PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 48-53 (1979)

# A Soluble Bone Morphogenetic Protein Extracted from Bone Matrix with a Mixed Aqueous and Nonaqueous Solvent (40616)[1]

MARSHALL R. URIST[2] AND ANDRZEJ J. MIKULSKI[3]

*Bone Research Laboratory, UCLA Medical School, Los Angeles, California 90024*

Development of bone occurs in intramuscular or subcutaneous implants of dentin (1, 2) and bone (3–6) matrix in postfetal life in an extraskeletal site under the influence of a bone morphogenetic protein (BMP). BMP is transferred from bone matrix to a responsive mesenchymal-like cell population within 24 hr after implantation (7). BMP is bioassayed by measurements of the quantity of histologically valid new bone per unit weight of preimplanted matrix 28 days after the operation (8). At earlier intervals, BMP activity can be estimated by the accumulation of the following specialized cell products in the implant: hyaluronate within 24 hr (9); hyaluronidase within 48 hr (10); $^{35}$S-sulfated substances within 7 days (11); alkaline phosphatase within 10 days (12); $^{45}$Ca uptake by mineralizing tissue within 10 to 14 days (13). All of these data on BMP activity are based on observations on implants of demineralized bone matrix or of insoluble bone matrix gelatin (14–18). Recently, with the aid of a modification of the methods of Herring *et al.* (19, 20) and Ashton *et al.* (21), we separated a soluble BMP from bone matrix digested with purified bacterial collagenase (22). We report here on a method of solubilization of BMP without digestion of the bone matrix.

*Materials and methods.* Eighty grams of rabbit cortical bone was excised from the midshaft of the long bones, pulverized manually under aseptic conditions with a mallet, washed 3× in 1 liter of cold distilled water to remove the old blood and bone marrow, and then demineralized in cold 0.6 *N* HCl within a period of 24 hr. After the acid was removed by dialysis against 5 mmol/liter of $NaN_3$ at 2°, the demineralized fragments were extracted in a solution of 50% ethylene glycol (EG) in 0.05 *M* phosphate-buffered 0.15 *M* sodium chloride (EG-PBS) at 25° as outlined in five steps shown in Fig. 1. The supernatant solution was separated from the matrix powder by centrifugation at 40,000g for 30 min, decanted, and desalted by dialysis against 0.01% EG in cold distilled water in membrane tubing (Spectrophor, MW 2000). The nondialyzable desalted substances were lyophilized, and analyzed by standard methods for hydroxyproline (23), hexosamine (24), total phosphorus (25), total Lowry protein (26), and amino acids (27). The experiment was repeated substituting 0.025 *M* Tris buffer for phosphate buffer to avoid phosphate binding and eliminate artifacts from measurements of total phosphorus.

For controls, EG-PBS extracts were similarly prepared from bone autolyzed (28) or denatured by demineralization in a solution of 70% alcohol in 0.6 *N* HCl to abolish matrix BMP activity (4).

The substances in solution obtained by Step 5 (Fig. 1) were dialyzed for 8 hr first against 100 mmole/liter of calcium chloride, then against phosphate buffer for 24 hr pH 7.2, to produce a protein calcium phosphate coprecipitate. The coprecipitate was washed three times in a 0.1% EG solution at 2°, in cold water and lyophilized. The protein was recovered by mechanical disaggregation and extraction of the coprecipitate with a 50% EG-PBS solution, and desalted by dialysis against 0.1% EG in cold water.

*Analytical sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.* Samples of the lyophilized substances obtained by the procedure shown in Figs. 1 and 2 were incubated for 2 hr in 0.01 *M* sodium phosphate buffer, pH 7.0, containing 1% SDS and 1% β-mercaptoethanol prior to application to a 10% gel. Slab gel electrophoresis was carried out by the method of Weber and Osborn (29).

[1] Supported by USPHS Grant DE02103.

[2] To whom reprint requests should be addressed to: 1000 Veteran Avenue, Room A3-34, Los Angeles, Calif. 90024.

[3] Andrzej J. Mikulski died in Warsaw, Poland on December 7, 1978.



0037-9727/79/090048-06$01.00/0

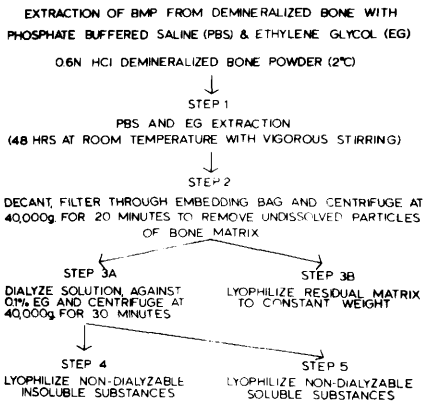



FIG. 1. Flow diagram of five steps in the ethylene glycol phosphate-buffered salt solution (EG-PBS) extraction of hydrophobic noncollagenous proteins from bone matrix. The dry weight of the substances obtained at each step is shown in Table I.

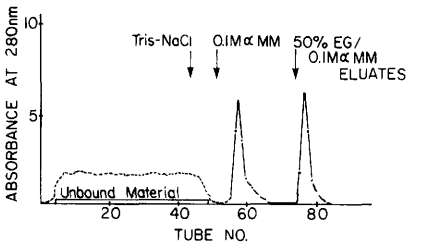



FIG. 2. Chromatography of EG-PBS solution applied on a Con-A-S column and eluted in sequence with α-MM and EG. A filtered and centrifuged EG-PBS extract (350 ml in volume) was dialyzed against 0.05 *M* PBS–HCl (pH 7.4) in 0.15 *M* NaCl and applied to a Con-A-S column (1.6 × 10 cm) equilibrated with the same buffer. The unbound fraction contained nearly all of the EG-PBS-soluble collagen peptide chains and a small quantity of various hydrophilic noncollagenous proteins. The α-MM and EG elutes contained only trace quantities of hydroxyproline-containing collagen peptides and significant quantities of hydrophobic glycoproteins.

The gels were stained in a 1:1 mixture of Coomassie brilliant blue G-250 and alcian blue stain. For determination of apparent molecular weights with the following standards (Bio-Rad Lab, Richmond, Calif.) were used: phosphorylase, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lyosozyme, 14,300.

*Concanavalin A (Con-A-S) Sepharose chromatography.* The soluble nondialyzable components were desalted by dialysis against 5% ethylene glycol in water and applied to a Con-A-S (1.6 × 10-cm) column equlibrated with phosphate-buffered saline (PBS), pH 7.4, as described by Davey *et al.* (30). When the absorption of the washings dropped to almost zero, the column was eluted with 150 ml of either 0.1 *M* α-methyl mannoside (α-MM) in PBS or 50% EG until two more 280-nm absorbing components were detected. The fractions collected from each peak were lyophilized for chemical analysis.

*Bioassay.* Bioassays for BMP were performed by implantation of 1, 2, and 5 mg of lyophilized material obtained from Steps 3 to 5 were implanted in double-walled cellulose acetate (Millipore, 0.45-μm pore size) chambers (30). The chambers were implanted in the anterior abdominal wall of allogenic rabbits (New Zealand) for 21 days. The function of the chambers is to release the protein slowly and over a period of time sufficient for a population of mesenchymal cells to gather.

The yield was determined morphometrically by the percentage of radioopaque histologically valid deposits of new bone covering the outer membrane of each chamber in microradiographs and histological sections stained in hematoxylin eosin and azure II.

*Results.* Table I summarizes measurements of the quantity of various fractions obtained by EG-PBS extraction of 0.6 *N* HCl demineralized bone matrix and estimates of the bone morphogenetic activity associated with each fraction. The EG-PBS extract was decanted and centrifuged at 40,000*g* for 1 hr to separate insoluble from soluble substances. The sedimented material was lyophilized, weighed, and analyzed. The supernatant solution was desalted by dialysis against 0.1% EG for 24 hr and until a straw-colored precipitate formed. After centrifugation, the precipitate was washed in 0.1% EG and lyophilized. The dry weight of the precipitate was 18.0 μg per mg of lyophilized bone matrix. The 0.1% EG-soluble substances (Step 5), after lyophilization, were fluffy white and weighed 28.4 μg/mg of bone matrix. The residual matrix was washed, lyophilized, weighed, and twice reextracted with EG-PBS to remove all soluble substances and leave a residue devoid of all BMP activity.

The chemical composition of substances derived from Step 5 in μg/mg was: total protein, 200.0 ± 12.0; amino acids, 194.4 ±

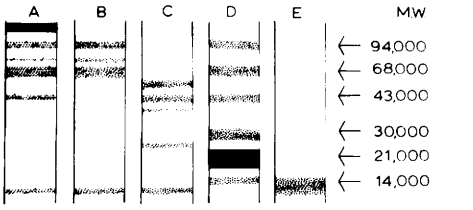
TABLE I. YIELD OF BMP AND ASSOCIATED SUBSTANCES EXTRACTED FROM BONE MATRIX WITH EG-PBS

| Substances obtained by step shown in Fig. 1 | Description | Yield (mg/g) dry demineralized bone matrix | Percentage of the outer membrane surface covered with new bone |
|---|---|---|---|
| 4 | Water insoluble, EG-PBS soluble | 18.0 | 0 |
| 5 | Soluble in 0.1% EG | 28.4 | 34.1 ± 9.0 |
| 5 Applied to Con-A column | Unbound protein | 0.027 | 0 |
| 5 Applied to Con-A column | α-MM eluate | 0.015 | 15.4 ± 2.0 |
| 5 Applied to Con-A column | EG eluate | 0.014 | 12.9 ± 2.5 |
| 5 Coprecipitated with calcium phosphate | EG-PBS extract of coprecipitate | 0.001 | 40.5 ± 6.0 |

21; hydroxyproline, 0.6 ± 0.1; hexosamines, 9.1 ± 1.2; total phosphorous, 4.9 ± 0.1. The error was eliminated from measurements of total phosphorus by substituting Tris-buffered NaCl for phosphate-buffered NaCl.

*Concanavalin A–Sepharose chromatography.* The above-described solution was applied to a Concanavalin A–Sepharose column equilibrated with Tris-buffered 0.15 *M* NaCl at pH 7.4. When the absorption dropped to almost zero, columns were eluted with α-methyl mannoside (α-MM) or α-MM in ethylene glycol (EG) and the eluates were collected for biochemical and biologic assays. The breakthrough fraction contained 274 μg protein/mg total product, 3.2 μg/mg hexosamine and 1.4% collagenous material (estimated on the basis of hydroxyproline content). The α-MM eluate contained 220.9 μg/mg total (Lowry) protein/mg product contaminated with 0.2 μg/mg of hydroxyproline. The EG eluate contained 188 μg/mg of protein contaminated with 0.4 μg/mg of hydroxyproline (Fig. 2).

Figure 3 is a diagram of the results of slab




A. EG-PBS TOTAL EXTRACT
B. CON A-S CHROMATOGRAPHY-α-MM ELUATE
C. CON A-S CHROMATOGRAPHY-EG ELUATE
D. STANDARDS
E. COPRECIPITATE, EG-PBS EXTRACT

FIG. 3. Diagrammatic representation of slab SDS–acrylamide gel electrophoresis patterns of β-mercaptoethanol-reduced fractions demonstrated in Figs. 1 and 2.

SDS–gel electrophoresis of the solution obtained by Step 5, and various subfractions. The total EG-PBS extract contained not less than six components with assigned molecular weights ranging from 100,000 to 12,000. The α-MM eluate contained about four stainable components and the EG about five with electrophoretic mobilities corresponding to standards ranging from 94,000 to approximately 12,000 in MW. Electrophoretic analysis of fraction E coprecipitate (Fig. 3) shows only one band. More recent experiments, however, with larger quantites of material applied to the gel, show three additional bands corresponding to components of higher MW. Based on uv absorbance, estimates of the weight of mixture of the four components of the α-MM eluate are about 15 mg, and of the five components of the EG eluate, 14 mg (Fig. 2).

Bioassays of lyophilized substances in diffusion chambers, as noted in Table I, showed that the total EG-PBS extract, and both the α-MM, the EG eluates, as well as the coprecipitated substances all induced transmembrane new bone formation (Figs. 4 and 5). The incidences of positive results were 3/4, 2/4, 2/2, and 3/4, respectively. Implants of the EG-PBS-insoluble substances, or control PBS-EG extracts of matrix prepared from autolyzed or acid alcohol denatured bone did not induce transmembrane bone morphogenesis. Neither did the denatured collagen and other soluble proteins collected in the unbound fractions of substances separated by Con-A column chromatography.

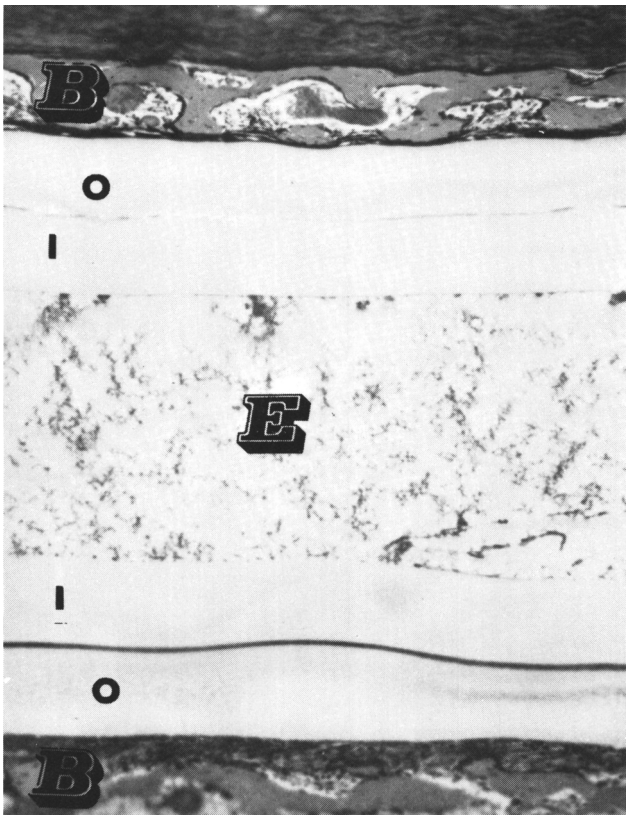



FIG. 4. Low-power magnification photomicrograph of deposits of new bone formed on both sides of a double-walled diffusion chamber containing 5 mg of EG-PBS-extracted bone matrix proteins 3 weeks after implantation. Note: remnants of extract (E); inner membrane (I); outer membrane (O); new bone (B).

*Discussion.* The working hypothesis of the above-described experiments is that the BMP is a hydrophobic protein molecule or protein aggregate that is dissociated from collagen and other hydrophilic substances by the use of EG, a water miscible nonpolar carrier. Conceptually, an EG·BMP complex is structurally stable and biologically active while BMP free in a purely aqueous medium is biologically inactive. Such EG·protein complexes are formed by many biologically active proteins which are not bound by covalent linkages into some generally insoluble matrix (31). According to Tanford (31) and Singer (32), hydrophobic proteins are difficult to dissolve and may even be expelled from aqueous solutions, yet may become miscible in certain nonaqueous solvents. The solvent must cause neither rupture nor formation of any covalent bonds within the macromolecule other than those within hydrogen atoms, and, therefore, should be chemically as inert as possible. Hydrophobic solvent mixtures sustain the biologically active conformation of proteins in aqueous media by clustering the hydrophobic amino acid components on the inside of a molecule away from the aqueous environment. In a mixture of water and EG, a weakly protic nonpolar solvent, hydrophobic interactions would protect the native conformation of BMP.

BMP·EG and other glycoprotein complexes may be bound to a Con-A column in two ways, one by carbohydrate recognition and another presumably by hydrophobic interaction. Analytical SDS–gel electrophoresis demonstrates that after mercaptoethanol reduction the $\alpha$-MM-eluted fraction includes at least four while an EG eluate about five stainable bands. By comparison of the electrophoretic mobilities of the component bands with standard purified proteins, the molecular weights range from 94,000 to less than 14,000. However, these are not protein

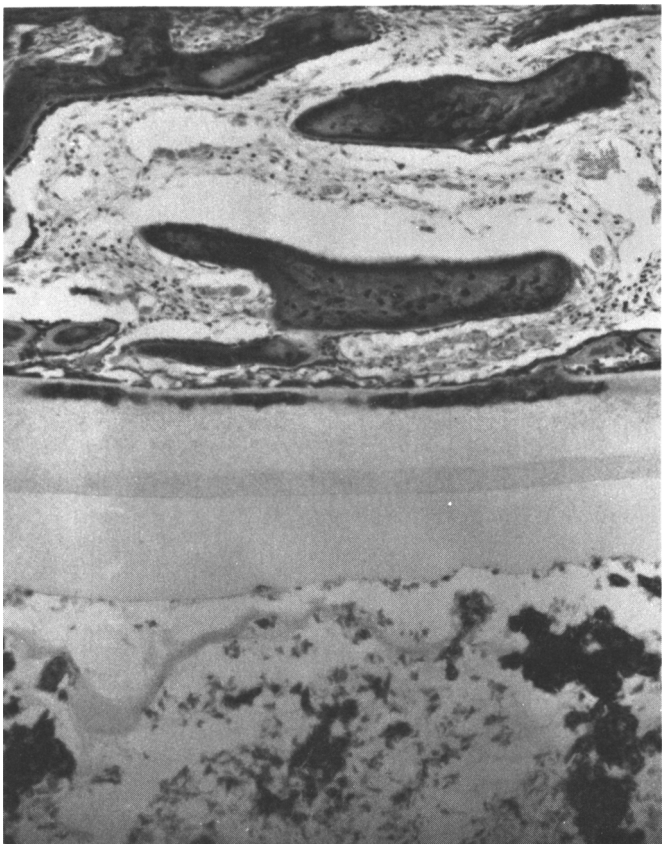


FIG. 5. High-power magnification photomicrograph of 4-week-old bone deposits (top) on the outside of diffusion chamber filled with a coprecipitate of calcium phosphate and the proteins in an EG-PBS extract (bottom). Undecalcified section, von Kossa stain.

MW estimates, but estimates based on electrophoretic mobilities of mercaptoethanol-reduced EG·protein complexes and must be corrected for the weight of EG bound to unreduced proteins by the use of $D_2O$ and the analytical ultracentrifuge (30). Furthermore, BMP is a disulfide-bonded molecule and is reversibly inactivated by mercaptoethanol reduction and oxidation (6).

The above observations indicate the combinations of experiments remaining to be done to ascertain which of the electrophoretically resolved components might have BMP activity. The principal problem is to define the smallest complete unit of BMP structure. Present observations on the binding of BMP active components to a Con-A simply affirms the view that BMP is a hydrophobic glycoprotein (22). BMP is now selectively separated from other proteins in the same way that coprecipitation with calcium phosphate separates serum $\alpha$-HS glycoprotein from albumin (33). Thus far, subfractionation of the Con-A chromatographed and of extracts of the calcium phosphate-coprecipitated proteins by Sephadex G-100 gel filtration, De-52 ion exchange, and other systems has not yet proven to isolate a BMP. The main difficulty is that each one subfraction weighed much less than 100 $\mu$g, a quantity of protein required for both bioassay and complete molecular characterization. However, to obtain the optimum quantity of starting material, we have recently substituted kilogram quantities of bovine bone for the 80-g batches of rabbit bone described in this report, and have a secured, biologically active crude bovine BMP. Bovine BMP-induced differentiation of bone in muscle pouches in both rats and rabbits. The composition of crude bovine BMP is under detailed investigation and will be reported in another communication.

*Summary.* A soluble bone morphogenetic protein (BMP) along with other noncollagenous proteins including only a relatively insignificant quantity of hydroxyproline-containing peptides were extracted from rabbit cortical bone by the action of an aqueous–nonaqueous solvent mixture. As it is bound by carbohydrate recognition and hydrophobic interaction with Con A, BMP has an essential characteristic of a glycoprotein. When $\alpha$-methyl mannoside and ethylene glycol elutes of proteins bound to a Con-A column are implanted inside of double-walled diffusion chambers, deposits of cartilage and bone develop on the outside. While estimates of the quantity of BMP are less than 0.01% of the dry weight of bone matrix, it is possible to concentrate and purify a biologically active BMP by application of the principles of hydrophobic protein chemistry.

1. Bang, G., and Urist, M. R., Arch. Surg. **94,** 781 (1967).
2. Yeomans, J. D., and Urist, M. R., Arch. Oral Biol. **12,** 999 (1967).
3. Urist, M. R., Science **150,** 893 (1965).
4. Urist, M. R., and Strates, B. S., J. Dent. Res. **50,** 1392 (1971).
5. Urist, M. R., Iwata, H., Ceccotti, P. W. L., Dorfman, R. L., Boyd, S. D., McDowell, R. M., and Chien, C., Proc. Nat. Acad. Sci. USA **70,** 3511 (1973).
6. Urist, M. R., Mikulski, A., and Conteas, C. N., Calc. Tiss. Res. **19,** 73 (1975).
7. Nakagawa, M., and Urist, M. R., Proc. Soc. Exp. Biol. Med. **154,** 568, (1977).
8. Urist, M. R., Jurist, J. M., Dubuc, F. L., and Strates, B. S., Clin. Orthop. **71,** 253 (1970).
9. Iwata, H., and Urist, M. R., Clin. Orthop. **90,** 236 (1973).
10. Nogami, H., and Urist, M. R., J. Cell Biol. **62,** 510 (1974).
11. Urist, M. R., Terashima, Y., Nakagawa, M., and Stamos, C., In Vitro **14,** 697 (1978).
12. Firschein, H. E., and Urist, M. R., Clac. Tiss. Res. **7,** 108 (1971).
13. Firschein, H. E., and Urist, M. R., Clin. Orthop. **84,** 263 (1972).
14. Urist, M. R., and Strates, B. S., J. Dent. Res. **50,** 1392 (1971).
15. Urist, M. R., Iwata, H., and Strates, B. S., Clin. Orthop. **85,** 275 (1972).
16. Iwata, H., and Urist, M. R., Clin. Orthop. **87,** 257 (1972).
17. Urist, M. R., Earnest, F., Kimball, K. M., DiJulio, T. P., and Iwata, H., Calc. Tiss. Res. **15,** 269 (1974).
18. Mikulski, A., and Urist, M. R., Prep. Biochem. **7,** 357 (1977).
19. Herring, G. M., Biochem. J. **159,** 749 (1976).
20. Herring, G. M., Calc. Tiss. Res. **24,** 29 (1977).
21. Ashton, B., Triffitt, J. T., and Herring, G. M., Eur. J. Biochem **45,** 525 (1974).
22. Urist, M. R., Mikulski, A. J., and Lietze, A., Proc. Nat. Acad. Sci. USA **76,** 1828 (1979).
23. Firschein, H. E., and Shill, J. P., Anal. Biochem. **14,** 296 (1966).
24. Chessi, C., and Piliego, F., Biochem. J. **77,** 508 (1960).
25. Chen, P. S., Torybara, T. Y., and Warnes, H., Anal. Biochem. **28,** 1756 (1956).
26. Lowry, O. H., Rosebrough, A. L., Farr, A. L., and Randall, R. S., J. Biol. Chem. **193,** 1756 (1951).
27. Rosen, H., Arch. Biochem. Biophys. **67,** 10 (1957).
28. Urist, M. R., Iwata, H., Boyd, S. D., and Ceccotti, P. L., J. Histochem. Cytochem. **22,** 88 (1974).
29. Wever, K., and Osborn, J., J. Biol. Chem. **244,** 4406 (1969).
30. Davey, M., Sulkowitz, E., and Carter, W. A., Biochemistry **15,** 704 (1976).
31. Tanford, C., Science **200,** 1012 (1978).
32. Singer, S. J., Adv. Protein Chem. **17,** 1 (1962).
33. Ashton, B. A., Hohling, H. J., and Triffitt, J. T., Calc. Tiss. Res. **22,** 27 (1976).

Received March 22, 1979. P.S.E.B.M. 1979, Vol. 162.