

## Solubilized and insolubilized bone morphogenetic protein

(cell differentiation/osteogenesis/noncollagenous protein)

MARSHALL R. URIST, ANDRZEJ MIKULSKI, AND ARTHUR LIETZE

Bone Research Laboratory, University of California School of Medicine, 1000 Veteran Avenue, Los Angeles, California 90024

Communicated by W. F. Libby, January 8, 1979

**ABSTRACT** A bone morphogenetic protein (BMP) obtained in solution by digestion of demineralized rabbit cortical bone matrix with bacterial collagenase retains its biologically active conformation in a neutral salt/ethylene glycol mixture. BMP may be insolubilized by coprecipitation with calcium phosphate and resolubilized by chemical extraction with a neutral salt in the same solvent mixture. Upon concanavalin A-Sepharose chromatography, BMP is bound by hydrophobic interaction and carbohydrate recognition and is recovered by elution with either  $\alpha$ -methyl mannoside or ethylene glycol solvent mixture. Implants of both eluates and the extracts of the coprecipitate in double-walled diffusion chambers induce transmembrane bone morphogenesis. BMP is not species specific; rabbit BMP induces new bone formation in the rat. The present observations indicate that BMP is a glycoprotein.

One of the most striking and consistently inducible forms of postfetal cell differentiation is the development of cartilage, bone, and bone marrow in an intramuscular implant of dentin or bone matrix (1-4). The initial deposits consist of cartilage and woven bone which are remodeled and replaced by an ossicle of lamellar bone and bone marrow. The quantity of bone is proportional to the mass of preimplanted, demineralized matrix (5). In previous communications (6-9), it was postulated that the new bone develops from somatic migratory mesenchymal type cells under the influence of a bone morphogenetic protein (BMP) that is released from acid-insoluble substance of bone matrix (10, 11) or insoluble bone matrix gelatin (12, 13). This is a preliminary report on a method of solubilization of BMP and of coprecipitation of BMP with calcium phosphate.

### MATERIALS AND METHODS

Fig. 1 summarizes the six-step procedure for separation of BMP from insoluble bone matrix. Rabbit cortical bone (100 g) was demineralized in HCl at 2°C for 24 hr and lyophilized. In step 1, the lyophilized matrix was sequentially extracted to decrease the content of lipid, proteoglycans, and sialoproteins and to convert the bone collagen to insoluble bone matrix gelatin in 8 M LiCl (12). In step 2, the bone matrix gelatin was incubated for 24 hr at 37°C at pH 7.2 in a 0.00054% purified bacterial collagenase (Worthington, CLSPA) in Hanks' solution (14) containing mM Tris, 300 mM CaCl<sub>2</sub>, and 3 mM NaN<sub>3</sub>. The collagenase was purified by the method of Peterkofsky and Diegelmann (15). A low enzyme-to-substrate ratio and a high concentration of Ca<sup>2+</sup> were used for suppression of contaminant proteases (16); the NaN<sub>3</sub> was used for antimicrobial activity. The pH was readjusted to 7.2 every 2 hr for the first 8 hr. After 24 hr, the total digest was centrifuged at 40,000 × G for 15 min (step 3). Step 4 produced a pellet of insoluble collagenase-resistant substances.

In step 5, the supernatant, a clear, slightly opalescent solution, was filtered through a cellulose acetate membrane (pore size,

0.30  $\mu$ m). In step 5A, the dialysate was lyophilized. One half of solution 5B was transferred, in step 5C, to a membrane sac (Spectrapor; 2000 M<sub>r</sub> assigned cutoff) and dialyzed for 24 hr against 0.1% ethylene glycol in distilled water at 2°C. The other half was dialyzed against 0.1 M phosphate buffer in 0.1% ethylene glycol in distilled water for 24 hr (step 5D). In step 5E, precipitated substances were separated from the soluble substances (supernatant 5F). In step 6, the coprecipitate obtained by step 5E was chemically extracted from the calcium phosphate by use of 1 M NaCl/2 mM Tris/5% ethylene glycol, pH 7.2 at 2°C. The extract was desalted by dialysis against 1% ethylene glycol and lyophilized. The substances separated at each of the above steps were lyophilized, weighed, and stored in sealed sterile containers.

**Chemical Analysis.** The proteins obtained by various steps in Figs. 1-3 were analyzed, by standard methods, for hydroxyproline (17), total protein (18), hexosamine (19), calcium (20), and phosphorus (21).

**Polyacrylamide Gel Electrophoresis.** The solution obtained by step 3 was fractionated by slab sodium dodecyl sulfate/polyacrylamide gel electrophoresis by the method of Weber and Osborn (22) and stained with a 1:1 mixture of Coomassie brilliant blue G-250 and alcian blue.

**Concanavalin A-Sepharose Chromatography.** The soluble nondialyzable components obtained at step 5B were desalted by dialysis against 5% ethylene glycol in water and applied to a concanavalin A-Sepharose column (1.6 × 10 cm) equilibrated with phosphate-buffered saline (P<sub>i</sub>/NaCl) (pH 7.4) by the method of Davey *et al.* (23). When the absorbance decreased to almost zero, the column was eluted with 150 ml of either 0.1 M  $\alpha$ -methylmannoside in P<sub>i</sub>/NaCl or 50% ethylene glycol until no further components absorbing at 280 nm were detected. The fractions collected from each elution were dialyzed against 1% EG in 1 mM P<sub>i</sub>/NaCl (pH 7.2), lyophilized, and analyzed for hydroxyproline, amino N, and hexosamine. The amino acid compositions were determined on acid hydrolysates of eluted proteins on a Beckman 120 B analyzer equipped with acidic and basic columns.

**Bioassay.** The lyophilized substances obtained at each step shown in Fig. 1 were bioassayed by implantation in double-walled cellulose acetate (Millipore) chambers in the anterior abdominal wall of allogeneic rabbits (24). For controls, empty chambers or chambers filled with purified rabbit albumin or calcium phosphate were implanted in the contralateral muscle of the animals with experimental implants. Substances obtained by steps 4, 5A, and 5F proved to be additional controls. Collagenase digests of autolyzed rabbit bone matrix and of rabbit Achilles tendon, comparable to the above-described digests of bone matrix gelatin, were implanted for matrix controls. Implants were also made in the rat to detect a cross-species response.

Abbreviations: BMP, bone morphogenetic protein; P<sub>i</sub>/NaCl, phosphate-buffered saline, pH 7.4.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

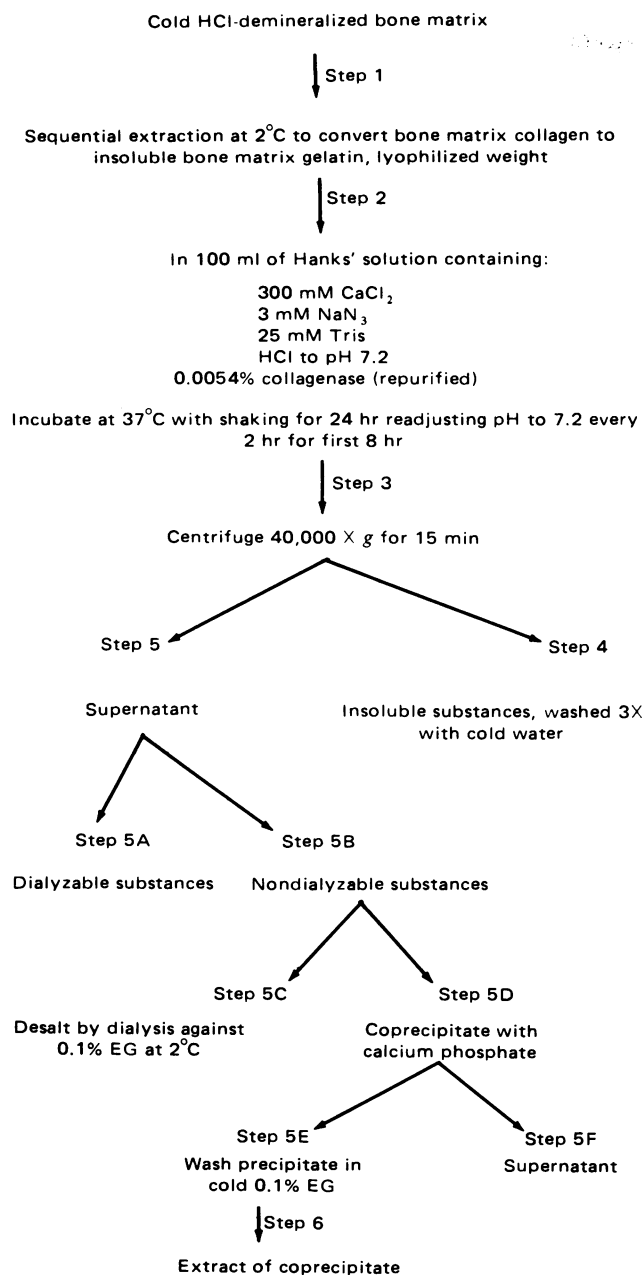


FIG. 1. Flow diagram of the procedure for preparation of soluble and insolubilized BMP. EG, ethylene glycol.

## RESULTS

The procedure described above produced six different groups of substances derived from bone matrix gelatin (Table 1). The general chemical composition of the four principal groups is shown in Table 2. The composition of the group obtained by step 4 was characteristic of the collagenase-resistant insoluble structural glycoproteins described by Robert and Compte (25). The relatively high levels of hydroxyproline and a small amount of hexosamine suggest that step 5A removed digestion products of collagenase, chiefly low molecular weight tripeptides and polypeptides. The relatively high levels of hydroxyproline, hexosamine, and proteins indicate that step 5B separated a selection of soluble nondialyzable collagen polypeptide chains and various noncollagenous proteins and polypeptides. Step 5D separated some of the nondialyzable noncollagenous protein (step 5E) from hydroxyproline-containing soluble collagen

Table 1. Dissolution of bone matrix gelatin

Group	Classification	Weight, g/100 g wet bone
1	Demineralized bone matrix	32.05
2	Bone matrix gelatin	25.60
4	Collagenase-insensitive substances, structural glycoproteins	1.97
5A	Dialyzable collagenase-released substances	12.82
5C	Nondialyzable collagenase-released substances (35% collagen peptides)	9.89
5C $\alpha$ -MM	Substances separated from 5C by $\alpha$ -methylmannoside elution of concanavalin A-Sepharose column	0.097
5C EG	Substances separated by elution with $\alpha$ -methylmannoside and then ethylene glycol	0.065
6	Separated from 5C, ethylene glycol extraction of calcium phosphate coprecipitate	0.040

peptide fragments (step 5F) by coprecipitation with calcium phosphate.

When chromatographed on a concanavalin A-Sepharose column, the centrifuged and filtered solution obtained at step 5B was distributed in three fractions. The distribution (Fig. 2) calculated by UV absorption was as follows: I, unabsorbed fraction (breakthrough), 93.1%; II,  $\alpha$ -methylmannoside, eluted fraction, 5.7%; III, 50% ethylene glycol eluted fraction, 1.2%. The breakthrough fraction contained 386.5  $\mu$ g of protein and 20.0  $\mu$ g of hydroxyproline per mg. The  $\alpha$ -methylmannoside eluate fraction contained 603.1  $\mu$ g of protein and 24  $\mu$ g of hydroxyproline per mg. The ethylene glycol eluate fraction contained 373.5  $\mu$ g of protein per mg and only trace amounts of hydroxyproline.

The coprecipitation of nondialyzable substances and calcium phosphate removed 85% (454  $\mu$ g/mg) of the total protein but only 8% of the hydroxyproline from solution 5B. Approximately 10% of the weight of the coprecipitate was extracted by the saline/Tris/ethylene glycol buffer. The residual calcium phosphate had a Ca/P molar ratio of 1.6. The hydrophobic noncollagenous proteins were selectively coprecipitated with calcium phosphate (step 5E), leaving hydrophilic collagen peptide fragments in solution (step 5F). The extract of the coprecipitate (step 6, Fig. 1) contained 391  $\mu$ g of protein per mg and only traces of hydroxyproline-containing peptides.

Sodium dodecyl sulfate gel electrophoresis of the  $\alpha$ -methylmannoside eluates demonstrated five bands corresponding to standards with molecular weights of 94,000, 68,000, 43,000, 21,000, and 14,300. The ethylene glycol eluate produced bands corresponding to molecular weights of 68,000, 43,000, 21,000, and 14,300. The densest staining band corresponded to a standard with a molecular weight of 68,000. However, the extract of the coprecipitated protein produced two faintly stained bands corresponding to the standards with molecular weights of 21,000 and 14,000. Amino acid analysis of the components eluted by  $\alpha$ -methylmannoside and by ethylene glycol showed that aspartic and glutamic acids were the predominant residues and leucine, valine, proline, and lysine were abundant. No hydroxyproline was detectable in either fraction. There was 10 mol % half-cystine in the  $\alpha$ -methylmannoside eluate and 16 in the ethylene glycol eluate.

Bioassay by implantation in double-walled diffusion cham-

Table 2. Chemical composition ( $\mu\text{g}/\text{mg}$ ) of substances obtained from total collagenase digestion of bone matrix gelatin by procedures illustrated in Fig. 1

Component $\mu\text{g}/\text{mg}$	Structural glycoproteins (step 4)	Dialyzable digestion products of collagen, $M_r$ 2000 (step 5A)	Nondialyzable substances (step 5B)	Substances coprecipitated with calcium phosphate (step 5E)
Hexosamine	9.2	1.7	10.9	9.0
Hydroxyproline	0.4	50.8	106.9	19.0
Total Lowry protein	675.7	123.3	523.5	454.0
Total P	3.7	0.2	1.8	35.7
Total Ca	0.3	95.5	6.9	76.2

bers demonstrated that transmembrane new bone formation was obtained in 8 of 10 trials of implants of substances obtained by step 5B in 7 of 10 from step 5C, and in 8 of 10 from step 6 (Fig. 3). Bone was also produced by  $\alpha$ -methylmannoside (two of four trials) or ethylene glycol (five of six trials) eluates of fractions II and III (Fig. 2) of solution 5B. Bone was produced by the coprecipitate obtained by step 5E in five of six implants and by the chemical extracts of the coprecipitate in three of four instances (Fig. 4). Control implants (10 each) of empty chambers, purified rabbit albumin, and calcium phosphate invariably produced negative results as did control implants of soluble and calcium phosphate insolubilized fractions of collagenase digest of bone matrix gelatin prepared from autolyzed bone or Achilles tendon. The substances obtained by steps 5B, 5C, and 6 produced a cross-species bone morphogenetic response in the rat.

The above-described coprecipitation procedure should not be equated with hydroxyapatite column chromatography. We applied the above-described collagenase digests to columns of hydroxyapatite gels and were unable to isolate fractions with BMP activity.

## DISCUSSION

Under the conditions specified above, a soluble BMP is released from the bone matrix by conversion of bone collagen to insoluble gelatin and by digestion with purified collagenase. The use of gelatin eliminates 95% of bone matrix noncollagenous proteins—i.e., phosphoproteins, sialoproteins, proteoglycans, and lipoproteins which have no BMP activity. The glycoproteins including BMP in bone gelatin are conveniently separated from collagen degradation products by concanavalin A-affinity

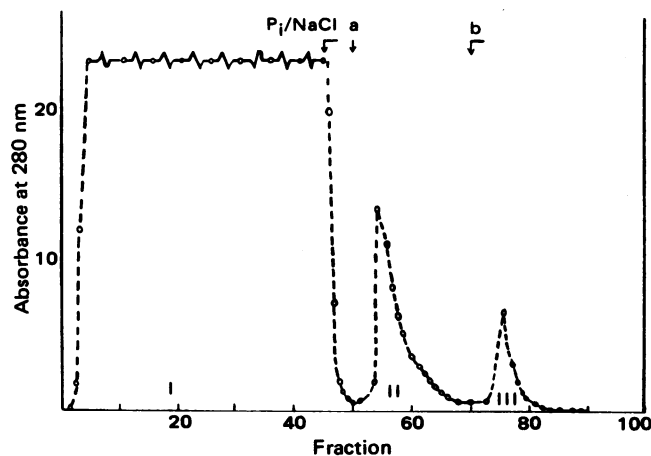


FIG. 2. Three fractions of the collagenase digest shown in Fig. 1 isolated by concanavalin A-Sepharose chromatography. The  $\alpha$ -methylmannoside (a, 0.1 M in  $\text{P}_i/\text{NaCl}$ ) and the ethylene glycol (b, 50% in  $\text{P}_i/\text{NaCl}$ ) eluate fractions (II and III, respectively), but not the breakthrough fraction (I), produced transmembrane bone deposits.

chromatography. Coprecipitation with calcium phosphate also separates BMP from other proteins and, at the same time, provides it with an inorganic solid support. Furthermore, a soluble BMP is *partially* recovered by extraction of the solid support with ethylene glycol in  $\text{P}_i/\text{NaCl}$ .

BMP is stabilized in an ethylene glycol/ $\text{P}_i/\text{NaCl}$  mixture that preserves hydrophobic molecular conformations (25, 26). No part of the helical segment of the hydrophilic bone collagen molecule is associated with BMP because the biologically active molecule is in an ethylene glycol solution containing only trace amounts of hydroxyproline-containing peptides and can be further purified by coprecipitation with calcium phosphate. The small quantities of protein in these biologically active fractions suggest that ethylene glycol-soluble BMP represents considerably less than 0.05% of the wet weight of cortical bone. When the ethylene glycol-soluble BMP is implanted inside a diffusion chamber, differentiation of new bone is induced on the outside. Ethylene glycol and  $\alpha$ -methyl mannoside eluates

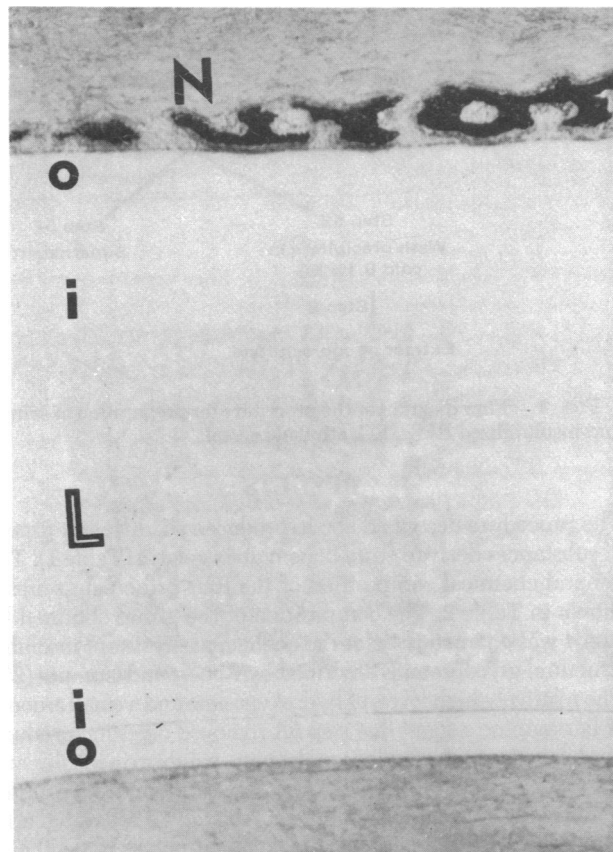


FIG. 3. Double-walled diffusion chamber showing deposits of new bone (N) on the outer membrane (o). Interstitial fluid (L) containing soluble BMP fills the space below the inner membrane (i). (Uncalcified section, von Kossa stain;  $\times 75$ .)

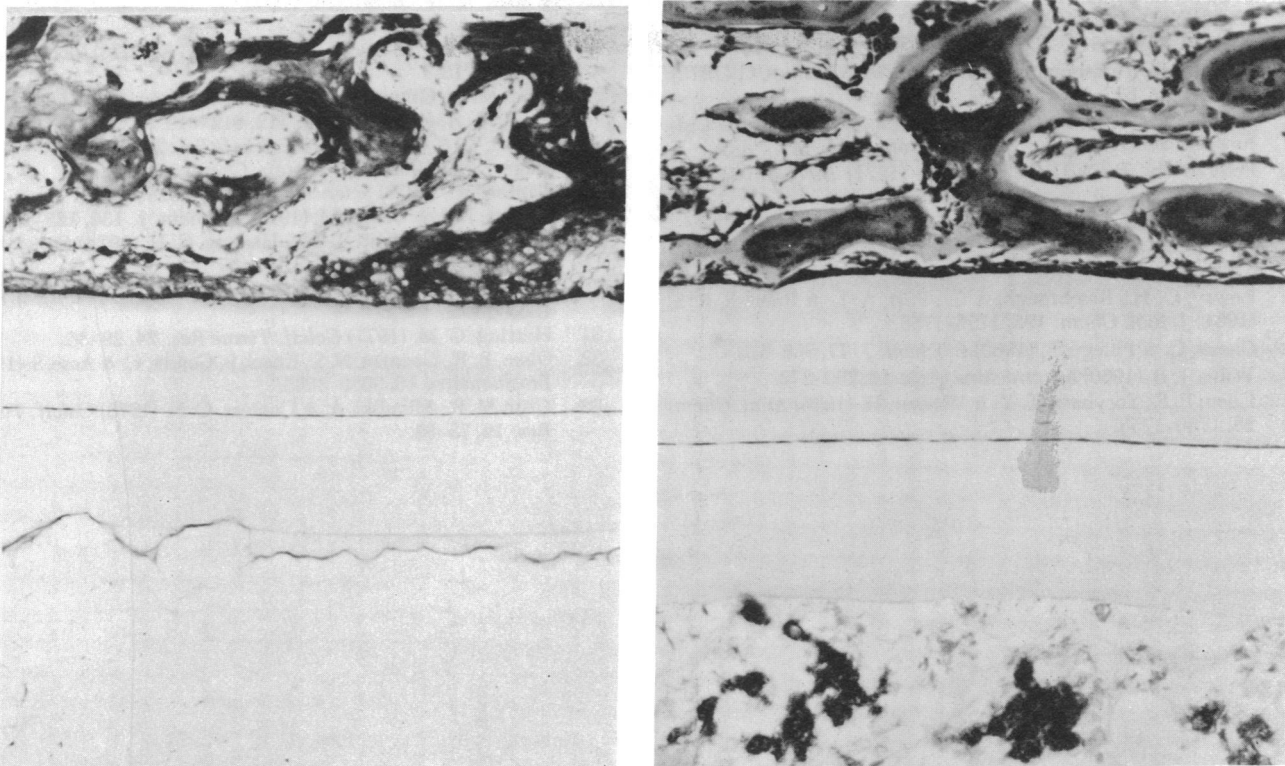


FIG. 4. Double-walled diffusion chamber. (Left) Deposits of new bone (top) and coprecipitate (bottom) of calcium phosphate and soluble noncollagenous proteins obtained by step 7 of the chemical procedures diagrammed in Fig. 1. (Undecalcified section, Mallory stain;  $\times 68$ .) (Right) Another section of the same chamber, stained with hematoxylin/eosin and azure. Note the outline of the coprecipitate (bottom) in the inside of the chamber and deposits of new woven bone (top) 3 weeks after implantation. ( $\times 69$ .)

of a concanavalin A-Sepharose column and coprecipitated BMP all induced transmembrane bone formation similarly.

Characteristically, BMP and other bone matrix glycoproteins are bound to a concanavalin A-Sepharose column by means of carbohydrate recognition and hydrophobic interaction. Five of these with molecular weights ranging from 94,000 to 14,300 were eluted with  $\alpha$ -methylmannoside. Four with molecular weights of 68,000 to 14,300 were eluted with ethylene glycol. Two with molecular weights of 21,000 and 14,300 coprecipitated with calcium phosphate. All three groups have comparable BMP activity. An obvious explanation for two groups is that BMP eluted by  $\alpha$ -methylmannoside may have been predominantly bound by carbohydrate recognition whereas the BMP eluted by ethylene glycol could have been chiefly bound by hydrophobic interaction. The BMP activity in the third group suggests that a low molecular weight biologically active hydrophobic molecule carried by glycoprotein may dissociate and coprecipitate with calcium phosphate. Ethylene glycol is present in all three systems and binds firmly to hydrophobic proteins so as to alter electrophoretic mobility. The complex precludes identification of a single stainable band common to both  $\alpha$ -methylmannoside and ethylene glycol eluates and requires a  $^2\text{H}_2\text{O}$  analytical centrifuge method for molecular weight determinations (27). This could explain why previously described bone glycoproteins released by collagenase without ethylene glycol in the system have a different range of assigned molecular weights (28–31). There is also the possibility that BMP activity may be associated not with bone glycoprotein as described by Ashton *et al.* (29) but with carbohydrate-containing scission products of the bone collagen hydroxyproline free COOH terminus described by Olsen *et al.* (32).

Although BMP could be a single glycoprotein molecule, there is the possibility that biologic activity may be a function of a

protein aggregate. Whatever its state of aggregation may be, the cysteine in both concanavalin A-bound fractions and previously reported observations on reversible mercaptoethanol extinction of biologic activity suggest that BMP is a disulfide-bonded structure (33). Whatever its structure may prove to be, present observations on biologic activity of the solid support invite investigations on BMP/calcium phosphate coprecipitates for repair of bone defects from injury, tumors, congenital malformations, and old infections. Patently, a physicochemically characterized BMP would be a propitious tool for fundamental research on cell differentiation.

This research was supported by grants from the U.S. Public Health Service (DE-02103), Max Factor Family Foundation, Solo Cup Foundation, and Marion and Eugene Bailey Fund.

1. Urist, M. R. (1965) *Science* **150**, 893–899.
2. Urist, M. R., Dowell, T. A., Hay, P. H. & Strates, B. S. (1968) *Clin. Orthop. Relat. Res.* **59**, 59–96.
3. Huggins, C. B. & Urist, M. R. (1970) *Science* **167**, 896–898.
4. Reddi, A. H. & Huggins, C. B. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1601–1605.
5. Urist, M. R., Jurist, J. M., Dubuc, F. L. & Strates, B. S. (1970) *Clin. Orthop.* **68**, 279–293.
6. Urist, M. R., Silverman, B. F., Buring, K., Dubuc, F. L. & Rosenberg, J. M. (1967) *Clin. Orthop. Relat. Res.* **53**, 243–283.
7. Urist, M. R., Hay, P. H., Dubuc, F. L. & Buring, K. (1969) *Clin. Orthop.* **64**, 194–220.
8. Urist, M. R. & Nogami, H. (1970) *Nature (London)* **225**, 1051–1052.
9. Urist, M. R. (1970) *Symp. Soc. Dev. Biol., Suppl.* **4**, 125–163.
10. Urist, M. R. & Strates, B. S. (1971) *J. Dent. Res., Suppl.* **6**, 50, 1392–1406.
11. Urist, M. R., Iwata, H., Boyd, S. D. & Ceccotti, P. L. (1974) *J. Histochem. Cytochem.* **22**, 88–103.

12. Urist, M. R., Iwata, H., Ceccotti, P. L., Dorfman, R. L., Boyd, S. D., McDowell, R. M. & Chien, C. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3511-3515.
13. Urist, M. R., Terashima, Y., Nakagawa, M. & Stamos, C. (1978) *In Vitro* **14**, 697-706.
14. Hanks, J. H. (1955) in *An Introduction to Cell and Tissue Culture*, ed. Scherer, W. F. (Burgess, Minneapolis, MN), p. 5.
15. Peterkofsky, B. & Diegelmann, R. (1971) *Biochemistry* **10**, 988-993.
16. Herring, G. M. (1976) *Biochem. J.* **159**, 749-755.
17. Firschein, H. E. & Shill, J. P. (1966) *Anal. Biochem.* **14**, 296-304.
18. Lowry, O. H., Rosebrough, A. L., Farr, A. L. & Randall, R. S. (1951) *J. Biol. Chem.* **193**, 1756-1758.
19. Chessi, C. & Piliego, F. (1960) *Biochem. J.* **77**, 508-510.
20. Willis, J. B. (1960) *Spectrochim. Acta* **16**, 259-272.
21. Chen, P. S., Torybara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756-1758.
22. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412.
23. Davey, M. W., Sulkowski, E. & Carter, W. A. (1976) *Biochemistry* **15**, 704-713.
24. Urist, M. R., Granstein, R., Nogami, H., Svenson, L. & Murphy, R. (1977) *AMA Arch. Surg.* **112**, 612-619.
25. Robert, L. & Compte, P. (1968) *Life Sci.* **7**, 493-498.
26. Singer, S. J. (1962) *Adv. Protein Chem.* **17**, 1-68.
27. Tanford, C. (1978) *Science* **200**, 1012-1018.
28. Triffitt, J. T. & Owen, M. (1973) *Biochem. J.* **136**, 125-134.
29. Ashton, B. A., Triffitt, J. T. & Herring, G. H. (1974) *Eur. J. Biochem.* **45**, 525-533.
30. Owen, M. & Schetlar, M. R. (1968) *Nature (London)* **220**, 1335-1336.
31. Herring, G. M. (1977) *Calcif. Tissue Res.* **24**, 29-36.
32. Olsen, B. R., Guzman, N. S., Engel, J., Condit, C. & Aase, S. (1977) *Biochemistry* **16**, 3030-3035.
33. Urist, M. R., Mikulski, A. & Contreas, C. N. (1975) *Calcif. Tissue Res.* **19**, 73-83.