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Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography

(cell differentiation/osteogenesis/development)

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ABSTRACT **Bovine bone morphogenetic protein (bBMP) induces differentiation of mesenchymal-type cells into cartilage and bone. bBMP has an apparent M_r of 18,500 ± 500 and represents <0.001% of the wet weight of bone tissue. A M_r 34,000 protein resembling osteonectin is separated by extraction with Triton X-100. A M_r 24,000 protein and about half of a M_r 22,000 protein are disassociated from bBMP by precipitation in 1.5 M guanidine hydrochloride. Aggregates of bBMP and a M_r 14,000 protein are insoluble in aqueous media; the bBMP becomes soluble when the M_r 14,000 protein is disassociated in 6 M urea and removed from the solution by ultrafiltration. Three separate molecular species with apparent M_rs 18,500, 17,500, and 17,000 are eluted at 0.10, 0.15, and 0.20 M phosphate ion concentrations, respectively, from a hydroxy-apatite column. The M_r 18,500 protein has the amino acid composition of acidic polypeptide and includes four half-cystine residues; the pI is 4.9–5.1. The M_r 22,000 component is a chromoprotein resembling ferritin. The NH_2-terminal amino acid sequence of the M_r 17,500 protein simulates histone H2B. The M_r 17,000 protein may possess calmodulin activity. Aggregates of the M_r 18,500 and other proteins induce formation of large deposits of bone; the M_r 18,500 protein alone is rapidly absorbed and induces formation of small deposits. None of the other proteins induces bone formation.**

Under the influence of bone morphogenetic protein (BMP), perivascular mesenchymal-type cells (pericytes) differentiate into cartilage and woven bone. BMP is an acidic polypeptide (1, 2) embedded in a complex assortment of intra- and extracellular protein aggregates derived from dentin (3), bone (4, 5), and osteosarcoma tissues (6–9). We report here on the purification of BMP by means of a combination of differential precipitation, ultrafiltration, and hydroxyapatite chromatography.

MATERIALS AND METHODS

Ten-kilogram batches of 1-year-old steer long bones were obtained from an abattoir. After the epiphyseal ends were cut away with a band saw, the diaphyses were mechanically scraped clean of soft tissues and extensively washed in cold water solution of 3 mM NaN_3. The washed bone was frozen in liquid N_2, ground in a Wiley mill to a particle size of 1 mm^3, defatted in chloroform/methanol (1:1), and again washed in 10 liters of cold water (step 1). The bone particles were demineralized in 0.6 M HCl at 4°C for 48 hr and again extensively rewashed in NaN_3 solution (step 2). The demineralized washed bone particles were chemically extracted to remove soluble noncollagenous protein (i.e., sialoproteins, plasma proteins, γ-carboxyglutamyl proteins, and phosphoproteins), simultaneously converting the collagen to insoluble bone matrix gelatin (step 3) by previously described procedures (10). Ten kilograms of whole wet bone produced ≈1.4 kg of freeze-dried insoluble bone matrix gelatin. The BMP was extracted from the insoluble bone matrix gelatin in an inorganic/organic solvent mixture of 0.5 M $CaCl_2$ in 6 M urea at 28°C for 24 hr containing 10 mM *N*-ethylmaleimide (NEM) to protect BMP against endogenous degradative enzymes.

The undissolved matrix and other substances were removed by filtration through cheesecloth. The supernatant solution was decanted, dialyzed against 23 vol of deionized water at 4°C, and allowed to stand overnight in the cold while a precipitate formed. The precipitate was collected by centrifugation (Sorvall RC-5B refrigerated superspeed) at 40,000 × *g* for 20 min, washed in cold deionized water, lyophilized, and weighed (Table 1, step 4).

Sixty grams of the fraction obtained by step 4 (Table 1) was redissolved in the original 0.5 M $CaCl_2$/6 M urea solution and dialyzed against 11 vol of 0.25 M citrate buffer (pH 3.1) at 4°C. After 24 hr, a grayish-white precipitate was collected by centrifugation at 40,000 × *g* for 1 hr. The precipitate was extensively washed, defatted in chloroform/methanol (1:1), and evaporated to dryness (step 5).

Twenty-two grams of the fraction obtained by step 5 (Table 1) was redissolved in 4 M guanidine hydrochloride (Gdn·HCl) and diluted to 1.5 M Gdn·HCl by dialysis at 28°C for 12 hr or until formation of a precipitate. The 1.5 M Gdn·HCl-soluble fraction was dialyzed against water for 24 hr, until precipitation was complete. The water-insoluble precipitate was centrifuged, extensively washed in cold water, lyophilized, and weighed (step 6, Table 1). The 1.5 M Gdn·HCl-insoluble fraction was centrifuged at 50,000 × *g* for 1 hr, washed in cold water, lyophilized, and weighed (step 7). The protein fraction obtained by step 7 was redissolved in 0.5 M $CaCl_2$/6 M urea and dialyzed against 0.2% Triton X-100 in 0.10 M Tris·HCl buffer solution (pH 7.2) containing 6 M urea for 24 hr at 28°C. The retentate was dialyzed exhaustively against deionized water at 4°C until precipitation was complete and then lyophilized (step 8). The Triton X-100-soluble proteins were also lyophilized and weighed (step 9).

The precipitate from step 8 was redissolved in 6 M urea and further purified by ultrafiltration (Amicon; pore size, M_r 10,000). When dialyzed against water, most of the retentate did not precipitate and the soluble portion was lyophilized and collected for chromatographic fractionation.

Ultrafiltration. Two-liter batches of the $CaCl_2$/urea-soluble proteins in 0.01 M phosphate buffer (pH 7.0) in 6 M urea were filtered through an H1P10-8 hollow fiber cartridge (Amicon; M_r 10,000, approximate cutoff). The retentate was washed by passing an additional 6 liters of 6 M urea through the hollow fibers and was dialyzed against deionized water at

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Abbreviations: BMP, bone morphogenetic protein; bBMP, bovine BMP; Gdn·HCl, guanidine hydrochloride; IEF, isoelectric focusing.

4°C. The precipitate and supernatant were lyophilized separately.

$NaDodSO_4$/Polyacrylamide Slab Gel Electrophoresis. The above described protein fractions were solubilized by incubation for 24 hr in 0.06 M Tris·HCl (pH 6.8) containing 2 M urea and 0.2% $NaDodSO_4$. Five microliters (2.5 mg/ml) of each sample was applied to a 12.6% gel with a 3% stacking gel and was electrophoresed at 25 mA. The gels were stained with 0.25% Coomassie brilliant blue R-250 in methanol/acetic acid/H_2O (5:1:5). The molecular weights were determined by using low molecular weight standards (Pharmacia), with a range of M_r 94,000–14,400.

Preparative Gel Electrophoresis. Electrophoresis also was performed with protein fractions applied to tube gels. Stained and unstained gel cylinders were electrophoresed in parallel and sliced to separate the individual bands. Selected slices were applied to fresh disc gels with 3% agarose and re-electrophoresed to verify the homogeneity of the protein samples. The individual protein constituents were eluted with 6 M urea, dialyzed against water, and lyophilized.

Preparative Flatbed Electrofocusing in a Granulated Gel. Two-hundred-milligram samples were dissolved in 95 ml of 4 M urea and added to 5 ml of ampholyte (Biolyte, Bio-Rad). After electrofocusing, each band was cut out of the gel separately with a spatula. Each of the gel samples was transferred to columns and eluted with 5 ml of 6 M urea in 0.5 M $CaCl_2$ for a solvent. The eluate was collected in tubes and the pH was determined for each sample.

Hydroxyapatite Chromatography. Hydroxyapatite columns of Bio-Gel HTP (Bio-Rad) were prepared by adding 15 g of hydroxyapatite to 100 ml of 0.05 M phosphate buffer (pH 7.3) containing 6 M urea. After 10 min the solvent was decanted and the hydroxyapatite was resuspended in 100 ml to give a settled bed height of 9 cm (volume, ≈55 ml). The column was equilibrated with 100 ml of starting buffer, using a gravity flow rate of 0.6 ml/min. Samples of 100 mg of the preparation obtained by step 8 were dissolved in 6 M urea, applied to the column, and separated into fractions.

Hydrophobic Column HPLC. Further purification of the protein fraction isolated by hydroxyapatite chromatography was accomplished by HPLC using a hydrophobic column (Ultrapore, reverse-phase Spherogel column, Beckman). One-milligram samples were dissolved in 0.1% phosphoric acid/0.1 M sodium perchlorate/5% trifluoracetic acid. The solution was applied to the column and eluted with a gradient of (*i*) 0.1% H_3PO_4/0.1 M $NaClO_4$ in water and (*ii*) 0.1% H_3PO_4/0.1 M $NaClO_4$ in a mixture of acetonitrile/H_2O (70:30), in 100 min with a flow rate of 1.0 ml/min.

Amino Acid Analysis. The individual proteins isolated by preparative gel electrophoresis were hydrolyzed at 110°C for 24 hr in 6 M HCl in evacuated sealed tubes. Analysis for tryptophan was performed after hydrolysis at 110°C for 24 hr in 2.5 M KOH/cystine, following performic acid oxidation; γ-carboxyglutamic acid was determined by P. A. Price (11). Amino acid analysis was performed on an amino acid analyzer (Beckman 119 C) equipped with a Spectra Physics 4000 data reduction system.

Amino Acid Sequences. The M_r 18,500 and M_r 17,500 components, further purified by hydrophobic column chromatography, were analyzed in the gas/liquid phase protein sequenator (12).

Bioassay. BMP activity was determined by implantation of isolated protein fractions and individual proteins in the hindquarter muscles of Swiss–Webster strain mice and skull trephine defects in monkeys, dogs, and rats.

RESULTS

Table 1 summarizes nine steps in the chemical extractions of BMP and associated proteins by differential precipitation and lists the yields of new bone from implants of the various protein fractions.

Changes in Solubility Produced by Ultrafiltration. A protein and protein aggregate with high BMP activity, obtained by step 8, are soluble only in 6–8 M urea or 4 M Gdn·HCl and insoluble in water. After large quantities of the M_r 14,000 component (but not all of the M_r 14,000 protein and some lower molecular weight proteins) were removed by ultrafiltration of a 6 M urea solution, the M_r 18,500, 17,500, and 17,000 proteins became soluble in water.

Triton X-100-Soluble Proteins. The Triton X-100-soluble, M_r 34,000 protein (isolated by step 9) was the single most abundant noncollagenous protein of the group lacking BMP activity. Specimens sent to J. D. Termine (5) for analytical

Table 1. Yield of BMP from bovine bone

Step	Fraction*	Weight, g	$M_r \times 10^{-3}$ of major electrophoretic components	Induced bone formation†		
				Incidence, %	*n*	Yield, mm^3/mg
1	Wet bone, fresh, pulverized	10.0‡	—	0	10	0
2	Dry, fat-free demineralized§	3.0‡	—	0	30	0
3	Bone matrix gelatin	1.4‡	—	48	40	0.5
4	0.5 M $CaCl_2$/6.0 M urea soluble, 0.25 M urea insoluble	60.0	—	78	50	1.5
5	0.5 M $CaCl_2$/6.0 M urea¶	22.0	18, 68, 45, 34, 17–18, 14, 12, 5	80	50	20
6	4 M Gdn·HCl soluble, 1.5 M Gdn·HCl soluble, water insoluble	7.23	45, 34, 24, 22, 18–17, 14, 12	88	30	2.5
7	1.5 M Gdn·HCl insoluble	12.0	34, 22, 17	8	20	0.5
8	0.5 M $CaCl_2$/6.0 M urea soluble‖	1.5	22, 12–18, 14, 12, 5	96	60	8.0
9	Dialyze supernatant against water and lyophilize	5.3	65, 45, 34, 22, 12	0	20	0

*BMP activity = 0.001% of wet bone.
†Implants in rump of athymic, cortisone-immunosuppressed, or untreated mice. *n* = number of implants.
‡Weight in kg.
§BMP activity = 0.003% of demineralized lyophilized fat-free bone matrix.
¶Dialyze against 0.25 M citrate buffer (pH 3.1). Wash precipitate in water, centrifuge, redefat in chloroform/methanol (1:1). Evaporate to dryness.
‖Dialyze against 0.2% Triton X-100/0.10 M Tris·HCl in 6 M urea (pH 7.2) for 24 hr. Then dialyze against deionized water until precipitation is complete.

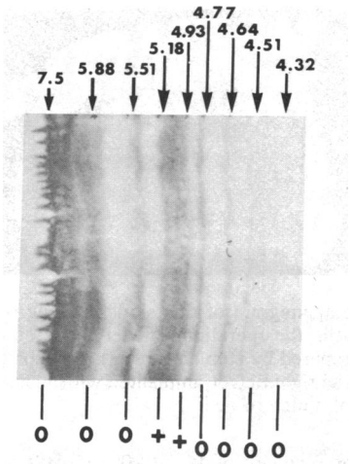


FIG. 1. Staining pattern of proteins fractionated by IEF. Estimates of the pI are indicated by the scale across the top. The results of bioassay of each fraction are shown by the symbols across the bottom. The two fractions with pI values ranging from 4.93 to 5.18 induced bone formation in the mouse's thigh muscle pouch; all of the fractions with pI values above 5.18 or below 4.93 induced formation of fibrous tissue only.

immunoprecipitation were found to crossreact with a M_r 32,000 protein named osteonectin.

Flatbed Isoelectric Focusing (IEF). Fig. 1 illustrates the results of fractionation of proteins obtained by step 8 (Table 1) by IEF. Two fractions with pI values of 4.9 and 5.3, collected by elution of the gel slices with 0.5 M $CaCl_2$/6 M urea, contained an aggregate of three proteins—i.e., M_r 22,000, 18,500 ± 500, and 14,000 components—and had BMP activity. Seven other fractions in the pI range of 4.3–7.5 had no M_r 18,500 component and no BMP activity.

Preparative Gel Electrophoresis. Slices of stained and unstained tube gels were extracted with 6 M urea to isolate the individual proteins and re-electrophoresed on slab gels. The M_r 24,000, 17,500, 17,000, and 14,000 components migrated the same distances as in the unsliced gels. The M_r 18,500 component also migrated the same distance but, in addition, produced three lower molecular weight components, M_r 14,000, 6,000, and 4,000.

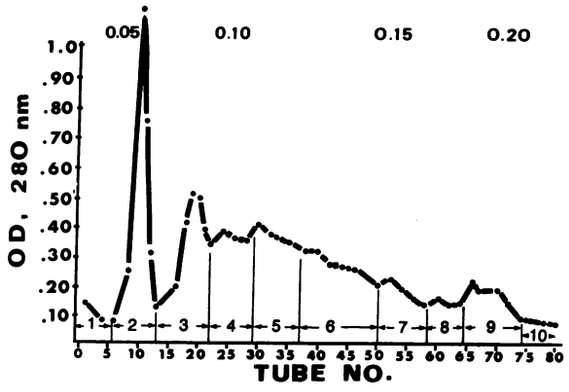
Hydroxyapatite Chromatography. Fig. 2 illustrates the absorbance patterns of various protein fractions eluted by a gradient of 0.05–0.3 M phosphate buffer at pH 7.3. Three proteins in the range of M_r 17,000–18,000 were separated from the M_r 22,000, 14,000, and other low molecular weight proteins by means of hydroxyapatite chromatography. A M_r 22,000 protein and other proteins lacking BMP activity were yellowish brown in color and were recovered in solutions of phosphate ions ranging from 0.05 to 0.10 M in concentration. Fractions containing components in the range of M_r 17,000–18,000 (with traces of M_r 14,000 and M_r 22,000 proteins) were generally eluted at concentrations of phosphate buffer of 0.18–0.2 M. By rechromatographing this fraction, it was possible to purify each of the three with variable degrees of contamination with M_r 14,000 and other low molecular weight proteins (Fig. 2 *Center* and *Right*). The protein with BMP activity was white in color and fluffy in texture and had an apparent M_r of 18,500 ± 500; the M_r 17,500, 17,000, and lower molecular weight proteins did not induce bone formation.

Amino Acid Analysis. The M_r 18,500 ± 500 BMP had the composition of an acidic polypeptide and may include one residue of hydroxyproline and possibly γ-carboxyglutamic acid (Table 2). Three γ-carboxyglutamic acid (Gla) residues were found in the M_r 14,000 protein, whereas none was detected in the M_r 22,000 or 34,000 proteins (13).

Hydrophobic HPLC. A protein with a M_r of ≈17,500 was isolated by hydrophobic gel HPLC. Isolated from the M_r 18,500 component, the M_r 17,500 protein failed to induce bone formation.

Amino Acid Sequence. Insofar as the NH_2-terminal amino acid was unblocked, the M_r 17,500 component was selected for amino acid sequence analysis. The first 25 amino acids appeared in the following sequence resembling that of histone H2B:

Pro-Glu-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro(10)
Lys-Lys-Gly-Ser-Lys-Lys-Ala-Val-Thr(Thr)(20)
Ala-Gln(Lys)(Lys)Asp(25)



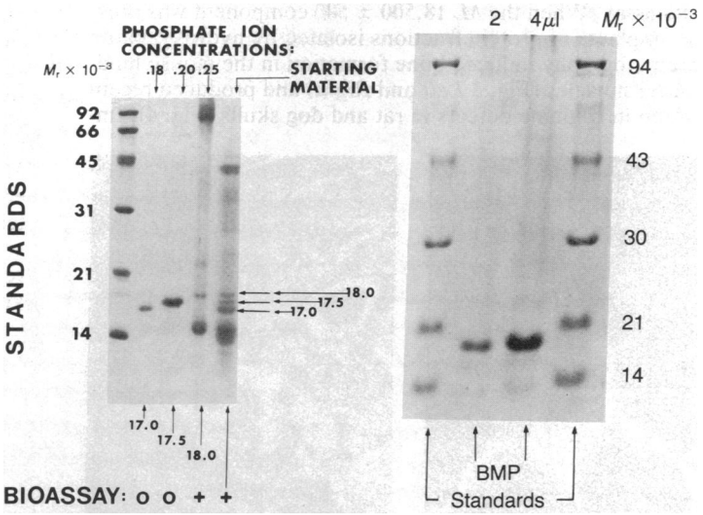


FIG. 2 (*Left*) Absorbance curve obtained by hydroxyapatite chromatography of 1.5 M Gdn·HCl-soluble, water-insoluble proteins (obtained in step 6, Table 1). The proteins with BMP activity were eluted at phosphate ion concentrations (shown across the top) ranging from 0.18 to 0.20 or 0.25 M (pH 7.3). (*Center*) Electrophoretic patterns of protein fractions obtained by hydroxyapatite chromatography. Protein fractions with apparent M_rs of 17,000, 17,500, and 18,000 were eluted at phosphate concentrations of 0.18, 0.20, and 0.25 M, respectively. Only samples containing the M_r 18,500 component (including the starting material and the fraction eluted at 0.25 M phosphate) induced bone formation. (*Right*) Electrophoretic patterns of a purified M_r 18,500 protein after removal of most of the M_r 14,000 protein by ultrafiltration. The M_r 18,500 protein was further purified by hydroxyapatite chromatography in 6 M urea and eluted at 0.22 M phosphate ion concentration. A small amount of M_r 14,000 protein is still observed in the overloaded sample.

Table 2. Amino acid analysis of M_r 18,500 ± 500 BMP

Residue	nmol	mol %	Residues/ 18,500*	Assumed residues	Weight
Asx†	23.7	10.5	17.6	18	2,071
Thr	9.20	4.1	6.89	7	708
Ser‡	22.5	10.0	16.8	17	1,480
Glx†	33.6	14.9	25.0	25	3,228
Pro	13.5	6.0	10.1	10	971
Gly	20.7	9.2	15.5	16	913
Ala	14.3	6.3	10.6	11	782
Val§	12.5	5.5	9.2	9	892
Met	2.53	1.1	1.85	2	262
Ile§	5.65	2.5	4.20	4	453
Leu	15.3	6.8	11.4	11	1,245
Tyr	8.39	3.7	6.22	6	979
Phe	8.62	3.8	6.38	6	883
Lys	8.62	3.8	6.38	6	769
His	4.29	1.9	3.19	3	411
Arg	16.1	7.1	11.9	12	1,874
Cys¶	6.32	2.8	4.70	5	515
Trp	ND	—	—	—	—
Total		100.0		168	18,436‖

ND, not determined.
*Calculations on these samples assume M_r = 18,500.
†Asx = Asp and Asn; Glx = Glu and Gln.
‡Ser values corrected by 10% due to destruction during acid hydrolysis.
§Val and Ile values may be low because only 24 hr of hydrolysis was used.
¶Half-cystine determined as cysteic acid after performic acid oxidation.
‖Plus possibly some γ-carboxyglutamic acid residues and one hydroxyproline residue.

The M_r 18,000 ± 500 putative BMP had a blocked NH_2-terminus.

Mercaptoethanol Reduction. No significant measurable differences in the electrophoretic mobility of the three components with M_rs in the range of 17,000–18,000 were noted before and after mercaptoethanol reduction. Nevertheless, BMP activity was destroyed by mercaptoethanol reduction.

Bioassay. When the M_r 18,500 ± 500 component was present, implants of protein fractions isolated by hydroxyapatite chromatography induced bone formation in the mouse hindquarter muscles (Fig. 3 *Left* and *Right*) and produced regeneration in trephine defects in rat and dog skulls (Fig. 4). Implants of 10 mg or more of M_r 14,000, 17,500, 17,000, 22,000, or 34,000 proteins, alone or in various combinations, failed to induce bone formation. Association of the M_r 18,500 protein with the M_r 14,000 protein decreased solubility *in vitro*, increased resorption time *in vivo*, and improved both the incidence and yield of new bone from implants of as little as 1 mg. Implants of combinations of M_r 18,500 protein with M_r 34,000 protein or M_r 34,000 protein and M_r 17,500 protein did not improve and may even have suppressed the bone formation. Implants of seven parts of M_r 22,000 protein, two parts of M_r 18,500 ± 500 protein, and one part of M_r 14,000 protein *weighing 3 mg* induced formation of bone consistently and in quantities exceeding the space the mouse thigh could contain (Fig. 5).

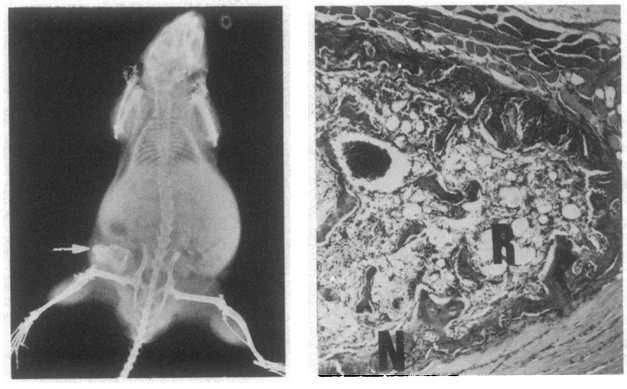


FIG. 3 (*Left*) Roentgenogram of a deposit of bone (arrow) 3 weeks after implantation of 5 mg of a M_r 18,500 ± 500 bovine BMP (bBMP) (isolated by hydroxyapatite chromatography) in a mouse thigh muscle. The contralateral thigh (control) was implanted with 5 mg of bovine albumin. (*Right*) Photomicrograph of a cross section of the deposit shown on the *Left*. N, new bone; R, bone marrow. (×100; hematoxylin/eosin and azure II stains.)

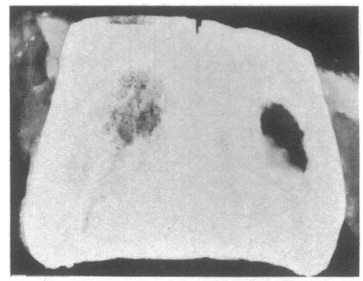


FIG. 4. Roentgenogram of dog skull showing two trephine defects, 8 weeks after the operation. The left defect, implanted with 50 mg of bBMP prepared by step 8 (Table 1), was completely repaired by new bone. The right defect, implanted with 50 mg of bovine albumin as a control, failed to heal.

DISCUSSION

Due to the densely packed bone matrix structure, the relatively small quantity compared with other noncollagenous proteins, and an inclination to form hydrophobic aggregates, BMP is difficult to isolate and purify. The first evidence that isolation was possible came when BMP was released from bone matrix gelatin by digestion with gelatinase, separated from the digest by coprecipitation with calcium phosphate (14), and proven to be a noncollagenous protein in nature. However, it was not until it was quantitatively extracted from bone matrix by means of a $CaCl_2$/urea solvent mixture and the hydrophobic low molecular weight proteins were solubilized and disassociated from each other in 4 M Gdn·HCl that BMP was isolated in significant quantities for detailed analysis (4, 5, 15). The combination of procedures reported here demonstrate: (*i*) three separate proteins with M_rs of 18,500, 17,500, and 17,000; (*ii*) three proteins of higher M_r (34,000, 24,000, and 22,000); and (*iii*) one or more proteins of lower M_r (14,000). One procedural improvement separates most of a M_r 34,000 protein, osteonectin (16) (representing about half of the total noncollagenous proteins), by virtue of its solubility in 0.2% Triton X-100; BMP is insoluble

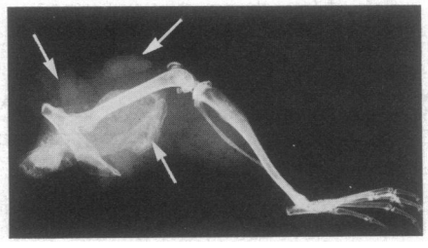


FIG. 5. Roentgenogram of large deposits of bone (arrows) overflowing the limbs of the mouse thigh in response to an implant of two parts of 18,500 protein, seven parts of M_r 22,000 protein, and one part of M_r 14,000 protein weighing a total of only 3 mg, 21 days after the operation.

in Triton X-100. Another improvement is in the use of ultrafiltration to separate the M_r 14,000 protein and other low molecular weight proteins that have a high affinity for BMP; by removal of large quantities of M_r 14,000 protein, BMP becomes soluble in water. Another improvement is obtained by the use of hydroxyapatite chromatography, which separates a M_r 18,500 BMP from a M_r 17,500 protein resembling histone H2B (identified from its NH_2-terminal amino acid sequence), from a M_r 17,000 protein (which may have calmodulin activity), and from a M_r 22,000 chromoprotein (possibly ferritin identified by immunoprecipitation from horse antiferritin). With the exception of the M_r 22,000 component, these proteins are generally contaminated with small quantities of M_r 14,000 protein or traces of M_r 6,000 and M_r 4,000 constituents. The M_r 22,000 protein is found in the unbound fraction and is relatively easy to purify. In contrast, the separation of the proteins of M_rs 18,500, 17,500, and 17,000 from each other and from other lower molecular weight proteins is arduous and is a painstaking process.

Preparative IEF indicates the pI of the M_r 18,500 molecule to be in the range of 4.9–5.1 but does not produce clean separation of bBMP from the M_r 17,500, 17,000, or 14,000 proteins. The M_r 17,500 histone H2B-like protein has no BMP activity. We have implanted 2 mg of histone H2B, prepared from Dunn osteosarcoma (6), a tissue with higher BMP activity than bone itself, and observed no BMP activity. Present evidence is against any protein other than the M_r 18,500 protein as inducing bone formation. Osteoinductive activity is extinguished from bone matrix by mercaptoethanol reduction (17). This observation suggests that the BMP is a disulfide-bonded molecule. Special investigations are required to determine the number of disulfide bonds and the position with respect to one or the other end of the molecule.

The affinity and capacity of BMP for calcium binding has not yet been measured but preliminary experiments indicate each to be on the order of serum proteins. However, Ca^{2+} alterations in the solubility of low molecular weight proteins associated with BMP are important and could influence the delivery of BMP to target cells. Upon further characterization, the M_r 14,000 protein is designated as matrix Gla protein (MGP); through formation of a MGP–Ca^{2+}–BMP aggregate, MGP could regulate local transfer of BMP from matrix to mesenchyme (13). BMP also adsorbs to bone mineral and coprecipitates with calcium phosphate, but it is completely recovered by extraction of the mineral with 4 M Gdn·HCl. A summary of physicochemical properties of BMP prepared from experiments previously and presently performed by our research group follows: (*i*) acidic polypeptide; (*ii*) apparent M_r = 18,000 ± 500 and pI = 5.0 ± 0.2; (*iii*) binds to hydroxyapatite; (*iv*) no carbohydrate detected; (*v*) soluble in neutral salt solution at pH 7.2; (*vi*) degraded by acid alcohol solutions; alkali sensitive; (*vii*) insolubilized by forming aggregates with M_r 14,000 proteins; (*viii*) may contain one residue of hydroxyproline; (*ix*) insoluble in chloroform/methanol, absolute alcohol, and acetone; (*x*) BMP M_r 14,000 complex: insoluble in Triton X-100 (non-ionic detergent); insoluble in 0.6 M HCl; soluble in 6 M urea or 4 M Gdn·HCl; soluble in 0.1% $NaDodSO_4$; soluble in 0.02% M HCl; partially soluble in ethylene glycol; (*xi*) nuclease (RNase and DNase) resistant; (*xii*) trypsin and chymotrypsin labile; (*xiii*) resistant to: chondroitinases A, B, and C, amylase, neuraminidase, hyaluronidase, alkaline phosphatase, acid phosphatase, chymopapain, collagenase, tyrosinase, and thermolysin.

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