CHEMICAL STRUCTURE OF STEROIDS IN RELATION TO PROMOTION OF GROWTH OF THE VAGINA AND UTERUS OF THE HYPOPHYSECTOMIZED RAT

By CHARLES HUGGINS, M.D., ELWOOD V. JENSEN, Ph.D., and ANNE STACK CLEVELAND

(From the Ben May Laboratory for Cancer Research of the University of Chicago, Chicago)

Plates 28 and 29

(Received for publication, May 3, 1954)

The experiments to be described were designed to correlate the growth-promoting activity of steroids with their molecular structure. The steroids had 2 or more functional centers and the biological indicators were the uterus and vagina of the rat. The analysis of the results furnishes some insight into the mechanism of action of steroids in the induction of growth—a problem which has not been elucidated. The state of oxidation of the ring structure and of the side groups of steroids were found to have high significance in the promotion of growth.

It will be demonstrated that the vaginal epithelium consists of 2 distinct layers of cells which differ profoundly in their growth in response to steroids, depending on the chemical structure present in those compounds. These differences in the rate of growth of the separate layers in response to different molecular groups provide 3 separate patterns in the vaginal mucosa—mucification, keratinization, and stratification.

Mucification of the vagina can be elicited in ovariectomized rats with testosterone (1) and related compounds; its characteristics are the proliferation of mucous cells which secrete a clear fluid that is rich in protein conjugated with carbohydrate. In keratinization there is growth of the deeper cells of the vaginal epithelium, which secrete a thick white fluid containing fibers of keratin. This effect can be reproduced by certain phenolic compounds, for example, estrone, as was first demonstrated by Doisy *et al.* (2), and by 1 member of the C_{19} series of steroids, 5-androstene-3β,17β-diol, as was found by Tschopp (3) and by Deanesley and Parkes (4). The third pattern, vaginal stratification, is characterized by vigorous growth of the deeper cells of the vaginal epithelium which secrete an opalescent watery fluid, but neither kera-

* This study was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, the Damon Runyon Memorial Fund for Cancer Research, the United States Public Health Service, and Mr. C. P. Winslow.

We are indebted to Georgiana Psik and Eustus Lauagan for technical assistance.

tin fibers nor mucus is produced; this pattern is elicited by a class of steroids to be defined.

For the precise measurement of the effects of growth-promoting substances on cells it is required that growth other than that resulting from the agent under assay shall be reduced to a minimum. Hitherto this axiom has been largely neglected in biological assays of steroids. Important extraneous factors stimulating growth can come both from without, and from within the animal.

In the intact rat, steroids commonly set in motion a complex set of physiologic changes related only indirectly to the compound being studied. Whereas phenolic estrogens such as estrone (5) in small amount depress the rate of body growth, testosterone and related steroids accelerate it. The great complications in the evaluation of steroids in the intact animal arise from the sensitivity of the hypophysis to these substances. The size of this gland and its functions are considerably increased in the presence of certain steroids whilst being greatly decreased by other compounds. For this reason hypophysectomized rats were used in the present experiments.

Certain foods contain steroids or substances with similar physiologic properties (6, 7). During preliminary experiments it was found that rations from 2 commercial sources induced estrus prematurely in adolescent rats, so that these foods could not be used. Difficulties of this sort were circumvented by the use of a synthetic diet free from estrogenic compounds (8). When hypophysectomized rats were maintained on this artificial diet bodily growth was greatly depressed and the endocrine glands were very small. Yet the animals were active and vigorous and have been maintained for more than a year without medicinal support.

In the past the most extensive studies of the effects of steroids on the female genital tract have been made in immature (9) or ovariectomized rats (10). Data will be presented comparing the effects of steroids on hypophysectomized rats with ovariectomized controls.

Very few studies have been reported of the effects of steroids on the genital tract of female hypophysectomized animals. It is known that testosterone induces canalization of the vaginal plate of hypophysectomized rats (11). There is complete disagreement concerning the efficacy of steroids in promoting growth of the uterus and vagina of hypophysectomized rodents, compared with ovariectomized mates. Mühlbock and van Maurik (12) found that estrone was more effective in hypophysectomized mice than in ovariectomized controls. Hill and Parkes (13) reported that the growth response to estriol was similar in both classes in the ferret. Müller (14) stated that estradiol was more effective in ovariectomized than in hypophysectomized rats.

Butenandt and Kudszus (9) observed that testosterone, dehydro*epi*androsterone, and androstenedione promoted the growth of the comb of the capon and the prostate of the rat and also induced premature opening of the vaginal plate of immature female

rats, a property not shared by androsterone and progesterone. Deanesley and Parkes (4) discovered that dehydro*epi*androsterone, in addition to promoting growth of the comb and the prostate, induced estrus in the immature rat and caused the appearance of the female type of plumage in the Sebright bantam capon, the last effect being caused by estrone but not androsterone. Tschopp (3) observed that 5-androstene-3β,17β-diol likewise induced both comb growth in the capon and estrus in ovariectomized rats. Korenshevsky and Dennison (1) found that both testosterone and estrone caused enlargement of the uterus of spayed rats.

Methods

Biological.—Albino rats from the Sprague-Dawley (Holtzman) strain were weaned at age 21 days and fed thereafter on the synthetic ration, designated 4C, of Wissler *et al.* (8), supplemented with one-quarter of an orange for every 4 rats. They were kept in an air-conditioned windowless room at a temperature of 25° ± 1° and were in darkness exactly 10 hours of each day. The animals were caged in stainless steel boxes which, with the food containers and water bottles, had been boiled in a strong solution of detergent before use to remove steroids. Special precautions were taken to maintain the colony free from disease. The rats were weighed and measured every 2 days. The perineum was inspected daily for the opening of the vaginal plate. Vaginal smears were purposely omitted to avoid disturbing the cytologic appearance of the vaginal epithelium in fixed sections.

The experiments were carried out on an undeviating schedule. The rats were received from the breeder at age 22 days and were operated upon at 24 days. The surgical operations performed under ether anesthesia were either hypophysectomy, ovariectomy, or adrenalectomy; adrenalectomized rats were given 0.9 per cent sodium chloride as a drinking fluid. Hypophysectomized rats which weighed more than 72 gm. at age 38 days were not used in the assay for fear that some pituitary function remained; for the same reason experiments were discarded if the combined weight of either the adrenal glands or the ovaries was 12 mg. or more at necropsy.

A solution (0.2 ml.) in sesame oil of the steroid under test was injected subcutaneously for 7 days, beginning at 38 days and using 4 rats or more at each dose level; the control animals received solvent alone. A highly refined preparation of growth hormone from bovine anterior pituitary glands was dissolved in 2 per cent sodium bicarbonate and 0.5 cc. was injected subcutaneously twice daily. At necropsy, performed at age 45 days, the adrenal glands, ovaries, the uterus, and the vagina were excised, blotted lightly on filter paper and weighed promptly on a torsion balance. The average weight of the uterus of hypophysectomized rats injected with compounds (at a daily dose of 1 mg.) was divided by the average for rats injected with the solvent alone and the results are expressed as the *ratio of uterine weight.* Segments of the vagina were fixed in formalin, and paraffin sections were stained with hematoxylin and eosin, and with celestin blue (15) for mucoprotein.

Chemical.—Most of the steroids were obtained from other laboratories;[1] the sources are listed in Table II. 4-androstene-3β,17β-diol (m.p. 152°) and 5-androstene-3,17-dione (m.p. 159°) were prepared as described by Butenandt (16, 17).

[1] We acknowledge with gratitude generous gifts of compounds used in these experiments from Professor Adolf Butenandt, University, Tübingen, Germany; Dr. E. A. Doisy, St. Louis University; Dr. T. F. Gallagher, Sloan-Kettering Institute for Cancer Research; Dr. Edward Henderson, Schering Corporation; Dr. C. D. Kochakian, Oklahoma Institute for Medical Research; Dr. C. H. Li, University of California; Dr. J. R. Mote and Dr. A. White, Chemical Specialties Co.; Dr. D. H. Peterson, The Upjohn Co.; Dr. F. A. Travers, Ciba Pharmaceutical Products, Inc.; Dr. A. E. Wilhelmi, Emory University; and Dr. W. A. Wright, Charles Pfizer and Co., Inc.

RESULTS

Our normal female rats at age 24 days weighed 51 ± 5 gm. Following hypophysectomy on this day there was a gradual increase of weight for 2 weeks —the average weight of 90 hypophysectomized rats at age 38 days was 65 ± 3.7 gm. The rats did not gain more than 3 gm. between age 38 and 45 days while the injections were being given.

At age 45 days the weight of the uterus of 34 hypophysectomized rats was 19.2 ± 2.8 mg., and the vagina weighed 20.4 ± 2.4 mg.; these animals had been injected with sesame oil daily from age 38 to 44 days. This uterine weight value was less than that of intact rats at age 24 days (when the operations were performed) or of rats subjected to ovariectomy either alone or with the removal of the adrenal glands (Table I). In addition to differences in uterine weight other facts proved that the female genital tract of hypophysectomized rats is far more atrophic than it is in their ovariectomized sisters.

The opening of the vaginal plate is an indicator of the presence of active steroids in the body although the indicator is not sensitive and these compounds must be present in considerable amounts for canalization to occur. In our colony the vaginal orifice is established in intact rats at 39 ± 5 days (range 28 to 49 days). In 145 rats ovariectomized at 24 days, the vaginal plate opened in 42 cases (29 per cent) before 40 days and in all of the remainder before 140 days. The vagina did not open in any of 1269 hypophysectomized rats observed in the present experiment before 38 days and only in those injected with certain active steroids thereafter. The vaginal plate remained imperforate in 16 untreated hypophysectomized rats observed for 365 days, establishing confidence that the experimental conditions including the diet did not contribute extraneous growth-promoting factors to these rats.

The vaginal epithelium (18) of hypophysectomized rats consists of a sheet of atrophic epithelium, 2 to 3 cells in thickness (Fig. 1). The superficial cells differ in histochemical properties from the deeper cells; the superficial layer has a thin layer of mucoprotein on its surface while the deeper cells are devoid of this protein as was demonstrated in sections stained with celestin blue (15). In comparison the vaginal epithelium of ovariectomized rats, though consisting of the same two layers, is much thicker, the superficial cells are tall (Fig. 2) and much mucoprotein is present; removal of the adrenals in addition to the ovaries did not increase the vaginal atrophy.

It was apparent that the removal of the ovaries and the adrenal glands failed to remove all of the endogenous factors causing growth of the female genital tract, whereas this was accomplished successfully by hypophysectomy. The stimulating agent remaining in ovariectomized rats proved to be the pituitary growth hormone.

Treatment of hypophysectomized rats with pituitary growth hormone, 0.2 mg. daily, increased the weight of the uterus (Table I) and produced tall

columnar mucous cells in the vagina (Fig. 3) with result that the uterus and vagina of the treated animals appeared identical with those of ovariectomized rats. The growth hormone was equally effective in hypophysectomized rats deprived of the ovaries and adrenal glands as in rats possessing these glands.

Hypophysectomized rats were compared with ovariectomized mates with respect to uterine growth induced by estrone and testosterone. In the lower dose range (Text-fig. 1) estrone was more effective in hypophysectomized rats. Testosterone, after an initial lag, caused greater uterine growth in hypophysectomized than in ovariectomized animals (Text-fig. 2). As a measure of the validity of the assay procedure, testosterone, 1 mg. daily, was administered to 31 hypophysectomized rats, age 38 days, for 7 days; the average weight of the uterus was 115.9 ± 9.2 mg. at age 45 days.

TABLE I

Influence of Growth Hormone on the Uterine Weight of Hypophysectomized Rats

Procedure	No. of rats	Age at necropsy	Uterine weight
		days	*mg.*
None; normal rats	8	24	29.6 ± 6.1
Ovariectomy	14	45	36.0 ± 6.0
Ovariectomy and adrenalectomy*	9	45	31.6 ± 3.3
Hypophysectomy	34	45	19.2 ± 2.8
Hypophysectomy and Growth Hormone‡	8	45	30.2 ± 3.1

Operations performed at age 24 days.

* Maintained with saline drinking fluid, without hormones.

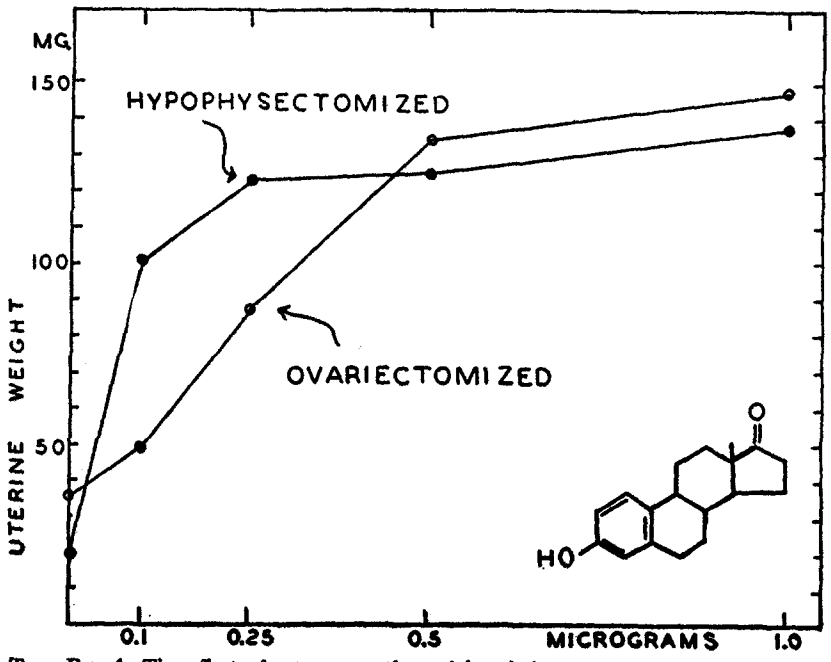
‡ 0.1 mg. of growth hormone injected subcutaneously twice daily from age 38 to 44 days.

The cytology of the vaginal epithelium is a more delicate indicator of activity of steroids than the increment of uterine weight. A clear cytologic response was evident in hypophysectomized rats after the injection of estradiol-17β, 0.005 μg. daily for 7 days, an amount which did not cause an increase of uterine weight.

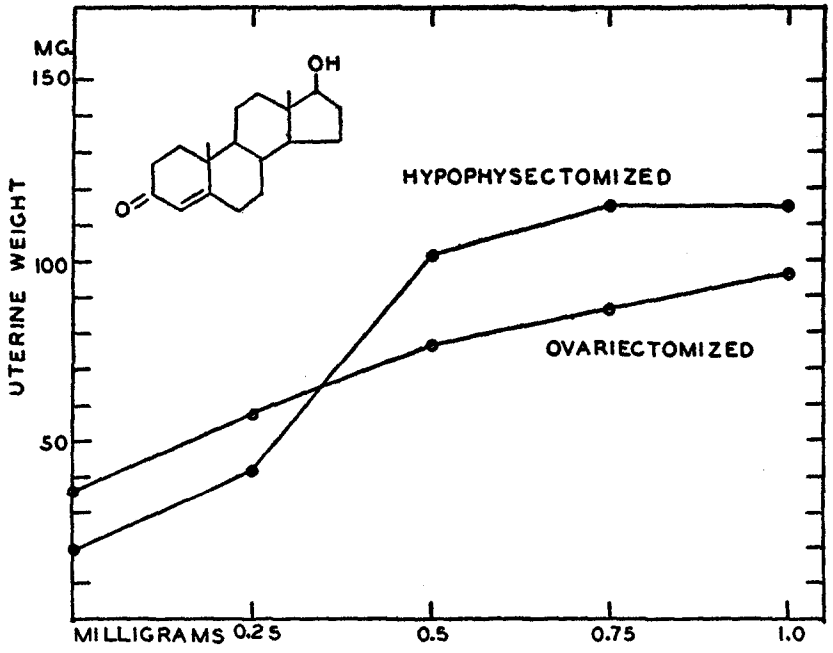
Effect of Steroids on Uterine Weight

(*a*) *C_3 and C_{17} Diones.*—Androstane-3,17-dione (IV)[2] was mildly active in promoting the growth of the uterus (Table II). The activity was increased, and to the same extent, by the introduction of one double bond into this molecule at either C_1 (II) or C_4 (III). Activity was further intensified by the presence of double bonds at both C_1 and C_4 as in 1,4-androstadiene-3,17-dione (I) but this caused a significant change in the vaginal epithelium as will be seen. The presence of double bonds at C_4 and C_6 as in 4,6-androstadiene-3,17-dione (V) eliminated activity. Likewise an additional hydroxyl group at C_{11} in either the

[2] The chemical formulae of the steroids are given in Text-fig. 4.



TEXT-FIG. 1. The effect of estrone on the weight of the uterus of ovariectomized and hypophysectomized rats.



TEXT-FIG. 2. The effect of testosterone on the weight of the uterus of ovariectomized and hypophysectomized rats.

TABLE II

Uterine Weight and the State of the Vaginal Epithelium Following the Injection of 19-Carbon Steroids

No.	Compound	Uterine weight ratio*	Vaginal epithelium‡
	C_3 and C_{17} Diones		
I	1,4-Androstadiene-3,17-dione§	5.8	Stratified
II	1-Androstene-3,17-dione§	3.8	Mucus
III	4-Androstene-3,17-dione§	3.4	Mucus
IV	Androstane-3,17-dione§	1.7	Mucus
V	4,6-Androstadiene-3,17-dione§	1.0	Inactive
VI	4-Androsten-11β-ol-3,17-dione‖	1.0	Inactive
VII	4-Androsten-11α-ol-3,17-dione‖	0.9	Inactive
	3-Hydroxysteroids		
VIII	5-Androstene-3β,17β-diol§	6.5	Keratin
IX	4-Androstene-3α,17β-diol§	5.7	Mucus
X	4-Androstene-3β,17β-diol	5.2	Mucus
XI	Androstane-3β,17β-diol¶	5.7	Stratified
XII	Androstane-3α,17β-diol¶	4.5	Mucus
XIII	5-Androsten-3β-ol-17-one (dehydro*epi*androsterone)§	3.1	Stratified
XIV	Androstan-3β-ol-17-one§	1.4	Stratified
XV	Androstan-3α-ol-17-one (androsterone)**	1.1	Mucus
XVI	Androstane-3β-17α-diol¶	1.0	Inactive
	17-Hydroxysteroids		
XVII	4-Estren-17β-ol-3-one‡‡ (19-*nor*-testosterone)§	7.7	Mucus
XVIII	4-Androsten-17β-ol-3-one (testosterone)§	5.8	Mucus
XIX	Androstan-17β-ol-3-one§	5.5	Mucus
XX	4-Androstene-11β,17β-diol-3-one‖	3.3	Mucus
XXI	4-Androstene-2β,17β-diol-3-one§	3.3	Mucus
XXII	4-Androstene-2α,17β-diol-3-one§	2.2	Mucus
XXIII	Etiocholan-17β-ol-3-one§	1.0	Inactive
XXIV	4-Androstene-11α,17β-diol-3-one‖	1.0	Inactive
XXV	4-Androstene-6β,17β-diol-3-one‖	1.0	Inactive
XXVI	4-Androsten-17α-ol-3-one (*epi*testosterone)¶	0.8	Inactive

* Average weight of uteri of 4 rats injected with the compound at dosage of 1 mg. *per diem* divided by the value obtained from a similar group injected with solvent alone.

‡ Histologic appearance of the surface cells following a daily dosage of 3 mg. of the compound.

§ Chemical Specialties Co., Inc., New York, N. Y.

‖ The Upjohn Company, Kalamazoo, Michigan.

¶ Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

** Dr. T. F. Gallagher, Sloan-Kettering Institute for Cancer Research, New York, N. Y.

‡‡ An 18-carbon steroid.

α (VII) or the β (VI) orientations rendered 4-androstene-3,17-dione (III) inactive.

(*b*) *3-Hydroxysteroids.*—Androstane-3β,17β-diol (XI) was considerably more active in causing uterine growth than androstane-3,17-dione (IV) and the introduction of a double bond at C_5 (VIII) enhanced its activity still more. Compounds with a hydroxyl in the 17β-position (VIII; XI) were more active than the corresponding ketones (XIII; XIV). Most compounds with a 3β-hydroxyl group (XI; XIV) were more active than corresponding steroids with a 3α-hydroxyl (XII; XV). No activity was associated with steroids having a 17α-hydroxyl group (XVI; XXVI).

(*c*) *17-Hydroxysteroids*—Testosterone (XVIII) was very effective in increasing uterine weight and the removal of its 19-methyl group (XVII) resulted in a more active compound. The activity of testosterone was decreased slightly by reduction of the double bond to the 5α-configuration (XIX). The activity was moderately decreased by the introduction of an additional hydroxyl in the molecule in the 11β- (XX), the 2β- (XXI) or the 2α- (XXII) positions. The metrotrophic activity of testosterone (XVIII) was destroyed by the introduction of an additional hydroxyl group at the 11α-position (XXIV) or at the 6β-site (XXV). Compounds having the 5β-structure such as etiocholan-17β-ol-3-one (XXIII) were inactive.

(*d*) C_{21} *Steroids.*—The replacement of the 17β-hydroxyl group of testosterone with an acetyl or an α-ketol group resulted in complete loss of activity on the uterus and vagina. The following compounds were found to be ineffective at a dose level of 1 mg. daily: progesterone; 17-hydroxyprogesterone; corticosterone; desoxycorticosterone; 11-dehydrocorticosterone; cortisone; hydrocortisone; 11-desoxy-17-hydroxycorticosterone.

Activity of Steroids on Vaginal Epithelium

In the presence of steroids with certain molecular features the superficial cells of the vaginal epithelium enlarged and secreted large quantities of mucoprotein (Fig. 8). Other groups in steroids stimulated the growth of the deeper cells preferentially; in such instances the mucous layer was shed whereupon the remaining cells produced keratin in place of mucus (Fig. 9). Still other combinations of chemical groups caused stratification of the vaginal mucosa unaccompanied by keratin or mucoprotein (Figs. 6, 7) at the relatively high dosage of 3 mg. daily of the steroid.

In hypophysectomized rats, *threshold* amounts of either testosterone (0.1 mg.) or estrone (0.1 μg.) induced growth of both layers of the vaginal epithelium with some growth of the mucous cells but the histologic patterns under these circumstances are not identical. Estrone in this small amount produced greater growth of the basal cells (Fig. 4) than occurred with testosterone from 0.1 mg. to 4 mg. *per diem.* The mucifying effect of estrone in small

dosage on the vaginal mucous cells of ovariectomized rats has been described previously (19).

The important differences of estrone and testosterone with respect to their action on the vagina were brought out by augmentation of dosage. When the daily amount of testosterone was increased to 1 mg. (or even 4 mg.) a progressive increase of mucoprotein occurred in the superficial cells without a trace of keratin production while the growth of the deeper cells was very slight. On the other hand an increase of estrone to 1 μg. daily caused the superficial layer to disappear, the basal cells increased considerably in size and number, and keratin was formed in quantity on the surface of the cells; a further increase of estrone to 100 μg. caused only an intensification of basal cell growth and of keratin formation. The simultaneous administration of testosterone (1 mg.) and estrone (1μg.) caused stratification (Fig. 5) with a pronounced decrease of mucoprotein and the cessation of keratin production. The designation (Table II) of the principal patterns induced by 19-carbon steroids in the vaginal epithelium applies to the effects occurring after large dosage (3 mg. daily). The outstanding characteristics of the epithelial surface were classified as the formation of mucous cells, stratified cells, or keratin fibers. It has been reported (10) that steroids in ovariectomized rats sometimes produce areas of cornification and mucified epithelium in the same vagina. Mixed patterns of this sort were not seen in hypophysectomized rats in the present experiments.

Keratinization.—Of the 19-carbon steroids which were investigated only 5-androstene-3β-17β-diol (VIII) induced keratin formation and this occurred in large amount at a daily dosage level of 1 mg.

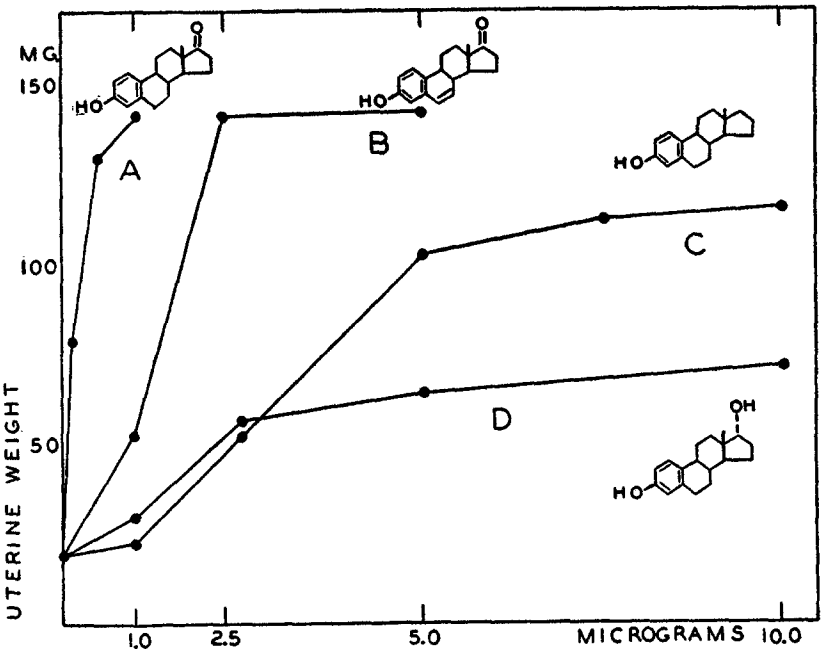
Stratification.—Stratification followed the administration of 4 steroids: androstane-3β,17β-diol (XI), androstan-3β-ol-17-one (XIV), 5-androsten-3β-ol-17-one (XIII), and 1,4-androstadiene-3,17-dione (I) (Fig. 6).

Mucoprotein Formation—Many steroids in this series produced vaginal mucification (Table II). The type of chemical group at C_{17} and its steric configuration were highly significant in producing mucification. Nine compounds possessed a 17β-hydroxyl group while a 17-ketone group was present in 4 steroids. Androstane-3,17-dione (IV) produced mucification and the introduction of 1 double bond in this compound at C_1 (II) or C_4 (III) intensified the effect, but double bonds at both C_1 and C_4 (I) blocked mucification (Fig. 6). Providing no inactivating group or configuration (to be described) was present mucification occurred with all steroids with 1 double bond in ring A. Of the saturated androstane compounds a hydroxyl group in the 3α-position produced mucification (XII) (XV) whereas similar compounds with a 3β-hydroxyl (XI) (XIV) caused stratification (Fig. 10) instead.

With reference to position C_3 in difunctional steroids a ketone group or an α-hydroxyl induced extensive mucification.

Inactive Compounds.—Certain molecular changes deprived otherwise active steroids of the ability to promote growth of the vaginal epithelium or of the uterus. The changes which inactivated androstane compounds follow: (*a*) The presence of an 11α-hydroxyl (VII) (XXIV); (*b*) The presence of a 6β-hydroxyl (XXV); (*c*) A double bond at C_6 as in 4,6-androstadiene-3,17-dione (V); (*d*) Inversion of the 17β-hydroxyl group of testosterone (XVIII) to the 17α-configuration (XXVI); (*e*) The 5β-steroid structure (XXIII).

Modification of the Estrone Structure.—Studies were made of 3 derivatives of estrone with structural factors which had been found to abolish activity in

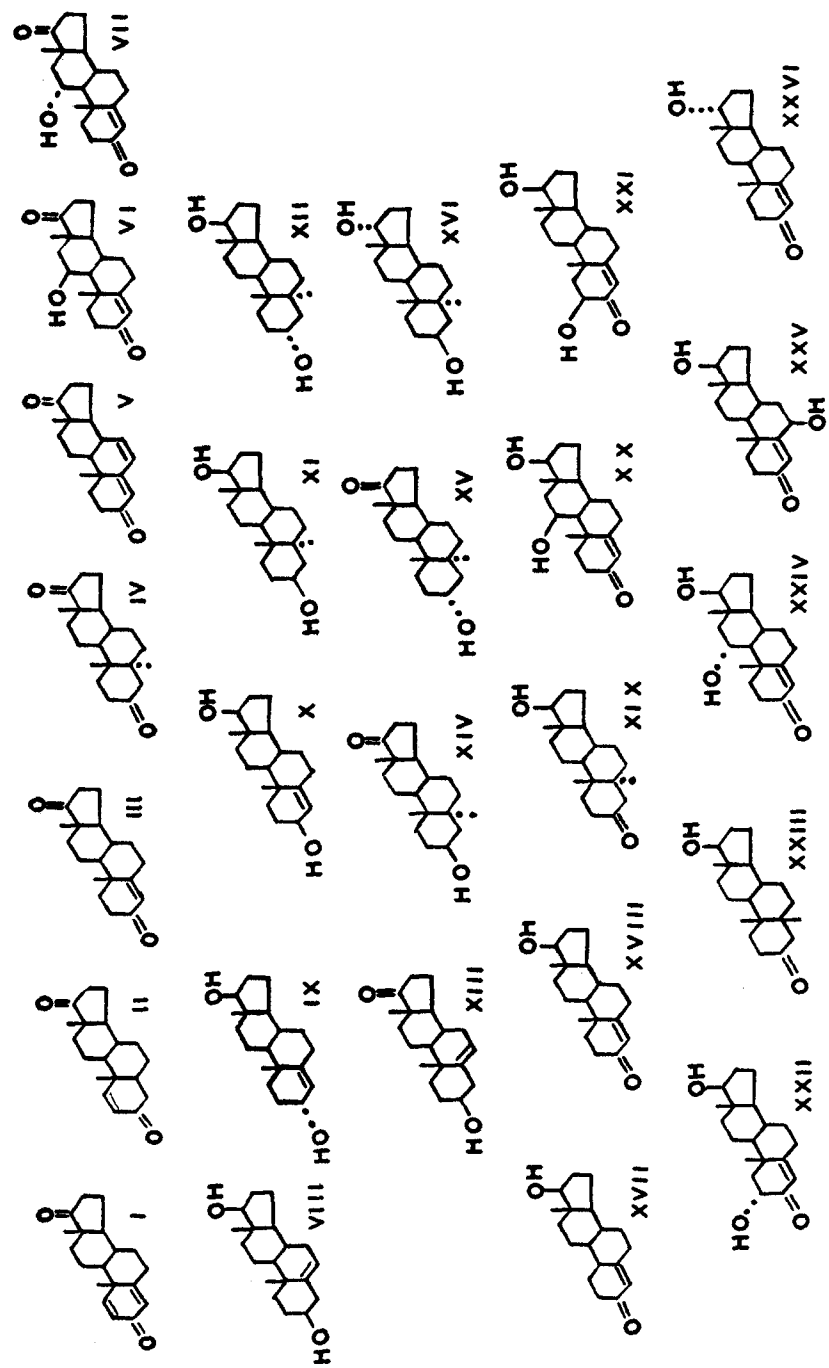


TEXT—FIG. 3. The effect of estrone (*A*), 6-dehydroestrone (*B*), 17-desoxyestradiol (*C*), and estradiol-17α (*D*) on the uterine weight of hypophysectomized rats.

C_{19}-steroids. These compounds differed from estrone in the following ways: 17-desoxyestradiol lacked a functional group at C_{17}; estradiol-17α had a hydroxyl at C_{17} in the α-position; 6-dehydroestrone had an additional double bond at C_6. In contrast to the compounds in the androstane series considerable activity in causing uterine growth was retained in the presence of the designated modifications although in each case it was less than that of estrone (Text-fig. 3.). All of these compounds caused vaginal keratinization.

DISCUSSION

The hypophysectomized rat is in a basal endocrine state when maintained on the synthetic diet employed in this study, and is protected from the con-


I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
XXV
XXVI

TEXT-FIG. 4

sumption of active steroids in the ration. The uterus and vagina are profoundly atrophic, but they retain such great sensitivity to growth-promoting hormones that weakly active compounds can be detected. The salient advantage for assay purposes of hypophysectomized rats over ovariectomized animals lies in the absence of the pituitary growth hormone. The pituitary growth hormone has the same growth-stimulating ability as active steroids in threshold quantities, and elevates the growth of the uterus and vagina of the hypophysectomized rat to the less atrophic state of its ovariectomized mate. Thus two hormones of widely different chemical characteristics, protein and steroid, can have a common property; in this case both induce significant growth of the female genital tract.

The superficial layer of the vaginal mucosa forms mucous cells which produce protein conjugated with carbohydrate, whereas the cells of the deeper layer produce keratin but only after the superficial layer has been cast off. Mild stimulation with threshold amounts of steroids of many types causes a growth response of both layers; only strong stimulation with appropriate compounds serves to bring out the profound differences of reactivity of the 2 layers.

When the compounds were administered at the high daily dosage level of 3 mg., uniform clear-cut patterns permitting discrimination of the principal growth effects were produced by all of the C_{19}-steroids which were investigated.

Three structural factors in the steroid molecule are of high significance in the promotion of growth—the position of the functional groups, the geometry of the molecule, and the state of oxidation at critical sites.

The functional groups which endow the androstane molecule with the ability to promote uterine growth are hydroxyls and ketones, and these are effective when located at positions 3 and 17. Additional hydroxyl groups are inhibitory; the introduction of 2α-, 2β-,or 11β-hydroxyl groups into the testosterone structure results in a moderate decrease of activity, whereas a 6β- or 11α-hydroxyl group completely abolishes physiologic action. Among the estrogens a 16α-hydroxyl group is likewise inhibitory, since estriol is known to be less effective than estradiol in promoting uterine growth in immature rats (20).

The geometry of the steroid molecule at certain centers of asymmetry is known to be a critical factor in hormonal activity (3, 21). This fact is substantiated by the present experimental results. The induction of growth in the uterus and the vagina is abolished if there is a cis-fusion of the A:B ring; if the 17β-hydroxyl group is inverted to the 17α-configuration; or if a β-oriented side chain is present at C_{17}. The removal of the 19-methyl group of testosterone, as in 19-*nor*-testosterone (XVII), increases the activity. In view of the importance of spatial considerations in determining activity of C_{19} steroids, it is reasonable to assume that close approximation to a specific

surface is involved in exciting physiologic activity. If this be the case, the fit is ruined by a hydroxyl group on the back of the molecule at C_{11} or by one on the front of the molecule at C_6. Groups at position 2 have a smaller influence, whereas the elimination of the angular methyl group at C_{19} enhances the interaction.

The state of oxidation of the steroid molecule is of great importance as a determinant of the efficacy of steroids in promoting growth. In experiments involving the living animal, it is difficult to say whether the compounds administered are active *per se* or whether they are first changed by metabolic processes. In the absence of further knowledge, however, it is reasonable to assume that the most active compounds in a closely related series possess groups in a state which actually functions biologically, whereas the less active compounds may first be transformed to the active structures. Lieberman and Teich (22) have made the generalization that, when changes occur during metabolism, steroids are usually transformed chemically to a more highly reduced state.

In accord with previous findings (23, 24) maximum growth stimulation requires a β-oriented hydroxyl group at C_{17}. Oxidation of this group to a ketone results in a significant decrease in activity. In contrast to C_{17} the oxidative state at C_3 is less critical in the promotion of uterine growth. For a given structure in the rest of the molecule, the activity is approximately the same in steroids possessing at C_3 either a ketone group or an α- or a β-hydroxyl group. However, the type of effect observed in the vaginal epithelium is profoundly influenced by the substituent at C_3. 3α-hydroxysteroids, as well as 3-keto compounds, cause preferential stimulation of the superficial cells to produce mucus, whereas, in general, the deeper layers respond to compounds with a 3β-hydroxyl group. The superficial cells of the vagina resemble the chicken comb in being far more reactive to α-hydroxyls than β-hydroxyl groups (3, 9). Talalay and his coworkers (25, 26) have discovered that certain bacteria contain 2 separate steroid alcohol dehydrogenases which, like the two vaginal layers, respond differently to steroids containing either β-hydroxyl groups or 3α-hydroxyl groups, and they have demonstrated that both types of enzymes are present in the tissues of the rat vagina and uterus (27). It is premature to ascribe the differential physiologic effects of steroids with 3α- or 3β-hydroxyl groups to the action of these enzymes.

With respect to the ring structure, the extent and the site of dehydrogenation are critical. Double bonds in ring A increase the efficiency in promoting growth, and these increases are in direct relationship to the number of double bonds introduced. In ring B the introduction of a double bond at C_5 increases the effectiveness of a steroid as compared with the corresponding saturated compound, but a similar structure at C_6 abolishes activity.

The relative significance of the ring structure and of the substituent groups

(at C_3 and C_{17}) in determining cellular response is brought out by the investigation of a series of androstenediols (Figs. 8 to 13). The shift of a double bond from C_5 to C_4 has a profound influence on physiologic effect. Steroids with a double bond at C_5 (VIII; XIII) stimulate the deeper cells preferentially, whereas those with a double bond at C_4 (IX; X) produce growth of the superficial layers. Thus 5-androstene-3β,17β-diol (Fig. 9) causes keratinization, but 4-androstene-3β,17β-diol and 4-androstene-3α,17β-diol induce mucification (Figs. 8, 13). Compounds with a hydroxyl group in the 3α-position (Figs. 12, 13) stimulate the superficial cells of the vagina preferentially with the production of mucus, whereas the deeper layer tends to respond preferentially to compounds with a hydroxyl in the 3β-orientation (Fig. 10). However, when a double bond at C_4 (favoring mucous cells) is associated with a 3β-hydroxyl group (favoring growth of deep cells) the determinant appears to be the double bond, since mucification results from the injection of 4-androstene-3β,17β-diol (Fig. 8).

In seeking an explanation for the interesting difference in the physiological actions *of* Δ^4 and Δ^5 steroids, certain chemical differences should be noted. Δ^4-3-Hydroxysteroids are allylic alcohols, and as such they are much more sensitive to dehydration than the corresponding Δ^5 compounds (28). Moreover, the allylic type alcohols can be oxidized readily to the corresponding ketones by reagents such as manganese dioxide which have no effect on Δ^5-3-hydroxysteroids (29).

5-Androstene-3β,17β-diol is unique among the compounds tested in that it alone shares the property of the phenolic estrogens in causing keratin formation in significant amounts by the deeper layer of vaginal epithelium. The present experiments show that this unusual physiologic property of the compound is dependent on at least 2 features of the molecule, the β-hydroxyl at C_{17} together with the double bond at C_5. The capacity of the compound to induce keratinization is lost either by the oxidation of its hydroxyl group at C_{17} to a ketone (Fig. 11), or by the reduction of its double bond to form androstane-3β,17β-diol (Fig. 10), and in each case stratification results. These effects emphasize the high significance of hydrogen atoms for the promotion of growth by C_{19} steroids.

CONCLUSIONS

In the hypophysectomized albino rat which is protected from contact with steroids in the ration and environment the uterus and vagina are highly atrophic but are sensitive indicators of activity of substances which promote their growth. Both the pituitary growth hormone and certain steroids have the common property of inducing growth of these tissues.

The vaginal epithelium consists of 2 layers of cells which differ profoundly in their growth in response to steroids, depending on the molecular struc-

ture of these compounds. The differential response to modifications of chemical structures of steroids permits evaluation of the importance of the intramolecular components for the process of growth.

The number and site of functional groups, the geometry of the molecule and the state of oxidation are of high importance in determining physiologic activity of steroids in the androstane series; these features are less specific in the estrane series.

Side groups at positions C_3 and C_{17} are of importance in the promotion of growth by steroids in the androstane series, but these active centers are not equivalent in their physiological influence. As a generalization, hydrogenation of the oxygen function at C_{17} (but not at C_3) and dehydrogenation at critical areas of the ring structure increase the quantitative efficacy of steroids in promoting growth. The position of double bonds and the state of oxidation at both C_3 and C_{17} determine the qualitative *type* of growth—cellular pattern, which a compound in the androstane series induces in the vaginal epithelium.

BIBLIOGRAPHY

1. Korenchevsky, V., and Dennison, M., *J. Path. and Bact.*, 1936, **43,** 345.
2. Doisy, E. A., Veler, C. D., and Thayer, S., *Am. J. Physiol.*, 1929, **90,** 329.
3. Tschopp, E., *Arch. internat. pharmacod.*, 1936, **52,** 381.
4. Deanesley, R., and Parkes, A. S., *Brit. Med. J.*, 1936, **1,** 257.
5. Wade, N. J., and Doisy, E. A., *Proc. Exp. Biol. and Med.*, 1931, **28,** 714.
6. Zarrow, M. X., Lazo-Wasem, E. A., and Shoger, R. L., *Science*, 1953, **118,** 650.
7. Cheng, E., Story, C. D., Yoder, L., Hale, W. H., and Burroughs, W., *Science*, 1953, **118,** 164.
8. Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R., *J. Immunol.*, 1946, **52,** 267.
9. Butenandt, A., and Kudszus, H., *Z. physiol. Chem.*, 1935, **237,** 75.
10. Clarke, E., and Selye, H., *Am. J. Med. Sc.*, 1942, **204,** 401.
11. Nathanson, I. T., Franseen, C. C., and Sweeney, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39,** 385.
12. Mühlbock, O., and van Maurik, G., *Acta Physiol. et Pharmacol. neerl.*, 1951, **2,** 80.
13. Hill, M., and Parkes, A. S., *Proc. Roy. Soc. London, Series B*, 1941, **113,** 541.
14. Müller, C., *Endokrinologie*, 1938, **20,** 8.
15. Gomori, G., Microscopic Histochemistry. Principles and Practice, Chicago, The University of Chicago Press, 1952, 73.
16. Butenandt, A., and Schmidt-Thomé, J., *Ber. chem. Ges.*, 1936, **69,** 198.
17. Butenandt, A., and Heusner, A., v. Dresler, D., and Meinerts, U., *Ber. chem. Ges.*, 1938, **71,** 182.
18. Smith, P. E., *Am. J. Physiol.*, 1932, **99,** 349.
19. Meyer, R. K., and Allen, W. M., *Science*, 1932, **75,** 111.
20. Dorfman, R. I., Gallagher, T. F., and Koch, F. C., *Endocrinology*, 1935, **19,** 33.
21. Deanesley, R., and Parkes, A. S., *Biochem. J.*, 1936, **30,** 291.
22. Lieberman, S., and Teich, S., *Pharmacol. Rev.*, 1953, **5,** 285.

23. Butenandt, A., and Tscherning, K., *Z. physiol. Chem.*, 1935, **234**, 224.
24. Ruzicka, L., Goldberg, M. W., and Meyer, J., *Helvet. chim. acta*, 1935, **18**, 994.
25. Talalay, P., and Dobson, M. M., *J. Biol. Chem.*, 1953, **205**, 823.
26. Talalay, P., and Marcus, P. I., *Nature*, 1954, **173**, 1189.
27. Talalay, P., personal communication.
28. Schoenheimer, R., and Evans, E. A., Jr., *J. Biol. Chem.*, 1936, **114**, 567.
29. Sondheimer, F., Amendolla, C., and Rosenkranz, G., *J. Am. Chem. Soc.*, 1953, **75**, 5930.

EXPLANATION OF PLATES

The photomicrographs are of paraffin sections of the vaginal mucosa stained with hematoxylin and eosin. The compounds had been injected from age 38 to 44 days inclusive, with necropsy at 45 days.

PLATE 28

FIG. 1. Vaginal mucosa from a hypophysectomized rat injected with sesame oil. Both the superficial and the deeper cells are highly atrophic. × 275.

FIG. 2. From an ovariectomized rat injected with sesame oil. Neither the superficial or the deep cells are as atrophic as in hypophysectomized rats (Fig. 1); the superficial layer consists of tall mucous cells. × 275.

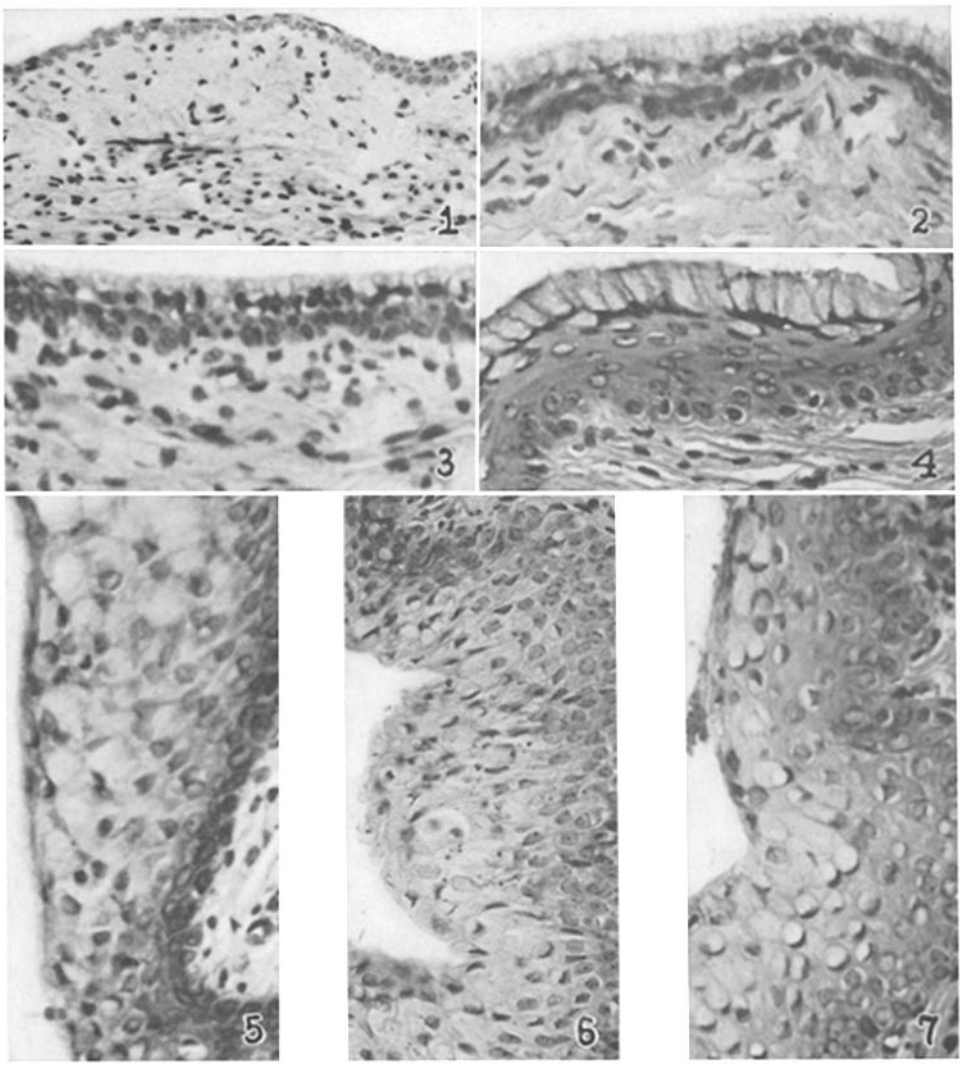
FIG. 3. Vaginal mucosa of a hypophysectomized rat, age 45 days. This rat had been injected with 0.2 mg. of pituitary growth hormone daily, and the vaginal epithelium resembles that of ovariectomized controls as shown in Fig. 2. × 275.

FIG. 4. From a hypophysectomized rat injected with estrone, 0.1 μg. daily. Growth of both the superficial mucous layer and the deep cells is demonstrated. × 275.

FIG. 5. From a hypophysectomized rat injected with testosterone, 1 mg., and estrone, 1 μg. daily. While considerable growth of the epithelial cells has occurred neither mucus nor keratin is produced. × 375.

FIG. 6. From a hypophysectomized rat injected with 1,4-androstadiene-3,17-dione, 3 mg. daily. Vaginal stratification resulting from this compound resembled that occurring after simultaneous administration of testosterone and estrone as in Fig. 5. × 275.

FIG. 7. From a hypophysectomized rat injected with androstan-3β-ol-17-one, 3 mg. daily. Stratification of the vaginal epithelium is demonstrated. × 375.



(Huggins *et al.*: Steroids and growth in hypophysectomized rat)

PLATE 29

The effects of relatively small changes in the chemical structure on the patterns of the vaginal epithelium of hypophysectomized rats.

FIG. 8. Preferential stimulation of the mucous cells occurred following administration of 4-androstene-3β,17β-diol, 1 mg. daily. $\times$ 270.

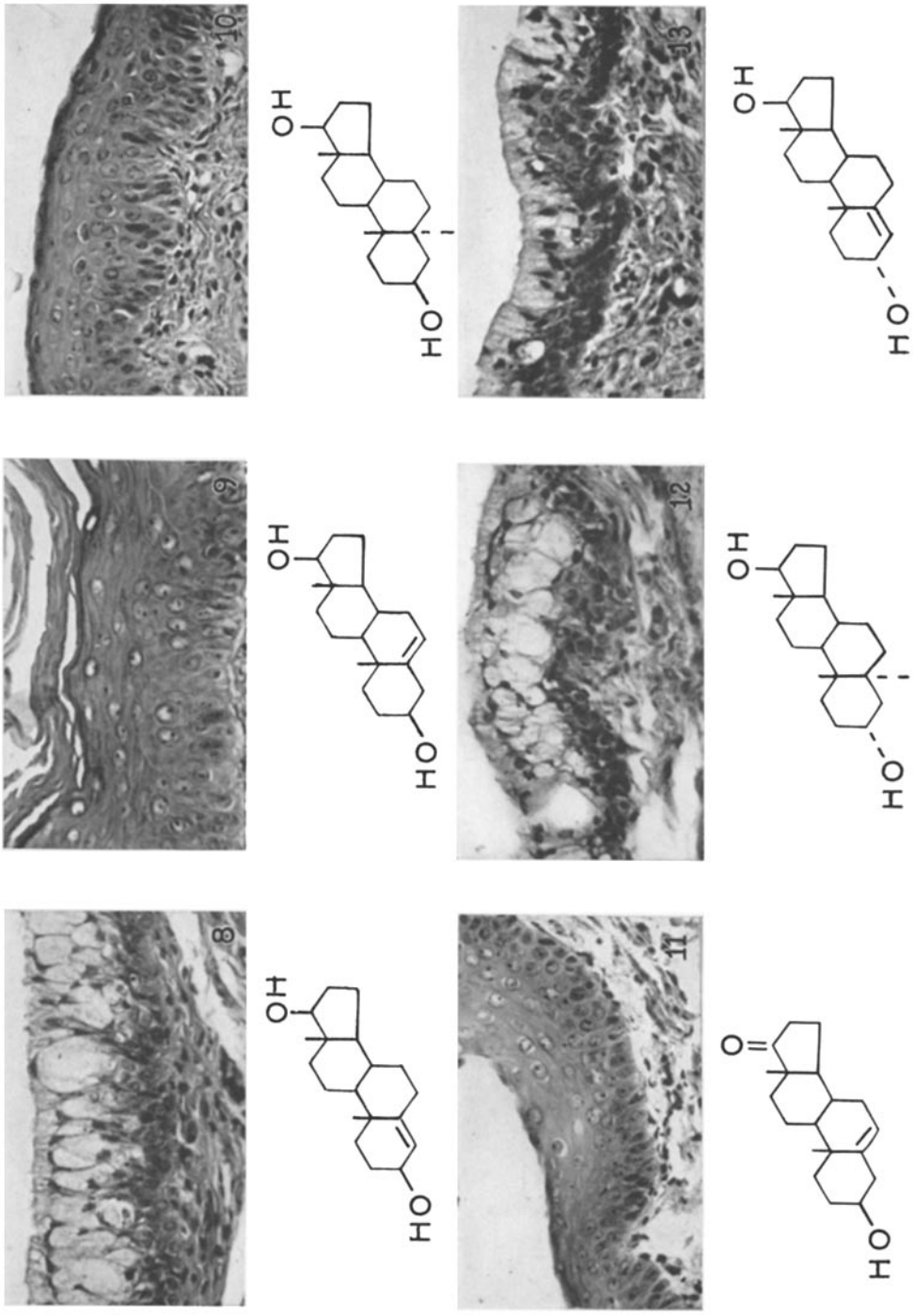
FIG. 9. Keratinization of the vaginal epithelium resulted from the injection of 5-androstene-3β,17β-diol, 1 mg. daily. $\times$ 270.

FIG. 10. Stratification of the vaginal epithelium was induced by androstane-3β, 17β-diol, 1 mg. daily. $\times$ 270.

FIG. 11. Stratification of the vaginal epithelium induced by 5-androsten-3β-ol-17-one, 3 mg. daily. $\times$ 270.

FIG. 12. Mucification of the vaginal epithelium following the injection of androstane-3α,17β-diol, 2 mg. daily. $\times$ 270.

FIG. 13. Mucification of the vaginal epithelium was induced by 4-androstene-3α,17β-diol, 1 mg. daily. $\times$ 270.



(Huggins *et al.*: Steroids and growth in hypophysectomized rat)