soluble and -insoluble fractions were then dialyzed against 50 mM ϵ-amino-*n*-caproic acid, 2.5 mM benzamidine hydrochloride, 0.5 mM NEM, and 0.3 mM PMSF, pH 7.5, to remove EDTA.

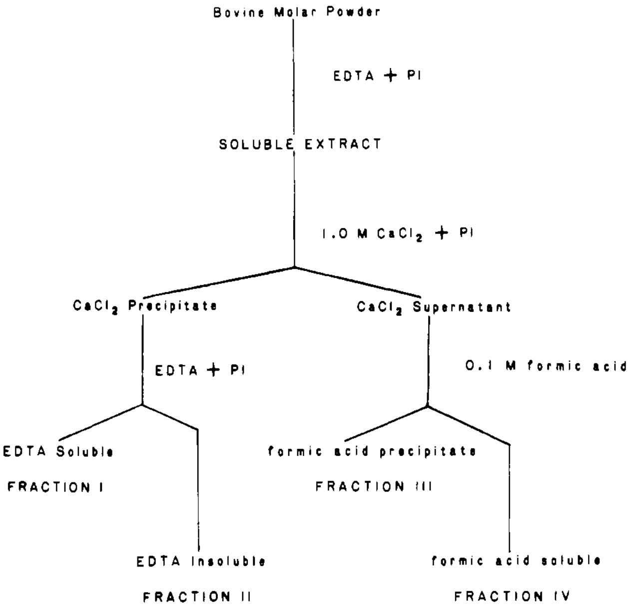

FIGURE 1: Flow diagram for isolation of bovine dentin phosphophoryn.

The contents of the dialysis sacs were removed, and the particulate residue was allowed to settle. The supernatant was decanted. The residue was washed with distilled H_2O; the wash and supernatant were combined and centrifuged in a Sorvall RC-2B centrifuge, using a GSA rotor, at 10 000 rpm for 30 min at 4 °C. The pellet was combined with the residue of the decanting step, frozen, and lyophilized. The soluble EDTA extract was dialyzed against H_2O by using Spectra Por 6 dialysis membranes (MWCO = 3500) and freeze-dried.

Precipitation of Bovine Dentin Phosphophoryn. Phosphophoryn was isolated from the soluble EDTA extract by the procedure summarized in Figure 1. Crude phosphophoryn was precipitated from the soluble EDTA extract by the method of Kuboki et al. (1979) as modified by Butler et al. (1981). The EDTA extract was dissolved (25 mg/mL) in cold 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.3, containing the same protease inhibitors as previously described, by stirring on ice. Solid $CaCl_2$ was slowly added to the solution until a final concentration of 1.0 M $CaCl_2$ was reached. The suspension was allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation in a Sorvall RC-2B centrifuge using an HB-4 rotor at 13 000 rpm for 15 min at 4 °C. The $CaCl_2$ precipitate was washed twice with 5 mL of 1.0 M $CaCl_2$ and 20 mM Tris-HCl, pH 8.3, containing protease inhibitors and then was resuspended in cold 0.5 M EDTA + PI. After the mixture was stirred for 2 h on ice, the supernatant was collected by centrifugation at 13 000 rpm as above. This supernatant (fraction I, Figure 1) was concentrated by ultrafiltration through an Amicon PM10 membrane. The buffer was changed to 0.34 M glycine, 50 mM Tris, and 0.1% (w/v) NaDodSO$_4$, pH 8.3 (GTS buffer), by diafiltration (Amicon PM10 membrane, MWCO = 10 000). The pellet that was not resolubilized by 0.5 M EDTA + PI (fraction II, Figure 1) was resuspended in distilled H_2O and dialyzed against distilled H_2O by using Spectra Por 6 membranes (MWCO = 3500) and then frozen, lyophilized, and stored at −20 °C.

The supernatant from the $CaCl_2$ precipitation step was further fractionated by dialysis against 0.1 M formic acid at 4 °C in Spectra Por 6 dialysis tubing (MWCO = 3500). This resulted in the formation of another precipitate in the dialysis bag. This precipitate (fraction III, Figure 1) was separated from the 0.1 M formic acid supernatant (fraction IV, Figure 1) by centrifugation at 13 000 rpm as described above. Fractions III and IV were frozen, lyophilized, and stored frozen at −20 °C.

Preparative Gel Filtration Chromatography. The GTS buffer solutions of crude phosphophoryn were applied directly to Sephacryl S-300 columns (Pharmacia, 2.0 × 105 cm) which were eluted with GTS buffer at room temperature. The flow was monitored continuously for the absorbance at 230 nm. Appropriate fractions were pooled, concentrated, and desalted by diafiltration (Amicon PM10 membrane) or gel filtration on a Sephadex G-100 column (Pharmacia, 2.0 × 95 cm) in 50 mM NH_4HCO_3, pH 8.0. Fractions were desalted by lyophilization and stored in the dry state at −20 °C.

DEAE-cellulose Chromatography. The desalted phosphophoryn peak from Sephacryl S-300 chromatography was dissolved in 50 mM Tris-HCl, pH 8.3, and the solution was applied to a DEAE-cellulose column (Whatman DE-52, 2.0 × 5.0 cm) at room temperature. The column was eluted by using a linear NaCl gradient in starting buffer (50 mM Tris-HCl, pH 8.3). The buffer was pumped through the column at a flow rate of 50 mL/h, the eluate was continuously monitored for absorbance at 230 nm, and 5-mL fractions were collected. The NaCl concentration was determined by conductivity measurements on every tenth fraction. Pooled fractions were desalted by diafiltration (Amicon PM10 membranes) and lyophilized.

Semipreparative and Analytical Gel Filtration Chromatography. Two dissociative chromatography systems were used for the final purification of BDPP. Sepharose CL-6B columns (Pharmacia, 1.5 × 96 cm) were eluted with 4 M guanidine hydrochloride and 50 mM Tris-HCl, pH 7.5 (4 M Gdn·HCl buffer). Elution was monitored by measuring the 230-nm absorbance of each 2-mL fraction. Bio-Sil TSK-400 columns [Bio-Rad, 0.75 × (2 × 30) cm] were also eluted with 4 M Gdn·HCl buffer. The absorbance at 230 nm was monitored continuously with a Waters Model 450 variable-wavelength detector. Sample applications were made by using a Waters U6K injector. The buffer was pumped at a flow rate of 0.5 mL/min by using an Eldex Labs Model A-30-S pump. These column systems were calibrated by using the standard globular proteins phosphorylase *b*, bovine serum albumin, ovalbumin, soybean trypsin inhibitor, cytochrome *c*, and insulin B chain and the phosphoproteins phosvitin and casein (all standards were purchased from Sigma Chemical Co., St. Louis, MO). The standards were reduced and alkylated by the method of Fish et al. (1969). Data were treated according to the method for flexibile polymers of Porath (1963) as described by Fish et al. (1969). This results in a linear relationship between the elution parameter (K_d) and the molecular weight (M_r) when $K_d^{1/3}$ is plotted vs. $M_r^{0.555}$. The elution parameter K_d is defined as

$$K_d = \frac{V_e - V_0}{V_i - V_0} \qquad (1)$$

where V_e is the elution volume of the peak concentration for the solute of interest, V_0 is the void volume, and V_i is the total included volume. Blue dextran (Sigma Chemical Co.) and dinitrophenylalanine (DNP-alanine) (Sigma Chemical Co.) were used as V_0 and V_i markers, respectively. Both of these chromatography systems gave excellent results for standard proteins with M_r values from 3000 to 95 000 as has previously been reported (Ansari & Mage, 1977; Ui, 1979).

Polyacrylamide Gel Electrophoresis. Three gel systems were used in this study. These were the following: (a) the method of Laemmli (1970); (b) a 5–15% linear acrylamide gradient system based on the Laemmli buffer system as described by Butler et al. (1981); (c) a modification of the Weber & Osborn system (1969) as described by Termine et al. (1980a).

Laemmli-type $NaDodSO_4$ gels were run at a 37.5:1 acrylamide:bis(acrylamide) cross-linking ratio. Running gels (slabs 0.15 × 9.0 × 14.0 cm) were prepared in 50 mM Tris-HCl and 0.1% $NaDodSO_4$, pH 8.3, at acrylamide concentrations of 6, 8, 10, and 12%. The stacking gels of 3% acrylamide (0.15 × 0.5 × 14 cm) were prepared in 50 mM Tris-HCl–0.1% $NaDodSO_4$, pH 6.8. Electrode buffer contained 50 mM Tris, 0.34 M glycine, and 0.1% (w/v) $NaDodSO_4$, pH 8.3. Samples were dissolved in 50 mM Tris-HCl, pH 6.8, 1.0% $NaDodSO_4$, 6% sucrose, 5% 2-mercaptoethanol, and 0.006% bromophenol blue and then were heated at 100 °C for 1–2 min before application to the stacking gel. Gels were run at 30 mA per gel until the dye front had migrated to within 0.5 cm of the bottom of the gel.

For routine analysis, the buffer system just described was used to prepare 5–15% linear polyacrylamide gradient gels.

Weber–Osborn $NaDodSO_4$ gels were also run at a 37.5:1 acrylamide:bis(acrylamide) cross-linking ratio. Running gels (slabs 0.15 × 9.5 × 14 cm) were prepared in 50 mM Tris–glycine–0.1% $NaDodSO_4$, pH 8.8, at acrylamide concentrations of 5, 6, 7, 8, 9, and 10%. Samples were dissolved in 0.1 M Tris–glycine, pH 8.8, 0.1% $NaDodSO_4$, 1.0% 2-mercaptoethanol, 12% sucrose, and 0.03% bromophenol blue and then heated at 100 °C for 1–2 min before application to the running gel. Gels were run at 30 mA until the dye front had migrated to within 0.5 cm of the bottom of the gel.

Staining with Coomassie brilliant blue was done in 0.5% Coomassie brilliant blue R-250 (Bio-Rad) in 25% (v/v) 2-propanol–10% (v/v) acetic acid; gels were then destained in 25% (v/v) 2-propanol–10% acetic acid. For the demonstration of phosphoproteins, which do not stain with Coomassie brilliant blue, separate gels were stained with Stains All (Bio-Rad) according to the method of Green et al. (1973).

Retardation coefficients (K_R) and free electrophoretic mobilities (M_0) were calculated according to the equation of Ferguson (1964):

$$\log R_f = \log M_0 - K_R[\mathrm{A}] \qquad (2)$$

where R_f is the relative electrophoretic mobility in a gel of acrylamide concentration [A] in grams per 100 mL. The data were fit by the method of least squares.

Sedimentation Analyses. Ultracentrifugation was carried out in a Beckman Spinco Model E analytical ultracentrifuge equipped with a RTIC temperature control unit, electronic speed control, and a UV scanner system. Aluminum- or charcoal-filled Epon centerpieces, sapphire windows, and an AN-H rotor were used for all experiments. Molecular weights were measured by the short-column meniscus-depletion equilibrium method of Yphantis (1964). A stock solution of BDPP in 10 mM Tris-HCl–0.5 M KCl, pH 8.3 (TKCl buffer), was prepared and dialyzed against TKCL buffer overnight (Spectra Por 2, MWCO = 10 000–12 000). Running solutions were prepared by dilution of a stock BDPP solution with dialysate. Yphantis six-channel centerpieces were used. Equilibrium sedimentation experiments were conducted at 12 000–13 000 rpm, proceeded by a short period of overspeeding at 18 000 rpm. The distribution of protein was followed by the absorbance at 245 nm. Absorbancies (y) and radial distances (r) obtained from scans were plotted as log

y vs. r. The slope of the resulting straight line is proportional to the apparent weight-average molecular weight.

Sedimentation velocity experiments were carried out at 60000 rpm by using standard 12-mm double-sector cells. The protein solutions in TKCL buffer were prepared by dilution of a stock BDPP solution with dialysate as described above. Cells were scanned at 245 nm at regular intervals starting at the time the rotor reached two-thirds of its maximum speed and continuing for a period longer than 1 h. The radial positions of the boundaries were measured at half the plateau height. Sedimentation coefficients (s) were calculated from the least-squares slope of the logarithm of the boundary radial position vs. time. Diffusion experiments were carried out in the ultracentrifuge in TKCL buffer using 12-mm capillary-type synthetic boundary cells at 12000 rpm. Absorbance scans at 245 nm were made at regular intervals. Diffusion coefficients (D) were calculated from the least-squares slope of the square of the difference between the radial positions of the three-quarter plateau height and the one-quarter plateau height (Δr^2) vs. time (Shumaker & Schachman, 1957).

Viscosity. Viscosity experiments were performed at 20 ± 0.1 °C by using a Cannon-Ubbelhode viscometer with a flow time for water of about 300 s. BDPP samples in either TKCL buffer or 4 M Gdn·HCl buffer were prepared and dialyzed overnight. Measurements were made on protein samples (1.5 mL) obtained by sequential dilution of the most concentrated sample with dialysate.

The data were treated according to the following equation:

$$\eta_{\mathrm{sp}}/c = (1/c)\left(\frac{t - t_0}{t_0}\right) = [\eta] + k[\eta]^2c \qquad (3)$$

where η_{sp} is the specific viscosity, c is the protein concentration in grams per deciliter, t_0 and t are the flow times of the solvent and solution, respectively, $[\eta]$ is the intrinsic viscosity (deciliters per gram), and k is the dimensionless Huggins' constant (Huggins, 1942). The value of t for a given dilution was taken as the average of three readings reproducible within ±0.3 s. The correction of the kinematic viscosity for the difference in density between solvent and protein solution was deemed unnecessary because of the high values obtained for $[\eta]$ (Tanford, 1955).

Analytical Methods. Phosphorus concentrations were determined after wet ashing the sample as described by Kirkpatrick & Bishop (1971), either by the ultramicro method of Bartlett (1959) or by the method of Dryer et al. (1957). Amino acid analyses were performed on a JEOL 6AH amino acid analyzer after the samples had been hydrolyzed in 6 N HCl for 22 h.

Measurement of protein concentration was based on phosphorus determinations. The weight percent phosphorus was determined for purified BDPP by submitting aliquots of a solution to phosphorus and amino acid analyses. The weight percent phosphorus was calculated by utilizing the amino acid analysis data to determine the amount of protein present. Thereafter, protein concentration was determined by using the weight percent phosphorus to convert phosphorus content to protein content.

Results

Isolation and Composition of BDPP. The calcium chloride precipitation and subsequent dialysis steps shown in Figure 1 yielded four principal fractions. Fraction II is an insoluble pellet left after EDTA extraction of the $CaCl_2$ precipitate. It was not solubilized by repeated EDTA extraction. This is in contrast to the total solubilization of the $CaCl_2$ precipitate reported by both Butler et al. (1981) and Kuboki et al. (1979). Fractions II, III, and IV contained proteins with high contents of prolyl and glutamyl residues. These were not studied further. Fraction I, containing the $CaCl_2$-precipitable, 0.5 M EDTA soluble components of dentin, was the material of major interest. $NaDodSO_4$–polyacrylamide gradient gel electrophoresis (PGGE) of this material showed that it contained a number of Coomassie blue staining bands with molecular weights below 68000 (data not shown). Preliminary studies (Veis et al., 1981) had demonstrated that undegraded BDPP eluted in the void volume of Sephacryl S-300 columns. Hence, the $CaCl_2$ precipitates were further purified by chromatography on Sephacryl S-300 under dissociative conditions. The void volume fraction from such chromatography was collected from a number of preparations. After the void volume material was desalted and lyophilized it was dissolved in pH 8.3 buffer, applied to a DEAE-cellulose (DE-52) column, and eluted with a linear NaCl gradient. As anticipated (Veis et al., 1972; Dickson et al., 1975; Lee et al., 1977), the major component eluted between 0.25 and 0.30 M NaCl, but in contrast to these earlier studies of BDPP, use of $CaCl_2$ precipitation and Sephacryl S-300 chromatography eliminated most of the EDTA-soluble components otherwise found eluting at lower ionic strength (Figure 2a). The material under the bar in Figure 2a was collected and dissolved in 4 M Gdn·HCl. This fraction produced a single major peak when run over Sepharose CL-6B (Figure 2b). The material eluting in the central region of this peak (bar, Figure 2b), the product of two gel filtration steps under different dissociative conditions and one ion-exchange chromatography step, was collected, desalted, and lyophilized for use in all subsequent studies.

Every fractionation step was monitored by linear gradient $NaDodSO_4$ gel electrophoresis. Intensely blue "Stains All" bands denoting the BDPP were evident in all preparations (Figure 3). The data in Figure 3 clearly demonstrate that the final BDPP fraction obtained after dissociative Sepharose CL-6B chromatography had exactly the same electrophoretic mobility in the $NaDodSO_4$ gel system as that in the initial crude EDTA extract. Thus, the precautions taken against proteolysis appeared to have been successful so that the BDPP isolated has the size characteristic of the protein in the initial extract and, presumably, in the tissue itself. Moreover, the gel in lane 8 of Figure 3 shows the preparation to be homogeneous.

It has previously been demonstrated for both bovine (Lee et al., 1977) and rat (Richardson et al., 1978) dentin phosphophoryns that essentially all of the phosphorus is present as phosphoseryl residues. During hydrolysis for amino acid analysis, some phosphoseryl and seryl residues are lost. Corrections for these hydrolytic losses were made by determining the concentration of organic phosphorus in a sample after wet ashing and assuming that this was equal to the concentration of phosphoserine. Then, utilizing the observed serine content from amino acid analysis data following 22-h HCl hydrolysis, we solved the equation of Richardson et al. (1978) to find the nonphosphorylated serine content. These data are presented in Table I along with the uncorrected results of the amino acid analysis. These results show a typical phosphophoryn composition in which 40% of the residues are aspartic acid, 46% of the residues are phosphoserine, and only 2.5% of the total residues are nonpolar in nature. These data agree well with those of Termine et al. (1980b) for fetal bovine dentin phosphophoryn with the exception that whereas we find 95% of the seryl residues phosphorylated, they found only 50% phosphorylation in the fetal material. We have confirmed this

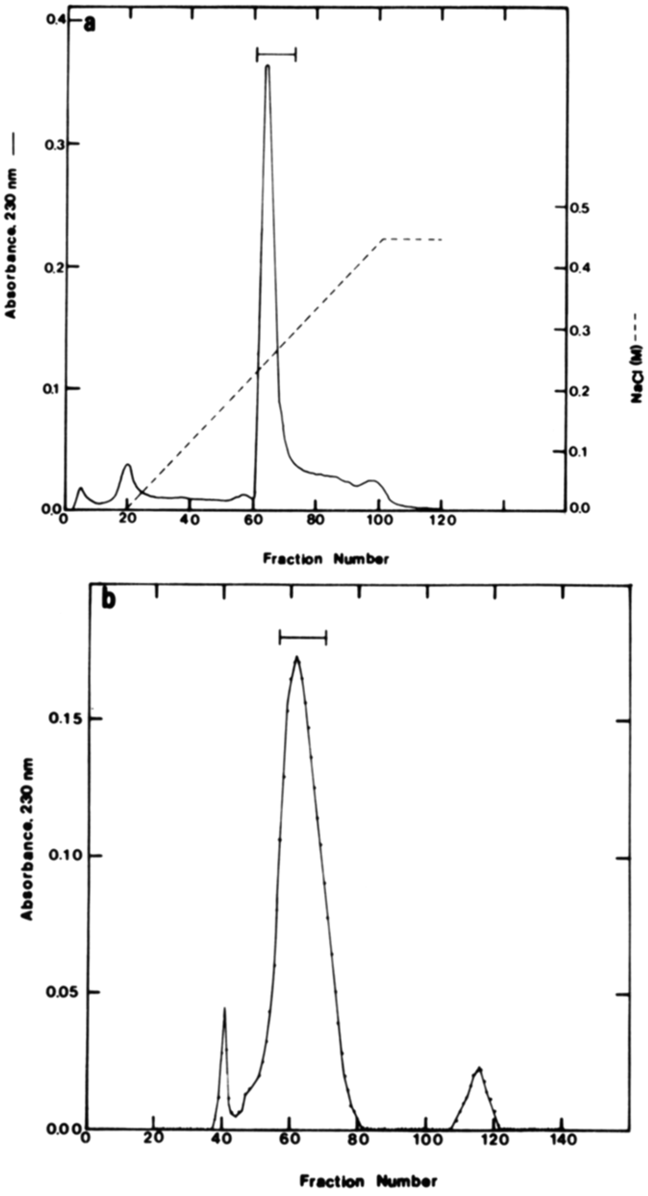

FIGURE 2: Chromatographic purification of bovine dentin phosphophoryn. (a) DEAE-cellulose chromatography (Whatman DE-52, 2.0 × 5.0 cm) of the pooled void fraction from Sephacryl S-300 chromatography. The column was eluted with a linear NaCl gradient in 50 mM Tris-HCl, pH 8.3. The fractions under the bar were pooled and desalted. (b) Sepharose CL-6B (1.5 × 96 cm) column equilibrated with 4.0 M guanidine hydrochloride–50 mM Tris-HCl, pH 7.5, and loaded with 2 mg of pooled and desalted fractions from DEAE-cellulose chromatography. The fractions under the bar were pooled and desalted.

lower value in their preparation. The mature BDPP organic phosphorus content is 10.4% by weight on the basis of the protein content determined from the corrected amino acid analysis. Tyrosine and phenylalanine are definite constituents of BDPP.

Molecular Weight. (*A*) *Gel Filtration.* Analytical gel filtration under dissociative conditions on Bio-Sil TSK-400 columns in 4 M Gdn·HCl is shown in Figure 4. Bovine dentin phosphophoryn had an apparent M_r = 100 000 by this method, in excellent agreement with that reported for fetal bovine dentin phosphoprotein (determined by utilizing a standard chromatographic system: Termine et al., 1980b). However, when our HPLC system was used, the apparent molecular weight of a sample was determined in 1 h, whereas conventional liquid chromatography on a CL-6B column required a minimum of 24 h.

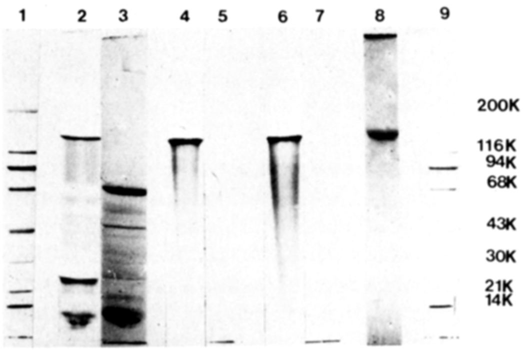

FIGURE 3: NaDodSO$_4$–5–15% linear polyacrylamide gradient gel electrophoresis of bovine dentin phosphophoryn at various stages of purification. Lanes 1, 3, 5, 7, and 9 were Coomassie brilliant blue stained; lanes 2, 4, 6, and 8 were Stains All stained. Lanes 2 and 3, EDTA-soluble extract from bovine molar powder; lanes 4 and 5, Sepharcyl S-300 void fraction; lanes 6 and 7, pooled fractions from DEAE-cellulose chromatography (bar in Figure 2a); lane 8, pooled fractions from Sepharose CL-6B chromatography (bar in Figure 2b); lanes 1 and 9, NaDodSO$_4$–polyacrylamide gel electrophoresis standards (Bio-Rad).

Table I: Amino Acid Composition of BDPP

residue	nmol[a]	residues/ 1000[a]	corrected nmol[b]	corrected residues/ 1000[b]
Lys	36.9	44	36.9	40
His	4.1	5	4.1	4
Arg	2.8	3	2.8	3
Asp	374.2	447	374.2	447
Thr	5.9	7	5.9	6
Ser	266.5	319	21.3	23
Glu	11.5	14	11.5	14
Pro	5.2	6	5.2	6
Gly	22.3	27	22.3	24
Ala	5.3	6	5.3	6
$^1/_2$-Cys	T[c]	T[c]	T[c]	T[c]
Val	2.8	3	2.8	3
Met	T[c]	T[c]	T[c]	T[c]
Ile	2.6	3	2.6	3
Leu	3.3	4	3.3	4
Tyr	1.9	2	1.9	2
Phe	2.0	2	2.0	2
P-Ser	89.4	107	428.9	461
Glu NH_2	T[c]	T[c]	T[c]	T[c]
total	836.6	999	931.0	1001

[a] Mean of triplicate determinations. [b] Corrected for phosphoserine and serine hydrolysis as described by Richardson et al. (1978). [c] T indicates trace.

(*B*) *Gel Electrophoresis.* BDPP behaves in an anomalous fashion in gel electrophoresis, as expected for a molecule with such high charge density (Banker & Cotman, 1972; Tung & Knight, 1971; Termine et al., 1980a). As shown by the plot of log R_f vs. [A] in Figure 5a,b, the free electrophoretic mobility of BDPP is reduced in comparison with the mobility of the standards in both NaDodSO$_4$ gel electrophoretic systems tested. Thus, neither the Weber–Osborn nor the Laemmli system can be used at a single gel concentration for BDPP molecular weight determinations. However, studies by Lambin et al. (1976) have shown that PGGE can give reliable estimates of the molecular weight for a protein even if the isoelectric pH of the protein is very low. NaDodSO$_4$–PGGE gels, previously used to monitor for proteolysis of BDPP (Figure 3), were calibrated for molecular weight determination by using reduced globular standards (Bio-Rad). In this system, BDPP had an apparent M_r = 156 500 ± 2100, markedly higher than the gel filtration value.

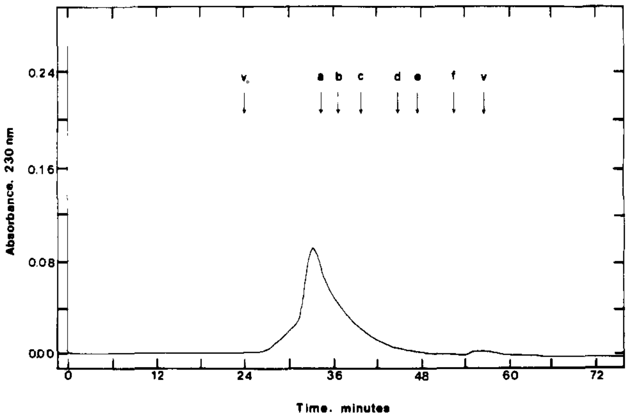

FIGURE 4: Dissociative analytical gel filtration of bovine dentin phosphophoryn (BDPP). Bio-Sil TSK 400 columns [Bio-Rad, 0.75 × (2 × 30) cm] were equilibrated with 4.0 M guanidine hydrochloride–50 mM Tris-HCl, pH 7.5, at a flow rate of 0.5 mL/min; 80 μg of BDPP was injected into the columns by using a Waters U6K injector. Void volume (V_0), total included volume (V_i), and the elution position of standards are indicated. Reduced and alkylated standards: (a) phosphorylase *b*; (b) bovine serum albumin; (c) ovalbumin; (d) soybean trypsin inhibitor; (e) cytochrome *c*; (f) insulin B chain.

(*C*) *Sedimentation Equilibrium.* The weight-average molecular weight was determined for BDPP in TKCL as a function of protein concentration by the short-column meniscus-depletion technique (Yphantis, 1964). It has been demonstrated (Johnson et al., 1954; Roark & Yphantis, 1971) that at any finite concentration the apparent molecular weight of a polyelectrolyte complex in a three-component system, such as BDPP in TKCL buffer, will be lower than the true molecular weight of the complex due to the Donnan effect. According to Scatchard's (1946) definition, the mass of the macromolecular polyelectrolyte complex (M_i) is given by

$$M_i = M_{pz} + z/[(v_+ + v_-)(M_{B^+} - M_{X^-})] \quad (4)$$

where M_{pz} is the mass of the anionic polyion alone, z is the valence of the polyion, v_+ and v_- are the absolute values of the valencies of the cation and anion of the added salt, respectively, M_{B^+} is the mass of the microcation, and M_{X^-} is the mass of the microanion. Using Scatchard's definition of components, it can be shown that the Donnan effect decreases as the macromolecular concentration approaches zero, and the experimentally obtained value of the weight-average molecular weight approaches the value for the ideal weight-average molecular weight of the polyelectrolyte complex as defined by eq 4 (Johnson et al., 1954; Roark & Yphantis, 1971).

Figure 6 shows the concentration dependence of the inverse of the apparent molecular weight for BDPP in TKCL buffer. The line represents the least-squares fit for these data and yields a zero concentration extrapolated value of M_i = 154 000 ± 11 000 for BDPP, in excellent agreement with the value obtained from NaDodSO$_4$–PGGE. From this value and the amino acid composition, we estimate z = 1465 at pH 8.3. With the difference in atomic weights between potassium and chloride ions at 4 daltons, the correction factor in eq 4 would amount to about 3000 daltons, a value less than 2% of the estimated molecular weight and even less than the experimental error. Thus, we feel that the zero concentration extrapolated value of M_i represents a reasonable estimate for M_{pz}, the molecular weight of the BDPP polypeptide backbone.

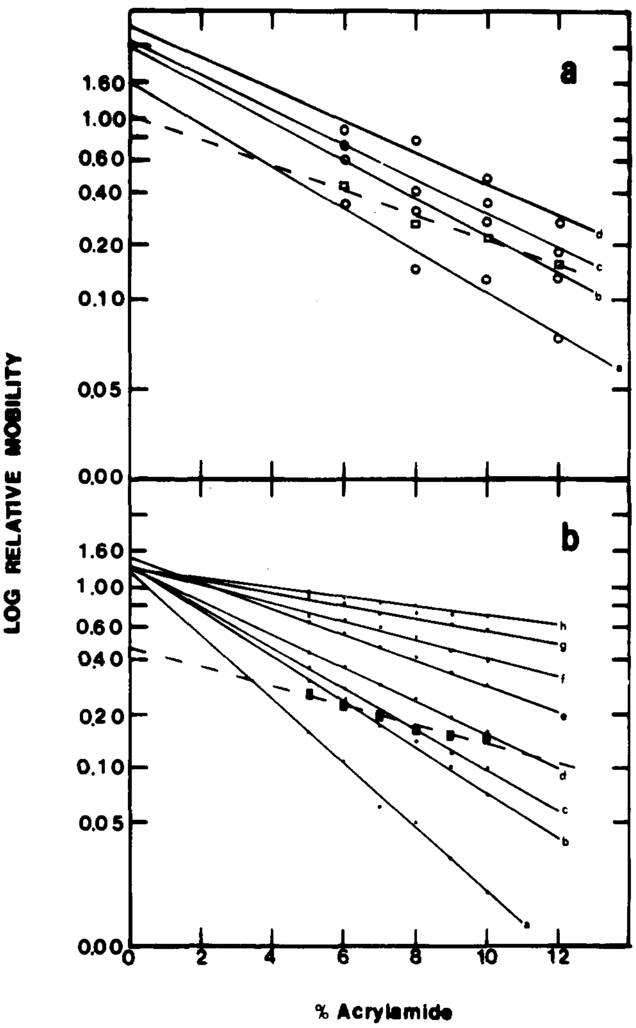

FIGURE 5: Ferguson plots of BDPP in NaDodSO$_4$–polyacrylamide gel electrophoretic systems. (**a**) Ferguson plot of Laemmeli (1970) gel system. (---) BDPP; (—) standards: (a) myosin; (b) β-galactosidase; (c) phosphorylase *b*; (d) bovine serum albumin. (**b**) Ferguson plot of modified Weber–Osborn (Termine et al., 1980a–c) type gel system. (---) BDPP; (—) standards: (a) myosin; (b) β-galactosidase; (c) phosphorylase *b*; (d) bovine serum albumin; (e) ovalbumin; (f) carbonic anhydrase; (g) soybean trypsin inhibitor; (h) lysoezyme.

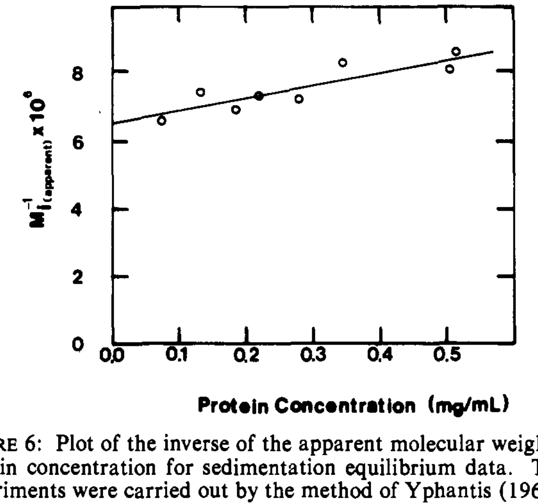

FIGURE 6: Plot of the inverse of the apparent molecular weight vs. protein concentration for sedimentation equilibrium data. These experiments were carried out by the method of Yphantis (1964) in 0.5 M KCl–10 mM Tris-HCl, pH 8.3, at 12 000–13 000 rpm. Extrapolation to zero concentration yields a value of 154 000 for the molecular weight of BDPP.

Direct determination of the partial specific volume (v) was not made due to the small quantity of protein available. The value of v was computed from the amino acid composition data, the residue v values of Lee & Timasheff (1979), and the residue v value of phosphoserine (McMeekin et al., 1949) by the procedure of Cohn & Edsall (1943). A value of v = 0.56 mL/g was obtained, reflecting the high phosphoserine content. This value compares favorably with the v value of 0.54 mL/g

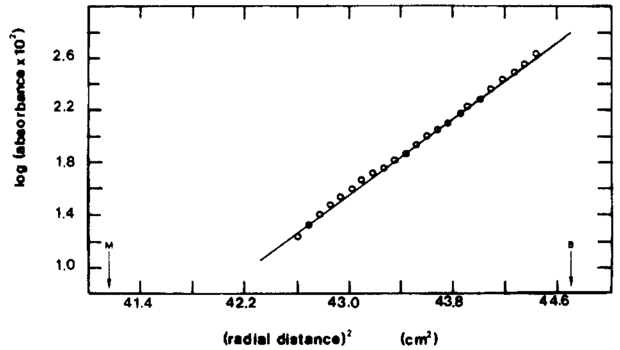

FIGURE 7: Plot of log absorbance vs. the square of the radial distance. This experiment was carried out by the Yphantis method (1964). The initial protein concentration was 0.34 g/L in 0.5 M KCl–10 mM Tris-HCl, pH 8.3. Centrifugation was performed at 12 000 rpm and at 20 °C; absorbance scans at 245 nm were performed 24 and 32 h. M denotes the position of the meniscus; B denotes the position of the bottom of the cell.

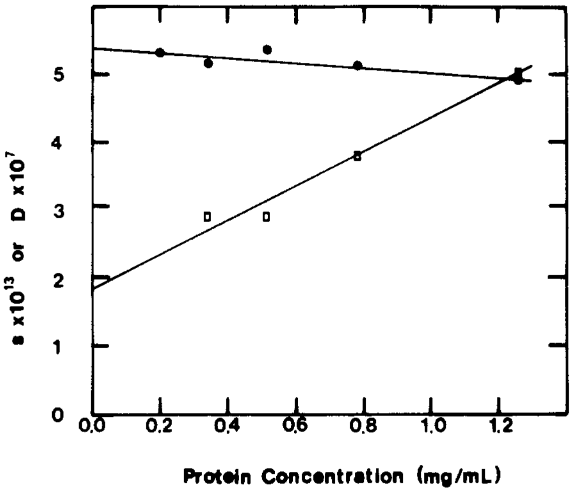

FIGURE 8: Influence of protein concentration on sedimentation and diffusion coefficients. Sedimentation coefficients (●) were measured at 60 000 rpm, 20 °C, in 0.5 M KCl–10 mM Tris-HCl, pH 8.3. Diffusion coefficients (□) were measured at 12 000 rpm, 20 °C, by using the same buffer as in the sedimentation velocity experiment. Least-squares extrapolation to zero concentration yields values of $s^\circ = 5.4 \times 10^{-13}$ s^{-1} and $D^\circ = 1.84 \times 10^{-7}$ cm^2 s^{-1}.

calculated by Jontell et al. (1982) for rat incisor phosphophoryn.

The molecular weight heterogeneity was tested in the sedimentation equilibrium experiments. Figure 7 shows a basic data plot of log absorbance (y) vs. the square of the radial distance (r^2) for an initial protein concentration of 0.34 mg/mL. These plots were linear at all initial protein concentrations examined, 0.076, 0.135, 0.187, 0.220, 0.283, 0.341, 0.505, and 0.514 mg/mL (correlation coefficients from linear least-squares analyses were always greater than 0.99). The linearity of these plots demonstrates the molecular weight homogeneity of BDPP as well as the absence of nonideal or concentration-dependent behavior other than the Donnan effect discussed previously. In this respect, the electrophoretic data showing homogeneity of BDPP are also in good agreement. It is evident that in spite of the high charge density along the polypeptide backbone of BDPP, 0.5 M KCl is sufficient to overcome the long-range electrostatic repulsive interactions.

(*D*) *Sedimentation Velocity and Diffusion Coefficient.* Sedimentation velocity and diffusion experiments were carried out in TKCL buffer. The plots of the observed sedimentation and diffusion coefficients are shown in Figure 8. Extrapolation to zero concentration was done by the method of least squares and yields zero concentration values of 5.4×10^{-13} s^{-1} and 1.84×10^{-7} cm^2 s^{-1} for the sedimentation (s°) and diffusion (D°) coefficients, respectively. The apparent molecular weight of BDPP obtained from these data, using the value $v = 0.56$ mL/g, is $M_{\mathrm{app}} = 167\,000$. We consider this value to be in fair agreement with the data from sedimentation equilibrium and NaDodSO$_4$–PGGE, considering the experimental and threoretical difficulties in the application of these techniques to solutions of flexible highly charged polymer molecules (Tanford, 1961; Pedersen, 1958). Values for $s^0_{20,\mathrm{w}}$ and $D^0_{20,\mathrm{w}}$ are 5.41×10^{-13} s^{-1} and 1.79×10^{-7} cm^2 s^{-1}, respectively.

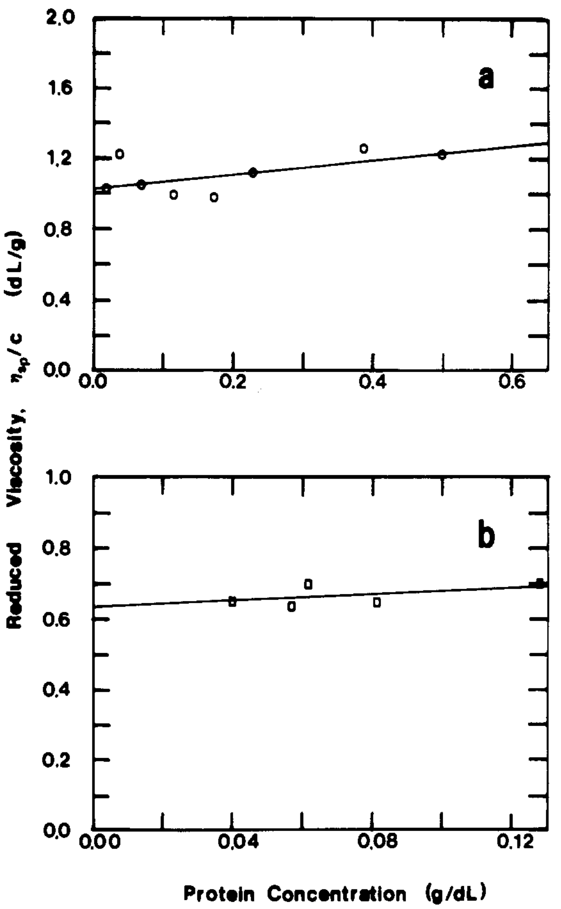

FIGURE 9: Dependence of reduced specific viscosity, η_{sp}/c, of bovine dentin phosphophoryn (BDPP) on protein concentration. (a) Reduced specific viscosity of BDPP in 0.5 M KCl–10 mM Tris-HCl, pH 8.3. Least-squares treatment yields a value for the intrinsic viscosity, $[\eta]$, of 1.006 dL/g and 0.4 for the Huggins constant. (b) Reduced specific viscosity of BDPP in 4 M guandinine hydrochloride–50 mM Tris-HCl, pH 7.5. Least-squares treatment yields a value for $[\eta] = 0.62$ dL/g and 1.02 for the Huggins coefficient.

(*E*) *Viscosity.* The viscosity of BDPP solutions in TKCL buffer was measured at 20 °C. Figure 9a shows these data. The intrinsic viscosity from linear least-squares analysis is 1.006 dL/g, and the Huggins constant is 0.4. A value for the Huggins constant around 0.35 is indicative of a flexible polymer in a good solvent system (Huggins, 1942). The molecular weight and intrinsic viscosity of a flexible polymer are related by (Flory, 1969)

$$[\eta] = K M_{\mathrm{r}}^{0.5} \Phi \qquad (5)$$

where $K = (C_\infty l_\mu^2/M_{\mathrm{b}})^{3/2}$. In these relationships, $[\eta]$ is the intrinsic viscosity, M_{r} is the polymer molecular weight, C_∞ is the characteristic ratio, l_μ is the virtual peptide bond length (3×10^{-8} cm), M_{b} is the average residue molecular weight, and Φ is a universal constant ($\Phi = 2.6 \times 10^{21}$). Polypeptides have C_∞ values in the range of 8.5–9.5. The maximum value

Table II: Molar Amino Acid Composition of BDPP[a]

residue	mol of residue/mol of BDPP
Lys	45
His	6
Arg	3
Asp	452
Thr	8
Ser	27
Glu	14
Pro	6
Gly	27
Ala	7
$^1/_2$-Cys	1
Val	3
Met	1
Ile	3
Leu	4
Tyr	2
Phe	2
P-Ser	518
Glu NH_2	1
total	1130

[a] Calculated on the basis of a molecular weight of 155 000 for BDPP.

of 9.5 was used for the C_∞ of BDPP to compensate for nearest-neighbor electrostatic repulsion and steric interaction between phosphoseryl and aspartyl residue side chains along the backbone which would tend to keep the chain in an extended conformation locally. M_b computed from the amino acid composition data was 137.6. A minimum apparent molecular weight value of 151 000 was obtained by this method, and this value is in excellent agreement with the sedimentation equilibrium and $NaDodSO_4$–PGGE results.

Figure 9b shows the viscosity data for BDPP in 4 M Gdn·HCl buffer. The Huggins constant was increased to 1.02, and the intrinsic viscosity was reduced to 0.62 dL/g compared to values in TKCL buffer. These data clearly indicate that BDPP does not assume a fully random conformation in 4 M Gdn·HCl buffer.

Discussion

The use of mild conditions for extraction and isolation, coupled with the use of protease inhibitors at all stages, resulted in the rapid and relatively inexpensive preparation of a highly purified sample of undegraded BDPP. The final purified sample showed no change in molecular size when compared with the high molecular weight Stains All staining material in the crude soluble EDTA extracts. We therefore conclude that, isolated in the manner described, the purified BDPP is in a state comparable to that in situ. The principal advantages of this method for purification are the rapidity and selectivity of the calcium chloride precipitation technique, as previously demonstrated (Kuboki et al., 1979; Butler et al., 1981), and the inexpensiveness in comparison to techniques utilizing guanidine hydrochloride at all stages of isolation.

Table III: Summary of Molecular Weight Data

method	mol wt[a]
dissociative analytical gel filtration	100 000
$NaDodSO_4$-PGGE	156 500
equilibrium sedimentation[b]	154 000
sedimentation and diffusion coefficient data[b,c]	167 000
intrinsic viscosity data[b,d]	151 000

[a] Apparent molecular weight. [b] Experiments were performed in 0.5 M KCl–0.01 M Tris-HCl, pH 8.3. [c] Calculations based on s^0/D^0 ratio from data of experiments conducted in 0.5 M KCl–0.01 M Tris-HCl, pH 8.3. Corresponding $s_{20,w} = 5.4 \times 10^{-13}$ s^{-1} and $D_{20,w} = 1.79 \times 10^{-7}$ cm^2/s. [d] Calculations are based on a intrinsic viscosity $[\eta] = 1.006$ dL/g and a characteristic ratio $C_\infty = 9.5$.

The homogeneity of the BDPP preparation was assessed by electrophoretic and sedimentation criteria. The final BDPP fraction was found to be homogeneous with varying acrylamide concentrations in two different gel electrophoretic systems and was homogeneous in sedimentation velocity and sedimentation equilibrium experiments. These data, the exhaustive purification scheme, and the excellent agreement of the amino acid composition data reported here with that of Termine et al. (1980b) would indicate the apolar amino acids, shown on a mole per mole BDPP basis in Table II, are an integral part of the BDPP molecule and are not electrostatically bound as has been suggested (Munksgaard et al., 1977; Kuboki et al., 1979). Lechner et al. (1981) have used a mild acetic acid degradation scheme (Krippner & Nawrot, 1977) to study the distribution of aspartyl residues in BDPP. They concluded that BDPP has a domain structure in which polyphosphoseryl and polyaspartyl sequences are separated by short intervening sequences of less polar amino acid residues. Thus, the amino acid compositions and low molecular weights of sulfonated polystyrene purified rat incisor phosphophoryns, which have been shown to be undegraded during purification (Munksgaard et al., 1977; Linde et al., 1980; Jontell et al., 1980), must be a result of species differences and not hydrolytic degradation as has been suggested by Veis et al. (1981). The differences between the amino acid composition reported here, as well as that reported by Termine et al. (1980b), for BDPP and the composition reported by Kuboki et al. (1979) for BDPP can be explained by the use of protease inhibitors and denaturing solvents in both this study and the one by Termine et al. Certainly another striking species variation between rat and bovine phosphophoryns is in lysine and glutamic acid contents. Rat incisor dentin phosphophoryns (Butler et al., 1981) have relatively high glutamic acid contents and relatively low lysine contents. As shown in the amino acid compositions (Tables I and II), BDPP has a much higher content of lysyl than of glutamyl residues. Also, recent investigations have shown that rat incisor dentin may contain at least two different species of phosphophoryns (Dimuzio & Veis, 1978a,b; Linde et al., 1980; Butler et al., 1981), whereas in bovine dentin, the present study and all previous studies of this tissue have always found only a single phosphophoryn species.

It was anticipated that any attempt at determining the molecular weight of BDPP would be complicted by nonideal or anomalous behavior because of its high charge density. In order to overcome this problem, we used conditions to minimize or remove the effects of the high charge density whenever possible. Further, we have compared the results from a number of different techniques based on the measurement of different properties. As shown in Table III, most of the measurements provide a consistent data set for the molecular mass of BDPP, in the range of 151 000–167 000 daltons. However, a distinctly different result was obtained by gel filtration chromatography in 4 M Gdn·HCl buffer. Moreover, Ferguson plots did not show ideal electrophoretic behavior.

In nongradient $NaDodSO_4$ gel electrophoresis, the apparent molecular weight is dependent on the acrylamide concentration. This has been reported for rat incisor phosphophoryns by Jontell et al. (1982). Termine et al. (1980b), using acrylamide concentrations of 7–8%, determined that the relative molecular weight of BDPP was 100 000. Unfortunately or fortuitously, this value was in excellent agreement with the value obtained from analytical gel filtration in 4 M Gdn·HCl in their work as well as in the present study. Because of the consistency of all other values, it is evident that BDPP also behaves in an anomalous fashion in 4 M Gdn·HCl. One possibility for the low value of the molecular weight in the analytical gel filtration in 4 M Gdn·HCl is that the solution conformation of BDPP is much different than that of the calibration standards. Viscosity studies have demonstrated that all proteins previously examined in concentrated guanidine hydrochloride solutions behave as random-chain polymers and that the Huggins constant for such protein solutions ranges from 0.16 to 0.67 (values typical of flexible polymers in good solvents; Tanford et al., 1967; Reisner & Rowe, 1969). Our data show that the Huggins constant for BDPP increases to 1.02 and that the intrinsic viscosity decreases to 0.62 dL/g in 4 M Gdn·HCl buffer. These changes clearly indicate that BDPP does not assume a fully random conformation in 4 M Gdn·HCl buffer but is instead in a more compact or folded conformation! This explains why analytical gel filtration in 4 M Gdn·HCl underestimates the value for the molecular weight of BDPP as a more compact or folded particle would be retarded longer on the column. Jontell et al. (1982), who found rat incisor phosphophoryn to have an anomalously *high* molecular weight in 6 M Gdn·HCl on gel filtration, have also attributed the discrepancy to some nonideal behavior but have not investigated the phenomenon further.

As we have pointed out, a recent study by Jontell et al. (1982) has shown that rat incisor phosphophoryns also behave anomalously on NaDodSO$_4$ gel electrophoresis and gel filtration chromatography in 6 M Gdn·HCl. These investigators also used sedimentation equilibrium to analyze the molecular weight of their phosphophoryn preparations. However, they were unable to obtain ideal behavior of the phosphorylated phosphophoryn in their experiments when 1 M NaCl–50 mM Tris-HCl, pH 7.4, was used as solvent. To obtain ideal behavior, they found it necessary to dephosphorylate the protein. Upon doing so, they obtained a value of only 28 000 for the zero concentration extrapolated value of the molecular weight. Addition of the phosphate gave a value of 38 000 for the phosphorylated species. This value is roughly 60% of the value obtained by these investigators when gel filtration chromatography in 6 M Gdn·HCl on Sepharose Cl-6B was used and is roughly 50% of the value obtained in an earlier study when 3 M Gdn·HCl was used on Sepharose CL-6B (Dimuzio & Veis, 1978a). It is also less than 50% of the value obtained for the molecular weight of rat incisor phosphophoryn obtained in a recent study utilizing NaDodSO$_4$–PGGE (Butler et al., 1981). This comparison is in sharp contrast to the present study where the physical techniques give values for the molecular weight of BDDP that are 150% of that obtained by gel filtration chromatography using Sepharose CL-6B in 4 M Gdn·HCl. Also, the present study shows excellent agreement between the molecular weight obtained from the primary physical methods and the estimate of M_r obtained by NaDodSO$_4$–PGGE (vide infra).

The range in M_r values obtained in the present study can be considerably narrowed by comparing and contrasting the various methods. The sedimentation equilibrium data are highly reliable as this technique has a sound thermodynamic basis for its application to proteins and polyelectrolytes. In contrast, sedimentation velocity and diffusion coefficient experiments are fraught with both theoretical and experimental difficulties in their application to flexible polyelectrolytes. NaDodSO$_4$–PGGE is particularly valuable as it removes the dependence on the rate of electrophoretic migration (free electrophoretic mobility), which is in turn dependent on the charge density (charge/mass ratio). Separation is accomplished solely by the sieving effect of the gel and is independent of the total net charge on the proteins. As a result, the validity of this technique as a method for estimating the molecular weight of a protein is dependent on how well the conformations of the standards used for calibration approximate that of the material of interest, just as it does for gel filtration chromatography. This technique has been used with great success to accurately estimate the molecular weight for a variety of proteins, including acidic proteins (Lambin et al., 1976) and other proteins that are known to bind only traces of NaDodSO$_4$ (Lambin, 1978). The agreement of the value we obtained by this method with that of the other physical methods we used suggests that the rodlike or flexible prolate ellipsoid models of NaDodSO$_4$–protein complexes (Reynolds & Tanford, 1970; Wright et al., 1975; Mattice et al., 1976) are good approximations for the solution conformation of BDPP.

In the calculation of the molecular weight from intrinsic viscosity data for BDPP in TKCL buffer, the value is dependent upon the choice of the characteristic ratio (C_∞). Decreasing the value of C_∞ from 9.5 to 9.0 increases the apparent M_r to 178 000. The validity of our choice of 9.5 for C_∞ is borne out by the consequent agreement of the M_r value obtained by this method with those from sedimentation equilibrium (s°/D°) and NaDodSO$_4$–PGGE. The observation that $C_\infty = 9.5$ results in the agreement of these molecular weight data also supports the suggestion that the molecular conformation of BDPP is rather extended and asymmetric.

As a result of these considerations, we conclude that the most reasonable estimate for the molecular weight range of undegraded BDPP is 154 000–156 500. This is much higher than that reported in other studies of phosphophoryns from the same tissue source (Termine et al., 1980b; Lee et al., 1977; Veis et al., 1972) and other species (Linde et al., 1980; Dimuzio & Veis, 1978a; Richardson et al., 1977; Lyaruu et al., 1982; Jontell et al., 1982). As a result of the anomalous behavior of BDPP in nongradient NaDodSO$_4$–polyacrylamide gel electrophoresis and analytical gel filtration in 4 M Gdn·HCl buffer, these methods are not suitable techniques for the molecular size characterization of phosphophoryns.

References

Ansari, A. A., & Mage, R. G. (1977) *J. Chromatogr. 140*, 98–102.

Banker, G. A., & Cotman, C. W. (1972) *J. Biol. Chem. 247*, 5856–5861.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Butler, W. T. (1972) in *Comparative Molecular Biology of Extracellular Matrices* (Slavkin, H. C., Ed.) pp 255–265, Academic Press, New York.

Butler, W. T., Bhown, M., Dimuzio, M. T., & Linde, A. (1981) *Collagen Relat. Res. 1*, 187–199.

Carmichael, D. J., & Dodd, C. M. (1973) *Biochim. Biophys. Acta 317*, 187–192.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, pp 370–377, Reinhold, New York.

DeSteno, C. V., Feagin, F., & Butler, W. T. (1975) *Calcif. Tissue Res. 17*, 161–163.

Dickson, I. R., Dimuzio, M. T., Volpin, D., Ananthanarayanan, S., & Veis, A. (1975) *Calcif. Tissue Res. 19*, 51–61.

Dimuzio, M. T., & Veis, A. (1978a) *Calcif. Tissue Res. 25*, 169–178.

Dimuzio, M. T., & Veis, A. (1978b) *J. Biol. Chem. 253*, 6845–6852.

Dryer, R. L., Tammes, A. R., & Routh, J. I. (1957) *J. Biol. Chem. 225*, 177–183.

Ferguson, K. A. (1964) *Metab. Clin. Exp. 13*, 985–1002.

Fish, W. W., Mann, K. G., & Tanford, C. (1969) *J. Biol. Chem. 244*, 4989–4994.

Flory, P. J. (1969) *Statistical Mechanics of Chain Molecules*, pp 30–48, Wiley, New York.

Glimcher, M. J. (1981) in *The Chemistry and Biology of Mineralized Connective Tissues* (Veis, A., Ed.) pp 617–673, Elsevier/North-Holland, New York.

Green, M. R., Pastewka, J. V., & Peacock, A. C. (1973) *Anal. Biochem. 56*, 43–51.

Huggins, M. L. (1942) *J. Phys. Chem. 64*, 2716–2718.

Johnson, J. S., Kraus, K., & Scatchard, G. (1954) *J. Phys. Chem. 58*, 1034–1039.

Jontell, M., & Linde, A. (1977) *Calcif. Tissue Res. 22*, 321–324.

Jontell, M., Linde, A., & Lundvik, L. (1980) *Prep. Biochem. 10*, 235–253.

Jontell, M., Pertoft, H., & Linde, A. (1982) *Biochim. Biophys. Acta 705*, 315–320.

Kirkpatrick, D. S., & Bishop, S. H. (1971) *Anal. Chem. 43*, 1707–1709.

Krippner, R. D., & Nawrot, C. F. (1977) *J. Dent. Res. 56*, 873.

Kuboki, Y., Fujisawa, R., Aoyama, K., & Sasaki, S. (1979) *J. Dent. Res. 58*, 1926–1932.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lambin, P. (1978) *Anal. Biochem. 85*, 114–125.

Lambin, P., Rochu, D., & Fine, J. M. (1976) *Anal. Biochem. 74*, 567–575.

Lechner, J. H., Veis, A., & Sabsay, B. (1981) in *The Chemistry & Biology of Mineralized Connective Tissues* (Veis, A., Ed.) pp 395–398, Elsevier/North-Holland, New York.

Lee, J. C., & Timasheff, S. N. (1979) *Methods Enzymol. 61*, 49–57.

Lee, S. L., Veis, A., & Glonek, T. (1977) *Biochemistry 16*, 2971.

Linde, A., Bhown, M., & Butler, W. T. (1980) *J. Biol. Chem. 255*, 5931–5942.

Lyaruu, D. M., Belcourt, A., Fincham, A. G., & Termine, J. D. (1982) *Calcif. Tissue Int. 34*, 86–96.

Maier, G. D., Lechner, J. H., & Veis, A. (1983) *J. Biol. Chem. 258*, 1450–1455.

Mattice, W. L., Riser, J. M., & Clark, D. S. (1976) *Biochemistry 15*, 4264–4272.

McMeekin, T. L., Groves, M. L., & Hipp, N. J. (1949) *J. Am. Chem. Soc. 71*, 3298–3300.

Munksgaard, E. C., Butler, W. T., & Richardson, W. S. (1977) *Prep. Biochem. 7*, 321–331.

Nawrot, C. F., Campbell, D. J., Schroeder, J. K., & Van Valkenberg, M. (1976) *Biochemistry 15*, 3445–3449.

Pedersen, K. O. (1958) *J. Phys. Chem. 62*, 1282–1290.

Porath, J. (1963) *Pure Appl. Chem. 6*, 233–240.

Reisner, A. H., & Rowe, J. (1969) *Nature (London) 222*, 558–559.

Reynolds, J. A., & Tanford, C. (1970) *J. Biol. Chem. 245*, 5161–5165.

Richardson, W. S., Beegle, W. F., Butler, W. T., & Munksgaard, E. C. (1977) *J. Dent. Res. 56*, 233–237.

Richardson, W. S., Munksgaard, E. G., & Butler, W. T. (1978) *J. Biol. Chem. 253*, 8042–8046.

Roark, D. E., & Yphantis, D. A. (1971) *Biochemistry 10*, 3241–3249.

Scatchard, G. (1946) *J. Am. Chem. Soc. 68*, 2315–2319.

Shumaker, V. N., & Schachman, H. K. (1957) *Biochim. Biophys. Acta 23*, 628–639.

Tanford, C. (1955) *J. Phys. Chem. 59*, 798–799.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.

Tanford, C., Kawahara, K., & Lapanje, S. (1967) *J. Am. Chem. Soc. 89*, 729–736.

Termine, J. D., & Conn, K. M. (1976) *Calcif. Tissue Res. 22*, 149–157.

Termine, J. D., Belcourt, A. B., Christner, P. J., Conn, K. M., & Nylen, M. U. (1980a) *J. Biol. Chem. 255*, 9760–9768.

Termine, J. D., Belcourt, A. B., Miyamoto, M. S., & Conn, K. M. (1980b) *J. Biol. Chem. 255*, 9769–9772.

Termine, J. D., Eanes, E. D., & Conn, K. M. (1980c) *Calcif. Tissue Int. 31*, 247–251.

Tung, J.-S., & Knight, C. A. (1971) *Biochem. Biophys. Res. Commun. 42*, 1117–1121.

Ui, N. (1979) *Anal. Biochem. 97*, 65–71.

Veis, A. (1978) in *Ions in Macromolecular and Biological Systems* (Everett, P. H., & Vincent, B., Eds.) pp 259–272, Scientechnica, Bristol, England.

Veis, A., Spector, A. R., & Zamioscianyk, H. (1972) *Biochim. Biophys, Acta 257*, 404–413.

Veis, A., Stetler-Stevenson, W. G., Takagi, Y., Sabsay, B., & Fullerton, R. (1981) in *Chemistry & Biology of Mineralized Connective Tissue* (Veis, A., Ed.) pp 377–387, Elsevier/North-Holland, New York.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Weinstock, M., & Leblond, C. P. (1973) *J. Cell Biol. 56*, 838–845.

Wright, A. K., Thompson, M. R., & Miller, R. L. (1975) *Biochemistry 14*, 3224–3228.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Zanetti, M., deBernard, B., Jontell, M., & Linde, A. (1981) *Eur. J. Biochem. 113*, 541–545.