STUDIES ON CARTILAGE

II. Electron Microscope Observations on Rabbit Ear Cartilage following the Administration of Papain

HUNTINGTON SHELDON, M.D., and ROBERT A. ROBINSON, M.D.,
with the technical assistance of ULLA F. AFZELIUS

From the Johns Hopkins University School of Medicine, Departments of Pathology and Orthopaedic Surgery, Baltimore

ABSTRACT

Electron microscope observations on rabbit ear cartilage following the administration of papain show that both the elastic component of the matrix and the amorphous material disappear leaving a matrix which consists of delicate fibrils which are presumed to be collagen. This unmasking of fibrils coincides with the appearance of an abnormal component in the electrophoretic pattern of the rabbit's serum. The chondrocytes show vacuoles in their cytoplasm which appear at the same time that the cells appear crenated in the light microscope. A ruffly appearance of the cell surface membrane coincides with this vacuolization, and vacuoles often appear open and in continuity with the extracellular space. The resurgence of the rabbit ear is accompanied by a reconstitution of both the amorphous material and the elastic component of the matrix. During this period numerous dilated cisternae of the endoplasmic reticulum which contain a moderately dense material are present in the chondrocyte cytoplasm. We have been unable to demonstrate a direct relationship between the elastic component of the matrix and a particular component of the chondrocyte cytoplasm, but it is clear that changes occur in the cartilage cell cytoplasm during both the depletion and reconstitution of the matrix. Previous studies on the effect of papain on elastic tissue are noted and the possible relationships between changes in the cells and matrix of this elastic cartilage are discussed.

The phenomenon of collapse of the ears of young rabbits after papain is administered intravenously was observed by Thomas (1) who reported the reversibility of this phenomenon. He described the appearance of auricular and other cartilage in the light microscope during the phase of collapse, and commented on a loss of basophilia in the cartilage matrix. Further studies on this phenomenon have been reported by Spicer and Bryant (2, 3), McCluskey and Thomas (4), Tsaltas (5), and Bryant, Leder, and Stetten (6), who describe the appearance of an abnormal acid component in the electrophoretic pattern of serum after the administration of papain. The present authors thought that this phenomenon of collapse and recovery might prove suitable for an electron microscope

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Dr. Sheldon is a Markle Scholar in Medical Science, Department of Pathology, McGill University School of Medicine, Montreal, Canada.

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study of the relationship between the matrix and the cellular constituents of cartilage. This communication reports electron microscope observations on rabbit ear cartilage taken at intervals from 30 minutes to 48 hours after the administration of papain. An electron microscope study of the ear cartilage of normal rabbits has been reported previously (7).

MATERIALS AND METHODS

One cc. of 1 per cent solution of crude papain[1] was administered intravenously to 12 young rabbits each of which weighed less than 1000 grams. Specimens of ear cartilage were removed 30 minutes, 2, 4, 8, 12, 18, 24, and 48 hours later. To avoid the effects of trauma no more than 2 interval biopsies were performed on any one ear. The tissue was examined with the light and electron microscope. Tissue for light microscopy was fixed in 10 per cent neutral formalin. Tissue for electron microscopy was prepared in the same manner as in the study on normal rabbit ear cartilage (7). Thin sections were cut either on a Sjöstrand or Porter-Blum microtome with a glass knife and were examined in either an RCA EMU-2B or 3C electron microscope.

OBSERVATIONS

In the electron microscope study of the ear cartilage of normal rabbit the *matrix* is described as consisting of two components: a uniformly distributed moderately dense substance which appears as a fine meshwork without any particular pattern extending from cartilage cell border to cartilage cell border; and a second component, a three-dimensional network of more dense material, which is best described as "felt-like," lying midway between the cells. Since the characteristic difference between this "elastic" cartilage and cartilage from the epiphyseal plate (8) is the presence of this "felt-like" material, for the sake of brevity, this material will be referred to subsequently as the *elastic component*. The normal chondrocyte *cytoplasm* shows, in addition to mitochondria, lipid spherules, glycogen, and components of the endoplasmic reticulum, a material which had not been described previously in light microscope studies and which appears as a relatively dense felt-work (7). The relationship in normal elastic cartilage between this cytoplasmic material and the elastic component of the matrix is not clear.

The observations in the present communication are correlated with light microscope observations and with the electron microscope study of the ear cartilage of normal rabbits.

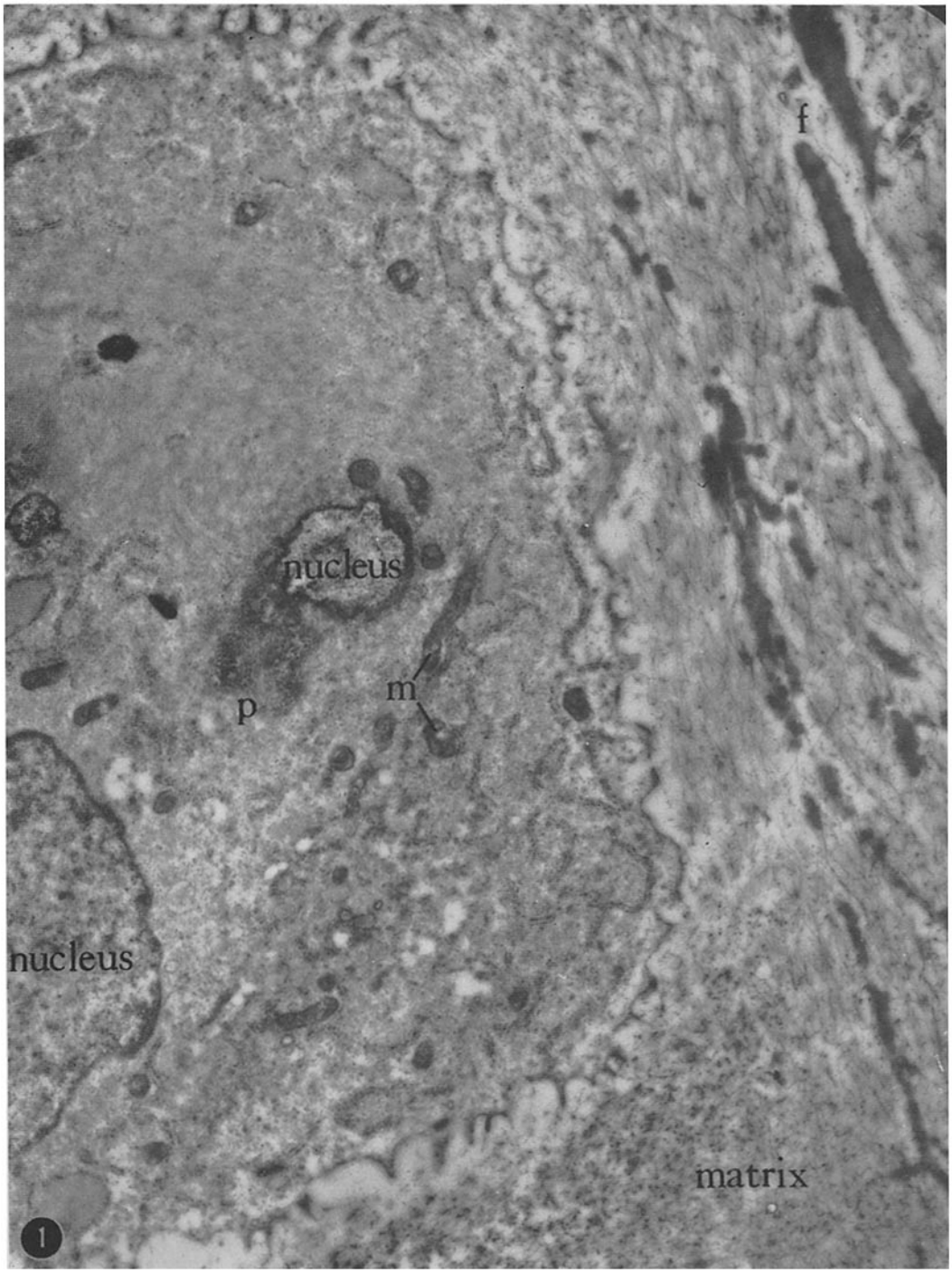
Thirty minutes after papain is injected, before any changes in the normal posture of the rabbit ear are apparent, there is an evident increase in the eosinophilia of the cartilage *matrix* when sections are stained with hematoxylin and eosin. At this early time, in areas where the extracellular matrix normally shows an amorphous fine meshwork, the electron microscope now reveals abundant delicate fibrils (Fig. 1). In areas where the elastic component normally lies, one sees this material, but each large fibril of it appears surrounded by a clear halo (Fig. 1). The chondrocytes show no significant changes in the appearance of their cell surface membrane, cytoplasm, or nucleus.

Two hours after papain has been given, the *matrix* continues to show fibrils, and we have the impression that the elastic component may have diminished in amount. The cytoplasm of many *chondrocytes* shows numerous vacuoles (Fig. 3) which are not present in normal cartilage cells. In sections from cells which contain many vacuoles

[1] Papain obtained from Delta Chemical Works, Inc., New York.

FIGURE 1

Electron micrograph of a portion of a chondrocyte and adjoining matrix *30 minutes* after the administration of papain. There is no gross evidence of ear collapse at this early stage. The cartilage matrix (right hand portion of figure) still shows the dense elastic component which is present in the normal auricular cartilage, but a clear space or halo appears about each large fibre (*f*). The rest of the matrix shows small fibrils of indeterminate length which appear similar to collagen fibrils at this low magnification, although without clearly apparent subbanding, a positive identification is not warranted. The appearance of fibrils in this matrix is in marked contrast to the usual amorphous appearance of this cartilage. The chondrocytes do not appear remarkable. The nucleus, cut in a tangential plane, shows many small pores (*p*). Mitochondria (*m*) and elements of the endoplasmic reticulum are visible in the cytoplasm. × 4,000.


f
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1

the cell surface membrane shows many fine extensions (Fig. 3) which are much larger than those described in normal chondrycytes or in cells which do not have vacuoles at this time (Fig. 2). There does not appear to be any significant difference in the appearance of the chondrocytes or matrix between the 2- and 4-hour material except that we have the impression that there is progressively less of the elastic component in the matrix. The changes seen with the light microscope in the period up to 8 hours can be summarized as:(1) a crenation of the chondrocytes and a decrease in the intracellular glycogen; (2) a disruption of elastic fibres and apparent swelling of the interlacunar septa; (3) a transient increase in metachromasia of the matrix with disperison of metachromatic granules.

Eight hours after papain, at a time when ear collapse is pronounced and light microscopy shows a marked increase in the eosinophilia of the matrix, there is complete loss of metachromasia (2). At this time the chondrocytes appear less crenated. The electron microscope shows a virtual absence of the elastic component from the matrix between the chondrocytes (Fig. 4), which is a striking change from the normal. Vacuoles are present in many chondrocytes and occasionally appear to be in continuity with the matrix (Fig. 4, inset). It is impossible to know from a picture whether these vacuoles actually represent the transport of material from the cell to the matrix and extracellular spaces or *vice versa*, but the appearance of the vacuoles suggests these possibilities.

Electron microscope observations at *12 and 18 hours*, a time when the ears apparently continue to lie flaccid, do not demonstrate any striking differences in the appearance of the *matrix* from the 8-hour period. However, the *chondrocytes* show an increase in the granular components of the endoplasmic reticulum over the normal chondrocyte, and we observe that not only are the cisternae more abundant but that they are often strikingly dilated with a moderately dense material (Figs. 6 to 8).

At *24 hours* after the administration of papain, a time at which the collapse phenomenon has passed its low point and the ears are beginning to regain their erect position, the electron microscope shows two findings of note in the *chondrocytes*. The first is that the nucleoplasm often appears much denser than in the normal cell (Fig. 5) without any discernible pattern of distribution of nuclear chromatin such as can be seen in the study on the normal chondrocyte (7) or in experimentally induced pathologic processes in other tissues (9). A second unusual finding is the presence of fine filaments in the cytoplasm of the chondrocytes (Fig. 5). This component appears to be the same material which appears felt-like in the normal chondrocyte. In a study of the normal cell we were unable to resolve any fine structure in this peculiar and hitherto undescribed cytoplasmic component. At this time, 24 hours after papain, small amounts of the elastic component can again be seen in the *matrix*.

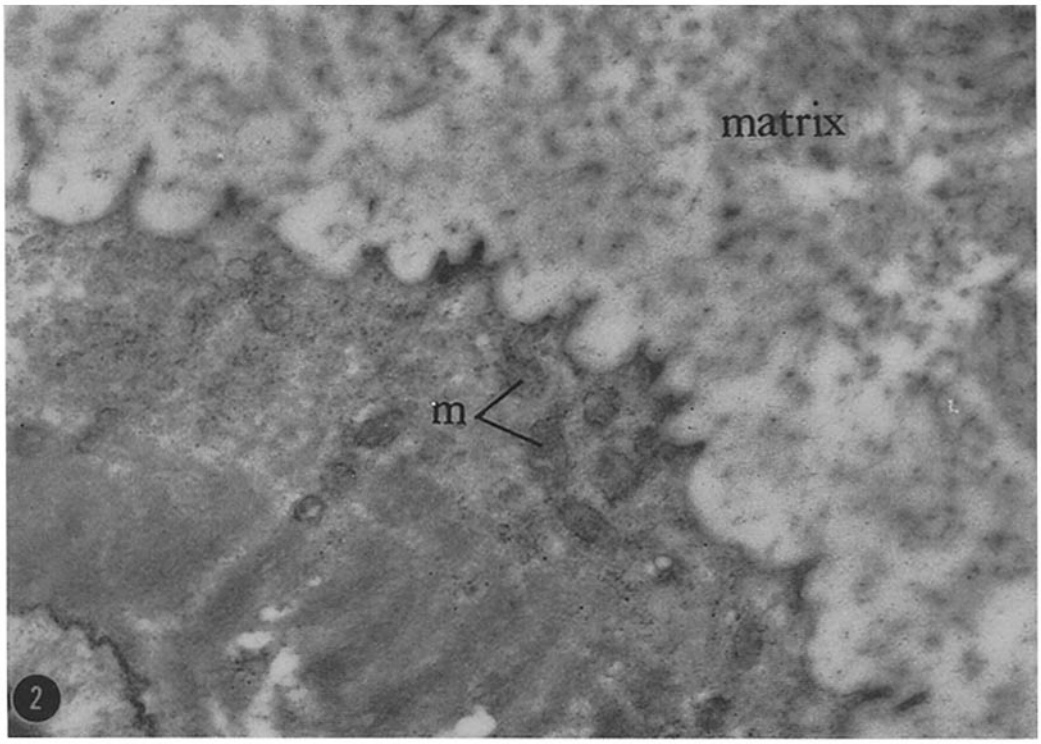
At *48 hours* after papain, electron micrographs show large amounts of the elastic component in the *matrix* between the chondrocytes, more than

FIGURE 2

The cytoplasm of this particular chondrocyte *2 hours* after the administration of papain does not appear remarkable. The cell surface membrane is delicately serrated; small mitochondria (*m*), a fat spherule (in one corner of which the figure 2 is placed), and small amounts of endoplasmic reticulum are present. This picture is shown for comparison with Fig. 3. × 20,000.

FIGURE 3

The appearance of this chondrocyte, *2 hours* after the administration of papain, is more typical of the changes that occur. The cell surface membrane is ruffled and shows abundant large microvilli while the cytoplasm is markedly vacuolated (*v*). If the vacuoles are either entering or leaving the cell one can imagine the effect such movement would have on the configuration of the cell surface membrane. The arrows indicate areas where vacuoles may have left the cell. It seems likely that the crenation of chondrocytes seen in the light microscope is the image of this effect. Delicate fibrils are present in the matrix and we have the impression that less of the elastic component is present at this time than at the 30 minute period. × 12,000.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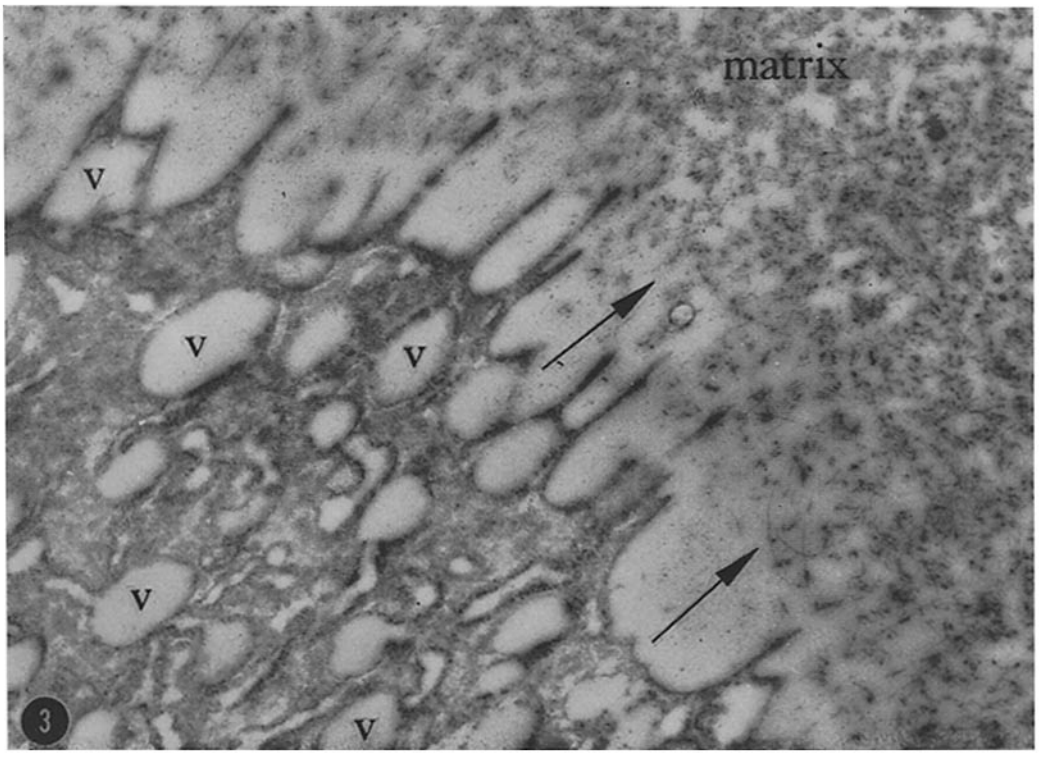
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is present under normal conditions. Furthermore, the fine fibrils which appeared to be the main constituent of the matrix shortly after papain was given are again obscured and the matrix reverts to a normal appearance. The *chondrocytes* at this stage also contain large amounts of cytoplasmic feltwork as well as numerous cisternae. These cisternae of the endoplasmic reticulum contain a material which has the same density as the elastic component of the matrix (Fig. 9). A curious finding is that a large number of chondrocytes at this period have two nuclei.

DISCUSSION

The observations recorded here are concerned with the *in vivo* effect of an enzyme, papain, on elastic cartilage. Electron microscopy has shown that this cartilage under normal conditions consists of cells and a complicated matrix, and that following the administration of papain, changes occur in all components of this tissue. The discussion will review observations made by other investigators on the papain effect and include a brief comment on previous studies on elastic tissue and the nature of papain.

Spicer and Bryant (3) have suggested that an acid polysaccharide released from the cartilage by papain combines with a basic plasma protein to form the acid-circulating component. They observed that dried plasma smears from papainized animals show a frosted glass appearance in which rounded vesicular bodies may be seen, and they demonstrated a change in the electrophoretic pattern of serum. Tsaltas (5), using rabbits injected with S_{35}, found a reduction in the S_{35} content of cartilage matrix at the time when there was a marked increase in the S_{35} in serum and urine. On the basis of changes in the $\frac{N}{\text{Hexosamine}}$ ratio in cartilage, he concludes that the chemical composition of the chondromucoprotein is temporarily altered. He suggests that the collapse of the cartilage is due to the loss of chondromucoprotein from the cartilage and a reduction of chondroitin sulfate in the chondromucoprotein which remains.

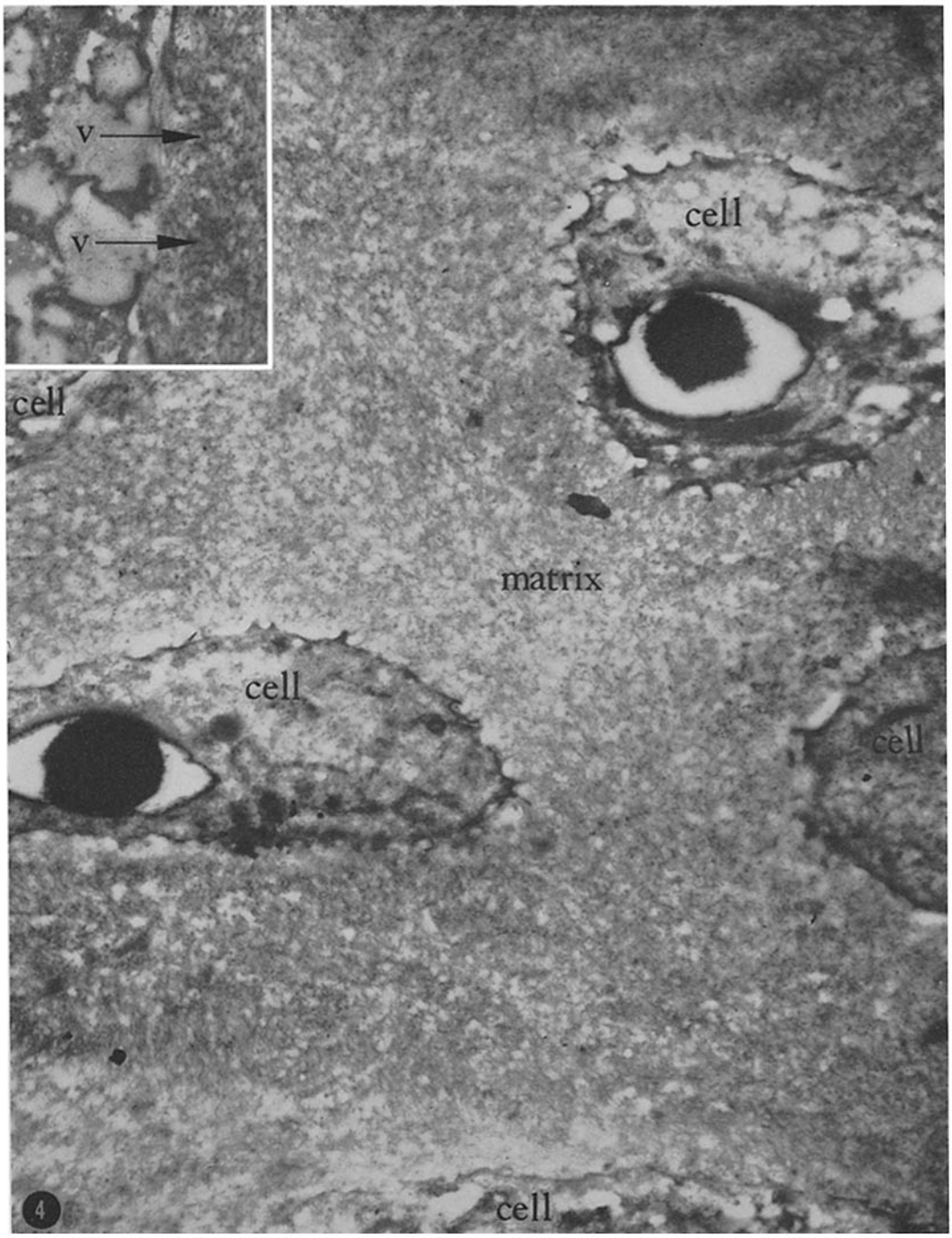
Early efforts to determine whether the loss of water from the cartilage causes the ear collapse showed that the ratio of wet to dry weight was identical for the control and experimental animals, excluding this possibility (4).

Regardless of the precise nature of the material which appears in the serum and urine and is deposited as small globules in vessels, we are left with the problem of the nature of the fibrils which remain in the matrix. At the time of this writing,

This is a drawing which represents the early changes in the gross appearance of the ears of a rabbit which has received papain *8 hours* previously. The ears begin to droop at the distal end.

FIGURE 4

A survey electron micrograph of portions of five chondrocytes taken from rabbit ear cartilage *8 hours* after the administration of papain at a time when the rabbit's ears have begun to droop (see drawing above). At this time when the elastic fibres are noted to be "fragmented" in the light microscope, many sections for electron microscopy fail to demonstrate any evidence of the elastic component. The inset shows a portion of a chondrocyte (8 hours after papain) in which two vacuoles (*v*) apparently are open to the matrix (arrows). Some fibrillar material appears in both the vacuoles. While one cannot tell from a picture whether such vacuoles are entering or leaving a cell, such continuities indicate a mechanism whereby material from certain cells can be secreted into the matrix.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we have not demonstrated them to have the characteristic fine structure of collagen fibrils with phosphotungstic acid staining or by diffraction methods, nor have we done hydroxyproline or other chemical analyses before and after papain. From circumstantial evidence, however, and from the low magnification electron microscope observations of the present study it seems likely that these fibrils belong to the collagen family.

The second change, in addition to the unmasking of fine fibrils, is the disappearance of the elastic component from the cartilage matrix. One might ask whether this is simply an extension of the unbinding process or if this change means that an additional material has been dissolved from the matrix. If there is an additional material then is this substance "elastin"?[2] It is interesting to note that Shatton and Schubert (10) have reported the presence of another protein in cartilage in addition to collagen, however, this new protein resembles serum albumin rather than elastin and, furthermore, their studies were done on bovine nasal cartilage which is considered fibrocartilage rather than elastic cartilage, and which presumably contains none of the elastic component. It may be of interest that the marked increase in the presence of S_{35} in serum and urine following papain shown by Tsaltas (5) is in keeping with changes one might expect during the dissolution of elastic tissue. Especially is this so when one recalls that Banga and Schuber (11) have shown that appreciable quantities of "sulfuric acid" are released during the action of elastase on elastic tissue. Other studies on elastic tissue of various kinds have shown that elastic tissue is closely related to the collagen moiety of this tissue. Romhanyi (12) showed that the protein filaments embedded in the matrix of the elastic fibres are in close structural relation to the collagen-type protein, an observation which is supported by electron microscope studies of Karrer on the aorta (13), and on the tunica propria of bronchioles (14), where collagen fibrils appear at the periphery of and are partially embedded in the homogeneous elastic component. Unpublished observations, by one of us (H.S.), on thin sections of bovine ligamentum nuchae, the classic source of elastic tissue, show the same homogeneous appearance with a scattering of collagen fibrils.

Studies of Baló and Banga (15) demonstrate that elastic tissue is readily attacked by an enzyme, elastase, first obtained by Eijkman from bacteria (16), but later shown to be present in pancreatic extracts. This enzyme was said to be distinct from ordinary proteases, such as trypsin, which do not

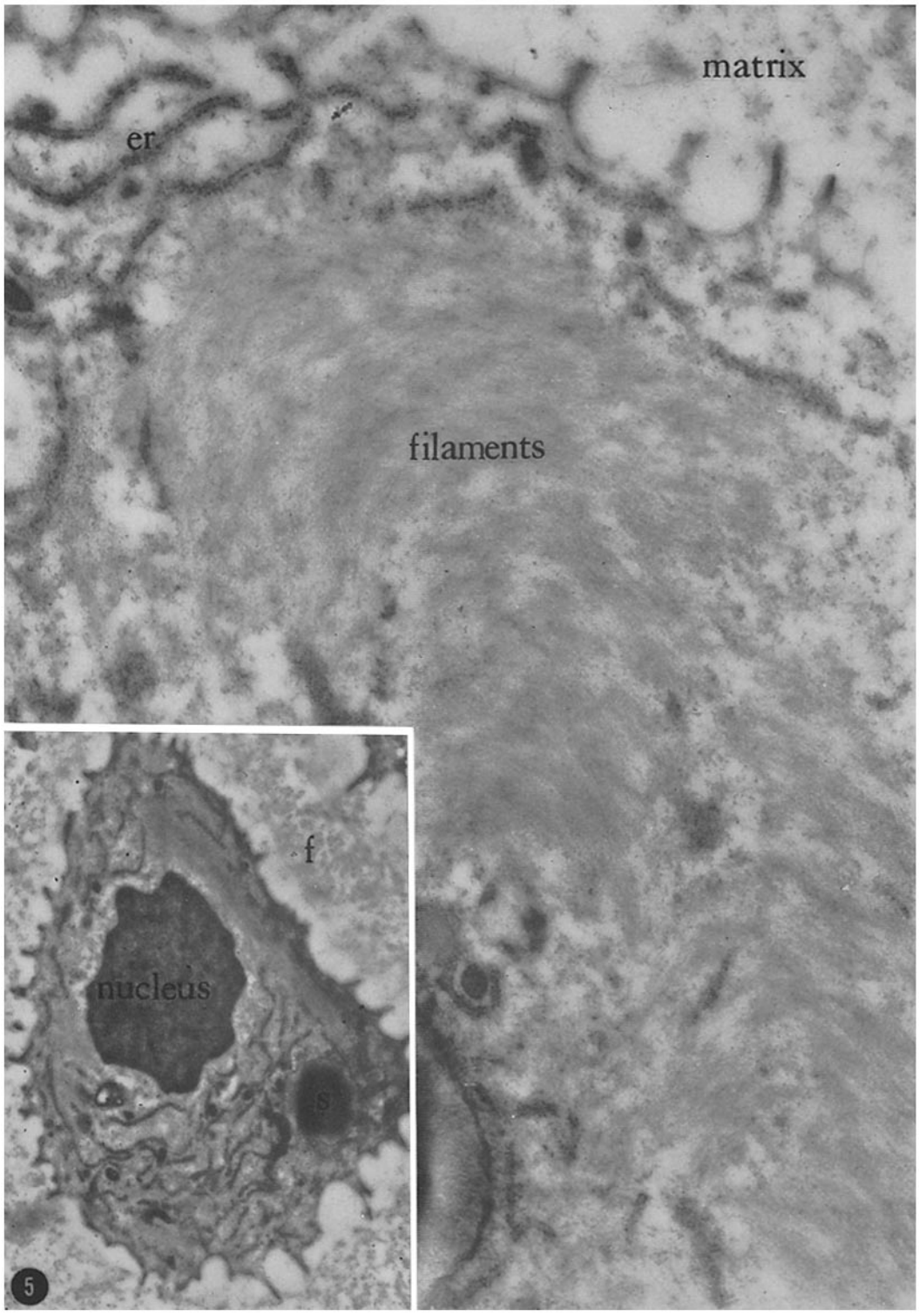
[2] Unfortunately there is no satisfactory chemical definition for the material elastin in the sense that insulin from various sources has a defined amino acid sequence.

This is a drawing which shows the rabbit's ears when they are completely flaccid.

FIGURE 5

Electron micrographs of chondrocytes and matrix *24 hours* after the administration of papain at a time when the rabbit's ears resemble those of a cocker spaniel (see drawing above). Filaments are present in the cytoplasm of these chondrocytes at this time in areas where the normal chondrocyte contains a dense felt-work in which no filaments could be resolved. The matrix between cells does not yet reveal any quantity of the elastic component that has disappeared and begins to reappear during the second 24 hours. Abundant elements of the granular component of the endoplasmic reticulum are present in the upper left corner (*er*). × 20,000.

The *inset* shows a low power image of a cell in which the nucleus appears much more dense and homogeneous than in normal chondrocytes. A lipid spherule is present in the lower right hand corner of the cell (*s*), and the fibrils in the matrix are indicated between the cell and its neighbor at *f*.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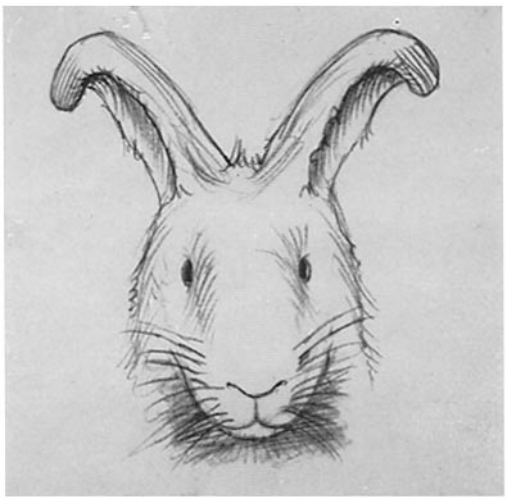
attack elastic tissue. The elastic tissue was broken down without the production of amino acids. To explain these results, both Banga (17) and Hall, Reed, and Tunbridge (18) proposed that elastic tissue was made up of globular subunits. A mucopolysaccharide supposedly is intimately bound to a protein moiety and elastase is believed by them to be a mucase. The nature of the substrate seems to be a point of some consequence in all these experiments, yet the enzyme itself is only defined in terms of the substrate.

Papain, a vegetable pepsin, is obtained from the juice of the fruit and leaves of *Carica papaya* and is commonly used as meat tenderizer. The potency of papain varies according to the process of preparation. Crystalline papain which catalyzes the hydrolysis of peptide bonds, has a molecular weight of 27,000 ± 2,000, is stable at 30°C., rapidly inactivated below pH 2.5 or above pH 12, and has a broad zone of electrophoretic mobility between pH 4 and 6 (19). McCluskey and Thomas (4) report that crystalline papain does not produce the same effect on rabbit ear cartilage as the crude material unless the crystalline papain is inactivated first. They suggest that the reason for this failure of activated papain is that it probably reacts with a substrate in the blood while the inactivated papain may be rapidly taken up by cartilage and there converted to its active form. Partridge (20) states that some preparations of papain contain an elastase of quite high activity. Odin (21) has reported that a considerable part of the carbohydrate moiety of submaxillary mucin is rendered dialysable by treatment with papain. It is interesting that papain attacks cytochrome c yielding autooxidizable pigments without any of their original enzymic activity (22). Papain also has been reported to digest wool keratin (23). Generally, in studies on the effects of an enzyme one may learn about the nature of both the enzyme



This is a drawing which represents the gross appearance of the rabbit's ears during the period of recovery from the papain effect.

FIGURE 6

Electron micrograph of a portion of a chondrocyte *18 hours* after the administration of papain. This field shows a portion of the Golgi complex with adjacent areas of the endoplasmic reticulum (*er*) in which cisternae show various degrees of dilatation. In the region of the Golgi complex rounded areas are present containing a moderately dense material. × 14,000.

FIGURE 7

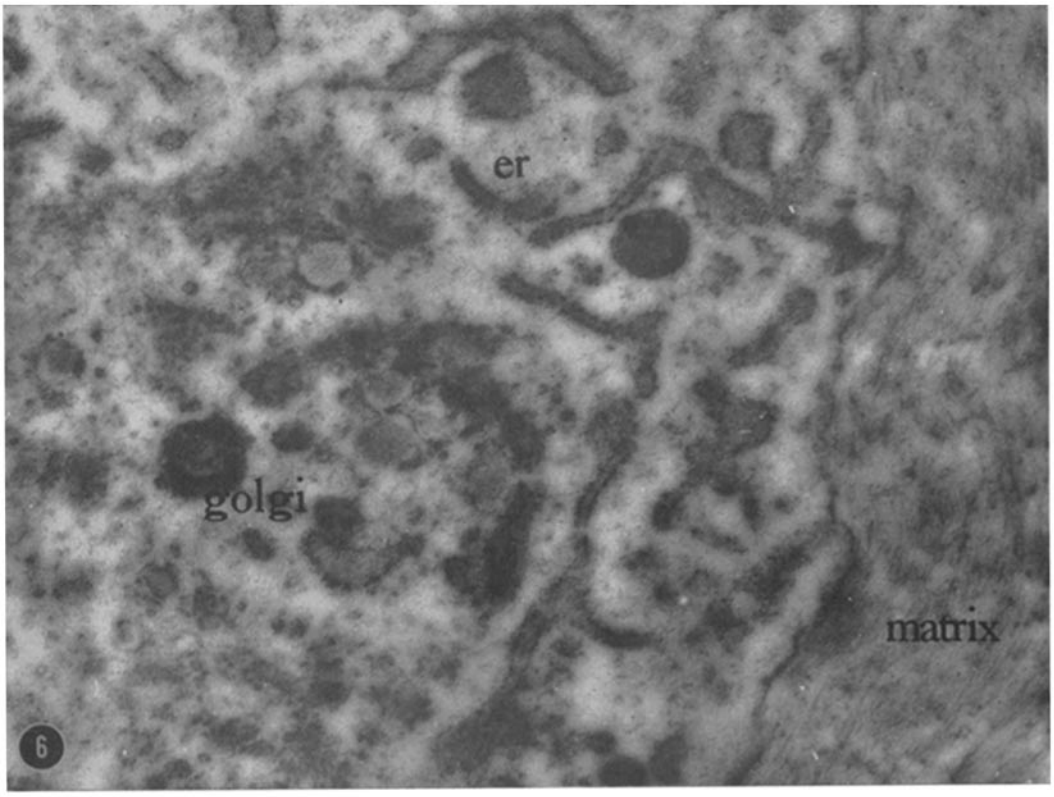
Electron micrograph of another area in a chondrocyte *18 hours* after papain showing more of the rounded dense agglomerates in which suggestions of fine structure may be seen (*c*). × 13,000.

FIGURE 8

Electron micrograph of a portion of a chondrocyte *24 hours* after papain showing dilated cisternae of the endoplasmic reticulum (*c*). × 19,000.

FIGURE 9

Electron micrograph of a portion of a chondrocyte *48 hours* after papain (see drawing above) showing very large dilated cisternae (*c*) containing a material which has a similar density to the elastic component of the matrix (*f*). Notice that the fibrils of the matrix are now obscured. × 10,000.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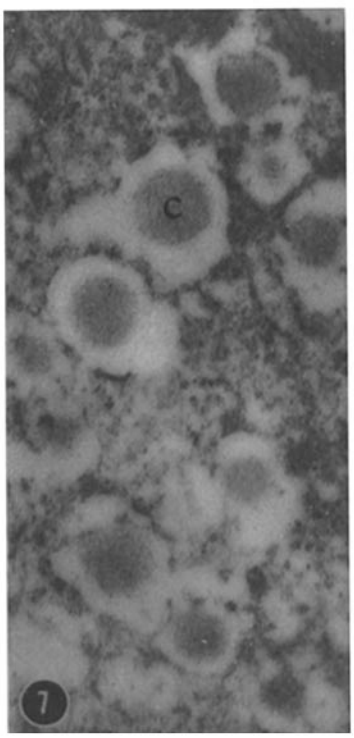
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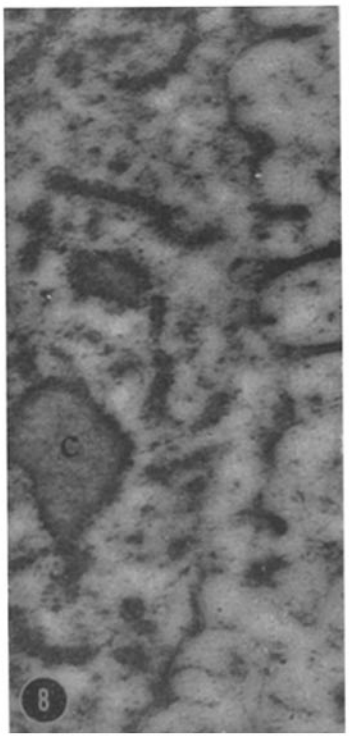
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and substrate. In this instance neither the enzyme or enzymes nor the substrate appears to be sufficiently well characterized so that we can clearly define one in terms of the other.

Light microscope observations indicate that glycogen disappears from the chondrocyte cytoplasm during the first 4 hours. This coincides with the crenation of the chondrocytes and the appearance in electron micrographs of vacuoles in the chondrocyte cytoplasm. These changes in the cytoplasm occur at a later time than the earliest disappearance of material from the matrix. It is not clear whether the intracellular changes are due to a direct effect of papain on the chondrocyte or whether the loss of glycogen and concomitant vacuolization represent early efforts on the part of the cell to restore the matrix to its normal state.

The changes in the chondrocyte which occur during later periods (after 12 hours), at which times there are increasingly enlarged cisternae in the endoplasmic reticulum, are related presumably to the reappearance of the amorphous material and the elastic component in the matrix.

Since we do not know the chemical nature of the material which is being reconstituted, whether it is a mucoprotein, or simply the protein-free carbohydrate moiety, or whether as Labella has suggested (24) it is a metabolically active lipoprotein, it is impossible to state whether the morphologic changes in the cell during the reconstitution of the matrix are peculiar to the synthesis of protein or carbohydrate or indeed, more likely, a more complex substance. It seems wiser to defer any generalizations about cell morphology and secretion until the nature of the reconstituted material is clearer. But the present observations suggest that the whole cell is involved in the process of matrix repletion following the papain effect. Although we have done no counts of mitoses, the changes in the pattern of the nucleus, and the impression that there is a large number of binucleate cells following the restoration of the ear, both suggest that the nucleus as well as the cytoplasm is involved in the process of reconstitution of the matrix. We have no direct morphologic evidence other than that which has been presented to support the hypothesis that the matrix of cartilage is maintained by secretion from various cytoplasmic compartments into the extracellular space. Perhaps further examination of cells during the period of recovery may demonstrate a direct relationship between the components of the cytoplasm and the particular components of the matrix.

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