

THE DEPRESSION OF ESTRONE-INDUCED UTERINE GROWTH  
BY PHENOLIC ESTROGENS WITH OXYGENATED FUNCTIONS  
AT POSITIONS 6 OR 16: THE IMPEDED ESTROGENS\*

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This paper is concerned with special effects which ketonic or hydroxyl groups respectively at position 6 or 16 in the steroid nucleus exert on the growth of the uterus of the rat.

It has been known for some time that estriol differs from other estrogenic compounds in its influence on growth of the female genital tract. Curtis and Doisy (1) found that estriol is more potent than estrone in causing opening of the vaginal plate of infant rats but is less active in evoking vaginal cornification. Secondly, whilst estriol in small quantity stimulates uterine growth, Dorfman, Gallagher, and Koch (2, 3) observed that a considerable increase of dosage does not cause maximal growth of this structure. More recently Hisaw, Velardo, and Goolsby (4) have shown that estriol reduces the effectiveness of estrone or estradiol in causing uterine growth when these steroids are administered together; this effect is remarkable since here one estrogenic substance is inhibiting the growth effect of another estrogen.

Heretofore estriol has been considered to be a unique compound in possessing these special physiologic influences. It will be demonstrated in this paper that certain other estrogenic agents exhibit effects on growth similar to those induced by estriol. The common molecular feature of these unusual estrogens is the presence of oxygenated functions at specific sites, position 6 or 16 of the estrane molecule.

The present experiments were carried out on hypophysectomized rats maintained on a ration which is free from growth-promoting steroids to minimize extraneous growth factors both of endogenous and exogenous origin. The steroids which were investigated were closely related in structure.

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### Methods

*Biological.*—Hypophysectomized albino rats maintained under controlled environmental conditions (5) were injected with steroids according to an unvarying schedule. The rats were obtained from the breeder at age 22 days and maintained thereafter on a synthetic diet.<sup>1</sup> Hypophysectomy was performed at age 24 days. The steroids were dissolved in ethyl alcohol and diluted with sesame oil to make the final alcoholic concentration 10 per cent. When steroids were injected in combination, they were administered in the same solution, which was always freshly prepared. The solution (0.2 ml.) was injected subcutaneously for 7 days, beginning at age 38 days; 6 or more rats were tested at each dosage level of every compound. The perineum was observed each day for opening of the vaginal plate. Necropsy was performed at age 45 days. All rats with body weight above 75 gm. or with weight of the spleen above 200 mg. were discarded to avoid the risk of residual pituitary activity from incomplete hypophysectomy. At necropsy, the spleen, preputial glands, vagina, and the uterus were excised, blotted lightly, and weighed promptly on a torsion balance. The nitrogen content of the uterus was determined by a micro-Kjeldahl technique. Histological preparations of the vaginal epithelium were made in each case.

In studies of inhibition of estrone by other steroids, the statistical probability, *P*, of a significant effect was derived from Fisher's (6) table of *t* values. The results were considered statistically significant when *P* values less than 0.05 were obtained for both the weight of the uterus and its nitrogen content.

*Chemical.*—Many of the steroids were obtained from other laboratories.<sup>2</sup> Certain monofunctional steroids were synthesized in this laboratory by methods to be described (7) elsewhere: 17-desoxyestradiol,  $\Delta$ -16,17-desoxyestradiol, 3-desoxyestradiol-17 $\alpha$ -ol, 3-desoxyestradiol-17 $\beta$ -ol, 3-desoxyestradiol-17-one. A monofunctional steroid by definition (8) possesses an oxygenated function at either position 3 or position 17 but not at both sites.

### RESULTS

The weight of the preputial glands of 34 hypophysectomized rats, age 45 days and uninjected with steroids was  $9.6 \pm 1.4$  mg. None of the phenolic estrogens caused an increment of weight of these structures. Furthermore the vestigial prostate was not visible in any case.

In Fig. 2, the growth of the uterus is related to the dosage of phenolic estrogens which differ in number of substituent groups and their state of oxidation; the amount of steroids investigated covered a 10,000-fold range. These data form a family of curves with one notable exception—estriol (XX)<sup>3</sup> has anomalous behavior as described previously (2, 3). In the related curves a small increment of steroid dosage above the threshold amount results in a sharp increase of uterine growth succeeded by a gentle terrace-like rise until

<sup>1</sup> This diet contains about 18.8 per cent protein. The formula is casein 254 gm.; dextrin 468 gm.; corn oil 38 gm.; alphacel 50 gm.; mixed vitamins 10 gm.; salt mixture 40 gm.; oleum percomorphum 3 drops; water 140 cc.; vitamin K 50 mg.

<sup>2</sup> We acknowledge with gratitude generous gifts of compounds used in these experiments from Dr. Max N. Huffman, Oklahoma Institute for Medical Research; Dr. Oscar Wintersteiner, The Squibb Institute for Medical Research; Dr. G. C. Mueller, McArdle Institute for Cancer Research; Dr. V. Prelog, Eidgenossische technische Hochschule, Zurich; and Dr. A. Zaffaroni, Syntex, S.A., Mexico, D. F.

<sup>3</sup> The chemical formulae of the steroids are given in Fig. 1.

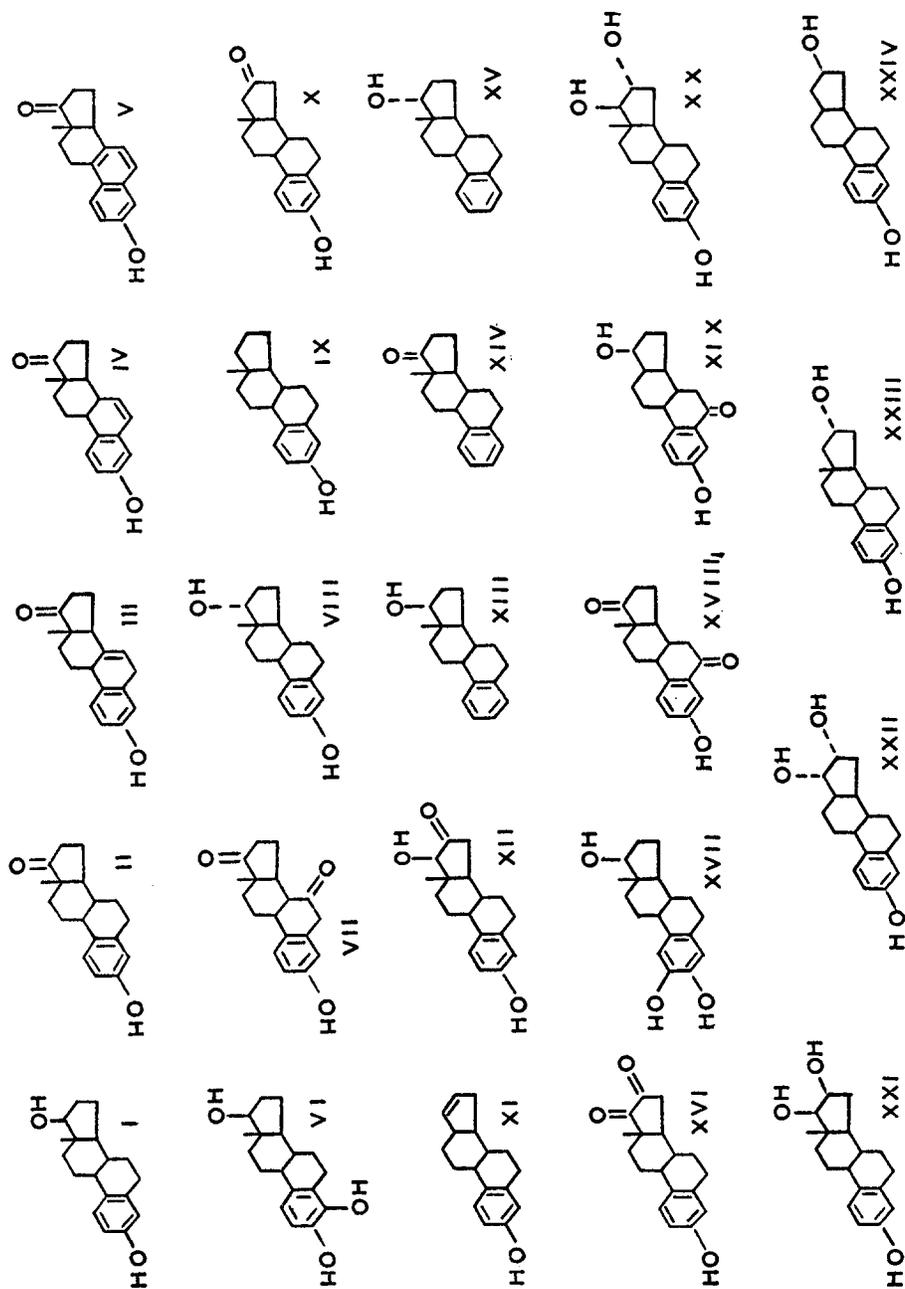


FIG. 1. Chemical formulae of the steroids.

maximal growth is attained. Estrogenic compounds of this type will be referred to as unimpeded estrogens and for steroids of this kind the minimal daily dosage required to initiate the high growth plateau (terrace point, *TP*) is stated (Table I).

In the case of estriol the steep increment of growth is lacking; instead a prolonged gradual increase results from augmented steroid dosage, after uterine growth has been initiated, until full size is reached. Estriol and compounds to be identified, with similar physiologic activity, will be designated *impeded*

TABLE I  
*Quantitative Effect of Unimpeded Estrogens in Promoting Uterine Growth*

The terrace point is the lowest amount of steroids causing the steep increment of uterine growth to be superseded by the final high plateau of growth.

No.	Steroid	Terrace point dosage*
		$\mu\text{g.}$
I	Estradiol-17 $\beta$	0.025
II	Estrone	0.25
III	Equilin	0.25
IV	6-Dehydroestrone	2.5
V	D-Equilenin	5
VI	4-Hydroxyestradiol-17 $\beta$	10
VII	7-Ketoestrone	10
VIII	Estradiol-17 $\alpha$	10
IX	17-Desoxyestradiol	10
X	Estrone-16	10
XI	$\Delta$ -16,17-Desoxyestradiol	20
XII	16-Ketoestradiol-17 $\beta$	25
XIII	3-Desoxyestradiol-17 $\beta$	25
XIV	3-Desoxyestrone	50
XV	3-Desoxyestradiol-17 $\alpha$	100
XVI	16-Ketoestrone	>100

\* This amount was administered daily for 7 days.

estrogens. After a moderate increase of uterine weight (about one-third of maximal growth) has been induced by steroids in this class, a tenfold increase of dosage causes little or no increment of growth.

*State of Oxidation of Unimpeded Estrogens in Relation to Uterine Growth.*—Estradiol-17 $\beta$  (I) was the most efficient of the unimpeded estrogens (Table I). The lack of either of its hydroxyl groups weakened its activity but to an unequal extent; 17-desoxyestradiol (IX) was more efficient as a promoter of growth than 3-desoxyestradiol-17 $\beta$  (XIII) which in turn was more efficient than 3-desoxyestrone (XIV) or 3-desoxyestradiol-17 $\alpha$  (XV). In accord with earlier findings (9) oxidation of the 17-hydroxyl group of estradiol-17 $\beta$  to form estrone (II) reduces the activity by a factor of 10.

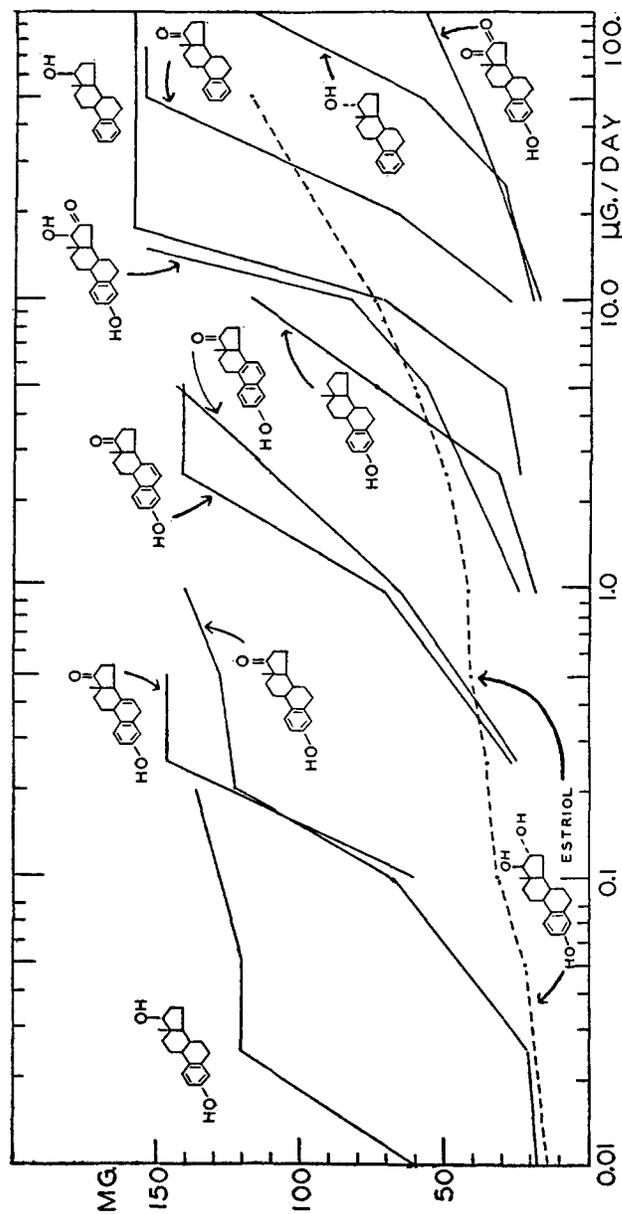


FIG. 2. The influence of dosage of 11 unimpeded estrogens compared with estriol with reference to the rate of increase of uterine weight. Ordinates: weight of the uterus in milligrams. Abscissae: daily dosage of steroids in micrograms plotted logarithmically.

The presence of 2 hydrogen atoms at C<sub>6</sub> appears to be required for full physiologic activity of estrone. No loss of efficiency of estrone occurs when the molecule is unsaturated at positions 7 or 8 as in equilin (III) but a considerable decrease of activity follows the removal of a hydrogen atom from the C<sub>6</sub> and C<sub>7</sub> positions of estrone as in 6-dehydroestrone (IV). No significant decrease of activity of 6-dehydroestrone results from the introduction of additional unsaturation in ring B as in D-equilenin (V).

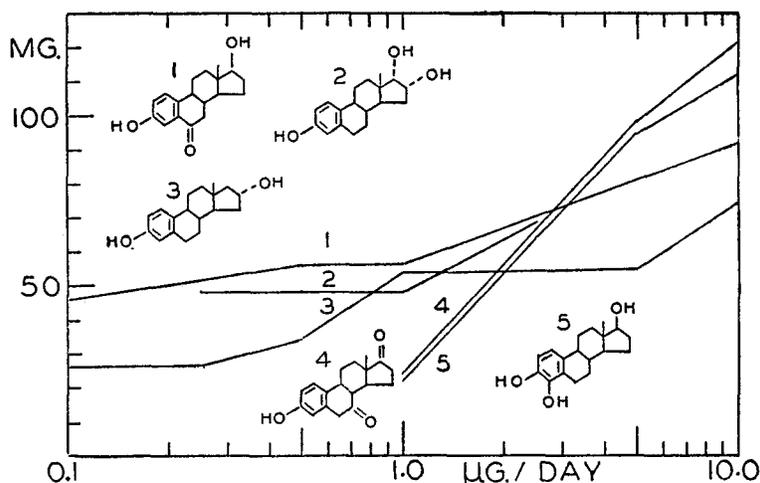


FIG. 3. Relationship of dosage to uterine weight of 3 impeded estrogens (curves 1, 2, 3) contrasted with 2 unimpeded estrogenic compounds (curves 4, 5). Ordinates: weight of the uterus in milligrams. Abscissae: daily dosage of steroids in micrograms plotted logarithmically.

The introduction of a ketonic group at C<sub>16</sub> in estradiol-17 $\beta$  to form 16-ketoestradiol-17 $\beta$  (XII) decreases its activity profoundly. Similarly 16-ketoestrone (XVI) is less than one-thousandth as efficient as estrone in promoting growth. Confirming earlier work, introduction of a 4-hydroxyl group as in 4-hydroxyestradiol-17 $\beta$  (VI) causes a marked decrease in potency (10) and 2-hydroxyestradiol-17 $\beta$  (XVII) is inactive (11).

*The Impeded Estrogens.*—The relationship between the dosage of impeded estrogens and the growth response of the uterus is shown in Figs. 2 and 3. The compounds recognized as impeded estrogens have either a ketone group at position 6 or a hydroxyl group at position 16; they are

- 1,3,5(10)-estratriene-
- 3,17-diol-6-one (6-ketoestradiol-17 $\beta$  (XIX))
- 3-ol-6,17-dione (6-ketoestrone (XVIII))
- 3,16 $\alpha$ -17 $\beta$ -triol (estriol (XX))

- 3,16 $\beta$ ,17 $\beta$ -triol (16-*epiestriol* (XXI))  
 3,16 $\alpha$ ,17 $\alpha$ -triol (17-*epiestriol* (XXII))  
 3,16 $\alpha$ -diol (estradiol-16 $\alpha$  (XXIII))  
 3,16 $\beta$ -diol(estradiol-16 $\beta$  (XXIV))

In earlier studies it has been reported that 17-*epiestriol* (12), estradiol-16 $\alpha$ , estradiol-16 $\beta$  (13) and 6-ketoestradiol-17 $\beta$  (14) are estrogenic but their similarity to estriol was not observed.

A close similarity was observed in the slope of the curves of uterine growth and in the quantities of all of the impeded estrogens required to produce this

TABLE II  
*Inhibition of Estrone-Induced Uterine Growth by Supplementary Steroids  
 Effects on Weight and Nitrogen Content of the Uterus*

In all cases estrone 0.5  $\mu$ g. was administered daily.  $\Delta$  indicates the change induced by the added steroid.

Added steroid	Optimal dose	Uterine weight		$\Delta$	Nitrogen content		$\Delta$
		Estrone alone	Estrone + added steroid		Estrone alone	Estrone + added steroid	
	$\mu$ g.	mg.	mg.	per cent	mg.	mg.	per cent
Estriol	2.5	155 $\pm$ 30	103 $\pm$ 9	-33	3.7 $\pm$ 0.4	2.4 $\pm$ 0.3	-35
16- <i>Epiestriol</i>	2.5	144 $\pm$ 30	86 $\pm$ 12	-40	3.4 $\pm$ 0.5	2.2 $\pm$ 0.2	-37
17- <i>Epiestriol</i>	2.5	137 $\pm$ 21	95 $\pm$ 10	-31	3.4 $\pm$ 0.5	2.4 $\pm$ 0.2	-29
Estradiol-16 $\alpha$	5.0	153 $\pm$ 30	96 $\pm$ 4	-37	3.7 $\pm$ 0.4	2.3 $\pm$ 0.3	-38
Estradiol-16 $\beta$	5.0	172 $\pm$ 20	128 $\pm$ 28	-26	4.2 $\pm$ 0.5	3.1 $\pm$ 0.7	-26
6-Ketoestrone	5.0	166 $\pm$ 28	95 $\pm$ 33	-43	4.0 $\pm$ 0.6	2.3 $\pm$ 0.7	-42
6-Ketoestradiol	1.0	131 $\pm$ 14	90 $\pm$ 13	-32	3.3 $\pm$ 0.3	2.3 $\pm$ 0.3	-28

$\pm$  indicates standard deviation.

The *P* values in each case were less than 0.01 except for estradiol-16 $\beta$  for which the value was 0.05.

growth. In contrast to the foregoing steroids with oxygen functions at positions 6 or 16, it was found that 4-hydroxyestradiol-17 $\beta$  and 7-ketoestrone (VII) are unimpeded estrogens (Fig. 3). Furthermore 16-ketoestradiol-17 $\beta$  and 16-ketoestrone do not cause an impeded growth response.

*Inhibition of Estrone-Induced Uterine Growth by Impeded Estrogens.*—When administered simultaneously with estrone, all the impeded estrogens tested caused a moderate depression of uterine growth below that induced by estrone alone. In this experiment various phenolic estrogens were added to a constant large quantity of estrone, 0.5  $\mu$ g. daily—an amount which induced nearly maximal uterine growth and which was considerably larger than the terrace point dosage of estrone. The depression of uterine growth manifested itself both in a decrease of weight and of total nitrogen content (Table II) of this

organ. Only partial blockade of the estrone effect was achieved; the maximal inhibition of uterine growth was 26 to 43 per cent. In the inhibition of estrone-induced uterine growth by phenolic estrogens critical quantities (stated in Table II) of the blocking agent are involved. An increase of the inhibitor above the critical range resulted in renewed growth of the uterus (Fig. 4) obviously from the estrogenic properties of the inhibitor itself.

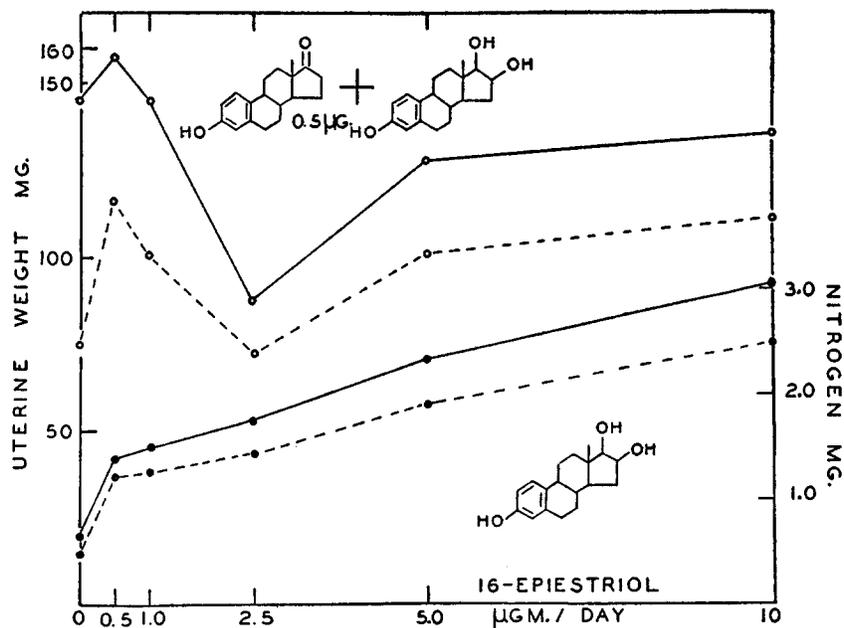


FIG. 4. The effect of increasing amounts of 16-epiestriol administered with a constant daily amount of estrone (0.5 µg.) on the weight (—) and nitrogen content (----) of the uterus. The 2 upper curves indicate the effect of the combination of estrone and 16-epiestriol; the 2 lower curves indicate growth induced by 16-epiestriol alone. Ordinates: left, uterine weight in milligrams; right, nitrogen content of uterus in milligrams. Abscissae: daily dosage of 16-epiestriol.

Whereas partial inhibition of the growth-promoting effect of estrone on the uterus was achieved, no depression of estrogenic effects on the vagina was observed. The time of opening of the vaginal plate, induced by estrone, was never retarded by estriol or related inhibiting compounds. Moreover, the weight of the vagina and the amount of cornification estimated from histological sections were not decreased by estrogenic inhibitors.

The following unimpeded estrogens failed to inhibit the growth-promoting action of estrone: estrone-16 (X); 2-hydroxyestradiol-17β; 4-hydroxyestradiol-

17 $\beta$ ; 7-ketoestrone; 16-ketoestradiol-17 $\beta$ ; estradiol-17 $\alpha$  (VIII); 17-desoxyestradiol; 3-desoxyestradiol-17 $\alpha$ ; 3-desoxyestradiol-17 $\beta$ .

Although estriol caused a significant depression of estrone-induced uterine growth, it did not inhibit the growth promoting effects of testosterone on the uterus (Fig. 5).

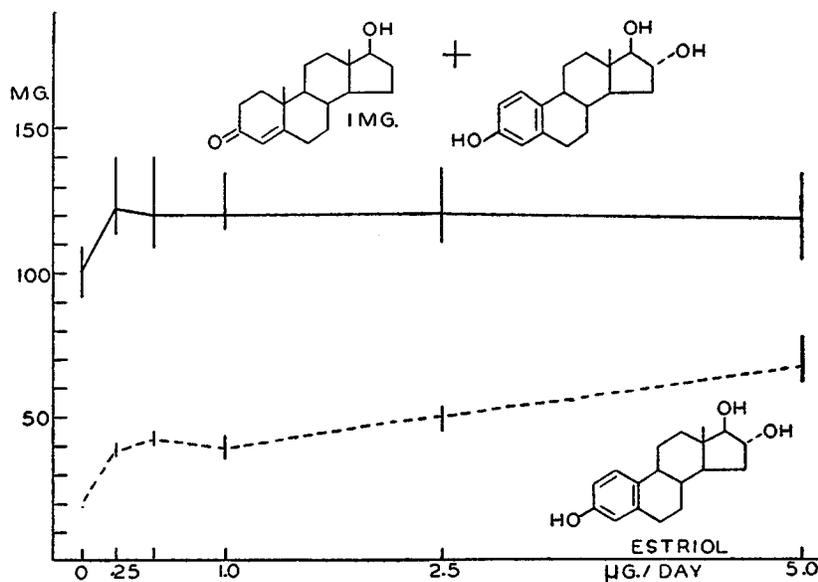


FIG. 5. Failure of estriol to inhibit growth of the uterus induced by testosterone, 1 mg. daily. The upper curve shows the effect of testosterone plus estriol on uterine weight; the lower curve indicates the increase of uterine weight caused by estriol alone. Ordinates: uterine weight in milligrams. Abscissae: the daily dosage of estriol in micrograms.

#### DISCUSSION

The impeded estrogens comprise a small group of steroids which differ from the majority of estrogenic compounds in 2 physiologic activities. Although full growth of the uterus can be evoked by sufficiently large doses of impeded estrogens, the *slope* of the curve of increment of uterine weight in response to increased dosage of these compounds is very gradual rather than steep. Secondly, impeded estrogens can partially inhibit the growth-promoting effects of estrone. The impeded estrogens are steroids which possess oxygenated groups at special sites, position 6 or 16. The explanation of the physiologic influences of impeded estrogens requires consideration of the molecular structure of estrane derivatives in terms of its effect on growth.

In general the structural requirements of growth-promoting steroids in the

estrane series include: (a) the site and (b) number of functional groups, (c) their steric orientation, and (d) the state of oxidation at select positions of the steroid nucleus.

Members of the estrane series are powerful stimulators of growth when they bear oxygenated functions (especially hydroxyl groups) at specific sites of the carbon skeleton, which thereby become active centres. The active centres are positions 3, 17, and 16 but they are not equivalent in promoting growth. The phenolic hydroxyl is considerably more effective than the highly active 17 $\beta$ -alcoholic hydroxyl in exciting uterine growth; thus 17-desoxyestradiol was more effective in this regard than 3-desoxyestradiol-17 $\beta$ . The 17 $\beta$ -hydroxyl group in the androstane series has been shown (8) to be an active centre in eliciting growth of the uterus and it exerts a similar effect in the estrane steroids. A hydroxyl group in the  $\alpha$ -orientation at C<sub>17</sub> is devoid of influences on growth; estradiol-17 $\alpha$  and 17-desoxyestradiol had identical effects on uterine growth. The alcoholic hydroxyl in the  $\beta$  orientation at C<sub>17</sub> adds to the effectiveness of the phenolic hydroxyl in promoting growth. A third oxygenated group supplementary to those at C<sub>3</sub> and C<sub>17</sub> always weakened the quantitative efficiency of the parent compound in its growth effects. It was found in the present studies that additional oxygenated groups at C<sub>2</sub>, C<sub>4</sub>, C<sub>6</sub>, C<sub>7</sub>, or C<sub>16</sub> lessened or destroyed the growth effects of estradiol-17 $\beta$ . It has been shown (5) that the uterine growth stimulation induced by testosterone also is affected adversely by supplementary hydroxyls at positions 2 ( $\alpha$  or  $\beta$ ), 6 ( $\beta$ ), and 11 ( $\alpha$  or  $\beta$ ).<sup>4</sup> It appears that certain effects of supplementary groups at C<sub>16</sub> are related to their influence on oxidation of the 17 ( $\beta$ ) hydroxyl group.

An oxygenated group at position 16 can exert two opposite effects on the growth process—it can serve as an active center in promoting uterine growth or under other circumstances it can retard growth; these opposite effects depend on the state of oxidation of the supplementary group at C<sub>16</sub> and on the presence, nature, and orientation of the substituent at C<sub>17</sub>.

The growth efficiency is enhanced somewhat when a hydroxyl group ( $\alpha$  or  $\beta$ ) is supplied at position 16 of those estrane compounds with no group or an  $\alpha$ -hydroxyl at C<sub>17</sub>. Hydroxylation at position 16 lowers the threshold dosage required for initiating growth by these steroids although they are converted to impeded estrogens. In this regard the impeded estrogens, estradiol-16 $\alpha$  and estradiol-16 $\beta$ , began to induce uterine growth in lower dosage than the unimpeded compound, 17-desoxyestradiol; similarly 17-*epi*estradiol has a lower threshold effect on growth than estradiol-17 $\alpha$ .

However, in the presence of a  $\beta$ -oriented hydroxyl group at C<sub>17</sub>, a profound

<sup>4</sup> More recently we have found that the following testosterone derivatives, at a daily dosage of 1 mg, fail to induce growth of the uterus: 8 $\beta$ -hydroxytestosterone; 14 $\alpha$ -hydroxytestosterone; 15-hydroxytestosterone; 16 $\alpha$ -hydroxytestosterone.

decrease of growth-promoting activity results from an adjacent hydroxyl group at C<sub>16</sub>; both estriol and 16-*epi*estriol are less active in exciting uterine growth than estradiol-17 $\beta$ . Likewise, the presence of a ketone group at C<sub>16</sub> decreases the efficiency of estrane compounds with ketone or hydroxyl groups at position 17; 16-ketoestrone and 16-ketoestradiol-17 $\beta$  are weak estrogens. Such effects are reminiscent of the influence of an oxygenated function at C<sub>16</sub> upon the oxidation of the 17 $\beta$ -hydroxyl group by a highly purified enzyme of bacterial origin, the  $\beta$ -steroid dehydrogenase of Talalay and Dobson (15). The bacterial enzyme can oxidize the 17 $\beta$ -hydroxyl group of estradiol-17 $\beta$  but this dehydrogenation does not occur when estriol or 16-ketoestradiol-17 $\beta$  are employed as substrates.

Other evidence was gathered that atoms bonded to C<sub>6</sub> or C<sub>16</sub> are highly significant in the promotion of growth. The loss of one of the hydrogen atoms at C<sub>6</sub> results in a considerable decrease of growth activity; 6-dehydroestrone is less active than estrone in exciting growth. This indicates an important relationship of hydrogen atoms to specific carbon sites in the steroid nucleus, since a hydrogen atom is dispensable at positions 7, 8, or 9 without change in the growth activity of the unsaturated compound. Similarly hydrogen atoms at position 16 are involved in the excitation of growth;  $\Delta$ -16,17-desoxyestradiol (XI) was found to be less efficient than 17-desoxyestradiol in promoting growth, confirming earlier findings (12).

The similarity of the quantitative relationship of dosage to the resultant uterine growth of all the impeded estrogens, irrespective of the site of the supplementary function at C<sub>6</sub> or C<sub>16</sub>, suggests a common biochemical mechanism to explain their unusual influences on growth. We postulate that both the inhibition of estrone action and impeded uterine growth are related to interaction of oxygenated functions at special centers of the estrane molecule with a specific surface possessing critical import in promoting growth; this interaction possibly is related to the binding of the steroids to a protein surface. It is known that phenolic estrogens, such as estrone (16) and estradiol-17 $\beta$  (17), can bind firmly to serum and hepatic liver proteins. Similarly, congeners of the 1,3,5 (10)-estratrien-3-ol family have a strong affinity (18) for the surface of the  $\beta$ -steroid dehydrogenase of Talalay and Dobson (15). This hypothesis would implicate oxygenated groups at positions 6 and 16 as significant loci in the estrane molecule in the binding process. In this regard no inhibition of estrone-stimulation or impedance results when a hydroxyl group at C<sub>4</sub> or a ketonic group at C<sub>7</sub> is added to estradiol-17 $\beta$ .

The inhibition of estrone-induced growth by impeded estrogens appears to be restricted to the uterus. Under conditions in which uterine growth was depressed, no decrease in the growth or cornification of the vagina was detected.

## CONCLUSIONS

Two small groups of steroids in the estrane series—here designated as impeded estrogens,—represent a class of compounds which differ from the majority of estrogenic substances in exerting certain unusual influences on growth of the uterus. The induction of these effects previously was considered to be peculiar to estriol. The unusual growth properties common to impeded estrogens are twofold: (a) after the threshold dosage required to initiate growth has been reached, the slope of the curve of increment of uterine weight in response to increased steroid dosage is very gradual rather than steep; (b) these compounds possess the ability to inhibit to a limited extent the uterine growth induced by estrone administered concurrently.

The partial inhibition of estrone-induced growth of the uterus is confined to a critical dosage of the impeded estrogen and is overcome by increased dosage of the inhibitor. Estrone-induced growth of the vagina is not inhibited by impeded estrogens. Furthermore the simultaneous administration of impeded estrogens and testosterone does not lessen the amount of uterine growth evoked by the latter.

The impeded estrogens so far encountered are 3-hydroxyestratriene derivatives possessing either a ketone group at position 6 or a hydroxyl group at position 16. Oxygenated functions at these positions in phenolic estrogens have special significance in the excitation and restraint of uterine growth unshared by similar groups at certain other sites of the estrane molecule.

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