RADIOAUTOGRAPHIC STUDIES OF SULFATE-SULFUR (S^{35}) METABOLISM IN THE ARTICULAR CARTILAGE AND BONE OF SUCKLING RATS*

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It has been reported (1) that following the intraperitoneal administration of S^{35}-labeled sulfate-sulfur to 7-day-old rats, the sulfur-35 was demonstrable by radioautography throughout the entire epiphyseal cartilage of humeri from the 24th to the 290th hour. At the 24th hour the concentration of the sulfur-35–containing component(s) was highest at the epiphyseal-diaphyseal junction. Then its concentration there decreased so that by the 216th hour its distribution throughout the epiphyseal cartilage was almost uniform.

The experiments reported here reveal the pattern in which sulfate-sulfur is deposited in the articular cartilage of suckling rats as early as 15 minutes after intraperitoneal injection, and the changes in the pattern of the deposition during the following 24 hours. Certain unsuspected aspects of sulfate-sulfur metabolism in the bone and bone marrow are also presented.

Method

Suckling rats of the Whelan strain were used. In one series of experiments approximately 5 μc. of carrier-free sulfur-35 in the form of sodium sulfate[1] in 0.1 ml. of distilled water was injected intraperitoneally into each of 8 7-day-old animals of the same litter. Two animals were sacrificed by decapitation 15, 30, 60, and 120 minutes after injection.

In a second series of experiments approximately 1.25 μc. of carrier-free sulfur-35 in the form of sodium sulfate in 0.1 ml. distilled water was injected intraperitoneally into each of 24 7-day-old animals of 3 litters. One rat from each litter was sacrificed by decapitation 2, 4, 6, 10, 24, 48, 72, and 96 hours after injection.

Both humeri, tibiae, and femurs were removed from each animal. One of each of these bones was fixed for 48 hours at 25°C. in a 3.7 per cent solution of formaldehyde (1 volume of 37 per cent U.S.P. formaldehyde, Merck and Co., was diluted with 9 volumes of distilled water). The second bone was fixed for 48 hours at 25°C. in a 3.7 per cent solution of formaldehyde saturated with barium hydroxide. This solution was prepared 24 hours before use. The subsequent preparation of radioautographs was carried out as previously described (1).

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[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 relative sulfur-35 concentration in different areas of the cartilage of the bones fixed in formaldehyde only was assessed from the radioautographs of the bones with a Weston photographic analyzer, Model 877.

RESULTS

Figs. 1 *a* to 4 *b* comprise a series of representative stained sections (*a*) and the corresponding radioautographs (*b*) of formaldehyde fixed humeri removed from rats of the same litter 15, 30, 60, and 120 minutes, respectively, after intraperitoneal administration of the S^{35}-labeled sulfate-sulfur. It is obvious in Fig. 1 *b* that even after 15 minutes some of this radioisotope has been deposited throughout the entire epiphyses. However, it is present in highest concentration at the junction of the epiphysis with the diaphysis. Figs. 2 *b*, 3 *b*, and 4 *b* show that during the next 105 minutes the pattern of deposition is not altered, except for intensification.

Representative radioautographs and the corresponding stained sections of humeri removed from the same animals but fixed in a solution of formaldehyde saturated with barium hydroxide are given in Figs. 5 *a* to 8 *b*. It will be seen that by the end of 15 minutes (Fig. 5 *b*) the bone shaft and bone marrow have already acquired a large fraction of the tracer isotope which is to be deposited therein during the following $1\frac{3}{4}$ hours (Figs. 6 *b*, 7 *b*, and 8 *b*). From these figures it appears that sulfur-35 is deposited transitorily but to a large extent in or on some component or components of the bone marrow during the first 30 minutes at least (Fig. 6 *b*). By the end of 60 minutes (Fig. 7 *b*) the discrete localization of the radioisotope in the bone marrow is less apparent, and after 2 hours it is almost indiscernible (Fig. 8 *b*).

It is of interest to note that not only the radioautographs, but also the staining characteristics of the humeri are dependent upon the method of fixation. The epiphyses of humeri fixed in a solution of formaldehyde take up toluidine blue and are stained a deep purple. On the other hand, the epiphyses of humeri fixed in a solution of formaldehyde saturated with barium hydroxide take up the dye to a very slight extent in the same period of time, and are stained a faint blue. It has been claimed that toluidine blue stains chondroitin sulfate specifically (2–4). It has been shown that the barium salt of chondroitin sulfate is soluble in water (5). Therefore, the marked loss of S^{35} from cartilage and of affinity for toluidine blue by the cartilage of the bones fixed in a solution of formaldehyde saturated with barium hydroxide may be due to the extraction of chondroitin sulfate during fixation.

Figs. 9 *a* through 14 *b* represent the stained sections (*a*) and the radioautographs (*b*) of corresponding humeri fixed in a solution of formaldehyde after removal from litter mates that were sacrificed 2 to 48 hours following the administration of S^{35}-labeled sulfate-sulfur. In this series one sees again that the radioisotope has been deposited throughout the entire epiphyses, but that its concentration is highest at the epiphyseal-diaphyseal junction. Up to the

24th hour (Figs. 10 *b* to 13 *b*), the pattern of deposition remains unchanged, except for intensification. A tendency to a more uniform distribution of the tracer element throughout the epiphyses is apparent in humeri removed 48 hours after injection (Fig. 14 *b*). The more uniform distribution appears to be the result of a decrease in the concentration of the sulfur-35 in the cartilage adjacent to the diaphysis. This might be interpreted as indicating a greater rate of turnover of the sulfur-35–bearing component at the epiphyseal-diaphyseal junction than in the rest of the epiphysis.

Figs. 15 *a* to 22 *b* show the stained sections (*a*) and the corresponding radioautographs (*b*) of opposite humeri from the same animals that furnished the materials shown in Figs. 9 *a* to 14 *b*.[2] These bones were fixed in a solution of formaldehyde saturated with barium hydroxide. In this series it can be seen that sulfur-35 is present in some component(s) of the bone marrow even 96 hours after administration. In the original autographs it appeared that between the 2nd and 24th hour the concentration of the sulfur-35–containing component(s) of the bone marrow increased and thereafter slowly decreased. These observations are similar to those reported on the bone marrow of adult rats (6).

One sees, also, that in the bone shaft the S^{35}-labeled component(s) is rather diffusely deposited from the 2nd to the 10th hour after administration (Figs. 15 *b* to 18 *b*). By the 24th hour and thereafter (Figs. 19 *b* to 22 *b*), the shaft is more sharply outlined.

In all instances radioautographs of humeri fixed in a solution of formaldehyde saturated with barium hydroxide indicate a relatively high concentration of labeled component(s) in newly calcified bone at the ends of the diaphysis. Here the tracer is deposited diffusely up to the 24th hour. Thereafter, detail of structure is sharpened and intensified.

In Figs. 21 *b* to 22 *b* one sees also that the S^{35}-labeled component(s) is present in centers of secondary ossification. As the centers develop, an increasing amount of the tracer isotope is incorporated in or attracted to some component(s).

These observations made on the humeri were substantiated by examination of the radioautographs of the tibiae and femurs of the animals.

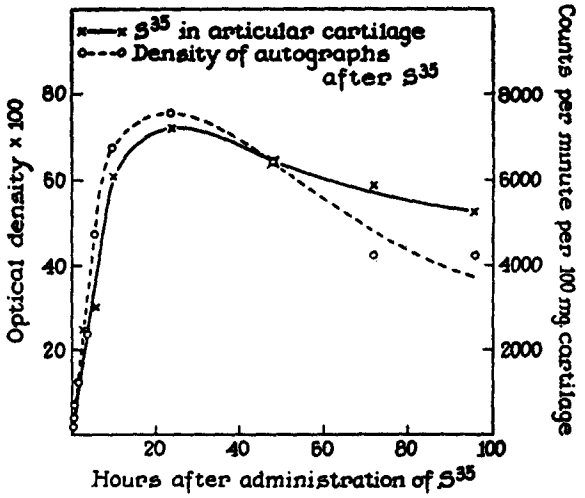
The change in the concentration of S^{35}-labeled components in the articular cartilage of the knee joints after intraperitoneal administration to 7-day-old rats has been reported (7). It was shown that there is a progressive increase in the concentration of the radioisotope up to about the 24th hour after its injection. Thereafter, up to the 160th hour the concentration slowly decreased.

It is known that the number of developable silver halide grains of a photographic emulsion, within certain limits, is dependent upon the concentration of

[2] Radioautographs of bones removed 72 and 96 hours after labeled sulfate-sulfur administration and fixed in a solution of formaldehyde were also prepared. They were similar to those previously reported (1).

the radioactive source to which the emulsion is exposed (8). Further, the number of silver grains present in a given area of a developed film is directly reflected in the optical density of that area.

Therefore, to ascertain if the observations previously reported (7) were in agreement with those made in the course of the present study, a background reading of each film bearing an autograph of a humerus, tibia, or femur, that had been fixed in formaldehyde, was taken with a Weston photographic analyzer, Model 877. This background reading was then subtracted from half the sum of



TEXT-FIG. 1. Relationship of the intensity of the radioautographic reaction of epiphyseal cartilage to time after the intraperitoneal administration of sulfur-35–labeled sodium sulfate to 7-day-old rats. The intensity of the reaction was measured as optical density with a Weston photographic analyzer, Model 877. Each point after any time interval represents the average value obtained from all the radioautographs of humeri and tibia-femur combinations which had been fixed in a solution of formaldehyde on removal from the animals at the end of that time interval. For comparison data are plotted that were previously reported (7) on the accumulation of sulfur-35 in the knee joint cartilage after intraperitoneal injection of labeled sodium sulfate to 7-day-old rats.

the average of three density readings taken in the area of highest concentration, and the average of three readings taken in the rest of the epiphysis. The radioautographs in the second series of experiments, represented by Figs. 9 *b* to 14 *b*, were not prepared at the same time, nor were the animals given the same amount of sulfur-35, as those in the series represented by Figs. *1 b* to 4 *b*. However, the results of the two series were made comparable as follows: The average of the values, obtained from the radioautographs of the epiphyses removed at the end of the 2nd hour in the first series, was divided by the corresponding average from the second series to give a factor by which each of the remaining averages in the second series was then multiplied.

The mean relative optical density of all radioautographs of the epiphyses of humeri, tibiae, and femurs removed after a given time interval was then plotted against time since injection. The result is shown in Text-fig. 1. For comparison, there has been included a plot of the data previously obtained by radiochemical analysis (7). The two curves lead to an identical conclusion as regards rate of increase and decrease of the sulfur-35 concentration in some component(s) of the articular cartilage in the suckling rat following intraperitoneal injection of S^{35}-labeled sulfate-sulfur.

DISCUSSION

The sulfur-35 which is deposited in the cartilage, bone, and bone marrow after administration as sulfate-sulfur to suckling rats may be considered as part of components which are either (*a*) insoluble in an acidic solution of formaldehyde, pH 3.8–3.9; (*b*) insoluble in a solution of formaldehyde saturated with barium hydroxide; or (*c*) soluble in both. To a large extent those components which are insoluble in the acidic formaldehyde appear to be soluble in a formaldehyde solution saturated with barium hydroxide. Furthermore, the S^{35}-bearing components which are insoluble in formaldehyde solution saturated with barium hydroxide are largely soluble in acidic formaldehyde.

It is suggested that the *formaldehyde-insoluble sulfur-35–bearing component* is primarily chondroitin sulfate, and that, therefore, the observed changes in concentration of this component are a reflection of the rate of chondroitin sulfate turnover in the articular cartilage of the suckling rat. This suggestion is based on the following observations and conclusions:—It has been found that sulfur-35, when given as sodium sulfate to the suckling rat, is incorporated into chondroitin sulfate of the articular cartilage (9). The major portion of the S^{35}-labeled sulfate-sulfur which is retained in the articular cartilage is probably retained therein as chondroitin sulfate (7). Since the barium salt of chondroitin sulfate is soluble in water, one would expect that during fixation in a solution of formaldehyde saturated with barium hydroxide, chondroitin sulfate would be extracted from tissue. Indeed, most of the sulfur-35–bearing components were extracted from the epiphyseal cartilage by this fixative. Furthermore, this loss was paralleled by loss of ability of the cartilage to react with toluidine blue to produce the characteristic metachromasia ascribed to chondroitin sulfate.

If the formaldehyde insoluble sulfur-35 represents chondroitin sulfate, its deposition indicates that the synthesis of chondroitin sulfate takes place throughout the entire epiphyseal cartilage. The rate of chondroitin sulfate turnover, however, is not the same in all parts of the cartilage. It is probably highest in the epiphyseal region adjacent to the diaphysis and in the cartilage which immediately surrounds developing centers of secondary ossification. This leads to the inference that calcification is associated with an accelerated synthesis and removal of chondroitin sulfate in the adjacent cartilage.

Sulfur-35–bearing components which are insoluble in a solution of formaldehyde

saturated with barium hydroxide probably include inorganic sulfates in addition to sulfur in some other forms insoluble as barium salts at an alkaline pH. Sulfur so characterized is demonstrable in increasing concentration during the development of secondary ossification centers. In the diaphysis, where new bone is being laid down, there is also a progressive increase in this form of sulfur. The sulfur-containing components laid down in these areas of calcification may obtain the sulfur from the chondroitin sulfate of the adjacent cartilage as well as from circulating inorganic sulfate-sulfur. This would be compatible with the opinion expressed by Siffert (10) as regards utilization of cartilage matrix in the formation of bone matrix, and with the opinion of Rubin and Howard (4) that chondroitin sulfate plays an important role in calcification.

Sulfur in components which are insoluble as barium salts is present in the bone shaft, and, once accumulated, is demonstrable therein for at least 12 days.

The autographs of bone marrow can be interpreted as indicating the presence of sulfur, for the most part in forms which are insoluble as barium salts. The rapid and relatively large transitory deposition of some of the sulfate-sulfur in discrete foci suggests that inorganic sulfate-sulfur enters the bone marrow, and that part is rapidly removed and part is incorporated in a more complex component. From the complex component the sulfur is then removed slowly.

The radioautographs in this and the previous paper (1) when viewed in the light of the above discussion enable one to visualize the disposition of sulfate-sulfur in the articular cartilage and leg bones of the suckling rat from age 7 days to 19 days.

SUMMARY

Fifteen minutes after intraperitoneal injection of sulfur-35 as sodium sulfate to 7-day-old rats the concentration of the isotope was highest in the cartilage at the epiphyseal-diaphyseal junction of long bones but was demonstrable throughout the entire epiphysis. Up to the 24th hour the pattern of deposition did not change as the concentration continued to increase.

As centers of secondary ossification developed, there occurred in them an increased concentration of some form of sulfur, insoluble as the barium salt. This sulfur was probably derived from the cartilage which the center of secondary ossification replaced.

Up to about the 30th minute after injection the sulfur-35 was deposited transitorily in a high concentration in discrete loci of the bone marrow. Excluding this transitory deposition, the highest concentration of the radioisotope in the bone marrow was seen in the specimens removed 24 hours after administration.

In the bone shaft the sulfur-35, in a form which was insoluble in an alkaline solution of barium ions, was deposited diffusely up to the 24th hour after its administration. Thereafter, the radioisotope decreased in concentration in the

middle portion of the bone shaft. However, the concentration of a similarly characterized sulfur-35–bearing component in the ends of the diaphysis continued to increase up to at least the 96th hour after injection.

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EXPLANATION OF PLATES

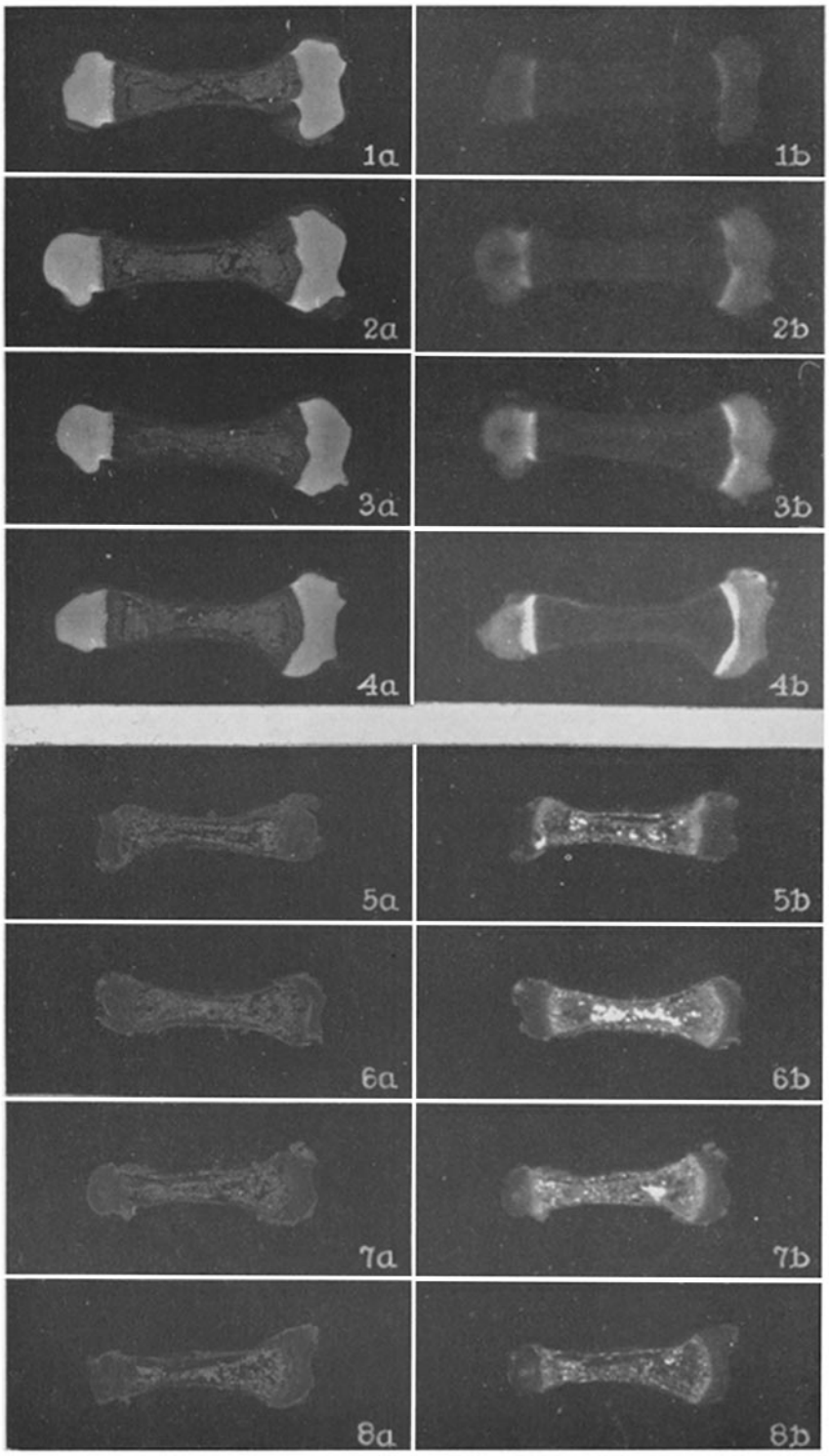
PLATE 46

FIGS, 1 *a*, 2 *a*, 3 *a*, and 4 *a* are reproductions of sections of rat humeri stained with toluidine blue. These humeri were removed 15, 30, 60, and 120 minutes, respectively, after intraperitoneal administration of about 5 μc. of sulfur-35 in the form of sodium sulfate to 7-day-old litter mates. The bones were fixed in a solution of formaldehyde. The reproductions were made by using the histological sections as negatives. × 3.9.

FIGS. 1 *b*, 2 *b*, 3 *b*, and 4 *b* are from the radioautographs produced by the sections 1 *a*, 2 *a*, 3 *a*, and 4 *a*, respectively, before the sections were stained. The film, Kodak contrast process ortho, was exposed for 1 week. 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. A progressive deposition of sulfur-35 in a characteristic pattern in the epiphyseal cartilage can be seen. The shaft is only faintly outlined. × 3.9.

FIGS. 5 *a*, 6 *a*, 7 *a*, and 8 *a* are reproductions of sections of rat humeri stained with toluidine blue. These humeri are from the same rats as the humeri used to obtain the sections shown in Figs. 1 *a*, 2 *a*, 3 *a*, and 4 *a*, but they were fixed in a solution of formaldehyde saturated with barium hydroxide. The reproductions were made by using the histological sections as negatives. It can be seen that the epiphyseal cartilage is only faintly stained. × 3.6.

FIGS. 5 *b*, 6 *b*, 7 *b*, and 8 *b* are from the radioautographs produced by the sections 5 *a*, 6 *a*, 7 *a*, and 8 *a*, respectively, before the sections were stained. The film, Kodak contrast process ortho, was exposed for 3 weeks. Enlargement and reproduction of the autographs were made by using the films as negatives. A deposition of the sulfur-35 in the bone shaft, newly formed bone, and bone marrow is apparent. It can also be seen that a relatively large amount of the isotope accumulated transitorily in the bone marrow. The epiphyseal cartilage gave only a faint reaction. × 3.6.

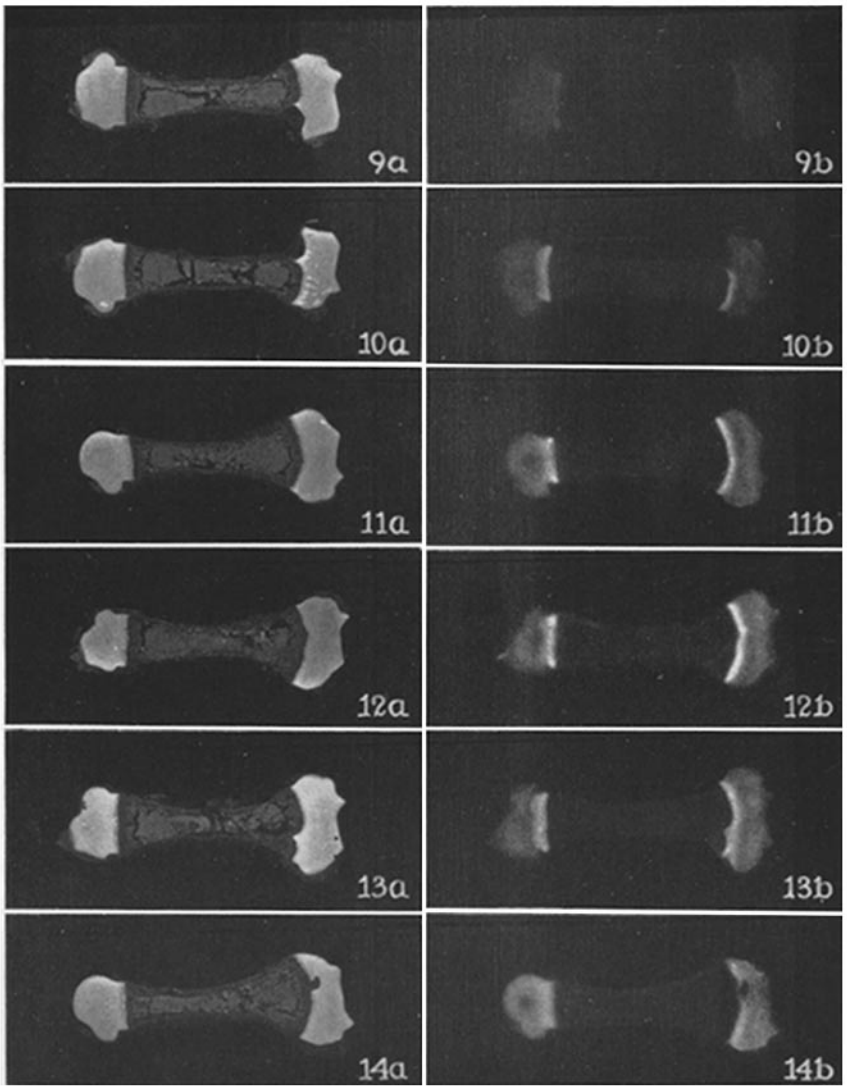


(Dziewiatkowski: Radioautographic studies of sulfate-sulfur metabolism)

PLATE 47

FIGS. 9 *a*, 10 *a*, 11 *a*, 12 *a*, 13 *a*, and 14 *a* are reproductions of sections of rat humeri removed 2, 4, 6, 10, 24 and 48 hours, respectively, after intraperitoneal administration of about 1.25 μc. of sulfur-35 in the form of sodium sulfate to 7-day-old litter mates. These humeri were fixed in a solution of formaldehyde and stained with toluidine blue. The reproductions were made by using the histological sections as negatives. × 3.9.

FIGS. 9 *b*, 10 *b*, 11 *b*, 12 *b*, 13 *b*, and 14 *b* are from the radioautographs produced by the sections 9 *a*, 10 *a*, 11 *a*, 12 *a*, 13 *a*, and 14 *a*, respectively, before the sections were stained. The film was exposed for 1 week. Enlargement and reproduction of the autographs were made by using the films as negatives. Here again, a progressive deposition of the sulfur-35 in a characteristic pattern in the epiphyseal cartilage can be seen up to about the 24th hour after injection. A fading of the zone of highest concentration at the epiphyseal-diaphyseal junction is seen to have occurred by the 48th hour after injection (Fig. 14 *b*). × 3.9.

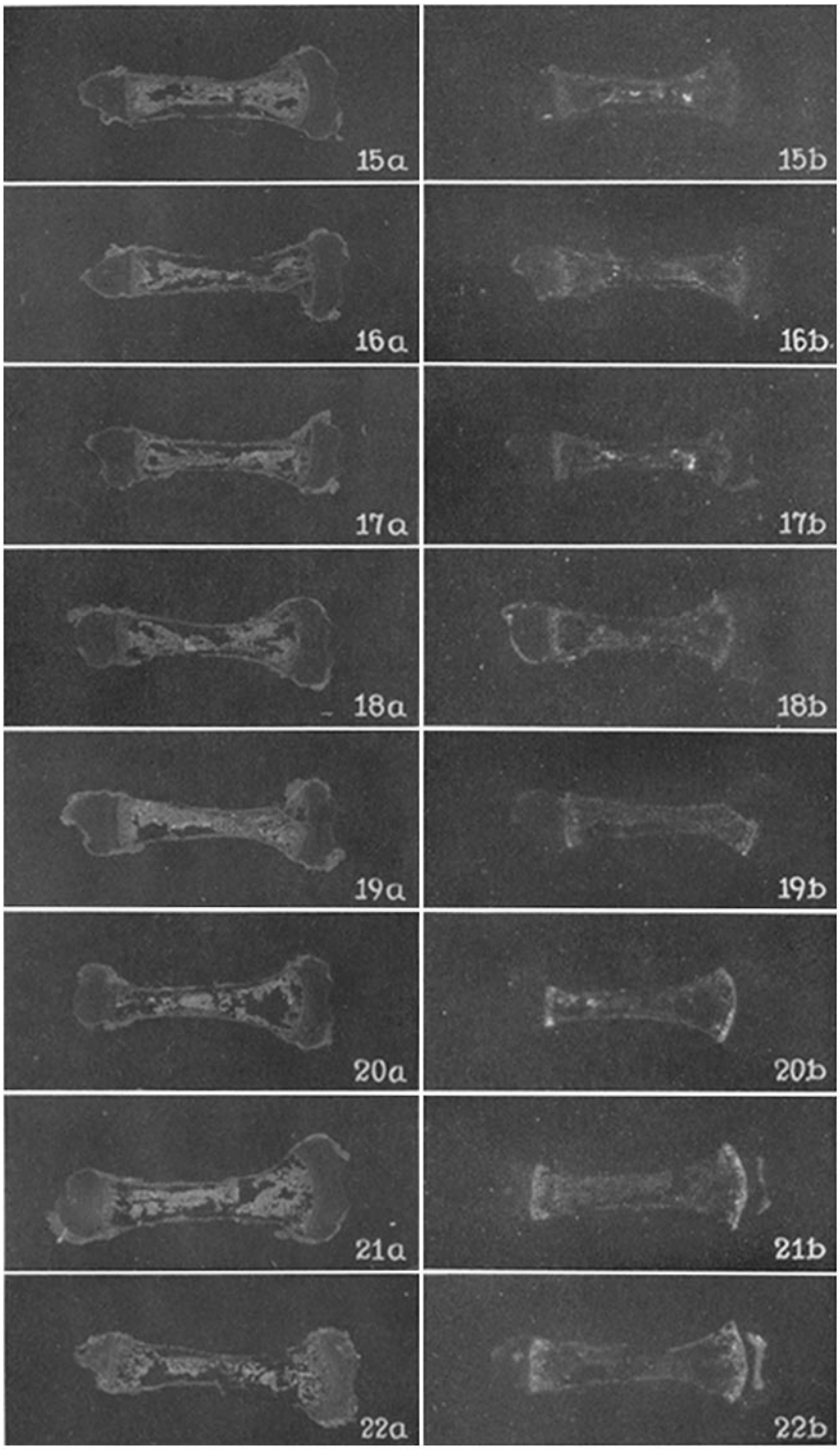


(Dziewiatkowski: Radioautographic studies of sulfate-sulfur metabolism)

PLATE 48

FIGS. 15 *a*, 16 *a*, 17 *a*, 18 *a*, 19 *a*, 20 *a*, 21 *a*, and 22 *a* are reproductions of sections of rat humeri removed 2, 4, 6, 10, 24, 48, 72, and 96 hours, respectively, after intraperitoneal administration of about 1.25 μc. sulfur-35 in the form of sodium sulfate to 7-day-old litter mates These are sections of humeri fixed in a solution of formaldehyde saturated with barium hydroxide and stained with toluidine blue. They are from opposite humeri of the same rats which furnished the materials for Figs. 9 *a* to 14 *b*. The reproductions were made by using the sections as negatives. × 3.9.

FIGS. 15 *b*, 16 *b*, 17 *b*, 18 *b*, 19 *b*, 20 *b*, 21 *b*, and 22 *b* are reproductions of the radioautographs produced by the sections of Figs. 15 *a* to 22 *a*, respectively, on Kodak contrast process ortho film. The film was exposed for 4 weeks. Enlargement and reproduction of the autographs were made by using the films as negatives. It can be seen from this series of autographs that the sulfur-35 was deposited in the bone shaft and bone marrow. The concentration of the isotope appears to be increasing in the areas of new bone (the ends of the diaphysis) up to at least the 96th hour after administration of the labeled sodium sulfate. The centers of secondary ossification appear to be acquiring an increasing amount of the sulfur-35 as the centers develop. × 3.9.



(Dziewiatkowski: Radioautographic studies of sulfate-sulfur metabolism)