Isolation and Chemical Characterization of the Phosphoproteins of Chicken Bone Matrix: Heterogeneity in Molecular Weight and Composition†

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ABSTRACT: Ethylenediaminetetraacetic acid and HCl extracts of calcified chicken bone were fractionated by a variety of techniques, including molecular sieving in guanidinium chloride, ion-exchange chromatography on DEAE-cellulose, high-performance liquid chromatography (HPLC), reverse-phase HPLC, and preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Using several different experimental schemas, we isolated 14 apparently homogeneous components varying in molecular weight from ~150K to ~4K–5K. The compositions of all of the phosphoproteins were characterized by high concentrations of Asp, Glu, Ser, Gly, and Ala. Seven of the components which were analyzed contained concentrations of carbohydrate varying from ~4% to ~17%. Three of the components containing *O*-phosphoserine which behaved as single bands on SDS–PAGE with molecular weights of ~150K, ~90K, and ~70K contained Hyp and Hyl or Hyl alone and may represent covalently bonded or strongly associated collagen–phosphoprotein complexes or hydroxylated Pro and/or Lys residues of the phosphoproteins. The findings that the amino acid compositions of several of the components were very similar and that N-terminal partial amino acid sequences of the ~90- and ~60-kilodalton (kDa) and of the ~150- and ~32-kDa components, respectively, were identical make it clear that some of the lower molecular weight components are derived by proteolysis from higher molecular weight species. In addition to proteolysis, we speculate that it is possible, from the N-terminal amino acid sequence data and preliminary cross-reaction studies of antibodies to four of the phosphoproteins, that the heterogeneity observed in the phosphoprotein components may also be due in part to there being more than one independent gene product for chicken bone phosphoproteins.

Phosphoproteins, one of the major constituents of the noncollagenous proteins found in all vertebrate calcified tissues, have been postulated to play an important role in the initiation and regulation of calcification (Veis, 1978; Glimcher, 1984). *O*-Phosphoserine [Ser(P)][1] has been identified as the almost exclusive phosphorylated amino acid in the proteins of enamel and dentin (Seyer & Glimcher, 1977; Linde et al., 1980; Lee et al., 1983) while the proteins of bone, cementum, calcified cartilage, and calcified avian tendon have been demonstrated to contain significant amounts of *O*-phosphothreonine [Thr(P)] in addition to Ser(P) (Cohen-Solal et al., 1978; Glimcher et al., 1979a–c). In addition to the bone from 8 species initially reported to contain Thr(P) (Cohen-Solal et al., 1978), we have since identified Thr(P) and Ser(P) in the bones of 12 additional species: monkey, mouse, guinea pig, dog, goose, duck, pheasant, partridge, codfish, pickerel, swordfish, and herring (M. J. Glimcher, B. Lefteriou, and D. Kossiva, unpublished results), making it quite clear that the presence of significant amounts of Thr(P) is a distinguishing feature of bone phosphoproteins compared with the phosphoproteins of dentin and enamel.

EDTA-soluble phosphoproteins were first identified in bovine and chicken bone in 1972 (Spector & Glimcher, 1972). In that and subsequent studies (Lee & Glimcher, 1981), phosphoproteins with molecular weights of ~4000–5000, ~12 000, and ~28 000–30 000 were isolated which were characterized by their high contents of aspartic and glutamic acids and an overall composition which distinguished them from the phosphoproteins of dentin and enamel. Ser(P) and Thr(P) were chemically identified in the M_r ~12 000 and ~28 000–30 000 phosphoproteins, and the phosphomonoester nature of the organic phosphorus components was further verified by ^{31}P NMR of the intact proteins (Lee & Glimcher, 1981). In both studies (Lee & Glimcher, 1979, 1981), however, a number of other phosphoprotein components were noted to be present in the EDTA extracts, but these were not further fractionated and isolated.

Bone matrix phosphoproteins have been isolated from other species as well. Two phosphoprotein components containing Ser(P) and Thr(P) with M_r ~33K and >100K have been identified in mouse bone (Gotoh et al., 1983) and one in rat bone of M_r 55K (Oosawa et al., 1985). Three protein components have been isolated from bovine bone containing phosphorus (Termine et al., 1981). We have analyzed several samples of one of these components (osteonectin) prepared by Dr. John Termine and have identified the presence of a very small quantity of Ser(P) and possibly a trace of Thr(P).

In the present study, we report the isolation and purification to apparent homogeneity of 14 phosphoprotein components

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[1] Abbreviations: GdmCl, guanidinium chloride; Ser(P), serine phosphate; Thr(P), threonine phosphate; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid.

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with molecular weights ranging from ~4000–5000 to ~150 000.[2] Three of the components (M_r 150K, 90K, and 70 K) contain Hyp and/or Hyl which may signify that they are covalently bonded to or are very strongly associated with collagen. Alternatively, it may be due to the hydroxylation of Pro and/or Lys residues of the phosphoproteins (Drickamer et al., 1986; Reid, 1983; Rosenberry & Richardson, 1977; Benson et al., 1985; Urushizaki & Seifter, 1985). Amino acid compositions, partial amino acid sequencing, and preliminary cross-reaction studies with antibodies to several of the phosphoproteins make it clear that the heterogeneity of the bone matrix phosphoproteins is due in part to proteolysis of higher molecular weight components. It is also possible that there is more than one gene for the phosphoproteins. A preliminary report has already been presented (Uchiyama et al., 1985).

Materials and Methods

Tissue and Tissue Extractions. Diaphyses of the metatarsal bones of 14-week-old chickens were cleaned, ground in a liquid N_2 cooled mill, and extracted in 0.5 M EDTA (200 mL/g of bone), pH 7.8 at 2 °C, for 2 weeks, or longer, with changes of EDTA every 3 days; the extract was dialyzed free of salt against 0.01 M NH_4HCO_3 using hollow fiber ultrafiltration with a molecular weight cutoff of 5000 (Amicon Corp., Lexington, MA) and lyophilized (Lee & Glimcher, 1981; Glimcher et al., 1979c). Protease inhibitors (Cohen-Solal et al., 1979; Lee & Glimcher, 1981) and the alkaline phosphatase inhibitor levamisole (0.01 mM) (Sigma Chemical Co., St. Louis, MO) (Fallon et al., 1980; Van Belle, 1972, 1976) were present during the preparation of the tissue and of the tissue extracts (Cohen-Solal et al., 1979; Strawich et al., 1985; Strawich & Glimcher, 1985). In other instances, the bone was extracted in prechilled 0.1 N HCl at 2 °C on ice, approximately 500 mL of HCl/40 g of dry bone for from 4 to 24 h. During the first 4–6 h, the pH was maintained at 1.0 by the addition of 12 M HCl. The HCl in the extracted solution was removed either by dialyzing directly against water or by raising the pH to 7.8 which precipitated the Ca–P solid phase and reduced the time the proteins were exposed to an acid pH as well as facilitating the recovery of the extracted proteins. Analyses for Ser(P) showed that well over 95% of the Ser(P) was recovered with the Ca–P solid phase. The precipitate was harvested by centrifugation, dissolved in 0.5 M EDTA, pH 7.8 at 2 °C, and dialyzed free of salts in the Amicon dialyzer apparatus (Lee & Glimcher, 1981) containing the protease inhibitors and levamisole.

Isolation and Purification of the Phosphoproteins. The two major schema for the isolation of the phosphoproteins are shown in Figure 1. Minor variations are described in the text.

Schema of Techniques Used in Isolating Phosphoproteins. In method I (Figure 1A), EDTA and HCl extracts were separated by molecular sieving (G-100 Sephadex). Nine fractions, each containing Ser(P) and Thr(P), were then individually chromatographed on DEAE-cellulose or Sephacel and assessed for homogeneity by SDS–PAGE stained with both Coomassie Blue and Rhodamine B. The DEAE samples were then molecularly filtered in 6 M GdmCl by HPLC or Sephacryl S-300, rerun at least once. The fractions obtained by molecular sieving were then examined by SDS–PAGE.

[2] The molecular weights listed in this paper are approximate. There has been reported to be a great variance between the molecular weights of phosphoproteins determined by SDS–PAGE and by equilibrium ultracentrifugation (Strawich et al., 1985; Stetler-Stevenson & Veis, 1983; Jontell et al., 1982; Creamer & Richardson, 1984). The approximation sign (~) will not be used hereafter.

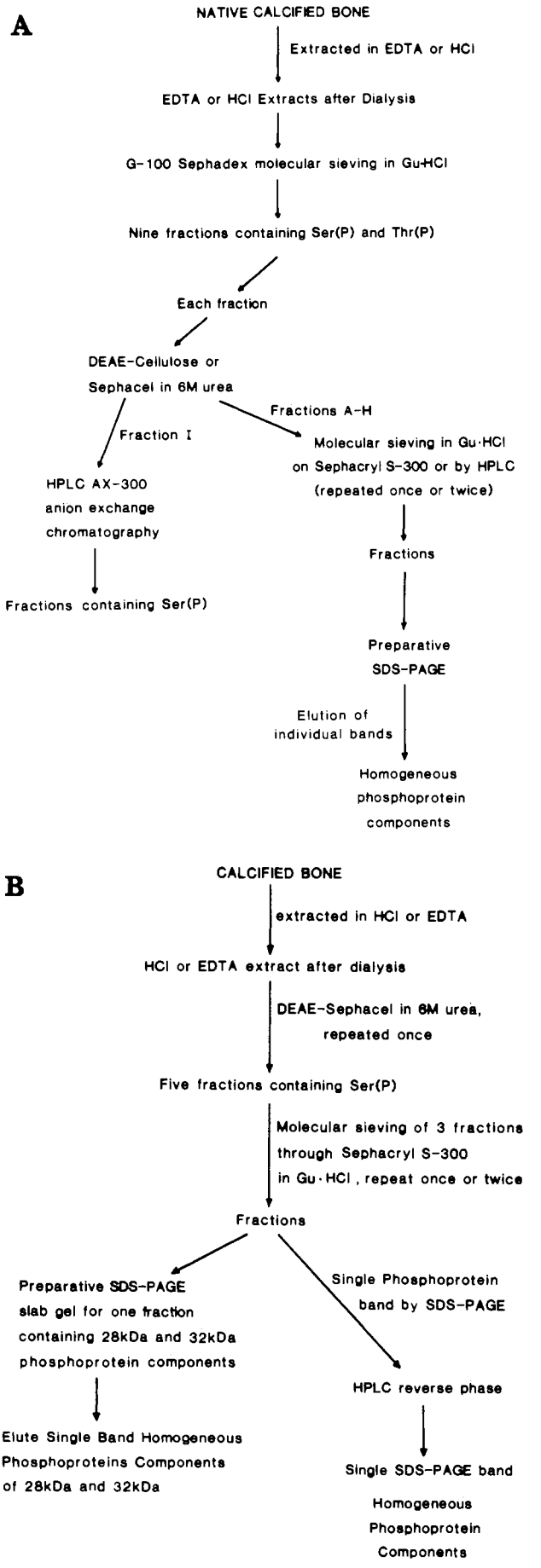

FIGURE 1: (A and B) Two major schema used to isolate chicken bone matrix phosphoproteins.

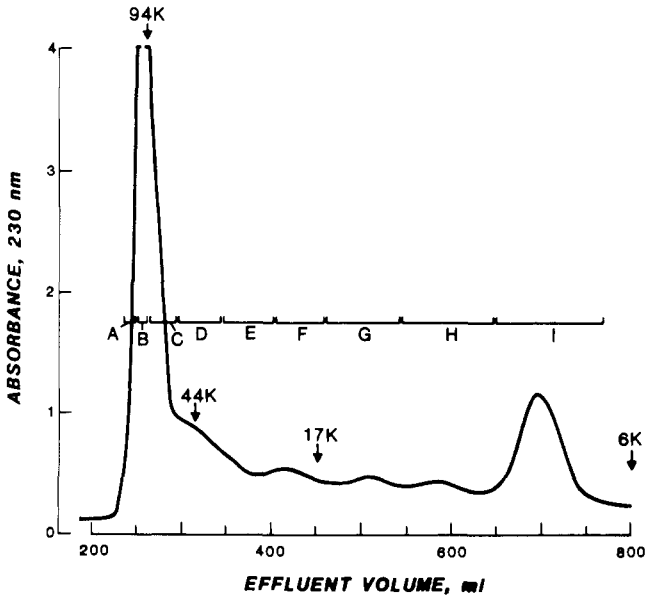

FIGURE 2: Elution pattern of EDTA-soluble proteins of chicken bone sieved on two columns (2.6 cm × 100 cm each) of G-100 Sephadex run in series in 4 M GdmCl, pH 7.5 at room temperature.

Homogeneity of the phosphoprotein components from HPLC and Sephacryl S-300 sievings were assessed, with one exception, by running them on SDS–PAGE slab gels, and the single phosphoprotein components were eluted from the gel. Analytical SDS–PAGE of these samples was carried out to assure that only single-band components had been eluted from the preparative SDS–PAGE. In a few instances, single-band components by SDS–PAGE were obtained from repeated molecular sieving after DEAE anion-exchange chromatography, but these samples were nonetheless further purified by preparative SDS–PAGE. A modification of schema I was used to isolate the lowest molecular weight (4K–5K) components. The ninth fraction from the Sephadex G-100 molecular sieve columns which contained osteocalcin as its principal protein component (fraction I, Figure 2) was chromatographed on DE-52 cellulose followed by additional anion-exchange chromatography by HPLC on an AX-300 Brownlee column (Rainin Instrument Corp., Inc., Woburn, MA). Two components eluting well after osteocalcin on the AX-300 column were isolated.

In schema II (Figure 1B), EDTA and HCl extracts were anion-exchange chromatographed on a 2.5 cm × 50 cm column of DEAE-Sephacel, pH 8.2, and 6 M urea at room temperature using a salt gradient from 0 to 0.5 M NaCl. The bone phosphoproteins were eluted between 0.2 and 0.5 M NaCl. Ser(P) was present in five of the fractions with most of the Ser(P) contained in the last two fractions (fractions 6 and 7), which were used for further purification. Fraction 3 was also used because it contained high molecular weight phosphoprotein components. The fractions containing Ser(P) were rechromatographed on DEAE-Sephacel and then molecularly sieved through two columns (each 1.5 cm × 100 cm) of Sephacryl S-300 in 6 M GdmCl, pH 7.4, run in series. The fractions were rerun on Sephacryl S-300 at least once. Five of the fractions (150, 90, 60, 32, and 15 kDa) from Sephacryl S-300 sieving were further purified by reverse-phase HPLC. Several fractions from the DEAE-Sephacel ion-exchange chromatography contained two Rhodamine B staining bands on SDS–PAGE after being sieved through Sephacryl S-300 (32- and 28-kDa components and 70- and 45-kDa components). In these cases, the samples were subjected to preparative SDS–PAGE on slab gels and the individual phosphoprotein bands eluted from the gels.

The difficulty encountered in isolating the highest molecular weight phosphoproteins observed by SDS–PAGE and Rhodamine B staining (Spector & Glimcher, 1972; Lee & Glimcher, 1981; Y, Gotoh, personal communication) was also experienced in the present experiments using schema I. However, with schema II, two high molecular weight components were isolated (150 and 90 kDa) from extracts first separated by anion-exchange chromatography on DEAE-Sephacel, then molecularly sieved twice on Sephacryl S-300 in 6 M GdmCl, and further purified by reverse-phase HPLC. The individual techniques are as follows.

(*A*) *Sephadex G-100 Chromatography.* EDTA and HCl extracts were dissolved in 4 M GdmCl, pH 7.4, and applied to two columns (2.6 cm × 100 cm each) of G-100 Sephadex (Pharmacia) run in series in 4 M GdmCl, pH 7.4 at room temperature. Eluent was monitored at 230 nm by using a flow cell spectrophotometer (Gilson, Holochrome), and 5-mL fractions were collected. The eluent was separated into nine fractions according to the elution profile. Each fraction was dialyzed against deionized, distilled water with inhibitors at 4 °C using Spectra-Por-3 dialysis tubing (molecular weight cutoff of 3500; Spectrum Medical Industries). Aliquot samples of each of the nine fractions were taken for total amino acid analyses and for determination of Ser(P) and Thr(P). The nine separate fractions containing Ser(P) and Thr(P) from each of several G-100 sievings were pooled and used for further procedures to isolate homogeneous preparations of the phosphoproteins.

(*B*) *DEAE-cellulose or Sephacel Anion-Exchange Chromatography.* Samples from either the G-100 molecular sieving (schema I) or the crude EDTA and HCl extracts (schema II) were applied to a 2.6 cm × 35 cm column of DE-52 cellulose or DEAE-Sephacel in 6 M urea at room temperature and 0.05 M Tris, pH 8.2. The column was eluted with a 0–0.5 M NaCl linear gradient at room temperature at a flow rate of ~60 mL/h. Fractions of ~10 mL were collected. The bone phosphoproteins were eluted between 0.2 and 0.5 M NaCl. Each peak obtained by anion-exchange chromatography was dialyzed, lyophilized, and analyzed for total amino acid composition and for Ser(P) and Thr(P).

(*C*) *Sephacryl S-300.* Samples of protein obtained by anion-exchange chromatography containing Ser(P) and Thr(P) were dissolved in 6 M GdmCl, pH 8.2, and 0.05 M Tris and passed through two columns (each 1.5 cm × 100 cm) of Sephacryl S-300 run in series in 6 M GdmCl, pH 7.4. The flow rate was ~19 mL/h, and ~3-mL fractions were collected.

(*D*) *High-Performance Liquid Chromatography (HPLC).* (*a*) *Molecular Sieving.* Some of the Ser(P)- and Thr(P)-enriched fractions obtained from DEAE anion-exchange chromatography were molecularly sieved by HPLC using appropriate combinations of TSK-SW 4000, 3000, and 2000 columns (Beckman Instruments) equilibrated and eluted with 6 M GdmCl, pH 6.5.

(*b*) *Ion-Exchange Chromatography.* Several fractions, in particular the peak containing osteocalcin as its major protein component obtained by G-100 Sephadex molecular sieving and DEAE-cellulose anion-exchange chromatography, were subjected to further purification by HPLC anion-exchange chromatography using a Brownlee Aquapore AX-300 column (Rainin Instrument Co., Inc., Woburn, MA). The column is equilibrated in a starting buffer of 0.05 M Tris, pH 7.5. The sample is dissolved in the same buffer and centrifuged, and the supernatant is applied through the injector at a concentration of ~1 mg/100 μL. The flow rate of the column is kept

at 1 mL/min. The HPLC program runs for 5 min with 100% starting buffer to remove void material. A stepwise gradient is then begun to a final concentration of 0.05 M Tris and 1 M NaCl, pH 7.5. The most highly phosphorylated phosphoproteins are not removed until a concentration of 1 M NaCl is reached. Fractions are taken at 1-mL intervals; the appropriate tubes are pooled, dialyzed, and lyophilized. Running time varies between ~95 and 115 min.

(*c*) *Reverse-Phase Chromatography.* Both C_{18} (μ Bondapak; Waters Associates, Milford, MA) (for all but the 150-kDa component) and C_3 (Ultrapore Analytical; Beckman Instruments, Inc., Fullerton, CA) (for the 150-kDa component) HPLC columns were used. Protein samples were dissolved in 0.1% TFA and the columns equilibrated in 0.1% TFA. A starting buffer of 0.1% TFA and a limiting buffer of 0.1% TFA and 70% acetonitrile were used. A gradient of 0–49% acetonitrile in 0.1% TFA was utilized and the column stripped with 70% acetonitrile before reequilibration in 0.1% TFA.

In one set of experiments, the 90-kDa phosphoprotein which was found to contain Hyp and Hyl was further subjected to reverse-phase HPLC using a C_8 Brownlee Aquapore RP-300 column (Rainin Instrument Co., Inc., Woburn, MA) (Eyre & Wu, 1983; Wu & Eyre, 1984). In this system, collagen and collagen peptides are very clearly separated from noncollagenous proteins which are eluted much earlier than collagen.

(*E*) *SDS–Polyacrylamide Gel Electrophoresis* (*Eyre & Wu, 1983*; *Wu & Eyre, 1984*). (*a*) *Analytical Gels.* Acrylamide gels (5% or 15%) of 1.5-mm thickness were used with a 3% acrylamide stacking gel and wells for samples. Samples were applied at a concentration of 2 mg/mL in a sucrose buffer (Na_2CO_3, SDS, and sucrose). The gels were run in a Tris–glycine–SDS buffer. Two identical gels were run at the same time and after electrophoresis were prepared as follows: one gel was stained with Coomassie Brilliant Blue R-250 in 2-propanol and acetic acid (25:10) overnight and destained with a methanol–acetic acid mixture. The other gel was fixed in a 25:10 mixture of 2-propanol–acetic acid overnight and then stained with Rhodamine B (Debruyne, 1983). We have found as reported by DeBruyne (1983) that phosphoproteins are specifically stained by Rhodamine B while other proteins are not.

(*b*) *Preparative Gels.* Preparative gels were made in the same way as analytical gels except that instead of wells in the stacking gel, the sample was applied to the entire length of the gel, ~6 mg/1.5-mm gel for a heterogeneous sample (Laemmli & Favre, 1973), and stained with Coomassie Brilliant Blue R-250 according to the method of Scott et al. (1976). After the position of the phosphoproteins on the analytical gel was determined, the appropriate band on the preparative gel was sliced and extracted according to the method described by Sreekrishna et al. (1980). The extracted samples were then dialyzed against a 0.005% SDS solution and lyophilized. Approximate molecular weights of the phosphoprotein components eluted were calculated from SDS–PAGE using regular protein standards (Bio-Rad) (Strawich et al., 1985).

(*F*) *Amino Acid Analyses.* Except for some of the Ser(P) and Thr(P) analyses which were carried out by ion-exchange HPLC (Etheredge & Glimcher, 1986), all analyses were carried out on an automatic amino acid analyzer (Beckman 121-M). Ser(P) and Thr(P) were determined chromatographically on partial acid hydrolysates (Cohen-Solal et al., 1978), γ-carboxyglutamic acid after alkaline hydrolysis (Hauschka, 1977), and complete amino acid analyses after hydrolysis of samples with 6 N HCl at 110 °C for 24 h in vacuo.

Table I: Distribution and Concentrations of Ser(P) and Thr(P) and Protein Content of Fractions Collected from G-100 Sephadex Molecular Sieving (See Figures 1A and 2)

	%			residues/1000 residues	
fraction	Ser(P)	Thr(P)	protein	Ser(P)	Thr(P)
A	5.8	1.7	23.7	1.7	0.2
B	7.9	6.8	15.3	2.2	0.3
C	7.9	0.7	9.3	3.3	0.04
D	19.2	12.8	9.1	6.7	0.6
E	9.1	16.1	7.0	5.8	1.5
F	19.5	24.2	9.8	8.8	1.7
G	23.7	27.8	7.9	13.0	2.3
H	3.5	2.4	6.0	2.6	0.3
I	3.5	7.6	12.7	1.0	0.3

(*G*) *Amino Acid Sequencing.* Protein samples obtained by reverse-phase HPLC were sequenced on a Model 470A Gas-Phase Protein Sequencer (Hewick et al., 1981). The phenylthiohydantoin amino acids from each cycle were identified by high-performance liquid chromatography (Hunkapiller & Hood, 1983).

(*H*) *Carbohydrate Analyses.* Seven of the phosphoproteins were analyzed for their carbohydrate content by methanolysis, re-N-acetylation, and formation of their trimethylsilyl derivatives. The samples are then injected into a Perkin-Elmer 900 gas chromatograph on a 3-m column packed with 3% OV-17 on Gas-Chrom Q, 80–100 mesh. The column is programmed from 120 to 290 °C with a gradient of 8 °C/min. The method is slightly modified from that of Reinhold (1972).

Results

The results of a typical Sephadex G-100 molecular sieving (Figure 1A, schema I) of an EDTA extract are shown in Figure 2. The eluent was divided into nine fractions (A–I, Figure 2), all of which contained Ser(P) and Thr(P). The distribution of Ser(P) and Thr(P) is given in Table I. On the basis of the Ser(P) concentrations, the majority of the phosphoproteins (62%) were eluted in three peaks corresponding to elution molecular weights from the G-100 Sephadex columns having mid values of 44K, 19K, and 15K. The three fractions from the G-100 Sephadex sieving contained only ~27% of the eluted protein. Fraction I is in the position of osteocalcin and contained γ-carboxyglutamic acid. SDS–PAGE of the individual G-100 Sephadex fractions obtained from EDTA or HCl extracts stained with Coomassie Blue and Rhodamine B, the latter specific for the phosphoproteins (DeBruyne, 1983), showed a large number of protein bands including four major, Rhodamine B staining phosphoprotein species having molecular weight of 32K, 28K, 15K, and 5K (Figure 3A).

All four fractions from the G-100 Sephadex molecular sieving (D, E, F, and G) containing the major Rhodamine B staining phosphoprotein species also contained a number of additional Coomassie Blue staining, apparently unphosphorylated proteins (Figure 3B).

DEAE-cellulose or Sephacel anion-exchange chromatography of the nine fractions isolated by G-100 Sephadex molecular sieving showed a generally characteristic elution profile with the phosphoproteins [determined from Ser(P) concentrations of the fractions] eluted at NaCl concentrations ranging from 0.2 to 0.5 M NaCl. Figure 4a–d demonstrates the elution pattern of the four fractions obtained from G-100 Sephadex filtration (Figure 2) which contained the majority of the extracted phosphoproteins. However, DEAE-cellulose or Se-

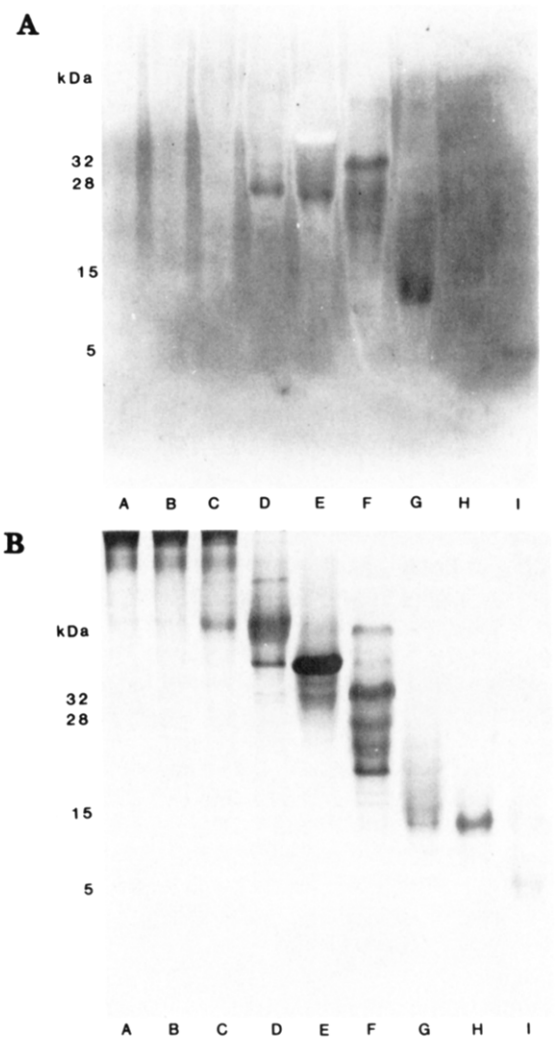

FIGURE 3: (A) SDS–PAGE of fractions obtained from G-100 Sephadex molecular sieving stained with Rhodamine B. A–I refers to fractions shown in Figure 2. (B) SDS–PAGE of fractions obtained from G-100 Sephadex molecular sieving stained with Coomassie Blue. A–I refers to fractions shown in Figure 2.

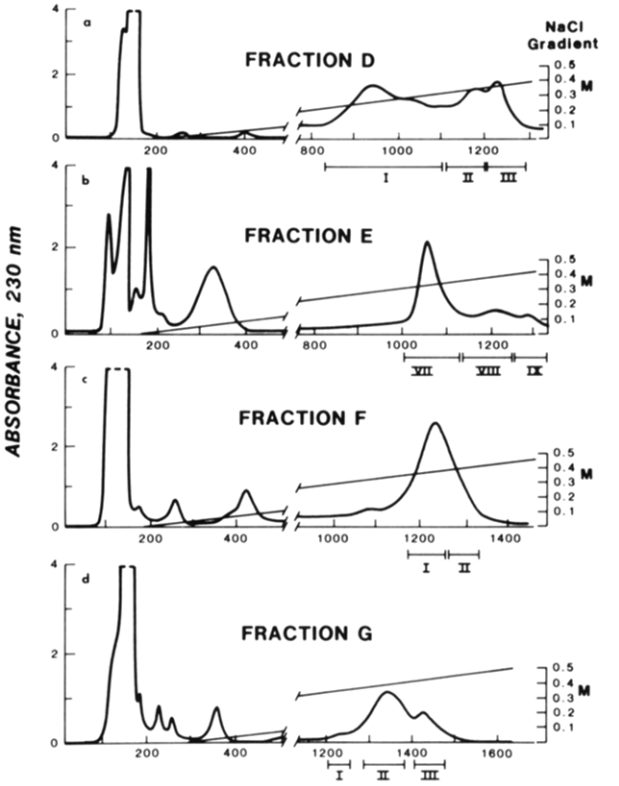

FIGURE 4: Elution patterns from DEAE-Sephacel of the four peaks obtained from G-100 Sephadex molecular sieving (Figure 2 and Table I) containing the majority of the bone phosphoproteins.

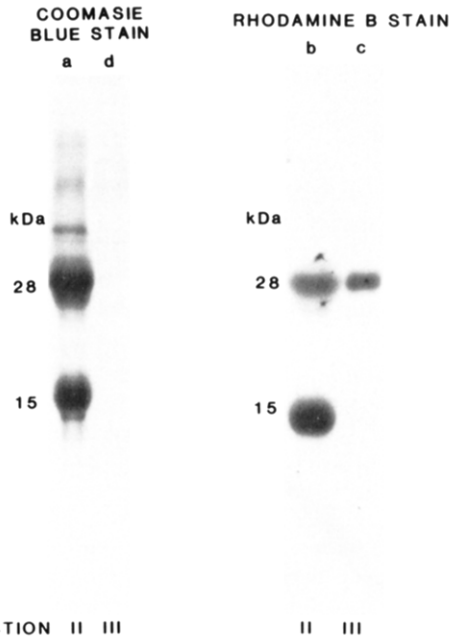

FIGURE 5: SDS–PAGE of two of the peaks obtained by DEAE-Sephacel anion-exchange chromatography of fraction D eluted by G-100 Sephadex molecular sieving. (a) Coomassie Blue and (b) Rhodamine B staining of peak II (Figure 4a). (c) Coomassie Blue and (d) Rhodamine B staining of peak III (Figure 4a).

phacel anion-exchange chromatography of the fractions obtained by G-100 Sephadex molecular sieving, even when repeated 1 or more times, failed to provide a single homogeneous, Rhodamine B staining phosphoprotein component without the presence of unphosphorylated Coomassie Blue staining proteins. For example, fractions D and G from the Sephadex G-100 molecular sieving (Figures 2 and 4a,d) each produced two major DEAE-cellulose peaks containing Ser(P) and Thr(P) [fractions II and III of D (Figure 4a); fractions II and III of G (Figure 4d)]. In addition to the presence of Coomassie Blue staining bands (Figure 5a), DEAE-cellulose peak II of fraction D (Figure 4d) obtained from the G-100 Sephadex sieving (Figure 2) contained two Rhodamine B staining bands with molecular weights of 28K and 15K, respectively (Figure 5b). Although DEAE-cellulose peak III from fraction D (Figure 4a) showed only one Rhodamine B staining band of M_r 28K (Figure 5c), it too contained Coomassie Blue staining bands in addition to the Rhodamine B band (Figure 5d). The front half (fraction I) of the slightly asymmetrical single Ser(P)-containing DEAE-cellulose peak from fraction F (Figure 4c) of the G-100 Sephadex sieving also contained a major Rhodamine B staining band of 28 kDa, as well as other bands staining faintly with Rhodamine B and having molecular weights of 32K and 33K and several of M_r 13K–15K. The two DEAE-cellulose peaks (II and III) (Figure 4d) of fraction G from G-100 molecular sieving (Figure 2) had approximately four major and several minor Rhodamine staining bands ranging from approximately 12 to 15 kDa (Figure 6) with several of the bands broadened, one considerably.

Molecular sieving (twice) in 6 M GdmCl by HPLC of the fractions obtained by DE-52 cellulose anion-exchange chromatography (twice) was successful in obtaining one fraction (M_r 28K) containing only a single Rhodamine B staining band

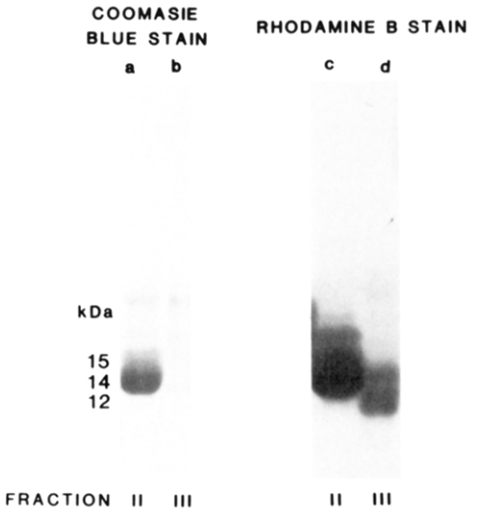

FIGURE 6: SDS–PAGE of peaks II and III eluted from DEAE-Sephacel anion chromatography of fraction G (Figure 4d) obtained by G-100 Sephadex filtration (Figure 2). (a) Fraction II stained with Coomassie Blue and (c) Rhodamine B; (b) fraction III stained with Coomassie Blue and (d) Rhodamine B.

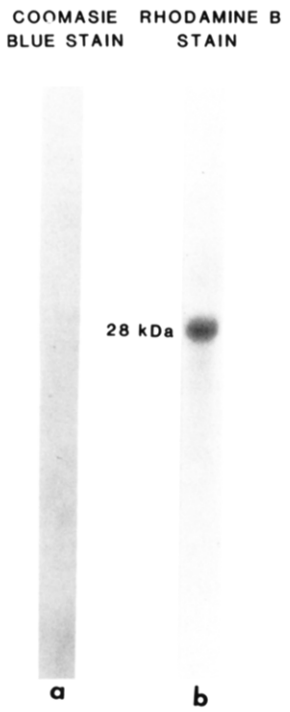

FIGURE 7: SDS–PAGE of fraction obtained from HPLC sieving in 6 M GdmCl (4000 TSK and 3000 TSK in series) of fraction III from DE-52 cellulose anion-exchange chromatography of fraction D obtained by G-100 Sephadex molecular sieving. (a) Rhodamine B staining; (b) Coomassie Blue staining.

without apparent Coomassie Blue staining proteins (Figure 7): fraction III of the DE-52 cellulose anion-exchange chromatography (Figure 4a) and fraction D from G-100 Sephadex molecular sieving (Figure 2). Despite this isolation of an apparent homogeneous phosphoprotein, this component, like the others obtained by other techniques, and not further purified by reverse-phase HPLC, was subjected to preparative SDS–PAGE and a 28-kDa component eluted (Figure 1A, Schema I). Analytical SDS–PAGE of two components (28 and 32 kDa) eluted from single bands of preparative SDS–PAGE is shown in Figure 8 (Rhodamine B stained). No additional bands were observed with Coomassie Blue staining.

When the second schema (Figure 1B, schema II) was used, viz., DEAE-cellulose or Sephacel anion-exchange chroma-

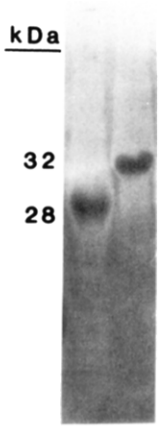

FIGURE 8: SDS–PAGE of two phosphoproteins eluted from two bands cut from a single preparative gel. The phosphoproteins were obtained from fraction D of G-100 Sephadex filtration and further purified by DE-52 cellulose anion-exchange chromatography. Stained with Rhodamine B.

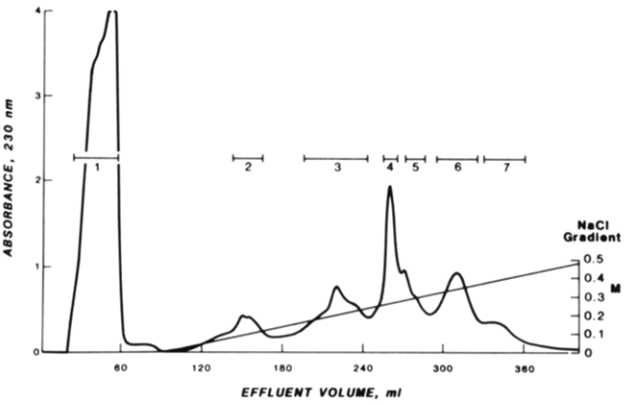

FIGURE 9: Elution pattern of EDTA-soluble phosphoproteins of chicken bone when subjected to anion-exchange chromatography on DEAE-Sephacel in 6 M urea, room temperature, pH 8.2.

Table II: Distribution of Ser(P), Thr(P), and Total Protein in Fractions Eluted by DEAE-Sephacel Anion-Exchange Chromatography (See Figures 1B and 2)

	%		
fraction	Ser(P)	Thr(P)	protein
1	0	0	38.0
2	0	0	6.9
3	5.7	Tr[a]	9.4
4	3.2	Tr	19.4
5	7.0	6.9	6.6
6	54.2	70.5	12.9
7	30.0	24.8	6.9

[a] Tr, trace.

tography in 6 M urea first, seven fractions were collected from the eluent, five of which (fractions 3–7) contained Ser(P) and Thr(P) (Figure 9). Two of the fractions (DEAE fractions 6 and 7), which contained the vast majority of the Ser(P) (Table II), and DEAE fraction 3, which by SDS–PAGE contained several high molecular weight components, were rechromatographed on DEAE-Sephacel, and the fractions obtained were used for further purification, first by molecular sieving on Sephacryl S-300 in 6 M GdmCl. Typical profiles of several samples from molecular sieving on Sephacryl S-300 of fractions obtained by DEAE-Sephacel anion-exchange chromatography are shown in Figure 10. After one to two runs on DEAE-Sephacel and two or more sievings through Sephacryl S-300, five major representative phosphoproteins

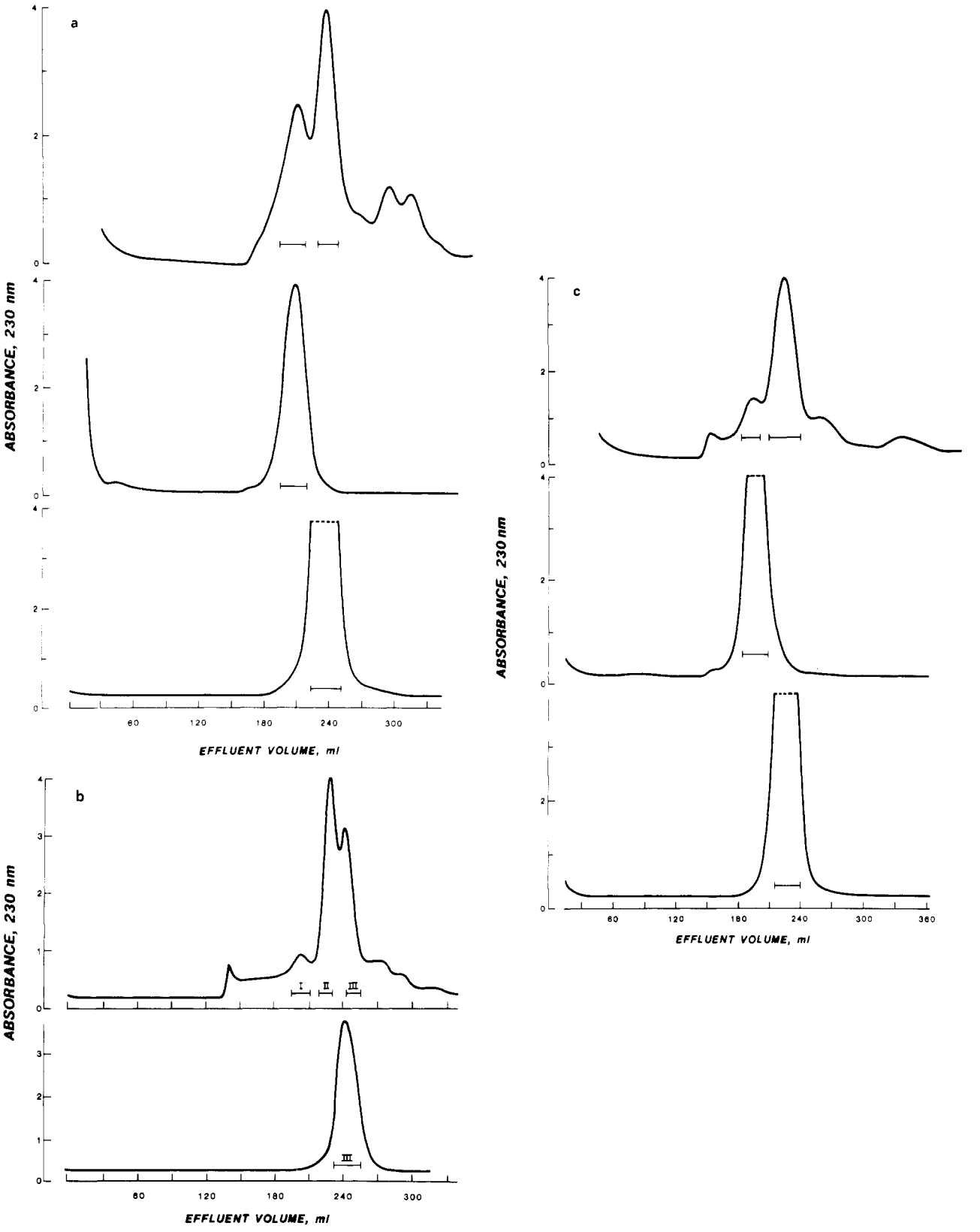

FIGURE 10: Molecular sieving through two columns of Sephacryl S-300 in 6 M GdmCl of fractions 3 (a), 6 (b), and 7 (c) obtained by DEAE-Sephacel anion-exchange chromatography (Figure 9).

spanning the breadth of molecular weights, and running as single bands on SDS–PAGE, were obtained in sufficient amounts for further purification by reverse-phase HPLC (150, 90, 60, 32, and 15 kDa). A representative elution pattern from reverse-phase HPLC and SDS–PAGE of the five components obtained by HPLC is shown in Figures 11 and 12, respectively. All of the components except for M_r 150K appeared as single, but broad band components possibly because of their relatively high content of carbohydrates. The relatively sharp 150-kDa band may be due to its very short excursion in the gel. All five of the components had a single major N-terminal amino acid sequence so the broadening is unlikely to be due to impurities. One Sephacryl fraction contained a 45- and a 70-kDa phosphoprotein, another, a 28- and a 32-kDa phosphoprotein.

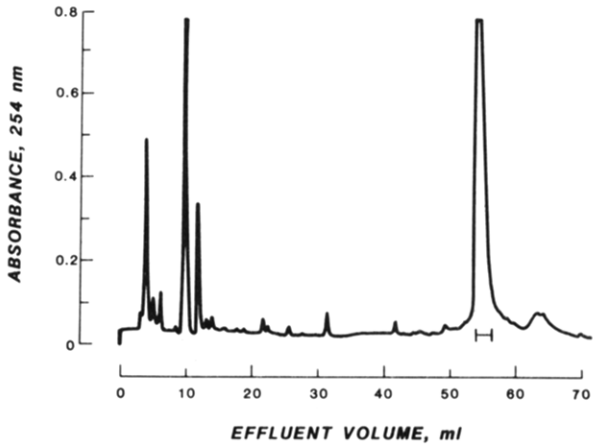

FIGURE 11: Representative elution pattern of a bone phosphoprotein (60 kDa) from reverse-phase HPLC (schema II; Figure 1B).

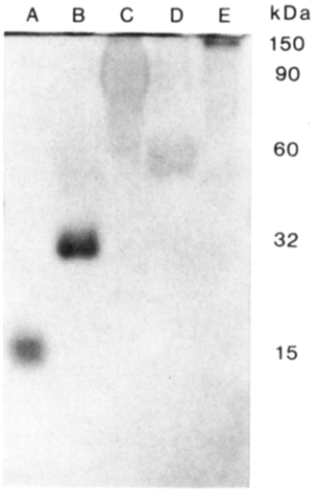

FIGURE 12: SDS–PAGE of five phosphoproteins each purified by reverse-phase HPLC as a final step (schema II; Figure 1B). Except for the 150-kDa component, all of the bands are relatively broad, possibly due to high carbohydrate content. The 150-kDa species has migrated very little into the gel which may account for its relative sharpness.

These could not be separated by repeated DEAE-Sephacel chromatography and molecular sieving. The two components in each of the fractions were individually eluted from preparative SDS–PAGE. Analytical SDS–PAGE of these components eluted from the preparative SDS is shown in Figure 13.

The amino acid compositions of 14 phosphoprotein components which were isolated and which migrated as single bands on SDS–PAGE are shown in Table III.

SDS–PAGE of the remaining homogeneous, single band phosphoproteins whose amino acid compositions are shown in Table III and which had not been previously illustrated is shown in Figure 14. All of them are stained with Rhodamine B. Three phosphoproteins which also stained with Coomassie Blue are also shown.

The partial amino acid sequences of the N-terminal portions of four of the phosphoprotein components are shown in Table IV.

The carbohydrate contents of seven of the phosphoprotein components are shown in Table V.

Discussion

Previous work on matrix phosphoproteins of chicken bone has succeeded in isolating only two homogeneous phosphorylated components of M_r 6K (Spector & Glimcher, 1972) and

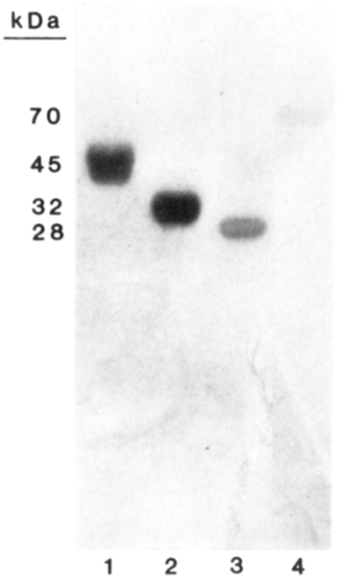

FIGURE 13: SDS–PAGE of four phosphoproteins eluted from two preparative SDS–PAGE gels. Components for the two preparative SDS–PAGE gels were obtained by DEAE-Sephacel anion-exchange chromatography and Sephacryl S-300 molecular sieving. One fraction contained the 28- and 32-kDa components, the other the 45- and 70-kDa components. Lane 1, M_r 45K; lane 2, M_r 32K; lane 3, M_r 28K; lane 4, M_r 70K. Stained with Rhodamine B. No additional components when stained with Coomassie Blue.

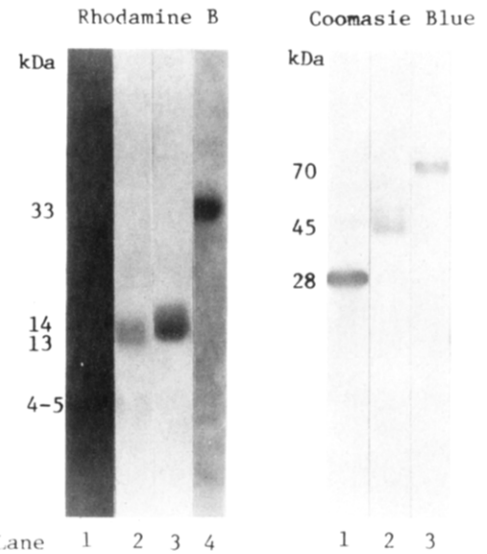

FIGURE 14: SDS–PAGE of homogeneous phosphoprotein components listed in Table III and not previously illustrated in the text. (Left panel) Four of the phosphoprotein components which stained with Rhodamine B. Lane 1, M_r 4K–5K; lane 2, M_r 13K; lane 3, M_r 14K; lane 4, M_r 33K. (Right panel) Three of the phosphoprotein components which stained with Coomassie Blue. Lane 1, M_r 28K; lane 2, M_r 45K; lane 3, M_r 70K.

12K (Lee & Glimcher, 1981) and one fraction which contained principally a phosphoprotein of M_r 28K–30K (Lee & Glimcher, 1981). Thr(P) as well as Ser(P) was identified in the M_r 12K and 28K–30K components and the phosphomonoester nature of the organic phosphorus moiety verified by ^{31}P NMR of the intact proteins (Lee & Glimcher, 1981). In both studies, however, it was clear on the basis of the Ser(P) contents of fractions eluted by molecular sieving and ion-exchange chromatography that there were many more EDTA-soluble Ser(P)-containing proteins in the EDTA extracts. At that time, efforts to isolate additional homogeneous phosphoprotein components were unsuccessful. In the present

Table III: Amino Acid Composition (Residues/1000 Total Residues) of 14 Homogeneous Phosphoprotein Components Isolated from Chicken Bone

M_r:	4K–5K$_1$[a]	4K–5K$_2$[a]	13K	14K	15K	28K$_1$[b]	28K$_2$[b]	32K	33K	45K	60K	70K[d]	70K[c] – collagen	90K[e]	90K[c] – collagen	150K[f]
schema:	I	I	I	I	II	I	II	II	II	II	II	II		II	II	II
source:	ion-ex HPLC	ion-ex HPLC	SDS–PAGE	SDS–PAGE	rev-ph HPLC	SDS–PAGE	SDS–PAGE	SDS–PAGE	SDS–PAGE	SDS–PAGE	rev-ph HPLC	SDS–PAGE	SDS–PAGE	rev-ph HPLC	rev-ph HPLC	rev-ph HPLC
amino acids																
CysO$_3$[g]	3	0	0	5	12	0	0	33	3	0	23	2	2	8	9	1
Asp	123	94	166	171	193	217	180	145	183	141	148	89	119	85	87	112
Thr	9	10	39	30	29	48	50	35	34	51	44	32	42	33	33	72
Ser	57	102	172	116	123	98	118	106	111	147	93	98	143	195	202	62
Glu	192	157	214	181	181	171	199	234	172	115	89	99	112	105	106	352
Pro	98	81	43	33	28	66	44	36	38	51	84	72	32	61	58	30
Gly	99	206	98	58	54	47	73	144	66	135	69	248	198	154	148	129
Ala	120	96	71	126	124	73	81	75	118	74	86	94	74	93	92	93
Cys	19	0	0	0	0	0	2	0	2	6	14	2	3			2
Val	59	38	31	64	48	53	45	49	60	24	56	25	33	41	42	26
Met	0	0	6	2	0	4	3	0	3	0	2	7	9			1
Ile	20	11	11	20	16	16	15	Tr[h]	18	17	22	16	23	19	20	1
Leu	52	42	22	67	62	26	32	25	58	48	74	36	48	41	42	20
Tyr	38	22	13	2	0	25	19	Tr	5	19	20	12	18	8	9	31
Phe	21	21	14	11	9	29	23	Tr	16	23	32	17	20	17	17	
Lys	16	47	48	35	34	28	38	30	32	41	45	31	28	31	31	17
His	19	21	26	19	22	56	40	23	22	30	53	19	30	35	36	20
Arg	54	35	29	59	64	44	37	65	58	68	45	59	68	66	67	32
Ser(P)	0.7	7	19	15	9	16	11	19	7	16	1.4	13	22	6	6	6
Thr(P)	0.5	0.8	5	6	4	5	3	5	2	5	Tr	4	6	Tr	Tr	Tr
Asp + Glu	315	251	380	352	374	388	379	379	355	256	237	188	231	190	193	464
Asp + Glu + Ser + Ala + Gly	591	655	721	652	675	606	651	704	650	612	485	628	646	632	635	748

[a] These components, 4K–5K$_1$ and 4K–5K$_2$, were isolated by Schema I methodology using HPLC AX-300 anion-exchange chromatography as the final step. [b] Two 28-kDa phosphoprotein components, one isolated by Schema I and the other by Schema II, had very different amino acid compositions. The subscripts 28 kDa$_1$ and 28 kDa$_2$ are used to identify the two components, 28 kDa$_1$ from Schema I and 28 kDa$_2$ from Schema II. [c] Theoretical amino acid compositions of phosphoproteins, subtracting contribution of collagen based on Hyp content. [d] Hyp, 42 residues/1000 residues; Hyl, 2 residues/1000 residues. [e] Hyp, 3 residues/1000 residues; Hyl, 2 residues/1000 residues. [f] Hyp, 0 residue/1000 residues; Hyl, 0.5 residue/1000 residues. [g] Cysteic acid. [h] Trace.

Table IV: Partial N-Terminal Amino Acid Sequences of Five Phosphoprotein Components of Chicken Bone Matrix

	150 kDa	32 kDa	90 kDa	60 kDa	15 kDa
1	Tyr	Tyr	Ala	Ala	Lys
2	Ala	Ala	Pro	Pro	Ala
3	Tyr	Tyr	Ala	Ala	Ala
4	Pro	Pro	Ala	Ala	Lys
5	Pro	Pro	Pro	Pro	Lys
6	Leu	Leu	Pro	Pro	Leu
7	His	His	Pro	Pro	Ile
8		(His)	Pro	Pro	Glu
9		Tyr	Leu	Leu	Asp
10		Lys	Gly	Gly	Ala
11		Gly			Ala
12		(Gly)	(Pro,Asp)	Asp	Ala
13			Asp	Asp	Ala
14			Pro	Pro	Glu
15			Glu	Glu	Val
16			Ile	Ile	Gly
17			Glu	Glu	Asp
18			Ala	Ala	Ser
19			Ala	Ala	Leu
20			Ala	Ala	Leu
21			Glu	Glu	(Ala)
22			Phe	Phe	Gly
23			Ala	Ala	Leu
24			Val	Val	Trp
25			Ile	Ile	
26				(Tyr,Ile)	
27				(Ile,Lys)	
28				(Asn)	
29				(Gly)	
30					
31					
32				(Lys)	

study, similar difficulties were encountered. For example, when the EDTA or HCl extracts of chicken bone were molecular sieved on two long G-100 Sephadex columns in series of GdmCl, each of nine eluted fractions contained a number of phosphorylated and nonphosphorylated protein components with a relatively broad range of molecular weights. In this regard, we note in passing that the use of Rhodamine B staining of SDS–PAGE gels to identify the phosphoprotein components in each of the eluted fractions was invaluble, since as many as 10 or more Coomassie-staining bands were present in many of the fractions eluted from the G-100 Sephadex columns. Thus, the only way we could identify and follow the phosphoprotein components in the various G-100 and subsequent fractions during the purification procedures was by Rhodamine staining. Taking sharper cuts of the eluted fractions and repeating the G-100 molecular sievings did little to limit the number of phosphorylated and nonphosphorylated species in the individual G-100 fractions. DEAE-cellulose or Sephacel anion-exchange chromatography in 6 M urea of each of the nine fractions obtained by G-100 Sephadex sieving also failed to isolate single, homogeneous phosphoprotein components, even when repeated. Homogeneous, single Rhodamine B staining bands without additional Coomassie Blue staining bands were finally achieved by a variety of techniques detailed under Materials and Methods and under Results, most commonly elution of single bands after preparative SDS–PAGE or by reverse-phase HPLC. By a combination of these techniques and schema (Figure 1A,B), 14 apparently homogeneous phosphoproteins which migrated as single bands on SDS–PAGE were eventually isolated which ranged in molecular weight from 150K to 4K–5K. To our knowledge, this is the first time that such a large number of homogeneous phosphoprotein components have been isolated from bone. All of the phosphoprotein components contained Thr(P) as well as Ser(P), consistent with our findings that EDTA extracts or whole calcified bone matrices from 20 species all contained significant amounts of Thr(P) as well as Ser(P). It is interesting that the highest molecular weight component isolated had approximately the same molecular weight as the highest molecular weight phosphoprotein isolated from dentin (Stetler-Stevenson & Veis, 1983).

Two of the phosphoproteins isolated contained Hyp and Hyl, and one only Hyl. The molecular weights of these Hyp-containing phosphoproteins were 90K and 70K, and the 150-kDa component contained only Hyl. Hyl without Hyp has previously been noted in a phosphorylated peptide released from bovine bone by treatment with periodate (Shuttleworth & Veis, 1971). The Hyl/100 Hyp ratio in the 90-kDa phosphoprotein component was also very elevated, viz., 45, a value similar to those found in phosphoproteins released from the EDTA-insoluble residues of bovine bone by bacterial collagenase and purified by molecular sieving and anion-exchange chromatography (Glimcher et al., 1986). This is the first time that Hyp and/or Hyl has been identified in the EDTA- or HCl-soluble phosphoproteins of bone (or dentin) without prior treatment (viz., cyanogen bromide, periodate, etc.) and/or enzymatic degradation of the EDTA-insoluble tissues.

It has been assumed in the literature (Carmichael et al., 1971; Lee & Veis, 1980; Curley-Joseph & Veis, 1979; Maier et al., 1983; Wohllebe & Carmichael, 1979; Butler et al., 1983; Cohen-Solal et al., 1979; Francois et al., 1967) that the finding of Hyp and/or Hyl in mineralized tissue phosphoproteins and Ser(P) in mineralized tissue collagens indicates the presence of covalent phosphoprotein–collagen complexes. However, there are equally plausible alternative explanations, namely, that the Pro and/or Lys residues of the phosphoproteins per se are hydroxylated and, in a like manner, certain of the serine, threonine, or other hydroxylated amino acid residues of collagen are phosphorylated (Drickamer et al., 1986; Reid, 1983; Rosenberry & Richardson, 1977; Benson et al., 1985; Urushizaki & Seifter, 1985).

If one assumes that the Hyp and/or Hyl represents collagen or peptides derived from collagen, the findings support the data and conclusions of Veis and his colleagues (Carmichael et al., 1971; Lee & Veis, 1980; Curley-Joseph & Veis, 1979; Maier et al., 1983; Wohllebe & Carmichael, 1979) that some fraction of the extracellular phosphoproteins of dentin and bone is covalently bound to collagen. While we were not able to quantitatively calculate what proportion of the total phos-

Table V: Carbohydrate Content (Percent Weight) of Selected Phosphoproteins of Chicken Bone Matrix

sample	Fuc	Xyl	Man	Gal	Glc	GalN	GlcN	NANA[a]	% CHO
150 kDa	0.35	0.07	1.4	3.9	0.36	3.2	2.4	5.9	17.6
90 kDa	0.11	0.05	3.0	3.0	0.25		4.5	3.3	14.2
60 kDa	0.09	0.09	3.4	3.4	0.32		5.4	4.5	17.2
33 kDa		0.17	0.3	1.3		1.4	0.1	3.1	6.4
32 kDa	0.47	0.05	1.1	3.4	0.20	2.2	1.7	3.7	12.8
28 kDa_1		0.24	0.2	2.8		2.6		4.9	10.7
15 kDa		0.03	0.2	0.9	0.17	1.0	0.3	1.7	4.3

[a] *N*-Acetylneuraminic acid.

phoprotein content these putatively covalent complexes of phosphoprotein and collagen represent, they clearly appear to involve only a small fraction of the total phosphoprotein pool. Our earlier studies failed to detect any collagen-bound Ser(P) or Thr(P) in chicken bone (Cohen-Solal et al., 1978). However, in more recent work using larger samples and more sensitive methods to detect Ser(P) and Thr(P), we have identified a very small number of what appear to be covalent or strongly associated phosphoprotein–collagen complexes in GdmCl-extracted proteins of decalcified chicken bone (Glimcher et al., 1986). However, in like manner, these findings could just as well be interpreted as phosphorylation of certain Ser and Thr residues of collagen (Urushizaki & Seifter, 1985).

All of the seven phosphoprotein components analyzed contained significant amounts of carbohydrate. Phosvitin (Grizzuti & Perlmann, 1975), the phosphorylated protein of hen egg yolk, and the salivary phosphoproteins (Shainkin & Perlmann, 1971) have also been found to contain significant amounts of carbohydrate (Oppenheim et al., 1982). Considering the relatively high carbohydrate content of the chicken bone phosphoproteins, they are more accurately described as phosphorylated glycoproteins or phosphoglycoproteins than as simple phosphoproteins. The high carbohydrate content may have accounted in part for the relatively broad bands of the phosphoproteins on SDS–PAGE.

We have no data as to the number or possibly different kinds of carbohydrate chains which are bound to the various phosphoprotein components. Thus, it is not possible to draw specific conclusions about the carbohydrate components from their overall composition. Nonetheless, the presence of xylose which is found in almost all of the proteoglycans together with the presence of hexosamines is highly suggestive that some of the carbohydrate is present as proteoglycans. On the other hand, the presence of glucose in the carbohydrate portions of some of the phosphoproteins is unusual for glycoproteins. Further data are necessary in order to more fully characterize the carbohydrate components.

Although there are marked differences in the contents of specific amino acids in the 14 phosphoprotein components, there are certain general compositional features which are characteristic of them all. All of the phosphoproteins are rich in Asp and Glu (Lee & Glimcher, 1981), the combined values of Asp and Glu varying from ~18% to ~40% of the total amino acid content. Ser, Gly, and Ala constitute the other major amino acids: together with Asp and Glu, these five amino acids constitute from ~50% to ~75% of the total amino acids present in the proteins. The extent of phosphorylation also varies considerably in the components isolated in this and previous studies (Lee & Glimcher, 1981).

The heterogeneity in composition, molecular weight, and extent of phosphorylation, and the presence of a very large number of phosphorylated proteins, seems clearly to be due in part to proteolysis: the same N-terminal amino acid sequence for the 90- and 60-kDa proteins and for the 150- and 32-kDa components.

Although the number of independent gene products for the phosphoproteins cannot be determined from the data in hand, we speculate on the basis of the N-terminal amino acid sequences reported here in conjunction with preliminary cross-reaction studies of monoclonal and polyclonal antibodies to four of the phosphoproteins that there is more than one independent gene product for the chicken bone phosphoproteins.

A similar situation has been found in dentin (Veis et al., 1981; Lee et al., 1983; Richardson et al., 1978), in which the presence of several phosphoprotein species has been attributed both to independent genomes (rat dentin) (Richardson et al., 1978) and to proteolysis of a high molecular weight precursor (bovine dentin) (Lee et al., 1983), the proteolysis apparently a function of age and maturation. Degradation of an initially higher molecular weight precursor to lower molecular weight components with time has also been demonstrated in vivo in the case of the phosphoproteins of dental enamel (Strawich & Glimcher, 1985).

Resolution of the problem of how many primary phosphoprotein products are synthesized by the osteoblasts, the nature of the primary phosphoprotein gene products, and their intracellular and extracellular degradative pathways must await results from cell-free translation and other molecular biology studies.

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Registry No. L-Ser(P), 407-41-0; L-Thr(P), 1114-81-4.

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