EFFECT OF HORMONES ON THE TURNOVER OF POLYSACCHARIDES IN CONNECTIVE TISSUES

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ABSTRACT A number of hormones somehow modify the turnover of the polysaccharides in a variety of connective tissues. In hypophysectomized animals the turnover of chondroitin sulfate and hyaluronic acid is decreased; when such animals are given growth hormone the turnover of chondroitin sulfate is enhanced but that of hyaluronic acid is unaltered. The effect of parathyroid extracts may be of a dual nature: in some connective tissues there may be an increase in the rate at which chondroitin sulfate is catabolized, in other tissues its synthesis may be stimulated. Thyroxine effectively restores toward normal the depressed synthesis and breakdown of polysaccharides in hypothyroid animals. Estradiol, in addition to inhibiting the resorption of the metaphyses in rats, inhibits the synthesis of chondroitin sulfate in cartilage and aorta. Cortisone too inhibits the synthesis of chondroitin sulfates and hyaluronic acid; its effect on their catabolism is not as striking.

Cells of the connective tissues are capable apparently of synthesizing the uridine diphosphate derivatives of glucuronic acid and iduronic acid and of *N*-acetyl glucosamine or *N*-acetyl galactosamine. Whether it be the hexosamine derivative or uronic acid derivative, glucose is the precursor. Somehow the requisite uridine diphosphate derivatives are enticed to react to build up heterologous polysaccharides, some of which in the presence of 3′-phospho-adenosine-5′-phosphosulfate and transferases are sulfated. A variety of heterologous polysaccharides are thus produced: hyaluronic acid, keratosulfate, heparin, and the chondroitin sulfates. A more detailed presentation of the reactions involved has been made earlier in this Symposium. Additionally, it has been suggested that these polysaccharides are only parts of much more complex macromolecules, in which they are combined with protein. Despite their size, however, they are synthesized intracellularly and secreted into the extracellular spaces to become part of the "ground substance." In his discussion at this Symposium on the fine structure of cells, Dr. Porter considered some of the observations bearing on this point. Additional evidence in support of the conclusion that protein-polysaccharides are synthesized intracellularly is available. From the observations which have been made along these lines the following (1) are relevant.

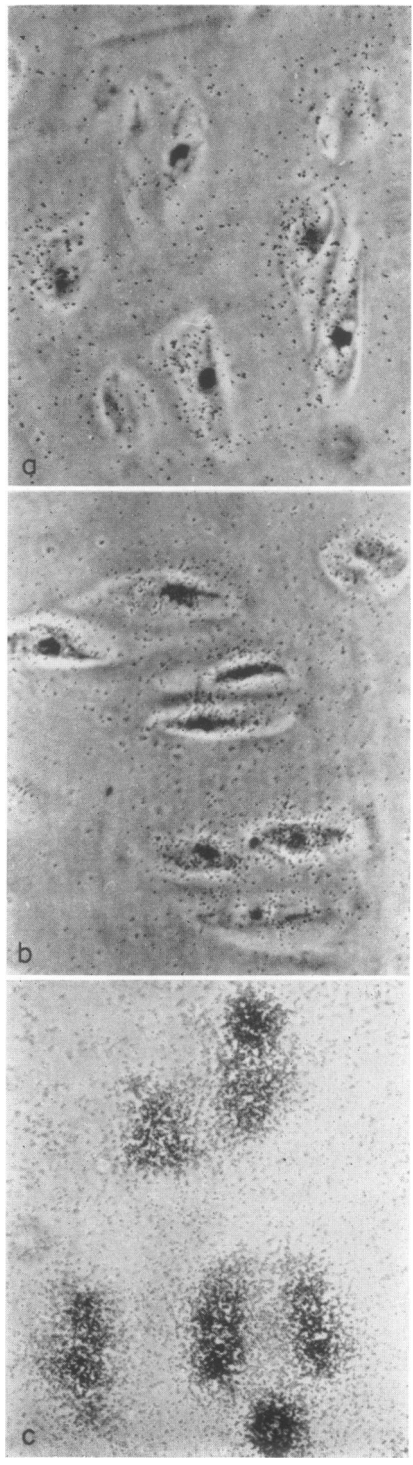

FIGURE 1 Autoradiograms of slices of bovine costal cartilage incubated for 2 hours in buffered solutions of salts containing (*a*) C^{14}-L-phenylalanine, (*b*) C^{14}-L-leucine, or (*c*) S^{35}-sulfate. The slices were fixed in a 10 per cent solution of formalin for 24 hours and dehydrated in graded concentrations of ethanol before imbedding in paraffin. Deparaffinized sections, 7 μ thick, affixed to microscope slides were coated with colloidon and dipped in NTB_3 emulsion. In each case the tracer is localized predominantly in the chondrocytes. The reaction over the matrix is only slightly greater than background. This figure originally appeared in *J. Biol. Chem.*, 1962, **237**, 2729.

Autoradiograms of slices prepared from bovine costal cartilage and incubated in a buffered solution of salts, containing, in addition, either S^{35}-sulfate, C^{14}-L-leucine, or C^{14}-L-phenylalanine, show that the radioactive tracers are predominantly localized in the chondrocytes (Fig. 1). It is pertinent that the radioactive tracers thus visualized in the cartilage slices at the end of 2 or 4 hours of incubation were not removed by washing with solutions of unlabeled Na_2SO_4, L-leucine, or L-phenylalanine; they were not removed by the process of fixation in formalin or by dehydration of the tissues in ethanol, initially at a concentration of 30 per cent. From cartilage slices thus incubated protein-polysaccharides were isolated according to Malawista and Schubert (2) and they were fractioned by differential centrifugation (3). The specific activities of the protein-polysaccharides were found to increase at parallel rates with time of incubation (Fig. 2), suggesting that the protein and the polysaccharides are synthesized concurrently (1).

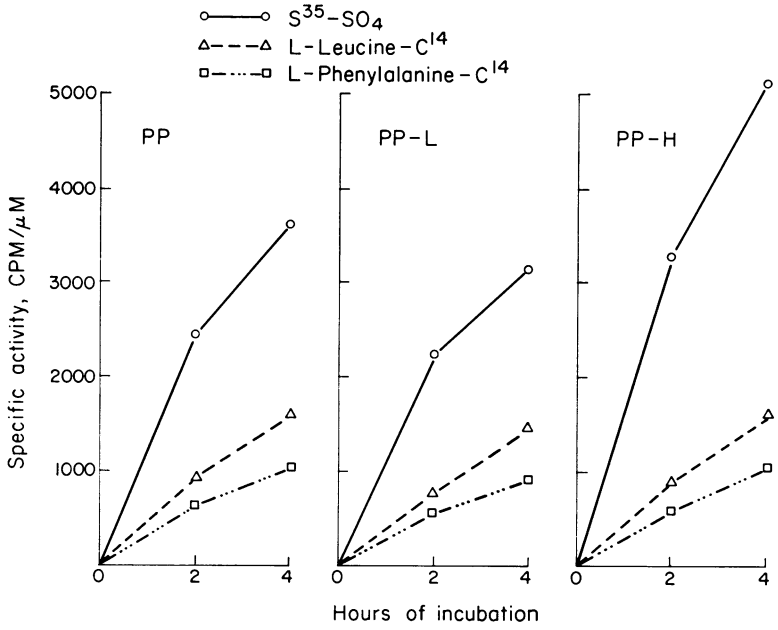

FIGURE 2 Increase in specific activities of protein-polysaccharides with time. The protein-polysaccharides were isolated from slices if bovine costal cartilage after these had been incubated in buffered solutions of salts containing S^{35}-sulfate, C^{14}-L-leucine, or C^{14}-L-phenylalanine. The specific activities are given per μM of sulfate, leucine, or phenylalanine, as the case may be. This figure originally appeared in *J. Biol. Chem.*, 1962, **237**, 2729.

Additionally, the accumulation of C^{14} from L-leucine and L-phenylalanine and of S^{35} from inorganic sulfate in the matrix surrounding chondrocytes of the epiphyses has been visualized (4). On such evidence, which is in accord with the observations

of a number of investigators (5–8), and the results obtained by the use of hyaluronidase on demineralized sections of the cartilage it has been suggested that the protein-polysaccharides are synthesized and secreted by the chondrocytes. It is noteworthy that interpretations of the functions of fine structures in chondrocytes (9) are also in accord with the suggestion that extremely large macromolecules are synthesized intracellularly and secreted without disruption of the whole cell.

Before embarking on a discussion of the effects of hormones on the turnover of polysaccharides in connective tissues, a consideration of the experimental approaches used in the studies may be helpful. In many of the studies normal animals have been treated with a hormone and then injected with a labeled precursor; *i.e.,* C^{14}-glucose, C^{14}-acetate, or S^{35}-sulfate. At intervals of time thereafter, the tissue of interest was excised and the polysaccharides were isolated to ascertain whether their specific activities were greater than or less than the specific activities of the polysaccharides isolated from like tissues of appropriate control animals. Alternatively, tissues of interest were excised from hormone-treated animals and incubated in buffered media containing labeled precursors. In experiments of the latter type, because of practical considerations, incubation was usually for short periods of time, a matter of a few hours. Consequently, only differences in rates of synthesis could be assessed. To this end also the polysaccharides were isolated to evaluate their specific activities.

Obvious variations on the above theme have also been used. Animals were deprived of an endocrine gland under study and some were subsequently treated with the hormone or hormones normally secreted by the gland for comparison with untreated and with intact litter mates. Another approach has been to label the polysaccharides *in situ* with a radioactive tracer and then to treat the animals with a hormone so as to determine whether there was any change in the catabolism of the polysaccharides.

The most direct and usually unequivocal approach to the study of the effect of a hormone on the turnover of polysaccharides involves the isolation and purification of the polysaccharides before a determination of their specific activity is attempted. This, however, is not an easy task. Furthermore, in most instances, particularly when dealing with small laboratory animals, like tissues from large numbers of animals have to be pooled to provide sufficient amounts of material from which enough of the polysaccharides can be isolated for an adequate analysis. Less direct means have been sought. In the case of the chondroitin sulfates a less direct approach has been used. In many studies the amount of sulfate "fixed" by a tissue has been determined and hopefully equated with chondroitin sulfate (5). The designation fixed has been assigned to the S^{35}-sulfate so bound in a tissue that it was neither washed out readily nor was it removed by dialysis. Justification for equating fixed S^{35}-sulfate with S^{35}-chondroitin sulfate (5) derives from studies in which it was shown that S^{35} administered as inorganic sulfate exists only temporarily in the tissues and circulation as inorganic sulfate, nearly all of the S^{35}-inorganic sulfate is rapidly

removed from the animal, particularly by way of the urine. In cartilage, for example, within 24 hours after administration the fraction of S^{35} present therein as inorganic sulfate is of the order of 2 per cent of the total concentration of S^{35} (Figs. 3 and 4). The remainder is non-dialyzable and most of it can be isolated as chondroitin sulfate (10). What happens can be visualized autoradiographically (Fig. 5 and 6). S^{35}-

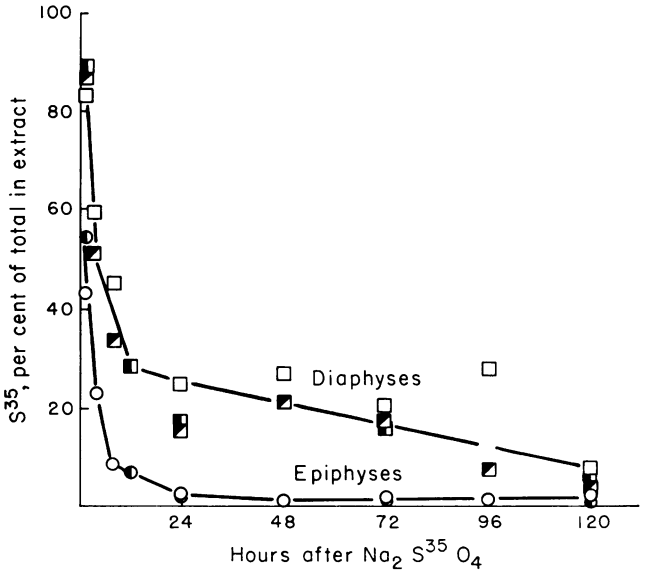

FIGURE 3 Recovery of S^{35} as inorganic sulfate from extracts of epiphyses and diaphyses removed from suckling rats at intervals of time following administration of $Na_2S^{35}O_4$. The extracts were prepared by the use of a 2.5*N* solution of NaOH. The inorganic sulfate was separated from other S^{35}-labeled materials by chromatography on columns of Dowex — 2 × 10. This figure originally appeared in *J. Exp. Med.*, 1957, **106,** 509.

labeled inorganic sulfate presented to the cartilage is rapidly taken up by the chondrocytes and used for the synthesis of chondroitin sulfate (11). The increase in the concentration of the label in the certilages is rapid. First it is seen predominantly in the chondrocytes and in time in the matrix as well. The concentration of S^{35}-labeled inorganic sulfate, however, is never very striking in the cartilage, even very soon after administration (Fig. 5). In the course of time (Fig. 6) some of the chondroitin sulfate synthesized by the chondrocytes of the epiphyses is partially utilized as the cartilage gives way to bone, be it that of the secondary ossification center or that of the metaphyses.

It should be obvious, then, that autoradiography is another technique by which the effects of hormones on the polysaccharides in specific tissues can be ascertained. With this technique one may obtain much additional information not only as to differences in concentration of labeled materials in the tissues of one animal as

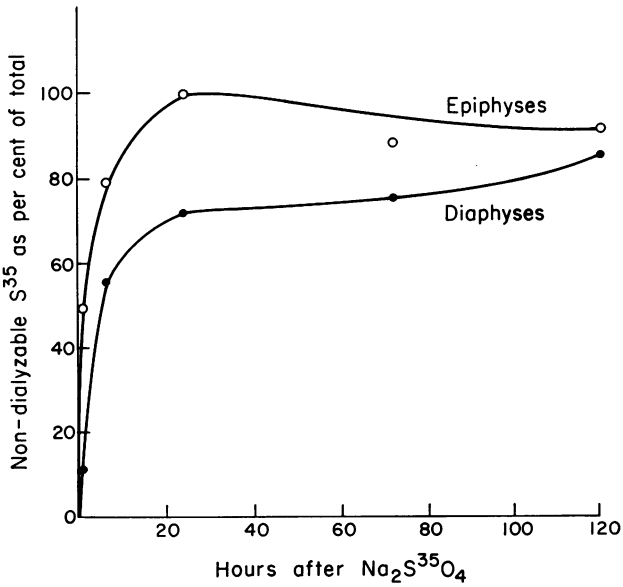

FIGURE 4 Increase with time in the fraction of S^{35} which was not removed from homogenates of epiphyses and diaphyses by dialysis. The tissues were from rats each of which had received 100 μc of S^{35}-sulfate when they were 7 days old. The tissues were homogenized into a 5 per cent solution of sodium 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. This figure originally appeared in *J. Exp. Med.*, 1957, **106,** 509.

compared to that in the tissues of another animal, but also as to whether these differences are the result of a difference in concentration in part or the whole of a tissue. It is even feasible to quantitate any differences found by the use of a densitometer (Fig. 7) (12). Autoradiography is, relatively speaking, exceedingly simple. It should be used whenever possible in conjunction with chemical analyses.

By the use of the above-described methods of assay the effects of hormones on the turnover of polysaccharides in a variety of tissues have been investigated. In the case of some hormones a number of studies are on record.

HYPOPHYSECTOMY AND GROWTH HORMONE

A rather striking decrease in the amount of S^{35}-sulfate fixed by costal cartilage of hypophysectomized rats was noted by Ellis, Hublé, and Simpson (13). At no time up to 25 hours after injection did the cartilage take up more than 11 per cent as much S^{35}-sulfate as the cartilage in control animals, even though the blood levels in the hypophysectomized animals were about 4 times higher than the blood levels in the control rats. The injection of 25 γ of growth hormone daily for 3 days partially restored the ability of the costal cartilage in the hypophysectomized rats to fix sul-

fate: in the hormone-treated rats 25 hours after S^{35}-sulfate there was 3 times as much S^{35}-sulfate in the costal cartilage as in the costal cartilage of the untreated hypophysectomized rats. That is, under the influence of the hormone, the hypophysectomized rats showed a concentration of the radioisotope in their cartilage which was 33 per cent of the concentration in the intact rats.

These observations were confirmed and extended by Denko and Bergenstal (14). They found that in hypophysectomized rats the uptake of S^{35}-sulfate by costal cartilage was only about one-half of that in the intact controls. Following on the administration of 100γ of growth hormone daily for 8 days to hypophysectomized rats the uptake of S^{35}-sulfate by the costal cartilage was increased three- to fourfold. It was about that of the intact control animals. Comparable doses of growth hormone when given to normal rats produced no stimulation of S^{35}-sulfate fixation. In fact, a suggestion of a decreased uptake was obtained. The uptake of S^{35}-sulfate by the tibial caps and xiphoid cartilage of the hypophysectomized rats was not depressed as markedly as that in the costal cartilage. The fixation of S^{35}-sulfate by these tissues, however, was enhanced also in the hormone-treated rats.

Murphy, Daughaday, and Hartnett (15) found that growth hormone stimulates the uptake of S^{35}-sulfate by both epiphyseal cartilage and nasal cartilage of hypophysectomized rats. The response of the tibial epiphysis was proportional to the logarithm of the dose of growth hormone between 10 γ and 250 γ given during the 24 hours prior to the administration of S^{35}-sulfate. This action of the growth hormone was not entirely specific: thyroxine alone increased the incorporation of sulfate and thyrotropin augmented the action of growth hormone.

Since the maximal effect of growth hormone was found to occur within 24 hours, at a time when the cartilage plate had not as yet increased to its greatest width, it was suggested that growth hormone acts to increase both the amount of cartilage and its metabolic activity.

In subsequent work (16) it was found that sera from normal rats contain a factor which promotes the uptake of sulfate *in vitro* by cartilage from hypophysectomized rats. This Salmon and Daughaday (16) termed the "sulfation factor." The sulfation factor appears to be induced by growth hormone, since it appears in the plasma of hypophysectomized rats after the administration of growth hormone. The sulfation-promoting activity of sera from normal rats could not be ascribed to their glutamine or insulin contents.

A year later, Salmon and Daughaday (17) reported that their sulfation factor was in reality a mixture of dialyzable and non-dialyzable components. Sera from hypophysectomized rats do not contain the non-dialyzable component; this then is more precisely the sulfation factor. In part, the dialyzable components, effective in stimulating the uptake of sulfate *in vitro,* are amino acids. Of thirteen amino acids tested, thereonine was most effective, but not as effective as the mixture of thirteen amino acids. When Eagle's mixture of amino acids was used, valine was the limiting

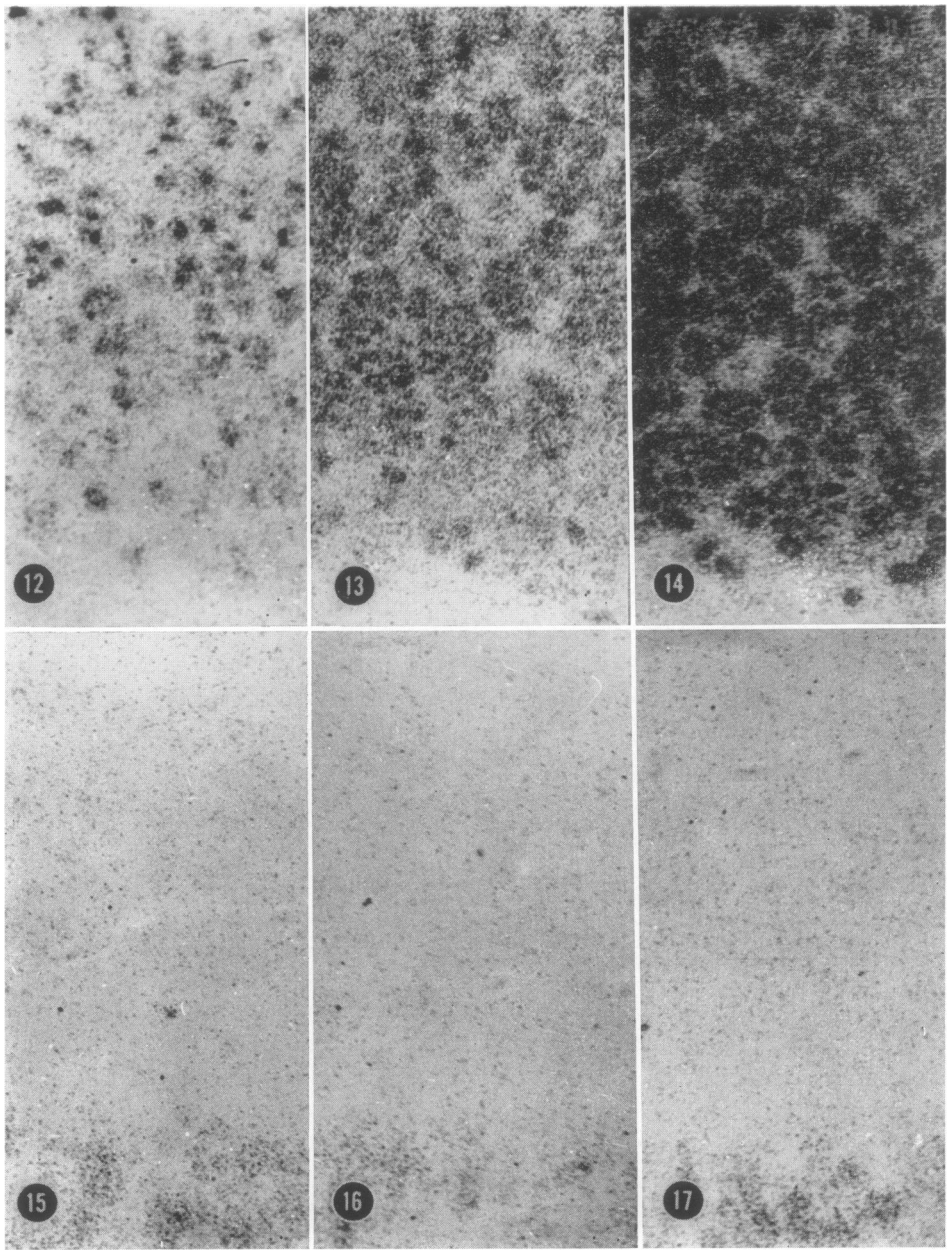

FIGURE 5 The reproductions 12, 13, and 14 are autoradiograms of regions in the epiphyseal plates of knee-joints removed 30, 60, and 120 minutes, respectively, after administration of 25 μc of S^{35}-sulfate to 7-day-old rats. The knee-joints were fixed in 10 per cent formalin. The autoradiographic reaction is predominantly given by the

amino acid (18). Some nine vitamins, when added to the incubation medium, were without effect (17).

In the studies thus far enumerated, an effect on the synthesis of chondroitin sulfate in the cartilages of hypophysectomized rats was of prime concern. The catabolism of hyaluronic acid and chondroitin sulfate in the skin of normal, hypophysectomized, and growth hormone–treated hypophysectomized rats has been examined by Schiller and Dorfman (19). The polysaccharides were isolated, separated, and analyzed for their specific activities 1, 3, 5, and 9 days after administration of a mixture of acetate-1-C^{14} and $Na_2S^{35}O_4$. In normal animals, half-life times of 2.9 and 4.8 days were calculated for hyaluronic acid and chondroitin sulfate, respectively. As a consequence of hypophysectomy these mucopolysaccharides were removed from the skin at a much slower rate, as indicated by half-life time of 5.1 days for hyaluronic acid and 11.7 days for chondroitin sulfate. Treatment of the hypophysectomized rats with growth hormone resulted in an accelerated catabolism of chondroitin sulfate, half-life time of 5.5 days, but the catabolism of hyaluronic acid was not increased, half-life time of 7.9 days.

PARATHYROIDECTOMY AND PARATHORMONE

Over the years it has been repeatedly suggested that extracts of the parathyroid gland, presumably parathormone, affect the components of bone matrix as well as the mineral phase. Because of these suggestions a few attempts have been made to ascertain whether the turnover of sulfated polysaccharides is altered. The administration of extracts of the parathyroid to animals previously given S^{35}-sulfate provoked a rise in the level of S^{35} in the blood and this was reflected in an increased excretion of the radioisotope in the urine (20, 21). An attempt by autoradiography to visualize loci in bones from which these S^{35}-labeled materials might have come (20) was in a sense unsuccessful. Indeed, it was found that the metaphysis of the proximal end of the tibia in hormone-treated rats produced more intense autoradiograms than that from control animals. This may be a suggestion that parathormone has an inhibitory effect on the resorption of the metaphysis or that it stimulates the produc-

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cells. There is, however, also an increase with time in the fixed S^{35} (chondroitin sulfate–S^{35}) of the matrix. × 148.

Reproductions 15, 16, and 17 are autoradiograms of regions in the epiphyseal plates removed 30, 60 and 120 minutes, respectively, as above but fixed in a 10 per cent solution of formalin, which was saturated with barium hydroxide. Fixation by this means results in the removal of chondroitin sulfate but not of inorganic sulfate. A comparatively small amount of S^{35} is in evidence. An examination of the lower portion of each frame reveals that the reaction of the metaphysis is greater than that of the cartilage. This figure originally appeared in *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 401.

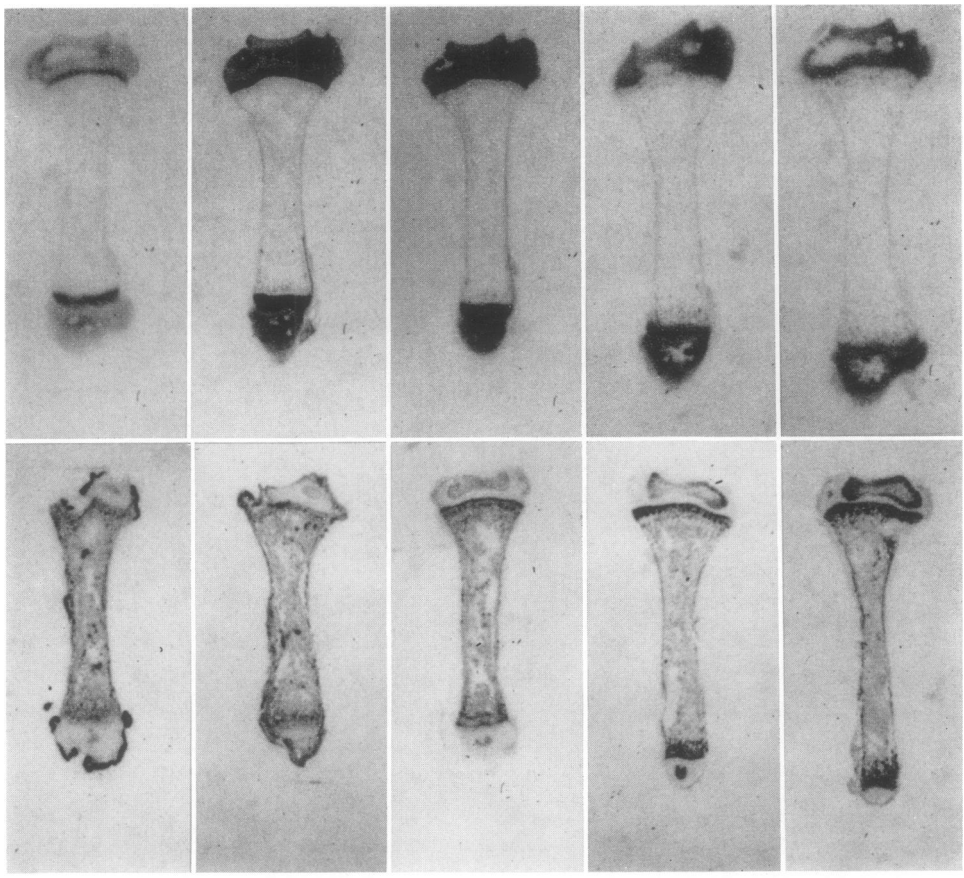

FIGURE 6 Contact autoradiograms of humeri from suckling rats given S^{35}-sulfate. The autoradiograms shown in the upper part of the figure were produced on Kodak contrast process ortho film by humeri fixed in 10 per cent formalin. A progressive accumulation of S^{35} up to 24 hours in the epiphseal cartilage can be seen. Thereafter, the concentration of S^{35} in the epiphyses decreases. Concurrently with this decrease there is an increase in the concentration of the tracer in the metaphyses and secondary ossification centers, as seen in the autoradiograms shown in the lower part of the figure. The latter were produced by sections of contralateral humeri fixed in a 10 per cent solution of formalin saturated with barium hydroxide. The S^{35} thus visualized was subsequently shown to be associated for the most part with material resembling chondroitin sulfate. In making any comparison between the two series of autoradiograms it is necessary to bear in mind that the film was exposed to the sections of humeri fixed in formalin saturated with barium hydroxide for a period of time which was twice as long as that used with sections of humeri fixed in formalin alone. × 4.8. This figure originally appeared in Radioisotopes and Bone, (edited under the direction of F. C. McLean by P. Lacroix and A. M. Budy), Oxford, Blackwell Scientific Publications, 1962, 277

tion of chondroitin sulfate in the epiphyseal plate, from which much of the material akin to chondroitin sulfate in the metaphysis derives. In line with the second of these two suggestions is the recent work of Johnston, Deiss, and Miner (22), who found an increased rate of incorporation *in vitro* of C^{14} from glucose-2-C^{14} into the hexosamines of calvaria from young rats given an extract of the parathyroid. No

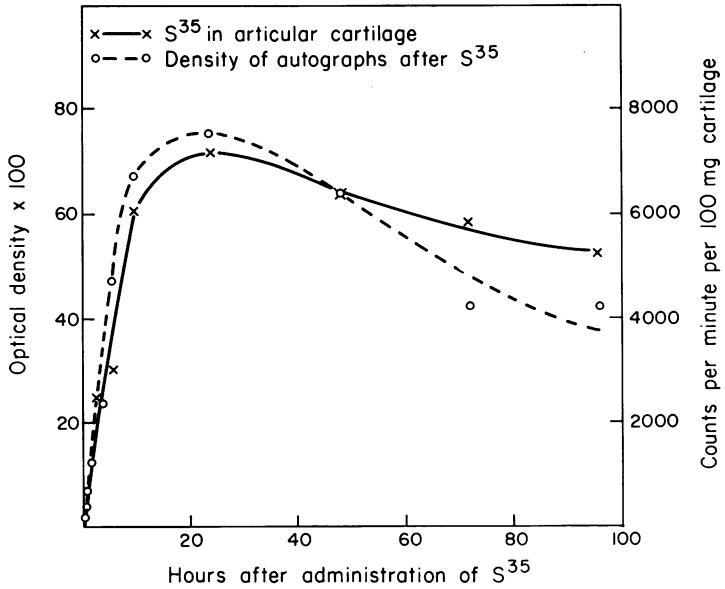

FIGURE 7 Change in the concentration of S^{35} with time in the epiphyses of suckling rats given S^{35}-sulfate. The change was determined by counting samples of $BaSO_4$ and by measurement of the optical density of autoradiograms produced by sections of comparable tissue. This figure originally appeared in *J. Exp. Med.*, 1952, **95**, 489.

significant difference was discernible in a comparison of calvaria from parathyroidectomized and sham-operated rats. If instead of hexosamines relatively crude mucoproteins were extracted from femurs, which had been incubated as the calvaria, the specific activities of the hexosamines in such mucoproteins from the animals given an extract of the parathyroid were higher than those from the control animals.

THYROIDECTOMY AND THYROXINE

Thyroxine is known to promote over-all differentiation and maturation of animals. In the case of cartilage, thyroxine primarily influences its maturation (23). Some years ago, the effect of thyroxine on the metabolism of sulfate in the epiphyseal cartilage of suckling rats was examined (24). It was found that thiouracil inhibited the uptake of S^{35}-sulfate and that this inhibition could be counteracted by thyroxine. Furthermore, if into suckling rats, previously given S^{35}-sulfate, thiouracil or thyroxine

was injected, the former decreased the rate and the latter increased the rate at which S^{35}-labeled components in the cartilage were catabolized (Fig. 8).

In an extension of this study (25), rats were thyroidectomized, either by administration of iodine-131 on the 1st day after birth or surgically when they were 28 days old. Starting with the 28th day of life until the experiment was terminated, the animals were fed a diet low in iodine. Some were given a supplement of L-thyroxine and all received intraperitoneally 1 μc of S^{35} as sulfate per gram of body weight 12 hours before sacrifice.

The concentration of sulfur-35 in the sera of the untreated animals thyroi-

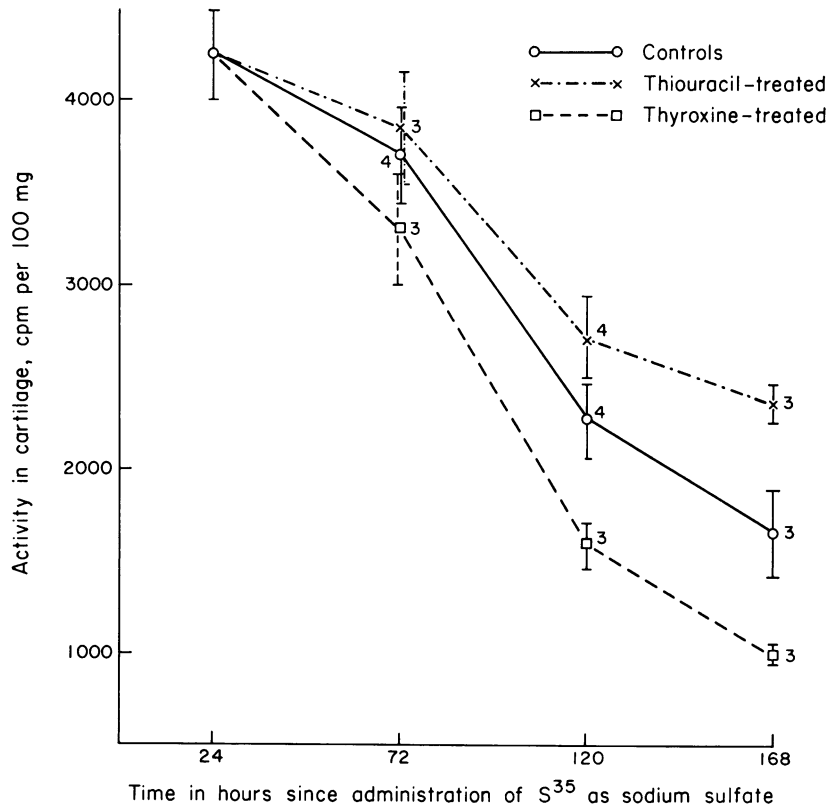

FIGURE 8 Effect of thyroxine and thiouracil on the disappearance of S^{35} from the cartilage of the knee-joints of suckling rats. S^{35}-sulfate was injected intraperitoneally on the 7th day of life. Into some of the rats 4 mg of thiouracil was then injected daily, intraperitoneally, starting with the 8th day of life. Other animals received 200 γ of thyroxine intraperitioneally on the 8th day of life and subsequently 100 γ doses on the 10th and 12th days of life. The number of animals used in obtaining a particular average value is shown by the numeral directly to the right of a plotted value. This figure originally appeared in *J. Biol. Chem.*, 1951, **189**, 717.

dectomized by the use of iodine-131 (Group 1A of Table I) was much higher than in the sera of the animals in the other groups (Groups 1B, 1C, and 1D). Despite this apparently favorable situation, the specific activities of the crude sulfomucopolysaccharides isolated from the skeletons and the pelts of the thyroidectomized and

TABLE I

CONCENTRATION OF SULFUR-35 IN SERA AND FEMURS, AND IN THE SULFOMUCOPOLYSACCHARIDES OF SKELETONS AND PELTS OF THYROIDECTOMIZED RATS

Group	Treatment	Serum	Femurs		Sulfomucopolysaccharides	
			Ends	Shafts	Skeleton	Pelt
		CPM/ *ml*	CPM/ *mg*	CPM/ *mg*	CPM/*mg* SO_4-*S*	CPM/*mg* SO_4-*S*
	Experiment I, thyroidectomy with I-131					
IA	Without thyroxine	7,250	13.4	12.6	9,275	4,950
IB	5γ thyroxine daily	1,050	12.0	5.4	15,840	8,700
IC	Controls, low iodine	2,765	20.4	9.2	31,130	29,500
ID	Controls, stock diet	975	11.3	3.9	18,990	26,800
	Experiment II, surgical thyroidectomy					
IIA	Without thyroxine	3,110	16.7	8.5	21,250	20,300
IIB	5γ thyroxine daily	3,030	16.1	6.9	40,120	29,000
IIC	10γ thyroxine daily	2,675	15.7	6.6	27,210	25,400
IID	Controls, low iodine	2,690	20.3	8.8	35,960	28,500
IIE	Controls, stock diet	1,160	13.8	4.7	22,200	26,500

The tissues were removed for analysis when the rats were 42 days old, 12 hours after an intraperitoneal injection of 1 μc of S^{35}-sulfate per gm of body weight. In experiment I, the thyroids were destroyed as a consequence of the administration of 100 μc of iodine-131 on the 1st day of life. In Experiment II, parathyroidectomy-thyroidectomy was performed on the 28th day of life. This table originally appeared in *J. Exp. Med.*, 1957, **105,** 69.

untreated rats were significantly lower than the specific activities of like samples from the skeletons and pelts of the animals with thyroids or the thyroidectomized animals given L-thyroxine. That the synthesis of sulfomucopolysaccharides was depressed in the thyroidectomized rats would not have been deduced if one had been simply satisfied to determine the uptake of S^{35}-sulfate by the ends and shafts of femurs (columns 4 and 5 of Table I).

Autoradiograms of the proximal ends of the tibiae and of the tail skin (Fig. 9) accord with the analytical data on the specific activities of the sulfomucopolysaccharides isolated from the skeletons and pelts. The tibiae and the samples of tail skin were fixed in formalin at pH 3-4 so that the inorganic sulfate was leached out. It can be seen that the concentration of sulfur-35 in the epiphyseal plates of the thyroidectomized rats (Fig. 9-1) was lower than in the epiphyseal plates of rats

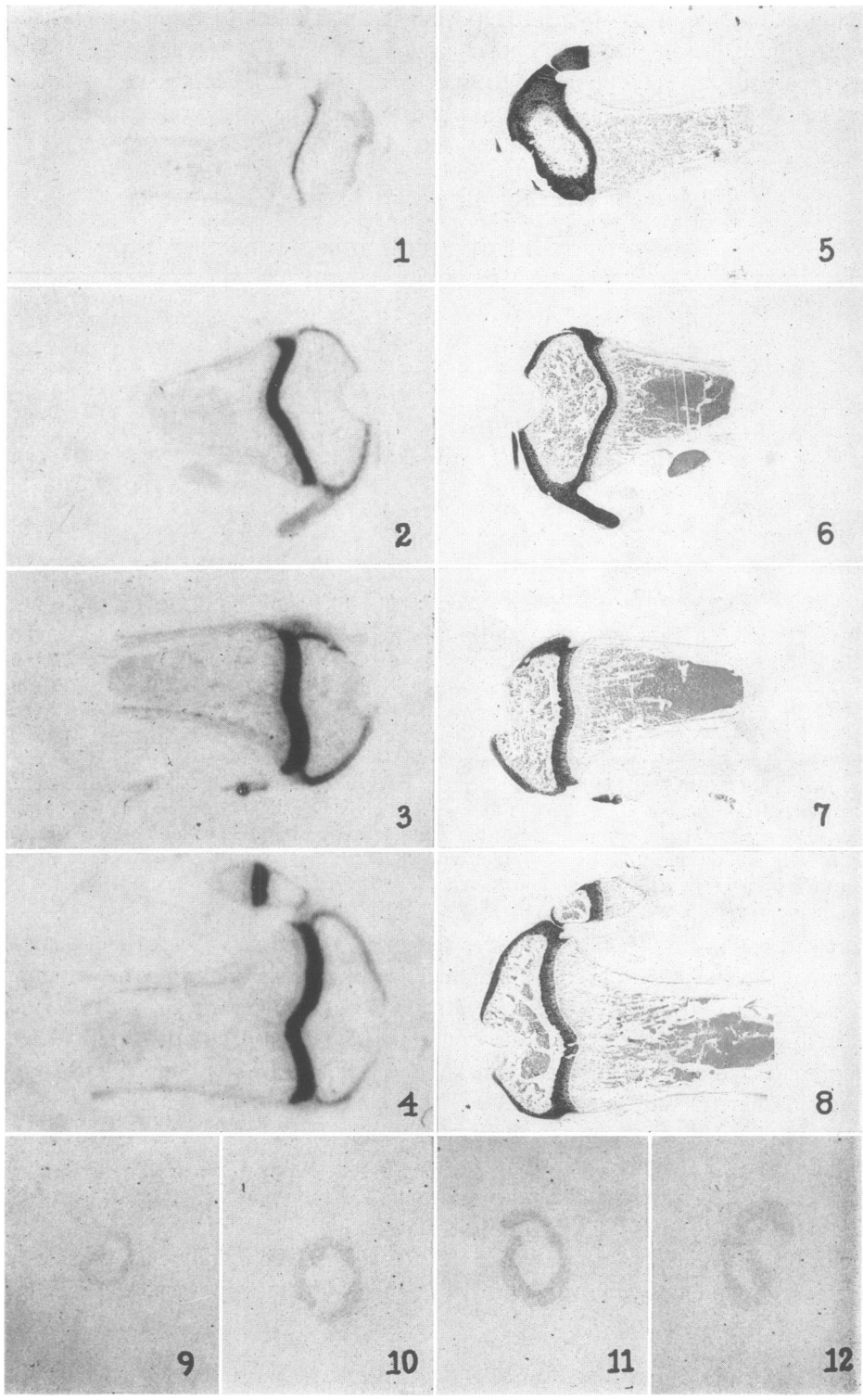

FIGURE 9

thyroidectomized and then treated with thyroxine (Fig. 9-2), or of intact rats on a low iodine diet (Fig. 9-3), or of intact rats on a stock diet (Fig. 9-4). This, even though the epiphyses of the untreated thyroidectomized animals were as those from animals much younger than the controls (Fig. 10); it has been shown that the uptake of S^{35}-sulfate by the epiphyses of bones in young rats is greater than the uptake by the epiphyses of bones in old rats.

Qualitatively similar results were obtained using surgically thyroidectomized rats.

More recently, Schiller, Slover, and Dorfman (26) administered acetate-1-C^{14} to normal rats and to rats fed a diet containing propylthiouracil; Some of the rats in the latter group were supplemented with thyroxine. The disappearance of C^{14} from hyaluronic acid and chondroitin sulfate fractions of the skin was determined. The C^{14} disappeared much more slowly from both mucopolysaccharide fractions isolated from the hypothyroid rats than from comparable samples isolated from the normal animals or the animals fed propylthiouracil and given thyroxine to counteract its effects. Of some interest is their finding that the concentration of hyaluronic acid was higher and that of the chondroitin sulfates, the sum of CSA-A and CSA-B, was lower in the hypothyroid animals than in the normal animals. Digestion of the chondroitin sulfate fraction with hyaluronidase suggested that in hypothyroid animals the ratio of chondroitin sulfate B to A is decreased.

The possibility that thyroid-stimulating hormone was responsible for the effects noted was eliminated: the changes found in the hypothyroid rats were also observed in hypophysectomized rats and, furthermore, it was demonstrated that two different preparations of thyrotropic hormone had no effect on the levels of either hyaluronic acid or of the chondroitin sulfates in intact rats.

FIGURE 9 Effect of thyroidectomy with iodine-131 on the uptake of S^{35}-sulfate by tibiae and skins as reflected by contact autoradiograms produced by sections of these tissues. The tissues were fixed in 10 per cent formalin. Directly to the right of an autoradiogram of a proximal end of a tibia in a photograph of the stained section which produced the autoradiogram. The sections were stained with 0.1 per cent toluidine blue in 30 per cent ethanol.

Frame 1. Produced by a section of a tibia from a rat whose thyroid has been destroyed by 100 μc of iodine-131, administered on the 1st day of life.

Frame 2. The tibia which produced this autoradiogram was removed from a rat, a litter mate, which was similarly thyroidectomized by the use of iodine-131, subsequently given 5 γ of L-thyroxine daily for 14 days.

Frame 3. Autoradiogram of a section of tibia from a control rat fed a low iodine diet, the same diet on which the thyroidectomized rats were maintained after weaning.

Frame 4. Autoradiogram produced by a section of tibia from a normal rat fed the stock diet.

Frames 9, 10, 11, and 12 are autoradiograms of tail skin from rats whose tibiae produced the autoradiograms shown as frames 1, 2, 3, and 4, respectively. This figure originally appeared in *J. Exp. Med.*, 1957, **105**, 69.

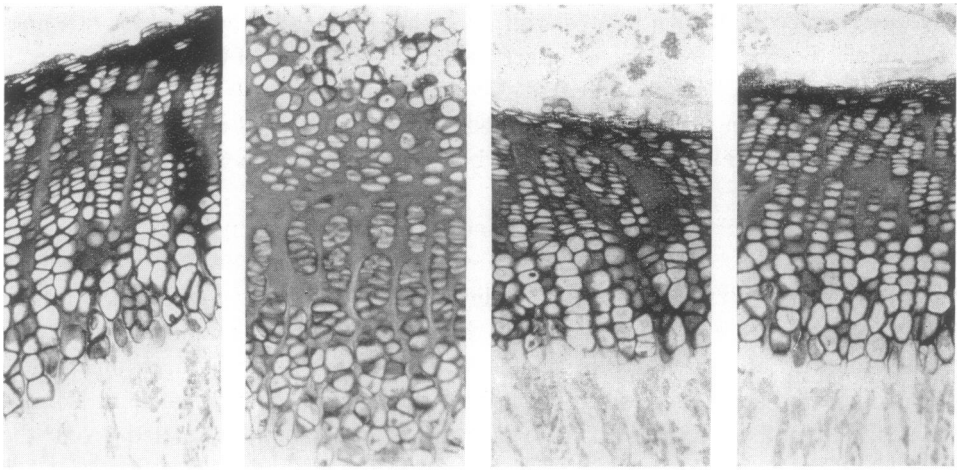

FIGURE 10 In rats thyroidectomized by the administration of iodine-131 on the 1st day of life, the development of the epiphyseal plate of the proximal end of the tibia was retarded, as compared to that in intact rats on a stock diet or a low iodine diet. The administration of 5 γ of L-thyroxine daily, starting on the 28th day of life, promoted maturation of the epiphyses. × 109.

INSULIN

In alloxan-diabetic rats (27) the incorporation of C^{14}-acetate into the hyaluronic acid and chondroitin sulfates of the skin was found to be about one-third of that in the skin of either normal or partially fasted rats. A comparable decrease in the utilization of S^{35}-sulfate for the synthesis of chondroitin sulfates was found. Although the catabolism of hyaluronic acid was also decreased, the catabolism of the chondroitin sulfates was apparently not affected.

In vitro, insulin in concentrations likely to be found under physiological conditions does not stimulate the uptake of sulfate by cartilage (18). It has been found, however, to act synergistically with a balanced mixture of amino acids, but not with glucose, as a stimulant for the uptake of sulfate by cartilage from hypophysectomized rats.

STEROIDS

Following on the work of Ragan *et al.* (28), who showed that cortisone delayed the development of elements of the connective tissue, Layton (29) on the basis of studies with S^{35}-sulfate suggested that the synthesis of chondroitin sulfate was inhibited. This suggestion has been amply verified by *in vivo* and *in vitro* studies (30–35), among others, not only by noting that there was a decreased uptake of sulfate by various

tissues in or from animals treated with cortisone, but also by an evaluation of the specific activities of chondroitin sulfates isolated from various tissues.

Additionally, the synthesis of hyaluronic acid is inhibited in the skin of rats injected with cortisone acetate (32), and the catabolism of both hyaluronic acid and the chondroitin sulfates is gradually suppressed by daily injections of hydrocortisone acetate (32).

Under some circumstances, the effect of cortisone on the uptake of sulfate by healing fractures can be counteracted in part by 17-ethyl-19-nortestosterone but not by testosterone propionate or methyltestosterone (33). Pretreatment of rats with 17-ethyl-19-nortestosterone alone stimulates the uptake of S^{35}-sulfate by fractured humeri (33).

Resorption of metaphyseal bone in immature rats is inhibited by massive doses of estrogens in proportion to the dose (36). As a consequence, the spongiosa is

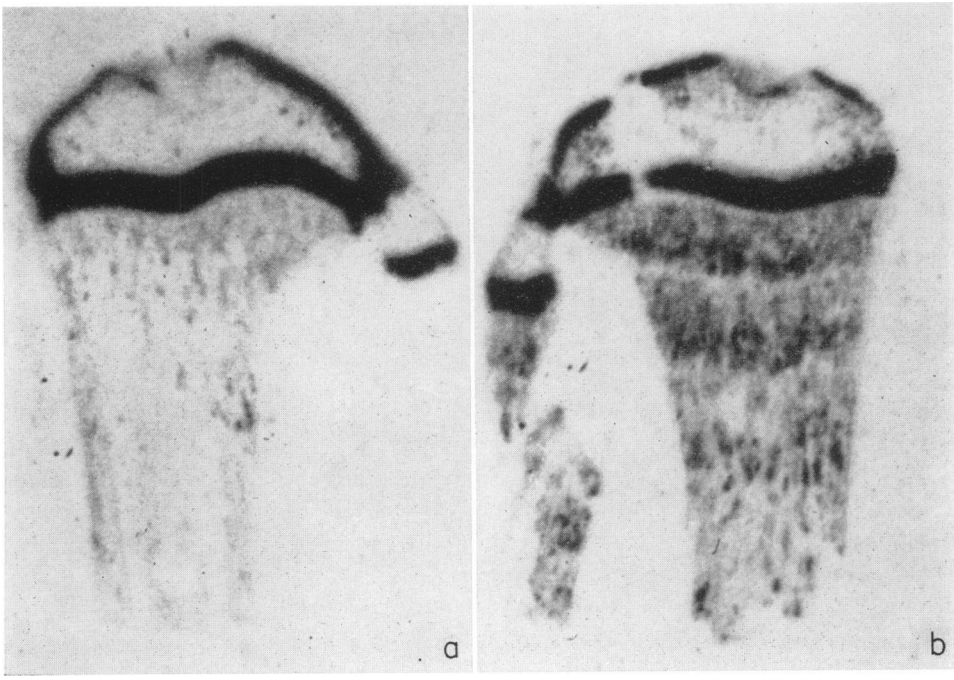

FIGURE 11 *a.* An autoradiogram of a section of a proximal end of a tibia from a 49-day-old rat which had received 100 μc of S^{35}-sulfate by intraperitoneal injection at weekly intervals for 4 weeks. The rat was sacrificed 24 hours after the last dose of S^{35}. The bone was fixed for 48 hours in 10 per cent formalin, embedded in paraffin, and sectioned at 7 μ.

b. Same as for frame *a*, 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 S^{35}-sulfate. $\times$ 9. This figure originally appeared in *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 151.

many times more abundant in the estrogen-treated rats than in normal rats. In an experiment done some years ago (37) weanling rats were given 2 mg of 17-β-estradiol benzoate at weekly intervals for 4 weeks and 100 μc of S^{35}-sulfate was injected intraperitoneally 24 hours after each dose of the estrogen. The animals were examined 24 hours after the last dose of the isotope. Autoradiograms (Fig. 11) of the proximal ends of the tibiae showed that the S^{35} had been deposited in the metaphysis in strata; each weekly dose had left its mark. The S^{35}-labeled components in such metaphyses as well as those in the metaphyses of litter mates that had received the same amount of S^{35}-sulfate but no 17-β-estradiol benzoate were

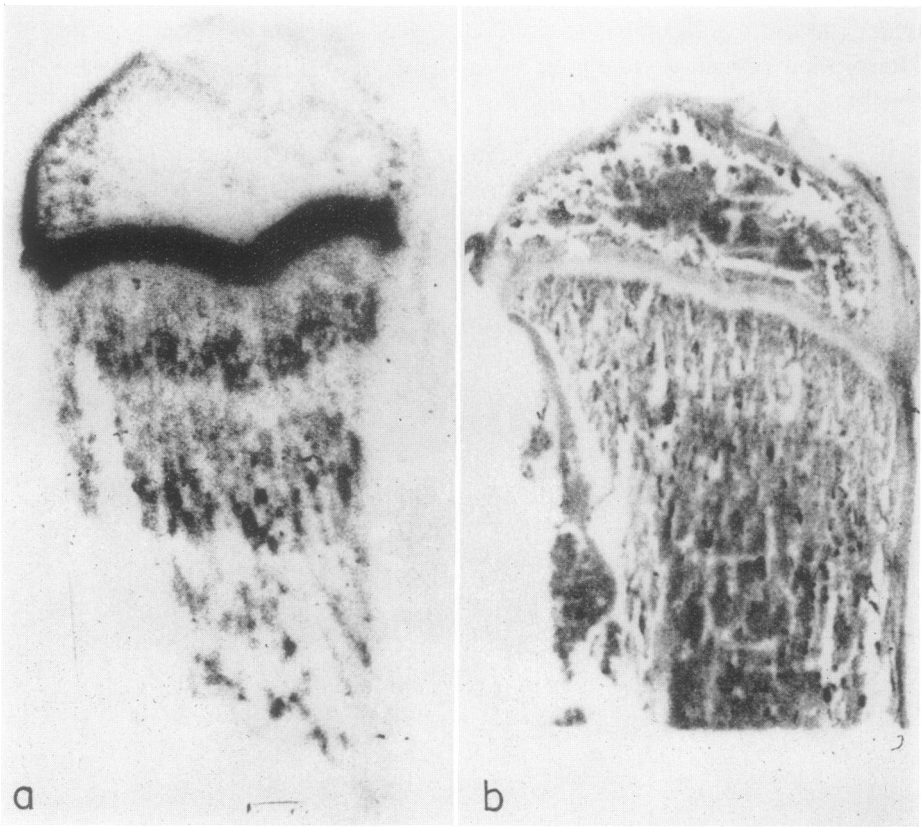

FIGURE 12 Contact autoradiograms of sections of the proximal ends of tibiae from rats into which 17-β-estradiol benzoate, 2 mg each time, was injected at weekly intervals for 4 weeks. Twenty-four hours after each dose of steroid the rats received (*a*) 120 μc of S^{35}-sulfate or (*b*) 10 μc of C^{14}-L-phenylalanine. They were sacrificed 24 hours after the last dose of radioactive materials. The sections were demineralized with a 4 per cent solution of acetic acid. It can be seen in (*a*) that repeated doses of S^{35}-sulfate are reflected as strata of S^{35} in the metaphysis. Similar strata of C^{14} are not discernible in the metaphyses of the rats given C^{14}-L-phenylalanine. This figure originally appeared in *J. Biophysic. and Biochem. Cytol.*, 1963, **18,** 19.

separated by chromatography: most of the S^{35}, between 80 to 90 per cent of the total, was found in material which resembled chondroitin sulfate, as judged, among other criteria, by chromatography and electrophoretic mobility. The over-all impression from these results and previous observations (10) was that most of this material, which resembled chondroitin sulfate, had been derived from the epiphyseal plate, wherein it had been synthesized by the chondrocytes.

Since the indications are that the chondrocytes of the epiphyses synthesize and secrete protein-polysaccharides and not free chondroitin sulfate, experiments like those with S^{35}-sulfate in estrogenized rats were repeated using C^{14}-L-leucine and C^{14}-L-phenylalanine (4). The results of these latter experiments suggest that the protein moiety of the protein-polysaccharides is somehow split away before the septa of epiphyseal cartilage are calcified. No strata of C^{14} in the metaphyses were

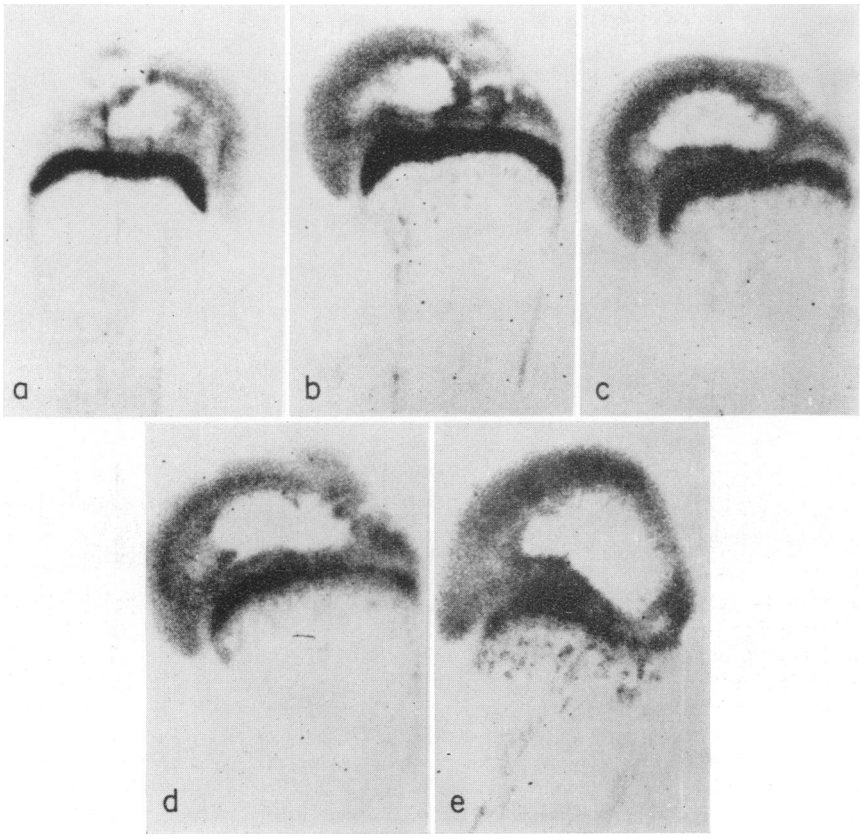

FIGURE 13 Contact autoradiograms of demineralized sections of the proximal ends of humeri from suckling rats at (*a*) 4 hours, (*b*) 24 hours, (*c*) 48 hours, (*d*) 72 hours, and (*e*) 96 hours after intraperitoneal injection of S^{35}-sulfate. The sections were demineralized by the use of a 4 per cent solution of acetic acid.

seen, as had been the case with S^{35}-sulfate (Fig. 12). This finding alone would be insufficient for the suggestion just made. Further evidence stems from observations on the turnover of C^{14}, given as C^{14}-L-leucine or C^{14}-L-phenylalanine, in the bones of suckling rats. With time after the administration of S^{35}-sulfate to suckling rats one sees that the whole of the epiphyseal plate is labeled and that eventually the metaphysis immediately subjacent to the plate is labeled, although less intensely than the plate (Figs. 6 and 13). With time after the administration of the C^{14}-labeled

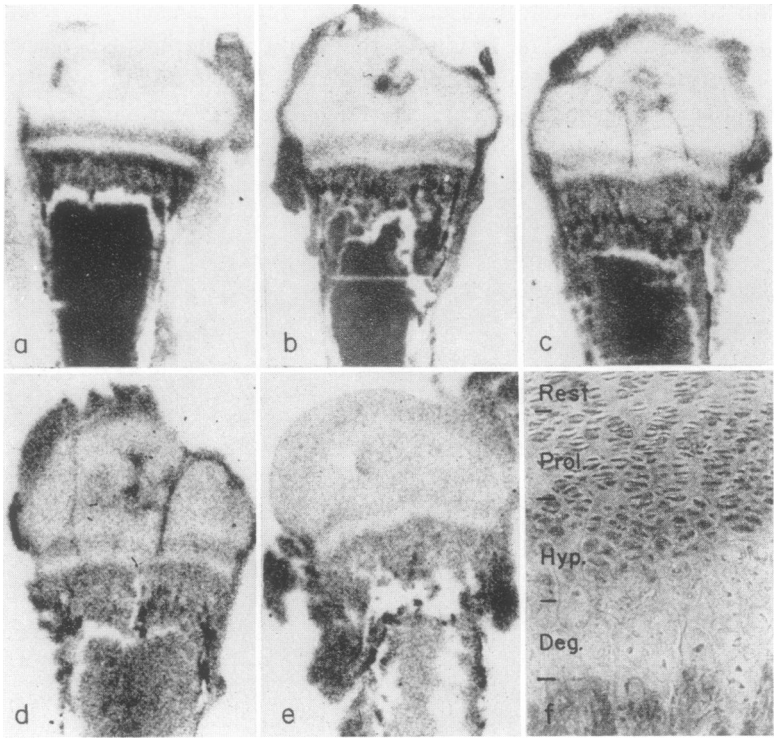

FIGURE 14 Contact autoradiograms of demineralized sections of the proximal ends of humeri from suckling rats at (*a*) 4 hours, (*b*) 24 hours, (*c*) 48 hours, (*d*) 72 hours, and (*e*) 96 hours after intraperitoneal injection of C^{14}-L-phenylalanine. The sections were demineralized by the use of a 4 per cent solution of acetic acid. A band of C^{14} in the metaphysis, just underneath the epiphyseal plate, can be seen in (*a*). Later, (*b*) and (*c*), it can still be discerned but at some distance from the epiphyseal plate.

(*f*). This is a coated autoradiogram of the epiphyseal plate of a humerous removed 4 hours after injection of C^{14}-L-phenylalanine into a suckling rat. It can be seen that at this time the highest concentration of C^{14} was present in the proliferating and mature chondrocytes. This autoradiogram is shown to delineate the position of the band of C^{14} across the epiphyses as seen in (*a*) through (*e*). As the concentration of C^{14} in this region of the epiphyseal plate decreased with time, there was no marked increase in the concentration of C^{14} in the region of the very hypertrophic and degenerate chondrocytes.

amino acids, on the other hand, there is no perceptible increase in the concentration of C^{14} in that region of the epiphyseal plate which abuts most intimately on the metaphysis (Fig. 14). Indeed, at higher magnification it was seen that there was a decrease in the concentration of C^{14} in the septa which are about to be calcified; C^{14} was discernible only in the chondrocytes which are about to be eliminated. Furthermore, attempts to extract protein-polysaccharides from metaphyses of normal and estrogenized rats, and from the metaphyses of normal rabbits and calves, have been unsuccessful so far (4).

Of some interest, in view of recent work (38) on the effects of estradiol on the uptake of sulfate by cartilages, is an incidental observation. In rats given massive doses of estradiol benzoate the epiphyseal plate of the proximal tibia was narrower than that in litter mate controls; it looked more senescent (Fig. 15).

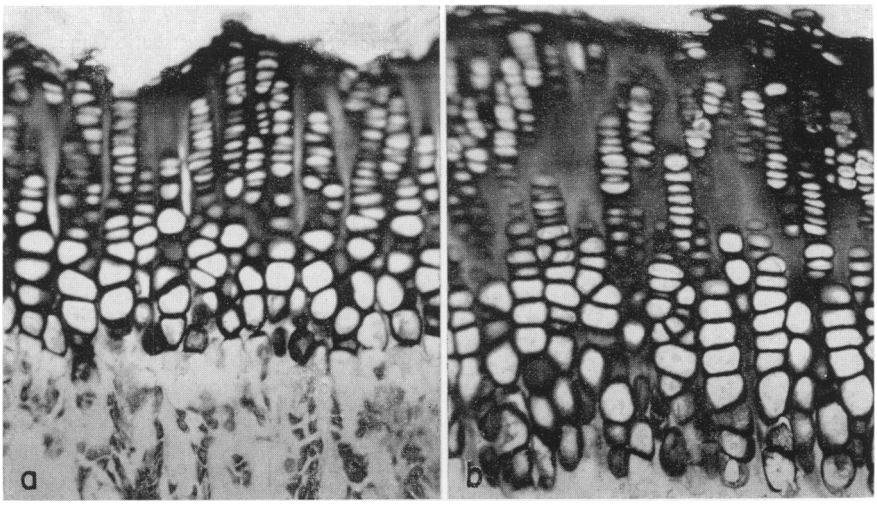

FIGURE 15 *a.* Microphotograph of the cartilage plate at the proximal end of a tibia from a 49-day-old rat that had received 2 mg of 17-β-estradiol benzoate in 0.2 ml of corn oil at weekly intervals for 4 weeks. Toluidine blue stain.

b. 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 whose cartilage plate is shown in Fig. 15 *a.* Toluidine blue stain. This figure originally appeared in *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 151.

Priest, Koplitz, and Benditt (38) found that estradiol benzoate, again administered to rats in large, unphysiological amounts, reduces the incorporation of S^{35}-sulfate by cartilage and aortas, *in vivo* and *in vitro,* to about 34 per cent of that in control animals. This effect of estradiol benzoate becomes apparent within 3 days for cartilage and 3 weeks for the aorta. The effect is very probably not a result of the suppression of testosterone. In the absence of the hypophysis the effect of the estradiol benzoate can still be elicited. That the reduced incorporation of sulfate

represents a reduced synthesis of sulfated polysaccharides is borne out by the finding that after treatment with estradiol benzonate the specific activity of the isolated polysaccharides was lower than that from control animals; the reduction was comparable whether S^{35}-sulfate or C^{14}-glucose was used (39).

On the basis of the work which has been done to date it is apparent that hormones do have an effect on the turnover of polysaccharides in connective tissues. The effects which have been observed, however, are on a macroscopic level. It remains for the future to determine exactly what mechanisms on the molecular level are inhibited or stimulated, as the case may be. The question still remains, how do the hormones act?

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