Identification of Organic Phosphorus Covalently Bound to Collagen and Non-Collagenous Proteins of Chicken-Bone Matrix

THE PRESENCE OF *O*-PHOSPHOSERINE AND *O*-PHOSPHOTHREONINE IN NON-COLLAGENOUS PROTEINS, AND THEIR ABSENCE FROM PHOSPHORYLATED COLLAGEN

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Non-collagenous phosphoproteins, almost all of which can be extracted in EDTA at neutral pH in the presence of proteinase inhibitors, are identified in the matrix of chicken bone, and are therefore not covalently bound to collagen. Similarly, all the peptides containing γ -carboxyglutamic acid are present in the EDTA extract and none in the insoluble residue, confirming that none is covalently linked to chicken bone collagen. However, organic phosphorus is also found to be present in chicken bone collagen, principally in the α 2-chains. Of the total protein-bound organic phosphorus present in chicken bone matrix, approx. 80% is associated with the non-collagenous proteins and 20% with collagen. The soluble non-collagenous proteins contain both *O*-phosphoserine and *O*-phosphothreonine and these account for essentially all of their organic phosphorus content. In contrast, collagen contains neither *O*-phosphoserine nor *O*-phosphothreonine. Indeed, no phosphorylated hydroxy amino acid, phosphoamidated amino acid or phosphorylated sugar could be identified in purified components of collagen, which contain approximately four to five atoms of organic phosphorus per molecule of collagen. Peptides containing organic phosphorus were isolated from partial acid hydrolysates and enzymic digests of purified collagen components, which contain an as-yet-unidentified cationic amino acid. These data, the very high concentrations of glutamic acid in the phosphorylated peptides, and the pH-stability of the organic phosphorus moiety in intact collagen chains strongly suggest that at least part of the organic phosphorus in collagen is present as phosphorylated glutamic acid. This would indicate that the two major chemically different protein fractions in chicken bone matrix that contain organic phosphorus may represent two distinct metabolic pools of organic phosphorus under separate biological control.

Phosphoproteins have been identified as part of the non-collagenous components of the organic matrices of mineralized vertebrate tissues such as enamel (Seyer & Glimcher, 1971; Papas *et al.*, 1977; Seyer & Glimcher, 1977a), dentine (Veis & Schlueter, 1964; Veis & Perry, 1967; Butler, 1972; Butler *et al.*, 1972; Veis *et al.*, 1972; Carmichael & Dodd, 1973; Pieri *et al.*, 1975; Menanteau *et al.*, 1977) and bone (Spector & Glimcher, 1972; Shuttleworth & Veis, 1972) [see Leaver *et al.* (1975b) for a review]. There still exists, however, at least two major points of controversy with regard to bone and dentine: (1) how much, if any, of the phosphorylated protein(s) is covalently bound to the collagen, and (2), if present,

the nature of the organic phosphate moiety covalently bound to the collagen.

Although evidence has been presented that none of the phosphoprotein is covalently bound to the collagen of rat dentine (Butler, 1972; Butler *et al.*, 1972, 1976), equally substantial and convincing data have been reported (Schlueter & Veis, 1964; Veis & Perry, 1967; Carmichael & Dodd, 1973; Dickson *et al.*, 1975; S. Lee & A. Veis, unpublished work) establishing that some of the phosphoprotein in the matrix of bovine dentine is covalently linked to the collagen. Although this apparent discrepancy may represent a species difference, it has also been suggested that the difference may have been due in part to the enzymic cleavage of the phosphoproteins of rat dentine from the collagen during their preparation and extraction from the tissues (Dickson *et al.*, 1975). Similarly in the case of bone, although organic

Abbreviation used: P_o, organic phosphate.

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phosphorus has also been identified in EDTA-soluble proteins of chicken (Spector & Glimcher, 1972) and bovine bone (Shuttleworth & Veis, 1972), definitive proof that bone collagen contains organic phosphorus has been difficult to establish because of the marked insolubility of this protein, which limits the amount of homogeneous components that can be isolated and purified. The evidence for the presence of organic phosphorus covalently linked to the collagen of bone and dentine is provided by the findings that α -chains extracted in denaturants (Francois *et al.*, 1967) and peptides liberated by CNBr and periodate degradation, and enzymic digestion (Shuttleworth & Veis, 1972; Dickson *et al.*, 1975; S. Lee & A. Veis, unpublished work) contain organic phosphorus. However, the relative proportion of the two phosphorylated fractions (covalently and non-covalently linked to the collagen) in bone and dentine matrix has not been elucidated.

As far as the nature of the phosphorylated components in the matrix of mineralized tissues is concerned, *O*-phosphoserine has been identified in the enamel proteins (Glimcher & Krane, 1964*b*; Levine *et al.*, 1967) and in the non-collagenous non-covalently bound phosphoproteins of bone (Shuttleworth & Veis, 1972; Spector & Glimcher, 1973) and dentine (Veis & Perry, 1967; Veis *et al.*, 1972; Butler *et al.*, 1976). Recently *O*-phosphothreonine has also been identified in the non-collagenous EDTA-extractable phosphoproteins of bone (Cohen-Solal *et al.*, 1978*a,b*), but was found to be absent (or present in exceedingly small concentrations) from enamel and dentine (Butler *et al.*, 1976; Cohen-Solal *et al.*, 1978*a,b*). Although the amino acid composition of peptides containing *O*-phosphoserine that were liberated by enzymic and chemical degradation strongly suggested that they were derived from collagen (Shuttleworth & Veis, 1972; Dickson *et al.*, 1975; S. Lee & A. Veis, unpublished work), *O*-phosphoserine was not detected in the purified intact α -chains of chicken bone and bovine dentine that contained organic phosphorus (Francois *et al.*, 1967). Indeed, the nature of the organic phosphate moiety in the intact α -chains of bone and dentine was not elucidated.

Since the phosphoproteins have been postulated to play a significant role in facilitating the nucleation of the first fragments of a solid phase of calcium phosphate during tissue calcification (Veis & Schlueter, 1964; Glimcher *et al.*, 1964; Glimcher & Krane, 1964*b*, 1968; Veis & Perry, 1967; Glimcher, 1976), and since there is also a close ultrastructural relationship between the mineral phase and the collagen fibrils (Robinson & Watson, 1952, 1955; Glimcher & Krane, 1968; Höhling *et al.*, 1971, 1974; Glimcher, 1976) and between the phosphoproteins and mineral deposition in dentine (Weinstock & Leblond, 1973), clarification of the chemical and

structural characteristics of the phosphoproteins of bone matrix, and the relationships between those phosphoproteins of bone matrix that are covalently and non-covalently linked to bone collagen, is potentially of great physiological significance.

In the present group of experiments, the problem of proteolytic cleavage of the phosphorylated peptides from the collagen was avoided by using bone removed immediately after the death of the animal and by using a variety of proteinase inhibitors in all of the solutions. Chicken bone was used in the experiments because the insoluble bone collagen can be brought into solution as the gelatin in denaturant solvents (Glimcher & Katz, 1965; Eyre & Glimcher, 1975; Glimcher, 1975) and individual homogeneous components isolated and purified. Thus the presence or absence of organic phosphorus in the purified α -chains and other components of collagen (β - and γ -polymers) could be determined directly.

The present paper reports the presence of organic phosphorus in both the collagenous protein components of the organic matrix of chicken bone, its distribution between the two components, and information concerning the chemical nature of the organic phosphorus components. Both *O*-phosphoserine and *O*-phosphothreonine were identified in the non-collagenous proteins, but neither of these components was found in the purified phosphorylated collagen components. Instead, preliminary compositional evidence of peptides derived from collagen containing the organic phosphate moiety, and the stability characteristics of the phosphate group, suggest that the organic phosphorus in bone collagen is linked to glutamic acid. Thus the major chemically different macromolecular components in chicken bone matrix that contain organic phosphorus are likely to represent two distinct metabolic pools of organic phosphorus under separate biological control.

Experimental

Tissue

The diaphyses of the metatarsal bones of 10-, 12- and 14-week-old chickens were dissected immediately after the death of the animals, washed and kept in cold 1M-NaCl/0.01M-Tris/HCl buffer, pH 7.4, while they were quickly split and cleaned of marrow. The bones from each of the age groups were analysed separately. For one batch of bones, 0.1mM-*p*-chloromercuribenzoate and 10 μ M-toluene-sulphonyl *p*-fluoride were added to all solutions including the 1M-NaCl solution used for the initial washing. The bones were cut into pieces, patted dry, frozen in liquid N₂ and freeze-dried before being ground in a Spex nitrogen mill to a fine powder that passed through a no. 140-mesh screen (10 μ m particle size). The bones were ground for no longer

than 20–30s at any one time to avoid excessive heating. In preliminary experiments, bones were hand-cut to 1–2mm-sized pieces to ascertain the effect of mechanical grinding of the bone to a fine powder on the extractability of the phosphoproteins. Aside from a lower rate of extraction, no differences were noted and the remaining experiments were all carried out on powdered bone samples.

Extraction of phosphoproteins

Bone powder was demineralized in 0.5M-EDTA, pH7.5, either at 2°C or at room temperature (approx. 21°C) in the presence and absence of *p*-chloromercuribenzoate and toluene-*p*-sulphonyl fluoride as noted. A few crystals of thymol and a few drops of toluene were also added to the solutions. One group of experiments was carried out in which a solution of 80% pyridine in EDTA was used for decalcification (Seyer & Glimcher, 1977b). Approx. 500ml of EDTA solution was used per gram of dry whole bone. The solutions were changed every 2–3 days and the extracts pooled at the end of each week. The total time of extraction varied from 3 to 4 weeks. Within each of the experiments, however, all of the conditions and extraction procedures were kept identical for those samples in which the solvents contained proteinase inhibitors and for those samples in which no inhibitors were added.

After each extract, the supernatant was centrifuged at 30000rev./min (75000g) at 4°C for 30min, carefully decanted and filtered through a coarse-mesh sintered-glass funnel, so as to avoid contamination of the solution with small amounts of the powdered insoluble bone.

After extraction in EDTA, the powdered decalcified bone matrix was washed extensively with first 0.16M-NaCl, pH7.4, secondly 1M-NaCl, pH7.4, and finally water, and these solutions added to the last extract. The insoluble residue was then either freeze-dried if the sample was to be used within 1 week, or frozen and kept frozen at –10°C until used at a later date. The EDTA extracts and the final NaCl and water washes were dialysed extensively against water at 2°C and freeze-dried.

Fractionation of the EDTA-soluble proteins

The non-diffusible organic constituents extracted in EDTA (300mg) were dissolved in 20ml of 0.1M-NH₄HCO₃, pH8.0, and applied to a column (25cm×100cm) of Sephadex G-100 equilibrated in the same buffer (Spector & Glimcher, 1972). Elution was performed with 0.1M-NH₄HCO₃, pH8.0, at a flow rate of 12ml/h, 6ml fractions being collected. Each tube was read manually for A₂₃₀ in a Zeiss spectrophotometer. Further purification of the fractions obtained by Sephadex G-100 molecular-sieving was carried out by DEAE-cellulose DE 52

ion-exchange chromatography as described by Spector & Glimcher (1972). Polyacrylamide-gel electrophoresis of the whole EDTA extract and of the fractions obtained by Sephadex G-100 molecular-sieving and DEAE-cellulose ion-exchange chromatography was carried out in 10% polyacrylamide gels by the method of Ornstein (1964) and Davis (1964). The gels were stained with either Coomassie Brilliant Blue or Stains-All (Eastman Organic Chemicals, Rochester, NY, U.S.A.) by the procedure of Green *et al.* (1973) for the detection of phosphoproteins.

Extraction of bone collagen as the gelatin in denaturant salts and its fractionation

The bone matrix that is insoluble after 3–5 weeks of extraction in 0.5M-EDTA, pH7.4, followed by a thorough wash in 0.1M-NaCl, pH7.5, 1M-NaCl, pH7.5, and water, contains collagen as its almost sole protein constituent (Glimcher & Katz, 1965). This insoluble residue was then extracted in 4M-CaCl₂, pH7.5, at 2°C or at room temperature with and without the presence of *p*-chloromercuribenzoate (0.1mM) and toluene-*p*-sulphonyl fluoride (10μM); 200ml of solution per gram of dried, decalcified bone matrix was used. In most experiments the residue was extracted four times for 3 days each. In others, the extraction period was increased to solubilize more of the collagen. The CaCl₂ extracts were then dialysed against water at 2°C and freeze-dried. The total calcium and phosphorus contents of the decalcified bone matrix and of the components extracted in CaCl₂ were determined as described and after ashing at 600°C. The organic phosphate content of the CaCl₂ extracts and of the purified collagen components obtained by molecular sieving and ion-exchange chromatography was determined by the method of Dryer *et al.* (1957) or Chen *et al.* (1956).

Separation and purification of collagen components

Approx. 250–300mg of the freeze-dried extracts was dissolved in 30ml of 2M-CaCl₂/0.05M-Tris/HCl buffer, pH7.5, at room temperature, and subjected to molecular-sieve filtration at room temperature through two columns (5cm×9cm) of agarose A15m, 100–200-mesh resin equilibrated in 2M-CaCl₂/0.05M-Tris/HCl, pH7.5, connected in series. Flow rate was maintained at 25ml/h. The A₂₃₀ of 12.5ml fractions of the eluate was measured in a Zeiss spectrophotometer. The homogeneity of the α-, β- and γ-fractions was monitored by polyacrylamide-gel disc electrophoresis in 5% (w/v) polyacrylamide gels equilibrated in 0.1M-sodium dodecyl sulphate (Furthmayr & Timpl, 1971). Samples were run for 4h at 6mA per tube and stained with Coomassie Blue. Appropriate fractions representing the α-, β- and γ-

fractions were pooled on the basis of the polyacrylamide-gel disc electrophoresis and if necessary subjected to molecular sieving through agarose once or twice more to obtain homogeneous α -, β - and γ -components.

The homogeneous α -components obtained by molecular-sieving were further separated to the $\alpha 1$ - and $\alpha 2$ -chains by ion-exchange chromatography on a column (2 cm $\times$ 11 cm) of CM-cellulose (CM 52, Whatman) equilibrated in 0.06 M-sodium acetate, pH 4.8, as the starting buffer (Piez *et al.*, 1963). The homogeneity of the $\alpha 1$ and $\alpha 2$ fractions was evaluated by polyacrylamide-gel disc electrophoresis using 5% polyacrylamide in 6 M-urea (Nagai *et al.*, 1964; Sakai & Gross, 1967) or in 5% (w/v) polyacrylamide gels equilibrated in 0.1 M-sodium dodecyl sulphate (Furthmayr & Timpl, 1971).

Additional purification of the collagen components was accomplished as follows. Approx. 200–300 mg of the freeze-dried samples of the α -, β - and γ -components obtained from agarose filtration, and of the $\alpha 1$ - and $\alpha 2$ -components obtained by CM-cellulose chromatography, were extracted twice in large volumes of 30% (w/v) KCl, pH 7.5, 2°C for 48 h each, in 0.5 M-EDTA, pH 8.0, at 2°C for 48 h. After thorough washing with water, the insoluble residue was dissolved in 0.2 M-NaCl, 0.05 M-Tris/HCl, pH 7.5, and further purified by chromatography on DEAE-cellulose (Miller, 1971).

The purified collagen components isolated by the methods described above moved as single bands on polyacrylamide-gel electrophoresis, and neither pentoses (Dische, 1955; Brown, 1946) nor hexosamines (Boas, 1953; Cessi & Piliago, 1960) were detected. These data indicate that the components were free of DNA, RNA and components of the glycosaminoglycans. Thus the successive purifications assured the homogeneity and purity of the components used for organic phosphorus and other analytical procedures.

Analytical determinations

Weight and composition of original bone samples. Samples of the freshly dissected and cleaned bone were dried at room temperature in a vacuum desiccator until a constant weight was achieved, and then ashed at 600°C for 18–20 h. Another sample of freshly dissected and cleaned bone was dried in an oven at 105°C until a constant weight was achieved (usually 4–6 h), and similarly ashed at 600°C for 18–24 h. [The true dry weight of native whole bone is difficult to determine very accurately because of the presence of bound water, which is not wholly released except at very high temperatures (Francois & Herman, 1961; Herman & Dallemagne, 1961). The small errors introduced in subsequent calculations as a result of using the dry weight of the bone determined

as described are not significant for the purposes of these experiments.] From the dry weight of the whole mineralized bone and its weight after ashing, the weight of the organic components in the native calcified bone could be calculated. Calcium (Clark & Collip, 1925) and total phosphorus (Chen *et al.*, 1956) determinations were carried out on the ashed samples from which the calcium and total phosphorus contents (inorganic and organic phosphorus) of the native whole bone could be calculated. To avoid changes in the organic matrix due to drying at high temperature, samples of native bone used for the extractions experiments were weighed after drying at room temperature in a vacuum desiccator. Corrections for the true dry weight and organic weight of the original starting material were made from the data obtained by drying a sample at 105°C as described above and from its ash content.

Samples of weighed whole bone were dissolved in 6 M-HCl at room temperature. Additional 6 M-HCl was added and the material hydrolysed for 24 h at 105°C. Both the total protein content of the original native bone and the collagen content were determined from these hydrolysates by complete amino acid analyses and from their hydroxyproline contents calculated from the amino acid composition. For confirmation, the hydroxyproline content was measured separately on an automatic analyser by using the method of Grant (1964). The hydrolysates were either analysed directly or after precipitation of most of the calcium and phosphorus by adjusting the pH (Glimcher & Levine, 1966).

Samples of weighed whole bone were also dissolved in large volumes of cold 4 M-HCl and left to demineralize at 2°C for approx. 16–24 h. Measured samples were then hydrolysed for 6 h at 105°C, from which their contents of *O*-phosphoserine and *O*-phosphothreonine were determined, and other samples dried and brought to 6 M-HCl and subsequently hydrolysed for 24 h at 106°C were used to determine the total protein content from their amino acid content and composition. Thus the content of *O*-phosphoserine and *O*-phosphothreonine per 1000 amino acids of original native bone could be calculated.

Weight and composition of the EDTA extracts. The freeze-dried EDTA extracts were dried in a vacuum desiccator at room temperature and weighed as described for whole bone. Ashed samples indicated that the preparations were NaCl-free and contained no detectable calcium. The weights of the extracts were therefore taken to represent the total non-diffusible organic contents extracted from the whole bone tissue. The protein content of the extracts was determined by quantitative amino acid analyses of weighed samples.

To calculate the fraction of the total P_o contributed by protein-bound P_o , the following procedures

and calculations were used. The total P_o content of the extracts was determined in weighed samples digested in 70% perchloric acid either by the method of Dryer *et al.* (1957) with *p*-semidine and ammonium molybdate as the reagents, or by the method of Chen *et al.* (1956) with ascorbic acid and ammonium molybdate as reagents. Phospholipids, RNA and DNA were extracted from weighed samples of the EDTA extracts by the method of Edelman *et al.* (1969) by using 100% ethanol instead of 95% ethanol for extraction of the phospholipids. The total P_o content of the phospholipid extracts was determined by the method of Chen *et al.* (1956). In some instances a small correction for the phospholipid-phosphorus content was necessary, owing to the presence of a small amount of *O*-phosphoserine and *O*-phosphothreonine in the phospholipid extract. The corrected value was obtained by subtracting the *O*-phosphoserine and *O*-phosphothreonine values from the total P_o content of the phospholipid extracts. Measurements of the P_o contributed by DNA and RNA directly were difficult, owing to the solubilization of large amounts of protein by the extraction procedures and by the hydrolysis of the phosphoproteins in the perchloric acid solutions. The P_o contributed by DNA and RNA was therefore calculated on the basis of their concentrations as determined by the presence of their respective sugars. The DNA content was measured as deoxyribose after reaction with diphenylamine as described by Dische (1955) and RNA as ribose by the orcinol reaction described by Brown (1946). The amount of protein-bound P_o was calculated by subtracting the P_o contributed by phospholipids, DNA and RNA from the total P_o content of the samples.

Good qualitative agreement was found between the values calculated for protein-bound P_o content of the EDTA extracts by using the above method, and those obtained by using a value for nucleotide P_o calculated from the total phosphorus content of the RNA and DNA extracts and from the contents of *O*-phosphoserine and *O*-phosphothreonine in the DNA and RNA extracts.

Amino acid analysis. A Beckman Spinco model 121M amino acid analyser equipped with a single column (0.28 cm $\times$ 33 cm) of Beckman AA-20 resin was used in these studies. Protein nitrogen content of all samples and complete amino acid analysis was obtained on samples hydrolysed *in vacuo* in 6M-HCl for 24 h at 106°C. Tyrosine was determined by hydrolysis *in vacuo* in 6M-HCl at 105°C for 24 h in the presence of 0.01% phenol.

γ -Carboxyglutamic acid was determined by ion-exchange chromatography of samples hydrolysed in 2M-KOH for 22 h at 106°C under N_2 as described by Hauschka (1977).

Identification and quantification of *O*-phosphoserine and *O*-phosphothreonine. Samples of the components

to be analysed were hydrolysed in 4M-HCl at 105°C for 6 h and chromatographed on a Beckman 121M automatic amino acid analyser by using 0.2M-sodium citrate, pH 1.50, as the eluting buffer. *O*-Phosphoserine and *O*-phosphothreonine (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used to standardize the elution positions of these amino acids on the amino acid analyser. To confirm further the identity of components that were eluted in the position of *O*-phosphoserine and *O*-phosphothreonine, 25–50 mg samples of the proteins in question were hydrolysed in 4M-HCl for 6 h and chromatographed on a preparative amino acid analyser consisting of MR-201 resin (Mark Instrument Co., Villanova, PA, U.S.A.) in a column (0.9 cm $\times$ 60 cm) equilibrated and eluted with 0.2M-sodium citrate buffer, pH 1.5. Fractions (1 ml) were collected every 1.5 min. Fractions eluted in the region of *O*-phosphoserine and *O*-phosphothreonine were collected. Samples of these fractions were rechromatographed on the Beckman 121M amino acid analyser by using 0.2M-sodium citrate buffer at pH 1.5 as the eluting buffer to confirm their chromatographic identity as *O*-phosphoserine and *O*-phosphothreonine. The remaining portion of the fraction was then further hydrolysed for 24 h at 106°C in 6M-HCl, and its complete amino acid analysis as well as its total phosphorus content (Dryer *et al.*, 1957) were determined. The identity of the fractions eluted in the positions of *O*-phosphoserine and *O*-phosphothreonine was confirmed by the liberation of serine and threonine respectively (Spector & Glimcher, 1974). Samples of aspartic acid, *O*-phosphoserine and *O*-phosphothreonine (Sigma Chemical Co., St. Louis, MO, U.S.A.) were similarly chromatographed and hydrolysed to confirm the adequacy of these procedures (Spector & Glimcher, 1973). In view of the very close elution positions of these and a number of the other phospho amino acids, these additional procedures are necessary to permit the positive and unequivocal identification of *O*-phosphoserine and *O*-phosphothreonine as components of the protein(s) in question.

Because *O*-phosphoserine and *O*-phosphothreonine as well as serine and threonine are destroyed during acid hydrolysis, corrections were therefore made for these degradations in order to calculate quantitatively the contents of *O*-phosphoserine and *O*-phosphothreonine in the proteins analysed. This was accomplished for the EDTA-soluble phosphoproteins in the following fashion. Samples (50 mg) of the EDTA-soluble proteins were hydrolysed in 4M-HCl at 106°C for periods of 4, 6, 8 and 24 h respectively. The contents of *O*-phosphoserine, *O*-phosphothreonine, serine and threonine in each of the hydrolysates were determined from amino acid analyses. From these data the contents of serine and threonine obtained from amino acid analyses of samples hydrolysed in 6M-HCl, 106°C for 24 h, and

the calculated values of the expected number of moles of *O*-phosphoserine and *O*-phosphothreonine that should have been recovered, graphs were constructed of the rates of hydrolysis of *O*-phosphoserine and *O*-phosphothreonine and extrapolated to zero time. From these charts the true content of *O*-phosphoserine and *O*-phosphothreonine of the proteins could be calculated from the values found on amino acid analysis given the conditions of hydrolysis used. Thus approx. 20, 29, 42, and 90% of the *O*-phosphoserine and 36, 55, 73, and 92% of the *O*-phosphothreonine were degraded after 4, 6, 8 and 24 h respectively of hydrolysis in 4M-HCl at 105°C.

Other O-phospho amino acids. Samples of the proteins were hydrolysed in 4M-HCl at 105°C for 6 h and chromatographed on the Beckman 121M automatic amino acid analyser and on Dowex 1 (Cl⁻ form) (Glimcher & Krane, 1964a) and compared with standards of *O*-phosphotyrosine, *O*-phosphohydroxyproline and *O*-phosphohydroxylysine prepared as described by Plimmer (1941), and those purchased from Vega-Fox Chemical Co. (Tucson, AZ, U.S.A.).

Presence of alkali-stable phospho amino acids and of hexose phosphates. Samples of proteins were hydrolysed in 3M-KOH for 3 h at 100°C in sealed glass tubes. The hydrolysates were desalted by precipitation with 2M-perchloric acid to pH 3.0 and analysed for P_i by the method of ChandraRajan & Klein (1976). Although essentially 100% of the P_o was converted into P_i under these conditions, the presence of small quantities of phosphoamidated amino acids was sought for by subjecting samples of the hydrolysates to high-voltage paper electrophoresis at pH 8.25 (Zetterqvist & Engström, 1967), by ion-exchange column chromatography on columns of Dowex 1 (X8, HCO₃⁻ form) resin (Mårdh *et al.*, 1971). The elution positions of the alkali-stable amino acids 1(3)-phosphohistidine, 3(1)-phosphohistidine and *N*- ϵ -phospholysine were determined by using standards obtained from Vega-Fox.

To determine whether there were hexose phosphates present (Benson, 1957), samples were partially hydrolysed in mild acid and alkali and subjected to high-voltage paper electrophoresis and paper and column chromatography (Benson, 1957; Glimcher & Krane, 1964a). In addition to staining of the paper strips to identify the various hexose phosphates, regions of the paper corresponding to standards of glucose 1-phosphate and galactose 1-phosphate (Sigma) were eluted from several strips with 2M-HCl, pooled and analysed for hexoses (Roe, 1955; Judd *et al.*, 1962) and total phosphorus (Dryer *et al.*, 1957).

Characterization and attempted identification of the organic phosphate moiety in chicken bone collagen

Presence of O-phosphoserine, O-phosphothreonine and other hydroxy and phosphorylated amino acids.

Samples of the purified β - and γ -polymers as well as the α 1- and α 2-chains of collagen were hydrolysed in 4M-HCl at 105°C for 6 h and chromatographed on the 121M Beckman automatic amino acid analyser with 0.2M-sodium citrate as the eluting buffer. In addition, larger samples (50–75 mg) were chromatographed on the preparative amino acid analyser column as described above in the section on the EDTA-soluble proteins, and the fractions corresponding to the positions of *O*-phosphothreonine and *O*-phosphoserine collected.

pH-stability of the organic phosphorus covalently bound to collagen. The stability of the organic phosphate bound to collagen was assayed as a function of pH by a modification of the method described by Anthony & Spector (1972). Approx. 50 mg samples of purified γ -components were incubated for 100 min at 37°C at various pH values and the P_i released was measured directly by the method of ChandraRajan & Klein (1976).

Isolation and characterization of phosphorylated peptides obtained from acid hydrolysates of collagen components. (a) Analytical. Purified β -, γ - and α 2-components respectively were partially hydrolysed in HCl and fractionated by ion-exchange chromatography by a modification of the method described by Seyer & Glimcher (1969) as follows. Approx. 100–300 mg samples of the individual protein components were hydrolysed in 11M-HCl (10 mg of collagen/ml of HCl) under vacuum for 40 h at 37°C. The acid was removed by evaporation at 40°C in a rotary evaporator and completed in a desiccator under NaOH pellets at room temperature. The dry residue was dissolved in a small volume of 0.01 M-HCl and applied to a column (1.5 cm $\times$ 28 cm) of Dowex 50 resin (X8; H⁺ form; 200–400 mesh) equilibrated in 0.01 M-HCl. Elution was performed at room temperature with 2 column-volumes of 0.01 M-HCl and 2 column-volumes of water followed by 1 M-NH₃ to strip the remaining peptides from the resin. Flow rate was adjusted to 1 ml/min and the A₂₃₀ of the 3 ml fractions that were collected was measured. All fractions were analysed for P_o and P_i by the method of Chen *et al.* (1956).

Samples of the fraction enriched in P_o were analysed directly for the presence of *O*-phosphoserine and *O*-phosphothreonine on the Beckman 121M automatic amino acid analyser by using 0.2M-sodium citrate, pH 1.5, and after further hydrolysis of samples for 1 h and for 4 h respectively in 4M-HCl at 105°C. Other samples were hydrolysed in 6M-HCl at 105°C for 1, 4 and 24 h and then subjected to complete amino acid analysis on the Beckman 121M by using the standard program for collagen. In other experiments, the phosphorus-enriched fractions obtained from the Dowex 50 ion-exchange chromatography were dissolved in a small volume of water, the pH was adjusted to 7.0 with 1.0M-NaOH and they were

subjected to ion-exchange chromatography on a column (0.9 cm $\times$ 20 cm) of Dowex 1 (X8; Cl⁻ form; 200–400 mesh) resin equilibrated with water. Elution was carried out at a flow rate of 0.8 ml/min by a linear gradient of HCl (0–0.8 M). The total volume used was 600 ml. The column was run at 4°C; 2.4 ml fractions were collected and their A_{230} values measured. All fractions were analysed for P_o and P_i (Chen *et al.*, 1956). Samples of the major fraction enriched in P_o were analysed directly for the presence of *O*-phosphoserine and *O*-phosphothreonine on the Beckman 121M automatic amino acid analyser and after further hydrolysis of samples in 4M-HCl at 105°C for 1 and 6 h respectively. Complete amino acid analysis was carried out on samples hydrolysed in 6M-HCl at 105°C for 24 h.

(b) Preparative. Approx. 500 mg–1 g portions of purified β - and γ -components respectively were hydrolysed in 11M-HCl under vacuum for 40 h at 37°C, samples fractionated on Dowex-50 resin as described and the peaks enriched in P_o pooled. This material was then subjected to ion-exchange chromatography on a preparative amino acid analyser (column size, 0.9 cm $\times$ 57 cm) at pH 1.5, with 0.2M-sodium citrate as the eluting buffer. All fractions were analysed for P_o and P_i. A peak enriched in P_o was eluted early that corresponded to the unknown peak (peak X) observed on analytical amino acid analyses (see below). This was confirmed by subjecting a small sample of this fraction to analysis on the Beckman 121M automatic amino acid analyser by using the 0.2M-sodium citrate, pH 1.5, program. After desalting the fraction on a column (0.9 cm $\times$ 6 cm) of Dowex 50 (X2) resin from which the P_o-enriched sample was eluted early with water, other samples of the phosphorus-enriched fraction obtained by preparative amino acid analysis and chromatography were hydrolysed in 4M-HCl at 105°C for 6 h and analysed at pH 1.5 on the Beckman 121M automatic amino acid analyser programmed for the detection of *O*-phosphoserine and *O*-phosphothreonine. Complete amino acid analyses were carried out on samples hydrolysed in 6M-HCl at 105°C for 24 h and analysed on the Beckman 121M automatic amino acid analyser with the standard program for collagen. The presence of pentose was measured by the method of Brown (1946), with ribose as a standard. The remaining sample was then subjected to high-voltage paper electrophoresis at pH 1.9, by using Whatman 3MM paper (46 cm $\times$ 56.5 cm) in a 7% (v/v) formic acid buffer. Electrophoresis was carried out at 500 V for 30 min for desalting and then for 60 min at 1500 V for separation of the components. P_i, *O*-phosphoserine, glutamic acid and a mixture of amino acids were used as standards. After electrophoresis, the paper was dried and strips corresponding to the position of the standards were stained with ninhydrin (0.5% ninhydrin in acetone containing 1% acetic acid

and 1% pyridine) and for phosphorus by the ascorbic acid reagent of Chen *et al.* (1956). Strips 1 mm wide on each side of the experimental runs were also stained with ninhydrin and for phosphorus. The major portion of the paper strip was divided into four areas corresponding to components migrating toward the anode. The cut-out strips were eluted with 2 ml of 6M-HCl and further hydrolysed for 24 h at 105°C. Total phosphorus and complete amino acid analyses were carried out on all fractions. Blanks were prepared by eluting similar sized strips of paper with 2 ml of 6M-HCl.

Isolation and characterization of phosphorylated peptides obtained from enzymic digestion of collagen components. Samples (50–150 mg) of freeze-dried preparations of purified β -, γ - and α 2-components respectively were suspended in 5 mM-Tris/HCl buffer, pH 7.5 (4 mg of protein/ml), containing bacterial collagenase (EC 3.4.4.19; type IV; Sigma) (approx. 20 mg of substrate protein/mg of enzyme protein) for 24 h at 35°C. The pH was then adjusted to 5.2 with dilute acetic acid and papain (EC 3.4.4.10; Sigma) (40 mg of substrate/mg of enzyme) added. The digestion was continued for 24 h at 37°C, at the end of which time the mixture was centrifuged and the supernatant adjusted to pH 7.4 with Tris buffer. Leucine aminopeptidase (EC 3.4.1.1; Sigma; 105 units/mg) (20 mg of substrate/mg of enzyme), carboxypeptidase B (EC 3.4.12.3; Sigma; 185 units/mg) (100 mg of substrate/mg of enzyme) and prolidase (EC 3.4.13.9; Sigma; 190 units/mg) (10 mg of substrate/mg of enzyme) were added to the clarified supernatant and digested for an additional 24 h at 37°C. At the completion of the enzymic digestion, the solution was clarified by centrifugation at 8000/rev. min (7700g) for 10–15 min at 4°C and the clarified supernatant freeze-dried.

Samples were analysed for total P_o content, amino acid composition and the presence of *O*-phosphoserine and *O*-phosphothreonine, and the peptides enriched in P_o were isolated by chromatography on Dowex 50 and Dowex-1 ion-exchange resins as described in the preceding section concerned with the peptides isolated from partial acid hydrolysates. Peaks enriched in organic P_o were subsequently analysed for the presence of *O*-phosphoserine and *O*-phosphothreonine and by complete amino acid analyses.

Results

Composition of native whole undecalcified bone and the extraction of EDTA-soluble non-diffusible components from bone

General. The organic weight of undecalcified native cortical diaphysial bone from the metatarsals of 10-, 12- and 14-week-old chickens varied from 35.0 to 36.5%. The variation was not directly

related to age in the age groups studied, but appeared to be more a function of the exact site from which the cortical bone was taken, and the amount of periosteal and endosteal surface bone removed during cleaning. The total calcium content was approx. 19% and the total phosphorus content 9% of the whole native undecalcified bone. The total protein content of the native bone was approx. 25%. Protein accounted for approx. 67–68% of the total organic content of the bone tissue. Collagen accounted for approx. 90–93% of the total protein content, and non-collagenous components about 6.5–9.5% (Table 1).

Table 1. *Composition and extraction of cortical diaphysal bone from the metatarsals of 12- and 14-week-old chickens*
Freshly dissected powdered bone was extracted eight times for 3 days each at 2°C. Proteinase inhibitors were added.

Age ...	Composition	
	Week 12	Week 14
Starting dry wt. (g)	2.33	4.90
Ash wt. (%)	64.0	63.5
Organic wt. (%)	36.0	36.5
Protein* (% w/w)	24.3	24.5
Collagen* (% w/w)	22.0	22.6
Organic material that is protein (% w/w)	67.5	67.1
Protein that is collagen (% w/w)	90.5	92.2
Organic material that is collagen (% w/w)	61.2	61.9
Protein that is non-collagenous protein (% w/w)	9.5	7.8
Extraction		
Original whole bone extracted and non-diffusible (% w/w)	11.2	11.1
Original organic weight of bone extracted (% w/w)	31.1	30.5
Protein of bone extracted as non-diffusible components (% w/w)	6.3	7.7
Collagen extracted as non-diffusible collagen (% w/w)	0.6	0.8
Non-collagenous protein of bone extracted in EDTA (% w/w)	~98–100	~98–100
Extracted non-diffusible protein that is non-collagenous (% w/w)	91.7	91.2
Extracted non-diffusible protein that is collagen (% w/w)	9.3	9.8
Decalcified insoluble residue that is protein (% w/w)	87	87
Protein in decalcified insoluble residue that is collagen (% w/w)	~98–100	~98–100
Non-collagenous protein-bound P _o in EDTA extract (% w/w)	80.1	79.8
Collagen-bound P _o in EDTA insoluble residue (% w/w)	19.9	20.2

* From direct amino acid analyses of whole undecalcified bone.

Table 2. *Composition of cortical diaphysal bone of the metatarsals of 10-week-old chickens and the extraction of organic components in 0.5M-EDTA, NaCl and water*
Dry calcified bone (16.60g) was used as the starting material for each of the experiments. Six extractions were carried out over a 4-week period at 23°C. All percentages are expressed by weight.

Compositional data		
Original native dry bone		
Ash (%)		64.0
Organic (%)		36.0
Protein (%)		24.8
Organic matter that is protein (%)		68.9
Collagen (%)		23.2
Organic wt. that is collagen (%)		64.4
Protein that is collagen (%)		93.5
Non-collagenous proteins (%)		6.5
Extraction data		
	Composition	
	Proteinase inhibitors present	Proteinase inhibitors absent
Original organic weight of the bone extracted in EDTA as non-diffusible components (%)	30.5	28.2
Total protein content of the bone extracted as non-diffusible protein components (%)	7.6	7.1
Original collagen content extracted in EDTA (%)	0.8	0.6
Non-diffusible proteins represented by collagen (%)	9.6	7.9
Non-diffusible proteins represented by non-collagenous protein (%)	90.4	92.1
Original non-collagenous proteins of bone extracted in EDTA (%)	~98–100	~98–100
EDTA-insoluble bone that is protein (%)	88	89
EDTA-insoluble bone protein that is collagen (%)	~98–100	~98–100
Non-collagenous protein-bound P _o (% of total protein-bound P _o)	78.4	79.3
Collagen-bound P _o (% of total protein-bound P _o)	21.6	20.7

There was no significant difference in the total amount of organic components or of the proteins of whole bone that could be solubilized at 2°C compared with those extracted at room temperature (approx. 21°C), although the rate of extraction was faster at the higher temperature. Otherwise no significant differences were found either in the amount of material extracted or in the nature of the components when the extractions were carried out at 2°C or at room temperature. No significant differences were found in the total amount of organic

components, of protein, of total non-diffusible P_o , or of protein-bound P_o in EDTA extracts of freshly dissected bone with or without the addition of proteinase inhibitors to all of the solutions (Table 2).

Less than 1% of the bone collagen is solubilized in EDTA and other neutral solutions of NaCl and water. It should be noted that, when bone powder is used for extraction experiments, the supernatants must be centrifuged at sufficient g values and for long enough time and must be carefully decanted and filtered so as to avoid contamination of the solution with small amounts of the powdered insoluble bone. If this is not done carefully, the supernatants will contain small amounts of insoluble bone powder, which will result in erroneously high values for the amount of collagen solubilized.

Almost all of the non-collagenous proteins of bone can be solubilized by prolonged extraction with EDTA, followed by washing in neutral salt solutions and water. On the basis of the amino acid analyses and the hydroxyproline contents of the EDTA-insoluble residues, the protein remaining in these residues consists almost entirely of collagen (~98–100%). This was confirmed by collagenase digestion of the EDTA-insoluble residue (Jones & Leaver, 1974; Leaver *et al.*, 1975a; Holbrook & Leaver, 1976; Thomas & Leaver, 1977). Thus the 90–95% or more of the collagen that is extracted in denaturing salts (Glimcher & Katz, 1965; Eyre & Glimcher, 1975; Glimcher, 1975) is therefore quite free of non-collagenous components, especially after it has been molecularly sieved one or more times through agarose. After further successive purifications including EDTA and KCl extractions, and CM-cellulose and DEAE-cellulose chromatography, the collagen components moved as single bands on polyacrylamide-gel electrophoresis, and neither pentoses nor hexosamines were detected, indicating they were free of nucleic acids and components of glycosaminoglycans.

Identification and content of γ -carboxyglutamic acid in bone, and its distribution in the soluble and insoluble components. The amount of γ -carboxyglutamic acid in whole undecalcified chicken bone varied from 0.6 to 0.9 residue per 1000 total amino acid residues and did not change significantly in the absence of proteinase inhibitors. Essentially all of the γ -carboxyglutamic acid could be accounted for in the EDTA-soluble extracts. No γ -carboxyglutamic acid was detected in very large samples (approx. 50 mg) of purified α -, β - or γ -components of collagen.

Distribution of total P_o and of protein-bound P_o . Approx. 80% of the total protein-bound P_o of bone matrix is recovered in the EDTA, in non-diffusible components, whereas approx. 20% remains with the insoluble residue. On the basis of the collagen content of the insoluble residue and the P_o content of the purified collagen components, roughly the same

Table 3. *Distribution of organic phosphorus in various components of the EDTA extract of cortical diaphysal metatarsal bone of 10-week-old chickens*

Bone powder was extracted twice a week for 3 weeks at 23°C.

	Organic phosphorus			
	Proteinase inhibitors present		Proteinase inhibitors absent	
	(μ g/100mg of extract)	(%)	(μ g/100mg of extract)	(%)
Total extract	245.0	100	242.0	100
Phospholipids	2.1	0.9	2.5	1.0
RNA	65.6	26.8	63.2	26.1
DNA	5.3	2.2	4.7	2.0
Protein	174.0	70.4	171.6	70.9

Table 4. *Distribution of organic phosphorus in various components of the EDTA extract of cortical diaphysal metatarsal bone of 14-week-old chickens*

Bone powder was extracted twice a week for 1.5 weeks at 23°C and pooled. The residual bone powder was then re-extracted twice a week for an additional 1.5 weeks. Proteinase inhibitors were present throughout.

Fraction	Distribution of organic phosphorus					
	First extract		Second extract		Total*	
	(μ g/100mg)	(%)	(μ g/100mg)	(%)	(μ g/100mg)	(%)
Total P_o	329.0	100	72.0	100	284.3	100
Phospholipids	3.9	1.1	7.7	10.7	4.5	1.6
RNA	89.9	27.3	55.8	77.5	84.0	29.5
DNA	3.6	1.1	1.6	2.2	3.3	1.3
Protein	231.5	70.4	7.0	9.7	191.4	67.3

* Values are calculated from the data that 82.6% of the total non-diffusible organic material extracted in EDTA was present in the first extract, and 17.4% in the second extract.

distribution of the total protein-bound P_o occurs, that is ~80% in the non-collagenous protein components and ~20% in the collagen components (see Tables 3–5 for typical experiments).

Organic phosphorus content of chicken bone collagen. Approx. 90–95% of the insoluble collagen was extracted as the gelatin in 4M-CaCl₂. The γ - and β -components isolated from the CaCl₂ extract by molecular sieving were found to contain approx. 2.3 atoms of P/1000 amino acid residues and the α -components 1.5 atoms of P/1000 amino acid residues. Further purification of the α -components on CM-cellulose revealed that the α 1-component

Table 5. *Distribution of protein-bound organic phosphorus of chicken bone between EDTA-soluble and -insoluble components*

The cortical diaphysal bone of metatarsals was used.

	Protein-bound P _o			
	Proteinase inhibitors present		Proteinase inhibitors absent	
	(mg/100 g of whole bone)	(%)	(mg/100 g of whole bone)	(%)
(a) 10-week-old chickens				
Recovered in non-diffusible components of EDTA extracts	29.2	77.3	28.1	77.4
Recovered in EDTA-insoluble residue	8.6	22.7	8.2	22.6
(b) 14-week-old chickens				
Recovered in non-diffusible components of EDTA extracts	21.4	85.0		
Recovered in EDTA-insoluble residue	3.7	15.0		

Table 6. *Organic phosphorus content of chicken-bone collagen*

Freshly dissected diaphysal portions of metatarsal bones of 10-week-old chickens were used. Proteinase inhibitors were present during preparation.

Species	No. of phosphorus atoms/ 1000 total amino acid residues
γ -Trimer	2.3
β -Dimer	2.2
α -Chain	1.5
$\alpha 1$ -Chain	0.3
$\alpha 2$ -Chain	3.3

contained approx. 0.3–0.4 atom of P/1000 total amino acid residues, whereas the $\alpha 2$ -component had approx. 3.3–4.5 atoms of P/1000 total amino acids. A typical experiment is shown in Table 6. Part of the variation in the P_o content of the α -chains arises as a result of the cleavage of some of the P_o to P_i during chromatography on CM-cellulose (Francois *et al.*, 1967). On the basis of the composition of the bone collagen molecule [$\alpha 1(I)_2\alpha 2$], the collagen macromolecule of chicken bone can be calculated to contain approx. 4–5 atoms of P. The results are slightly higher, but are consistent with previous findings (Francois *et al.*, 1967). There was no significant difference in the P_o content of the collagen components in freshly dissected bone when proteinase inhibitors were omitted from all of the solutions used. On the basis of the P_o contents of the $\alpha 1$ -, $\alpha 2$ - and the β - and γ -components, it would appear that many of the β -component dimers have the com-

position $\beta 1$ –2, and many of the γ -component dimers have the composition $\gamma 1$ –2–2, with at least one of the cross-linkages in the latter clearly being inter-molecular.

Identification of the P_o components in whole undecalcified bone and in the EDTA-extractable proteins

Both *O*-phosphoserine and *O*-phosphothreonine were identified in hydrolysates of whole undemineralized bone (Cohen-Solal *et al.*, 1978a) and in EDTA non-diffusible extracts by their elution positions on automatic amino acid analysis. Essentially all (>95%) of the *O*-phosphoserine and *O*-phosphothreonine in whole bone was recovered in the non-diffusible EDTA-extractable proteins.

By using relatively large samples of EDTA-soluble material and of samples from which DNA, RNA and phospholipids had been extracted (Glimcher *et al.*, 1964), both analytical, preparative and a combination of both methods indicated that essentially all of the protein-bound P_o could be accounted for by *O*-phosphoserine and *O*-phosphothreonine. In a typical experiment, approx. 98% of the total protein-bound P_o content of the non-collagenous EDTA-soluble proteins was recovered as *O*-phosphoserine and *O*-phosphothreonine, of which *O*-phosphoserine constituted 69% and *O*-phosphothreonine constituted 29%. On the basis of the total serine and threonine contents of the EDTA-extractable non-diffusible proteins, it is calculated that approx. 9% of all of the serine residues and approx. 5% of the threonine residues of the EDTA-extractable proteins are phosphorylated. Since the non-collagenous proteins extracted from bone in EDTA consist of both phosphorylated and non-phosphorylated serine-containing components (Herring, 1972; Spector & Glimcher, 1973; Leaver *et al.*, 1975a,b), it is clear that some of the phosphopeptides are undoubtedly considerably more phosphorylated. This is confirmed by complete amino acid analyses of the fractions from the whole EDTA extract of the soluble proteins separated by molecular sieving on Sephadex G-100 (Table 7). As Table 7 shows, fraction C, which was enriched in γ -carboxyglutamate, accounted for approx. 20% of the protein present and 6% of the phospho amino acids in the EDTA extract. Further purification of this γ -carboxyglutamate-rich fraction on DEAE-Sephadex A-25 separated the γ -carboxyglutamate protein (osteocalcin) from other acidic components. This fraction contained 75 γ -carboxyglutamate residues/1000 amino acids and less than one residue of *O*-phosphoserine per 1000 amino acids (Hauschka & Gallop, 1971). Recent studies with isoelectric focusing have now isolated osteocalcin free of *O*-phosphoserine (P. Hauschka, personal communication).

Table 7. *Distribution of potential calcium-binding amino acids in EDTA-soluble proteins of chicken bone matrix*
Values are expressed as residues/1000 total amino acid residues.

Amino acid	EDTA extract	Sephadex G-100 fractions ...	A	B	C	D	E
Asp	108		126	149	129	103	103
Glu*	130		156	162	110	113	148
Gla†	7		3	2	40	68	20
Ser‡	70.5		71	57	56	43	56
O-Phosphoserine§	6.5		6	22	11	3	9
Thr‡	57		59	41	39	9	14
O-Phosphothreonine§	2.0		1	9	3	1	2

* Corrected by subtraction of γ -carboxyglutamic acid.

† γ -Carboxyglutamic acid; determined in 2M-KOH hydrolysates (22h, 110°C).

‡ Corrected by subtraction of O-phosphoserine and O-phosphothreonine respectively.

§ Determined after 4M-HCl hydrolysis (6h, 105°C).

Nature of P_o in decalcified bone matrix and in collagen

As noted above, O-phosphoserine and O-phosphothreonine were detected (usually less than 5% of the total O-phosphoserine and O-phosphothreonine contents of the whole undecalcified bone, but as high as 10% in other instances) in the EDTA-insoluble bone matrix after decalcification with EDTA. Since most of the components containing the O-phosphoserine and O-phosphothreonine could be removed by further extractions with EDTA, and on the basis of the weight of the decalcified bone matrix used and on the O-phosphoserine and O-phosphothreonine contents of the EDTA extracts, it can be calculated that ~98% or more of the phosphoproteins containing O-phosphoserine and O-phosphothreonine are solubilized from the bone after extensive extraction in EDTA, and in the neutral salt solutions and water used to wash the decalcified bone powder.

Detection of phospho amino acids and hexose phosphates in purified collagen components. By using a variety of hydrolysis conditions and large samples (50–100mg) of the β -, γ - and α_2 -components of collagen so as to be able to detect very small quantities of the phospho amino acids, no evidence could be obtained for the presence of O-phosphoserine or O-phosphothreonine in the collagen components by preparative amino acid chromatography. O-Phosphohydroxyproline, O-phosphotyrosine and O-phosphohydroxylysine were not detected either by preparative amino acid chromatography or by ion-exchange chromatography on Dowex 1 (X8; Cl^- form). 1(3)-Phosphohistidine, 3(1)-phosphohistidine and ϵ -N-phospholysine were not found in alkaline hydrolysates. No hexose phosphates were detected. DNA, RNA and phospholipid were also not detected in large samples of the purified collagen components.

pH-stability curve of the organic phosphate moiety in collagen. The stability of the P_o moiety in purified

Fig. 1. *Stability of the organic phosphorus of collagen as a function of pH*

Samples of purified γ -components of chicken bone collagen were incubated at the appropriate pH for 100 min at 37°C and the P_i released was measured by the method of ChandraRajan & Klein (1976).

collagen components (β , γ and α_2) plotted as a function of pH was a U-shaped curve (Fig. 1). It is very similar to the stability curve of acyl phosphate bonds reported by others (Nagano *et al.*, 1965; Anthony & Spector, 1972).

Isolation of phosphorylated peptides from acid digests of purified collagen components. Most of the phosphorus from the partial acid hydrolysates of purified collagen components (principally γ -components) (11M-HCl, 40h at 37°C) was recovered in the first ninhydrin peak eluted from the Dowex 50 (X8) column. Approx. 30–35% of the phosphorus was present in this fraction as P_o . Ion-exchange

Fig. 2. Ion-exchange chromatography of partial acid hydrolysate of γ -components of chicken bone collagen. The first fraction of a partial acid hydrolysate of the γ -components of chicken bone collagen eluted from a Dowex-50 (X8) column was further chromatographed on a Beckman 121M automatic amino acid analyser in 0.2M-sodium citrate buffer, pH 1.5. The peak, enriched in P_0 (peak X), was further hydrolysed in 6M-HCl for 1h, 4h, and 6h at 105°C. No O-phosphoserine or O-phosphothreonine was detected. The amino acid composition and the amino acids liberated by the hydrolyses are presented in Table 9.

chromatography of this fraction on the Beckman 121M automatic amino acid analyser at pH 1.5 with 0.2M-sodium citrate as the eluting buffer revealed a major peak (X) eluted before cysteic acid and earlier than O-phosphoserine or O-phosphothreonine. Free O-phosphoserine and O-phosphothreonine were not present (Fig. 2) and were not liberated after further hydrolysis of a sample in 4M-HCl at 105°C for 1 and 4h. Peak X was still detectable in the sample hydrolysed for the additional 1h in 4M-HCl at 105°C, but was absent after 4h of hydrolysis. The amino acids liberated from this fraction after hydrolysis in 4M-HCl for 1h and 4h at 105°C as well as a complete amino acid analysis of a sample hydrolysed in 6M-HCl at 105°C for 24h are given in Table 8.

Table 8. Amino acid composition of P_0 -enriched peptides (fraction 1) obtained from the γ -components of chicken bone collagen

This first fraction containing a number of peptides was obtained by ion-exchange chromatography of an 11M-HCl/40h/37°C hydrolysate of purified γ -components of chicken bone collagen on a column (1.5cm $\times$ 28cm) of Dowex 50 (X8; H⁺ form) resin. Abbreviation used: 4Hyp, 4-hydroxyproline. Results are given as residues of amino acids per 1000 total amino acid residues.

Hydrolysis conditions of fraction 1 ...	Composition		
	4M-HCl, 1h, 105°C	4M-HCl, 4h, 105°C	6M-HCl, 24h, 105°C
4Hyp	15	32	43
Asp	4	4	9
Thr	—	2	2
Ser	2	6	7
Glu	924	878	774
Pro	10	4	25
Gly	17	22	68
Ala	19	28	43
Val	—	14	3
Ile	—	—	1
Leu	1	2	4
Tyr	2	2	2
Phe	2	2	2
His	1	1	1
Lys	2	3	8
Arg	—	—	7

Table 9. Amino acid composition and phosphorus content of fraction X obtained from the γ -components of chicken bone collagen

This material was obtained by preparative amino acid chromatography of fraction 1 (Table 8) in 0.2M-sodium citrate buffer, pH 1.5. The sample was hydrolysed in 6M-HCl for 24h at 105°C before amino acid analysis. P_0 and P_1 determinations were carried out by the method of Chen *et al.* (1956), and the pentose determination was by the method of Brown (1946). Abbreviation used: 4Hyp, 4-hydroxyproline.

	Amino acid residues or atoms of phosphorus per 1000 amino acids
4Hyp	89
Asp	49
Ser	30
Glu	507
Pro	24
Gly	183
Ala	70
Lys	26
Val	24
P_1	84
P_0	412
} 496	
Pentose (as ribose)	0
Hexosamine	0

A fraction obtained from the first peak eluted from the Dowex 50 (X8) column by preparative amino acid chromatography was found to contain peak X when chromatographed on the Beckman 121M amino acid analyser in 0.2M-sodium citrate buffer, pH1.5. The complete amino acid composition and phosphorus content of peak X obtained by preparative amino acid chromatography are shown in Table 9. Some of the P_i was probably liberated during chromatography and during the phosphorus analysis. The close correspondence between the phosphorus and glutamic acid contents is evident. No evidence for the presence of either *O*-phosphoserine or *O*-phosphothreonine was detected in partial acid hydrolysates of peak X when chromatographed on the Beckman 121M automatic amino acid analyser programmed for these amino acids.

Although no ninhydrin-staining spots were observed in the anionic portion of the paper strips after high-voltage paper electrophoresis of peak X, elution of regions of the paper adjacent to P_i (more and less negatively charged) showed the presence of small amounts of P_o in both regions compared with standards run only with buffer or standard amino acid mixtures. Complete amino acid analyses of the fractions containing the P_o showed the presence only of glutamic acid and some glycine. Quantification of the amounts of glutamic acid, glycine and P_o was not possible because of the small amounts present, but qualitatively the phosphorus contents paralleled the contents of glutamic acid. Two cationic species that stained with ninhydrin were observed close to the origin and both contained glutamic acid and glycine, but considerably less P_o than glutamic acid.

When the first fraction of the partial acid hydrolysate obtained by ion-exchange chromatography on Dowex 50 was rechromatographed on Dowex-1 resin, about 80% of the total phosphorus charged on the column was eluted with the second 230nm-absorbing fraction. This fraction had the highest A_{230} of any of the fractions eluted. The P_o in this fraction accounted for approx. 25% of the total P_o content of the sample. When chromatographed on the Beckman 121M automatic amino acid analyser at pH1.5, 0.2M-sodium citrate buffer, the major peak observed corresponded to peak X (Fig. 3). *O*-Phosphoserine and *O*-phosphothreonine were not detected after partial acid hydrolysis and ion-exchange chromatography on the Beckman 121M automatic amino acid analyser. Total amino acid analysis revealed that glutamic acid constituted over 90% of the amino acid content, with only trace amounts of hydroxyproline, glycine, alanine and valine present. Combinations of the preparative methods detailed above have failed to yield a single homogeneous compound, but the amino acid composition of the fractions enriched in P_o have all been characterized by their very high concen-

Fig. 3. Ion-exchange chromatography of a phosphorus-enriched fraction obtained from a partial acid hydrolysate of the γ -components of chicken bone collagen

Chromatography was performed on a Beckman 121M automatic amino acid analyser in 0.2M-sodium citrate, pH1.5. The starting material was the second peak eluted, when the first peak obtained from a Dowex-50 (X8) column was rechromatographed on a Dowex-1 (X8) column. The γ -components were initially hydrolysed in 11M-HCl at 37°C for 40h. *O*-phosphoserine and *O*-phosphothreonine were not detected when samples of this fraction were further hydrolysed in 4M-HCl for 1h and 6h at 105°C.

trations of glutamic acid (exceeding 90%) and the absence of *O*-phosphoserine and *O*-phosphothreonine. Similar findings have been obtained from partial acid digests of purified α 2-chains.

Isolation of phosphorylated peptides from enzymic digests of purified collagen components. (a) γ -Components. Eight fractions containing P_o were isolated by chromatography of the enzymic digests on Dowex 50 (X8; H^+ form) resin. Approx. 90% of

Table 10. *Amino acid compositions of P_o-rich fractions obtained from enzymic digestion of γ -components of chicken bone collagen by Dowex-50 chromatography*
Abbreviation used: 4Hyp, 4-hydroxyproline.

Fraction ...	Residues of amino acids per 1000 total amino acid residues			
	2	5	6	7
4Hyp	—	—	7	39
Asp	75	28	67	131
Thr	70	20	59	33
Ser	111	38	113	61
Glu	157	95	175	209
Pro	95	328	74	67
Gly	100	309	96	181
Ala	75	28	61	103
Cys	—	—	—	—
Val	61	22	53	30
Met	—	—	—	11
Ile	36	20	30	17
Leu	83	54	86	30
Tyr	—	9	31	19
Phe	31	16	28	36
Hyl	1.0	2.0	tr	3.0
Lys	31	16	35	14
His	10	6	10	6
Arg	88	9	71	8
Peak X*	5+	1+	3+	1+

* Relative values based on area under the curve and on the total amino acid content of the samples analysed.

Table 11. *Amino acid compositions of P_o-rich fractions obtained by chromatography on Dowex 1 of fraction 2 (Table 10) isolated from enzymic digestion of γ -components of chicken bone collagen by Dowex-50 chromatography*

	Residues of amino acids per 1000 total amino acid residues	
	Fraction 4	Fraction 5
Asp	100	—
Thr	46	—
Ser	78	—
Glu	180	282
Pro	80	128
Gly	143	301
Ala	94	128
Val	54	44
Ile	29	11
Leu	54	41
Tyr	27	9
Phe	7	11
Hyl	5	Trace
Lys	12	25
His	79	—
Arg	9	19
Peak X*	5+	2+

* Relative values based on total amino acid content of samples.

the P_o charged on the column was recovered in the eight fractions. The amino acid analyses of the four major P_o-containing peaks are shown in Table 10 (peaks 2, 5, 6 and 7). All four fractions contained peak X when analysed without further hydrolysis and after 1 h hydrolysis in 4M-HCl at 105°C. No *O*-phosphoserine or *O*-phosphothreonine was detected under these conditions or after hydrolysis in 4M-HCl for 6 h at 105°C.

The second fraction obtained from Dowex-50 chromatography was subjected to further chromatography on Dowex 1 (X8) resin from which two fractions enriched in P_o were eluted (fractions 4 and 5). Both fractions (Table 11) contained peak X without further hydrolysis and after 1 h of acid hydrolysis in 4M-HCl at 105°C. Hydroxylysine was present in relatively high concentrations, but no *O*-phosphoserine or *O*-phosphothreonine was detected in the samples as such or after partial acid hydrolysis in 4M-HCl at 105°C for 6 h.

(b) α 2-component. After enzymic digestion, five fractions were obtained by chromatography on Dowex 50 (H⁺ form) resin, only the first of which contained significant amounts of P_o. No free *O*-phosphoserine or *O*-phosphothreonine was detected in this fraction, or after partial acid hydrolysis, but the fraction did contain peak X before and after 1 h of hydrolysis in 4M-HCl at 105°C.

Discussion

The present studies have established that there are non-collagenous phosphoproteins in the matrix of chicken bone that can be extracted in 0.5M-EDTA at neutral pH in the presence of proteinase inhibitors from tissues removed immediately after the death of the animals. Under these circumstances, it is unlikely that proteolysis can account for their separation from the bone collagen. Rather, one may conclude that these phosphoproteins are not covalently bound to collagen *in vivo*. Although almost all of the non-collagenous proteins containing *O*-phosphoserine and *O*-phosphothreonine can be readily extracted from the bone matrix and from the collagen and are therefore not covalently bonded to the collagen, the last 5–10% or so of the total *O*-phosphoserine- and *O*-phosphothreonine-containing phosphopeptides are removed slowly only after prolonged and repeated extractions with EDTA and long after all of the Ca²⁺ and P_i ions of the mineral phase have been dissolved. This fraction may represent phosphoproteins that are more intimately bound physically and/or by stronger, but not covalent, chemical bonds than the bulk of the non-collagenous proteins. Although an even smaller proportion of the *O*-phosphoserine- and *O*-phosphothreonine-containing phosphopeptides often remains with the insoluble collagen residue despite repeated

and prolonged extraction with EDTA, it is also unlikely to be linked to the collagen by stable covalent bonds, since it can clearly be dissociated from the collagen by denaturants as evidenced by the fact that purified collagen components isolated in denaturants contain no *O*-phosphoserine or *O*-phosphothreonine. This very small (<5%) fraction of the *O*-phosphoserine- and *O*-phosphothreonine-containing phosphopeptides may not only be physically entangled and in close physical relationship to the collagen fibrils, but may be chemically linked to the collagen by more stable, but nonetheless denaturant-labile, bonds like the intermolecular cross-links of chicken bone collagen (Glimcher & Katz, 1965; Glimcher, 1975; Eyre & Glimcher, 1975).

The results of the present study are similar in some respects to those reported previously for dentine and bone, but differ significantly in certain other important aspects. All of the previous studies as well as the present ones have identified phosphoproteins in the organic matrix of dentine and of bone that are not covalently bound to the collagen and can be extracted in EDTA and in other solvents (Veis & Schlueter, 1964; Zamosciany & Veis, 1966; Carmichael *et al.*, 1971, 1975; Butler *et al.*, 1972; Spector & Glimcher, 1972; Shuttleworth & Veis, 1972; Veis *et al.*, 1972; Carmichael & Dodd, 1973; Dickson *et al.*, 1975). However, although the present results, like those reported for rat dentine, indicate that essentially all of the phosphoproteins containing *O*-phosphoserine (and *O*-phosphothreonine in the case of chicken bone) are not bound to the collagen by strong covalent bonds, there is strong evidence that a significant amount of the non-collagenous phosphoproteins are covalently linked by denaturant-stable bonds to bovine dentinal and bone collagen (Schlueter & Veis, 1964; Veis & Perry, 1967; Shuttleworth & Veis, 1972; Carmichael & Dodd, 1973; Dickson *et al.*, 1975). Although it has been argued that the absence of phosphoproteins covalently linked to collagen in rat dentinal collagen may have been due to enzymic cleavage of the phosphoproteins from collagen during preparation of the tissue (Dickson *et al.*, 1975), there appears to be little likelihood that this occurred in the present experiments. A more likely explanation is that there are species and tissue differences as well as possible changes in the nature of the bond between the phosphoproteins and collagen that occur with maturation in certain species (Spector & Glimcher, 1972). Thus the absence of *O*-phosphoserine- and *O*-phosphothreonine-containing phosphopeptides from the γ -components (and higher-molecular-weight components as well), as well as the α -components of chicken bone collagen, makes it unlikely (at least in the case of chicken bone) that the phosphopeptides are synthesized as components independent and free of collagen, only later to become covalently bonded

to the collagen as a function of maturation (Veis *et al.*, 1969).

The present results also clearly establish and confirm previous findings that the α -chains of chicken bone collagen contain covalently bound P_o , and that it is located principally in the $\alpha 2$ -chains and is not present as *O*-phosphoserine or *O*-phosphothreonine (Francois *et al.*, 1967). The finding that chicken bone collagen contains P_o and that it is not present as *O*-phosphoserine or *O*-phosphothreonine differs from the results reported for rat dentinal collagen (Butler, 1972; Butler *et al.*, 1972, 1976), bovine bone (Shuttleworth & Veis, 1972) and dentinal collagen (Dickson *et al.*, 1975); no P_o was found in rat dentinal collagen and the peptides presumably derived from collagen, and covalently attached to bovine bone and dentinal collagen did contain *O*-phosphoserine. On the other hand, no *O*-phosphoserine was detected in purified intact α -chains of bovine dentinal collagen containing P_o (Francois *et al.*, 1967).

The chemical nature of the P_o moiety covalently bound to chicken bone collagen has not been established. There is, however, considerable evidence that the compound is phosphorylated glutamic acid. Partial acid hydrolysis released a highly acidic component (peak X) that was eluted before cysteic acid and before *O*-phosphoserine or *O*-phosphothreonine. When peak X was isolated by preparative amino acid chromatography from partial acid digests of large samples of purified collagen, it was found to contain large amounts of hydroxyproline, indicating its probable derivation from collagen. It contained large amounts of glutamic acid and closely parallel amounts of P_o . Further partial acid hydrolysis released components that were rich in P_o and that contained 90–95% or more of glutamic acid. Similarly, peptides containing P_o , but no *O*-phosphoserine or *O*-phosphothreonine, and likewise enriched in glutamic acid and the unknown peak X, have also been isolated after enzymic digestion of purified collagen components including the $\alpha 2$ -chains. Thus, although it has not so far been possible to isolate a completely homogeneous preparation of the phosphate moiety containing the P_o and to positively identify the component, the compositional data and pH-stability characteristics strongly suggest that it is glutamyl phosphate, possibly located in an environment that increases its acid-stability as compared with many of the acyl phosphate bonds identified in other proteins (Nagano *et al.*, 1965; Anthony & Spector, 1972). Indeed, certain acyl phosphates such as succinyl (3-carboxypropionyl) phosphate or maleyl phosphate are much more stable in acid than is glutamyl phosphate (Walsh *et al.*, 1970). As has been pointed out, structural factors and the local chemical environment of the acyl phosphate bond may exert profound effects on the hydrolytic behaviour of this

Fig. 4. Schematic illustration of the current views on the phosphopeptide components of the organic matrix of bone and dentine (a) In bovine bone and dentine, some of the non-collagenous phosphoproteins are depicted as being covalently bound to the collagen by strong denaturant-stable bonds, whereas another fraction is not covalently bound to the collagen and can be easily dissociated in various aqueous solvents. This view has been championed principally by Veis and his colleagues (Carmichael *et al.*, 1971, 1975; Veis *et al.*, 1972; Carmichael & Dodd, 1973; Dickson *et al.*, 1975). *O*-Phosphoserine was the only phosphorylated amino acid identified in these tissues until recently, when *O*-phosphothreonine was also found (Cohen-Solal *et al.*, 1978b). (b) On the other hand, Butler and associates consider, on the basis of their experiments with rat dentine (Butler, 1972; Butler *et al.*, 1972, 1976), that none of the phosphoproteins is covalently linked to the collagen. Only *O*-phosphoserine has been identified in the matrix of rat dentine. (c) The present data indicate that essentially all the non-collagenous phosphoproteins in chicken bone matrix can be dissociated from collagen. A very small portion is very tightly bound, but can also be dissociated by strong denaturants. In addition to these non-collagenous phosphoproteins, which contain both *O*-phosphoserine and *O*-phosphothreonine, the α -chains of the chicken bone collagen *per se* also contain P_o , but this is not present as either *O*-phosphoserine or *O*-phosphothreonine. There is no evidence at the present time to suggest whether this P_o moiety is attached to an amino acid in the primary structure of the α -chains, or is part of a peptide that is itself covalently linked to the α -chain.

bond (Nagano *et al.*, 1965; Walsh *et al.*, 1970; Anthony & Spector, 1972). In any event, the finding that the components of chicken bone collagen are distinctly different from those associated with the non-collagenous peptides of the organic matrix suggests that the phosphorylation of these two major phosphorylated components in the organic matrix of bone may be under separate metabolic control and regulation.

It is not clear whether the P_o moiety covalently linked to chicken bone collagen is a component of a non-collagenous peptide that is covalently linked to the α -chains of collagen, as has been postulated for the phosphoproteins of bovine bone and dentine, or whether it is a phosphorylated amino acid in the primary structure of the α -chains (Glimcher, 1976). A diagrammatic summary of previous results and the present ones on the distribution, chemistry and organization of P_o in the organic matrices of bone and dentine is presented in Fig. 4.

The isolation of essentially all of the γ -carboxyglutamic acid in the EDTA extracts in the presence of proteinase inhibitors and its absence from the

purified components of collagen establishes that this unique calcium-binding peptide of bone matrix (Hauschka *et al.*, 1975; Price *et al.*, 1976) is also not covalently bound to collagen, at least in chicken bone. The presence of both γ -carboxyglutamic acid and phosphorus groups in non-collagenous peptides of bone matrix and the calcium-binding properties of these components may be of great significance as far as calcification of bone matrix is concerned. In addition to increasing the potential number of calcium-binding sites, a complex of carboxy and P_o groups would have a greater affinity constant for calcium ions, with the result that the bound calcium ions would be more stable than either a simple calcium-carboxyl or a calcium-phosphate bond alone. Moreover, the complex might also serve to fix the bound calcium ions in a specific and unique stereochemical array, an important point (Glimcher & Krane, 1968; Glimcher, 1976) when one considers the postulated role of the phosphoproteins in facilitating the initiation of calcification in mineralized tissues (Glimcher & Krane, 1968; Veis *et al.*, 1969; Weinstock & Leblond, 1973; Glimcher, 1976). In this

regard it is noteworthy that dentine, bone and enamel all contain phosphopeptides rich in aspartic acid and glutamic acid. Indeed, glutamic acid has been identified as being immediately before the *O*-phosphoserine residues of the enamel phosphopeptides (Seyer & Glimcher, 1969, 1971, 1977a; Papas *et al.*, 1977). Similarly aspartic acid residues appear to be located immediately before *O*-phosphoserine in the phosphopeptides of bovine dentine (A. Veis, personal communication). In the case of the enamel proteins, molecular models based on both amino acid sequence and on X-ray-diffraction data indicate that calcium ions may indeed be shared between protein-bound carboxy and phosphate groups both intra- and inter-molecularly (Glimcher, 1978). The recent studies *in vitro* of calcium binding to collagens, which has also strongly suggested that some of the calcium is bound as a tightly linked complex between carboxy groups and organically bound phosphate (Li & Katz, 1976), adds additional experimental evidence for the role of both the acidic amino acids and phosphorylated amino acids in the interaction between the components of organic matrices and the inorganic ions of the mineral phase in the calcification of skeletal and dental tissues.

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