RADIOAUTOGRAPHIC VISUALIZATION OF SULFUR-35 DISPOSITION IN THE ARTICULAR CARTILAGE AND BONE OF SUCKLING RATS FOLLOWING INJECTION OF LABELED SODIUM SULFATE

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PLATES 31 AND 32

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In a previous paper (1) it was reported that after intraperitoneal administration of sulfur-35 in the form of sodium sulfate to suckling rats, 7 days of age, the isotope was demonstrable in the knee joint cartilage during the following 164 hours. Up to the 24th hour the concentration of sulfur-35 was on the increase and thereafter up to the 164th hour its concentration decreased, but slowly. It was suggested in the same paper that most, if not all, of the sulfur-35 retained after 24 to 48 hours in the knee joint cartilage may have been retained therein as chondroitin sulfate. This suggestion was based on observations that the sulfur-35 concentration was not appreciably decreased in an aqueous solution of cartilage, which had been removed from suckling rats injected with labeled sodium sulfate by the addition to the solution of solid barium carbonate to saturation followed by heating for 6 hours on a steam-bath. Had the sulfur-35 been present as inorganic sulfate or in any form which was insoluble as the barium salt it is highly probable that it would have been removed from the solution by the centrifugation and filtration which followed the heating period with barium carbonate, whereas the barium salt of chondroitin sulfate would have remained in solution. This suggestion has been substantiated by work reported in a more recent paper (2) on the isolation of chondroitin sulfate with sulfur-35 incorporated therein after administration of this isotope as sodium sulfate.

Our curiosity was aroused as to how the isotope, sulfur-35, when given as sodium sulfate was taken up and distributed in the epiphyseal cartilage of suckling rats. An attempt to answer this question by the radioautographic technique has been made. The results are here reported.

Method

Suckling rats of the Whelan strain were used. In one series of experiments 2 mg. of S^{35}-tagged sodium sulfate[1]- (8.24×10^5 C.P.M.) in 0.1 ml. distilled water was injected intraperi-

[1] The sulfur-35 used in this investigation was supplied by the Oak Ridge National Laboratory, on allocation from the United States Atomic Energy Commission.

The activity was determined on the $BaSO_4$ precipitated from an aliquot of the solution

toneally into each of 30, 7-day-old animals of 3 litters. Two representative animals from each litter were sacrificed by decapitation at 24, 48, 96, 216, and 290 hours after administration of the isotope.

In a second series of experiments 4 mg. of labeled sodium sulfate (13.6×10^5 C.P.M.) in 0.1 ml. distilled water was injected intraperitoneally into four 15-day-old rats. These sucklings were sacrificed 24 hours later.

Within 3 minutes of the time when an animal was sacrificed its carcass was placed in a cold room at 0°C. Before an hour had elapsed the humeri, and the tibiae, still connected through the knee joints to the femurs, were removed, the soft tissue was trimmed away, except for cartilage and ligaments, and they were placed in fixative at room temperature. One of the humeri and a tibia-femur combination from each animal were fixed for 24 hours in 3.7 per cent formalin (one volume of 37 per cent U.S.P. formaldehyde, Merck, was diluted with nine volumes of distilled water). The opposite humerus and tibia-femur combination were fixed for 24 hours in 3.7 per cent formalin saturated with barium hydroxide. All bones were imbedded in paraffin, cut at 7μ, and the sections transferred to slides coated with egg albumin. It was unnecessary to decalcify the bones for sectioning. The paraffin was removed with xylol and the mounted sections were then covered in a dark room with Kodak ortho process film, cut to 3 inches by 1 inch size, with the emulsion facing the sections. The top surface of the sections was about 20μ from the surface of the emulsion, a separation achieved by placing a narrow strip of glassine paper[2] at each end of the microscope slide. A clean slide was used as a backing for the film. The film and the sections may be likened to the filling in a sandwich composed of glass slides. Each "sandwich" was held together by rubber bands and was wrapped individually in black paper.

After 3 to 7 weeks in the dark at 20°C., the film was developed in Kodak D-19 for 5 minutes, washed for 30 seconds in 1 per cent acetic acid, and fixed in Kodak F-5 for 20 minutes or more. The film was finally washed in running tap water for 30 minutes or more, rinsed in distilled water, and dried. Subsequently the sections were stained with hematoxylin-eosin or with toluidine blue, 0.1 per cent in 30 per cent ethanol.

Some sections of humeri were floated directly onto the emulsion of Kodak lantern slide plates, as described by Evans (3). At the end of the exposure time, 3 to 7 weeks at 20°C. in the dark, development of an image was effected in the emulsion by the process described above for the film.

Recently Boyd and Board (4) reported the production of images on photographic emulsions by the action of fresh histological specimens of normal tissue. Yagoda (5) also warns against pseudophotographic effects. With these warnings in mind, controls were run in which sections of humeri from uninjected suckling rats were used together with the emulsions employed for the radioautographs. The time of exposure to these control sections was similar to the time which sufficed to produce an image on development when sulfur-35 was present. No image was found on the emulsions in the control experiments.

RESULTS AND DISCUSSION

A series of radioautographs and the corresponding sections, stained with toluidine blue, of humeri from rats of the same litter is presented in Figs. 1*a*

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used. The determination was corrected to a weight of 10 mg. $BaSO_4$ in a ¼ oz. Buckeye ointment tin. A G-M tube with the mica window, 1.50 mg./cm.^{2}, 1 cm. from the surface of the $BaSO_4$ precipitate was used.

[2] Eli Lilly and Co., Indianapolis, powder paper (glassine) was used. The sections were not covered by this paper.

to 5*b*. These are sections of humeri fixed in 3.7 per cent formalin, pH 3.8–3.9, from rats that were 7 days old at the time the labeled sodium sulfate was administered. It is indicated by the radioautograph (Fig. 1*b*) of the humerus removed 24 hours after isotope administration that the sulfur-35 is present by this time throughout the entire epiphysis. The isotope, however, is not equally distributed; it is present in highest concentration at or near the junction of the cartilage with the diaphysis. That is, the sulfur-35 concentration is greatest in the area of cartilage where replacement by bone marrow and bone is about to occur. At the end of 48 hours (Fig. 2*b*) there appears to be no marked alteration in the distribution of the sulfur-35 in the epiphysis from that seen at the end of 24 hours. By the end of 96 hours (Fig. 3*b*) the radioautographs reveal that there has been a decrease in the ratio of sulfur-35 concentration in the cartilage adjoining the diaphysis to the concentration in the rest of the epiphysis. That is, the labeling isotope has become more uniformly distributed. That a process of equalization was operative is unquestionably indicated by the radioautographs (Figs. 4*b* and 5*b*) of humeri removed 216 hours and 290 hours after administration of the isotope.

There is undoubtedly some loss of the isotope from the epiphyseal cartilage between the 24th and 290th hour of observation. One would suspect that this loss would be most pronounced in the areas adjacent to the diaphysis, since it is here that bone marrow and bone are known to invade and replace the cartilage. Previous observations (1) have indicated that when sulfur-35 is administered as sodium sulfate to suckling rats on the 7th day of life the concentration of this isotope changes very slowly in the articular cartilage between the 8th and 15th day of life. Hence, it is probable that in the present experiments the equalization of the concentration of the isotope in the epiphyseal cartilage with the passage of time is due in large measure to an attainment of a more uniform distribution therein and that only a small fraction of the sulfur-35 was lost from the epiphyses of the humeri up to about the 200th hour after administration.

As centers of secondary ossification arise in the cartilage, the sulfur-35 appears to diminish in concentration and to disappear from these loci. That it does not actually disappear to the extent indicated in the radioautographs, Figs. 2*b*, 3*b*, 4*b*, and 5*b*, will be apparent from what is to follow in the discussion of radioautographs of humeri fixed in formalin saturated with barium hydroxide.

Conceivably the greater sulfur-35 concentration in the cartilage at its junction with the diaphysis 24 hours after administration of the labeled sodium sulfate may be due to a difference in either the composition, degree of metabolic activity, or blood supply in this area as compared with that further removed from the epiphyseal-diaphyseal border. At present it is impossible to indicate the reason for the preferential accumulation of the sulfur-35 at this border. Streeter's (6) classification of cartilage cells has proved useful in reporting its

distribution. According to Streeter those cartilage cells which are emerging or have just emerged from the skeletal blastema are classified as being in phase 1. They show no marked pattern in their arrangement. In phase 2 are the slender cartilage cells that are actively proliferating in cell tiers transverse to the long axis of the humerus. In phase 3, the cells are three or more times as large as in phase 2; they are now cuboidal with many vacuoles and cell division is still present. Cells of maximum size surrounded by opaque intercellular substance (the whole like a honeycomb) are classified as in phase 4. In the last phase, phase 5, the cartilage cells are disintegrating to varying degrees and in some areas only the intercellular support of the honeycomb remains. By thus labeling the cartilage cells in the epiphyses under discussion it becomes possible to state that the labeling isotope accumulated to a greater extent, by the 24th hour following its administration, in areas which contained cells in phases 3, 4, and 5. This conclusion is substantiated by radioautographs of sections of humeri which were fixed in formalin on removal from 15-day-old rats 24 hours after administration of labeled sulfate. One such autograph is presented in Fig. 6*b*. It should be noted that in this autograph there are two zones of relatively high sulfur-35 concentration: an area at or near the junction of cartilage and diaphysis and an area surrounding the fairly well developed centers of secondary ossification. These two zones have the most mature cartilage cells, namely cells in phases 3, 4, and 5.

It is further possible to say that the sulfur-35 concentration is not as great in the region of phase 5 cartilage cells as it is in the region of somewhat younger cells, those of phases 3 and 4. Evidence for this was obtained in radioautographs of humeri prepared according to the method of Evans (3). 24 hours after the administration of S^{35}-labeled sodium sulfate to 7-day-old suckling rats, their humeri were removed, fixed in formalin, embedded in paraffin, sectioned at 7μ, and then floated in the dark onto the emulsion of lantern slides. They were let stand in the dark at 20°C. for 4 weeks and remained on the emulsion during and following photographic development. A photomicrograph of part of such a resulting radioautograph, in which cartilage cells of phases 3, 4, and 5 are seen superimposed on the developed silver grains of the emulsion, is presented in Fig. 7. One sees in such magnified autographs that the concentration of silver grains is not as great beneath phase 5 cells as it is in the area covered by phase 4 and 3 cartilage cells. It would also appear that the sulfur-35 is present in both the cartilage cells and the intercellular substance, the matrix. One cannot be sure that grains of silver beneath the intercellular substance are not in part the result of the action of emanations arising from within the cells. With equal possibility those underneath the cells might have resulted from emanations originating within the matrix.

In all the radioautographs discussed thus far, whenever there was evidence of sulfur-35 localization in the diaphysis it was primarily in the periosteum

(see Figs. 1*a* to 6*b*). This would appear to be in contradiction of reports in previous papers (7, 8) to the effect that bone and bone marrow accumulate sulfur-35 administered as sodium sulfate. The major portion of the sulfur-35 found in bone marrow, however, was shown to be acid-soluble (8). It would appear, therefore, that during fixation of bones in 3.7 per cent formalin, which was acid in reaction, the sulfur-35 was washed out of the marrow. With this in mind, humeri and tibia-femur combinations were fixed in formalin saturated with barium hydroxide. A series of histological sections of humeri thus fixed, and the autographs produced by these sections are presented in Figs. 8*a* through 10*b*. These radioautographs, Figs. 8*b*, 9*b*, and 10*b*, can be compared with the radioautographs, Figs. 1*b*, 2*b*, and 3*b*, since they are respectively from opposite sides of the same rats.

It should be noted that the autographs, Figs. 8*b*, 9*b*, and 10*b*, do not indicate any appreciable concentration of sulfur-35 in the epiphyseal cartilage; only the perichondrium appears to have accumulated any significant amount of this isotope. The explanation for this may be as follows: It has been demonstrated that the sulfur-35 which is retained in epiphyseal cartilage of suckling rats is retained therein primarily as chondroitin sulfate (2). It seems likely that by fixation of these humeri in formalin saturated with barium hydroxide the chondroitin sulfate was washed out. This possibility is consistent with the solubility of the barium salt of chondroitin sulfate indicated by Bray *et al.* (9). These radioautographs do indicate that sulfur-35 is taken up by bone marrow and bone, particularly newly formed bone at each end of the diaphysis.

Earlier in this discussion it was stated that as centers of secondary ossification arise in the cartilage, the sulfur-35 appears to diminish in concentration and to disappear from these loci. This, however, was with tissues fixed in acid formalin. Studies with tissues fixed in formalin saturated with barium hydroxide indicate that this loss does not occur to more than a limited extent, as shown by autographs, Figs. 8*b*, 9*b*, and 10*b*. It would appear therefore that early in the development of centers of secondary ossification sulfur-35 merely changes its chemical affiliation. The sulfur-35 which is fixed in cartilage, very probably as chondroitin sulfate, is insoluble in acidic formalin. When centers of secondary ossification develop, the sulfur-35 becomes soluble in acid formalin but insoluble in formalin saturated with barium hydroxide.

One is led to wonder if there is any relationship between the metabolism of sulfate sulfur and phosphorus in loci where ossification is progressing. Leblond *et al.* (10) have shown by radioautography that phosphorus is deposited in relatively high concentration in or near cartilage regions where ossification is taking place.

The observations made on tibia-knee-femur combinations were similar in nature to those discussed above in the case of humeri.

SUMMARY

The localization of sulfur-35 administered intraperitoneally as sodium sulfate to 7-day-old rats was determined by radioautography of sections of humeri and tibia-knee-femur combinations removed 24, 48, 96, 216, and 290 hours after injection of this isotope.

Radioautography of sections of bone and cartilage that had been fixed in formalin indicated that the tracer isotope was present throughout the entire epiphysis. Its concentration therein was highest initially at the epiphyseal-diaphyseal junction where the more mature cartilage cells were present.

By the 96th hour post injection the sulfur-35 had become more uniformly distributed in the epiphyses and an even distribution of it throughout the epiphyseal cartilage was almost attained by the 216th hour post injection.

As centers of secondary ossification arose in the epiphyseal cartilage, the sulfur-35 appeared to diminish in concentration and disappear from these loci. However, radioautographs of cartilage fixed in formalin saturated with barium hydroxide, instead of in formalin only, disclosed the fact that the tracer isotope was still present in these loci.

When bone and bone marrow were fixed in formalin the autographs indicated the presence of sulfur-35 primarily in the periosteum. Only a negligible amount appeared to be present in the bone shaft and marrow. However, when these tissues were fixed in formalin saturated with barium hydroxide it was possible to demonstrate the presence of the tracer isotope in both bone and bone marrow.

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EXPLANATION OF PLATES

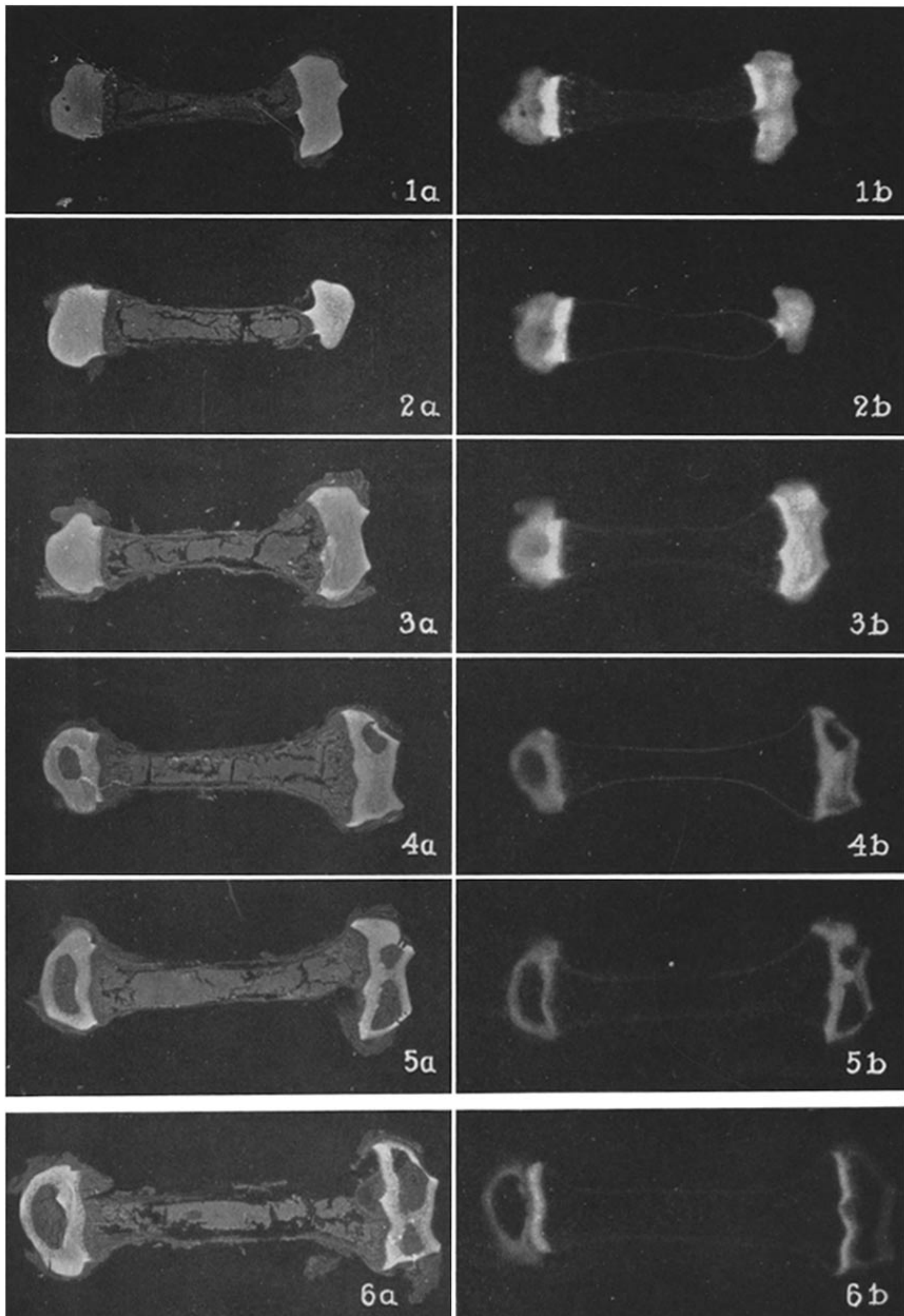
Plate 31

Figs. 1*a*, 2*a*, 3*a*, 4*a*, and 5*a* are reproductions of sections of rat humeri stained with toluidine blue. These humeri were removed 24, 48, 96, 216, and 290 hours, respectively, after 2 mg. of labeled sodium sulfate (8.24×10^5 c.p.m.) had been administered intraperitoneally to 7-day-old litter mates. The bones were fixed in formalin. The reproductions were made by using the histological sections as negatives. $\times$ 5.

Figs. 1*b*, 2*b*, 3*b*, 4*b*, and 5*b* are from the radioautographs produced by the sections 1*a*, 2*a*, 3*a*, 4*a*, and 5*a* respectively, before the same sections were stained. The film, Kodak ortho process, was exposed for 4 weeks. Enlargement and reproduction of the autographs were made by using the films as negatives. In these reproductions, therefore, the lighter areas correspond to the darker areas in the original radioautographs. $\times$ 5.

Fig. 6*a* illustrates a section of a humerus removed 24 hours after the intraperitoneal administration of 4 mg. (13.6×10^5 c.p.m.) of S^{35}-labeled sodium sulfate to a 15-day-old rat. The humerus was fixed in formalin and stained with toluidine blue. The section was used as a negative in making the reproduction. $\times$ 5.

Fig. 6*b* is from the radioautograph produced on Kodak ortho process film by the same section shown in Fig. 6*a* before the section was stained. The film was exposed for 4 weeks and the reproduction made by using this film as the negative. $\times$ 5.



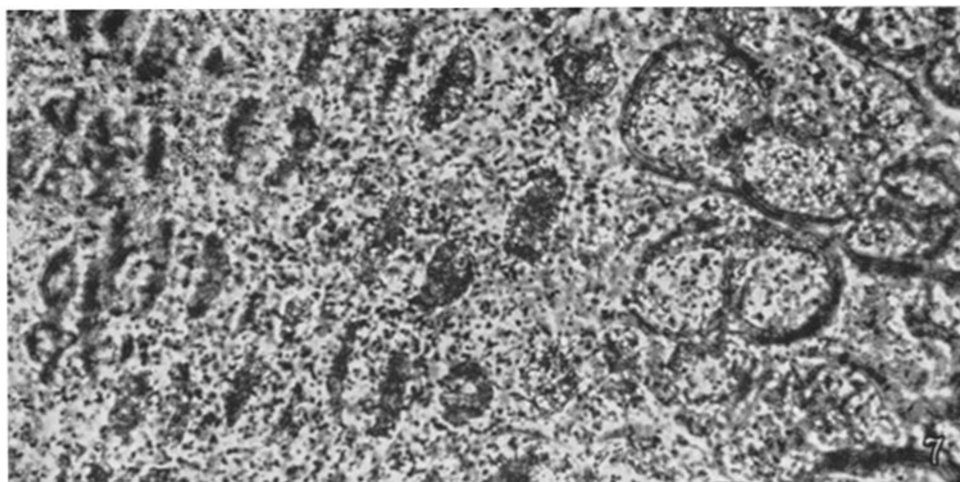
(Dziewiatkowski: Radioautographs of cartilage growth)

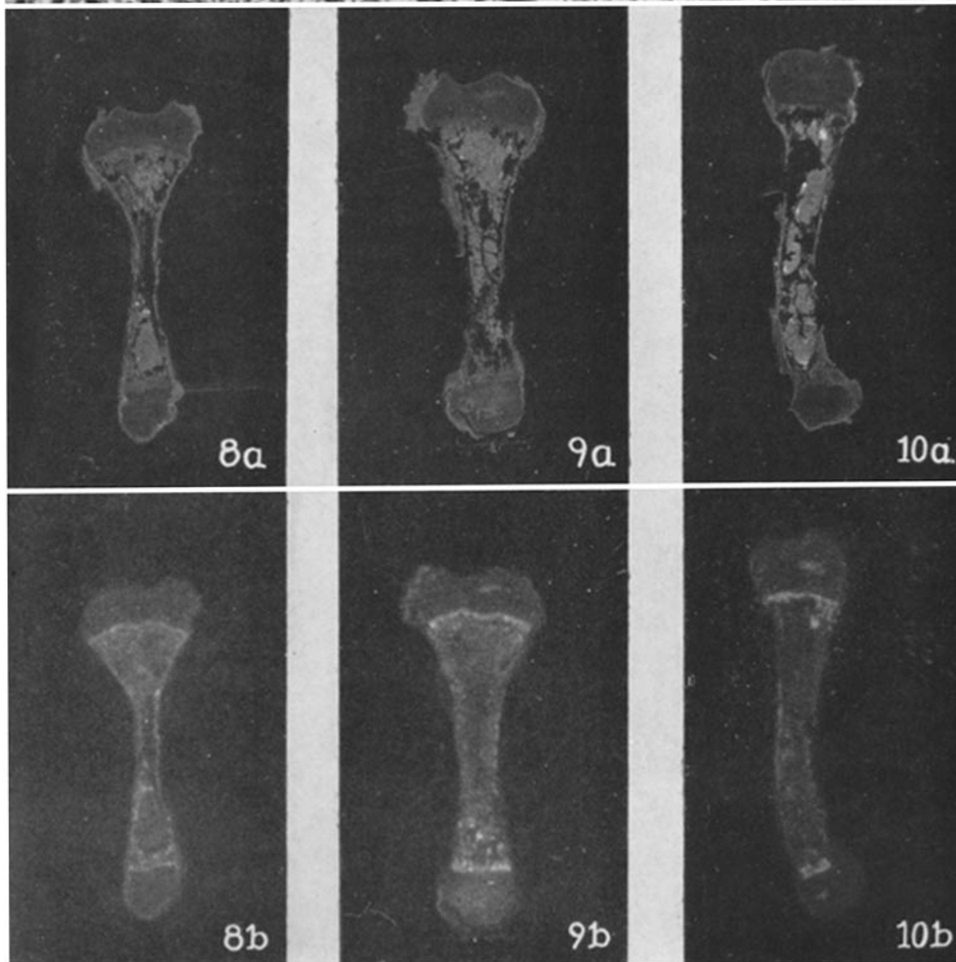
PLATE 32

FIG. 7 is from a photomicrograph of unstained epiphyseal cartilage at the junction of the cartilage and the diaphysis. The section of humerus used for this was cut adjacent to the section used to obtain the radioautograph reproduced in Fig. 1*b*. It was floated directly onto the emulsion of a Kodak contrast lantern slide plate. Exposure was of 4 weeks' duration. The emulsion was developed with the section in place. The number of reduced silver grains is greater in the area covered by cartilage cells in phases 3 and 4 than under that covered by the most mature cells, those in phase 5, seen on the right in the figure. × 527.

FIGS. 8*a*, 9*a*, and 10*a* are reproductions of sections of humeri removed from suckling rats 24, 48, and 96 hours, respectively, after the administration of S^{35}-labeled sodium sulfate to 7-day-old rats. The bones were fixed in formalin saturated with barium hydroxide. These sections were stained with toluidine blue. They are from opposite humeri of the same rats which furnished the materials for Figs. 1*a* to 3*b*. The reproductions were made by using the sections as negatives. × 5.

FIGS. 8*b*, 9*b*, and 10*b* are reproductions of radioautographs produced by the bones shown in Figs. 8*a*, 9*a*, and 10*a*, respectively, before the sections were stained. The film, Kodak ortho process, was exposed for 4 weeks. The original autographs were used as negatives to make the reproductions. These radioautographs suggest the presence of less sulfur-35 in the epiphyses and more in the diaphyses than is indicated in the corresponding Figs. 1*b*, 2*b*, and 3*b*. Centers of secondary ossification may be seen. × 5.





(Dziewiatkowski: Radioautographs of cartilage growth)