Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2213–2217, April 1984
Medical Sciences

Osteonectin, bone proteoglycan, and phosphophoryn defects in a form of bovine osteogenesis imperfecta

(congenital bone disease/noncollagenous matrix proteins)

J. D. TERMINE*, P. GEHRON ROBEY*†, L. W. FISHER*, H. SHIMOKAWA*, M. A. DRUM*, K. M. CONN*, G. R. HAWKINS*, J. B. CRUZ‡, AND K. G. THOMPSON‡

*Mineralized Tissue Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20205; and ‡Department of Veterinary Pathology, College of Veterinary Medicine, Texas A & M University, College Station, TX 77843

Communicated by Elizabeth F. Neufeld, December 22, 1983

ABSTRACT **Bovine osteogenesis imperfecta is a congenital disease in Holstein cattle having several characteristics in common with human osteogenesis imperfecta syndromes. In particular, affected calves have multiple bone fractures and friable teeth. Bone collagen isolated from the affected animals (Texas variant) showed slightly decreased α1(I) and α2(I) chain electrophoretic mobility and increased hydroxylysine content. Overall, collagen was present in the affected bones at 80–90% of normal values. However, osteonectin, a 32,000 M_r bone-specific protein found previously to promote collagen mineralization *in vitro* and present in abundance ($\approx$3% of total protein) in normal calf bone, was severely depleted (<2% of normal levels) in the osteogenesis imperfecta bone and dentin. The bone proteoglycan was similarly depleted. In contrast, the bone sialoprotein was not as severely affected. Further, the diseased teeth lacked (<10% of normal values) phosphophoryn, a dentin-specific protein normally present as 4–5% of the total calf dentin matrix. The data suggest multiple hard tissue matrix protein deletions, perhaps due to impaired cell development.**

Osteogenesis imperfecta (OI) is an inherited disease of human bone that is heterogeneous in expression with degrees of severity ranging from a perinatal lethal form to milder variants with minimal clinical manifestations (1, 2). Affected individuals usually show bone fragility either with or without concomitant growth deformities and can be classified as nondeforming or progressively deforming occurring from either dominant or recessive inheritance (3). The disease may also be accompanied by fragile, opalescent teeth [dentinogenesis imperfecta (DI)], blue sclerae, and joint laxity (1). OI collagens are often present at lower levels than normal, with apparently decreased fibril diameters (4, 5) and increased hydroxylysine content (6). Analyses of cultures of skin biopsies from OI patients have shown a wide range of collagen abnormalities (7, 8) with the most severely affected individuals (perinatal lethal form) having structural defects in type I collagen (9–13). However, the type and extent of the skin collagen abnormalities reported for other forms of OI in surviving individuals do not universally reflect the severity or extent of the underlying bone disease.

Until recently, no animal model for OI had been studied, although the disease had been reported occasionally in bovine and ovine offspring (14–16). The bovine form of the disease has been described in offspring of an Australian bull (17). More recently, a Texas bull has sired affected calves. The latter affected calves have multiple intrauterine or postnatal fractures (or both) of ribs, limbs, and mandibles. The ultrastructure of the bones is hypercellular and porous with characteristics of woven rather than lamellar bone. The osteoblasts were generally found to have dilated rough endoplasmic reticulum with no preferential orientation with respect to the mineralization front (unpublished data). In both cases, all affected calves have DI with markedly reduced dentin including few and irregularly arranged tubules (17). Joint laxity is common to all affected animals, whereas deep blue sclerae have been more predominant in offspring from the Texas sire (unpublished data). Dominant inheritance is likely for both models based on crossbreeding experiments, but disease frequency is higher (44% of progeny) in the Australian than in the Texan variety (50% of progeny). The Texas offspring appear to have the more severe osteopenia.

Previously, we established that several noncollagenous matrix proteins are apparently specific to bone tissue. These include osteonectin (18, 19), the bone proteoglycan (20), and the bone sialoprotein (21). Osteonectin, a 32,000 M_r bone glycoprotein, was shown to bind selectively to both hydroxyapatite and collagen. This protein also promoted mineral deposition onto type I collagen *in vitro* (19). Antibodies to these bone proteins were used to demonstrate their tissue specificity and matrix localization (19–21) and biosynthetic studies have shown them to be products of osteoblast-like cells (unpublished data). In this study, we examined bone and dentin from the Texas-bred OI animals and report specific alterations in the noncollagenous proteins of these affected tissues.

MATERIALS AND METHODS

Tissue Preparation. Affected offspring were obtained from artificially inseminated Holstein and Angus cows. Affected calves were identified at birth and sacrificed within 18 days. Subperiosteal and central cortical bone was dissected from long bones and mandibles of (*i*) OI Holstein–Holstein and Holstein–Angus calves and (*ii*) their normal, age-matched half-siblings or siblings. The dissected bone was washed extensively with phosphate-buffered saline containing protease inhibitors (22). Aliquots of the washed bone were lyophilized. Bone ash was determined after heating at 600°C for 24 hr. Dentin matrices were prepared from unerupted deciduous molar teeth as described (22).

Characterization of Collagen. Lyophilized bone fragments were powdered manually under liquid nitrogen and demineralized by dialysis against 10% acetic acid. Duplicate portions of the total demineralized bone from all calves were hydrolyzed in 6 M HCl at 110°C (24 hr) and amino acids were quantitated by automated methods (22). Collagen was solubilized sequentially from the demineralized bone first with 0.5 M

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.

Abbreviations: OI, osteogenesis imperfecta; DI, dentinogenesis imperfecta.

†To whom reprint requests should be addressed.

acetic acid and then by pepsin digestion (1 mg of pepsin to 20 mg of demineralized bone) at 4°C for 24 hr. Amino acid compositions of the purified collagens (in duplicate) were obtained as described above.

Extraction and Fractionation of Noncollagenous Proteins. Freshly dissected, washed hard tissue fragments were extracted sequentially first with 4 M guanidine·HCl/0.05 M Tris·HCl, pH 7.4, containing protease inhibitors (G extract) and then with an identical solution containing 0.5 M EDTA (E extract) as described (18). Extracts were concentrated by ultrafiltration (Amicon YM 5 or 10 filters). Aliquots were desalted by Bio-Gel P-2 (Bio-Rad) chromatography in 0.1 M formic acid and then lyophilized. The remainder of the bone extracts were chromatographed on tandem Sepharose CL-6B (Pharmacia) in 4 M guanidine·HCl as described (18). Corollary extracts of the dentinal matrices were chromatographed on DEAE-Sephacel (Pharmacia) in 7 M urea as described (23).

Analytical Methods. Aliquots of G and E extracts were electrophoresed on $NaDodSO_4$ gradient (4–20%) polyacrylamide slab gels as described by Laemmli (24) with only minor modifications (20). Five percent polyacrylamide slab gels for collagen analyses were used exactly as described by Laemmli (24). Quantitation of osteonectin, bone proteoglycan, and bone sialoprotein was performed by ELISA as described (19–21). Immunodetection of these bone-specific proteins after transfer by electroblotting onto nitrocellulose paper was performed by a modification of the method of Towbin *et al.* (25) as described (21). Further, 150-μg samples of extracts were prepared for bone proteoglycan immunodetection (electrophoretic transfer blot) after digestion of constituent glycosaminoglycans with 10 mU of chondroitinase ABC (Miles) for 1 hr at 37°C (20). (Intact proteoglycans do not always electroblot efficiently under standard conditions, whereas their core proteins transfer normally.)

RESULTS

The OI bones contained as much mineral as normal bones based on their ash contents (Table 1). This is not unexpected because woven and lamellar bones are known to be equal in mineral content (26). The OI bones were exclusively woven in structure and all woven bone is reported to mineralize via matrix vesicles in interfibrillar spaces (26). The normal specimens consisted entirely of lamellar bone in which mineralization occurs primarily within the collagen fibrils (26). However, the OI bones apparently contained less protein (Table 1). Amino acid analysis of the total demineralized bone protein showed that the OI bone contained slightly less collagen (6–10%) than normal, as indicated by reduced quantities of hydroxyproline, proline, and glycine (Table 1). Amino acid compositions from the purified normal and OI bone collagens (solubilized by pepsin digestion) were identical except that the OI samples contained more hydroxylysine and concomitantly less lysine (Table 1). The acetic acid- and pepsin-soluble bone collagens were electrophoresed on 5% $NaDodSO_4$/polyacrylamide slab gels (Fig. 1). The acid-soluble bone collagen migrated slightly slower than the standard,

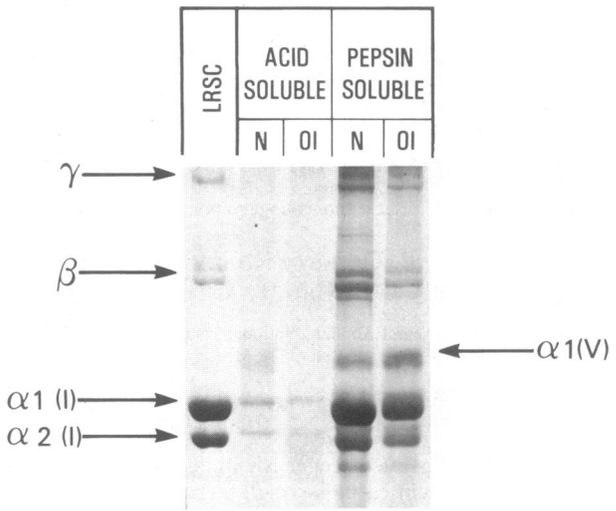

FIG. 1. 5% $NaDodSO_4$/polyacrylamide gel electrophoresis of normal (N) and OI bone collagens. Collagen liberated by acetic acid extraction or pepsin digestion of demineralized bone was electrophoresed in comparison with standard lathyritic rat skin collagen (LRSC). The bovine bone collagen migrated slightly slower than LRSC, and the α chains of the OI bone collagen had a lower mobility than those of normal bone collagen. Treatment of the demineralized bone with pepsin also liberated type V collagen as indicated by the presence of α1(V).

acid-soluble, lathrytic rat skin collagen. The OI calf bone collagens migrated somewhat slower than those from normal calf bone, probably due to their increased lysine hydroxylation (27). No type III collagen was detected in any OI or normal bone sample upon treatment with delayed reduction by dithiothreitol (data not shown). After pepsin digestion of total OI or normal demineralized bone, small amounts of type V collagen were observed (Fig. 1).

It was shown previously that sequential E extracts (guanidine·HCl/EDTA) are enriched in the bone-specific noncollagenous proteins (18, 20, 21). The extracts from femurs of both normal and age-matched half-siblings with bovine OI were chromatographed on molecular sieve columns. Typical elution profiles are seen in Fig. 2. Although the level of the serum-derived α_2HS glycoprotein appeared to have increased in the OI bone (also see Fig. 3), the most striking difference in the two profiles was a marked decrease in the amount of protein eluting in the osteonectin region. Although a slight decrease was seen in the column elution region of the 24,000 M_r bone phosphoprotein, this protein did not appear altered on $NaDodSO_4$ gels (Fig. 3). It should be noted that some bone matrix components (e.g., bone proteoglycan) do not have significant absorbance at 280 nm. Osteocalcin (a bone gla-containing protein) absorbance was variable from specimen to specimen.

The absence of osteonectin was confirmed on reduced, $NaDodSO_4$/polyacrylamide gradient gels as shown in Fig. 3. Osteonectin, which migrates in this particular gradient gel

Table 1. Compositional analysis of normal and OI bovine bone

Bone	*n**	Ash content, %	Protein content, %†	Amino acid composition, residues per 1000 residues						
				Demineralized bone				Collagen		
				Hyp	Pro	Gly	Hyp + Pro	Hyl	Lys	Hyl/(Hyl + Lys)
Normal	2	63 ± 2	26 ± 3	114 ± 1	119 ± 1	305 ± 1	233	12 ± 2	24 ± 1	0.33
OI	3	66 ± 2	15 ± 3	103 ± 2	106 ± 2	285 ± 2	209	17 ± 1	20 ± 2	0.46
OI/normal		1.05	0.58	0.90	—	0.93	0.94	—	—	1.39

Where indicated, values are expressed as mean ± SD.
*n = Number of calves studied.
†Nondialyzable material remaining after demineralization.

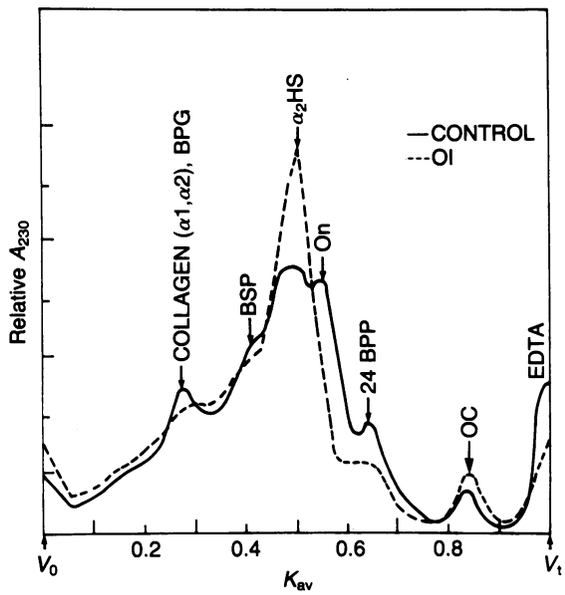

FIG. 2. Elution profiles of the E extracts from OI and half-sibling control, midshaft cortical bone chromatographed on Sepharose CL-6B columns in 4 M guanidine·HCl. Typical peak positions of authentic bone proteins are depicted for α1 and α2 collagens, bone proteoglycan (BPG), α_2HS glycoprotein (α_2HS), osteonectin (ON), 24,000 M_r bone phosphoprotein (BPP), bone sialoprotein (BSP), and osteocalcin (OC).

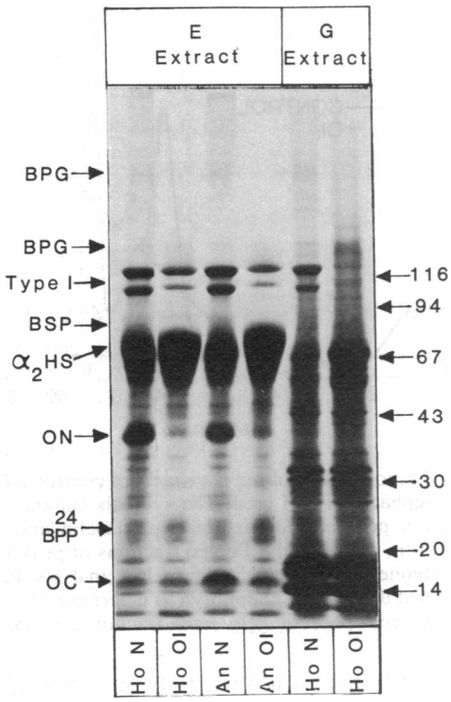

FIG. 3. NaDodSO$_4$ gradient (4–20%) polyacrylamide gels of normal (N) and OI cortical bone extracts. The initial guanidine·HCl (G) and the subsequent guanidine·HCl/EDTA (E) extracts were co-stained with Coomassie and Alcian blue. Band positions of authentic bone proteins (20) are shown for type I α1 (upper) and α2 (lower) collagen chains, bone proteoglycan (BPG), bone sialoprotein (BSP), α_2HS glycoprotein (α_2HS), osteonectin (ON), 24,000 M_r bone phosphoprotein (BPP), and osteocalcin (OC). The molecular weight standards (shown as $M_r \times 10^{-3}$) include (top to bottom): β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin. Ho, Holstein–Holstein; An, Holstein–Angus.

system as a 40,000 M_r species, was prominent in the E extract of both the Holstein–Holstein and the Holstein–Angus normal bones but was apparently missing in the E extracts from bones of their OI half-siblings. These results were consistent for the four Texas OI calves examined to date. Immunoreplicas (electrophoretic transfer blots) of these NaDodSO$_4$ gels showed that detectable amounts of osteonectin were still present in the OI bones but at a drastically reduced level (Fig. 4). After isoelectric focusing, these same extracts were electrotransferred onto nitrocellulose. Immunodetection of osteonectin in these replicas yielded identical doublet bands for both the normal calf osteonectin and the small amount of osteonectin present in the OI extracts, suggesting that the reactive OI protein was identical to normal osteonectin (data not shown).

The G extracts from both the normal and affected bone specimens were electrophoresed to determine whether the osteonectin missing from the OI bone E extracts was removed in the initial (G) extractions. Fig. 3 shows that the G-extract patterns were very similar, with no excess staining in the osteonectin region. In addition, electrophoretic transfer blots (immunoreplicas) of these gels actually showed a decrease in the small amounts of osteonectin released in the OI G extract relative to normal (Fig. 4), thus confirming the results seen for the E-extract proteins.

The amount of osteonectin missing from the OI bone matrices was quantitated by using competition ELISA analysis. Osteonectin levels in the total bone extracts (G + E) from the four affected calves were 1.2% (±0.9%) of the levels found in their normal, age-matched half-siblings. The bone proteoglycan was also affected significantly in the OI calves. Although alcian blue-staining bone proteoglycan bands (20) were observed in NaDodSO$_4$ gels of OI bone extracts, the intensity relative to normal was markedly diminished (Fig. 3). Immunodetection of the electroblotted proteoglycan core protein (Fig. 4) further demonstrated the marked decrease in the bone proteoglycan content found in the OI bone extracts. Competition ELISA analysis showed that OI bone extracts (G + E) contained only 6.5% (±2.7%) of the bone proteoglycan levels found in total bone extracts from the normal controls. The bone sialoprotein appeared less severely affected in the bovine OI tissues on gel filtration (Fig. 2) and on NaDodSO$_4$ gels (Fig. 3). Competition ELISA analysis of the total OI bone extracts (G + E) showed that bone sialoprotein levels were 48.9% (±2.7%) of normal.

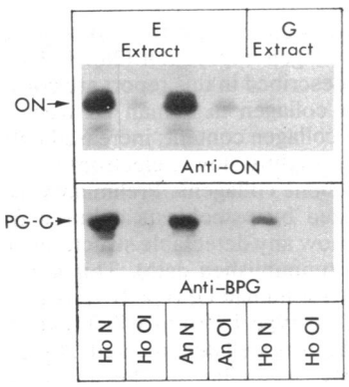

FIG. 4. Horseradish peroxidase-conjugated indirect immunodetection of osteonectin (ON) and the bone proteoglycan (BPG) core protein (PG-C, obtained after removal of glycosaminoglycan chains with chondroitinase ABC) on nitrocellulose electroblots of NaDodSO$_4$ gels containing both normal (N) and OI cortical bone extracts. Note the positive but decreased response in the lanes containing the OI samples. Arrows indicate the locations of authentic osteonectin and bone proteoglycan core protein. Ho and An, as in legend to Fig. 3.

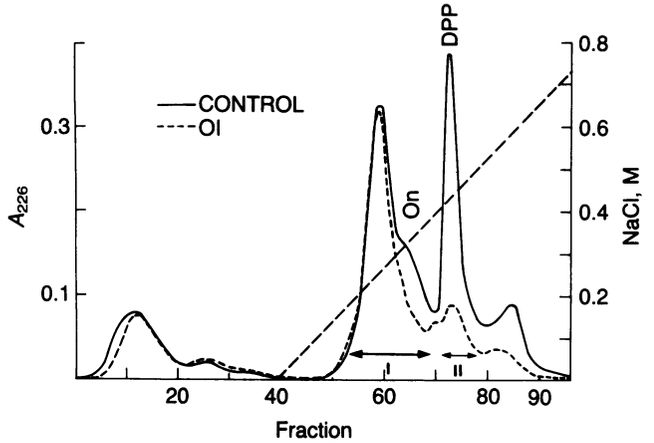

FIG. 5. Elution profile of the E extracts of control and OI dentin on DEAE-Sephacel in 7 M urea. The symbols On and DPP locate typical elution positions for dentinal osteonectin and the dentin phosphophoryn, respectively. Pooled fractions of peak I were analyzed for osteonectin-like proteins by ELISA analysis. Peak II fractions contained dentin phosphophoryn as determined by amino acid analysis and electrophoretic gels. Each fraction contained 1.3 ml.

The affected animals in this study all exhibited DI. The enamel protein distribution and content in the affected animals was found to be normal and was not studied further. The normal and affected dentin extracts (processed sequentially in a manner identical to that of the cortical bone) were chromatographed on DEAE-Sephacel as shown in Fig. 5. The DI profiles showed a marked decrease in dentin phosphophoryn, the major noncollagenous protein of dentin (Fig. 5, peak II). This was confirmed on amino acid analysis (dentin phosphophoryn contains serine and aspartate as 80% of total residues; ref. 23) and on $NaDodSO_4$ gel electrophoresis using Stains All (Bio-Rad) detection (data not shown). Normal dentin also contains an osteonectin-like protein that elutes in the peak I position of Fig. 5, a region also depressed in the DI extracts. This was confirmed by competition ELISA analyses. The osteonectin-like protein of dentin is at least 50% crossreactive with osteonectin (unpublished data). Peak I of the DI dentin had <1% of osteonectin-equivalent activity relative to the same fractions from normal controls.

DISCUSSION

Human OI has been thought to involve genetic alterations primarily in collagen expression (28). Indeed, many of the collagen data described in this report are consistent with earlier studies of collagen in human OI tissue, including decreased type I collagen content, increased hydroxylysine/lysine ratios, and slightly slower electrophoretic mobility relative to normal bone collagens. Preliminary data on procollagens synthesized by osteoblasts cultured from bovine OI bone did not show any detectable structural differences from control tissue (unpublished data). This is in contrast to results described for human OI type II variants (9, 11–13) and a single case of OI type I (10) in which defective collagen α chain biosynthesis is well documented (28). Thus, the bovine form of OI may be similar to other forms of human OI in which collagen structural gene abnormalities are not well understood (29).

This study demonstrates that several noncollagenous calf bone matrix proteins, each of which also exists in human bone, are severely depleted in bovine OI bone and dentin. The OI bone samples contained only 1.2% of the osteonectin and 6.5% of the proteoglycan found in normal tissue, whereas the sialoprotein was less affected (48.9% of normal). In the DI dentin, phosphophoryn and the osteonectin-like protein were each <10% of normal. Dentin phosphophoryn, a 100,000 M_r noncollagenous matrix protein accounting for 4–5% of the total protein in developing calf dentin (23), is characteristic of normal (tubular) dentin in all species studied. The phosphophoryn deficiency in human DI has been suggested earlier (30).

It is not clear, at present, how these three proteins are linked in this disease. Nevertheless, it is obvious that these are the major hard tissue proteins affected in the bovine OI syndrome and thus are significant manifestations of the disease. Although there was no biochemical evidence of matrix degradation, biosynthetic studies are needed to decide whether the osteonectin, bone proteoglycan, and phosphophoryn deficiencies noted reflect synthetic or degradative phenomena. However, in view of the apparent specificity of these deletions (α_2HS glycoprotein, for example is not degraded), synthetic defects are suspected.

One interesting hypothesis that may explain the data is that the abnormal bovine OI tissues may reflect arrested development. The overall OI bone morphology, for example, is that of primitive, woven bone. Embryonic collagens generally have increased lysine hydroxylation (27, 31) and small-diameter collagen fibrils similar to those seen for OI bone (4, 5), and it is possible that these changes might affect subsequent interaction with other bone proteins. However, only a slightly decreased osteonectin content was observed for the woven bone of early fetal calves (unpublished data). In an analogous fashion, dentin phosphophoryn is a normal constituent of mature, tubular dentin. It is not certain whether this protein is equally distributed in the atubular, mantle dentin formed earlier in tooth germ development. Thus, the hard tissue aspects of this disease may represent a failure of the osteoblast to mature and produce normal lamellar bone and of the odontoblast to produce mature dentin. Because more than one tissue system is clinically affected (e.g., bone, dentin, sclera), it is possible that a defect in a circulating factor(s) or its receptor(s) is responsible for differentiation or maturation (or both) of these diverse tissues and may underlie the observed abnormalities. In any event, the findings described above present new challenges in the evaluation of this inherited bone disease syndrome.

L.W.F. was supported by a grant-in-aid from the Shriner's Hospitals for Crippled Children (St. Louis Unit).

1. McKusick, V. A. (1972) in *Symposium on Heritable Disorders of Connective Tissue* (Mosby, St. Louis, MO), pp. 390–454.
2. Pinnell, R. S. & Murad, S. (1983) in *The Metabolic Basis of Inherited Diseases*, eds. Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L. & Brown, M. S. (McGraw-Hill, New York), pp. 1425–1449.
3. Sillence, D. (1981) *Clin. Orthop. Relat. Res.* **156,** 11–25.
4. Bailey, A. J. & Robins, S. P. (1973) in *Biology of the Fibroblast*, eds. Kulonen, E. & Pikkarainen, J. (Academic, New York), pp. 385–395.
5. Light, N. D. & Bailey, A. J. (1978) in *Biology of Collagen*, ed. Viidik, A. & Vuust, J. (Academic, San Francisco), pp. 15–38.
6. Trelstad, R. L., Rubin, D. & Gross, J. (1977) *Lab. Invest.* **36,** 501–508.
7. Rowe, D. W., Poirer, M. & Shapiro, J. R. (1981) *Dev. Biochem.* **22,** 155–162.
8. Spranger, J. (1982) *Prog. Clin. Biol. Res.* **104,** 223–233.
9. Barsh, G. S. & Byers, P. H. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5142–5146.
10. Byers, P. H., Shapiro, J. R., Rowe, D. W., David, K. E. & Holbrook, K. A. (1983) *J. Clin. Invest.* **71,** 689–697.
11. Williams, C. J. & Prockop, D. J. (1983) *J. Biol. Chem.* **258,** 5915–5921.
12. de Wet, W. J., Pihlajaniemi, T., Myers, J., Kelly, T. E. & Prockop, D. J. (1983) *J. Biol. Chem.* **258,** 7721–7728.
13. Chu, M.-L., Williams, C. J., Pepe, G., Hirsch, J. L., Prockop, D. J. & Ramirez, F. (1983) *Nature (London)* **304,** 78–80.

14. Holmes, J. R., Baker, J. R. & Davies, E. T. (1964) *Vet. Rec.* **76,** 980–984.
15. Kater, J. C., Hartley, W. J. & Dysart, T. H. (1963) *N. Z. Vet. J.* **11,** 41–46.
16. Jensen, D. T., Rasmussen, P. G. & Basse, A. (1976) *Nord. Veterinaermed.* **28,** 304–308.
17. Denholm, L. J. & Cole, W. G. (1983) *Aust. Vet. J.* **60,** 9–17.
18. Termine, J. D., Belcourt, A. B., Conn, K. M. & Kleinman, H. K. (1981) *J. Biol. Chem.* **256,** 10403–10408.
19. Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., McGarvey, M. L. & Martin, G. R. (1981) *Cell* **26,** 99–105.
20. Fisher, L. W., Termine, J. D., Dejter, S. W., Whitson, S. W., Yanagishita, M., Kimura, J. H., Hascall, V. C., Hassell, J. R. & Nilsson, B. (1983) *J. Biol. Chem.* **258,** 6588–6594.
21. Fisher, L. W., Whitson, S. W., Avioli, L. V. & Termine, J. D. (1983) *J. Biol. Chem.* **258,** 12723–12727.
22. Termine, J. D., Belcourt, A. B., Christner, P. J., Conn, K. M. & Nylen, M. U. (1980) *J. Biol. Chem.* **255,** 9760–9768.
23. Termine, J. D., Belcourt, A. B., Miyamoto, M. S. & Conn, K. M. (1980) *J. Biol. Chem.* **255,** 9769–9772.
24. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
26. Bernard, G. & Marvaso, V. (1981) in *Matrix Vesicles*, eds. Asconzi, A., Bonucci, E. & de Bernard, B. (Wichtig Editore, Milan, Italy) p. 1.
27. Crouch, E. & Bornstein, P. (1978) *Biochemistry* **17,** 5499–5509.
28. Eyre, D. R. (1981) *Clin. Orthop. Relat. Res.* **159,** 97–107.
29. Rowe, D. W. & Shapiro, J. R. (1982) in *Symposium on Heritable Disorders of Connective Tissue*, eds. Acheson, W., Bornstein, P. & Glimcher, M. (Mosby, St. Louis, MO), pp. 269–282.
30. Takagi, Y. & Veis, A. (1981) in *The Biochemistry and Biology of Mineralized Connective Tissues*, ed. Veis, A. (Elsevier/North-Holland, New York), pp. 233–243.
31. Kirsh, E., Krieg, T., Remberger, K., Fendel, H., Bruckner, P. & Muller, P. K. (1981) *Eur. J. Clin. Invest.* **11,** 39–47.