bond may possibly be regulated by the neighboring amino acid sequence, thereby explaining the preservation of this sequence in the different species. The mechanism for variations in the amino terminal sequence cannot be explained by our data. Perhaps there is more than one kappa chain cistron resulting from reduplication. Independently occurring mutations in several of these cistrons could be transmitted in the germ line and account for the observed amino acid differences. Regarding stability and economy of genetic material one might ask if some mechanism allows a common genetic message to be translated into different polypeptide chain products (*1*).

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Protein-Polysaccharide Loss during Endochondral Ossification: Immunochemical Evidence

Abstract. *Fluorescein-labeled antibodies to protein-polysaccharides from rat and calf cartilage were used for the histochemical localization of protein-polysaccharide in epiphyseal cartilage. There was less protein-polysaccharide in the zone of provisional calcification than in the proliferating and maturing zones, and none was demonstrable in the metaphysis. During or just preceding calcification, protein-polysaccharide or its protein component is lost or drastically altered.*

The anionic polysaccharides of cartilage are, for the most part, covalently linked to protein other than collagen (*1*); and they can be extracted with water from cartilages by high-speed homogenization and subsequently separated by differential centrifugation into, at least, a heavy fraction (PP-H) and a light fraction (PP-L). From its behavior in the ultracentrifuge, the light fraction may be relatively homogeneous, although polydisperse (*2, 3*).

During endochondral ossification, much of the polysaccharide is eliminated; analytical and autoradiographic data (*4, 5*) indicate that only 20 to 25 percent of the sulfate in the cartilage persists in the metaphysis. This significant change in sulfate concentration of the organic matrix may be related to the process of calcification (*4, 6*). In large measure, the sulfate in the metaphysis is part of material akin to chondroitin sulfate (*7*). Histological evidence of changes in the metachromatic properites of cartilage matrix in the hypertrophic zone (*8*) also suggests an alteration in the nature or state of combination of the mucopolysaccharides related to calcification. Extraction of protein-polysaccharides resembling those in cartilage has been unsuccessful when techniques applicable to cartilage were used on fresh or decalcified samples of metaphyseal bone (*9*). Possibly, in the process of endochondral ossification the protein in the protein-polysaccharides may be modified or eliminated. Indeed, there is a protease in cartilages which, in vitro, snips away about 25 percent of the protein in PP-L. This product is much more soluble than the PP-L from which it is derived. Conceivably, such a modification of PP-L may be an essential step for its elimination from cartilage or for further modifications leading to its partial retention in the developing metaphyseal trabeculae.

Precipitating antibodies have not been produced in animals injected with chondroitin sulfate or hyaluronate (*10*), but precipitating antibodies against PP-L from various cartilages (*11, 12*) and against hyaluronate-protein (*13*) have been produced in rabbits and guinea pigs. Therefore, the associated proteins seem to be necessary for antigenicity. The precipitin reaction of PP-L or of hyaluronate-protein with its antiserum is eliminated by prior treatment of the antigen with some proteolytic enzymes, but the reaction with antiserum proceeds after similar treatment with testicular hyaluronidase (*12–14*).

Accordingly, we have used the fluorescent antibody technique to test whether the concentration of PP-L decreases concomitantly with the calcification of the matrix of growth cartilages, and whether PP-L is present in the metaphyses.

Protein-polysaccharides were extracted into water by high-speed homogenization of costal cartilage of calves and of epiphyseal cartilage of young rats (12 to 14 days old) (*15*) and further purified (*2*). The sample of PP-L from the costal cartilage of calves decreased in weight by 12.11 percent

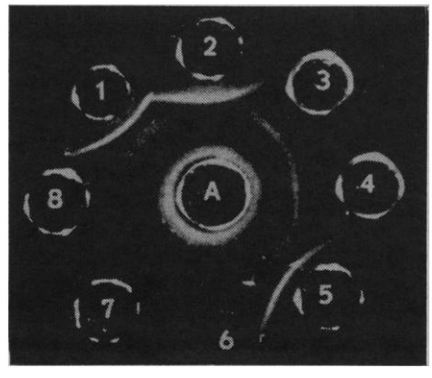

Fig. 1. Effect of incubation with hyaluronidase on PP-L. Well A, bovine antiserum to PP-L; wells 1 and 5, bovine PP-L incubated with hyaluronidase; well 2, bovine PP-L; well 3, rat PP-L; well 4, rat PP-L incubated with hyaluronidase; well 6, hyaluronidase alone; wells 7 and 8 not used.

when dried at 78°C for 3 hours at reduced pressure. It contained (*3*) uronic acid, 27.9 percent; hexosamines, 23.7 percent; sulfur, 5.1 percent; and nitrogen, 4.8 percent. The sample weighed 22.7 percent of its initial dry weight after it was ashed.

The homogeneity of the samples of PP-L used as antigens was checked by chromatography and electrophoresis. Electrophoresis of PP-L on cellulose acetate strips (0.025*M* sodium phosphate buffer of *p*H 7.4) demonstrated, on staining with toluidine blue (*16*), a major component at the origin and a minor component which migrated towards the anode. Chromatography on sheets of Whatman No. 1 paper with a mixture of 2-propanol and 0.04*M* ammonium formate–formic acid at *p*H 4.5 (45 : 55) (*17*) showed four metachromatic components—toluidine blue staining—of which two were present only in very small amount. Samples prepared elsewhere were comparably inhomogenous.

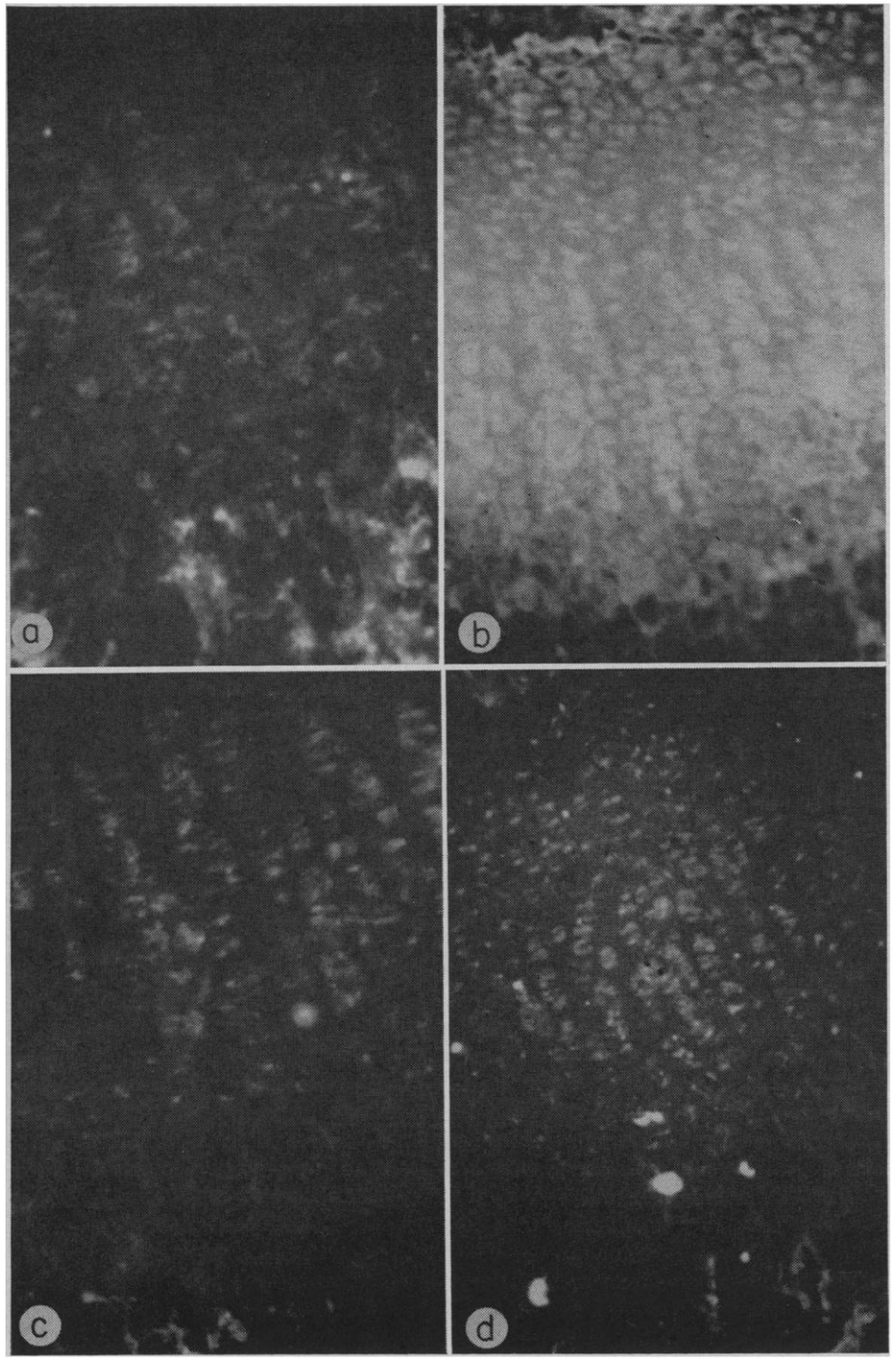

Fig. 2. Immunofluorescence studies of rat epiphyseal cartilage. Sections (8 μ) are fresh proximal ends of tibiae. (a) Section incubated in buffered saline only, to demonstrate intrinsic fluorescence; (b) incubation in fluorescein-labeled specific globulin, showing bright fluorescence in epiphyseal cartilage, although intensity is decreased approaching or in the zone of provisional calcification (or both); (c) preliminary incubation with unlabeled specific globulin, followed by incubation with labeled specific globulin. Decreased fluorescence as compared with section b illustrates the blocking reaction confirming specificity; (d) incubation with fluorescein-labeled nonspecific globulin demonstrating nonspecific staining (35-mm Kodak daylight high-speed Ektachrome film).

A solution of PP-L (5 to 10 mg in 1 ml of 0.8 percent sodium chloride) was mixed with an equal volume of Freund's adjuvant and injected intramuscularly into rabbits (2 to 3 kg) at intervals of 1 week to 1 month. One week after each injection the serums were examined for antibodies by the double diffusion method of Ouchterlony (*18*). The precipitin lines formed slowly, especially in the case of the PP-L from bovine costal cartilage and its antiserum; they could best be seen with oblique or dark-field illumination. The antiserums to calf PP-L also reacted with calf serum and the antiserums to rat PP-L reacted with rat serum. These interfering antibodies were removed by addition of small amounts of calf or rat serum respectively until no further visible precipitation occurred. The resulting antiserums were much more specific towards PP-L, but their potency was decreased.

Our preparation of bovine PP-L (10 to 20 mg/ml) and those donated by others gave three precipitin lines with the antiserums to bovine PP-L, even after absorption with normal calf serum (Fig. 1). This is in general agreement with the results of Loewi and Muir (*12*). Similar results were obtained when the PP-L from rat epiphyses was diffused against its antiserum, but the precipitin lines appeared more rapidly and were more intense.

To characterize the antigen-antibody system further, the effects of hyaluronidase and proteolytic enzymes on the antigens were studied. Bovine or rat PP-L (2 mg) was incubated with 0.2 mg of hyaluronidase (*19*) for 3 hours at 37°C in 0.04*M* acetate buffer, *p*H 5.5, containing sodium chloride (0.15 mole/liter). The total volume was 0.12 ml. The very high viscosity of the solution decreased rapidly, but the reactivity with the corresponding antiserum was essentially unchanged. A cross reaction between rat PP-L and antiserum to bovine PP-L was present with or without in-

cubation with hyaluronidase (Fig. 1); however, a cross-reaction between bovine PP-L and antiserum to rat PP-L was observed only after treatment of the PP-L with hyaluronidase. These results, suggesting that the protein of the PP-L is primarily involved in its antigenicity, agree with those of DiFerrante (*14*) and of Loewi and Muir (*12*). This suggestion is supported by results from incubation of the bovine PP-L with some proteolytic enzymes for 3 hours at 37°C. Bovine PP-L which had been incubated with trypsin (*20*) at *p*H 7 in 0.1*M* sodium phosphate buffer did not give any precipitin lines with antiserum to bovine PP-L. Very faint precipitin lines were observed with PP-L incubated with papain in the presence of cysteine, 2,3-dimercaptopropanol, and ethylenediaminetetraacetate. Weak precipitin lines were seen with PP-L incubated with carboxypeptidase A in 0.05*M* trishydroxymethylaminomethane (tris) buffer, *p*H 7.5, in 0.25*M* sodium chloride, whereas chymotrypsin in this buffer or in 0.1*M* phosphate buffer at *p*H 7.4 had very little effect. On incubation with each of these proteolytic enzymes, there was a rapid and marked decrease in the viscosity of the solution. Furthermore, comparable results were obtained in experiments in which the enzymes were presumably inactivated after the incubation with PP-L by heating at 80° to 90°C for 10 minutes. This precaution was taken to reduce the possibility of having the antibodies enzymatically degraded in the course of diffusion in the agar plates.

From the absorbed antiserums, the globulins were precipitated by the addition of an equal volume of saturated ammonium sulfate. The precipitates were isolated by centrifugation, redissolved in 0.8 percent sodium chloride, and dialyzed at 4°C against repeated changes of buffered saline (0.01*M* sodium phosphate buffer, *p*H 7.4, containing 0.8 percent sodium chloride) until the dialyzing buffer was free of ammonium sulfate. The nitrogen content (microkjeldahl) was used to calculate the concentration of protein in the nondialyzable portion, which was then subject to reaction with fluorescein isothiocyanate (*21*), 8 mg of the latter being used for 500 mg of globulin (*22*). The reaction mixture was passed through a column (2 by 20 cm) of Sephadex G-50 to separate the conjugated proteins from excess labeling reagent. The conjugated proteins were then fractionated on diethylaminoethyl cellulose columns with a gradient of sodium chloride. Fraction II from such columns, as defined by Goldstein *et al.* (*22*), after concentration and dialysis against buffered saline, was used for the fluorescence microscopy studies.

Frozen sections (8 μ) of the proximal ends of tibiae from young rats (21 to 28 days old) were cut within 10 to 30 minutes after removal from the animals. Costochondral junctions of calves were used to prepare similar sections within 2 hours of slaughter. All sections were transferred to rings of vaseline on microscope slides and two drops of fluorescent antibody solution (0.5 to 1.0 mg/ml) were applied to each; the slides were then rocked gently in a moist chamber for 1 hour. The sections were then washed with buffered saline, mounted in a mixture of equal parts of buffered saline and glycerol, and examined microscopically (Zeiss fluorescence microscope with high-pressure mercury lamp, BG12 exciting filter, and a yellow barrier filter) (Fig. 2).

The sections labeled with only the fluorescein-labeled specific globulins showed a characteristically brilliant yellow-green fluorescence (Fig. 2b). The cells of all zones of the growth cartilage were more intensely fluorescent than those in the controls. The walls of the lacunae of the proliferating and hypertrophying cells fluoresced strongly. The matrix of the reserve zone fluoresced only slightly. The matrix around the proliferative and upper hypertrophic cells showed moderate to strong fluorescence, but there was a marked diminution in the fluorescence of the matrix around the lower hypertrophic and degenerate cells (zone of provisional calcification). In addition, the fluorescence of the metaphysis treated with fluorescein-labeled specific globulin and the corresponding controls were similar in intensity. Here and in the zone of provisional calcification, the fluorescence was not enhanced by prior decalcification of the sections with 0.5*N* hydrochloric acid or with 0.7*N* acetic acid for 15 minutes.

The results with sections of the growth cartilage and adjoining bone of calf rib were similar to those obtained with the sections of tibiae from rats.

Sections of rat tibiae could be stained with fluorescein-labeled antiserums to bovine PP-L, and labeled antiserum to rat PP-L could stain sections of costochondral junction of calves. These results are in agreement with the cross reactions observed in the immunodiffusion studies.

Our results indicate that there is a decrease in the concentration of PP-L in the zone of provisional calcification during the process of endochondral ossification. Since the staining of the metaphyseal trabeculae was no greater than that in the control sections, the polysaccharides in the trabeculae may no longer be covalently linked to the protein with which they were linked in the cartilage, or the protein may be so drastically altered that it no longer bears a resemblance to the original.

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