SOME ASPECTS OF THE METABOLISM OF SULFATE-S\textsuperscript{35} AND CALCIUM-45 IN THE METAPHYESSES OF IMMATURE RATS

INFLUENCE OF \(\beta\)-ESTRADIOL BENZOATE

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PLATE 28

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Autoradiographic studies on the disposition of S\textsuperscript{35}-sulfate in the long bones of suckling rats showed that the concentration of S\textsuperscript{35} in the metaphyses increased as it decreased in the epiphyseal cartilage plates (1). This suggested that the S\textsuperscript{35} which accumulated in the metaphyses was derived from the chondroitin sulfate of the epiphyseal cartilage plates. In contrast with the chondroitin sulfate in the cartilage, the labelled material in the metaphyses was insoluble in formalin saturated with barium hydroxide; it therefore seemed likely that the S\textsuperscript{35} in the metaphyses was in either inorganic sulfate or/and in some compound (or compounds) insoluble in the presence of barium ions at pH above 10. This suggestion was supported by Engfeldt, et al. (2, 3) who concluded that 4 days after the administration of sodium sulfate-S\textsuperscript{35} to dogs the isotope in bone was partitioned between inorganic sulfate and organic sulfate. Kent, et al., (4) separated the S\textsuperscript{35}-sulfate in rabbit bones into a form removed by decalcifying solutions and a form which was shown to be S\textsuperscript{35}-labelled chondroitin sulfate or a substance akin to it.

Massive doses of estrogens inhibit resorption of metaphyseal bone in immature rats (5-7), and the degree of inhibition is proportional to the dose (5). As a consequence the spongiosa may be many times longer than that of normal rats.

It occurred to us that after intermittent administration of 17-\(\beta\)-estradiol benzoate and S\textsuperscript{35}-sulfate to rats one could more easily harvest a sufficient amount of metaphyseal bone from them, than from normal rats, for the determination of the nature of the S\textsuperscript{35}-labelled materials shown by autoradiograms to be deposited in the metaphyses. Moreover, if in the isolation of the metaphyses for analysis remnants of epiphyseal cartilage were included as a contaminant, this contamination would be relatively less significant in the case of the samples from estradiol-treated rats than in the samples from normal rats. A comparative study was therefore made of the S\textsuperscript{35}-labelled materials in the metaphyses of normal rats and estradiol-treated rats in the hope that if the

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results on the estradiol-treated rats were qualitatively similar to the results on
the normal rats, the results on the latter would be more readily interpretable.
Calcium-45 was administered to some of the rats to assess the effect of estradiol
benzoate on the metabolism of calcium in the skeleton.

Material and Methods

Fourteen albino rats of the Sherman strain at the age of 21 days were separated without
regard to sex into 2 experimental groups of 4 animals each, and 2 control groups of 3 animals
each. Throughout the experimental period all animals were maintained on Purina dog biscuits
ad libitum. Each rat in the experimental groups received weekly an intraperitoneal injection
of 2 mg. of 17β-estradiol benzoate in 0.2 ml. of corn oil for 4 consecutive weeks. The
animals in the control groups received only corn oil in the same dose and according to the
same time-table. Twenty-four hours after each injection of hormone the rats in one experi-
mental and one control group received by intraperitoneal injection 100 μc. of carrier-free S35
as sodium sulfate; those in the other experimental and control group received 11 μc. of Ca45
as calcium chloride.1 Twenty-four hours after the fifth injection of radioisotopes, roentgeno-
grams of the animals were taken, blood samples were withdrawn from their hearts under ether
anesthesia, and humeri, tibiae, parietal bones, and dorsal skin were removed for analysis.
The blood was allowed to clot and the serum was separated by centrifugation.

The alkaline phosphatase activity was determined (8) in one humerus from each rat and
in portions of pooled sera. To this intent, weighed samples of the humeri were homogenized
into a 0.9 per cent solution of sodium chloride by grinding with sand in the cold, 0–4°C. The
homogenates were used after suitable dilution with the saline solution.

The concentration of S35 in the tissues of the rats treated with S35-sulfate was determined
as follows: Weighed samples were hydrolyzed with 4 ml. of 8 N hydrochloric acid in sealed
tubes in a steam bath for 16 hours. The hydrolysates were then diluted with water to 300 ml.,
5 ml. of 0.05 N sodium sulfate solution was added to act as carrier, and the sulfate was pre-
cipitated as barium sulfate by the addition of 5 ml. of a 10 per cent solution of barium chloride.
For counting, the precipitates were isolated on filter paper discs (9).

The femurs from the rats given S35-sulfate were cleaned of all muscle, split lengthwise, and
the marrow was teased, washed, and blotted out. The epiphysis of each distal end was peeled
away and the distal halves of the diaphyses were then pooled for analysis: one pool for the
control animals, the other for those treated with estradiol benzoate. The bone samples were
cooled to −40°C and pulverized with a cold stainless steel mortar and pestle. The concentration
of S35 was determined in small portions of the pulverized materials as indicated above.

For chromatography of the S35-labelled materials about 300 mg. of the pulverized bone
from each of the two pools was extracted with 5 ml. of a 10 per cent solution of sodium hy-
droxide for 16 hours at 0°C. The insoluble residue was separated by centrifugation and reex-
tracted twice with 2 ml. of water in the centrifuge. The water extracts were added to the so-
dium hydroxide extract and, after adjusting the pH to 6.5–6.7 with glacial acetic acid, the
volume was made up to 10 ml. with water. A precipitate which formed following the addition
of glacial acetic acid was removed by centrifugation. Five ml. of the clear solution was then
delivered onto a 1 by 4 cm. column of dowex 2 x 10, (CI), 200 to 400 mesh. Sulfur-35-labelled
materials were eluted with a solution of increasing chloride concentration. Each 10 ml. frac-
tion of the eluate was subjected to acid hydrolysis following which its S35 content was deter-
mined as indicated above.

Two further portions of about 150 mg from each of the pools of pulverized bone were sus-

1 The sulfur-35 and calcium-45 were obtained from the Oak Ridge National Laboratory on
allocation from the United States Atomic Energy Commission.
pended in 2 ml. of a 5 per cent solution of sodium versenate, pH 7.1 at 25°, and dialyzed in rocking dialyzers (10) against frequent changes of 25 ml. portions of the same solution of sodium versenate for 72 hours at 0°. This was followed by dialysis against frequent changes of distilled water for 48 hours under the same conditions. After this procedure the insoluble material in the inner chamber of the dialysis unit was lumpy but soft. It was finely dispersed by homogenization in a small glass homogenizer and the homogenate was then diluted to 10 ml. with water. Small portions were used for S assay according to the procedure employed in the case of the humeri. A 5 ml. aliquot from each homogenate was mixed with 1 ml. of a 2.5 per cent solution of cetyltrimethylammonium bromide, which Scott (11) has found to be an effective precipitant of acid mucopolysaccharides. The mixtures were set aside at 0° for 4 hours and the precipitates were isolated by centrifugation. Washing the precipitates once with 5 ml. and twice with 2 ml. portions of 95 per cent ethanol saturated with sodium chloride was effective in removing nearly all of the precipitant. The centrifuge tubes were inverted at this point and allowed to drain for 10 minutes. The residue was then extracted twice with 1 ml. portions of a 0.05 M sodium hydroxide solution. These extracts were analyzed for uronic acid (12), hexosamine (13), sulfur-35, and were subjected to paper chromatography (14) and electrophoresis on paper (15).

A parietal bone, the proximal end of a tibia, and a piece of skin from each animal were fixed in a 10 per cent solution of formalin for 48 hours. The proximal end of the other tibia from each of the animals was fixed in a 10 per cent solution of formalin saturated with barium hydroxide for 48 hours. After dehydration in alcohol and embedding in paraffin, 7 μ sections were prepared for autoradiography (16). Sections of skin, parietal bones, and tibiae from the animals that had received Ca were similarly prepared, except that fixation was in 80 per cent ethanol saturated with magnesium carbonate.

The concentration of calcium in the tissues of the rats given Ca was determined by precipitation of the calcium as the oxalate and titration with perchloratoxeric acid (17). Calcium-45 was determined by counting the activity in calcium oxalate precipitates isolated by filtration onto filter paper disks (18). Where necessary, carrier calcium was added to bring the calcium content of the sample to 4.0 mg.

RESULTS AND DISCUSSION

In agreement with the report of Day and Follis (5) no striking change was found in the alkaline phosphatase activity of the sera and humeri following the administration of estradiol benzoate. Also in agreement with others (5), the density and length of the spongiosa at the most actively growing ends of the long bones were found to be much greater in the roentgenograms of rats given estradiol benzoate than in those of the control rats.

The data on the concentration of Ca and calcium in the tissues are presented in Table I. The rats which received estradiol benzoate received a higher dose of Ca per gram of body weight than the control rats, because the former grew less than the latter. For this reason no interpretation of the slight differences in the values for specific activity is attempted. The data on the concentration of calcium in the bones, on the other hand, suggest an effect of estradiol benzoate on calcium metabolism: the concentration of calcium in the proximal ends of the tibiae and humeri, in the distal ends of the humeri, and in the parietal bones of the estradiol-treated rats was slightly higher than in those of the untreated rats.

The data on the concentration of S in the tissues of the rats are presented
in Table II. A higher concentration of this isotope was found in the tissues of the estradiol-treated rats than in the tissues of the control rats. However, it again seems hazardous to ascribe much meaning to these findings because of the possibility that the difference may be a reflection of the dissimilar doses of S\textsuperscript{35}-sulfate per gram of body weight.

The primary objective of these experiments was to determine the nature and origin of the S\textsuperscript{35}-labelled materials in the metaphyses; this was in part realized. By extraction with a 10 per cent sodium hydroxide solution, 44 and 63 per cent

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Untreated rats</th>
<th>Estradiol-treated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal No.</td>
<td>1 2 3</td>
<td>4 5 6 7</td>
</tr>
<tr>
<td>Initial weight, gm.</td>
<td>34 26 40</td>
<td>45 48 33 29</td>
</tr>
<tr>
<td>Final weight, gm.</td>
<td>151 146 146</td>
<td>122 110 118 106</td>
</tr>
<tr>
<td>c.p.m. per mg. calc. (mg. Ca per 100 gm.)</td>
<td>(10.4) (10.5) (11.0)</td>
<td>(10.2) (10.4) (10.7) (9.9)</td>
</tr>
<tr>
<td>Serum</td>
<td>8610 9050 8610</td>
<td>10200 10960 10070 10490</td>
</tr>
<tr>
<td>Skin</td>
<td>7000 6400 6180</td>
<td>8220 6170 8820 9320</td>
</tr>
<tr>
<td>Humerus, proximal</td>
<td>9590 5740 10040</td>
<td>9990 10620 9580 10380</td>
</tr>
<tr>
<td>Humerus, middle</td>
<td>8110 4700 9520</td>
<td>(21400) (20600) (20300)</td>
</tr>
<tr>
<td>Humerus, distal</td>
<td>7930 6370 7640</td>
<td>(13900) (16200) (14800)</td>
</tr>
<tr>
<td>Tibia, proximal</td>
<td>9240 6760 9380</td>
<td>(15100) (15300) (17100)</td>
</tr>
<tr>
<td>Parietal bone</td>
<td>7930 4700 9290</td>
<td>(17800) (17100) (17900)</td>
</tr>
</tbody>
</table>

At weekly intervals for 4 weeks, weanling rats received 2 mg. of 17-\-estradiol benzoate in 0.2 ml. of corn oil by intraperitoneal injection. The control animals similarly received only the corn oil. Twenty-four hours after each injection of estrogen 11 \microc. of Ca\textsuperscript{46} as calcium chloride in water was injected intraperitoneally into each rat. The rats were sacrificed 24 hours after the last dose of Ca\textsuperscript{46}.

The values in parentheses are the calcium concentrations, expressed as milligrams per cent, wet weight.
of the S\textsuperscript{35} was removed from the distal metaphyses of the femurs from the estradiol-treated and control rats, respectively. Of the extractable S\textsuperscript{35}, 75 and 86 per cent, respectively, did not pass through a cellophane membrane when dialyzed against water. Chromatography on an anion exchange resin, Dowex 2, showed that 9 per cent of the S\textsuperscript{35} in the extract of the bone from the control rats was in the form of inorganic sulfate; 22 per cent of the S\textsuperscript{35} in the extract of the bone from the estradiol-treated rats was accounted for in this form (Text-fig. 1). The remainder of the S\textsuperscript{35} in both extracts behaved chromatographically as does the S\textsuperscript{35} in a similar extract of epiphyses removed from 8-day-old rats 24 hours after dosing with S\textsuperscript{35}-sulfate. This latter observation strongly suggests that some of the S\textsuperscript{35} in the metaphyses is present in material akin to chondroitin sulfate. If the extraction with sodium hydroxide solution is assumed to remove all of the material resembling chondroitin sulfate, but only a small fraction of the inorganic sulfate, then 33 to 54 per cent of the S\textsuperscript{35} in the metaphyses is present in forms other than inorganic sulfate.

It is more likely that about 80 per cent of the S\textsuperscript{35} in the distal metaphyses of the femurs is present in compounds other than inorganic sulfate. This higher value is indicated by the experiments in which the pulverized metaphyseal bone was dialyzed against a solution of sodium versenate and then against water. From the sample of bone from the control animals 6 per cent and from the sample of bone from the estradiol-treated animals 21 per cent of the S\textsuperscript{35} was removed by dialysis. These values for dialyzable S\textsuperscript{35} and the values for

### TABLE II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Untreated rats</th>
<th>Estradiol-treated rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.P.M. per 100 mg. tissue</td>
<td>C.P.M. per 100 mg. tissue</td>
</tr>
<tr>
<td>Serum</td>
<td>50 70 106</td>
<td>129 149 205 254</td>
</tr>
<tr>
<td>Skin</td>
<td>365 508 857</td>
<td>780 881 1000 930</td>
</tr>
<tr>
<td>Humerus, proximal</td>
<td>2640 4300 6770</td>
<td>7250 5960 6380 8040</td>
</tr>
<tr>
<td>Humerus, middle</td>
<td>834 1120 2330</td>
<td>2790 2110 2410 2920</td>
</tr>
<tr>
<td>Humerus, distal</td>
<td>1530 2190 3060</td>
<td>4330 2860 3340 4030</td>
</tr>
<tr>
<td>Parietal bone</td>
<td>702 1060 1370</td>
<td>1630 1380 1600 1870</td>
</tr>
</tbody>
</table>

2 mg. of 17β-estradiol benzoate in 0.2 ml. of corn oil was injected intraperitoneally into weanling rats at weekly intervals for 4 weeks. The control animals similarly received only the corn oil. Twenty-four hours after each injection of estrogen 100 μc. of S\textsuperscript{35} as sodium sulfate in water was injected intraperitoneally. The rats were sacrificed 24 hours after the last dose of S\textsuperscript{35}-sulfate.
S\textsuperscript{35} as inorganic sulfate, as determined by chromatography of extracts on dowex 2, suggest that relatively more of the S\textsuperscript{35} was in the form of inorganic sulfate in the metaphyses of the estradiol-treated rats than in the metaphyses of the control rats. Why this should be so is not apparent. Of the S\textsuperscript{35} which did not pass through the cellophane membrane, 83 to 87 per cent was precipitable by cetyltrimethylammonium bromide and was subsequently soluble in 0.05 \textit{N} sodium hydroxide. In the latter solutions, in addition to S\textsuperscript{35}-sulfate, uronic acid and hexosamine were present. Indeed, when these solutions were analyzed by paper chromatography and paper electrophoresis, material was found which behaved as did purified chondroitin sulfate, isolated from bovine nasal septa.
by the procedure of Boström and Månsson (19). The materials extracted from
the bones were metachromatic in the same way as the purified chondroitin
sulfate. Moreover, the areas with radioactivity on the papers, as determined by
autoradiography, coincided with the areas of metachromasia.

The experiments on the samples of bone decalcified by dialysis against a
solution of sodium versenate yielded an additional point of interest. The samples
of metaphyseal bone were a mixture of spongy and compact bone. The ratio,
mass of spongy bone to mass of compact bone, however, was greater for samples
from the estradiol-treated rats than for samples from the control rats. If, now,
the concentration of chondroitin sulfate in the spongiosa is greater than in the
compact bone, one should find more chondroitin sulfate per gram of sample
from the femurs of the estradiol-treated rats than in a sample from the femurs
of the control rats. Indeed, the distal metaphyses from the femurs of the experi-
mental animals contained 1.4 times more uronic acid and hexosamine per gram
of bone than the comparable metaphyses of the control rats. However, the con-
centrations of uronic acid and hexosamine in the extracts were not in a ratio of
1:1. It was found that per gram of bone there were 1.46 mg. of uronic acid and
0.32 mg. of hexosamine in the case of the control animals, and 2.00 mg. of
uronic acid and 0.48 mg. of hexosamine in the case of the experimental animals.
The reason for the deviation from a ratio of 1:1 is unexplained. Enough material
was not available for a determination of sulfate-sulfur.

The autoradiograms of the proximal metaphyseal region of tibiae from the
estradiol-treated rats show more S\(^{35}\) and Ca\(^{45}\) than do corresponding regions of
tibiae from the control rats. Autoradiograms of the tibiae from the rats given
estradiol benzoate show that deposition occurred in definite strata (Figs. 2, 4,
and 6). Particularly in the case of the S\(^{35}\)-sulfate–dosed rats (Fig. 2) it can be
seen that each weekly dose left its mark. Especially interesting is the difference
which can be made out as to the arrangement of the strata of S\(^{35}\) and Ca\(^{45}\). In
Fig. 6 it is seen that during the 24 hours following the last injection of Ca\(^{45}\) much
of this isotope has been incorporated in the metaphysis immediately subjacent
to the epiphyseal cartilage plate. This is not the case with S\(^{35}\)-sulfate (Fig. 2):
the same region in the metaphysis gives a relatively weak autoradiographic
reaction. These observations are in agreement with the thesis that Ca\(^{45}\) is taken
up into metaphyseal bone by exchange with newly deposited calcium as well
as by deposition. The S\(^{35}\)-sulfate, on the other hand, comes for the most part
from the chondroitin sulfate of the cartilage during the development of meta-
physeal bone.

In the opinion of some workers (5) estrogens in massive doses have no ap-
parent effect on endochondral ossification in the rat, although regressive
changes in the epiphyseal disk of the rat tibia have been observed by others
(6, 7). An examination of the stained sections of the proximal portions of tibiae
convinced us that there was an effect. Without exception, the epiphyseal
cartilage plates of the tibiae from the estradiol-treated rats (Fig. 7) were narrower than those of the tibiae from the control rats (Fig. 8). This was because there were fewer columnar and hypertrophic cartilage cells in the epiphysal cartilage plates of the treated animals compared with the numbers of these cells in the controls. Because of this difference in the cartilage plates, the rates of endochondral ossification may have differed in these two groups of animals.

SUMMARY

Weanling rats were given 2 mg. of 17-β-estradiol benzoate at weekly intervals for 4 weeks. Twenty-four hours after each intraperitoneal injection of the estrogen 100 μc. of S35-sulfate or 11 μc. of Ca45 was similarly injected. The animals were sacrificed 24 hours after the last dose of isotopes.

An effect of estradiol benzoate on calcium metabolism was deduced from the observation that the concentration of calcium in some tissues of the treated rats was higher than the concentration in the tissues of untreated rats.

Alkaline extracts of the distal metaphyses of femurs from the estradiol-treated and from control rats, given S35-sulfate, were shown by chromatography on an anion exchange resin to contain from 9 to 22 per cent of the S35 as inorganic sulfate. From similar bone samples, 6 to 21 per cent of the S35 was removed by decalcification with sodium versenate. Most of the remaining S35 was associated with uronic acid and hexosamine; on paper chromatograms and paper electrophoretograms S35 was shown to be part of material which migrated and was metachromatic in the same way as purified chondroitin sulfate.

Autoradiograms of the proximal ends of tibiae from the animals given estradiol benzoate showed that both the S35 and Ca45 were deposited in the metaphysis in strata. The arrangement of the strata of S35, however, was different from the arrangement of the strata of Ca45. This difference in arrangement is interpreted as indicating that most of the S35 in the metaphysis was derived from the chondroitin sulfate of the cartilage plate which the metaphysis had replaced.

BIBLIOGRAPHY

EXPLANATION OF PLATE 28

Fig. 1. A reproduction of an autoradiogram of a section of a proximal end of a tibia from a 49-day-old rat which had received 100 μc. of S35-sulfate by intraperitoneal injection at weekly intervals for 4 weeks. The bone was fixed for 48 hours in a 10 per cent solution of formalin, embedded in paraffin, and sectioned at 7 μ. Kodak contrast process ortho film was exposed to the section for 2 weeks. × 5.

Fig. 2. Same as for Fig. 1, except that the tibia was from a rat that received 2 mg. of 17-β-estradiol benzoate in 0.2 ml. of corn oil 24 hours before each injection of S35-sulfate. × 5.

Fig. 3. Same as for Fig. 1. The tibia was from the rat that furnished the material for Fig. 1, except that the bone was fixed for 48 hours at 25° C. in a 10 per cent solution of formalin saturated with barium hydroxide. × 5.

Fig. 4. Same as for Fig. 3, except that the tibia was taken from the rat that furnished the material for Fig. 2. × 5.

Fig. 5. Reproduction of an autoradiogram of a section of the proximal end of a tibia from a 49-day-old rat that received 11 μc. of Ca45 at weekly intervals for 4 weeks. The bone was fixed for 48 hours in 80 per cent ethanol saturated with magnesium carbonate, embedded in paraffin, and sectioned at 7 μ. Kodak contrast process ortho film was exposed to the section for 24 hours. × 5.

Fig. 6. Same as for Fig. 5, except that the tibia was from a rat given 2 mg. of 17-β-estradiol benzoate in 0.2 ml. of corn oil 24 hours before each injection of the Ca45. × 5.

Fig. 7. Microphotograph of the cartilage plate at the proximal end of a tibia from a 49-day-old rat that had received 2 mg. of 17-β-estradiol benzoate in 0.2 ml. of corn oil at weekly intervals for 4 weeks. Toluidine blue stain. × 171.

Fig. 8. Microphotograph of the cartilage plate at the proximal end of a tibia from a 49-day-old rat, an untreated litter mate of the rat the cartilage plate of which is shown in Fig. 7. Toluidine blue stain. × 171.
(Dziewiatkowski et al.: Sulfate-$S^{35}$ and calcium-45 metabolism in metaphyses)