A CONSIDERATION OF THE PERMEABILITY OF CARTILAGE TO INORGANIC SULFATE

ROBERT D. CAMPO and DOMINIC D. DZIEWIATKOWSKI, Ph.D.

From The Rockefeller Institute

ABSTRACT

On the basis of an examination of autoradiograms of knee-joints fixed so as to remove chondroitin sulfate or inorganic sulfate, or to minimize the loss of both, it is suggested that the cartilage is permeable to inorganic sulfate *in vivo* and *in vitro*. *In vivo* and *in vitro*, almost as rapidly as it enters the cartilage, inorganic sulfate is utilized by the cells in the synthesis of chondroitin sulfate. The net result is a continuing low concentration of inorganic sulfate in the cartilage.

The chondroitin sulfates of cartilaginous tissue are rapidly labeled with sulfur-35 when the latter is administered as inorganic sulfate (1, 2). For at least 24 hours, labeling of the chondroitin sulfates continues despite a precipitous fall in the concentration of the inorganic S^{35}-sulfate in the blood and in the cartilage (2, 3). Inherent in these observations is the necessary condition that cartilage, which is avascular, is permeable to either sulfate ions or to sulfate complexed so as to mask its negative charge.

Kantor and Schubert (4) suggested that cartilage excludes or repels anionic dyes because of the high density of the fixed negative charges on the polyanion chondroitin sulfate which is highly concentrated in cartilage. Although it has been demonstrated also that demineralized cartilage exhibits the properties of a cation exchanger (5) it does not follow necessarily that it behaves in this manner in an animal. Indeed, an interferometric study (6) on the diffusion of salts suggests that sulfate may diffuse into the nucleus pulposus, a tissue which resembles cartilage, at a rate which is about half the rate of diffusion in water.

With the above observations and suggestions in mind, an autoradiographic study on the microscopic level was made of the entry of inorganic S^{35}-sulfate into, and its utilization by, epiphyseal cartilages of young rats. The sulfur-35 present in the cartilage as inorganic sulfate was differentiated from the sulfur-35 present therein as chondroitin sulfate on the basis of the autoradiograms obtained after the use of a variety of fixatives.

MATERIALS AND METHODS

Twenty-five microcuries of carrier-free sulfur-35 as H_2SO_4 was injected intraperitoneally into each of six 7-day-old and six 22-day-old rats. The knee-joints were removed from two representative animals of each age group 30, 60, and 120 minutes later. The knee-joints were split lengthwise and each of the four "halves" representative of an animal was fixed separately and differently:

1. In a 10 per cent solution of formalin.
2. In a 10 per cent solution of formalin saturated with barium hydroxide.
3. In a 10 per cent solution of formalin which also contained 10 gm. of barium acetate ($Ba(C_2H_3O_2)_2$) per 100 ml.
4. In a mixture of one part absolute ethanol and one part of absolute methanol.

The tissue was fixed for 48 hours at 25°C. Following dehydration in ethanol and clearing in xylol the tissue was embedded in paraffin. Sections were cut at 7 μ. After removing the paraffin from the sections with xylol, contact (7), coated (8), and stripping film (9), autoradiograms were prepared.

In parallel with the above, the knee-joints from six 7-day-old rats and from six 22-day-old rats were removed and split lengthwise. The four "halves"

representative of each animal were then immediately incubated together in 10 ml. of a solution of salts labeled with 5 microcuries of S^{35}-sulfate and buffered to pH 7 with phosphate (10). The 25 ml. Erlenmeyer flasks were open to the air and were shaken at 60 strokes per minute in a water bath at 37°C. The tissue from two flasks containing the cartilage pieces of 7-day-old rats and the tissue from two flasks containing the cartilage pieces from 22-day-old rats was removed after 30, 60, and 120 minutes of incubation. After the tissue of each flask was rinsed in 10 ml. of an unlabeled solution of salts, the pieces were fixed separately and differently, as above, prior to the preparation of autoradiograms.

RESULTS AND DISCUSSION

The organization of the articular cartilage in the knee-joints of 22-day-old rats is shown in Fig. 1. Representative autoradiograms of similar regions of cartilages incubated in a solution of salts containing S^{35}-sulfate are reproduced in Figs. 6 through 11. An examination of Figs. 6 to 8 shows that, after fixation of the tissue in 10 per cent formalin, sulfur-35 is demonstrable in increasing amounts in the cells. There is evidence suggesting that most, if not all, of the sulfur-35 thus visualized is present in the cells as chondroitin sulfate. From cartilage slices similarly incubated and fixed in formalin as much as 89 per cent of the sulfur-35 has been recovered as chondroitin sulfate (11). On the other hand, very little if any, inorganic S^{35}-sulfate should be present in tissue fixed in this manner; one would expect the inorganic S^{35}-sulfate to have been extracted in the process of fixation in 10 per cent formalin and in the subsequent dehydration, initiated in 30 per cent ethanol.

Some evidence for the removal of chondroitin sulfate from cartilage by fixation in a solution of

FIGURE 1

A photomicrograph of a region in the articular cartilage of a knee-joint, representative of the regions which produced the autoradiograms shown in Figs. 6 to 11. The knee-joint was from a 22-day-old rat. It was fixed in 10 per cent formalin and the sections were stained with toluidine blue. × 148.

FIGURE 2

A photomicrograph of a region in an epiphyseal plate, typical of the regions which produced the autoradiograms shown in Figs. 12 to 17. The epiphyseal plate was from a knee-joint of a 7-day-old rat. The knee-joint was fixed in 10 per cent formalin and the sections were stained with toluidine blue. × 148.

FIGURE 3

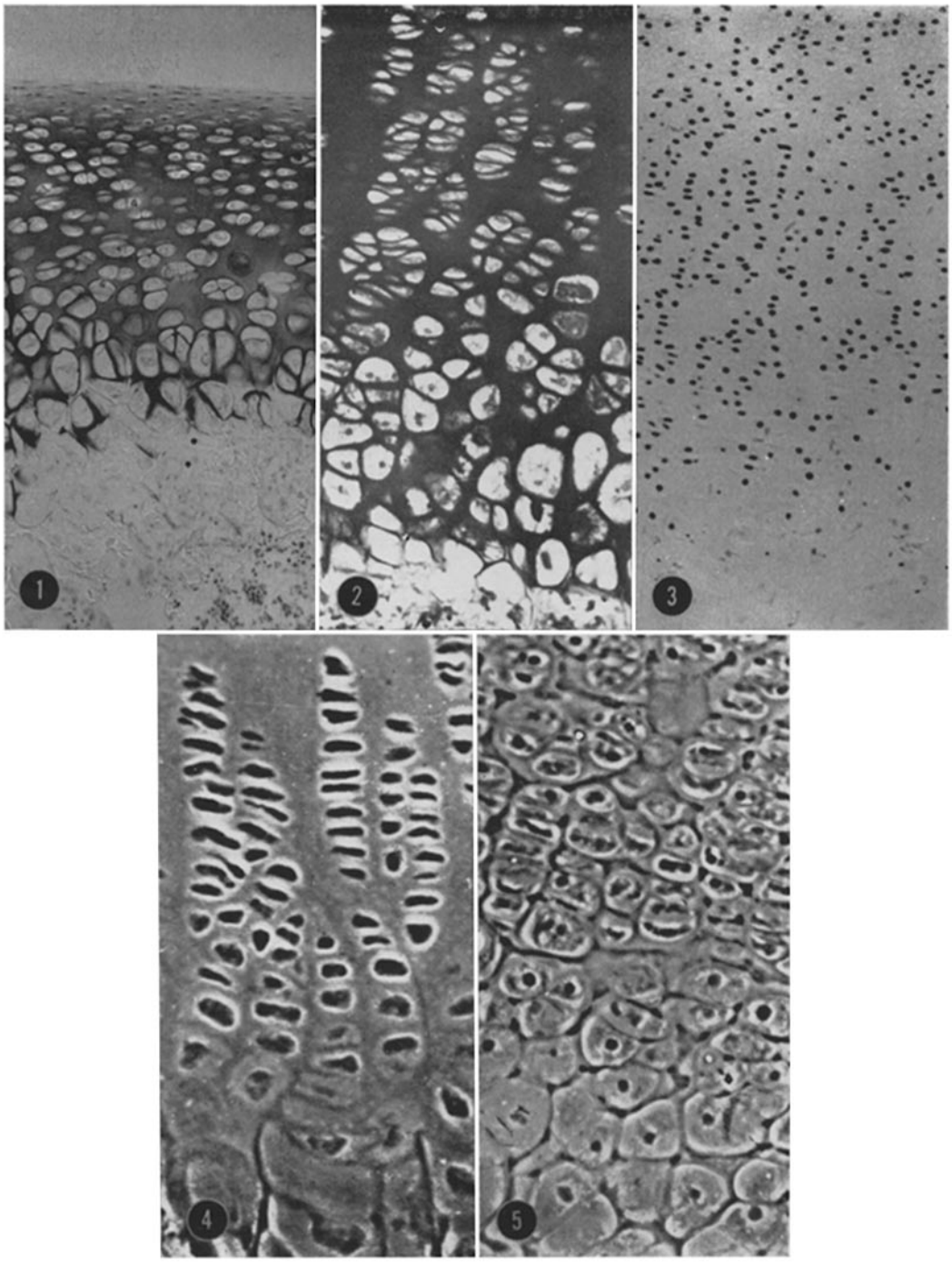
A photomicrograph of a region in an epiphyseal plate from a knee-joint of a 7-day-old rat. The region is comparable to that shown in Fig. 2; however, the section was prepared from a knee-joint fixed in a 10 per cent solution of formalin, which was saturated with barium hydroxide. It, too, was stained with toluidine blue. The nuclei stained orthochromatically, whereas in the rest of the tissue practically no stain was evident. × 148.

FIGURE 4

A photomicrograph of a region in the epiphyseal plate from a knee-joint of a 7-day-old rat, as seen in a phase contrast microscope. The knee-joint was fixed in 10 per cent formalin and the section was not stained. × 296.

FIGURE 5

A photomicrograph of a region in the epiphyseal plate from a knee-joint of a 7-day-old rat, as seen in a phase contrast microscope. The unstained section was from a knee-joint fixed in a 10 per cent solution of formalin saturated with barium hydroxide. It can be seen that the intercellular matrix is absent and consequently the swollen, distorted cells are in close proximity to each other. × 296.


1
2
3
4
5

formalin saturated with barium hydroxide has been recorded previously (7, 12–14). Further suggestions that this is so derive from an examination of the sections reproduced as Figs. 2 to 5. In Fig. 3 it can be seen that toluidine blue has stained only the nuclei—orthochromatically. The basophilia and metachromasia ordinarily associated with cartilage were abolished. In contrast, after fixation in formalin only (Fig. 2) the matrix was densely stained and exhibited its characteristic metachromasia. The reason for this is partly evident from a comparison of Fig. 4 with Fig. 5. During fixation in formalin saturated with barium hydroxide, the chondroitin sulfate of the intercellular matrix is removed and the cells swell until they come almost in contact with one another. The absence of sulfur-35 in the cells (Figs. 9 to 11 and Figs. 15 to 17) indicates that chondroitin sulfate is removed also from the cells. Whether the proteins of the matrix and of the cells are extracted is not known. A suggestion that the proteins are not removed completely comes from a comparison of Fig. 4 with Fig. 5. In the latter the material between the cells is optically denser than that between the cells after formalin fixation. This may reflect a concentration of the protein components due to compression.

The autoradiograms produced by sections of tissue fixed in formalin saturated with barium hydroxide presumably represent inorganic S^{35}-sulfate. The argument is that by the use of this fixative chondroitin sulfate is solubilized and removed, whereas the inorganic sulfate is precipitated *in situ* as barium sulfate. It then follows that though a considerable amount of inorganic sulfate enters cartilage rapidly, it is also rapidly swept into the cells and incorporated into chondroitin sulfate. The net result, as can be seen in Figs. 9 to 11, is that the concentration of inorganic sulfate remains low.

The autoradiograms of the knee-joints removed from animals given inorganic S^{35}-sulfate intraperitoneally were essentially similar to those prepared from knee-joints incubated in a labeled solution of salts. This is evident from a comparison of Figs. 12 to 17 with Figs. 6 to 11. The comparison of these two plates also brings out a difference. *In vitro*, the inorganic S^{35}-sulfate progressively increased in the cartilages (Figs. 9 to 11); on the other hand, in the *in vivo* experiments no differences were discernible in the concentration of inorganic sulfate with time (Figs. 15 to 17). This difference is explicable if one considers that *in vitro* the concentration of S^{35}-sulfate in the bathing medium is decreased only slightly because of the entry of the inorganic sulfate into the cartilage and, therefore, the incubation medium constitutes an essentially constant reservoir. *In vivo*, however, the concentration of inorganic S^{35}-sulfate in the circulating fluids drops precipitously (2, 3).

It is worthy of note that by the use of the fixatives

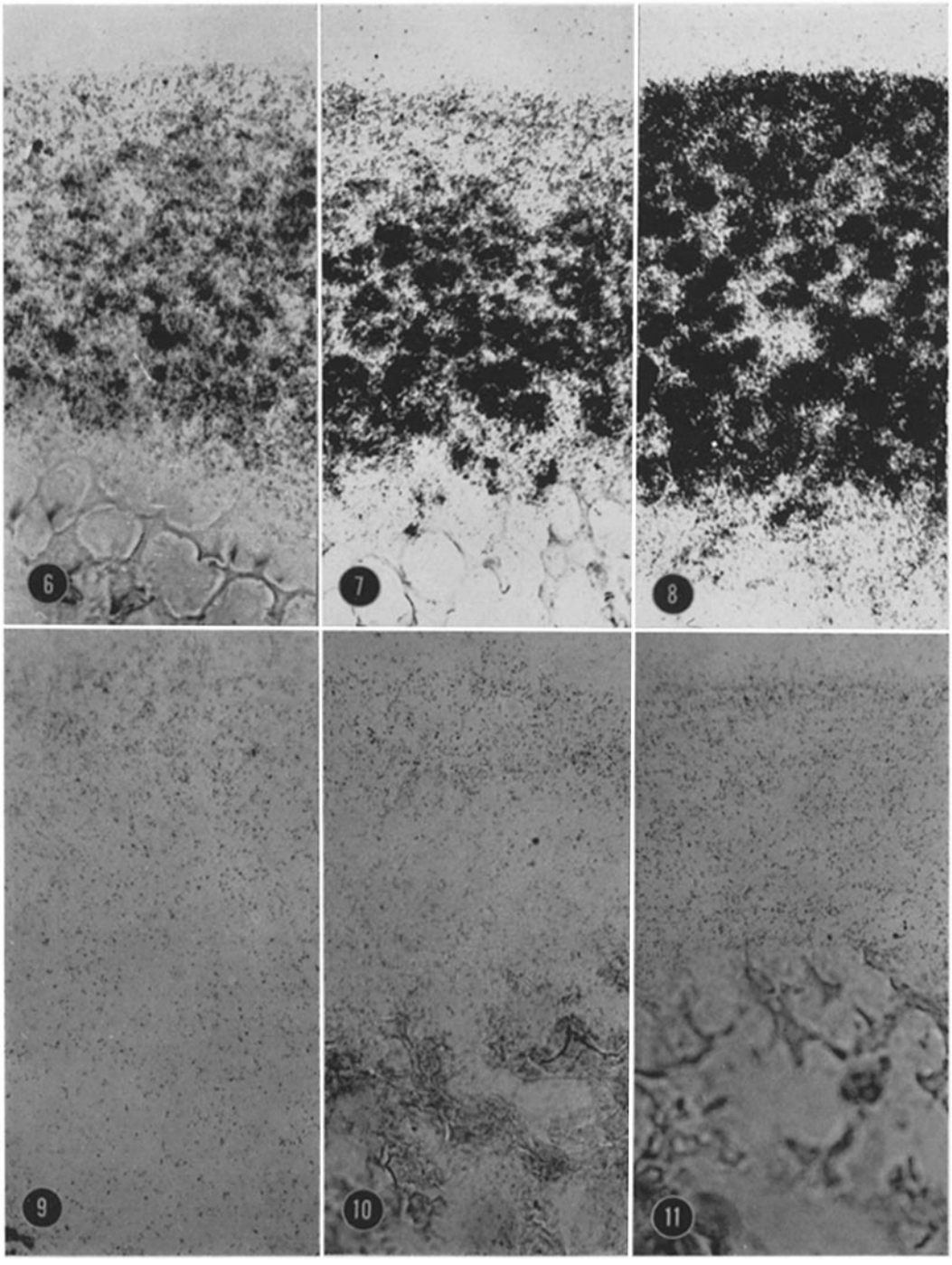
Reproductions of autoradiograms of regions in the articular cartilage of 22-day-old rats. The autoradiograms were prepared with Kodak NTB_3 liquid emulsion, using unstained sections. The articular cartilage is from knee-joints which were incubated in a solution of salts containing 5 microcuries of S^{35}-sulfate per 10 ml. of solution. × 148.

FIGURES 6 to 8

Autoradiograms of the articular cartilage after incubation for 30, 60, and 120 minutes at 37°C., respectively. The knee-joints were fixed in 10 per cent formalin. The autoradiographic reaction is primarily intracellular and shows that the cells rapidly and continuously "fix" S^{35}-sulfate over the period of 120 minutes.

FIGURES 9 to 11

Autoradiograms of the articular cartilage after incubation for 30, 60, and 120 minutes at 37°C., respectively, as above. However, in this case the knee-joints were fixed in a 10 per cent solution of formalin saturated with barium hydroxide. The exposure time was the same as that used in the preparation of the autoradiograms shown as Figs. 6 to 8. In contrast to the results seen in the latter figures, it can be seen that there is only a slight over-all reaction, which increases but little with time. There is some indication that the cartilage proximal to the articulating surface has more sulfur-35 than the rest of the cartilage.


6
7
8
9
10
11

which were expected to minimize the loss of inorganic sulfate and chondroitin sulfate, formalin with barium acetate or a mixture of equal parts of absolute ethanol and absolute methanol, more pronounced autoradiographic reactions were obtained. The densities of such autoradiograms were judged to be summations of the densities seen in the autoradiograms of tissue fixed in formalin and in formalin saturated with barium hydroxide.

How to reconcile the above observations on the entry of the sulfate ion into cartilage with the suggestion that cartilage is impermeable to anions (4) remains a question. It is unlikely that the charge on the sulfate ion is masked so as to allow it to enter cartilage. Though such a hypothesis might be tenable as a possible explanation of the *in vivo* results, it is unlikely as an explanation of similar results obtained *in vitro*. It is more likely that, if there is a masking of charges, the negative charges on the chondroitin sulfate are masked by an association of these polyanions with proteins. Cationic dyes then could enter cartilage if their affinities were greater than the affinities of the proteins for the chondroitin sulfate. Conversely, if the affinities of the anionic dyes were weaker than the affinity of chondroitin sulfate for the proteins, the anionic dyes would not be accepted by the cartilage. One might hypothesize further thus to explain the repeated observations that calcium does not enter cartilage except at the calcification front, where for some reason, as yet unknown, the anionic nature of chondroitin sulfate is revealed.

The S^{35} used in this investigation was supplied by the Oak Ridge National Laboratory.

Received for publication, September 21, 1960.

BIBLIOGRAPHY

1. Dziewiatkowski, D. D., 1951, *J. Biol. Chem.*, **189**, 187.
2. Boström, H., 1952, *J. Biol. Chem.*, **196**, 447.
3. Dziewiatkowski, D. D., 1949, *J. Biol. Chem.*, **178**, 197.
4. Kantor, T. G., and Schubert, M., 1957, *J. Histochem. and Cytochem.*, **5**, 28.
5. Boyd, E. S., and Neuman, W. F., 1951, *J. Biol. Chem.*, **193**, 243.
6. Paulson, S., Sylven, B., Hirsch, C., and Snellman, O., 1951, *Biochim. et Biophysica Acta*, **7**, 207.
7. Dziewiatkowski, D. D., 1951, *J. Exp. Med.*, **93**, 451.
8. Gross, J., Bogoroch, R., Nadler, N. J., and Leblond, C. P., 1951, *Am. J. Roentgenol. and Radium Therap.*, **65**, 420.
9. Pelc, S. P., 1947, *Nature*, **160**, 749.

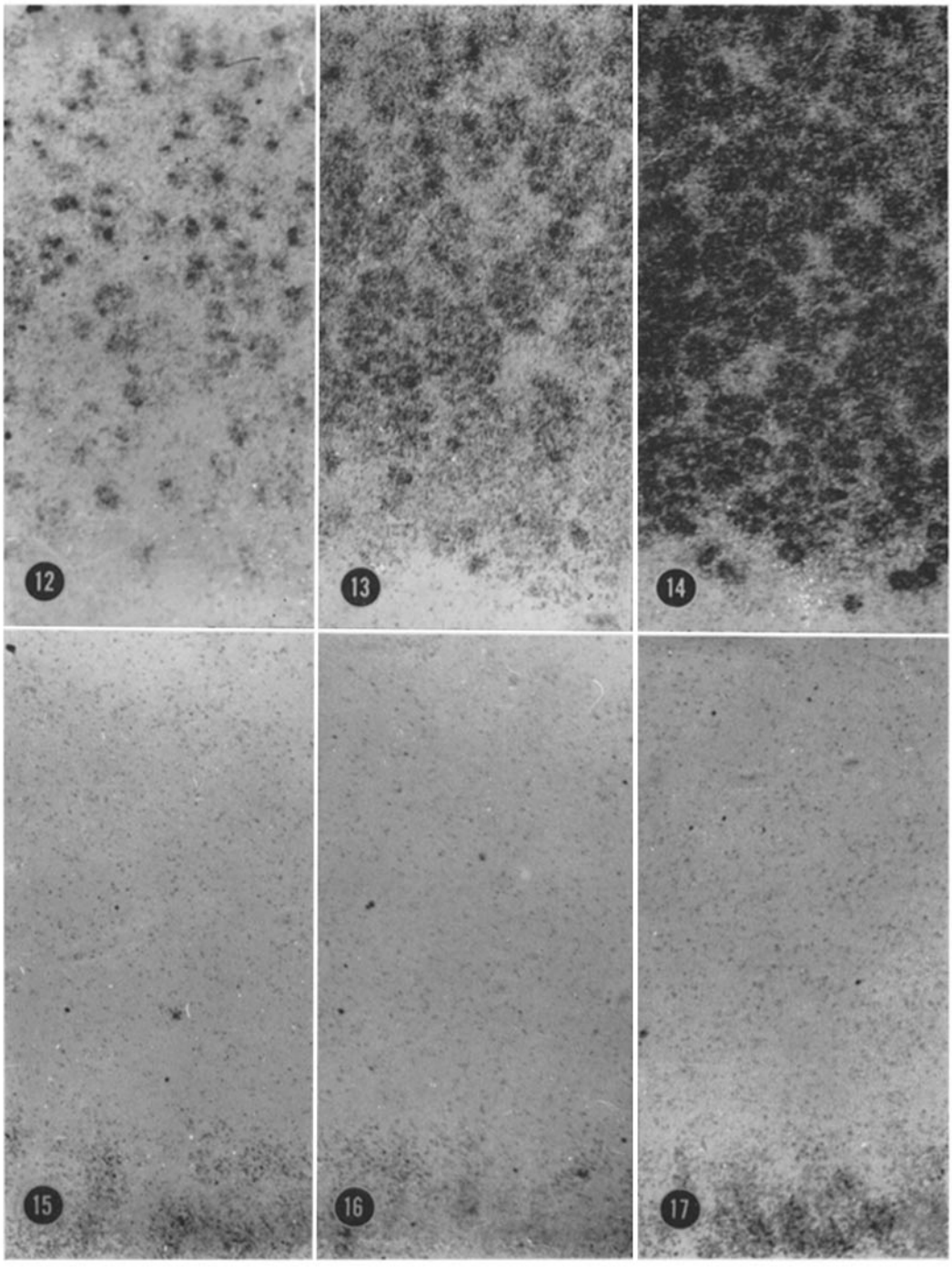
Reproductions of autoradiograms of regions in the epiphyseal plates of knee-joints. The autoradiograms were prepared using Kodak AR 10 stripping film. The sections were unstained and were prepared from the knee-joints of 7-day-old rats that had received 25 microcuries of S^{35}-sulfate intraperitoneally. × 148.

FIGURES 12 to 14

Autoradiograms of sections from knee-joints removed 30, 60, and 120 minutes, respectively, after administration of the S^{35}-sulfate. The knee-joints were fixed in 10 per cent formalin. As in the case of the articular cartilage of knee-joints incubated in a solution of salts containing S^{35}-sulfate, the autoradiographic reaction is predominantly given by the cells. There is, however, also an increase with time in the "fixed" S^{35} of the matrix.

FIGURES 15 to 17

Autoradiograms of sections from knee-joints removed 30, 60, and 120 minutes, respectively, after administration of the S^{35}-sulfate. The knee-joints were fixed in a 10 per cent solution of formalin, which was saturated with barium hydroxide. Again, as was the case with the knee-joints incubated in a solution of salts containing S^{35}-sulfate, a comparatively small amount of sulfur-35 is visualized. In contrast to the *in vitro* experiments, however, the concentration of the S^{35}-labeled material (presumably inorganic sulfate) does not increase with time. An examination of the lower portion of each of the figures reveals that the reaction of the metaphysis is greater than that of the cartilage.


12
13
14
15
16
17

10. KLEBANOFF, S. J., DZIEWIATKOWSKI, D. D., and OKINAKA, G. J., 1958, *J. Gen. Physiol.*, **42**, 303.
11. DZIEWIATKOWSKI, D. D., unpublished observations.
12. DZIEWIATKOWSKI, D. D., 1952, *J. Exp. Med.*, **95**, 489.
13. DZIEWIATKOWSKI, D. D., 1954, *J. Exp. Med.*, **99**, 283.
14. DZIEWIATKOWSKI, D. D., BRONNER, F., DIFERRANTE, N., and ARCHIBALD, R. M., 1957, *J. Biophysic. and Biochem. Cytol.*, **3**, 151.