CARTILAGE MATRIX DEPLETION BY RHEUMATOID SYNOVIAL CELLS IN TISSUE CULTURE*

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In chronic rheumatoid arthritis, the synovial membrane grows as a pannus over the articular cartilage and erodes it, producing irreversible changes in joint structure and function. On the basis of many experimental observations, hydrolytic enzymes from various sources in the rheumatoid joint have been thought to contribute to the dissolution of the cartilage matrix.

Barnett (1) showed that when hyaluronidase was injected into a rabbit joint which was then vigorously exercised, there was a loss of articular cartilage ground substance, presumably due to digestion of the polysaccharide chains of the protein-polysaccharide (PP) of the matrix. Since Dingle (2) first proposed that lysosomal enzymes might degrade the matrix of articular cartilage in the rheumatoid joint, considerable indirect evidence has accumulated to support this suggestion. Some of the evidence and literature citations are presented elsewhere (3, 4) and are briefly summarized here. The rheumatoid synovial membrane contains a much higher content of lysosomal enzymes than the normal membrane, as revealed by cytochemical stains using the light and electron microscope, and by chemical analyses of enzyme activities in homogenates. High levels of lysosomal enzymes are present in rheumatoid synovial fluids and presumably arise either from dissolution of leukocytes in the fluid, or from lining cells in the synovial membrane, or from both. Extracts of rheumatoid, but not normal, synovial membrane and preparations of lysosomal enzymes obtained from liver degrade viscous solutions of the ground substance extracted from cartilage matrix. Injection into rabbit joints of streptolysin S, which is thought to "labilize" lysosomal enzymes, causes a proliferative synovitis with pannus formation and cartilage erosion.

The experimental basis for reversible cartilage matrix depletion by proteolytic enzymes was provided by Thomas (5). He showed that papain injected intravenously into rabbits caused their ears to droop. After several days, the ears returned to their erect position. The depletion of the anionic polysaccharides of the cartilage matrix by

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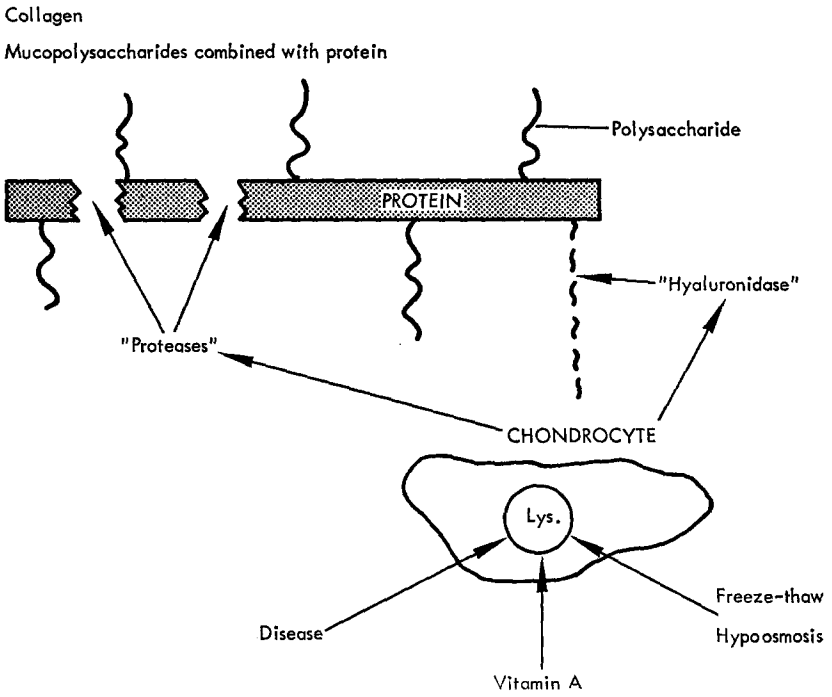
papain was manifested by loss of metachromatic staining of the affected cartilage, and by the appearance of chondroitin sulfate in the rabbits' blood and urine. Knee articular cartilage was similarly affected, although the results were less dramatic. Later it was shown that vitamin A administered parenterally to rabbits could reproduce the effects of papain (6). Vitamin A appears to disrupt lysosomes, and so it became evident that cartilage itself contained the seeds of its own destruction. Proteolytic enzymes (cathepsins), arising from lysosomes in chondrocytes or from exogenous sources and gaining



TEXT-FIG. 1. Diagram of the probable mode of attack on the protein-polysaccharide of cartilage matrix by hydrolytic enzymes released from lysosomes of chondrocytes.

access to cartilage, could hydrolyze the protein moiety of the PP of the matrix, liberating peptides bound to short chains of anionic polysaccharide (Text-fig. 1).

Recently, immunofluorescent methods have provided new means of studying depletion of cartilage matrix by hydrolytic enzymes. Antiserum to the cartilage PP appears to be directed to the protein moiety (7), and localizes immediately around the chondrocyte capsule (8, 9). Cartilage sections incubated in vitro with hyaluronidase show loss of metachromatic staining for polysaccharides but increased intensity and more diffuse fluorescent staining. This indicates that the protein core of the PP can persist in hyaluronidase-digested cartilage. Long incubations with proteolytic enzymes, as would be expected, cause loss of both metachromatic and fluorescent staining, but short incubations, such as 10 min with papain, result in changes similar to those produced with hyaluronidase (9).

In some early rheumatoid joints, there are articular cartilage changes similar to those observed in experimental studies where enzymatic digestion has occurred: The cartilage matrix is less basophilic; metachromasia is much diminished; and fluorescent staining with antiserum to cartilage PP appears intense and more diffuse. In some instances, a layer of synovial lining cells is seen covering the surface of the cartilage and, while there is no actual invasion of the cartilage, the matrix appears depleted, as judged by eosinophilic staining with hematoxylin and eosin and diminished metachromatic staining. These observations led us to investigate the possibility that cartilage matrix depletion could be reproduced in vitro using tissue cultures of rheumatoid synovial cells.

Materials and Methods

Preparation of Synovial Cells and Cartilage.—Synovial membranes were obtained at arthrotomy from patients with osteoarthritis and meniscoid injuries (normal synovia) three patients with rheumatoid arthritis, and one with Reiter's syndrome. All samples were immediately placed in sterile cold nutrient medium (Dulbecco-Vogt, modification of Eagle's medium plus 10% calf serum) in which they were transported to the laboratory. Specimens were "floated" in phosphate-buffered saline or in medium in a sterile dish so that small samples of the lining cell layer could be cut off and placed in a plastic Petri dish (60 × 15 mm) scored on the bottom with a sterile knife so that the tissue fragment would adhere to the plate. The explants were nourished initially in medium containing 25% calf serum; this was decreased to 10% when a confluent monolayer of cells had developed and were ready to be subcultured. Incubation was carried out at 37°C in an atmosphere of 10% CO_2 and room air. Each cell strain had been subcultured two to four times before these experiments, representing at least 10 generations in culture plus an unknown number of generations required to form the original monolayer plate from the explanted fragments.

Three samples of articular cartilage were obtained from the knee joint (Table I): one from a patient (L.R.) with Reiter's syndrome (Experiment 1); one from a patient (B.R.) undergoing meniscectomy (experiment 2); and one from the recently amputated limb of a patient with diabetes and localized gangrene of the toes (experiment 3). These cartilage samples were also transported from the operating room to the laboratory in cold sterile tissue culture medium and were then divided into fragments of approximately 2 mm^3 with a scalpel. One of these fragments was frozen immediately as described below in preparation of histological sections. The other fragments were kept in tissue culture medium at 37°C for a period not longer than 4 days until transferred to plates containing synovial cell cultures.

Three experiments were performed (Table I). In each, the rheumatoid and nonrheumatoid synovial membrane cell cultures were transferred to fresh Petri dishes using 0.25% trypsin to disperse the cells. Several hours later, the new cultures were adherent to the plates. The medium was decanted to remove any remaining trypsin, and the cells were washed twice with fresh medium. A fragment of cartilage was placed in each culture dish amidst the synovial cells. One cartilage fragment was placed in a dish containing medium but without cells and served as a control. Fresh medium was added to all dishes, and subsequent medium changes were made twice weekly. After 1 wk, the cartilage fragments were adherent to the culture dishes and showed tiny cellular sprouts visible at their edges. After 2½ wk, no further "sprouting" was observed from the cartilage pieces. The surrounding synovial cells proliferated in their customary fashion.

Preparation of Histological Sections.—After 17–19 days of incubation, the cartilage fragments were freed by dissection from surrounding culture cells, rapidly frozen on a Freon

plate, and stored at −20°C. Later, a set of 6 μ cryostat sections was prepared from each. The specimen of nonincubated cartilage, rapidly frozen soon after removal at surgery, as well as cartilage fragments incubated in medium alone, served as controls. These were sectioned and stained in the same manner as were the test cartilage fragments.

Metachromasia: Cryostat sections were air-dried for 30 min, fixed in 10% buffered formalin for 30 min, and stained for metachromasia by the safranin O–fast green technique (10).

Basophilia: Sections for routine hematoxylin and eosin stains were prepared in a manner similar to those for metachromatic staining.

Preparation of Antisera for Immunofluorescence.—Rabbits were immunized with the readily sedimenting, or "heavy" fraction, of purified human articular cartilage protein-polysaccharide, and antiserum was collected in a manner previously described (7). A globulin

TABLE I

Sources of Synovial Membrane Cells and Cartilage

Patient	Diagnosis	Sex	Age	Designation
Experiment I				
Cartilage from L.R.	Reiter's syndrome	M	22	Cart. A
Synovial cells from H.P.	Osteoarthritis	F	55	NSM-4
" " " R.A.	Rheumatoid arthritis	M	40	RSM-7
" " " L.R.	Reiter's syndrome	M	22	Reiter's
Experiment 2				
Cartilage from B.R.	Torn meniscus	M	40	Cart. B
Synovial cells from R.A.	Rheumatoid arthritis	M	40	RSM-7
" " " B.R.	Torn meniscus	M	40	NSM-6
Experiment 3				
Cartilage from H.S.	Amputation for diabetic gangrene	F	80	Cart. C
Synovial cells from J.K.	Fracture of patella	M	30	NSM-5
" " " D.B.	Torn meniscus	F	40	NSM-7
" " " O.C.	Rheumatoid arthritis	M	27	RSM-8
" " " C.S.	Rheumatoid arthritis	F	26	RSM-9

fraction was prepared in the cold by mixing equal volumes of antiserum and saturated $(NH_4)SO_4$. The precipitate obtained by centrifugation was washed once with cold half-saturated $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in phosphate-buffered saline (pH 7.2) and dialyzed in the buffer.

Immunofluorescence.—Cryostat sections of cartilage fragments were mounted on glass slides, air-dried for 30 min, fixed in 10% phosphate-buffered neutral formalin for 5 min, and then washed in phosphate-buffered saline for 5 min. Then the globulin fraction of the rabbit antiserum was applied to the sections and allowed to react for 30 min in a moist chamber at room temperature. The sections were washed four times, 5 min each, in phosphate-buffered saline, then covered with fluorescein isothiocyanate–labeled goat anti-rabbit gamma globulin[1] for 30 min, washed again as above, and cover slips were mounted over phosphate-buffered glycerol-saline. Slides were viewed by fluorescence microscopy using a Zeiss Standard-Universal microscope equipped with: HB 200 Osram mercury bulb; UG-2 and BG-12 exciter filters;

[1] Antibodies, Inc., Davis, Calif.

dark field condenser; and 410 mμ barrier filter. Black and white photographs were reproduced from 35 mm color transparencies (high speed daylight ektachrome film, ASA 160, 3 min exposures).

Specificity of immunofluorescent reactions was assured by parallel testing on serial cartilage sections with: (*a*) antiserum thoroughly absorbed with antigen; (*b*) normal rabbit globulin substituted for the specific antiserum; and (*c*) direct application of the fluorescein-labeled goat anti-rabbit globulin. Fluorescence was absent or minimal in these instances, but was not reduced when the antiserum was absorbed with human serum proteins or other unrelated antigens, such as calf serum proteins.

TABLE II

Reactions of Cartilage Matrix in Tissue Culture Cells

	APPH* fluorescence	Safranin 0 meta-chromasia	Basophilia	Architecture
Experiment 1				
Cart. A control	4+	4+	3+	Intact
" A in NSM-4	3+	2+	Trace	Intact
" A in RSM-7	0	0	0	Total collapse
" A in Reiter's	3+	2+	Trace	Intact
Experiment 2				
Cart. B control	3+	1+	Trace	Intact
" B in NSM-6	1+	Trace	0	Intact
" B in RSM-7	0	0	0	Intact
Experiment 3				
Cart. C control	4+	4+	1+	Intact
" C in NSM-5	4+	4+	1+	Intact
" C in NSM-7	4+	4+	1+	Intact
" C in RSM-8	1+	0	0	Intact
" C in RSM-9	1+	Trace	0	Peripheral collapse

* Globulin fraction of rabbit antiserum to "heavy" fraction of cartilage protein-polysaccharide.

Each set of slides was evaluated for degree of safranin 0 metachromasia, basophilia, and matrix fluorescence, and scored on a 0 (absent) to 4+ (maximum) scale.

RESULTS

Results are summarized in Table II. In the three experiments, the cartilage matrix of all fragments which had been implanted in rheumatoid synovial cells showed striking loss of metachromasia (Fig. 1 *b*) and markedly diminished or absent fluorescence (Fig. 2 *b*). In experiment 1, the fragment appeared shrunken and fibrous with loss of lacunae (Fig. 3). In the other experiments, architectural details were better preserved, although in experiment 3 there was collapse of the peripheral stroma in one of the cartilage fragments implanted in rheumatoid cells.

The cartilage fragments removed from an environment of normal synovial cells, or Reiter's cells, reacted more like the control cartilage sections. They exhibited safranin O metachromasia (Fig. 1*a*) and fluorescence (Fig. 2 *a*) which, in some instances, were indistinguishable from the control cartilages and, in other instances, very slightly decreased compared to the controls.

In general, matrix basophilia was diminished in all cartilage fragments, including the controls, but the subtle changes which were observed paralleled the results obtained with safranin O.

An attempt was made to demonstrate enzymatic activity in the medium. From the various synovial cell monolayer cultures containing cartilage fragments, medium was withdrawn after 72 hr of incubation and added directly to plates containing cartilage fragments alone. Medium changes were carried out twice more at intervals of 72 hr, and then the cartilage fragments were examined. It was not possible to observe a consistent effect of normal or rheumatoid cell medium on the cartilage fragments either by metachromasia or immunofluorescence. It is conceivable that incubation times of 72 hr may lead to inactivation of enzymes released from the synovial cells into the medium. Studies using shorter periods of incubation are in progress.

DISCUSSION

These results show that rheumatoid synovial cells grown in tissue culture for many generations are able to deplete the matrix of a piece of articular cartilage present in the same medium. The absence of fluorescent staining as well as metachromasia indicates depletion of both the protein and polysaccharide components of the matrix. No such staining changes were observed in the cartilage fragments incubated in the presence of normal synovial cells or cells derived from the inflamed synovial membrane of a patient with Reiter's syndrome. The source of the experimental cartilage fragment did not seem to be significant, as the cartilage from a patient with Reiter's syndrome was used in one experiment and two different normal cartilage specimens were used in the other experiments.

The rheumatoid synovial cells in culture did not "invade" the cartilage fragments, and so depletion of the cartilage matrix appears to be due to release of enzymes from the rheumatoid cells. Since both metachromatic and fluorescent staining were lost, the enzymatic activity appears to be more "proteolytic" than "hyaluronidase-like." Lysomal enzyme activity might be responsible; both normal and rheumatoid cells in culture contain lysosomal enzymes, and the rheumatoid cells usually show increased activity as revealed by stains for acid phosphatase and β-glucuronidase (11). Yet, unless the local pH around the cartilage is somehow lower, the pH of the medium is likely to be uniformly neutral, a pH where lysosomal enzymes do not have optimal activity. However, Fell and Dingle (12) have shown that liver lysosomes can decrease the metachromasia of

cartilage matrix at pH 7 and abolish it completely at pH 6. Other enzymes, nonlysosomal in nature and presumably proteolytic, could be released by the rheumatoid cells. Recently it has been shown that pieces of rheumatoid synovia placed on collagen gels can hydrolyze the collagen (13).

A number of interesting means of observing the release and local action of "hydrolytic" enzymes from tissues or cells in vitro have recently been introduced. These include: the collagenolytic activity associated with the resorption of the tadpole tail (14); the dissolution of bone chips by HeLa cells in the presence of parathyroid hormone (15); the release of ^{45}Ca from bone by ascites cells previously exposed to parathyroid hormone (16); and the digestion of gelatin by bone sections (17).

The present study is a new contribution to the mechanism of cartilage matrix dissolution and is of particular importance because it incriminates rather specifically the rheumatoid synovial membrane cells. It is of further interest because it demonstrates that a unique trait of the rheumatoid cells is perpetuated through many generations in tissue culture.

SUMMARY

Articular cartilage fragments were added to monolayer cultures of synovial membrane cells. After 3 wk of incubation, the cartilage fragments were examined histologically for metachromasia and basophilia, and for fluorescent staining using a rabbit antiserum to cartilage protein-polysaccharide. Cartilage incubated with cells derived from rheumatoid synovial membranes showed striking loss of metachromasia and basophilia as well as diminished to absent fluorescent staining. Cartilage fragments incubated with cells from normal synovia, or with cells from the synovial membrane of a patient with Reiter's syndrome, did not show these changes and resembled control cartilage incubated in tissue culture medium alone. It appears, therefore, that rheumatoid synovial cells in tissue culture are able to deplete the matrix of articular cartilage.

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BIBLIOGRAPHY

1. Barnett, C. H. 1956. Wear and tear in joints. An experimental study. *J. Bone Joint Surg.* **38B**:567.
2. Dingle, J. T. 1962. Lysosomal enzymes and the degradation of cartilage matrix. *Proc. Roy. Soc. Med.* **55**:109.
3. Barland, P., A. B. Novikoff, and D. Hamerman. 1964. Lysosomes in the synovial membrane in rheumatoid arthritis; a mechanism for cartilage erosion. *Trans. Assoc. Amer. Physicians.* **77**:239.
4. Weissmann, G. 1966. Lysosomes and joint disease. *Arthritis Rheumat.* **9**:834.

5. Thomas, L. 1956. Reversible collapse of rabbit ears after intravenous papain, and prevention of recovery by cortisone. *J. Exptl. Med.* **104**:245.
6. Thomas, L., R. T. McCluskey, J. L. Potter, and G. Weissmann. 1960. Comparison of the effects of papain and vitamin A on cartilage. I. The effects in rabbits. *J. Exptl. Med.* **111**:705.
7. Sandson, J., L. Rosenberg, and D. White. 1966 The antigenic determinants of the protein-polysaccharides of cartilage. *J. Exptl. Med.* **123**:817.
8. Loewi, G. 1965. Localization of chondromucoprotein in cartilage. *Ann. Rheumatic Diseases.* **24**:528.
9. Barland, P., R. Janis, and J. Sandson. 1966. Immunofluorescent studies of human articular cartilage. *Ann. Rheumatic Diseases.* **25**:156.
10. Lillie, R. D. 1965. Histopathological Technic and Practical Histochemistry. McGraw-Hill Book Co., New York. 3rd edition. 507.
11. Goldfischer, S., C. Smith, and D. Hamerman. 1967. Increased lysosomes in rheumatoid synovial cells in tissue culture. *Arthritis Rheumat.* **10**:281.
12. Fell, H. B., and J. T. Dingle. 1963. Studies on the mode of action of excess of vitamin A. 6. Lysosomal protease and the degradation of cartilage matrix. *Biochem. J.* **87**:403.
13. Evanson, J. M., J. J. Jeffrey, and S. M. Krane. 1967. Characterization of a collagenolytic enzyme from cultures of rheumatoid synovium. *Arthritis Rheumat.* **10**:277.
14. Gross, J., and Y. Nagai. 1965. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc. Natl. Acad. Sci. U.S.* **54**:1197.
15. Boyle, A. B., and W. F. Neuman. 1965. Effects of parathyroid hormone on HeLa cell cultures. *J. Cell Biol.* **24**:316.
16. Tenenhouse, A., R. Meier, and H. Rasmussen. 1966. Parathyroid hormone and bone mobilization *in vitro*. *J. Biol. Chem.* **241**:1314.
17. Bélanger, L. F., and B. B. Migicovsky. 1963. Histochemical evidence of proteolysis in bone: the influence of parathormone. *J. Histochem. Cytochem.* **11**:734.

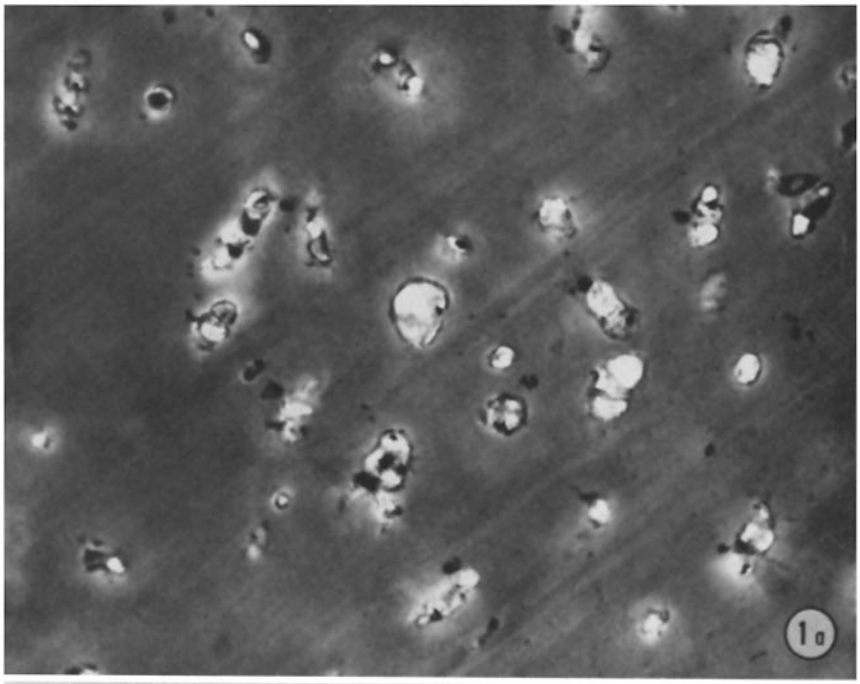
EXPLANATION OF PLATES

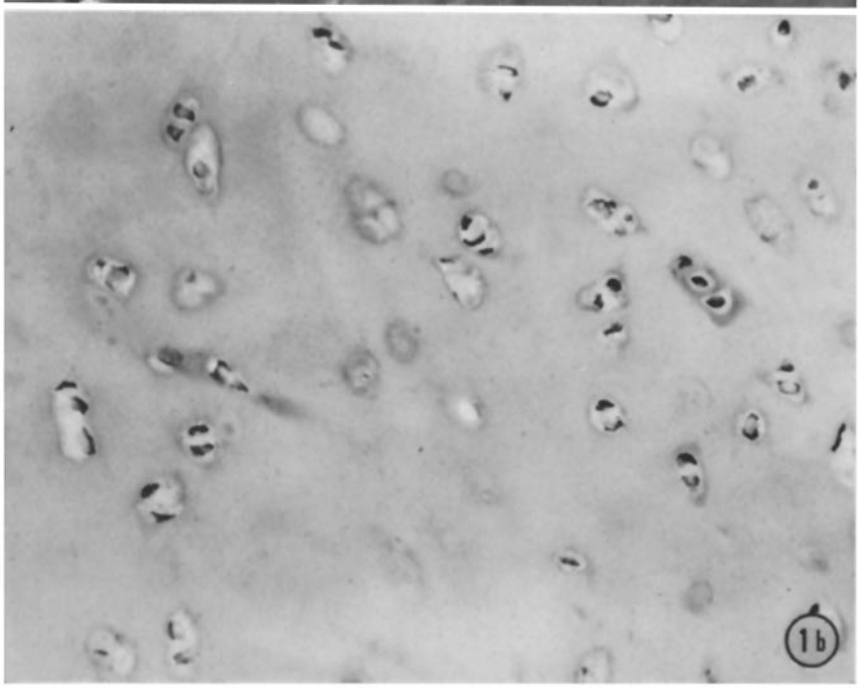
PLATE 77

FIG. 1. Cryostat sections of cartilage fragments previously incubated in synovial cell cultures for 19 days and stained with safranin O. × 210.

FIG. 1 *a*. Cartilage which was incubated in normal synovial membrane cells (cartilage C in NSM-7) and which exhibits strongly positive matrix metachromasia.

FIG. 1 *b*. Cartilage which was incubated in rheumatoid synovial membrane cells (cartilage C in RSM-9) and which shows only minimal matrix metachromasia. The closer spacing of the chondrocyte lacunae in this section suggests loss of matrix substance.





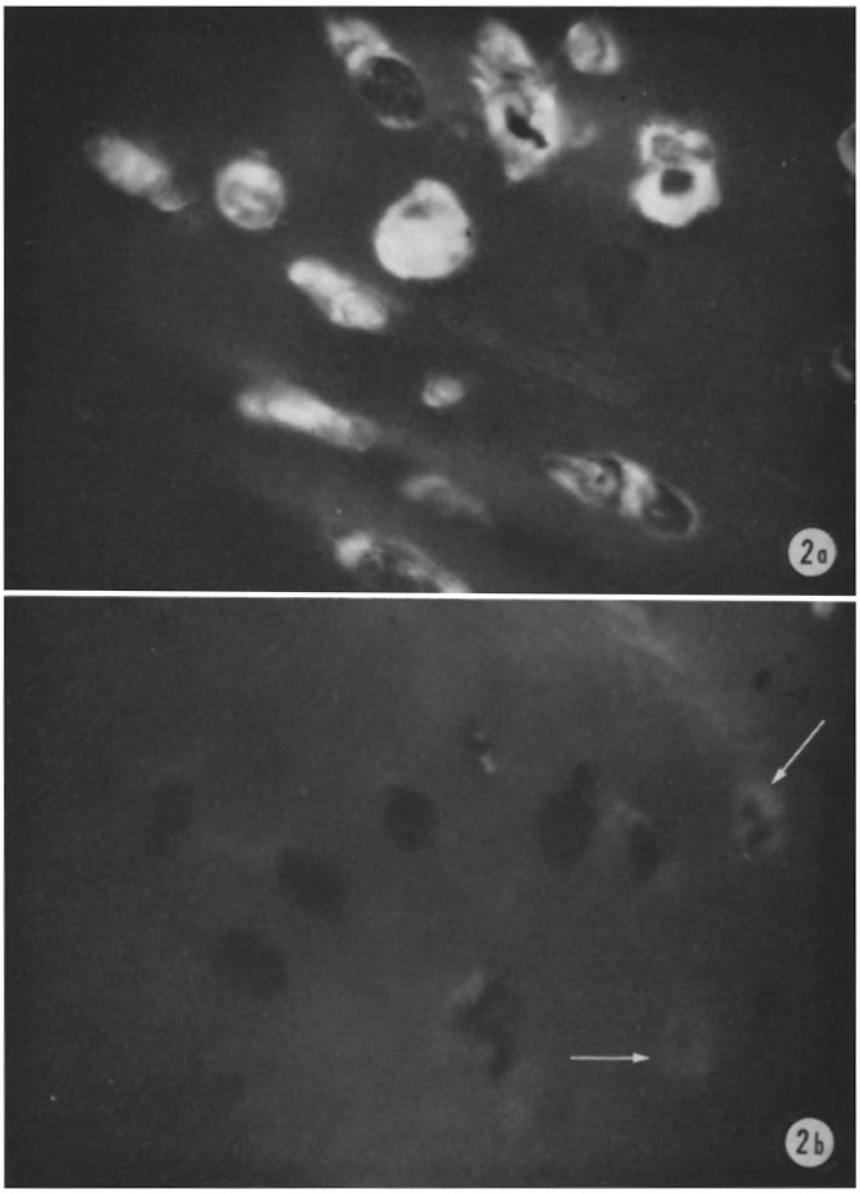
(Hamerman et al.: Cartilage matrix depletion by synovial cells)

PLATE 78

FIG. 2. Cryostat sections of cartilage fragments, previously incubated in synovial cell cultures for 19 days and reacted with rabbit antiserum to cartilage protein-polysaccharide and fluorescein-labeled goat anti-rabbit globulin. Enlarged approximately × 600.

FIG. 2 *a*. A cartilage fragment which had been incubated in normal synovial membrane cells (cartilage A in NSM-4) and which retains bright perilacunar matrix fluorescence.

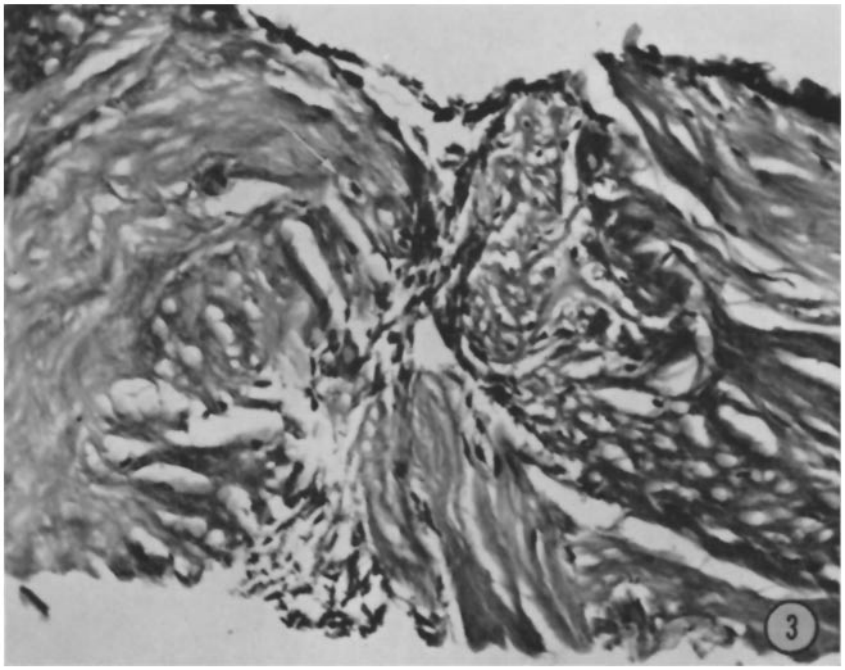
FIG. 2 *b*. A cartilage fragment which had been incubated in rheumatoid synovial membrane cells (cartilage C in RSM-8) and shows only trace amounts of perilacunar matrix fluorescence (arrows).



(Hamerman et al.: Cartilage matrix depletion by synovial cells)

PLATE 79

FIG. 3. This section reveals the total collapse of cartilage matrix after incubation of this fragment in rheumatoid synovial membrane cell culture for 19 days (cartilage A in RSM-7). Collagen fibers surround distorted lacunae (arrow). Hematoxylin and eosin. × 250



(Hamerman et al.: Cartilage matrix depletion by synovial cells)