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Organic phosphorous distribution in chicken bone matrix

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Summary. Non-collagenous, soluble proteins and collagen have been extracted quantitatively from freshly dissected chicken bone at 4 °C in presence of protease inhibitors, and their content in organic phosphorus assayed. We found that non-collagenous proteins were not covalently bound to collagen in chicken bone, as in some calcified tissues. They represented 30 p. 100 of the organic matrix and bound 75 p. 100 of the organic phosphorus. Collagen accounted for 70 p. 100 of the organic matrix and bound 25 p. 100 of the organic phosphorus. The presence of phosphate groups covalently linked to proteins in the bone matrix may play an important role as mineralization sites.

Introduction.

The mechanisms whereby mineral is deposited in certain collagenous tissues such as bone, dentin, calcified cartilage, but not in other connective tissues such as tendon and skin, are unknown. The cells or the organic matrix itself may play a catalytic role in the mineralization process.

Organic phosphate groups which have been identified in the proteins of calcified tissues could be important sites for the initiation of mineralization. Non-collagenous proteins have been identified in some calcified tissues such as enamel (Seyer *et al.*, 1971 ; Glimcher and Krane, 1964), dentin (Buttler, 1972) and bone (Shuttleworth *et al.*, 1972) (Spector *et al.*, 1972). In some tissues only, these phosphoproteins have been found to be covalently bound to the collagen (Carmichael *et al.*, 1971). This discrepancy may represent a species difference but also may be the result of the enzymatic cleavage of soluble phosphoproteins from collagen.

Although many studies on non-collagenous protein phosphorylation have been done and the presence of organic phosphorus on bone collagen detected (Glimcher *et al.*, 1964), the quantitative distribution of the organic phosphorus covalently bound to the proteins of bone matrix, has never been established.

Material and methods.

The diaphyses of metatarsal bone of 10-week old chickens were dissected immediately after slaughter. Two batches of bone were analyzed. For one batch protease

inhibitors were added to all solutions : 0.1 mM p-chloromercurylbenzoate, 10 μM p-toluene-sulfonylfluoride. The bones were scraped, washed in 1 M NaCl and H_2O at 4 °C, freeze-dried, and ground in a nitrogen mill. All the extractions were carried out at 4 °C.

1. *Protein extraction and purification.* Soluble non-collagenous proteins were extracted with 0.5 M EDTA, pH 7.5 (Spector *et al.*, 1972). Subsequent analyses were performed on the crude EDTA soluble extract.

The collagen from the decalcified EDTA insoluble residue was extracted by 4 M $CaCl_2$, pH 7.5, and fractionated by molecular sieve filtration on Agarose A 15 M (5 × 180 cm) eluted with 2 M $CaCl_2$ 0.05 M tris-HCl, pH 7.5. The alpha chains were further purified into alpha-1 and alpha-2 chains by ion-exchange chromatography on carboxymethylcellulose (Piez *et al.*, 1963).

2. *Analytical determinations.* The weight of organic components in native calcified bone was calculated from the weights of whole dry mineralized bone and ash. Protein concentrations were determined by amino acid analysis on a Beckman 121 M amino acid analyzer. Collagen was assayed by hydroxyproline determination (Grant, 1964). Phosphorus was assayed according to the method of Chen *et al.*, 1956. Organic phosphorus was obtained by deduction of inorganic phosphorus from the total phosphorus performed on a sample completely digested with 70 p. 100 perchloric acid. Phospholipid and nucleic acid extractions and their content in organic phosphorus were performed as described by Edelman *et al.* (1969).

TABLE 1

Compositions of cortical diaphyseal bone, organic non-diffusible components extracted in 0.5 M EDTA, and EDTA insoluble decalcified residue (p. 100)

		Protease inhibitors present	Protease inhibitors absent
Native dry bone	Ash wt	65.9	
	Organic wt	34.1	
	Protein wt	24.8	
	Organic wt protein	72.7	
EDTA soluble, non-diffusible components	Organic wt	30.5	28.2
	Protein content wt	90.4	92.1
	Protein as N.C.P.	100	100
	Protein as collagen	0.8	0.6
	Native bone as NCP *	7.6	7.1
EDTA insoluble components	Organic wt	69.5	71.8
	Protein content wt	88	89
	Protein as collagen	100	100
	Native dry bone as collagen	21.6	18.8

* NCP : non-collagenous protein.

Results.

The compositions of cortical diaphyseal bone, organic non-diffusible components extracted in 0.5 M EDTA, and EDTA insoluble decalcified residue are given in table 1. The organic matrix represented about 35 p. 100 of the native dry bone. EDTA soluble components, mainly proteins, represented 30 p. 100 of the organic matrix and insoluble collagen represented 70 p. 100.

The content of organic phosphorus in the crude EDTA soluble extract and in the insoluble EDTA residue, show that 70 p. 100 of the organic phosphorus is located in the soluble EDTA components (table 2).

TABLE 2

Distribution of organic phosphorus (Po) between the crude EDTA soluble extract and the insoluble EDTA residue

Components	Protease inhibitors present		Protease inhibitors absent	
	Po mg/100 g dry bone	p. 100 Po	Po mg/100 g dry bone	p. 100 Po
Soluble non-diffusible EDTA components	29.2	77.3	28.1	77.4
EDTA insoluble components	8.8	22.7	8.2	22.6

In order to evaluate the true amount of organic phosphate bound to the protein moities, phospholipids and nucleic acids were extracted and organic phosphorus measured. In EDTA soluble components, 70 p. 100 only of the organic phosphorus of the crude extract was bound to the proteins. The distribution is given in table 3.

TABLE 3

Distribution of organic phosphorus (Po) in the EDTA soluble components

	Protease inhibitors present		Protease inhibitors absent	
	Po µg/100 mg	p. 100 total Po	Po µg/100 mg	p. 100 total Po
Crude extract.....	245.0	100	242.0	100
Phospholipids	2.1		2.5	
RNA.....	65.6	29.6	63.2	29.1
DNA	5.3		4.7	
Protein.....	174	70.4	171.6	70.9

From all these data, no differences could be observed in the extracted components in presence or absence of protease inhibitors.

80-90 p. 100 of the collagen was extracted in 4 M $CaCl_2$, pH 7.5, from the EDTA insoluble residue. Only minute amounts of nucleic acids were present. After purifi-

cation of the collagen by gel filtration and separation of the alpha-1 and alpha-2 chains by ion-exchange chromatography, no nucleic acid was detected. The organic phosphorus distribution in chicken bone collagen is given in table 4.

TABLE 4

Organic phosphorus in chicken bone collagen

Collagen components	P atoms/1 000 residues amino acids
Gamma-trimer	2.3
Bêta-trimer	2.2
Alpha-chains	1.5
Alpha 1-chain	0.3
Alpha 2-chain	3.3

Discussion.

Non-collagenous phosphoproteins present in the matrix of chicken bone can be extracted from freshly dissected bone in 0.5 M EDTA at neutral pH, even in the presence of inhibitors. Under these conditions, it is unlikely that the phosphoproteins are the result of proteolysis following slaughter of the animal and/or during subsequent extractions. It may be concluded that the soluble phosphoproteins of chicken bone are not covalently bound to collagen *in vivo*. These proteins represent about 25-30 p. 100 of the organic matrix proteins and they are highly phosphorylated. In addition to phosphate groups, they contain a high percentage of acidic amino acids such as aspartic acid, glutamic acid (Spector *et al.*, 1972), gamma-dicarboxyglutamic acid (Hauschka *et al.*, 1975). Calcium ions could be very tightly linked to carboxyl groups and organic phosphate groups, to make a complex which would catalyse the initiation of mineralization.

Our results confirm earlier findings that the alpha chains of chicken bone contained covalently-bound organic phosphorus mainly located in the alpha-2 chain (François *et al.*, 1967). Although its phosphate content is low, its role in mineralization nucleation cannot be excluded.

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Résumé. Les protéines solubles dans l'EDTA, de nature non collagénique, ainsi que le collagène ont été extraits à partir d'os diaphysaires de poulet, immédiatement après la mort des animaux. Les résultats obtenus dans les conditions de préparation choisies (tissu frais, basse température, présence d'inhibiteurs des enzymes protéolytiques) permettent de conclure que ces protéines ne sont pas liées covalentiellement au collagène. Bien que ces protéines ne présentent que 25 à 30 p. 100 des protéines de la matrice osseuse, leur taux en phosphate organique représente 70 à 75 p. 100 du phosphore organique total. La richesse en groupements phosphates associée à leur composition en acides aminés caractéristiques, confèrent à ces composés des propriétés importantes dans la liaison du calcium et l'initiation possible de la minéralisation. Le collagène, environ 65 p. 100 des protéines, ne contient que 25 à 30 p. 100 du phosphore organique ; son rôle comme facteur d'initiation est également possible.

References

BUTTLER W. T., 1972. Dentinal phosphoproteins, 255-262. In SLAVKIN H. C. *The comparative molecular biology of extracellular matrices*, Acad. Press, Lond. and N. Y.

CARMICHAEL D. J., VEIS A., WANG E. T., 1971. Dentin matrix collagen : evidence for a covalently linked phosphoprotein attachment. *Calcif. Tiss. Res.*, **7**, 331-344.

CHEN P. S., Jr., TORIBARA T. Y., WARNER H., 1956. Microdetermination of phosphorus. *Anal. Chem.*, **28**, 1756-1758.

EDELMAN M., HIRSCH C. A., HIATT H. H., FOX M., 1969. Apparent changes in mouse liver DNA content due to interference by non-DNA diphenylamine-reacting cytoplasmic material. *Biochim. biophys. Acta*, **179**, 172-178.

FRANÇOIS C. J., GLIMCHER M. J., 1967. Organic phosphorus content of the alpha chains of chicken bone collagen. *Nature*, **214**, 621-622.

GLIMCHER M. J., FRANÇOIS C. J., RICHARDS L., KRANE S. M., 1964. The presence of organic phosphorus in collagens and gelatins. *Biochim. biophys. Acta*, **93**, 585-602.

GLIMCHER M. J., KRANE S. M., 1964. The identification of serine phosphate in enamel proteins. *Biochim. biophys. Acta*, **90**, 477-483.

GRANT R. A., 1964. Estimation of hydroxyproline by the auto analyser. *J. clin. Path.*, **17**, 685-686.

HAUSCHKA P. V., LIAN J. B., GALLOP P. M., 1975. Direct identification of the calcium-binding amino acid, gamma-carboxyglutamate, in mineralized tissue. *Proc. nat. Acad. Sci.*, **72**, 3925-3929.

PIEZ K. A., EIGNER E. A., LEWIS M. S., 1963. The chromatographic separation and amino acid composition of the subunits of several collagens. *Biochemistry*, **2**, 58-66.

SEYER J. M., GLIMCHER M.J., 1971. The isolation of phosphorylated polypeptide components of the organic matrix of embryonic bovine enamel. *Biochim. Biophys. Acta*, **226**, 279-291.

SPECTOR A. R., GLIMCHER M. J., 1972. The extraction and characterization of soluble anionic phosphoproteins from bone. *Biochim. biophys. Acta*, **263**, 593-603.

SHUTTLEWORTH A., VEIS A., 1972. The isolation of anionic phosphoproteins from bovine cortical bone *via* the periodate solubilization of bone collagen. *Biochim. biophys. Acta*, **257**, 414-420.