TURNOVER OF S^{35}-SULFATE IN EPIPHYSES AND DIAPHYSES OF SUCKLING RATS

NATURE OF THE S^{35}-LABELLED COMPOUNDS

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The maximal accumulation of S^{35} in the epiphyseal cartilage of suckling rats occurs about 24 hours after the intraperitoneal injection of S^{35}-sulfate (1), at which time the isotope is most highly concentrated in the epiphyseal cartilage plate (2). Thereafter, the concentration of S^{35} in the epiphyses decreases rather slowly, the decrease being most noticeable in the epiphyseal cartilage plate and in regions of the epiphyses where ossification centers develop. In the diaphyses, on the other hand, there is a rapid rise and fall in the concentration of the isotope so that by the 4th hour only a faint autoradiographic reaction is elicited. Beginning at about the 24th hour the concentration of S^{35} is seen to increase in the metaphyses. Because of the concurrent decrease of the concentration in the epiphyseal cartilage plate and increase in the metaphyses, it seemed likely that the S^{35} is transferred from the cartilage plates to the metaphyses, where it accumulates as inorganic sulfate or/and as some other compounds, of which the precursor is the chondroitin sulfate of the cartilage (2). Indeed, it has been reported that after the administration of S^{35}-sulfate to dogs (3, 4), rabbits (5), and rats (6) the isotope in the bones can be separated into two forms, one of which probably is inorganic sulfate and the other a material akin to chondroitin sulfate.

Experiments were undertaken to ascertain the nature, and the changes in concentration, of the S^{35}-labelled materials in epiphyses and diaphyses of suckling rats after administration of S^{35}-sulfate. The hope was that the findings would partially indicate the mechanisms involved in the development of the metaphyses. The results are presented here.

EXPERIMENTAL

Experiment I.—One hundred μc. of S^{35} as sodium sulfate[1] was injected intraperitoneally into each of twelve 7 day old rats, which were then returned to their mothers. Two animals

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[1] The S^{35} was obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission. The doses, ± 20 per cent, were calculated from the data furnished by the supplier.

were sacrificed after intervals of 1, 8, 12, 24, 72, and 120 hours. Humeri, tibiae, and femurs were removed for analysis. The epiphyses were separated from the diaphyses and the latter were split lengthwise so that the marrow could be teased, washed, and blotted out. Like materials were pooled, weighed, and homogenized into 5 ml. of a cold (0–4°C.) 2.5 N solution of sodium hydroxide. Aliquots were taken for the determination of S^{35} and the remainder of each homogenate was shaken in the cold (0–4°C.) for 16 hours. Insoluble material was then separated by centrifugation for 30 minutes at 7000 *G* and washed twice with 2 ml. of water in the centrifuge. The washes were added to the sodium hydroxide extract and the pH was adjusted to 6.0–6.5 with glacial acetic acid. Water was added to bring the volume up to 10 ml. A precipitate which formed on the addition of the acetic acid was removed by centrifugation. Aliquots of the clear supernatant solution were taken for the determination of S^{35} content, for dialysis against water, and for chromatography on columns of dowex-2.

Dowex-2, 10 per cent cross-linkage, 200 to 400 mesh, was prepared for chromatography as follows. One pound of the resin was suspended in 2 liters of 4 N hydrochloric acid at room temperature and allowed to settle for 1 hour, then the liquid was decanted. This was repeated once before the resin was repeatedly washed with distilled water. The wet resin was used to prepare columns 1 cm. in diameter and 4 cm. high. Before delivering an unknown sample onto a column, distilled water was passed through the column of resin until the effluent was chloride-free.

A 1 ml. aliquot of the 10 ml. of cleared extract was delivered onto a column of resin and, after washing down with 20 ml. of water, elution of the S^{35}-labelled materials was effected by a gradient change in the concentration of chloride. The effluent was collected in 10 ml. fractions.

The position of inorganic sulfate in the chromatograms was ascertained as follows. Extracts of epiphyses and diaphyses, removed from 7-day-old rats that had not received S^{35}-sulfate, were prepared as above. Just prior to delivery of an aliquot onto a column of resin, a known amount of carrier-free S^{35}-sulfate was added to the extracts. The S^{35}-sulfate was recovered quantitatively in fractions 5, 6, 7, and 8 (see insert in Fig. 3).

The concentration of S^{35} was determined in each fraction by delivering a 2 ml. portion into a pyrex tube, which after the addition of 2 ml. of 8 N hydrochloric acid was sealed and heated in a steam-bath for 6 hours. The hydrolysate was transferred to a 600 ml. beaker, which contained 5 ml. of 0.05 N sodium sulfate solution, the volume was adjusted to about 250 ml. with water, and the sulfate was precipitated by the addition of 5 ml. of a 10 per cent solution of barium chloride. Sixteen to 24 hours later the solution was reduced in volume to about 50 to 75 ml. by boiling down on a hot-plate. After cooling, the barium sulfate was isolated by filtration onto paper disks. The radioactivity in the barium sulfate was determined with a mica end-window (1.5 mg./cm.^{2}) Geiger-Mueller tube and scaler. A sufficient number of impulses was counted so that the probable error of this determination was ± 3 per cent. The concentrations of S^{35} in the homogenates, extracts, and dialysates were similarly determined after suitable dilution.

Duplicate 1 ml. portions of each extract were dialyzed against 25 ml. portions of water for 20 hours at 0–4°C. in rocking dialyzers (7). The concentration of S^{35} in the dialysate was determined.

Experiment II.—In a second experiment the extraction procedure was changed from that employed in the first experiment. Each of ten 7 day old rats received 100 μc. of S^{35}-sulfate. The epiphyses and cleaned diaphyses were homogenized into a 5 per cent solution of sodium versenate, the pH of which was 7.6. After removing aliquots for the determination of S^{35}, the homogenates were dialyzed with rocking for 72 hours at 0–4°C. against frequent changes of the same sodium versenate solution. Dialysis for 48 hours at 0–4°C. against frequent changes of distilled water followed. A fine suspension of the non-dialyzable material was prepared by homogenization in a glass homogenizer and the volume of each suspension was

adjusted to 20 ml. with water. The concentration of S^{35} was determined in these suspensions, which were also chromatographed as in the first experiment, using 1 ml. of each diaphyseal suspension but only 0.5 ml. of the epiphyseal suspensions.

Experiments III and IV.—Attempts to separate the S^{35}-labelled materials on a large scale also were made. To this intent, the long bones were removed from 78 rats, 7 to 8 days old, 24 hours after each had received intraperitoneally about 100 μc. of S^{35}-sulfate. Two pools of epiphyses and two pools of cleaned diaphyses were prepared. One of the pools of epiphyses (4 gm.) and one of the pools of diaphyses (2 gm.) were then extracted with a 2.5 N solution of sodium hydroxide as in Experiment I above, but the volumes were increased in proportion to the weight of the samples. Sodium versenate "extracts" of the other pools were prepared as in Experiment II above (6 gm. of epiphyses and 3 gm. of diaphyses were used), and heated for 15 minutes in a boiling water bath, cooled, and the pH was adjusted to 6.0 with 1 N acetic acid. The preparation from the epiphyses was incubated with 20 mg. of crude papain, and that from the diaphyses with 10 mg. of crude papain, for 24 hours at 37°C. The papain was inactivated by heating the digests for 15 minutes in a boiling water-bath. After cooling, the pH was adjusted to 7.6 with a 2.5 N solution of sodium hydroxide and the suspensions were incubated again for 24 hours at 37°C. with 40 and 20 mg. of crystalline trypsin, respectively. To retard bacterial growth, 5000 units of penicillin were added to each suspension prior to incubation with papain and trypsin. The digests were again heated for 15 minutes in a boiling water-bath after incubation with trypsin. The resultant solutions were clear. After cooling, the solutions were shaken repeatedly with 50 ml. of a 2:1 mixture of chloroform and amyl alcohol to reduce further their content of protein. The aqueous phases were set aside for chromatography.

The volumes of the sodium hydroxide extracts were adjusted to 110 ml. with water and 50 ml. portions were then delivered onto columns of dowex-2 resin, 3.3 cm. in diameter and 4 cm. high. Elution was achieved as already described, except that the volumes of eluting solutions and the effluent fractions were 10 times as large. As each fraction of 100 ml. was collected it was neutralized with solid sodium bicarbonate. On the basis of a determination of S^{35} in the fractions, these were pooled, and dialyzed against running tap water until nearly salt-free. The solutions were then concentrated in a flash evaporator to about 100 ml. each and were electrodialyzed against distilled water at 0–4°C. The salt-free solutions were evaporated to dryness in a flash evaporator and the residues were taken up in water and centrifuged. The concentrations of uronic acid (8), hexosamines (9), and nitrogen (10) in these supernatant solutions were determined. In some of the solutions, after oxidation with nitric acid, the concentration of sulfur was determined by a modification of the conductometric procedure of Paulson (11). The solutions were subjected to electrophoretic analysis, using Whatman 3 MM paper and a sodium acetate buffer, pH 5.2, μ 0.1. Paper chromatograms were prepared according to Kerby (12). Autoradiograms of some of the paper electrophoretograms and paper chromatograms were prepared on x-ray film, before the dried papers were stained with an 0.1 per cent solution of toluidine blue in 30 per cent ethanol. Excess dye was removed by washing the papers repeatedly in 30 per cent ethanol, which contained also 1 per cent acetic acid.

The fraction of S^{35} precipitable with cetyltrimethylammonium bromide (13) from each of the concentrates of pooled effluent fractions was determined.

Portions of the solutions, hydrolyzed as for the hexosamine determination (9), were evaporated to dryness and the residues were dissolved in 0.1 ml. of water. Aliquots of 0.01 ml. were used for chromatography on Whatman No. 1 paper. A 75-10-15 mixture of secondary butanol, water, and 88 per cent formic acid was allowed to ascend up the paper sheets for 24 hours. After drying at room temperature, the sheets were sprayed with an 0.1 per cent solution of ninhydrin in ethanol. Autoradiograms were prepared of some of the chromatograms on x-ray film before the solution of ninhydrin was applied.

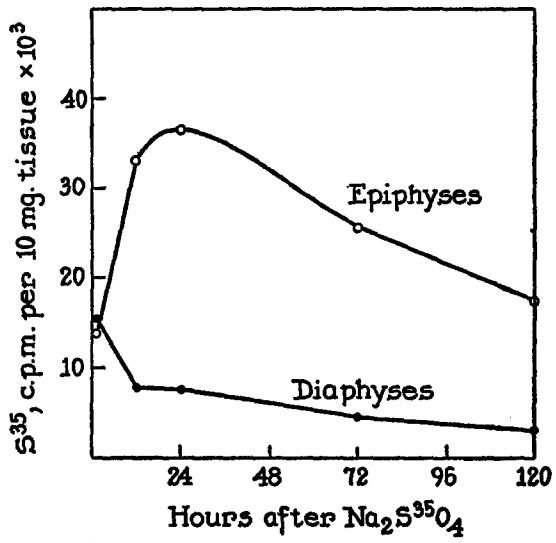

FIG. 1. Change in the concentration of S^{35} with time in the epiphyses and diaphyses of rats. The long bones were removed from 2 rats after the indicated intervals of time following interperitoneal injection of 100 μc. of S^{35}-sulfate into each of ten 7 day old rats. The bone marrow was removed from the diaphyses before analysis.

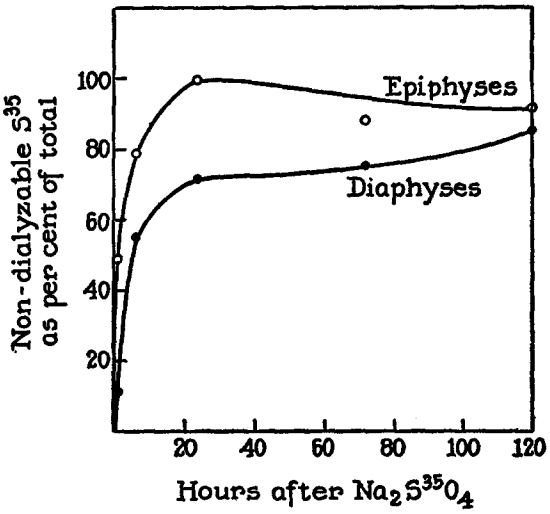

FIG. 2. Increase with time in the fraction of S^{35} which was not removed from homogenates of epiphyses and diaphyses by dialysis. The epiphyses and diaphyses were from rats each of which had received 100 μc. of S^{35}-sulfate when they were 7 days old. The tissues were homogenized into a 5 per cent solution of sodium versenate, pH 7.6, and the homogenates were dialyzed for 72 hours at 0–4°C. against frequent changes of the same sodium versenate solution and then for 48 hours against frequent changes of distilled water.

The volume of the sodium versenate extract of epiphyses was adjusted to 305 ml. with water, that of the diaphyses to 105 ml. Portions of 25 ml. were delivered onto columns of dowex-2, 3.3 cm. in diameter and 8 cm. high. The S^{35}-labelled materials were eluted from the columns with solutions of graded sodium chloride concentration until 60 fractions, each of 100 ml., were collected. The S^{35}-labelled materials which remained on the columns after this were stripped off with 200 ml. of 8 N hydrochloric acid. The fractions were pooled, concentrated, and the concentrates analyzed as in the first attempt, except that only the concentration of total hexosamines was determined. The concentration of sulfur was not determined.

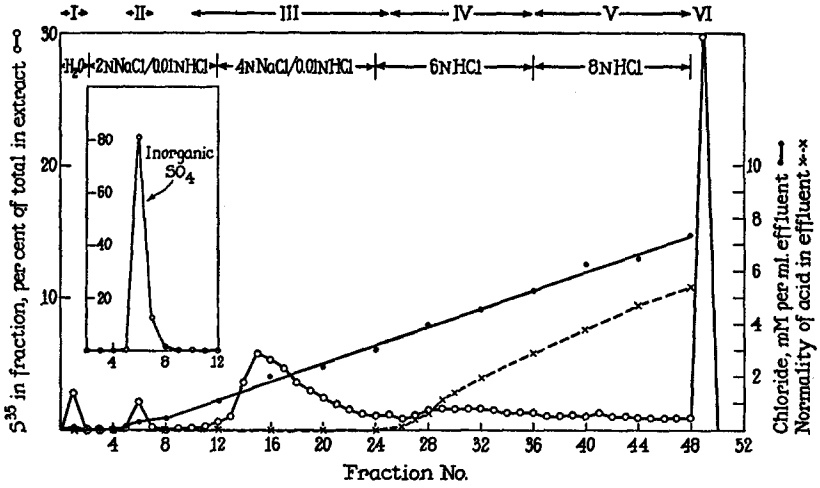

FIG. 3. Chromatograms of S^{35}-labelled materials present in extracts of epiphyses of suckling rats. The epiphyses were of long bones removed from 2 suckling rats 24 hours after each had an intraperitoneal injection of 100 μc. of S^{35}-sulfate. The tissues were extracted for 16 hours at 0–4° by shaking with a 2.5 N solution of sodium hydroxide. The pH of the extracts was adjusted to 6.5–6.7 with acetic acid and clarified by centrifugation. For chromatography, portions of the extracts were delivered onto 1 x 4 cm. columns of dowex-2 × 10, in the chloride form, 200 to 400 mesh. The compositions of the chloride solutions which flowed into the mixing chamber are shown at the top of the figure. Each fraction was of 10 ml.

The position of inorganic sulfate was ascertained by chromatographing extracts of epiphyses and diaphyses removed from suckling rats that had not received S^{35}-sulfate. Inorganic sulfate-S^{35} was added to the extracts just before chromatography. The added S^{35} was recovered nearly completely in fractions 5, 6, 7, and 8, as shown in the insert.

RESULTS

Determination of the Relative Concentrations of S^{35} in Inorganic Sulfate and in "Bound Forms".—

The changes in the concentrations of total S^{35} in the epiphyses and diaphyses, as found in Experiment II, are presented in Fig. 1. In agreement with previous observations (1, 2) there is a progressive increase of the concentration of S^{35} in the epiphyses up to about the 24th hour, followed, thereafter, by a slow

decrease. In contrast, S^{35} in bone (diaphyses) decreases rather rapidly up to about the 24th hour, after which time the concentration of S^{35} decreases at a slower rate.

The S^{35}-sulfate which enters the epiphyses is rapidly incorporated into materials which are not removed by dialysis against water. "Bound" S^{35} (subsequently shown to be sulfate) accumulates less rapidly in bone, Fig. 2. Further-

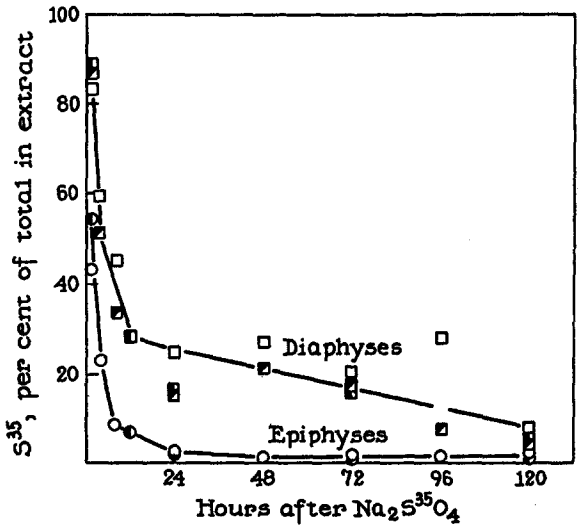

FIG. 4. Recovery of S^{35} as inorganic sulfate from extracts of epiphyses and diaphyses removed from suckling rats after intervals of time following administration of S^{35}-sulfate. The inorganic sulfate was separated from other S^{35}-labelled materials by chromatography on columns of dowex-2 × 10 as detailed in the text and shown in Fig. 3. In Experiment I, the values for the S^{35} in the inorganic sulfate fraction of the extracts of diaphyses are indicated by ◧; the values for the S^{35} in the inorganic fraction of epiphyses are indicated by ◐. The values from a preliminary experiment, in which the diaphyses were subdivided into metaphyses (◩) and "cylinders" (central portion of diaphyses) (□) are also shown; the notation (○) indicates the values for the epiphyses in this experiment.

more, in bone the ratio of S^{35}-sulfate in the non-dialyzable forms to the S^{35}-sulfate in the dialyzable forms continues to increase perceptibly up to the 120th hour, whereas in the epiphyses this ratio is nearly constant after the 24th hour.

In Experiment I, in which the epiphyses and diaphyses were extracted with a solution of sodium hydroxide, the extracts of the epiphyses contained 91.4 to 92.1 per cent and the extracts of the diaphyses 81.2 to 83.4 per cent of the S^{35} originally present in the homogenates of the tissues.

The S^{35} in the extracts as inorganic sulfate was separated by column chromatography from that present in other forms, Fig. 3. The amounts of S^{35} found as inorganic sulfate in the extracts of epiphyses and diaphyses after intervals

of time in Experiment I are shown in Fig. 4. These data are similar to the data on the removal of S^{35}-sulfate by dialysis of the extracts against water, Fig. 5. In a preliminary experiment it was found that when the diaphyses were subdivided into metaphyses ("funnels") and the cylindrical portions ("cylinders") the data were similar to those obtained when the whole diaphyses were used. The data of this preliminary experiment are included in Figs. 4 and 5.

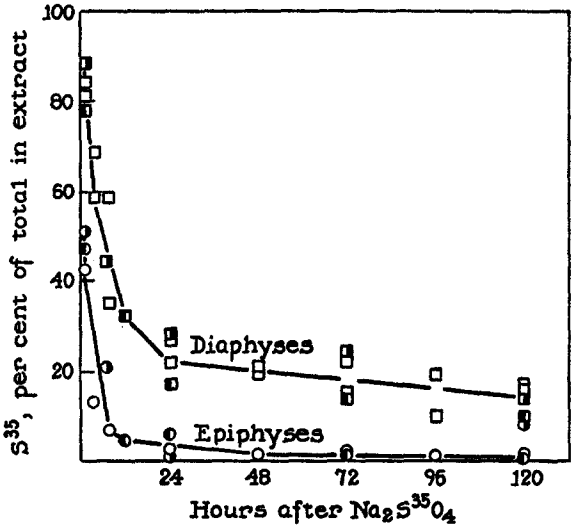

FIG. 5. The fraction of the total S^{35} in extracts of epiphyses and diaphyses which is removed by dialysis is dependent on the time which has elapsed since the S^{35}-sulfate was administered to suckling rats. In Experiment I (■, ◑), the extracts were dialyzed with shaking against 25 volumes of water for 16 hours at 0–4°C. In Experiment II (◨, ◑), homogenates of the tissues in 5 per cent sodium versenate, pH 7.6, were dialyzed with shaking at 0–4°C. against frequent changes (9 changes) of the same versenate solution for 72 hours, and then against frequent changes (5 changes) of water for 48 hours. Additional values (□, ○) are from a preliminary experiment, in which the diaphyses were subdivided into metaphyses and "cylinders" (central portions of diaphyses).

In Experiment I the inorganic sulfate was clearly separated from the rest of the S^{35}-labelled materials. Furthermore, about 70 to 90 per cent of the S^{35} was recovered in 48 fractions and the remainder was washed off the columns with an additional 20 ml. of 8 N hydrochloric acid, provided that, after the 48th fraction had been collected, elution was discontinued for 10 to 12 hours.

In Experiment II, even though suspensions were put on the columns, the chromatograms of the S^{35}-labelled materials, Fig. 6, resembled those in Experiment I, Fig. 3, with the exceptions that, (*a*) only a trace or no S^{35} appeared where inorganic sulfate was previously found and (*b*) little or no S^{35}-labelled material was washed off the columns by water (because in Experiment II the

loads placed on the columns of resin were much smaller than in previous experiments). Apparently the S^{35}-labelled materials (excepting inorganic sulfate), which were washed off the dowex-2 columns with water were in excess of the amount which the resin could hold. Indeed, when the column was overloaded so that a considerable amount of material was obtained by elution with water, no S^{35}-labelled material was eluted before fraction 12 or 13 on rechromatography

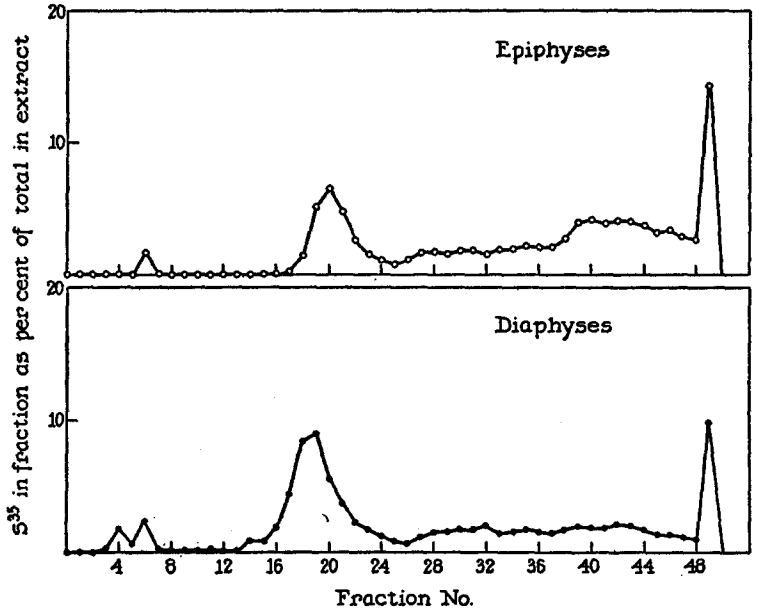

FIG. 6. Chromatograms of suspensions of epiphyses and of diaphyses removed from 13-day-old rats 120 hours after the rats had each received 100 μc. of S^{35}-SO_4 intraperitoneally. The tissues were homogenized into 5 per cent sodium versenate, pH 7.6, and the homogenates were dialyzed against frequent changes of the sodium versenate solution and then against frequent changes of distilled water before they were delivered onto 1 by 4 cm. columns of Dowex-2 × 10, chloride form, 200 to 400 mesh. The elution of materials from the columns of resin was as detailed in the text and in the legend to Fig. 3.

of a small portion of the water eluate. After fraction 13 the pattern resembled that seen when the load was excessive.

Attempts to Characterize S^{35}-Labelled Materials Other than Inorganic Sulfate.—

In the first attempt to separate the S^{35}-labelled materials on a large scale the fractions were pooled as indicated by the Roman numerals at the top of Fig. 3. The analytical data on the materials in the concentrates of the salt-free pools are given in Table I. In no instance was the ratio of uronic acid to hexosamine found to be 1 as would be expected if only chondroitin sulfate were present.

In the majority of cases the values for hexosamine were too low compared with the values for uronic acid. As indicated by chromatography on dowex-50 according to Gardell (9) galactosamine accounted for 93 per cent or more of the total hexosamine in the pools; small amounts of glucosamine were found in pools I and III.

TABLE I

Analysis of Materials in Concentrates of Pooled Effluent Fractions Collected in the Chromatography of Extracts of Epiphyses and Diaphyses of Suckling Rats

Pool No.	Uronic acid	Hexosamines	Sulfur	S^{35}
	μM	μM	μM	C.P.M. × 10^3/μM *hexosamine*
Epiphyses				
I	126.0	115.0	93.0	51.2
II	Trace	Trace	—	1.6 per cent of the S^{35} in extract recovered here
III	23.4	10.5	64.6	171.6
IV	16.1	7.6	33.0	172.3
V	6.6	3.2	22.0	108.6
VI	9.5	5.7	—	92.4
Diaphyses				
I	2.6	7.7	—	6.7
II	Trace	Trace	—	16.8 per cent of the S^{35} in extract recovered here
III	3.2	3.5	—	74.9
IV	2.0	1.6	—	70.9
V	2.8	1.8	—	33.3
VI	2.7	1.3	—	25.8

S^{35}-sulfate was injected intraperitoneally into 7-day-old rats and these were then sacrificed 24 hours later. The dose was 100 μc. of carrier-free S^{35} per rat. The epiphyses (4 gm.) and diaphyses (2 gm.) were extracted with a 2.5 N solution of sodium hydroxide by shaking for 16 hours at 0–4°C. The pH of the extracts was adjusted to 6.5–6.7 with acetic acid before they were delivered onto columns of dowex-2 × 10 resin in the chloride form. For further details consult the text and the legend to Fig. 3.

The numerals in column 1 of the table correspond to the numerals at the top of Fig. 3 and indicate the fractions which were pooled.

Based on the concentrations of hexosamine found in the pools, the concentrations of nitrogen were too high. This was explicable, for amino acids were found by chromatography of hydrolysates on paper. Furthermore, weak autoradiographic reactions were given by the regions of the papers to which cystine and methionine might have moved. The high molar ratios for sulfur in the concentrates of pools III, IV, and V, epiphyses, are probably due to the presence of cystine and methionine in these pools.

Most (73 to 100 per cent) of the S^{35} in the concentrate of pools I, III, IV, and V was precipitated by cetyltrimethylammonium bromide. The percentage

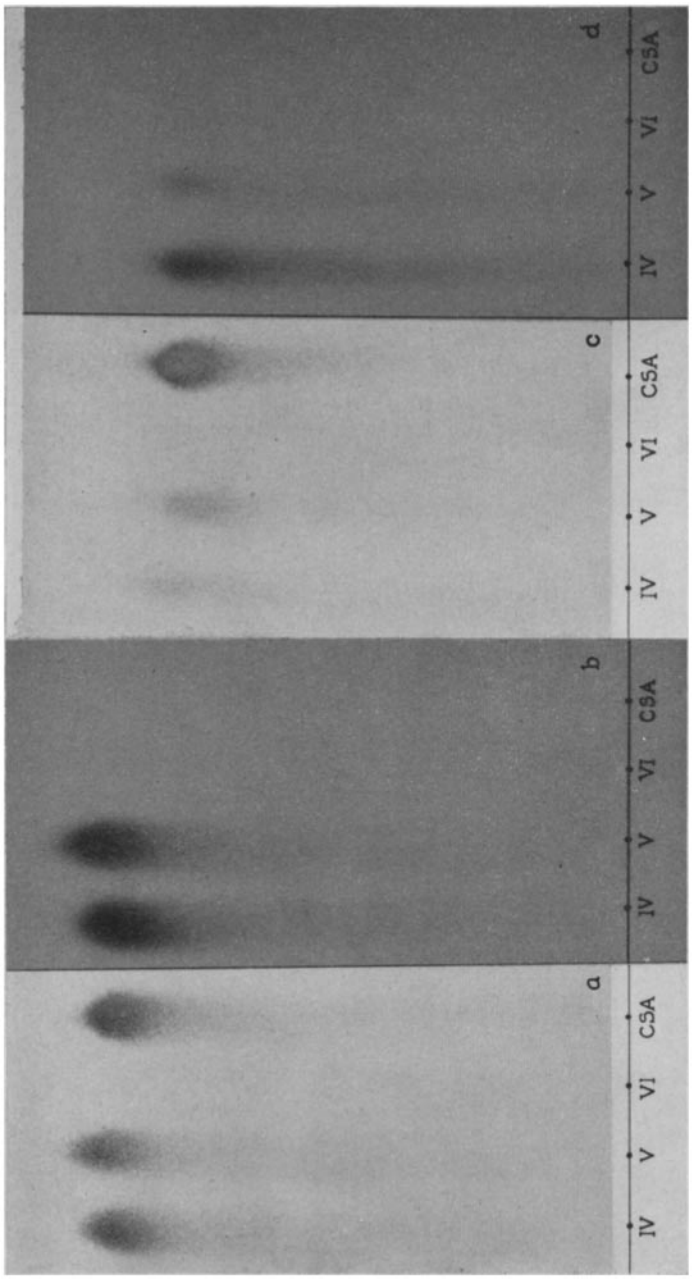

FIG. 7. Paper electrophoretograms of materials in concentrates of pooled effluent fractions collected when (*a*) an extract of epiphyses and (*c*) an extract of diaphyses were chromatographed on 3.3 x 4 cm. columns of Dowex-2 × 10, chloride form, 200 to 400 mesh. A 2.5 N solution of sodium hydroxide was used to extract the S^{35}-labelled materials from the epiphyses and diaphyses removed from suckling rats 24 hours after the intraperitoneal injections of S^{35}-SO_4. For electrophoresis, Whatman 3 MM paper, and a sodium acetate buffer, pH 5.2, μ 0.1 were used.

Autoradiograms of the paper electrophoretograms were prepared on x-ray film before the papers were stained by dipping them into a 0.1 per cent solution of toluidine blue in 30 per cent ethanol. Excess dye was washed away by repeated rinses of the paper in 30 per cent ethanol which also contained 0.5 per cent acetic acid. Autoradiogram (*b*) was produced by (*a*) and autoradiogram (*d*) by (*c*).

The Roman numerals indicate which pool of fractions (see Fig. 3) contained the materials applied at the origin. CSA is an abbreviation for purified chondroitin sulfate isolated from bovine nasal septa; 0.01 ml. of an aqueous solution, containing 2 mg. per ml., was applied to the paper.

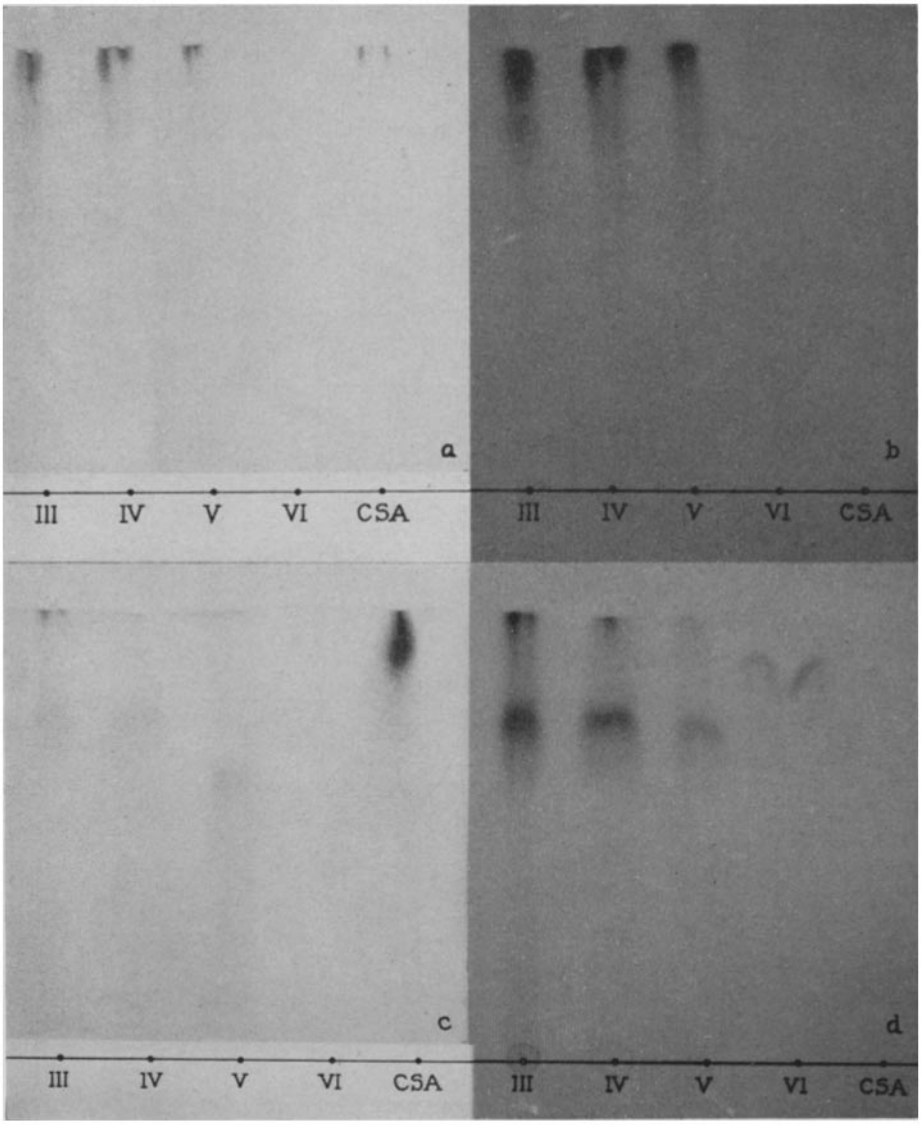

FIG. 8. Paper chromatograms of materials in concentrates of pooled effluent fractions collected when (*a*) an extract of epiphyses and (*c*) an extract of diaphyses were chromatographed on columns of Dowex-2 as indicated in the legend to Fig. 7 and in the text.

Autoradiograms of the paper chromatograms were prepared on x-ray film before the papers were stained with toluidine blue; (*b*) was produced by (*a*) (epiphyses) and (*d*) was produced by (*c*) (diaphyses).

CSA is an abbreviation for purified chondroitin sulfate isolated from bovine nasal septa; 0.01 ml. of an aqueous solution, containing 2 mg. per ml., was applied to the paper.

of S^{35} precipitable with this reagent decreased as the number of the pool increased. Indeed, only about 4 per cent of the S^{35} in pools VI was thus precipitable, probably because of degradation by the strong acid used to elute the materials from the columns of resin.

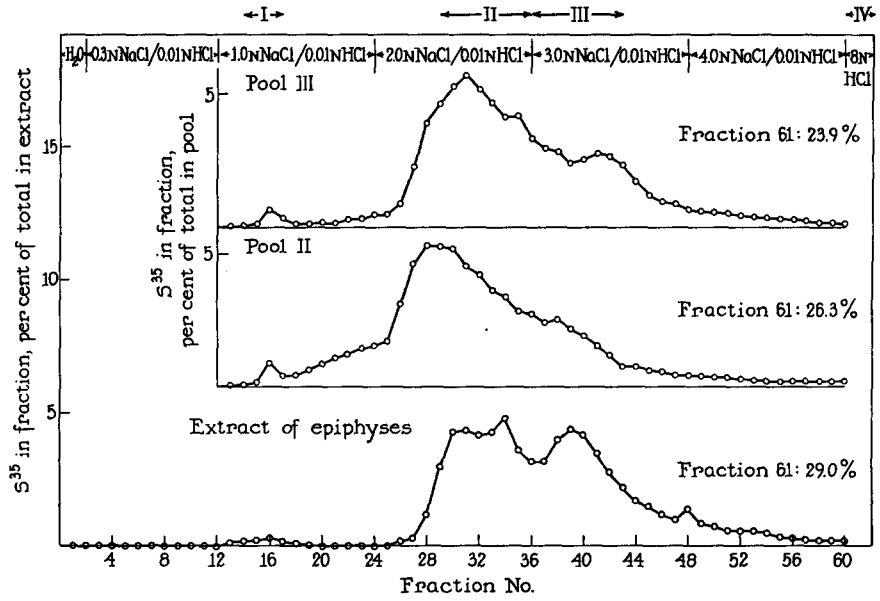

FIG. 9. Chromatogram of an extract of epiphyses removed from suckling rats 24 hours after the administration of S^{35}-sulfate. This extract was incubated with papain and trypsin before chromatography. Each effluent fraction was of 100 ml. Elution was by a change in chloride concentration affected by increasing the chloride concentration of the solutions which flowed into a mixing chamber which originally contained 1250 ml. of a 0.01 N solution of hydrochloric acid. The concentrations of the chloride solutions are indicated at the top of the figure. The Roman numerals indicate which fractions were pooled for analysis, the results of which are given in Table II.

The elution patterns obtained when salt-free concentrates of pools II and III were again chromatographed are also shown in the inserts.

Fraction 61 contained material washed off the columns with 8 N hydrochloric acid.

Autoradiograms of paper electrophoretograms, Fig. 7, and of paper chromatograms, Fig. 8, showed that in most of the concentrates there was S^{35}-labelled material which in its mobility resembled chondroitin sulfate isolated from bovine nasal septa by the procedure of Boström and Månsson (14). The autoradiograms of the paper chromatograms showed that in some of the concentrates there was also S^{35}-labelled material which differed from chondroitin sulfate in its mobility. The regions of the paper to which this latter S^{35}-labelled material migrated were stained a faint violet with toluidine blue, whereas the regions of

the paper occupied by the materials resembling chondroitin sulfate were stained metachromatically.

In the second attempt at large scale separation smaller amounts of S^{35}-labelled materials were found in the concentrates of the pooled fractions. The fractions were pooled as indicated by the Roman numerals at the top of Fig. 9. Some of the analytical data on these concentrates are given in Table II. These data and electrophoretic and chromatographic analyses again suggest the

TABLE II

Analysis of Materials in Concentrates of Pooled Effluent Fractions Collected in the Chromatography of Extracts of Epiphyses and Diaphyses of Suckling Rats

Pool No.	Uronic acid	Hexosamines	S^{35}
	μM	μM	C.P.M. $\times 10^3/\mu M$ *hexosamine*
Epiphyses			
I	1.3	1.2	33.0
II	61.0	31.0	169.5
III	36.9	26.4	170.5
IV	24.8	15.5	165.0
Diaphyses			
I	0.5	0.4	63.0
II	0.9	0.7	96.0
III	2.5	1.9	175.5
IV	2.6	2.5	132.0

The epiphyses (6 gm.) and diaphyses (3 gm.) were from 8-day-old rats that had each received 100 μc. of S^{35} as sulfate by intraperitoneal injection 24 hours previously. The tissues were homogenized into a 5 per cent solution of sodium versenate, pH 7.6. After dialysis against water, the homogenates were incubated with papain and then trypsin, whereby clear solutions were obtained. These were delivered onto columns of domex-2 × 10 resin in the chloride form. For further details consult the text and the legend to Fig. 9.

The Roman numerals in column 1 of the table correspond to the Roman numerals at the top of Fig. 9 and indicate the fractions which were pooled.

presence of material akin to chondroitin sulfate. It is interesting to note that when S^{35}-labelled material in certain pooled fractions, pools II and III, was rechromatographed, all of the material was not recovered in the effluent volumes where it was first found; the original chromatogram, as in Fig. 9, was simulated instead.

DISCUSSION

From the results obtained when extracts of epiphyses and diaphyses were dialyzed against water or chromatographed on an anion exchange resin it is apparent that the injected S^{35}-labelled sulfate did not accumulate in the epiphyses and diaphyses as inorganic sulfate. The S^{35} in this form decreased rapidly to

low levels. S^{35}-sulfate in a bound form, however, did accumulate and persist in these tissues; for the most part, it was incorporated into materials resembling chondroitin sulfate. It is suggested, therefore, that the S^{35}-materials which were demonstrated by autoradiography to be progressively deposited in the metaphyses as the concentration of S^{35} concurrently decreased in the epiphyseal cartilage plates (2) are akin to the chondroitin sulfate of the epiphyses and are derived from the latter. A probable explanation of why formalin-barium hydroxide almost completely removes S^{35} from the epiphyseal cartilage but not from the metaphyses (2) is as follows. This fixative is capable of dissolving the chondroitin sulfate of the cartilage. On the other hand, the trabeculae of the metaphyses can be visualized as projections of cartilage sheathed in mineral salts. The sheaths of mineral salts are insoluble in the alkaline fixative and act, therefore, as protective barriers against the solubilizing effect of the fixative on chondroitin sulfate.

Chromatography on dowex-2 of the sodium hydroxide extracts of epiphyses and diaphyses did more than separate inorganic sulfate from sulfate in bound form. A number of S^{35}-labelled components, from which S^{35}-sulfate was released on acid hydrolysis, were indicated. These were considered as possibly derived from chondroitin sulfate during the extraction with the 2.5 N sodium hydroxide. However, the sodium versenate "extracts," which were in essence homogenates from which salts had been removed by dialysis, also gave similar chromatograms of S^{35}-labelled materials. In the fractions, which contained the bound S^{35}-sulfate, uronic acid, and hexosamines, particularly galactosamine, were present. Indeed, in most of these fractions there was material which qualitatively resembled chondroitin sulfate. A second labelled component with an R_f value lower than chondroitin sulfate was also found by paper chromatography. The fact that these two were not separated by chromatography on the Dowex-2 and were contaminated by protein may be a partial explanation for the divergence of the analytical values for uronic acid, hexosamines, nitrogen, and sulfur from the values one would expect if only chondroitin sulfate were present.

An attempt to remove the protein from sodium versenate "extracts" by incubation with papain and trypsin prior to chromatography was not entirely successful. Though some protein was undoubtedly removed, the fractions containing bound S^{35}-sulfate still contained proteins or peptides. These were probably in association with chondroitin sulfate. Shatton and Schubert (14) have isolated a mucoprotein from cartilage, and others (for example 15, 16, 17, 18) have suggested the existence in tissues of protein-polysaccharide complexes. It may be because of the associated protein that S^{35}-labelled materials eluted in certain fractions did not reappear entirely in the same fractions on rechromatography, Fig. 9. It is, of course, possible that the polysaccharide as well as the protein are labile in the chromatographic system employed.

The overall impression from the present study and the work of others as to

the role of sulfate in the development of the metaphysis is as follows. A matrix produced by the cartilage cells of the epiphyseal plate region is somehow modified so that it can be calcified. The modified matrix still contains chondroitin sulfate. Among the ions which are precipitated during the calcification of the matrix, the sulfate ion is included. To a limited extent the sulfate ion thus included in the mineral deposit can exchange with circulating inorganic sulfate.

SUMMARY

S^{35}-sulfate was injected intraperitoneally into 7-day-old rats and their long bones were removed after intervals of time. The epiphyses were separated from the diaphyses for analysis.

From the diaphyses freed of bone marrow about 82 per cent of the S^{35} which they contained was extracted with a 2.5 N solution of sodium hydroxide. More, about 91 per cent of the S^{35}, was thus extracted from the epiphyses.

Dialysis of the extracts against water showed that the fraction of S^{35} which was dialyzable decreased rapidly with time. After 1 hour about 80 per cent and 50 per cent of the S^{35} in the extracts of diaphyses and epiphyses, respectively were found in the dialysates, after 24 hours about 20 per cent and 4 per cent, and after 120 hours 12 per cent and 1 per cent. Similar values for the S^{35} in inorganic sulfate were found when the extracts were chromatographed on an anion exchange resin, dowex-2.

The S^{35}, other than inorganic sulfate, was in the form of bound sulfate, which was released by acid hydrolysis. Uronic acid and hexosamines, primarily galactosamine, were associated with the S^{35}. Indeed, on paper electrophoretograms and paper chromatograms the major S^{35}-labelled component which was seen resembled chondroitin sulfate in its mobility. On the paper chromatograms, also a second S^{35}-labelled component with a mobility lower than that of chondroitin sulfate was found. It is unlikely that the latter is a breakdown product of chondroitin sulfate, produced in the course of extraction with the sodium hydroxide solution. In fact, both components were also found in sodium versenate homogenates which had been dialyzed extensively against water.

On the basis of these results it is suggested that the greatest part of the S^{35}-labelled materials previously demonstrated by autoradiography to be progressively deposited in the metaphyses after 24 hours,—as the concentration of S^{35}-sulfate concurrently decreased in the epiphyseal cartilage plates,—are akin to the chondroitin sulfate of the epiphyseal cartilage plates and are derived from the latter.

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