INHIBITION OF SYNTHESIS OF SULFATED MUCOPOLYSACCHARIDES BY ESTRADIOL*

By ROBERT E. PRIEST,‡ M.D., and R. MARLENE KOPLITZ

(From the Department of Pathology, University of Washington School of Medicine, Seattle, and the Seattle Veterans Administration Hospital, Seattle)

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In a previous report (1) evidence was presented that estradiol reduces the incorporation of radioactive sulfate into cartilage and aortas of normal rats. It was also observed that the thoracic segment of the aorta incorporated radioactive sulfate at a significantly greater rate than did the abdominal segment. These observations bear directly upon our understanding of the normal metabolism of connective tissues and could conceivably also bear upon sex differences in incidence and severity of disease in these tissues (2–6). Therefore, further exploration of these phenomena seems warranted, and this report presents evidence indicating that the effect of this estrogenic hormone is on the rate of synthesis of sulfated acid mucopolysaccharides by these tissues.

Materials and Methods

Animals.—All animals were young adult male rats of the Sprague-Dawley strain weighing 175 to 200 gm. They were housed individually under conditions of constant temperature and humidity and were supplied with Purina chow and tap water *ad libitum.*

Hormones and Administration.—1 ml ampules containing 1.67 mg of estradiol benzoate in sesame oil (progynon-B)[1] were used. Subcutaneous injections of 0.2 ml (0.333 mg) were given daily, 6 days each week, for 3 weeks, to each animal. Control animals were given 0.2 ml of sesame oil alone.

Incubation of Tissues.—Following sacrifice by exsanguination under ether anesthesia or by cervical fracture, thoracic and abdominal portions of the aorta were removed as well as samples of costal cartilage. These were cleaned of surrounding tissue and divided into segments 1 to 2 mm long. Usually tissues from each animal were treated as individual samples, although in some experiments like tissues from a group of animals were pooled and aliquots taken for incubation.

Samples were incubated in 5 ml of Tyrode's solution (7) in 20 ml beakers or 25 ml Erlenmeyer flasks in a shaking incubator at 37°C under 95 per cent oxygen and 5 per cent carbon dioxide for varying periods of time as indicated in the text. When incorporation of radioactive

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‡ Veterans Administration Clinical Investigator and Research Assistant Professor of Pathology.

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sulfate was to be measured, this medium contained 0.35 μc/ml of sulfate labeled with sulfur-35, obtained from the Oak Ridge National Laboratories at ±20 per cent of stated activity and was not further standardized. When glucose incorporation was to be measured, the concentration of glucose in the medium was reduced to 0.5 gm/liter and uniformly labeled glucose-C^{14} (Volk Radiochemical Co., Chicago) was included at 0.2 μc/ml. For experiments in which the acid mucopolysaccharides were extracted from the tissues following incubation, larger amounts of tissue from several animals were incubated in a volume of 50 ml of medium in 250 ml Erlenmeyer flasks under the same conditions.

Radioactive Assay.—Assay of radioactive sulfate was ordinarily done in a thin window, continuous gas flow counter as previously described (1, 8). However, when analyses of hydrolyzable sulfate and of sulfur-35 were performed on the same aliquot, the barium sulfate suspension used for the sulfate analysis (see below) was heated with additional carrier barium sulfate and counted at infinite thickness.

Carbon-14 or sulfur-35 content of isolated acid mucopolysaccharides labeled with only one isotope was measured by drying 0.1 ml aliquots of an aqueous solution on planchets and counting as at infinite thinness. Measurement of carbon-14 and sulfur-35 in isolated mucopolysaccharides labeled with both isotopes was carried out by counting both isotopes in a dried aqueous aliquot, removing the sulfate from a second aliquot, and counting the supernatant. Thus carbon-14 was determined following removal of the sulfate and sulfur-35 calculated by difference. Sulfate was removed following hydrolysis of a sample of mucopolysaccharide in a sealed tube containing 4 N HCl by autoclaving at 15 pounds per square inch for 16 hours and precipitating the sulfate as barium sulfate.

Analysis of Hydrolyzable Sulfate.—Following hydrolysis, sulfate content of samples of tissues or isolated mucopolysaccharides was carried out by a modification of the procedure of Toennies and Bakay (9). Measurements were made in a volume of 5 or 10 ml using a Coleman model 14 spectrophotometer equipped with a nephelometer attachment. Hydrolysis of the samples was effected in sealed tubes containing 4 N HCl by autoclaving for 16 hours at 15 pounds per square inch.

Other Analyses.—Extraction of acid mucopolysaccharides was done as outlined by Einbinder and Schubert (10), scaled down proportionately to accomodate smaller amounts of tissue. Hexosamine was measured by the Elson-Morgan reaction (11) and uronic acid by the method of Dische (12).

RESULTS AND DISCUSSION

When tissues from animals treated with estradiol are incubated *in vitro* in the presence of radioactive sulfate, incorporation of that isotope expressed as counts per minute per unit wet weight of tissue is significantly diminished (Fig. 1). Further information concerning this phenomenon can be obtained by measurements of content of water and of acid mucopolysaccharide in such tissues.

The content of water in cartilage, thoracic or abdominal aorta is not altered significantly following administration of estradiol for 3 weeks (Table I). Therefore, changes in incorporation of radioactive sulfate following administration of estradiol cannot be accounted for by changes in content of water in the tissues, and figures representing the ability of tissues to incorporate radioactive sulfate differ only in magnitude when expressed as radioactivity per unit wet weight or per unit dry weight (Fig. 1).

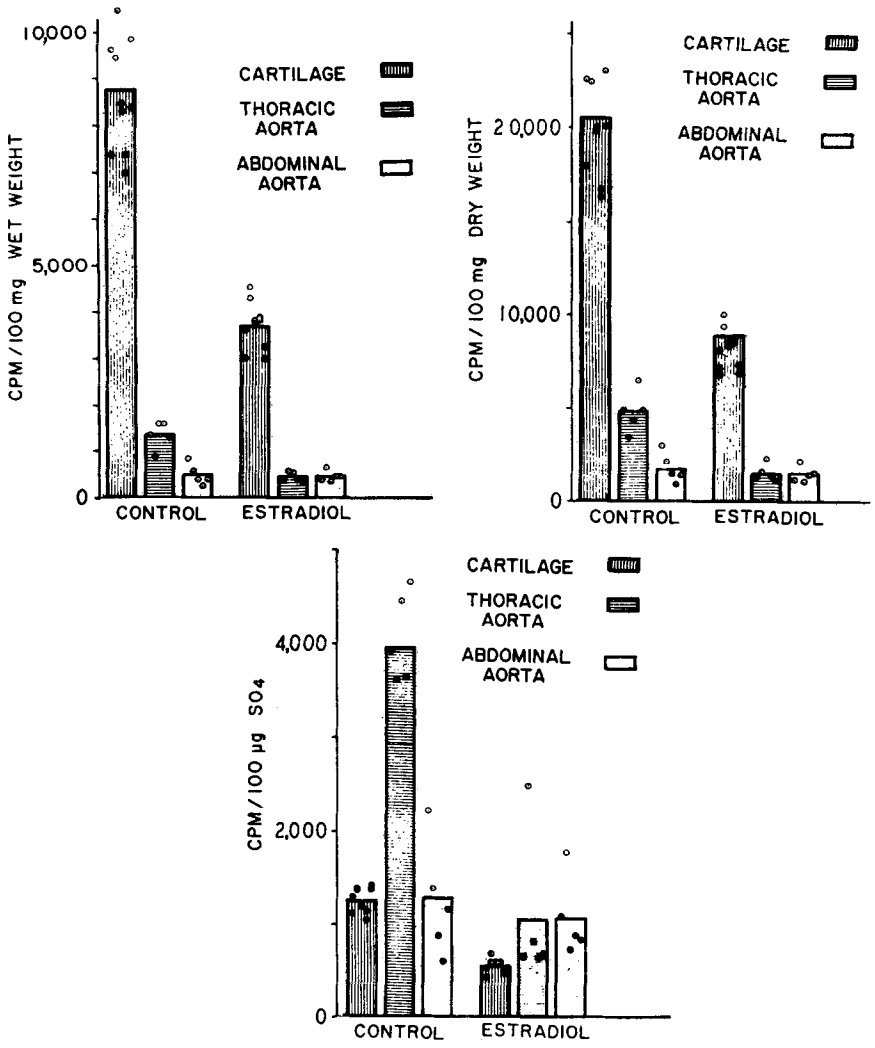

FIG. 1. Effect of pretreatment with estradiol for 3 weeks on *in vitro* incorporation of S^{35}-sulfate into cartilage, thoracic and abdominal aorta of rats when referred to wet weight, dry weight, and content of hydrolyzable sulfate of tissue.

The content of hydrolyzable sulfate in these tissues was determined as a measure of sulfated acid mucopolysaccharide and is recorded in Table II. The amount of hydrolyzable sulfate in cartilage is, of course, considerably greater than that in aorta, but there is no appreciable difference between the quantities found in thoracic and abdominal portions of aorta. The amount of hydrolyzable sulfate per unit weight of cartilage from the animals treated with estradiol

appears to be somewhat reduced, but the reduction is slight and is not statistically significant. Histologic sections of cartilage and aorta stained with hematoxylin and eosin, alcian blue, or toluidine blue showed no morphologic differences between control animals and those given estrogens. When incorporation of radioactive sulfate is calculated on the basis of content of hydrolyzable sulfate, the rate is found to be much more rapid in the thoracic aorta of normal animals than it is in either the abdominal portion of the same vessel or in cartilage (Fig. 1). Since the content of hydrolyzable sulfate in the thoracic

TABLE I

Content of Water in Tissues of Male Rats with and without Treatment with 0.333 mg Estradiol Benzoate for 3 Weeks

Treatment	No. Animals	Cartilage		Thoracic aorta		Abdominal aorta	
		Mean	SD	Mean	SD	Mean	SD
		per cent water		*per cent water*		*per cent water*	
Control	5	57.5	1.27	72.0	5.08	72.0	1.19
Estradiol	5	55.5	0.71	70.6	3.08	70.8	1.64

TABLE II

Content of Hydrolyzable Sulfate in Tissues of Male Rats with and without Treatment with 0.333 mg. Estradiol Benzoate for 3 Weeks

Treatment	No. Animals	Cartilage		Thoracic aorta		Abdominal aorta	
		Mean	SD	Mean	SD	Mean	SD
		μg SO_4 per 100 mg dry tissue		*μg SO_4 per 100 mg dry tissue*		*μg SO_4 per 100 mg dry tissue*	
Control	5	1632	172	123	32.6	138	20.4
Estradiol	5	1532	33.6	178	70.4	148	28.6

and abdominal portions of the aorta is not appreciably different, the turnover of this sulfate must be considerably more rapid in the thoracic than in the abdominal portion. Christie and Dahl (13) have observed that the consumption of oxygen *in vitro* is also more rapid in the thoracic portion of the aorta than in the abdominal portion. Such observations provide evidence that some metabolic processes proceed at different rates in different portions of the arterial vascular bed and raise the possibility that the differences in susceptibility to disease in these different portions could be related to their metabolic capabilities.

In order to establish whether or not the diminished incorporation of sulfate into these tissues following administration of estradiol represents diminished

incorporation into the sulfated acid mucopolysaccharides, extraction of the acid polysaccharides was carried out following incubation of the tissue in the presence of radioactive sulfate. Incorporation into acid mucopolysaccharides isolated from tissues of animals given estradiol was reduced to 45.8 per cent of the values obtained from control animals (Table III). This reduction parallels the reduction of incorporation of sulfate into the tissue itself. It was concluded that estradiol inhibits incorporation of sulfate into acid mucopolysaccharides.

There is unquestionably an effect of estradiol, under these conditions, on the metabolism of sulfated acid mucopolysaccharides. It is entirely conceivable, however, that hormonal influences on this metabolism measured by incorporation of sulfate could represent influences on the mechanism of sulfation without significant effects upon the polysaccharide backbone of the molecule. The

TABLE III

Radioactivity of Cartilage and Acid Mucopolysaccharide Extracted from Cartilage of Normal and Estradiol-Treated Rats after in Vitro Incubation in the Presence of S^{35}-Sulfate

Treatment	Radioactivity of AMP extracted from cartilage			Radioactivity of cartilage
	CPM/μg hexosamine	CPM/μg sulfate	CPM/mg cartilage extracted	CPM/mg dry cartilage
Control	12.7	69.7	27.2	205
Estradiol	5.4	31.9	11.3	83
$\frac{\text{Estradiol}}{\text{Control}}$	42.8 per cent	45.8 per cent	41.7 per cent	40.5 per cent

radioactivity of acid mucopolysaccharides isolated from tissues incubated in the presence of glucose with carbon-14 can, however, be regarded as a measure of synthesis of the polysaccharide portion of the molecule. Techniques were developed for measuring carbon-14 and sulfur-35 separately in acid mucopolysaccharides labeled with both isotopes. It was then possible to design an experiment to determine whether or not the synthesis of acid mucopolysaccharides is influenced by estradiol and whether or not it parallels the incorporation of sulfate.

As can be seen from Table IV, the incorporation of glucose-C^{14} into the acid mucopolysaccharides isolated from cartilage of rats under the influence of estradiol is markedly reduced from control values. Incorporation of sulfate-S^{35} is also reduced to about the same per cent of the control values. These experiments provide strong support for the view that the reduction in incorporation of radioactive sulfate into tissues of rats given estradiol is a reflection of a reduction of synthesis of sulfated acid mucopolysaccharides.

The sulfated acid mucopolysaccharides in aorta and cartilage are not, of course, a single molecular species. The techniques used in these experiments deal with these only as a group. It is therefore possible that synthesis is reduced more in some molecular species than in others, and is even possible, although unlikely in our view, that synthesis of one or another is increased. In the extraction experiments using cartilage, the material isolated exhibited electrophoretic and chromatographic properties (14) identical with those of commerical chondroitin sulfate and contained the same proportions of hexosamine, uronic acid, and sulfate. Since only small amounts were available they were not subjected to more elaborate analyses.

TABLE IV

Radioactivity of Acid Mucopolysaccharides Extracted from Cartilage of Normal and Estradiol-Treated Rats Following in Vitro Incubation in the Presence of Both S^{35}-Sulfate and C^{14}-Glucose

Treatment	Sulfur-35			Carbon-14		
	CPM/μg sulfate	CPM/*uronic acid*	CPM/*mg cartilage extracted*	CPM/μg *sulfate*	CPM/μg *uronic acid*	CPM/*mg cartilage extracted*
Control	45.5	370	162	3.16	25.8	11.3
Estradiol	21.1	168	75	1.54	12.3	5.5
$\frac{\text{Estradiol}}{\text{Control}}$	46.4 per cent	45.5 per cent	46.2 per cent	48.6 per cent	47.6 per cent	48.6 per cent

Since other hormones—adrenal steroids, growth hormone, and insulin—have been demonstrated to possess the capacity to alter the metabolism of sulfated acid mucopolysaccharides, we were concerned in a previous report with the possibility that the administration of estradiol might influence the secretion of one of these, and its effect on sulfate incorporation could be accounted for on that basis, but no supporting evidence was obtained. However, no significant and reproducible effects of estradiol in an entirely *in vitro* system have been found. Crystalline estradiol added directly to the incubation medium, serum exposed to estradiol, and serum from animals under treatment with estradiol have all produced variable effects on different occasions. Indeed, it has been found that some presumably normal sera can stimulate sulfate incorporation into cartilage from one animal and depress uptake into cartilage from a different animal. Cartilage from the same animal may be affected differently by different sera following exposure to crystalline estradiol. An example of this phenomenon is illustrated in Fig. 2.

The possibility that circulating estrogenic hormones may influence synthesis

of mucopolysaccharides has not been excluded by these experiments. Other circulating factors which influence sulfate incorporation (15, 16) could account for the variability in response elicited by sera from different individuals. The ease with which this effect of estradiol on synthesis of sulfated acid mucopolysaccharides can be demonstrated following injection into the whole animal contrasts sharply with the failure to demonstrate a consistent effect in an entirely *in vitro* system. It is therefore unlikely that if such a circulating factor does exist, the effects of administration of estradiol on mucopolysaccharide synthesis can be explained entirely on that basis. However, this influence of

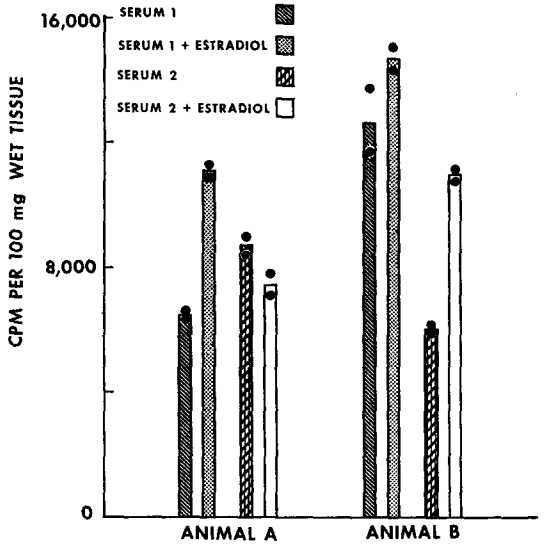

FIG. 2. Effect of two normal rat sera with and without exposure to estradiol on incorporation of S^{35}-sulfate into cartilage from two normal rats.

estradiol need not be a direct effect of the hormone on the enzymes involved in synthesis of the acid mucopolysaccharides. A previous finding that this influence was not demonstrable for more than a day following injection of the hormone (1) also suggests that the effect may not be a direct one. Indeed, this effect of estradiol on the biosynthesis of the sulfated mucopolysaccharides of connective tissue is quantitatively little different from its effect upon body weight of the animal. As can be seen from Table V, when varying amounts of estradiol benzoate are administered, the effects upon body weight and upon the ability of cartilage to incorporate radioactive sulfate *in vitro* are similar. The reduction in body weight is linear with the logarithm of the hormonal dose; however, the reduction in ability to incorporate sulfate departs from linearity at the highest dose.

One of the substrates thought to be important in biosynthesis of acid mucopolysaccharides is glutamine, which by transamination of a hexose 6-phosphate, provides the hexosamine of the polysaccharide. When biosynthesis proceeds *in vitro* this transamination usually is the step which limits the rate of synthesis

TABLE V

Effect on Body Weight and on in Vitro Incorporation of S^{35}-Sulfate of Different Amounts of Estradiol for 3 Weeks

Daily amount estradiol benzoate	No. Animals	Sulfate incorporation into cartilage			Body weight		
		Mean	SD	Control	Mean	SD	Control
mg		*CPM/100 mg dry wt*		*per cent*	*gm*		*per cent*
None	4	8103	2279	100	308	6.5	100
0.003	4	7315	441	90	265	0.8	86
0.033	4	5349	332	72	238	8.7	77
0.333	4	3215	737	40	211	11.7	68

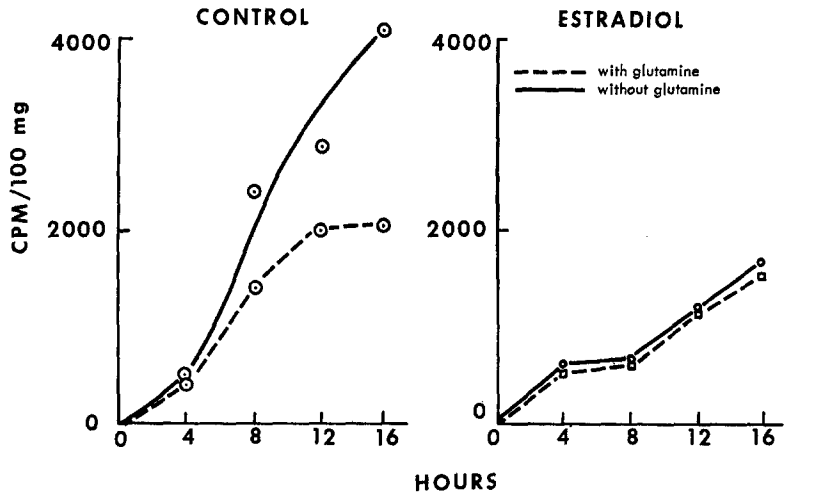

FIG. 3. The *in vitro* effect of glutamine on incorporation of S^{35}-sulfate into aortas of rats with and without pretreatment with 0.333 mg estradiol benzoate for 3 weeks.

and consequently, the rate is in part dependent upon supplies of glutamine in the incubation medium. Addition of glutamine to the incubation medium stimulates sulfate incorporation into aorta from normal animals, but has no demonstrable influence on incorporation into the same tissue from animals given estradiol (Fig. 3). While the presence of glutamine does not always stimulate incorporation into normal cartilage, it invariably fails to relieve the in-

hibition following administration of estradiol. While this transamination limits the rate of synthesis *in vitro* in tissues from normal animals it does not do so in tissues from animals given estradiol. This provides strong evidence that estradiol does not regulate the synthesis of acid mucopolysaccharides by causing changes in available glutamine. It has not been determined which step in biosynthesis is affected, however.

Although the exact mechanisms whereby estradiol influences the metabolism of connective tissue have yet to be identified, it seems clear that it reduces synthesis of sulfated mucopolysaccharides and does so to different degrees in different connective tissues.

SUMMARY

The influence of estradiol upon incorporation of isotopes into the sulfated acid mucopolysaccharides of connective tissues of the rat has been studied.

Administration of the hormone for 3 weeks significantly reduces the *in vitro* incorporation of S^{35}-sulfate into cartilage and thoracic aorta without significantly altering the amount of hydrolyzable sulfate in these tissues.

The reduction of sulfate incorporation into these tissues by estradiol represents reduced synthesis since the hormone reduces incorporation of sulfate into the acid mucopolysaccharides of the tissue to the same extent and since reduction in incorporation of C^{14}-glucose also parallels S^{35}-sulfate incorporation into acid mucopolysaccharides.

The rate of synthesis of sulfated acid mucopolysaccharides is greater in thoracic aorta than abdominal aorta or cartilage. Since the amount of hydrolyzable sulfate is the same in these two portions of aorta, the turnover is also more rapid in the thoracic aorta.

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