

Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation

(extracellular bone matrix/bone induction/cartilage differentiation)

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ABSTRACT Subcutaneous implantation of demineralized diaphyseal bone matrix in allogeneic rats results in the local induction of endochondral bone differentiation. We have explored the potential of three dissociative extractants, 4 M guanidine hydrochloride (Gdn·HCl), 8 M urea/1 M NaCl, and 1% NaDodSO₄ at pH 7.4, containing protease inhibitors to solubilize putative inductive molecules in the bone matrix. Extraction of bone matrix with any one of these extractants resulted in the loss of the bone inductive property. The solubilized extracts were then reconstituted with the residue by dialysis against water. The various reconstituted matrices were bioassayed for bone inductive potential by quantitation of alkaline phosphatase activity and ⁴⁵Ca incorporation on day 12 after implantation. There was complete recovery of biological activity after reconstitution of the residues with each of the three extracts. Polyacrylamide gel electrophoresis of the extracts revealed similar protein profiles. Gel filtration of the 4 M Gdn·HCl extract on Sepharose CL-4B showed a heterogeneous broad peak. When fractions of that peak containing proteins <50,000 daltons were reconstituted with inactive 4 M Gdn·HCl-treated bone matrix and then implanted, new bone was induced. These observations demonstrate the dissociative extraction and successful biological reconstitution of bone inductive macromolecules in demineralized bone matrix.

Subcutaneous implantation of demineralized diaphyseal bone matrix in allogeneic rats results in the local differentiation of fibroblasts to form endochondral bone (1–3). The developmental cascade of endochondral bone differentiation consists of mesenchymal cell chemotaxis, cell proliferation, differentiation and hypertrophy of chondrocytes, vascular invasion, calcification of cartilage matrix, formation and remodeling of bone, and, finally, differentiation of hematopoietic bone marrow in the newly formed ossicle (4). Although the cellular and biochemical events accompanying the sequential cell differentiation are well known (4), the precise molecular mechanisms underlying the action of matrix on cells are not clear. Anastasiades *et al.* (5) first showed that 4 M guanidine hydrochloride (Gdn·HCl) extracts of rat bone matrix increased the biosynthesis of glycosaminoglycans by cultured rat fibroblasts. Takaoka *et al.* (6) and Hanamura *et al.* (7) used 4 M Gdn·HCl in the solubilization of bone-inducing substances from osteosarcomas.

The present report describes the dissociative extraction and successful biological reconstitution of extracellular matrix components involved in induction of bone differentiation.

MATERIALS AND METHODS

Preparation of Demineralized Bone. Dehydrated diaphyseal shafts of rat femur and tibia were pulverized in a CRC micromill (Techni Laboratories, Vineland, NJ) and sieved to a

discrete particle size of 74–420 μ m. The powders were demineralized with 0.5 M HCl, extracted with water, ethanol, and ether, and prepared as described (1).

Dissociative Extraction of Matrix Components from Demineralized Bone. The demineralized bone matrix was initially washed (30 ml/g of matrix) in 0.02 M phosphate/0.15 M NaCl, pH 7.4, at room temperature for 10–20 min. It was then extracted (30 ml/g of matrix) with constant stirring at 4°C for 16 hr in different extractants which included a low-salt extractant (20 mM Na₂HPO₄/50 mM Tris, pH 7.4), essentially the same extractant but with 0.25% Triton X-100, and three different dissociative extractants: 4 M Gdn·HCl/50 mM Tris, pH 7.4; 8 M urea/1 M NaCl/50 mM Tris, pH 7.4; 1% NaDodSO₄/50 mM Tris, pH 7.4. All extractants contained a mixture of protease inhibitors: 5 mM benzamide/0.1 M 6-aminohexanoic acid/0.5 mM phenylmethylsulfonyl fluoride/5 mM *N*-ethylmaleimide. In addition, 50 mM Tris/0.15 M 2-mercaptoethanol, pH 7.4, with protease inhibitors was also used to extract proteins that were disulfide bonded to the matrix. The extracts were centrifuged (40,000 \times g, 30 min, 4°C) and the supernatants were dialyzed against water at 4°C in Spectrapor 3 tubing (M_r \approx 3500 cutoff) and lyophilized; the residues (insoluble demineralized bone matrix from that extractant) were washed three times in distilled water before lyophilization. Each residue and each extract was bioassayed for its potential to induce endochondral bone. The proteins of the demineralized bone matrix extracted by different extractants were characterized by NaDodSO₄/polyacrylamide gel electrophoresis.

Reconstitution of Solubilized Extracellular Matrix Components. The extracellular matrix components of demineralized bone solubilized by different dissociative extractants were reconstituted with their respective residues by the following methods and bioassayed for their potential to induce endochondral bone: (i) both the extract and the residue were transferred without separation to Spectrapor 3 tubing and dialyzed extensively against distilled water at 4°C for 3–4 days and then lyophilized; (ii) the extract and residue were separated by centrifugation (40,000 \times g, 30 min, 4°C) and the residue was washed with distilled water before the extract and residue were mixed, dialyzed against distilled water, and lyophilized; (iii) the residue and extract were dialyzed against distilled water, lyophilized individually, and then thoroughly mixed mechanically. Essentially the same method was used to reconstitute the solubilized extracellular matrix components extracted by the three extractants. However, in the case of NaDodSO₄, the dialysis was initially carried out against several changes of 25% isopropanol followed by 15% isopropanol to remove the detergent before dialysis against distilled water. Other experiments revealed that isopropanol had no effect on the bone inductive

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Abbreviation: Gdn·HCl, guanidine hydrochloride.

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potential of demineralized bone matrix. Column fractions obtained by gel filtration were lyophilized and reconstituted by dissolving 1 part (wt/wt) of sample in 2 ml of 4 M Gdn·HCl, to which 10 parts (wt/wt) of water-washed 4 M Gdn·HCl-insoluble demineralized matrix was added. The mixture was stirred for 2 hr at 4°C and then dialyzed against distilled water and lyophilized.

Bioassay. Demineralized bone matrix, variously reconstituted bone matrix preparations, and insoluble bone matrix residue after dissociative extractions were assayed for ability to induce endochondral bone by subcutaneous implantation (ether anesthesia) into male Long-Evans rats at bilateral sites located over the thorax (1). The day of implantation was designated as day 0 of the experiment. On day 12, ⁴⁵CaCl₂ (17 mCi/ μ g of Ca; 1 Ci = 3.7×10^{10} becquerels) in isotonic saline was injected intraperitoneally at a dose of 1 μ Ci/g of body weight 2 hr before the rats were killed. The subcutaneous button-like plaques (implants) were dissected out and cleaned of adherent tissue. The implants were weighed and homogenized in 2 ml of ice-cold 0.15 M NaCl/3 mM NaHCO₃. Alkaline phosphatase activity of supernatants and ⁴⁵Ca incorporation into the insoluble sediment and calcium content were determined as an index of bone formation as described (8). The histological appearance of the implants was also monitored. The Gdn·HCl extract was reconstituted with acid-extracted tendon matrix as described for bone matrix residue and bioassayed on day 12. Tendon matrix alone served as a control.

Polyacrylamide Slab Gel Electrophoresis. The components extracted from demineralized bone matrix by different dissociative extractants were characterized by polyacrylamide slab gel electrophoresis (9). Gradient gels at 5–20% were used as the separating gel and a spacer gel of 3% acrylamide/2 M urea was added to all the gels. Samples containing 100 μ g of protein, dissolved in 50 mM Tris/20% (wt/vol) glycerol/1% NaDodSO₄/2 M urea were heated at 90°C for 5 min with and without 0.1% mercaptoethanol before application to the gels. Electrophoresis was in 50 mM Tris glycine, pH 8.3/0.1% NaDodSO₄ at 15 mA per slab and 20°C for 12 hr. After electrophoresis, the gels were stained with: (a) 0.25% Coomassie brilliant blue R250 in 50% methanol/10% acetic acid for 30 min and then destained initially in 40% methanol/10% acetic acid followed by 10% methanol/7.5% acetic acid; (b) periodic acid-Schiff reagent (Sigma) after oxidation with 1% periodate to identify glycoprotein; (c) StainsAll (Eastman), by the procedure of Green *et al.* (10), to identify phosphorylated or sialic acid containing protein bands. Molecular weight standards (Pharmacia) were phosphorylase (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α -lactalbumin (14,400).

Gel Filtration. Solubilized bone matrix components extracted by 4 M Gdn·HCl were fractionated at 20°C on a Sepharose CL-6B column (1.5 \times 80 cm) for analysis and on a Sepharose CL-4B column (2.5 \times 120 cm) for preparative purposes. Both columns were equilibrated with 4 M Gdn·HCl/50 mM Tris, pH 7.4, and eluted with the same eluent at a flow rate of

15 ml/hr; 5-ml fractions were collected. The eluent was continuously monitored at 230 nm. Appropriate fractions were pooled, dialyzed against distilled water at 4°C, and lyophilized. Fractions were characterized by NaDodSO₄/polyacrylamide gel electrophoresis and bioassayed after reconstitution with 4 M Gdn·HCl-insoluble demineralized bone matrix. Gel filtration of 4 M Gdn·HCl-soluble extract of demineralized bone matrix was also carried out on an analytical column after (a) treatment with EDTA (20 μ mol/mg of protein per ml), (b) reduction and alkylation, (c) collagenase digestion, and (d) trypsin digestion.

Enzymatic Digestion. Lyophilized fractions of 4 M Gdn·HCl-soluble components of demineralized bone matrix were digested with: (a) chondroitinase ABC (0.25 unit/mg of sample) in 2.0 ml of 0.1 M sodium acetate/0.1 M Tris, pH 7.4 adjusted with acetic acid; (b) highly purified bacterial collagenase (11) (enzyme/substrate, 1:100) dissolved in 0.2 ml of 50 mM Tris, pH 7.4/5 mM calcium acetate/5 mM CaCl₂/5 mM *N*-ethylmaleimide; and (c) trypsin (10 μ g/mg of sample) in 50 mM Tris, pH 7.4/10 mM CaCl₂. The digested samples were dialyzed against distilled water and lyophilized. They were then bioassayed after reconstitution with the 4 M Gdn·HCl-insoluble residue and characterized by gel electrophoresis.

Analytical Procedures. Samples for amino acid analysis were hydrolyzed in 6 M HCl at 106°C for 24 hr in sealed tubes in an atmosphere of nitrogen. Samples for hexosamine analysis were hydrolyzed in 4 M HCl at 100°C for 10 hr in sealed tubes under nitrogen. The analyses were performed with a Durrum D500 analyzer by standard procedures. Protein content was determined by the procedure of Lowry *et al.* (12).

Histology. Matrix-induced plaques (implants) were fixed in Bouin's fixative and embedded in JB4 plastic embedding medium (Polysciences, Warrington, PA), and 1- μ m sections were cut and stained with toluidine blue.

RESULTS

Dissociative Extraction of Demineralized Bone Matrix Components. Extraction of washed demineralized bone matrix with low-salt extractant alone or containing 0.25% Triton X-100 had no influence on the ability of the matrix to induce endochondral bone differentiation. Bioassay of the respective water-washed residues revealed that the specific activity of alkaline phosphatase and the incorporation of ⁴⁵Ca and calcium content in day 12 plaques did not change from the control (data are not shown). Dissociative extraction of demineralized bone matrix with 4 M Gdn·HCl/50 mM Tris, pH 7.4, or 8 M urea/1 M NaCl/50 mM Tris, pH 7.4, or 1% NaDodSO₄/50 mM Tris, pH 7.4, all containing protease inhibitors, resulted in a complete loss of the ability of bone matrix to induce endochondral bone differentiation (Tables 1–3; Fig. 1). Alone, 8 M urea was ineffective in perturbing bone induction; however, 8 M urea in 1 M NaCl completely abolished new bone formation by the residue (Table 2). Extraction of demineralized bone matrix with 2-mercaptoethanol, a sulfhydryl-reducing reagent, resulted in the loss of

Table 1. Influence of 4.0 M Gdn·HCl extraction and reconstitution on alkaline phosphatase and ⁴⁵Ca incorporation in day 12 plaques

Group	Alkaline phosphatase, units/mg protein	⁴⁵ Ca incorporation, cpm/mg tissue	Calcium content, μ g/mg tissue	Bone histology
Control	3.61 \pm 0.72	3070 \pm 648	14.46 \pm 3.34	+ + + +
Residue	0.06 \pm 0.01*	30 \pm 3*	0.25 \pm 0.03*	—
Reconstitution by dialysis	4.95 \pm 0.60	4254 \pm 384	20.66 \pm 1.57	+ + + +
Reconstitution by mechanical mixing	3.51 \pm 0.71	2185 \pm 636	10.51 \pm 1.94	+ + + +

Values are mean \pm SEM of eight observations from four rats.

* For difference from control, $P < 0.05$.

Table 2. Influence of 8 M urea/1 M NaCl extraction and reconstitution on alkaline phosphatase and ⁴⁵Ca incorporation in day 12 plaques

Group	Alkaline phosphatase, units/mg protein	⁴⁵ Ca incorporation, cpm/mg tissue	Calcium content, μg/mg tissue	Bone histology
Control	2.20 ± 0.25	2395 ± 568	13.67 ± 1.65	+ + + +
Residue, 1.0 M NaCl	1.60 ± 0.27	1176 ± 204*	12.18 ± 0.82	+ + + +
Residue, 8.0 M urea	1.62 ± 0.33	1596 ± 353	12.92 ± 3.42	+ + + +
Residue, 8.0 M urea/1.0 M NaCl	0.09 ± 0.02*	33 ± 9*	0.33 ± 0.06*	-
Reconstitution by dialysis	1.66 ± 0.31	2064 ± 903	16.96 ± 6.42	+ + + +
Reconstitution by mechanical mixing	2.17 ± 0.28	1808 ± 387	13.74 ± 2.40	+ + + +

Values are mean ± SEM of eight observations from four rats.

* For difference from control, $P < 0.05$.

the ability of the matrix to induce endochondral bone, confirming a previous observation (13).

Reconstitution of Dissociatively Extracted Component(s) of Bone Matrix Involved in Endochondral Bone Differentiation. The total loss of biological activity of bone matrix after dissociative extraction was restored when the extracted soluble components were reconstituted with their respective inactive insoluble residue. Reconstitution achieved either by direct dialysis of the extractant or by mechanically mixing the lyophilized extract with the insoluble water-washed residue restored the biological activity of the matrix. When reconstituted with residue, 4 M Gdn·HCl-extracted bone matrix components were able to induce endochondral bone differentiation as measured by the specific activity of alkaline phosphatase, ⁴⁵Ca incorporation, and calcium content in day 12 plaques (Table 1). Reconstitution of bone matrix components extracted by 8 M urea/1 M NaCl or 1% NaDodSO₄ with the respective matrix residues exhibited complete biological activity as shown by the specific activity of alkaline phosphatase, ⁴⁵Ca incorporation, and calcium content (Tables 2 and 3). To reconstitute NaDodSO₄-extracted bone matrix components with the residue it was necessary to dialyze against 25% isopropanol several times, then against 15% isopropanol, and, finally, against distilled water. Reconstitution with NaDodSO₄ extracts without prior dialysis against isopropanol did not restore osteoinductive potential (data not shown).

When 4 M Gdn·HCl-solubilized bone matrix components were reconstituted with acid-extracted tendon matrix, endochondral bone differentiation was not observed. Collagenase and trypsin digestion completely abolished the biological activity of 4 M Gdn·HCl-soluble extract whereas chondroitinase digestion did not have any effect (data not shown). Reconstitution of 2-mercaptoethanol-soluble components to either its insoluble residue or to 4 M Gdn·HCl-insoluble residue did not restore the biological activity, indicating the importance of disulfide-bonded conformation for biological function.

Fractionation of 4 M Gdn·HCl-Solubilized Extracellular Bone Matrix Components. Gel filtration of solubilized components on Sepharose CL-4B revealed a broad peak which was pooled into four fractions (Fig. 2). Lyophilized components of

each fraction were reconstituted with 4 M Gdn·HCl residue and bioassayed. Fraction IV restored the biological activity to the matrix; the three other fractions did not show significant biological activity (alkaline phosphatase and ⁴⁵Ca incorporation). Fractionation of 4 M Gdn·HCl-soluble components on an analytical column (Sepharose CL-6B) revealed the similar pattern as shown in Fig. 2. Pretreatment of the soluble components with EDTA did not change the chromatographic profile.

Characterization of Dissociatively Extracted Bone Matrix Components. The amino acid compositions of the various dissociatively extracted fractions are summarized in Table 4. The Gdn·HCl and 8 M urea/1 M NaCl extracts were generally similar in amino acid composition. It is noteworthy that the NaDodSO₄ extract had a composition indicative of predominantly noncollagenous proteins. Components of extracellular bone matrix solubilized by dissociative extractants revealed similar protein profiles when electrophoresed on 5–20% polyacrylamide gels (Fig. 3). The 4 M Gdn·HCl and 8 M urea/1 M NaCl extracts showed identical protein bands which included α, β, and γ chains of type I collagen and several noncollagenous proteins. The NaDodSO₄ extract revealed proteins smaller than 94,000 daltons. Electrophoresis on Sepharose CL-4B showed that peak I contained predominantly collagenous peptides. Peak II contained mostly proteins of about 80,000 daltons. Peak III contained mainly proteins in the region of 70,000 daltons and smaller. Peak IV contained proteins smaller than 50,000 daltons (Fig. 2).

DISCUSSION

When implanted subcutaneously into allogeneic rats, demineralized bone matrix induces an invariant sequence of events resulting in *de novo* formation of cartilage, bone, and bone marrow (1–3). Although the cellular and biochemical changes accompanying the matrix-induced bone differentiation are well documented (4), the precise molecular mechanisms are not clear. The complete loss of endochondral bone differentiation by bone matrix after dissociative extraction and its restoration upon reconstitution demonstrate the extraction and survival of putative inductive molecules in dissociative extractants. In addition, this

Table 3. Influence of 1% NaDodSO₄ extraction and reconstitution on alkaline phosphatase and ⁴⁵Ca incorporation in day 12 plaques

Group	Alkaline phosphatase, units/mg protein	⁴⁵ Ca incorporation, cpm/mg tissue	Calcium content, μg/mg tissue	Bone histology
Control	1.62 ± 0.20	2156 ± 398	10.23 ± 1.39	+ + + +
25% isopropanol	1.75 ± 0.33	1956 ± 370	11.32 ± 1.16	+ + + +
Residue	0.19 ± 0.26*	107 ± 31*	0.55 ± 0.26*	-
Reconstitution by dialysis	1.37 ± 0.16	1986 ± 520	17.97 ± 1.18*	+ + + +
Reconstitution by mechanical mixing	1.30 ± 0.26	1202 ± 247*	3.80 ± 0.69*	+ + + +

Values are mean ± SEM of eight observations from four rats.

* For difference from control, $P < 0.05$.

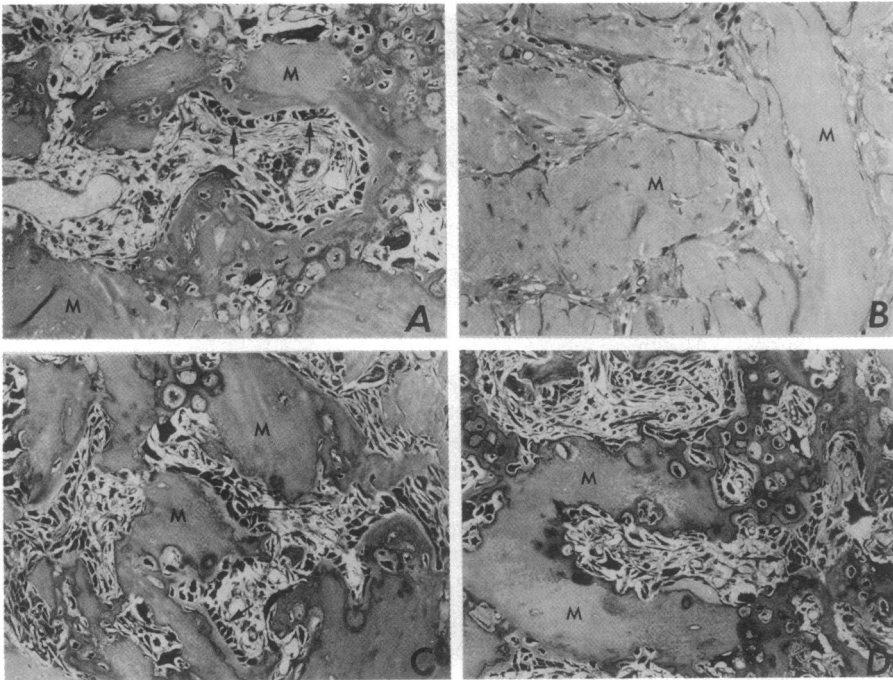


FIG. 1. Implants on day 12. ($\times 200$) (A) Control. Bone formation and vascular invasion are evident. M, implanted demineralized bone matrix particles. The arrows indicate osteoblasts. (B) Gdn-HCl-treated residue. Note the complete absence of new bone formation. The implant consists of bone matrix and surrounding mesenchymal cells. (C) Reconstitution of the Gdn-HCl residue with the Gdn-HCl extract. Note the complete restoration of bone formation. (D) Reconstitution of the Gdn-HCl residue with peak IV extract. Complete restoration of bone induction is evident.

study provides an experimental method to bioassay soluble proteins for osteoinductive potential in a reproducible and quantitative manner.

The role of collagenous matrix in endochondral bone formation is obscure. Digestion of lyophilized 4 M Gdn-HCl extract with highly purified bacterial collagenase before reconstitution resulted in loss of bone induction. The collagenous residue insoluble in 4 M Gdn-HCl was osteoinductively inactive showing the inability of collagenous bone matrix alone to induce endochondral bone differentiation. Dissociatively extracted os-

teoinductive components from bone matrix reconstituted with tendon matrix did not induce bone formation. This indicates the importance of bone-specific collagenous matrix despite the fact that the major collagen type in these two tissues is type I. It has been mentioned that collagen is not necessary to induce bone formation (14). It is likely that collagenous matrix provides a suitable substratum for attachment of mesenchymal cells (13, 15). The geometry of bone matrix with an extensive surface area (16) may be a more suitable substratum for anchorage-dependent proliferation and differentiation (17). Studies on enzymatic

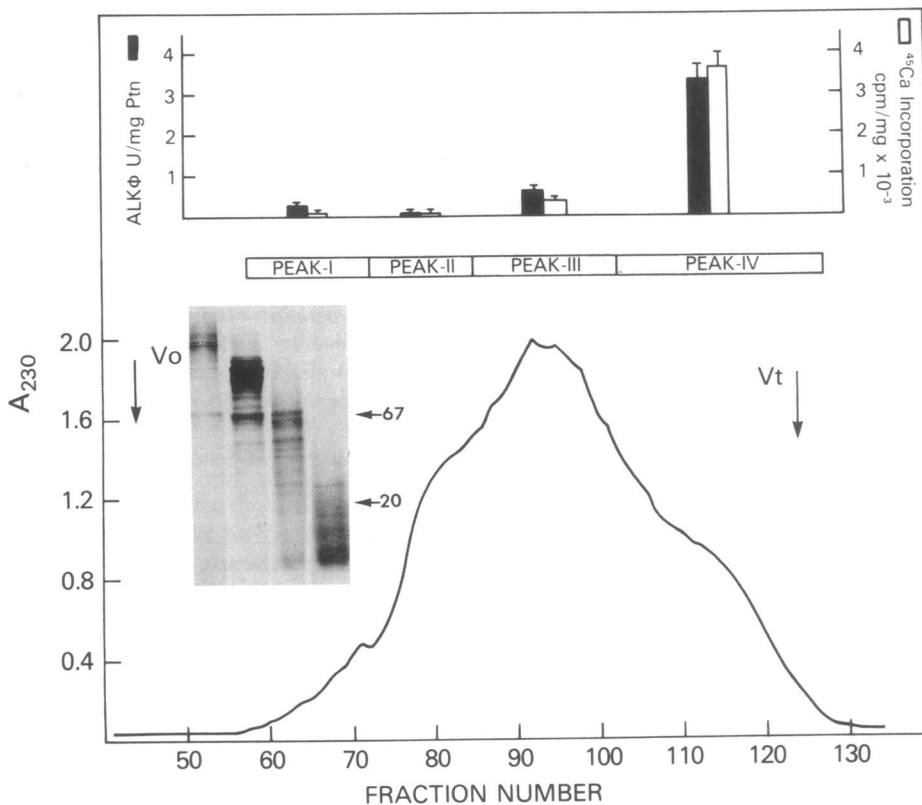


FIG. 2. Gel filtration of the 4 M Gdn-HCl extract of demineralized diaphyseal bone matrix on Sepharose CL-4B. The fractions were pooled as indicated, reconstituted with Gdn-HCl residue, and bioassayed. (Upper) Fraction IV has the most activity as quantitated by alkaline phosphatase (units/mg of protein) and ^{45}Ca incorporation. (Inset) polyacrylamide gel (5–20% gel) pattern of the four pooled peaks (I to IV), stained by Coomassie blue.

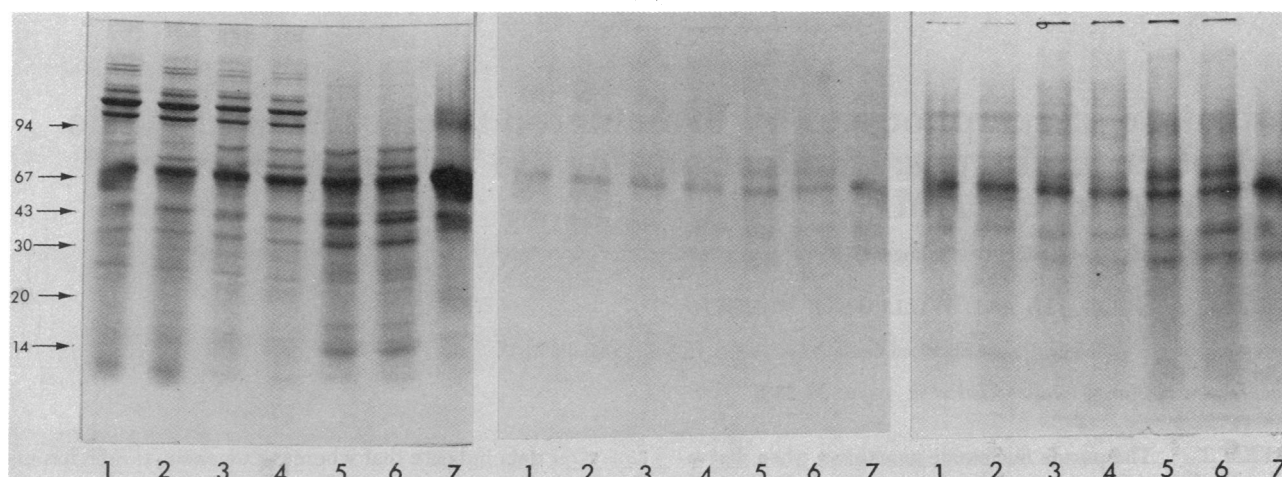


FIG. 3. Slab gel electrophoretograms (5–20% polyacrylamide) of dissociative extracts of the bone matrix stained by Coomassie blue (Left), periodic acid-Schiff reagent (Middle) for glycoproteins, and StainsAll (Right) to reveal phosphoproteins and sialic acid-containing proteins. Lanes: 1 and 2, Gdn·HCl extracts before and after reduction; 3 and 4, 8 M urea/1 M NaCl extracts before and after reduction; 5 and 6, 1.0% NaDodSO₄ extracts before and after reduction; 7, 0.15 M mercaptoethanol extract. Arrows, markers for molecular weight ($\times 10^{-3}$).

digestion showed that the osteoinductive molecule is sensitive to trypsin but resistant to chondroitinase. Gel filtration of 4 M Gdn·HCl extracts showed that osteoinductive proteins are smaller than 50,000 daltons.

These results imply that proteins tightly associated with ex-

tracellular bone matrix play a role in local regulation of bone differentiation. In conclusion, the present study demonstrates the extraction of bone inductive proteins by three different dissociative extractants—4 M Gdn·HCl, 8 M urea/1 M NaCl, and 1% NaDodSO₄—and the successful biological reconstitution of endochondral bone differentiation activity.

Table 4. Amino acid composition of various dissociatively extracted fractions*

Amino acid	Residues/1000 residues				
	A	B	C	D	E
4-Hyp	94	37	35	1	—
Asp	50	67	93	116	99
Thr	21	45	43	58	57
Ser	42	55	57	67	53
Glu	79	120	118	120	144
Pro	106	95	88	65	91
Gly	326	178	186	102	89
Ala	105	87	89	89	114
Val	21	50	35	49	66
Met	7	8	7	6	2
Ile	9	26	18	29	26
Leu	28	63	59	87	81
Tyr	9	20	22	34	36
Phe	16	30	26	38	42
His	5	21	18	26	36
Hyl	12	6	8	1	1
Lys	21	42	39	57	45
Arg	48	50	59	52	30

* A, demineralized bone matrix; B, 4 M Gdn·HCl extract; C, 8 M urea/1 M NaCl extract; D, 1% NaDodSO₄ extract; E, 0.15 M mercaptoethanol extract.

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