ESTRADIOL REDUCES INCORPORATION OF RADIOACTIVE SULFATE INTO CARTILAGE AND AORTAS OF RATS*

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Hormonal influences on connective tissues have been observed for a variety of hormones and a variety of sites. Gonadal hormones cause accumulation of intercellular ground substance in certain locations; the sex skin of monkeys (1) and the cock's comb (2) are well known examples. The metabolism of sulfated mucopolysaccharide components of connective tissue is known to be influenced by growth hormone (3, 4) and adrenal steroids (5, 6, 7), but only cursory metabolic studies have been reported using sex hormones (8, 9). An attempt has been made to determine whether one gonadal steroid, estradiol, influences the metabolism of connective tissue; and hyaline costal cartilage and aorta were chosen for examination. The incorporation of sulfate labeled with S^{35} into these tissues, both *in vivo* and *in vitro*, was used as a specific measure of metabolic activity of sulfated mucopolysaccharides. Justification for this view can be found in reports from several laboratories (10–13). Hyaline cartilage was chosen for study because it contains large amounts of chondroitin sulfate and because its response to other hormones is well documented (3–7). Aorta was chosen as an example of arterial connective tissue because the rate of incorporation of sulfate into it is impressive (14) and because some striking sex differences in incidence and severity of arterial disease, both spontaneous and experimentally induced (15–19), have been observed. The metabolism of the mucopolysaccharide components of arteries could conceivably have a bearing upon these differences in incidence of disease.

Materials and Methods

Animals.—All animals used in these experiments were adult rats of the Sprague-Dawley strain initially ranging in weight from 200 to 400 gm., but varying by no more than 20 gm. within any one experiment. These were housed in groups of six and supplied with Purina chow and tap water *ad lib.*, except where indicated in the text.

Hormones and Administration.—The following preparations of hormone were used: 1 ml.

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ampules of estradiol benzoate containing 1.67 mg./ml. in sesame oil (progynon B); and 10 ml. vials of testosterone propionate containing 1.00 mg./ml. in oil (oreton).[1] Estradiol was administered in 0.2 ml. quantities (0.333 mg.) daily, 6 days each week, by subcutaneous injection into the skin of the back. Injections were rotated among four quadrants so that any one site was used only for every fourth injection. Testosterone propionate was administered similarly in amounts of 0.25 ml. (0.250 mg.) per injection. Control animals were given an equal volume of sesame oil alone in the same manner.

Preparation of Tissues.—For *in vivo* experiments (*i.e.*, those in which radioactive sulfate was injected into the animals prior to sacrifice), animals were sacrificed under ether anesthesia and tissues immediately removed. Aortas were stripped free from adventitia and samples of costal cartilage stripped of muscle to the perichondrium. The samples were then promptly weighed and kept frozen until assayed for radioactivity.

For *in vitro* experiments the animals were sacrificed and the tissues cleaned in the same manner. Aortas were divided into thoracic and abdominal segments which were thereafter treated as separate samples. Each segment was then cut into pieces 1 to 2 mm. long. Samples of costal cartilage were also cut into pieces 1 to 2 mm. in length. The samples of tissue were transferred to 18 × 150 mm. culture tubes containing radioactive sulfate and incubated at 37°C., usually for 4 hours. Tissues were rinsed three times with about 5 ml. of isotonic saline with the sample remaining in the final rinse overnight. Tissues were then blotted dry, weighed, and kept frozen until assayed for radioactivity.

Incubation Medium and Radioactive Assay.—The samples of tissue were incubated in Tyrode's solution as described by Layton (20) for this purpose, except that 50,000 units of potassium penicillin G and 50 mg. of streptomycin sulfate per liter were included. No carrier sulfate beyond that included in the streptomycin sulfate (12.5 μg./ml.) was added. To this medium was added from 0.15 to 0.35 microcuries per ml. of carrier-free radioactive sulfate. Radioactive sulfate was obtained in several lots from the Oak Ridge National Laboratory at ±20 per cent of stated activity and was not standardized after receipt. Thus, comparisons of radioactivity can be made only within an experiment and it is not valid to compare amounts of radioactivity between different experiments.

Assay of radioactivity was done as described by Denko and Bergenstal (4). Briefly, this procedure consists of fusing the sample with Na_2CO_3 by heating in a platinum crucible, dissolving in 1 N HCl, neutralizing, adding 4 ml. of M/10 carrier Na_2SO_4 and precipitating the sulfate with an excess of $BaCl_2$. The precipitate was filtered, dried with ethanol, and counted at infinite thickness in a thin window, continuous gas flow ("Q" gas) counter, to an expected error of 3.5 per cent or less.

In order to determine whether the size of the sample influenced the counting rate, a statistical comparison of thirty-six pairs of samples was made by the method of individual comparison (21). No influence of size, within the range of size of samples used (30 to 75 mg.), on the counting rate was found.

RESULTS

Young adult male rats were divided into two groups of six on the basis of weight. Animals in one group were given daily injections of 0.33 mg. of estradiol benzoate in sesame oil, and in the other were given an equal volume of sesame oil. After 3 weeks each animal was given an intraperitoneal injection of 100 μc. of sulfur-35 as sulfate. Twenty-four hours later the animals were sacrificed, tissues removed, weighed and assayed for radioactivity.

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[1] These preparations were generously supplied by the Schering Corporation, Bloomfield, New Jersey.

From Table I it can be seen that the incorporation into costal cartilage of animals treated with estradiol averages 34 per cent of the amount found in animals not so treated. The difference between incorporation into aortas of treated and untreated animals is not impressive and could easily be due to chance. The differences observed between the animals treated with estradiol and the control group are highly significant for cartilage ($p < 0.001$) despite the wide dispersion of observations in the control groups.

The effect observed by administration of $S^{35}O_4$ *in vivo* could well represent differences in metabolic activity of the tissues sampled during the 24 hours or less during which they were exposed to radioactive sulfate. Such results could equally well represent differences between treated and untreated animals in renal excretion of sulfate, or differences in the volume of extracellular fluid, or even differences in concentration of sulfate ion within the body. Therefore,

TABLE I

The Effect of Estradiol on in Vivo *Incorporation of S^{35}-Sulfate into Cartilage and Aorta*

Treatment	Cartilage			Aorta		
	No. animals	Mean	S.D.	No. animals	Mean	S.D.
		c.p.m./*100 mg.*			c.p.m./*100 mg.*	
Control	5	753	213	5	396	45
Estradiol	6	254	215	6	257	70

Animals were pretreated with 0.333 mg. estradiol benzoate daily for 3 weeks, injected with 100 μc. S^{35}-sulfate, and sacrificed 24 hours later.

an *in vitro* technique for observing incorporation of sulfate into surviving tissues was devised in order to obviate the difficulties inherent in the *in vivo* system.

Again adult male animals were treated with estradiol benzoate for 3 weeks, tissues removed, and incubated for 4 hours in a medium containing labeled sulfate. The tissues were then assayed for radioactivity and compared with tissues from animals not treated with estradiol. These data are presented in Fig. 1.

The incorporation into costal cartilage of the treated group is approximately half that of the control group. In this and subsequent experiments the aorta was divided into thoracic and abdominal segments which were incubated and assayed separately. It can be seen that in the control group the thoracic segment incorporates sulfate at a rate significantly ($p < 0.001$) greater than the abdominal segment. This has been a consistent observation in many experiments using untreated animals. It is also evident that this difference is largely abolished in the animals treated with estradiol. In these animals the incorporation into the thoracic segment is reduced significantly ($p < 0.001$) from that in the control group.

The relationship between the duration of incubation and the effects of estradiol on sulfate metabolism in cartilage and aorta were then explored. Two groups of six adult male rats were selected and one group injected daily for 3 weeks with estradiol. A pool of costal cartilage was made for each group and aliquots incubated for varying periods of time up to 24 hours. The thoracic and abdominal aortic segments were not pooled. The samples were incubated separately, but for different periods of time. From Fig. 2 it can be seen that the

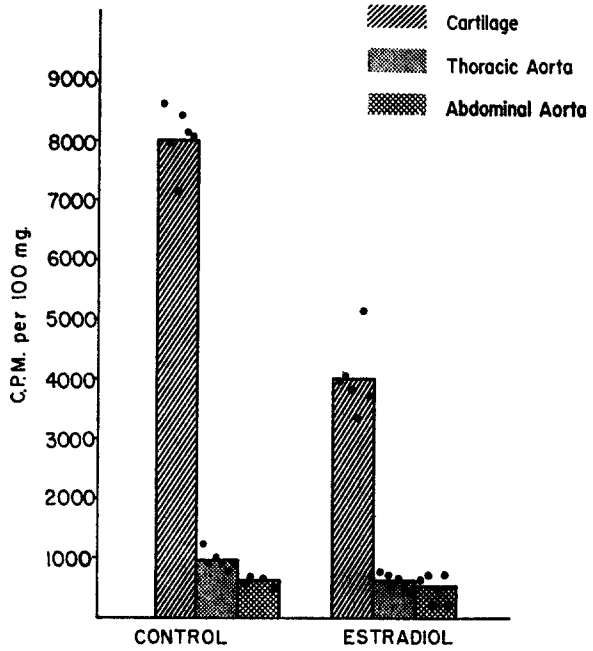

FIG. 1. The *in vitro* incorporation of S^{35}-sulfate into cartilage, thoracic and abdominal aortas of male rats with and without pretreatment with 0.333 mg. estradiol benzoate daily for 3 weeks.

rate of incorporation into costal cartilage from the treated animals is less than that of the controls. Moreover, the slope of the curve for the treated animals changes more rapidly than that for the controls. The difference in rate of incorporation into thoracic and abdominal aortas in control animals is apparent, but no such difference is demonstrated in the treated animals. Diminution in incorporation of sulfate is present in cartilage, thoracic aortas and abdominal aortas from the treated animals, but for the aortic samples this is not clearly apparent until after several hours of incubation. It was decided, however, to use 4 hours as a standard period of incubation since effects of estradiol on cartilage and thoracic aorta could be demonstrated using this period and since it fell near the maximum slope of the curve.

In order to approximate the time necessary for this effect of estradiol to become apparent, male animals were injected daily for varying periods of time and compared with untreated animals after the tissues were incubated for 4 hours. These results are depicted in Fig. 3. Comparing single animals, no diminution in incorporation of sulfate into cartilage was demonstrable at the end of 1 day, but was apparent by 3 days. Incorporation into aorta as well as cartilage seemed to be reduced after 2 and 3 weeks of treatment.

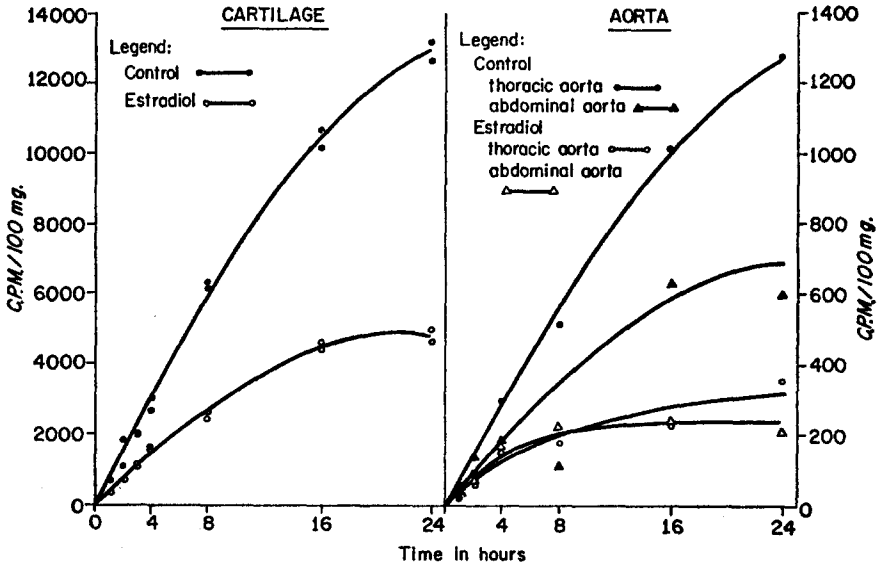

FIG. 2. The effect of time of incubation on incorporation *in vitro* of S^{35}-sulfate into cartilage and aortas of rats pretreated for 3 weeks with 0.333 mg. estradiol benzoate daily.

The delay of more than a day in the case of cartilage and more than 3 days in the case of aorta before the influence of estradiol became demonstrable by the techniques used, raised the possibility that the effect might be mediated through another hormone, or through some other secondary effect. Consequently, experiments were designed to investigate some of the possibilities.

Testosterone propionate in an amount (0.25 mg./day) sufficient to cause gain in weight was given to a group of six adult female rats over a 3 week period. No difference between the incorporation of sulfate *in vitro* into either cartilage or aortas of these animals was found when compared to control animals, as is shown in Table II.

Hypophysectomized male and female animals were started on experiment a week after operation. They were fed ground Purina rat chow and injected with 0.333 mg. of estradiol daily for 4 days rather than 3 weeks. Following this pretreatment period, cartilage incubated *in vitro* incorporated significantly less ($p < 0.001$) radioactive sulfate than did cartilage from hypophysectomized control animals. These figures are shown in Table III.

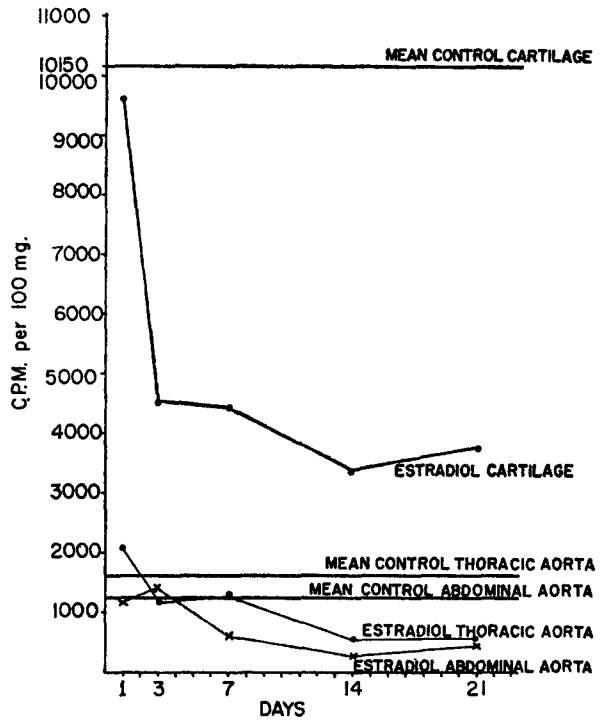

FIG. 3. Variation in incorporation of S^{35}-sulfate into cartilage and aortas of male rats with different periods of pretreatment with 0.333 mg. estradiol benzoate daily after *in vitro* incubation for 4 hours.

TABLE II

The Effect of Testosterone on in Vitro *Incorporation of S^{35} Sulfate into Cartilage and Aorta of Female Rats*

Treatment	Cartilage			Thoracic Aorta			Abdominal Aorta		
	No. animals	Mean	S.D.	No. animals	Mean	S.D.	No. animals	Mean	S.D.
		C.P.M./100 mg.			C.P.M./100 mg.			C.P.M./100 mg.	
Control.....	6	1927	219	6	535	142	6	342	92
Testosterone....	6	1952	282	6	525	131	6	473	114

Animals were pretreated with 0.25 mg. testosterone propionate daily for 3 weeks, sacrificed, and tissues incubated.

During these experiments using estradiol it was observed that both consumption of food and gain in total body weight was appreciably reduced in animals injected with 0.333 mg. daily. The question arose as to whether the effect on incorporation of sulfate into cartilage and aortas of animals so treated might

be due to reduction of food intake alone. Two experiments were designed to determine if this were so by controling food intake. In one experiment two groups of adult male rats were fed a constant diet of evaporated milk by gastric tube three times daily for 4 days. The amount of diet per animal per individual feeding was increased from 5 ml. initially to 7 ml. on the final day. *In vitro* incorporation of sulfate into cartilage, as seen in Table IV, was diminished in the group injected with estradiol over this period. A second experiment was done to control intake of food. Three groups of six adult male rats were

TABLE III

The Effect of Estradiol on in Vitro *Incorporation of S^{35}-Sulfate into Cartilage of Hypophysectomized Rats*

Treatment	No. animals	Mean	S.D.
		C.P.M./*100 mg.*	
Control	6	5859	1015
Estradiol	7	3763	407

Seven days following hypophysectomy animals were pretreated with 0.333 mg. estradiol benzoate for 4 days, sacrificed, and tissues incubated.

TABLE IV

The Effect of Estradiol in Rats on a Constant Diet on S^{35}-Sulfate Incorporation in Vitro *into Cartilage*

Treatment	No. animals	Mean	S.D.
		C.P.M./*100 mg.*	
Control	6	3250	223
Estradiol	6	2800	116

Animals were pretreated with 0.333 mg. estradiol benzoate daily for 4 days while on a constant force-fed diet, then sacrificed, and tissues incubated.

used. The first group was fed ground fox chow *ad lib.* and was injected with sesame oil alone. Food intake was measured daily. Another group was fed *ad lib.* and injected with estradiol daily. The final group did not receive estradiol but the amount of food offered to each animal was limited to the amount eaten on the previous day by an animal in the group treated with estradiol with which it had been paired according to weight at the beginning of the experiment. At the end of 3 weeks the animals were sacrificed, tissues incubated and assayed for radioactivity in the usual manner. The results are summarized in Fig. 4. The differences in incorporation of sulfate into cartilage and thoracic aorta between the animals given estradiol and the untreated ones are similar to those observed in other experiments. That these differences cannot be

explained by differences in amount of food consumed is apparent on comparing the data from the paired controls with that from the group given estradiol. Incorporation of sulfate into cartilage and thoracic aorta is significantly ($p < 0.001$ and $p < 0.025$, respectively) less in the group receiving estradiol than in the group not receiving estradiol, but restricted to the same amount of diet. That restricting diet effects sulfate incorporation is also probably true, at least in cartilage, for the amounts found in the cartilage from animals with restricted

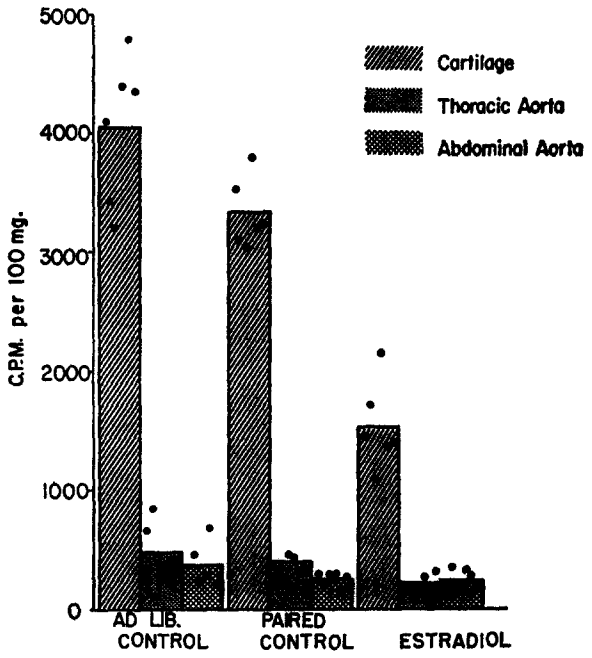

FIG. 4. The effect of controling diet on incorporation *in vitro* of S^{35}-sulfate into cartilage and aortas of rats pretreated for 3 weeks with 0.333 mg. estradiol benzoate daily.

diets is about 20 per cent less ($p < 0.025$) than in those fed *ad lib.* The differences in incorporation of sulfate into aortas from these two groups could be explained by chance.

With the demonstration that estradiol reduces the incorporation of sulfate into cartilage of animals injected with radioactive sulfate *in vivo*, and with the demonstration of similar effects on cartilage and aortas from pretreated animals with incubation *in vitro*, some preliminary attempts have been made with an entirely *in vitro* system. Addition of crystalline estradiol to the incubation medium failed to produce inhibition of incorporation of sulfate. When cartilage was incubated in the presence of serum, incorporation of sulfate seemed partially dependent upon the size of the sample of tissue—a dependency which

was not found when it was incubated in Tyrode's solution. It has thus far not been possible to determine whether estradiol in the presence of serum influences incorporation of sulfate by tissue when added to the incubation medium.

DISCUSSION

Sulfate incorporated into tissues is found in the sulfated mucopolysaccharides (10, 12) and sulfolipids (22). Sulfolipids have yet to be identified in aorta or cartilage and it is likely that the sulfate incorporated into these tissues goes into sulfated mucopolysaccharides alone. It is probable that this incorporation represents synthesis of mucopolysaccharide, rather than a transsulfation or a combination of transsulfation and synthesis. The effects of estradiol that have been demonstrated in these experiments are most likely on the synthesis of sulfated mucopolysaccharides of cartilage and aorta, although certain other possibilities have not been excluded. While alterations in water content of the tissues could explain the phenomenon under study, this seems an unlikely possibility since aortas from animals treated with estrogens are not increased in size. It is possible that the rate of transfer of sulfate across cell membranes could be reduced by administration of the hormone and the rate of cellular accumulation of inorganic sulfate thus reduced. There is, however, no experimental precedent for this view.

In cartilage the sulfated mucopolysaccharide is thought to be largely, if not exclusively, chondroitin sulfate, although in costal cartilage from older human beings Meyer has recently reported finding substantial amounts of keratosulfate (23). The sulfated polysaccharides of aortic tissue represent a mixture which includes chondroitin sulfate, chondroitin sulfate B, and since mast cells are found in some species, heparin or heparin-like polysaccharides are probably also present (24). It has not been determined whether estradiol—or other hormones—influence principally chondroitin sulfate metabolism or whether other polysaccharides are also effected. The techniques which we have used do not differentiate between incorporation of sulfate into the various classes of sulfated polysaccharides.

In all of the experiments reported here assays of radioactivity were done at infinite thickness, yielding a figure which is directly proportional to the total amount of radioactivity per sample. This figure was then referred to the wet weight of sample. Results obtained by this method yield no information about absolute or relative amounts of sulfated mucopolysaccharides present in the sample. It has not been determined, then, whether, with the striking reduction in incorporation of radioactive sulfate into these tissues under the influence of estradiol, the specific activity is also reduced. Estradiol is effective in reducing the incorporation of sulfate into cartilage both *in vivo* and *in vitro* following pretreatment. Aortic tissue responds similarly but the reduction is less striking. Neither response follows immediately upon the administration of estradiol

and the lag phase is different for the two tissues. This suggests that the effect need not be a direct one of the hormone on the tissue.

With the use of these techniques the failure to observe changes in the metabolism of sulfate following the administration of testosterone propionate is in contrast to the report of Kowalewski (8), who observed increase in incorporation of radioactive sulfate in the fracture sites of bones after treatment of animals with testosterone propionate. It should be pointed out that the metabolism of sulfated mucopolysaccharides in growing bone is probably controlled, at least in part, by different mechanisms than those which apply to mature or slowly growing cartilage. With the use of histochemical and radioautographic as well as chemical techniques, the hypothesis has been advanced (25, 26) that the radioactive sulfate which appears in bony metaphyses is derived from that previously incorporated into the epiphyses, and this transfer of radioactive sulfate from epiphyses to metaphyses is effected by processes which influence growth of bone. It is not surprising, then, that hormonal effects on incorporation of sulfate into diaphyseal bone and into hyaline costal cartilage might differ.

The observation that female sex hormones alter the metabolism of sulfate in arteries could have a bearing on the pathogenesis of arterial disease. The "loosening" of the ground substance in the arterial lesions of human atherosclerosis has been emphasized by Aschoff (27) and in more recent times the relation between the appearance of lipid in the lesions and the appearance of metachromatic material said to be acid mucopolysaccharides has been noted (28, 29), although some recent reports have denied this association (30). Coronary atherosclerosis and its consequence, myocardial infarction, are less frequent in young women than young men (15) and this difference has been related to gonadal function (31, 32). Inhibition of experimentally produced coronary lesions has been demonstrated in chickens (16) and rats (17) given estrogens. The mechanism by which estrogenic hormone results in this inhibition is usually attributed to alterations in the amount and partition of serum lipids, but with the demonstration that estradiol influences the metabolism of arterial sulfate and probably of sulfated mucopolysaccharides, which are prominent components of the atherosclerotic lesion, the possibility that estrogenic hormones influence the development of the atherosclerotic lesions through changes in the metabolism of the arterial wall itself should not be ignored.

SUMMARY

Estradiol in large amounts reduces the incorporation of radioactive sulfate into cartilage and aortas of rats.

This reduction becomes apparent within 3 days for cartilage and 3 weeks for aorta.

The effect is not mediated through suppression of testosterone secretion and the hypophysis is not necessary for the effect to be demonstrated.

The thoracic segment of aortas from normal rats incorporates more sulfate than does the abdominal segment and this difference is reduced following the administration of estradiol.

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