

HORMONAL INFLUENCES ON MAMMARY TUMORS
OF THE RAT*

II. RETARDATION OF GROWTH OF A TRANSPLANTED FIBROADENOMA IN
INTACT FEMALE RATS BY STEROIDS IN THE ANDROSTANE SERIES†

BY CHARLES HUGGINS, M.D., AND KLAUS MAINZER,§ M.D.

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

PLATE 42

(Received for publication, December 27, 1956)

It was found in the experiments to be described that many steroids in the androstane series profoundly retard the growth of a transplanted benign mammary tumor in adult intact female rats. This paper is concerned with the relationship of the molecular structure of these steroids to their inhibitory effects on the tumor and with mechanisms involved in the depression of neoplastic growth by these agents. A quantitative relationship was found to exist between the dosage of the steroid administered and the extent of the restraint of the tumor which ensued. In the variations of its growth rate in response to hormonal modifications the benign tumor of the rat resembles some human mammary carcinomas, and in this regard it is a serviceable laboratory model that is unique at the present time. The growth of the transplanted tumor is accelerated or retarded by hormones which already have been found through clinical practice to have like effects on carcinoma of the breast of the human being. The availability of quantitative methods of study of a hormone-responsive experimental tumors expands the scope of investigation of mammary neoplasms.

The mammary fibroadenoma employed in the experiments has two outstanding characteristics. It is a neoplasm. Secondly, the tumor possesses some of the responsiveness to hormones which is characteristic of normal mammary epithelium of the rat in so far as its growth rate is profoundly altered by the administration or withdrawal of appropriate hormones. With respect to the promotion of growth by steroid and protein hormones, it was shown earlier (1) that there are many similarities in the reactivity of both the transplanted

* This study was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the American Cancer Society, Inc., the Illinois Division of the American Cancer Society, and the United States Public Health Service.

† We are indebted to Eustus Lauagan, Gloria Torralba, Yolanda Torralba, Solomon Wiseman, and Joseph Yavit for assistance.

§ Dr. Mainzer is a Fellow of the American Cancer Society, Inc.

fibroadenoma and the normal mammary tree of its host to these agents but that striking differences of response also exist. It is evident that the neoplastic transformation of mammary epithelium has resulted in a loss of certain growth characteristics and in a gain of others; these matters will be considered further in this paper.

The benign mammary fibroadenoma studied in the present work grows to a considerable size within 50 days in normal adult female rats whereas its growth is slight after the ovaries have been removed; the retarding effect of ovariectomy upon the growth of a similar tumor has been described earlier (2). The growth of the tumor is enhanced profoundly in ovariectomized rats by the administration of phenolic estrogens in critical quantities, or progesterone (1). In hypophysectomized rats (1) the tumor grows indolently but after some weeks active growth commences despite the absence of growth-promoting hormones as determined by absence of growth in the atrophic tissues of the host—a sensitive biological test.

It has been learned in clinical practice (3) that testosterone can cause regression of the neoplasm in certain women with advanced mammary cancer and several related steroids, 17α -methyltestosterone and dihydrotestosterone, have been found to have a similar beneficial activity; the effectiveness of these compounds has been reviewed recently (4). The mechanisms involved in the restraint of human mammary cancer by androstane compounds are unclear. Segaloff *et al.* (5) observed that testosterone uniformly produced a decrease in the urinary excretion of gonad-stimulating hormones in the human; the consequent decrease of ovarian function would appear to be significant in some cases in the regression of the neoplasm. The decrease of gonadotrophin production does not account satisfactorily for the well established regression of mammary cancer induced by testosterone in many ovariectomized or postmenopausal women.

Heiman and Krehbiel (2) found that mammary fibroadenoma grew slowly in the intact male rat and that the rate of tumor growth increased slightly after orchietomy. Testosterone is the only compound in the androstane series which has been studied for its growth inhibitory effects on this tumor. Heiman (6) found that the administration of this steroid to male and female rats bearing grafts of mammary fibroadenoma reduced the number of "takes" and retarded the subsequent growth of the tumor. In the experiments of Millar and Noble (7) testosterone caused slight or no inhibition of growth of the transplanted rat mammary fibroadenoma which they employed. The inhibitory effects of testosterone and certain related compounds to be reported in this paper are of much greater magnitude than has been reported in experiments of other workers.

In the present experiments, the effects of hormonal modifications on the growth of transplanted mammary fibroadenoma were correlated in each case with growth in the mammary glands of its host. The determination of the alkaline phosphatase content of one of the breasts is a simple and valid technique for measuring growth quantitatively in this structure; the technique is

advantageous since the activity of the enzyme can be correlated by histochemical methods with its site in the contralateral mammary gland. Folley and Greenbaum (8) have found that the alkaline phosphatase content of the mammary gland increases rapidly and greatly during pregnancy in the rat. It has been shown (1) that the removal of the ovaries from adult rats results in a considerable decrease in the content of this enzyme in the mammary gland and, conversely, that the administration of increasing amounts of phenolic estrogens to these ovariectomized rats causes a progressive increase in the alkaline phosphatase content of the breast until a high plateau of the quantity of this enzyme is reached.

Methods

In brief, many fragments of similar size of benign mammary fibroadenoma were transplanted in groups consisting of many rats of the same age reared under similar laboratory conditions and the tumors were harvested 50 days later; the experimental variable was the altered steroid status of the host. The mammary fibroadenoma used in the experiments had arisen in our laboratory as previously described (1) and had been maintained by successive transplantations over a period of 2 years.

Albino rats of the Sprague-Dawley strain were obtained from the dealer at age 42 days and kept thereafter under controlled climatic conditions. The rats were fed a commercial ration except that hypophysectomized rats were maintained on a steroid-free high protein diet (9).

The experiments were carried out on 16 series of female rats. Each series contained 102 to 106 rats which were divided in 11 groups of 9 to 16 animals. In order to establish control parameters of tumor growth two of the groups were not injected with steroids; these consisted, respectively, of normal and castrate females. In addition, in many series the tumor was transplanted to intact and castrate males and hypophysectomized female rats. Characteristic variations in the growth rate of the tumor in untreated intact male and female rats as contrasted with its growth in animals from which the gonads or the hypophysis had been removed, always at age 42 to 44 days, were regarded as functional tests of the growth capacity and hormonal dependence of the tumor. The surgical operations and the transplantation of tumors were performed under ether anesthesia. Nine groups each consisting of 9 intact female rats were injected with steroids in every series.

The tumor was transplanted to the groups of recipients when they were 51 days old. The donor in each case was an intact adult female bearing a large tumor. The tumor was excised, sliced, and oval fragments were cut from the slices with a surgical punch. The implants measured about 8 x 5 x 5 mm. and weighed about 50 to 70 mg. Every tenth fragment was weighed and the standard deviation of these weights was calculated. Either four, or preferably eight, pieces of tumor were implanted subcutaneously in widely separated places through small incisions in the interscapular region and in the epigastrium.

The steroids¹ were dissolved in ethyl alcohol which was diluted with sesame oil to make the final alcoholic concentration 10 per cent. The solution (0.2 ml.) was injected subcutaneously 6 days each week for 7 weeks. *Throughout this paper dosage refers to the amount administered each day.*

¹ We acknowledge with gratitude generous gifts of compounds used in these experiments from Dr. H. J. Ringold, Syntex, S. A., Mexico City, Dr. Victor Drill, G. D. Searle & Co., Chicago; Dr. Edward Henderson, Schering Corporation, Bloomfield, New Jersey; Dr. Karl Junkmann, Schering, A. G., Berlin, Germany; and Dr. E. V. Jensen, The Ben May Laboratory for Cancer Research, Chicago.

At necropsy, always 50 to 52 days after transplantation, the tumors, preputial glands, prostates, ovaries, and uteri were weighed on a precision balance. Histologic preparations of tumors from each group were always studied. The site of alkaline phosphatase was determined in histological preparations of the tumor, the mammary glands and the vagina by the method of Gomori (10).

The content of alkaline phosphatase was determined in a mammary gland in the inguinal region: adipose tissue containing mammary tubules was excised from a triangle bounded by the inguinal ligament, the deep epigastric vein and the midline. This tissue was spread on filter paper and, for convenience, immersed in a large volume of ice-cold acetone for 2 to 9 hours. After evaporation of acetone in an evacuated desiccator for 15 hours each gland was homogenized in 5 ml. of water, centrifuged and the supernatant solution was filtered. The alkaline

TABLE I

Growth of Transplants of Two Mammary Fibroadenomas of Varying Degrees of Hormonal Dependence in Intact, Castrate, and Hypophysectomized Rats

There were 9 rats in each category; necropsy was performed, at age 101 days, 50 days after transplantation of the different tumors. The rats were untreated.

Category	Mean tumor weight
	<i>mg.</i>
(a) Tumor with high hormonal dependence	
Intact females.....	1464
Castrate females.....	163
Castrate males.....	102
Intact males.....	73
Hypophysectomized females.....	46
(b) Tumor with less hormonal dependence	
Intact females.....	2702
Castrate females.....	1731
Castrate males.....	476
Intact males.....	367
Hypophysectomized females.....	100

phosphatase content was determined by the method of King and Armstrong (11) and the results are expressed in the units of these workers. In preliminary experiments it was found that immersion of the mammary glands in chilled acetone for the brief periods used in this study did not result in a loss of content of alkaline phosphatase.

RESULTS

Histologic study of the tumor transplants did not reveal a change to carcinoma or sarcoma in any case in the present experiments and metastases were never observed at necropsy.

The transplanted mammary fibroadenoma was considered to possess high hormonal dependence when, in the absence of treatment with steroids, the transplants grew vigorously in intact females and much more slowly in ovariectomized and hypophysectomized sisters and in males (Table I *a*). At the end of

the experimental period the mean weight of tumors of this kind was 68 to 190 mg. in females deprived of ovaries, whereas the values in intact females weighed between 443 and 2217 mg. Quantitative values of this sort were obtained in nearly all of the experiments and from these considerations it was apparent that the growth rate of the tumor and its state of hormonal dependence had not changed during the experimental period.

But in 3 experiments to be designated the tumor was less hormone-dependent than the foregoing as the functional tests brought out (Table I *b*). The differences were quantitative. These tumors grew to a considerable size in ovariectomized rats and in males during the experimental period although their size was smaller than companion transplants growing in intact females.

In each of 5 experiments the tumors grew to larger size in castrate males than in the intact males (Table I). The differences, although not large, were consistently present.

Androstane Inhibitors of Tumor Growth

In Table II, data from 3 series of experiments are presented. The values show that many compounds in the androstane series retarded the growth of the fibroadenoma in adult female rats although with varying degrees of potency. The monofunctional steroid androstan-3-one did not retard growth but androstan-17 β -ol was a moderately effective inhibitor. The effects of androstane derivatives on tumor growth in intact rats were always related to the growth in untreated rats deprived of the ovaries in the same series. Steroids were considered to be powerful growth inhibitors of the mammary fibroadenoma when the size of the tumor in treated intact rats was equal to or less than the size in ovariectomized rats. Dihydrotestosterone and 2 α -methyl-androstan-17 β -ol-3-one fell in this class.

4-Androstene-3,17-dione, 1 mg., was considered to be a weak inhibitor of tumor growth because the restraint of growth (Table II), while confirmed in repeated experiments, was less than the inhibition resulting from ovariectomy. Furthermore, the growth-retarding effects of this steroid and of other inhibitors were intensified by an increase of dosage (Text-Fig. 1) of the compounds.

The inhibitory steroids were observed to produce restraint of growth in a direct proportional relationship (Text-Figs. 1 and 2) to the amount of the compound administered until maximal retardation of tumor growth was achieved.

Many compounds in the androstane series exceeded 4-androstene-3,17-dione in their potency as inhibitors of mammary fibroadenoma (Table II, Fig. 1). These included in ascending order of effectiveness (Table II) androsterone, testosterone, and dihydrotestosterone. No modification of the rate of tumor growth was observed from the administration of etiocholan-17 β -ol-3-one, 1 to 5 mg. or *epitestosterone*, 1-2 mg.

Experiments were carried out in 5 series of rats from which the ovaries had

been removed; in each study 1 group received dihydrotestosterone, 1 mg., while a similar lot was not treated with steroids. In each of these experiments the tumors grew at a slower rate in the ovariectomized rats receiving dihydrotestosterone than in their uninjected ovariectomized mates (Table III).

TABLE II

The Effect of Removal of the Ovaries Compared with Administration of Steroids to Intact Adult Female Rats in Retardation of Growth of Mammary Fibroadenoma

There were 9 rats in each group and in each experiment there were groups of intact and ovariectomized females which were not injected. The steroids were injected, 1 mg. daily, for 50 days; Δ is the gain in body weight of these rats during this period. Alkaline phosphatase indicates the content of this enzyme in the inguinal mammary gland. The mean weights of the tumors, ovaries, preputial glands, and the ventral prostate are given.

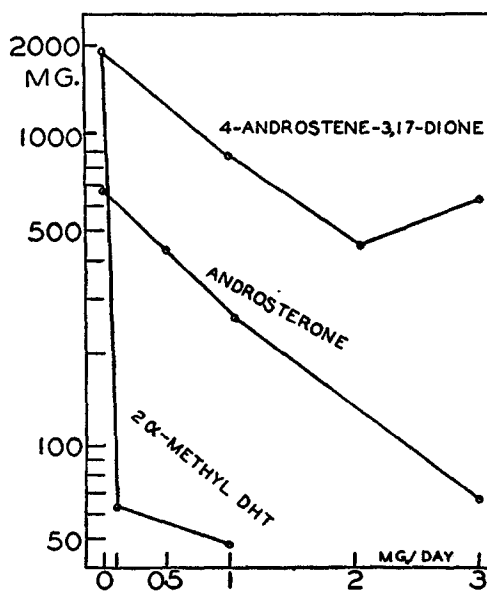
Compound	Δ	Tumor weight			Ovaries	Preputial glands	Ventral prostate	Alkaline phosphatase
		Uninjected		Injected				
		Intact	Ovariectomy	Intact				
	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>units*</i>
Androstan-17-one	88	917	190	1731	104	129	0	1.30 \pm 0.4
4-Androstene-3,17-dione	130	1920	120	873	35	241	116	1.59 \pm 0.3
Androstan-17 β -ol \ddagger	132	917	190	473	57	147	27	3.35 \pm 0.5
Androstan-3 α -ol-17-one (androsterone)	123	677	88	258	92	254	94	1.23 \pm 0.3
4-Androsten-17 β -ol-3-one (testosterone)	130	1117	68	114	33	262	103	2.10 \pm 0.3
Androstan-17 β -ol-3-one(dihydrotestosterone)	127	917	190	76	35	450	271	3.10 \pm 0.4
2 α -Methylandrostan-17 β -ol-3-one	153	1920	120	48	37	266	134	3.90 \pm 0.3

* King and Armstrong units with standard deviation.

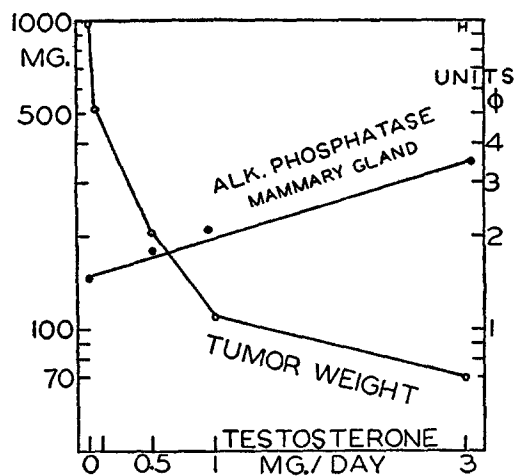
\ddagger Daily dosage, 2 mg.

Supplementary Oxygen Functions.—The addition of oxygen substituents, ketone or hydroxyl, at positions 6, 15 α , or 16 α , abolished the tumor inhibitory effects of testosterone and dihydrotestosterone.

Supplementary Methyl Groups.—With respect to the inhibition of growth of the mammary tumor, the addition of 1 or more methyl groups to potent inhibitory compounds in the androstane series considerably modified the effectiveness of the parent compounds, enhancing it in some steroids and destroying it in others. The effect on growth of the addition of methyl groups at positions 2, 3, 4, 16, or 17 to compounds in the androstane series was examined.



TEXT-FIG. 1. The weight of transplanted mammary fibroadenoma in rats treated with 3 members of the androstane series. Ordinates, mean weights of the tumors plotted logarithmically. Abscissae, daily dosage of compound in milligrams.



TEXT-FIG. 2. Depression of the weight of mammary fibroadenoma and increase of alkaline phosphatase in the inguinal mammary gland induced by increasing doses of testosterone. Ordinates on the left indicate the weight of the tumor and on the right the content of alkaline phosphatase in the mammary gland expressed in King and Armstrong units; these data are plotted logarithmically. Abscissae, daily dosage of compound in milligrams.

2 α -Methyldihydrotestosterone and 2 α ,17 α -dimethyldihydrotestosterone were found to be powerful inhibitors (Table III) of the growth of the mammary tumor. The presence of two methyl groups at C₂ destroyed the tumor-restraining effects of steroids; 2,2'-dimethyl-, and 2,2',17 α -trimethyldihydrotestosterone exerted no significant inhibitory activity on the neoplasm (Table III).

A methyl group situated at C₃ eliminated the tumor-inhibitory activity of steroids; 3 α -methylandrosterone-3 β ,17 β -diol, 1 mg., and 3 β -methylandrosterone-3 α ,17 β -diol, 1 mg., were ineffective in this regard. 4,4'-Dimethyldihydrotestosterone, 1 mg., 16 α -methylandrosterone, 1 mg., and 16-methylenandrosterone,

TABLE III

Effect of Additional Methyl Substituents in Androstane Compounds on the Retardation of Growth in Adult Intact Rats

The steroids, 0.1 mg. daily, were injected for 50 days with 9 rats in each group. The mean weights of the tumor, the ovaries, and the preputial glands are given. Alkaline phosphatase indicates the content of this enzyme in the inguinal mammary gland.

Compound and daily dose	Tumor weight	Ovaries	Preputial glands	Alkaline phosphatase
	mg.	mg.	mg.	units*
2,2'-17 α -Trimethyldihydrotestosterone	8867	82	103	1.48 \pm 0.1
Intact: no injections	2217	87	121	1.43 \pm 0.4
2,2'-Dimethyldihydrotestosterone	896	85	124	1.48 \pm 0.3
Dihydrotestosterone	486	68	168	1.83 \pm 0.4
2 α ,17 α -Dimethyldihydrotestosterone	271	70	148	2.84 \pm 0.4
2 α -Methyldihydrotestosterone	263	68	144	2.53 \pm 0.9
Ovariectomized: no injections	186	—	105	1.15 \pm 0.2
Ovariectomized: Dihydrotestosterone \dagger	101	—	276	3.55 \pm 0.3

* King and Armstrong units with standard deviation.

\dagger The daily dosage of dihydrotestosterone was 1 mg.

1 mg., possessed no inhibitory effects. 17 α -Methyltestosterone, 1 mg., was a potent inhibitor of growth of the tumor.

3-Hydroxysteroids.—The steric position of an hydroxyl group at C₃ in compounds in the androstane series exerted a remarkable influence on the growth of the neoplasm. Steroids with an hydroxyl group in the 3 β configuration were weak inhibitors or actually accelerated the growth of mammary fibroadenoma whereas related compounds with a 3 α -hydroxyl group retarded growth of the tumor to great extent. An acceleration of growth of the tumor resulted from the administration of 5-androstene-3 β ,17 β -diol, dehydro ϵ piandrosterone or ϵ piandrosterone (Table IV); 4-androstene-3 β ,17 β -diol was a weak inhibitor of tumor growth. The injection of androsterone considerably retarded the growth of the tumor and 4-androstene-3 α ,17 β -diol was a powerful inhibitor.

Dihydrotestosterone Restrained the Stimulation of Growth of Benign Mammary Fibroadenoma in the Presence of Estradiol-17 β or Progesterone

Earlier (1) it had been shown that progesterone, 4 mg., or estradiol-17 β , 1 μ g., enhances very considerably the growth of transplanted fibroadenoma in ovariectomized rats. In the present experiments the same effects were observed when these steroids were administered to intact rats (Table V). The profound stimulation of tumor growth induced by these steroids, administered separately (Table V) or together (Fig. 1) was completely abolished by the additional administration of dihydrotestosterone, 1 mg.

TABLE IV

Differential Effects of 3 α - and 3 β -Hydroxysteroids on Growth of Mammary Fibroadenoma in Adult Intact Female Rats

The steroids were injected for 50 days with 9 rats in each group. The mean weight of the tumor, ovaries, and preputial glands is given. Alkaline phosphatase indicates the content of this enzyme in the inguinal mammary gland.

Compound and daily dose	Tumor weight	Ovaries	Preputial glands	Alkaline phosphatase
	mg.	mg.	mg.	units*
5-Androstene-3 β ,17 β -diol, 2 mg.....	1600	35	307	2.33 \pm 0.4
Androstan-3 β -ol-17-one, 3 mg. (<i>epi</i> androsterone).....	1177	87	321	1.35 \pm 0.2
5-Androsten-3 β -ol-17-one, 3 mg. (Dehydro <i>epi</i> androsterone).....	915	54	293	2.24 \pm 0.4
Intact: no injections.....	520	92	125	1.46 \pm 0.4
4-Androstene-3 β ,17 β -diol, 0.5 mg.....	366	75	207	1.63 \pm 0.3
Androstan-3 α -ol-17-one, 1 mg. (androsterone)..	133	80	222	1.11 \pm 0.2
4-Androstene-3 α ,17 β -diol, 0.5 mg.....	92	31	309	3.09 \pm 0.3
Ovariectomized: no injections.....	86	—	109	0.97 \pm 0.2

* King and Armstrong units with standard deviation.

Dihydrotestosterone induced marked growth of the prostate, preputial glands, and the mammary glands with a pronounced increase of alkaline phosphatase in the latter. The simultaneous administration of estradiol-17 β , 1 μ g., and dihydrotestosterone did not modify significantly these growth processes or the content of alkaline phosphatase in the mammary gland (Table V).

Dihydrotestosterone Failed to Retard the Growth of Mammary Fibroadenoma of Low Hormonal Dependence

In this experiment, repeated on 3 occasions, the transplanted mammary fibroadenoma was more refractory to experimental hormonal changes than any other tumor utilized in the present study. The lessened responsiveness was

inferred from (a) equal growth in ovariectomized and intact female hosts and (b) uncommonly brisk growth in hypophysectomized rats (Table VI). From histologic examination no differences were evident between the refractory tumor and the ordinary sort. Dihydrotestosterone, 1 mg., failed to retard the growth

TABLE V

Inhibitory Effect of Dihydrotestosterone on the Growth of Mammary Fibroadenoma When Administered with Estradiol-17 β or Progesterone

The steroids were administered for 50 days with 9 intact female rats in each group. The mean weights of the tumor and the preputial glands are given. Alkaline phosphatase indicates the content of this enzyme in the inguinal mammary gland.

Steroids, with daily dosage	Tumor weight	Preputial glands	Alkaline phosphatase
	mg.	mg.	units*
Progesterone, 4 mg.	3647	123	3.23 \pm 1.09
Estradiol-17 β , 1 μ g.	1986	116	1.96 \pm 0.47
Intact females; no injections	443	106	1.56 \pm 0.58
Ovariectomized; no injections	91	119	1.27 \pm 0.11
Dihydrotestosterone, 1 mg.	60	264	3.54 \pm 0.25
Dihydrotestosterone, 1 mg. and estradiol-17 β , 1 μ g.	58	288	3.75 \pm 0.23
Dihydrotestosterone, 1 mg. and progesterone, 4 mg.	55	399	3.17 \pm 0.27

* King and Armstrong units with standard deviation.

TABLE VI

Failure of Dihydrotestosterone to Block the Growth of Mammary Fibroadenoma in Hypophysectomized Rats

Six fragments of tumor were transplanted in each rat, with 9 female rats in each class. One group of hypophysectomized rats was injected with dihydrotestosterone; the other groups were untreated.

Class	Surviving transplants	Mean tumor weight
	per cent	mg.
Intact	59	1127
Ovariectomized	75	1193
Hypophysectomized: uninjected	88	370
Hypophysectomized + dihydrotestosterone, 1 mg.	93	691

of this tumor in hypophysectomized rats (Table VI). This steroid caused extensive growth of the mammary glands in all of the hypophysectomized hosts of the tumor.

Effects of Tumor-Inhibitory Steroids on Growth of Normal Tissues

All of the steroids in the androstane series which inhibited the growth of the mammary fibroadenoma possessed common properties of increasing the size of

certain normal organs whilst decreasing that of others. Prominent amongst the augmented growth effects is a gain of body weight which exceeded that occurring in uninjected mates. Moreover, the steroids induced selective enlargement of preputial glands, the vestigial prostatic glands (when present), and the breasts. The ovaries were often but not invariably reduced in size.

During the experiment, 130 intact rats implanted with tumor but uninjected with steroids gained 76 to 114 gm.; the mean increase in their body weight was 95.1 ± 9 gm. In all groups of rats injected with potent tumor-inhibitory steroids in the androstane series (Table II) the increases of body weight exceeded these values. The greatest weight gain occurred in rats injected with 2α -methyl-dihydrotestosterone in which the mean increase in body weight was 153 gm.

At the end of the experiments the weight of the preputial glands of 50 uninjected tumor-bearing intact female rats was 109 ± 22 mg. In these untreated animals the vestigial prostate was visible in the gross in 18 rats; the mean weight was 5.1 ± 0.6 mg. In rats injected with each of the powerful tumor-inhibitory steroids the mean values of the weight of the preputial and prostatic glands were considerably increased (Table II) above the control values.

Growth of the mammary glands was induced by all of the compounds in the androstane series which restrained the growth of the mammary tumor. These compounds produced enlargement and redness of the nipples. The enlarged mammary glands were thick, orange in color, and of firmer consistency than the adipose tissue which encased them. On microscopic examination it was found that the large size consisted of an increase both in number and in size of the mammary tubules. There was also an augmentation in the secretion in the tubules, manifested by an increase in neutral fat and alkaline phosphatase as demonstrated by histochemical means. When growth of the mammary gland was induced by steroids the content of alkaline phosphatase in the mammary gland was considerably elevated above that of the breasts of uninjected rats (Tables II to V).

The growth of the mammary glands was not confined to rats in which the members of the androstane series were inhibitors of the tumor. Related steroids which promoted the growth of the tumor induced growth of the mammary gland equally as pronounced as that effected by the inhibitors; 5-androstene- $3\beta,17\beta$ -diol and dehydroepiandrosterone enhanced the growth of the tumor, induced the growth of large red nipples, and caused marked enlargement of the mammary glands with the formation of lobules of alveoli, lactation, and an increase of alkaline phosphatase.

Most of the tumor-inhibitory steroids induced a profound decrease in the size of the ovaries (Table II) and abolished the formation of keratin in the vagina. But the administration of androsterone, 1 to 3 mg. daily, whilst inducing progressive retardation of the growth of the tumor, did not result in a decrease in ovarian weight (Table II) and in many of these rats keratinization of the vaginal epithelium was present.

DISCUSSION

For steroids in the androstane series the molecular structure required for the *inhibition* of the hormonal dependent mammary fibroadenomas was identical with the requirements for the *promotion* of growth by these compounds of certain hormonal targets, notably the mammary glands. There was never restraint of tumor growth by any of the compounds without accompanying growth of some normal organs. Earlier, Selye *et al.* (12) discovered that testosterone causes proliferation of the mammary gland of the rat. A bizarre finding in the present experiments was the profound inhibition of growth of mammary tumor transplants by steroids which were producing vigorous growth of the breasts of the host. The prostate and the giant sebaceous preputial glands were also stimulated to grow by the tumor inhibitors. Furthermore, the various tumor inhibitors in the androstane series differed only in potency; qualitative differences were not observed between them *vis-à-vis* their effects on either tumor or host.

The structural factors in the androstane molecule which are of high significance in the retardation of growth of benign mammary fibroadenoma (and the promotion of growth of the mammary glands as well) are the number, the position, and the state of oxidation both of functional groups and alkyl groups.² These requirements are reminiscent of the steroid structure which is effective in promoting selective growth of the vagina, uterus (13), and prostate (14) of the female hypophysectomized rat.

The functional groups which endow these molecules with a tumor-inhibitory capacity are hydroxyls and ketones. These are effective when located at positions 3 and 17. The optimal number of these groups is 2. Additional oxygen functions, ketone or hydroxyl at positions 6, 15 α , or 16 α , destroyed the tumor-inhibitory effect of testosterone and dihydrotestosterone. Ketone groups at positions C₃ and C₁₇ are less effective than hydroxyls, in proper orientation, at these sites. The monofunctional steroid androstan-17 β -ol was an inhibitor of tumor growth, although less effective than dihydrotestosterone; androstan-3-one did not influence tumor growth.

For retardation of tumor growth a special steric orientation of the hydroxyl groups which are present is preferential or obligatory. An obligatory steric orientation for an hydroxyl group at C₁₇ is *beta*; whereas testosterone was a potent inhibitor, *epitestosterone* did not influence the growth of the tumor. With reference to position 3, the α -orientation of a hydroxyl group is highly preferential for tumor inhibition. Both ketone groups and, much more effectively, α -oriented hydroxyl groups contributed to the inhibition of tumor growth whereas most of the steroids with a 3 β -hydroxyl accelerated growth of the

² In unpublished experiments on the effect of 4-estrene compounds on growth of mammary fibroadenoma, we have found that 17 α -ethinyl-19-*nor*-testosterone, 0.5 mg., accelerated the growth of the tumor whereas 17 α -ethyl-19-*nor*-testosterone was a powerful inhibitor of its growth.

tumor. In this wise androsterone restrained growth of the tumor whilst *epi*-androsterone accelerated its growth. The site of unsaturation in the molecule exerted an important influence on its effect on tumor growth. The Δ^4 steroid, 4-androstene- 3β , 17β -diol retarded the growth of the tumor to a slight extent whilst its Δ^5 analogue, 5-androstene- 3β , 17β -diol enhanced its growth.

The presence of one or more methyl groups at special sites of the androstane molecule exerted an effect on the restraint of tumor growth. Among the compounds tested, the most powerful steroid for the restraint of mammary fibroadenoma was 2α -methyl dihydrotestosterone. The introduction of a 17α -methyl group in this compound or in testosterone did not weaken the tumor-inhibitory activity of the parent compound. The presence of two methyl groups at position 2 (or at position 4) destroyed the tumor-inhibitory activity of dihydrotestosterone. Steroids with a 3α - or 3β -methyl group likewise were inactive on growth processes.

Evidence was derived from the experiments that 2 biological mechanisms are operative in the restraint of mammary fibroadenoma by compounds in the androstane series in intact female rats. These are (a) the depression of hormone production by the ovary and (b) the blockade of activity of growth-promoting steroids at the level of the tumor cell.

The hormonal status of the normal adult female rat is very favorable for the growth of the tumor and the ovary contributes much to this conducive state since ovariectomy considerably retards the growth of the transplants. Testosterone causes atrophy of the ovary as McEuen *et al.* (15) showed; this steroid-induced gonadal atrophy is due to a selective inhibition of pituitary function since it does not occur when gonadotrophin is administered concurrently with testosterone (16). The depression of ovarian function by some steroids in the androstane series was seen often in the present experiments and, no doubt, it is a factor in the restraint of tumor growth in intact rats but its importance is not primary because of considerations which follow.

Although many of the androstane derivatives which retarded the growth of mammary fibroadenoma induced a profound decrease in ovarian size and abolished keratin formation in the vagina, these effects were not essential for the retardation of tumor growth. It was found that testosterone, 0.1 mg. and androsterone, 1 to 3 mg. brought about an extensive restraint of tumor growth while the size of the ovaries was not less than that of uninjected controls; moreover, in these rats the formation of keratin in the vagina was typical of estrus. It is certain that in these animals, inhibition of tumor growth had occurred in rats in which estrogenic function of the ovary was preserved despite the administration of the steroids in the dosage specified.

Moreover, it was discovered in the experiments that the administration of dihydrotestosterone to ovariectomized rats induced greater restraint of mammary tumor growth than was achieved by the simple removal of the ovaries.

It would appear from this observation that there are extra-ovarian factors in the rat, as in man, which stimulate growth of mammary tumors and that these are suppressed by the inhibitory androstane steroids. It has been shown that the adrenal glands (17) and the pituitary (18) secrete hormones that maintain human mammary cancer, since regression of the neoplasm follows the removal of these endocrine glands in certain cases.

The massive stimulation of growth of mammary fibroadenoma which attends the administration of certain steroids was overcome by the concurrent injection of dihydrotestosterone. In earlier work (1) it had been shown that critical doses of phenolic estrogens and large doses of progesterone stimulated the growth of the mammary tumor in ovariectomized rats. In the present experiments it was observed that the growth of this tumor in intact adult female rats was much accelerated by the administration of estradiol-17 β , 1 μ g. and progesterone, 4 mg. when these compounds were injected singly or together. The stimulation of tumor growth by these steroids was nullified by the administration of dihydrotestosterone, 1 mg. simultaneously, whereas the growth of the mammary gland was enhanced. The most reasonable explanation of these remarkable effects of the androstane compounds is differential physiologic activity at the cell surfaces of the tumor and the mammary epithelium respectively. Certainly in the benign mammary tumor dihydrotestosterone, while itself incapable of stimulating growth of the tumor cells, can prevent the growth-stimulating effects of estradiol-17 β and progesterone; in the normal mammary gland it promotes the growth of the epithelial cells regardless of the presence or absence of the powerful growth-promoting compounds of other steroid categories.

It is noteworthy that dihydrotestosterone was unable to prevent the growth of an unusually malignant mammary fibroadenoma in hypophysectomized rats.

SUMMARY

Many members of the androstane series profoundly retarded the growth of a transplanted benign mammary fibroadenoma of the rat; the restraint of tumor growth was in direct proportion to the amount of the administered compound until its maximal effect was achieved. Certain steroids closely related to the androstane inhibitors accelerated the growth of the tumor. These effects of divergent sort depend on the molecular structure of the steroid.

The molecular structure of androstane derivatives, which is of high significance in modifying the rate of growth of the benign mammary tumor, consists of multiple components. These include (a) the presence and number of ketone and hydroxyl groups in special orientation at specific sites, (b) the sites of dehydrogenation in the molecule, and (c) the presence, number, and state of

hydrogenation of alkyl groups at designated molecular positions. These multiple factors determine whether androstane compounds will inhibit growth of the tumor, enhance it, or fail to influence its growth.

The androstane compounds which caused either the restraint or the promotion of tumor growth had the common property of inducing proliferation of the normal mammary epithelium.

Two mechanisms are involved in the restraint of growth of mammary fibroadenoma by androstane inhibitors. The primary effect is the abolition of action of phenolic estrogens and progesterone when dihydrotestosterone is administered concurrently, presumably through direct action at the tumor cell level. A secondary contributory suppressive effect is the depression of ovarian activity, and consequently of the production of phenolic estrogens and progesterone, by these compounds.

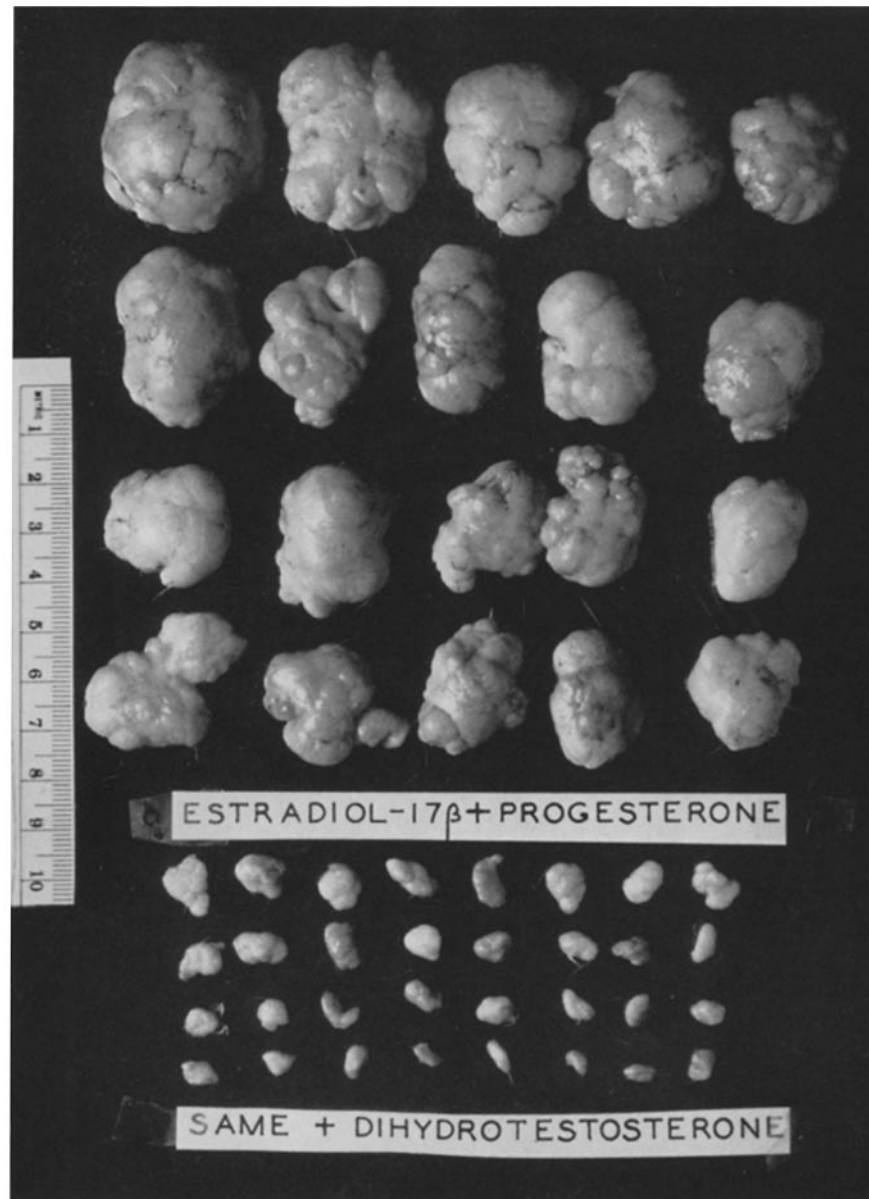
Transplanted mammary fibroadenoma in the rat possesses neoplastic traits and also some growth properties of normal mammary epithelium; inhibition of these latter by hormonal methods commonly retarded the growth of the tumor. But in hypophysectomized rats dihydrotestosterone failed to inhibit the growth of a mammary fibroadenoma with unusually low hormonal dependence as determined by functional tests of its growth.

BIBLIOGRAPHY

1. Huggins, C., Torralba, Y., and Mainzer, K., *J. Exp. Med.*, 1956, **104**, 525.
2. Heiman, J., and Krehbiel, O. F., *Am. J. Cancer*, 1936, **27**, 450.
3. Loeser, A. A., *Lancet*, 1941, **2**, 698.
4. Huggins, C., *J. Nat. Cancer Inst.*, 1954, **15**, 1.
5. Segaloff, A., Gordon, D., Horwitt, B. N., Schlosser, J. V., and Murison, P. J., *Cancer*, 1951, **4**, 319.
6. Heiman, J., *Am. J. Cancer*, 1940, **39**, 178.
7. Millar, M. J., and Noble, R. L., *Brit. J. Cancer*, 1954, **8**, 495.
8. Folley, S. J., and Greenbaum, A. L., *Biochem. J.*, 1947, **41**, 261.
9. Huggins, C., and Jensen, E. V., *J. Exp. Med.*, 1955, **102**, 335.
10. Gomori, G., *Microscopic Histochemistry, Principles and Practice*, Chicago, The University of Chicago Press, 1952, 184.
11. King, E. J., and Armstrong, A. R., *Canad. Med. Assn. J.*, 1934, **31**, 376.
12. Selye, H., McEuen, C. S., and Collip, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 201.
13. Huggins, C., Jensen, E. V., and Cleveland, A. S., *J. Exp. Med.*, 1954, **100**, 225.
14. Huggins, C., and Jensen, E. V., *J. Exp. Med.*, 1954, **100**, 241.
15. McEuen, C. S., Selye, H., and Collip, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 390.
16. Bottomley, A. C., and Folley, S. J., *J. Physiol.*, 1938, **94**, 26.
17. Huggins, C., and Bergenstal, D. M., *Cancer Research*, 1952, **12**, 134.
18. Luft, R., Olivecrona, H., and Sjögren, B., *Nord. Med.*, 1952, **47**, 351.

EXPLANATION OF PLATE 42

FIG. 1. Mammary fibroadenomas harvested from 2 groups of rats injected with estradiol-17 β , 1 μ g. and progesterone, 4 mg. (the upper tumors) and with the same compounds in identical dosage supplemented with dihydrotestosterone, 1 mg. (the lower tumors).



(Huggins and Mainzer: Hormonal influences on mammary tumors. II)