Reinterpretation of the Ultrastructure of Cartilage Matrix

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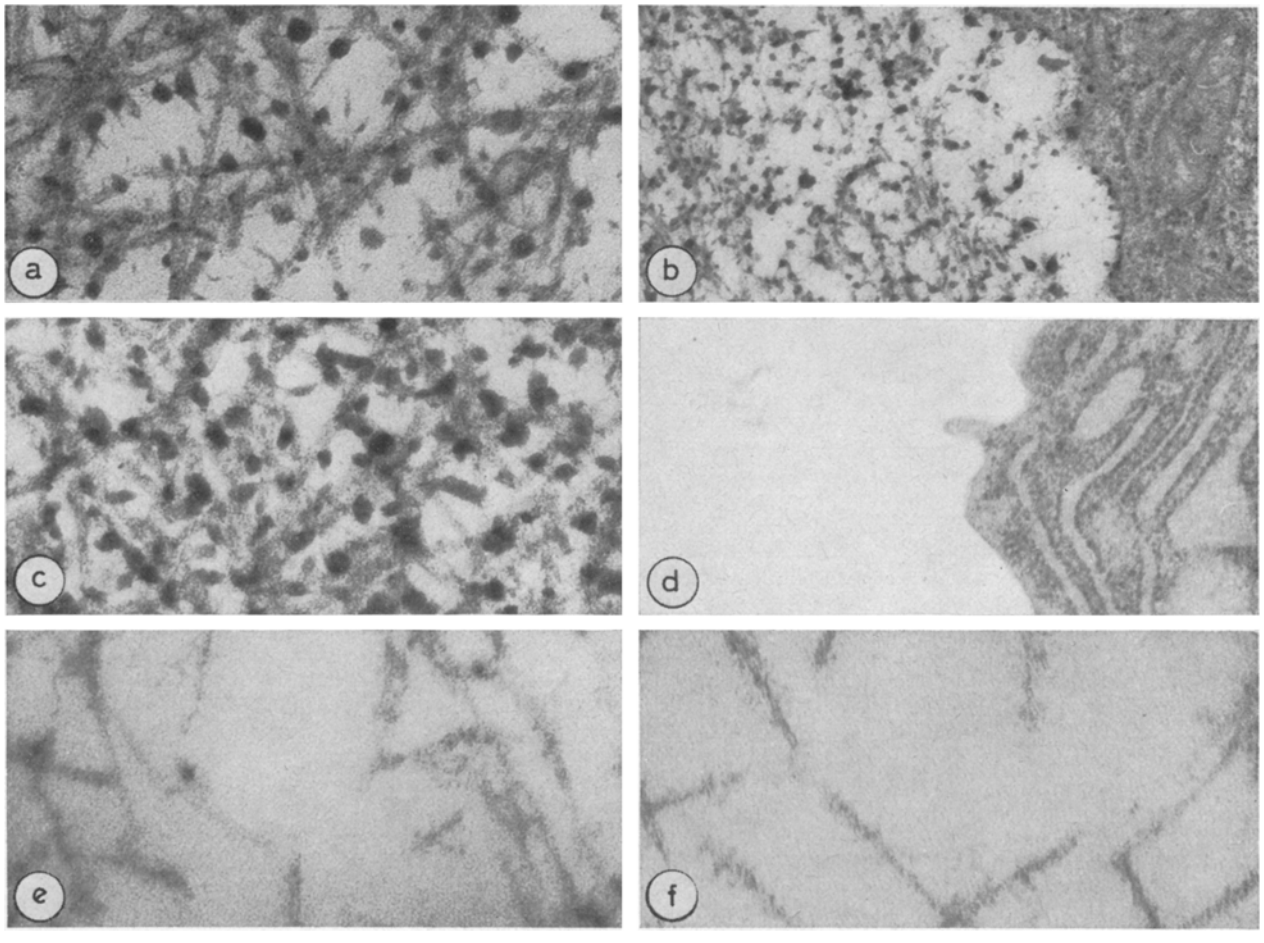
Summary. The epiphyseal cartilage from new-born mouse was treated with collagenase in two ways: either before fixation or after glutaraldehyde fixation. The electron dense granules of the matrix were not seen in the micrographs of cartilage treated with collagenase before fixation. It is concluded that collagen plays a definite role in the formation of the granules at the time of tissue fixation and that the granules are fixation artifacts.

The extracellular matrix of epiphyseal cartilage contains collagen both tropocollagen and polymeric collagen, non-collagenous proteins, some of which are complexed with sulphated glycosaminoglycans to form proteoglycans (also known as protein-polysaccharide complex) and various small macromolecules in the aqueous phase[2,3]. In electron micrographs the matrix reveals the following structural constituents: fibres, electron dense granules and amorphous electron translucent ground substance[2,3] (Figure a). Here we present evidence to show that collagen plays a definite role in the formation of the electron dense granules and that the granules are, in fact, fixation artifacts.

Epiphyseal cartilage of the toes from the new-born mice was dissected out in a physiological balanced salt solution[4]. The tissue pieces, about 1 mm^3 in size, were treated in two different ways. In one experiment, the unfixed pieces were treated for 2 or 4 h with a collagenase solution (chromatographically purified collagenase, specific activity 582 units/mg, dissolved in the physiological solution to give a concentration of 5000 units/ml, origin *Clostridium histolyticum;* from Worthington Biochemical Company, New Jersey) at 37 °C and fixed in glutaraldehyde (2.5% in 0.1 *M* phosphate buffer at 4 °C for 1 h. In the other experiment, the collagenase treatment (3.5 h) was preceded by fixation in the glutar-



Epiphyseal cartilage of mice. a) Extracellular matrix showing collagenous fibres, electron dense granules and amorphous electron translucent ground substance (×120,000). b) Extracellular matrix along with the part of a cell (×30,000). c) Extracellular matrix of cartilage which was prefixed in glutaraldehyde and treated with collagenase (3.5 h) to show electron transparency of the ground substance. Both fibres and granules are virtually unaffected (×120,000). d) Extracellular space and part of the cell from cartilage treated with collagenase (4 h) and then fixed with glutaraldehyde to show the effect on extracellular matrix. Notice that the cell remains unaffacted by collagenase treatment (×30,000). e) Extracellular matrix from the cartilage treated with collagenase for 2 h before being fixed in glutaraldehyde. Wherever present the matrix has fibres and some granule-like structures (×120,000). f) Extracellular matrix from the cartilage shown in d). Notice the complete absence of the electron dense granules (×120,000).

aldehyde (30 min). The tissue pieces were post-fixed in 1% osmium tetroxide for 1 h and processed for Araldite-Epon embedding. Sections were stained with lead and uranyl acetate.

The collagenase treatment of the living tissue produced very striking changes in the ultrastructure of the matrix but no changes were apparent in the cells (Figures b and d). After the treatment with collagenase for 2 h the matrix was seen to consist of some fibres and occasional granules; in addition, the latter were also different in appearance from those in the control (Figures a and e). After the 4-hour treatment with collagenase the matrix showed no granules and there was a still further reduction in the number of the fibres (Figures d and f). The presence of some fibres, which presumably are collagenous in nature[2], after this treatment indicates the relative resistance of the polymeric collagen, as compared with the tropocollagen, to collagenase.

On the other hand, the collagenase treatment of the prefixed tissue did not noticeably affect the ultrastructural appearance of the matrix constituents, even at the periphery of the tissue piece (Figure c). This indicates that collagenase has practically no effect on collagen after glutaraldehyde fixation of cartilage, perhaps because of the formation of complexes between collagen and glutaraldehyde[5] or proteoglycans[6]. However, the increased electron transparency of the ground substance suggests that some of the tropocollagen (or its procollagen precursor) may have been removed by the enzyme treatment.

In view of the fact that collagenase is known to be highly selective in its action there are two possible explanations for the virtual absence of the electron dense granules in the micrographs (Figures e and f) of the tissue treated with collagenase before fixation. One, that collagen is a major constituent of the granules, and two, that it plays a definite role in the formation of the granules at the time of tissue fixation. But others[2,3,7,8] have concluded from their work involving both specific staining procedures[2] and extraction of unfixed and fixed cartilage by hyaluronidase, guanidium chloride, $CaCl_2$ and trypsin[3,8] that the electron dense granules are largely made up of proteoglycans, and that collagen, if any, is only a minor constituent of the granules.

We conclude that collagen plays a definite role in the formation of the granules at the time of tissue fixation and that the granules are, therefore, fixation artifacts. This hypothesis further explains the association of the granules with the collagen fibres in electron micrographs, and is consistent with the observation that the granules from fixed cartilage tissue can be removed by treatment with hyaluronidase or trypsin[3].

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Protection by Methylcholanthrene Against Hepatic Carcinogenesis in Rats Ingesting N-4-(4′-Fluorobiphenyl)acetamide

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Summary. Methylcholanthrene protected against the development of carcinomas of the liver in Fischer rats ingesting N-4-(4′-fluorobiphenyl)acetamide.

N-2-fluorenylacetamide (2-FAA) induces tumors of the liver in rats[1–3]. Modification of the chemical structure by elimination of the methylene bridge to form N-(4-biphenyl)acetamide decreases carcinogenicity of the liver[4–6]. When the halogen, fluorine, is substituted in the 4′-position, i.e., N-4-(4′-fluorobiphenyl)acetamide (4′F-4-BAA), animals again develop tumors of the liver.

Methylcholanthrene (MCA) inhibits the development of carcinomas of the liver in male rats ingesting 2-FAA[7]. The present study was done to determine if MCA would also prevent the induction of carcinomas of the liver by 4′F-4BAA.

Methods. Inbred male Fischer strain (F-344) rats from the National Institutes of Health were used when they were 12 weeks of age and weighed 230 to 246 g. They were divided into 2 groups of 25 animals each. One group of rats received 4′-F-4BAA and the second group was given MC simultaneously with the 4′-F-4BAA.

The carcinogen was incorporated in Morris Diet No. 272[8]. 4′-F-4BAA was added in the amount of 0.04%; and MCA 0.033%. The two diets were fed ad libitum continuously for 36 weeks. Thereafter, the rats were given Purina laboratory pellets. 48 weeks after the start of the experiment animals that survived were killed by exsanguination. Complete autopsies were performed. Tissues were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin.

Results. Animals in both groups gained weight for 36 weeks, at which time the weights remained constant. Rats, with rare exceptions, survived for the 48 weeks duration of the experiment.

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