Steroid Excretion in Cancerous and Noncancerous Persons

II. Urinary Estrogens*

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In a previous paper (9) data were presented on the estrogenic titer of the "estrone," or weak phenolic fraction, and the "estriol," or strong phenolic fraction, of the urine from certain cancerous women before and after the injection of estrone and a combination of estrone and progesterone. In those experiments, the method of estrogen assay employed was a modification of the Astwood (1) technic. It seemed worthwhile to use a more conventional technic for the assay of estrogen. The patients previously studied were a selected group of women having carcinoma of the cervix and fundus. We were interested to see if these findings could be extended to cancerous persons generally, particularly because Ross and Dorfman (12) have since reported no unusual total urinary estrogen in the cases of 4 women with cancer of the breast.

Subjects and Methods

The urines from eight sets of subjects were collected in 48- to 72-hour lots, with toluene as the preservative. The cancer cases were patients at the Worcester City Hospital and consisted of men and women in early to middle stages of the disease. Urines from cachectic, moribund, and post-operative cases were not collected. All diagnoses were confirmed by either biopsy or autopsy. The age distribution of the patients and types of disease are given in Table I. The control groups consisted, except as noted in Table I, of noncancerous patients in roughly the same physical condition as the cancerous patients. No individual patient contributed more than 6 per cent of the total (except in the series II noncancerous males of Table I). As can be seen from Table I, miscellaneous cases contributed to the urine pools. This was desired in order to discover if any striking alteration of estrogen excretion characterizes cancerous persons generally. Patients with tumors of the adrenal cortex were not included.

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The method of hydrolysis and extraction employed has been previously described (10). The alkaline phenolic fractions obtained were neutralized, extracted with ether, and brought to dryness. The dried extracts from individual urines were pooled by redissolving in ether and the pooled sample was washed twice with a saturated solution of $NaHCO_3$ followed by two wash-

Table I: Sources of Urines Contributed to Urine Pools

Urine pool	No. liters	Types of contributors	Age range in years
Noncancerous ♀ I	124	¾ from nonhospitalized normal women; ¼ from cases of ovarian cyst, nonmalignant cervical erosion, neurosis	18 to 63
Noncancerous ♀ II	152	All from 10 advanced cases of pulmonary tuberculosis confined to bed	4 to 60
Cancerous ♀ I	208	Malignancies of uterus and cervix (36%), ear and antrum (21%), stomach and intestines (18%), breast (16%), lung (7%), spine (2%)	32 to 80
Cancerous ♀ II	121	Malignancies of cervix (62%), breast (17%), stomach and intestines (13%), and spine (8%)	32 to 79
Noncancerous ♂ I	210	All from nonhospitalized normal men	26 to 55
Noncancerous ♂ II	166	9/10 from nonhospitalized normal men; 1/10 from cases with arteriosclerotic heart disease and acute orchitis	16 to 60
Cancerous ♂ I	441	Malignancies of stomach and intestines (31%), bladder (31%), penis (18%), prostate (12%), lung (3%), throat (2%), brain (2%), pancreas (1%)	29 to 81
Cancerous ♂ II	191	Malignancies of mouth (30%), bladder (29%), bone (19%), prostate (14%), brain (6%), rectum (2%)	40 to 83

ings with distilled water. Ketonic material was removed by the use of the Girard reagent (7). The remaining nonketonic fraction was separated into weak phenolic and strong phenolic fractions by the method of Cohen and Marrian (3). Urinary estrone should be contained in the ketonic fraction since estrone is the only ketonic estrogen that has been isolated from human urines. Estradiol is the probable estrogen in the nonketonic weak phenolic fraction (10) and estriol in the nonketonic strong phenolic fraction (3).

All assays were conducted on spayed female rats. Our routine procedure involves injection of estrogen in 3 subcutaneous injections, 4 hours apart, once every 5 days. Only those animals showing positive vaginal smears at 48 hours after the first injection are employed for assay purposes at the succeeding injection period. Negative animals are primed by the injection of a total of 1.5 γ estrone in the 3 injections. Animals which show negative smears after two primings are discarded. By this method with our strain of animals 1 rat unit (50 per cent positive) is equal to 1 μgm. of estrone, 1 μgm. of estriol, and 0.12 μgm. of estradiol. In practice we employ a minimum of 16 animals for an assay and attempt to approximate the 50 per cent point. All materials are dissolved in olive oil. Standard curves for estrone, estriol, and estradiol in olive oil were constructed using recrystallized materials of constant melting point. In the second set of four types of subject, aliquots of each fraction were assayed; in the first set no assay was made of the total phenolic material but of every separation thereafter (Table II). Results are expressed in estrogenic activity per liter.

TABLE II: THE ESTROGENIC TITER OF VARIOUS PHENOLIC FRACTIONS OF POOLED URINE SAMPLES *

Type of subjects	Series	1. Total phenolic r.u. per liter	2. Ketonic phenolic (estrone) r.u. per liter	3. Nonketonic phenolic r.u. per liter	4. Nonketonic weak phenolic (estradiol) r.u. per liter	5. Nonketonic strong phenolic (estriol) r.u. per liter	6. Sum of assays of 2, 4, and 5 r.u. per liter
Noncancerous ♀	I	.....	1.13 ± 0.11	2.56 ± 0.31	2.98 ± 0.33	0.83 ± 0.10	4.94
Cancerous ♀	I	.....	0.19 ± 0.02	2.85 ± 0.23	0.75 ± 0.09	0.35 ± 0.04	1.29
Noncancerous ♀	II	2.35 ± 0.29	0.21 ± 0.02	1.15 ± 0.13	0.57 ± 0.05	0.68 ± 0.08	1.46
Cancerous ♀	II	2.06 ± 0.20	0.16 ± 0.01	1.30 ± 0.14	0.30 ± 0.03	0.28 ± 0.03	0.74
Noncancerous ♂	I	.....	0.39 ± 0.04	1.93 ± 0.23	0.17 ± 0.02	0.28 ± 0.03	0.84
Cancerous ♂	I	.....	0.33 ± 0.02	2.22 ± 0.26	0.74 ± 0.08	0.42 ± 0.05	1.49
Noncancerous ♂	II	2.16 ± 0.26	0.35 ± 0.04	1.26 ± 0.13	0.48 ± 0.04	0.16 ± 0.02	0.99
Cancerous ♂	II	1.71 ± 0.19	0.32 ± 0.04	2.18 ± 0.26	1.09 ± 0.11	0.28 ± 0.03	1.69

* The standard errors for each value given are calculated from the formula $S.E.=\pm\sqrt{\frac{p \cdot q}{n}}$ where p = the per cent positive smears, q the per cent negative smears, and n the number of animals.

RESULTS

The data on the various groups of subjects are summarized in Table II.

An inspection of these data clearly reveals the following differences: (a) the sum of the rat units of the three final fractions is higher in the noncancerous women than in the cancerous women; this is reversed in the case of the men's urines (Table II, column 6); (b) the strong phenolic nonketonic fractions show uniformly more activity in the noncancerous women's urines (Table II, column 5); (c) the weak phenolic nonketonic fractions of the cancerous men's urines are uniformly of higher titer than the same fractions from the urines of noncancerous men (Table II, column 4). Within the individual series other differences appear. Thus in series I the ketonic fraction of the urines of noncancerous women has about 6 times the activity of the same fraction from the urines of cancerous women (Table II, column 2). This large difference is markedly reduced in series II in which comparison is made between urines from tuberculous and cancerous women. Again, the titers of the nonketonic phenolic fractions of the women's urines do not differ significantly in the two series (Table II, column 3). But when these are separated into weak and strong phenolic fractions a higher titer is obtained for the extracts from noncancerous urines. Among the males' urines there is no significant difference between the titers of the ketonic fractions (Table II, column 2), the titers of the nonketonic fractions tend to be higher in the cancerous men (Table II, column 3), and further separation shows this difference even more strongly.

In Table III is a comparison between the titers of the total nonketonic material and the sum of the titers of weak phenolic and strong phenolic nonketonic fractions. These data indicate that the fractionation of the nonketonic extracts leads to an increase (Table III, series I) or no significant change (Table III, series II) in the estrogenic titers of noncancerous women, whereas the same extracts of cancerous women's urines show a decrease in titer. A similar decrease occurs on the fractionation of extracts of men's urines. This decrease appears to be restored on recombining the weak and strong phenolic fractions in their original proportions (Table III, noncancerous men, column 1). This implies that the men and the

noncancerous women excrete nonketonic materials which enhance the activity of certain urinary estrogens, and that these materials are fractionated by the Cohen and Marrian procedure; the noncancerous women, on the other hand, excrete no such substances, their urines containing, if anything, inhibitors of estrogenic activity. We have assayed various combinations of pure estradiol and estriol and find neither enhancement nor inhibition of activity.

TABLE III: A COMPARISON OF THE ESTROGENIC TITERS OF THE NONKETONIC EXTRACTS BEFORE AND AFTER SEPARATION INTO WEAK AND STRONG PHENOLIC FRACTIONS

Type of subjects	Series	1. Nonketonic phenolic r.u./liter	2. Nonketonic weak phenolic plus nonketonic strong phenolic r.u./liter	3. 2 as per cent of 1
Noncancerous ♀	I	2.56	3.81	149
Cancerous ♀	I	2.85	1.10	39
Noncancerous ♀	II	1.15	1.25	109
Cancerous ♀	II	1.30	0.58	45
Noncancerous ♂	I	1.93 (1.99)*	0.45	23
Cancerous ♂	I	2.22	1.16	48
Noncancerous ♂	II	1.26	0.64	51
Cancerous ♂	II	2.18	1.37	63

* The figure in parenthesis is the titer of the nonketonic fractions (weak and strong) upon recombination in the original proportions.

reverse occurs with the weak phenolic extracts. The 40:1 ratio reported for pure estriol is not obtained, being about 5:1 in one instance and 13:1 in another (Table IV, column 4). There is no evidence in these partitions for the further removal of enhancing material, in fact the data on the strong phenolic fractions suggest that inhibitory material is removed particularly from the extracts of the urines of cancerous men. The higher titer of the strong phenolic fraction of cancerous men previously noted thus becomes even more marked. The recoveries from the weak phenolic fractions are nearly quantitative, since there is no statistically significant difference between the titers of the unfractionated material and the sum of the titers of the two fractions.

DISCUSSION

The data of this paper are presented as indicative merely of possible modes of estrogen metabolism in cancerous persons generally. The differences in estrogen excretion noted may not be due to the presence of malignancies in the cancer subjects. We note first of all that most of our control groups contain on the average somewhat younger persons and a larger proportion of "healthy" individuals. Among the women

TABLE IV: THE PARTITIONING OF THE ESTROGENIC ACTIVITY OF CERTAIN NONKETONIC URINE FRACTIONS BETWEEN BENZENE AND 0.3 M Na_2CO_3

Type of subjects	Series	Fraction partitioned	1. Titer before partitioning r.u./liter	2. Na_2CO_3 fraction r.u./liter	3. Benzene fraction r.u./liter	4. Per cent of activity in Na_2CO_3 fraction 2 as per cent of 2 and 3
Noncancerous ♂	I	Strong phenolic	0.28	0.35	...	..
Cancerous ♂	I	Strong phenolic	0.42	0.60	0.12	83
Cancerous ♂	II	Strong phenolic	0.28	0.83	0.06	93
Cancerous ♂	I	Weak phenolic	0.74	0.20	0.74	21
Noncancerous ♀	I	Weak phenolic	2.98	0.21	2.45	8

This evidence for the presence of inhibitory and enhancing materials led us to attempt a further purification of certain of these urine fractions. We observed that the strong phenolic nonketonic extracts contained large amounts of tarry materials having a cresol-like odor. It has recently been reported (15) that estriol, the presumably native estrogen of these fractions, may be extracted from benzene solution by 0.3 M Na_2CO_3. By this method 40 parts of estriol are partitioned into the Na_2CO_3 solution to 1 part in the benzene. We have applied this partitioning to three strong phenolic and two weak phenolic nonketonic fractions. The result was a notable segregation of most of the tarry material into the benzene fraction. The data on estrogenic activity (Table II) accords roughly with expectation; *i.e.,* most of the activity of the strong phenolic extracts is found in the Na_2CO_3 fraction whereas the a somewhat higher proportion of postmenopausal ages is included in the cancerous group. This would imply that we must ascribe to differences in age and physical condition the three clear-cut differences noted; *i.e.,* decrease in the sums of the titers of the three principal fractions in cancerous women and an increase in cancerous men, higher "estriol" titers in noncancerous women's urines, and higher "estradiol" titers in cancerous men's urines. We can understand this in the case of the women wherein a decrease of estrogen excretion with illness and age is implied, but the opposite implication in the men is striking.

We should note, too, that the differences observed may be ascribable either to "normal" variations in estrogen excretion or variations in the assays themselves. The latter we may discount since our standard data have been repeatedly checked without finding sig-

nificant departures from expectation. The former we think is well covered by the quantities of starting material employed, the smallest urine pool (121 liters in series II, cancerous females) represents roughly 100 days' excretion of a single individual. The titers in series I and series II are, all things considered, in quite good accord. The exception is in series II where the various fractions of women's urines have lower titers than in series I, especially in the control group of tuberculous women. These latter were chosen as contributors purposely in order to obtain specimens from "very sick" persons. They were all confined to bed in very advanced stages of pulmonary tuberculosis. Even these women show the characteristic differences noted.

Finally, the peculiarities of these data, as those on all pooled urines, may be due to the inclusion in the urine pools of certain pathological individuals whose markedly abnormal estrogen excretion might change the general picture. The consistency in the outstanding differences from series to series would militate against this.

It is clear that if the observed peculiarities in estrogen excretion are ascribable to the cancerous condition of the subjects further investigation is required. Primarily, a checking of individual cases seems mandatory. The previous publication cited (9) represented such an attempt in a limited group of special cases. The chief difference between those data and the present study is an apparent lower titer in estrogenic activity in our pooled urines. We ascribe this difference in part to the difference in method of assay employed for we have found (unpublished data) that with urine extracts, particularly from cancerous persons, the Astwood method gives titers ⅓ to 3 times as high as the spayed rat test. In other words, the immature rat uterus is more sensitive to "enhancing" materials than the spayed rat vagina.

The absolute levels of estrogen output obtained conform roughly to those reported by previous investigators. In women with cancer of the breast, Ross and Dorfman (12) obtained values varying from less than 1.7 to 33.3 μgm. equivalent of estrone per day. Smith and Smith (13) have obtained somewhat higher values at most stages of the menstrual cycle in normal women, as have Dingemanse and Laqueur (5) in an individual woman's cycles. The latter find a ratio of ketonic to nonketonic estrogenic activity of 1:1 during the catamenia and of 1:2 during the intermenstrual period, whereas Smith and Smith find no estrone during bleeding and a ratio of about 1:2 during the intermenstrual period. In our own data the ratios for the noncancerous women's urines are: series I, 1:2.3; series II, 1:5.5; for the cancerous women these ratios are: series I, 1:15.0; and II, 1:8.1. In the men's urines we find the following ratios of activities in the ketonic and nonketonic fractions: noncancerous men, I, 1:4.9; II, 1:3.6; cancerous men, I, 1:6.7; II, 1:6.8. Dingemanse, Laqueur, and Mühlbock (6) report that ⅓ to ½ of the estrogenic activity of pooled normal male urine is in the ketonic fraction. They find an average of 7 μgm. of estrone equivalent per liter of total estrogenic activity. Later Dingemanse and Laqueur (4) report 2 to 27 μgm. equivalent per liter in men with testicular lesions other than chorionepithelioma.

The age grouping in both our men's and women's series included older persons than those reported by the various investigators cited and probably accounts for the somewhat lower values we obtained.

The definite indications that we obtained of the presence of estriol and estradiol in men's urines is, we believe, notable. Estrone is the only estrogen that has been isolated from the urine of normal men (6). The probable presence of estradiol is indicated by its isolation from testis tissue (2). But the possible origin of estriol is obscure since estriol has not been isolated from male tissue or organs. The concept of estriol as a product of estrone metabolism in the female (11, 13, 14) will require elaboration if estriol is derived from estrone in the male.

Finally, it is evident that a determination of "total" estrogenic activity is far from informative concerning estrogen metabolism. Thus our assays of the total phenolic material in series II give no statistically significant differences between the activity of extracts from the cancerous and noncancerous patients. Yet fractionation discloses several clear differences.

Summary

Pooled urines from 2 sets each of cancerous and noncancerous men and women were separated into three principal phenolic fractions containing presumably estrone, estradiol, and estriol. Bioassay of each fraction was performed, and it was found that (a) the sum of the estrogenic activity of the three fractions was higher in the noncancerous women's urines than in urine from cancerous women; (b) this was reversed in the case of the men's urines because of the higher titers of the nonketonic fractions; (c) the "estriol" fraction of noncancerous women's urines had a higher titer than the same fraction from cancerous women's urines; (d) the "estradiol" fraction of cancerous men's urines had a higher titer than the corresponding fraction of noncancerous men's urines.

A further separation of certain nonketonic fractions indicates that the difference in titers between cancerous and noncancerous men is real and due probably to the estrogens presumably segregated into these fractions.

A useful fractionation of estriol particularly is indicated.

The implications of these findings are discussed.

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Corrections

To the Proceedings of Scientific Sessions of the 34th Annual Meeting of the American Association for Cancer Research, Inc., Vol. 1, No. 9, pp. 729-753, add:

GLYCOGEN AND WALKER TUMOR 256. HOWARD A. BALL. (San Diego, Cal.)

Abstract not available.

To be published in *Cancer Research*.

THE ECTOPIC TESTIS AND TUMORIGENESIS. J. B. HAMILTON. (Yale University School of Medicine, New Haven, Conn.)

The relationship between ectopy of the testis and tumors of the testis was analyzed. Supplementary data regarding ectopy of the testis from experimental work with rodents was presented.

To the discussion of histological technic, p. 747, add:

DR. J. E. EDWARDS (Bethesda, Md.): In reference to Dr. MacCarty's criticisms and remarks about artifacts I wish to point out that all of these tissues were from animals autopsied within 5 to 10 minutes after death. The same sections were shown to Dr. Kenneth Mallory and Dr. Frederic Parker of the Mallory Institute, and they thought they were very good. Furthermore, knowing the excellent work that the Mallory Institute is famous for I was very much pleased to have them ask me if I would supply them with the technic we used.

At the request of the authors, the following correction of an error in the manuscript is published:

Page 804.—Insert Yeast Extract for Spleen Extract in the heading of Table IV in the article: Lewisohn, R., C. Leuchtenberger, R. Leuchtenberger, D. Laszlo, and K. Bloch. Action of Yeast Extract on Transplanted and Spontaneous Malignant Tumors in Mice. Cancer Research, **1**:799-806. 1941.