

# The Urinary Excretion of Estrogens, 17-Ketosteroids, Creatine, and Creatinine in High and Low Mammary Tumor Strains of Mice\*

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Since the development of strains of mice with variations in the incidence of spontaneous mammary tumors, attempts have been made to demonstrate anatomic or physiologic differences between them that might be etiologically related to tumor formation. We have searched for such a variation in the urinary excretion rates of the so-called sex hormones because of the many observations linking these agents with mammary tumor formation in susceptible strains of mice. In the present communication, comparative studies on the rates of excretion of 17-ketosteroids, estrogens, creatine, and creatinine in the urine of high (C3H) and low (C57) mammary tumor strains of mice are reported. Urinary creatinine determinations were done in order to check the accuracy of the urine collections and, since creatine excretion is influenced to some extent by the sex hormones (35), urinary creatinine-creatinine ratios were obtained.

Other attempts to detect metabolic variations between high and low mammary tumor strain animals are briefly reviewed.

Anatomical observations on high, intermediate, and low mammary tumor strains of mice have been almost completely limited to the mammary glands and the endocrine system. In strains developing mammary tumors, the mammary glands develop and involute in a patchy fashion during the estrous cycle, pregnancy, and after lactation. Persistent hyperplastic areas may go on to tumor formation (13, 20, 25). Injected estrogens cause considerable proliferation in the mammary gland of a high mammary tumor strain male or female mouse, but only a minimal and variable response in the low tumor strains (22). The ovaries of the high tumor strains may show more corpora lutea as com-

pared to the low tumor strains (26, 38). The X-zone of the adrenal cortex of a low tumor strain persists up to 100 days of age as compared to 200 days in a high tumor strain (12). A peculiar "brown degeneration" of the adrenal gland is said to occur chiefly in the high tumor strains (11), but this is not generally agreed (9). Ovariectomy in a high tumor strain female mouse at birth induces extensive adrenal hyperplasia and adrenocortical adenomas with evidence of estrogenic function at the age of about 8 months (14, 44), but this may also occur in low tumor strains (19).

Physiologic differences in hormonal sensitivity of high and low tumor strains have also been studied. Shimkin and Andervont (30) found that castrated C3H (high mammary tumor strain) mice required almost twice as much estrone as the C57 (low tumor strain) mice to produce vaginal estrus. They conclude, however, "that no correlation has been found between the manifestations of estrogenic stimulation, such as the duration of vaginal keratinization, number of estrous cycles and normality of the cycles, and the susceptibility of various strains of mice to mammary carcinoma."

Visscher and his group (42) demonstrated that high tumor strain mice (C3H) on a restricted caloric intake sufficient to prevent weight gain, did not develop any mammary tumors. Furthermore, low mammary tumor strain A mice given the same amount of food as the C3H mice gained weight. Tannenbaum (37) could prevent tumor formation in C3H mice by reducing the caloric intake at any age before the actual appearance of the tumor. It appears that some high mammary tumor strains require more food than the low tumor strains, and this increased caloric intake may possibly be related to tumor formation. In high tumor strain mice (C3H), even in the absence of mammary tumors, the red blood count and hemoglobin fall progressively with age (34). The xanthin oxidase (dehydrogenase content of the liver) is considerably lower (15) and susceptibility to the toxic effect of heptaldehyde is greater in the high tumor strains (33). The esterase

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activity of tumor susceptible mice (C3H and A) is significantly elevated in the serum and decreased in the feces as compared with a nonsusceptible strain (C57), whereas no difference appeared in the liver, kidney, and urinary esterase activity (10). The amount of fluorescent porphyrin in the lacrimal glands of mice was found to vary directly with their mammary susceptibility (16). No differences between strains have been found in the excretion of urinary protein (43), or in the ability of the livers to inactivate estrogens *in vitro* (40).

The discovery by Bittner (6-8) that the incidence of mammary tumors in the offspring of mice may be altered by the presence or absence of a factor in the mother's milk, and the subsequent studies of Anderfont (1, 2) and Twombly (39) have led to a further search for metabolic differences between animals of the same strain when the rate of tumor development is altered by regulating the milk factor. Thus far two strain differences have been tested after foster nursing. Shimkin and Anderfont (30) have shown that mice foster nursed by strains of opposing cancer susceptibility continue to show their strain differences in sensitivity to estrogens. The alveolar hyperplasia preceding mammary tumor formation was found by Van Gulik and Korteweg (41) to occur in the mammary glands of a low tumor strain litter foster nursed by a high tumor strain mother, and this hyperplasia was not present in the reverse situation.

#### MATERIALS AND METHODS

Twenty-four virgin C3H and C57 mice, 4 months old, were placed in metabolism cages that permitted the separation of urine and feces, in October, 1940, and 24 C3H mice, 6 months old and once-pregnant, were started in January, 1941. These groups were in the cages almost continuously until May, 1941, so that observations extended through a period of from 4 to 10 months. The metabolism cage, a modification of Gross and Connell's apparatus (21), consisted of a metal cage  $7\frac{1}{2} \times 6 \times 6$  inches, with a bottom composed of pyrex glass rods about  $\frac{1}{2}$  inch apart. The cage ordinarily housed 8 mice. A water bottle was fastened to the outside, and food was daily attached inside the cage in small bottles just large enough to admit the head of a mouse. The cage was placed over a glass funnel that connected to a side-arm device so that urine running down the side of the funnel would collect at its tip and run into the side-arm by means of capillary attraction, whereas the feces would fall straight down into a bottle at the bottom of the collecting tube. Urines were collected and measured daily, the apparatus was rinsed with water, and the urine and washings were pooled and stored in a refrigerator. Chemical determinations were made biweekly on each

group and calculations were based on the number of mouse days per specimen (that is, 14 day collection period  $\times$  24 mice in the group = 336 mouse days per specimen). Although this method of collection is very efficient for rats, several difficulties arise in mice that prevent a complete separation of urine and feces or a complete urine recovery. Mouse feces are fairly moist and will occasionally stick to the side of the funnel. A single mouse voiding is about 0.2 to 0.5 cc., and in running down the side of the funnel some of the urine may run into fecal matter that will absorb part of it and contaminate the rest. Small voidings may partially evaporate before they reach the collecting bottle. The presence of the proper number of mice in the cage (8 to 12) produces a greater urinary volume and therefore a more complete collection. The urine collected was clear, and rarely showed any gross fecal matter. It was estimated from creatinine determinations that about 5 per cent of the urine was recovered in apparatus washings, and total urine losses up to 30 per cent occurred. It seemed technically impossible to avoid these difficulties, so the hormonal data are also calculated per 10 mgm. creatinine in the urine. The errors in collection appear fairly constant between groups, and for comparative purposes these pitfalls do not detract from the essential observations. Incidentally, it may be noted that by offering a solution of 1 per cent NaCl and 5 per cent glucose as drinking water, urine volume could be increased up to 5 times. This technic was not used, however, since the possibility of glucose appearing in the urine would interfere with creatinine determinations.

Bills' diet (5), composed of yellow corn, 57 per cent; dried whole milk, 25 per cent; linseed oil meal, 12 per cent; crude casein, 3.7 per cent; alfalfa leaf meal, 1.5 per cent; table salt, 0.4 per cent; and calcium carbonate, 0.4 per cent, was fed daily. By adding one-half its weight of water, this diet was made into a paste that did not spill easily from the feed cups and the mice were able to gain weight on it.

Creatine and creatinine determinations were made by the technic of Folin (17) modified for the Evelyn photoelectric colorimeter. Estrogens and 17-ketosteroids were extracted from the urine after the technic of Smith and Smith (31). They were then separated from each other by the method of Gallagher and his associates (18). The 17-ketosteroids were measured colorimetrically by a modification of the Zimmerman reaction. Estrogens were measured by assay on castrated mice, and determinations were made on both an aliquot of the total benzene extract and on the NaOH-soluble fraction separated from the neutral fraction. These technics have previously been described in detail by Nathanson, Towne, and Aub (27). Estrogenic activity in mouse urine was checked

in 2 specimens by the use of Astwood's uterine weight technic (3). Zinc hydrolysis was carried out preliminary to extraction on 2 urine specimens by the method described by Smith and Smith (32).

Urines were collected over 24 hour periods on small groups of C3H and C57 males and females of various ages, and creatine-creatinine ratios determined on 87 of these specimens.

Table I summarizes the data obtained on the three groups. The average weight of the C57 mice rose slightly during the 4 to 10 month period, the virgin C3H mice remained unchanged, while the once-pregnant C3H mice showed a slight loss. It is therefore apparent that conditions within the cage did not permit entirely normal growth. The creatinine excretion in relation to body weight remained fairly con-

TABLE I: WEIGHT, URINE EXCRETION, CREATININE, 17-KETOSTEROID, AND ESTROGEN EXCRETION IN VIRGIN C57 AND C3H AND ONCE-PREGNANT C3H FEMALE MICE OVER THE AGE PERIOD OF 4 TO 10 MONTHS

Urinary creatinine excretion expressed as mgm. per mouse per month. 17-Ketosteroids expressed as mgm. per mouse per month, and as mgm. per 10 mgm. creatinine in the urine. Estrogens, expressed in international units, calculated per mouse per month and also per 10 mgm. creatinine in the urine.

Strain	Age, months	Number of mice per group	Average weight per mouse, gm.	Urine excretion per mouse per month, cc.	Creatinine excretion per mouse per month, mgm.	17-Ketosteroids.		Estrogens, international units	
						Excretion per mouse per month, mgm.	Excretion per 10 mgm. creatinine, mgm.	Excretion per mouse per month	Excretion per 10 mgm. creatinine
C57(V)	4	24	19.0	23.1	12.7	0.68	0.54	1.9	1.5
C3H(V)	4	24	21.6	46.2	18.2	0.86	0.47	1.8	1.0
C57(V)	5	24	20.2	17.7	16.4	0.79	0.48	1.5	0.9
C3H(V)	5	24	22.2	35.3	21.2	0.87	0.41	2.3	1.6
C57(V)	6	24	21.1	15.8	17.4	1.40	0.88	1.7	1.1
C3H(V)	6	24	23.5	24.6	18.8	1.93	1.05	1.2	0.7
C3H(P) †	6	24	26.2	29.0	21.4	0.46	0.22	2.3	1.1
C57(V)	7	22	21.1	10.0	16.4	1.13	0.65	2.2	1.4
C3H(V)	7	24	23.6	17.5	18.4	1.00	0.55	1.2	0.6
C3H(P)	7	24	25.1	21.4	21.4	1.23	0.63	1.4	0.7
C57(V)	8	21	21.5	18.5	15.1	1.10	0.72	2.1	1.4
C3H(V)	8	24	23.5	19.6	16.7	1.67	1.00	1.4	0.8
C3H(P)	8	23 <sup>1</sup>	25.6	20.3	16.4	0.88	0.53	1.2	0.8
C57(V)	9	19	20.4		9.3	0.75	0.94	2.6	3.3
C3H(V)	9	24	22.6		15.2	1.29	0.85	1.8	1.2
C3H(P)	9	19 <sup>2</sup>	23.9		12.7	0.82	0.65	2.3	1.8
C57(V)	10	13	22.0		9.2	0.67	0.73	3.3	3.6
C3H(V)	10	23 <sup>3</sup>	20.8		12.4	0.55	0.44	2.1	1.7
C3H(P)	10	18 <sup>2</sup>	23.1		8.1	0.70	0.87	2.7	3.3
C57(V)	Average for entire period	21	20.7	15.0	13.8	0.93	0.71	2.2	1.9
C3H(V)		24	22.5	30.4	17.3	1.17	0.68	1.7	1.1
C3H(P)		22	24.8	23.6	16.0	0.82	0.58	2.0	1.6

\* V = Virgin.

† P = Postpartum.

<sup>1</sup> 1 dead with a spontaneous mammary tumor.

<sup>2</sup> 4 dead with spontaneous mammary tumors.

<sup>3</sup> 1 dead with a spontaneous mammary tumor.

## RESULTS

Several general observations between the 2 strains were noted. Low tumor strain mice (C57) had a lesser food and water intake and urine excretion as compared with the C3H strain. During the winter, the urine excretion of all strains decreased (Table I). The C57 mice had a higher mortality rate: Nine out of 22 mice died between the ages of 9 and 11 months. Those available for autopsy had liver or kidney infections. The once-pregnant C3H mice developed only 4 of 22, and the virgin C3H mice 1 of 24 spontaneous mammary tumors at 11 months of age. This is lower than the incidence in our colony, but may be partially explained by their abnormal living conditions and a probable decrease in food intake (37).

stant from the fourth to eighth months of age, the C57 mice excreting an average of 15.6 mgm. the virgin C3H mice 18.7 mgm., and the once-pregnant C3H mice 19.7 mgm., per mouse per month. During the ninth and tenth months creatinine excretion was decreased in all groups. This may be due to less satisfactory urine collections resulting from a decreased urine volume due to the weight losses and death of some of the animals, although it is possible that the mice were excreting less creatinine. From the data obtained over the period of 4 to 8 months of age, however, it is calculated that the 20 gm. mouse excretes about 0.5 mgm. creatinine per day (25 mgm./kg.) and that no obvious strain difference exists.

Substances present in the neutral fraction of the ben-

zene extract of mouse urine react with *m*-dinitrobenzene in an alcoholic alkaline solution (Zimmerman reaction) to produce a color similar to, but not identical with, that obtained in corresponding fractions of human urine (23, 28, 36). Neutral extracts of human urine are known to contain ketonic and non-ketonic substances. The ketonic fraction includes the 17-ketosteroids. Although the non-ketonic fraction does not give the Zimmerman reaction, it contributes to the color when the crude extract is assayed. By treatment with Girard's reagent T, the ketonic substances may be separated. Thus assay of this ketonic fraction alone will eliminate partially, if not completely, the additional chromogen from the non-ketonic material present in the crude extract. It seemed possible that non-ketonic substances might explain the differences in reaction between mouse and human extracts. Accordingly, various mouse urine extracts were treated with Girard's reagent T. It was found that the crude mouse urine extracts contained both ketonic and non-ketonic substances, with ratios analogous to those obtained in human extracts. However, by employment of the Zimmerman reaction, it was not established that the ketonic fraction was identical with that of human urine, since the mouse urine extract did not give exactly the same color. This does not rule out the presence of 17-ketosteroids in mouse urine, but it is possible that other ketonic substances, non-steroid as well as other ketosteroids, may be present. These may be entirely responsible for, or may add to or mask, the typical color of the 17-ketosteroids. A similar situation exists to some extent in human urine. The ketonic material obtained in the mouse urine has not been identified further. However, because of the similarity in reaction, the material assayed in the mouse urine is referred to as 17-ketosteroid-like. The excretion levels of the 17-ketosteroid-like substances, measured in androsterone equivalents, varied from month to month in each group (Table I). In general, when calculated in terms of excretion per mouse per month, the levels for the C3H virgin females are somewhat higher than in the other two groups. However, when the excretion rate is calculated per 10 mgm. of creatinine in the urine, there is no appreciable difference in the groups. The highest levels are found between the sixth and ninth months. Assays of the material by the Holtorff-Koch technic gave values about 50 per cent lower than the modified Oesting method (23). This has been observed likewise in human urine. In the light of obvious difficulties in collection and analysis, one is forced to conclude that the differences in the various groups are not significant.

In contrast to the shortcomings in the assay and identification of the 17-ketosteroid-like substances, less difficulty was encountered in establishing the presence of estrogenic activity in the urine. The collection

time was sufficiently long to equalize the normal variations that occur in the estrous cycle. However, the inherent errors in most methods of bioassay and the difficulties in assaying accurately small quantities of estrogens are sufficiently great so that only fairly large variations in output should be considered significant. Injection of the extracts produced typical smears of cornified vaginal epithelium in castrated mice (Allen-Doisy technic), and considerable increase in uterine weight, up to 50 per cent, occurred in 21 day old female rats 6 hours after injection (Astwood technic). In most cases, both the crude benzene extracts and the NaOH-soluble (phenolic) fraction of these extracts containing the estrogens, were assayed on each urine specimen. The latter gave values about one-third lower, but the two types of extracts paralleled each other closely. The data reported in Table I represent the determinations on the crude benzene extract, since

TABLE II: EFFECT OF ZINC-HYDROCHLORIC ACID HYDROLYSIS ON ESTROGENIC ACTIVITY OF MOUSE URINE

Pooled specimens, untimed collection

Group No.	Strain	Hydrolysis	Estrogenic activity (international units)
102A	C57	HCl	28
102B	C57	Zn-HCl	24
111A	C57	HCl	32
111B	C57	Zn-HCl	26
103A	C3H	HCl	30
103B	C3H	Zn-HCl	22
112A	C3H	HCl	24
112B	C3H	Zn-HCl	26

it was desired to estimate the total estrogenic activity. Zinc hydrolysis did not perceptibly alter the estrogenic activity of mouse urine (Table II).

Data are calculated on the basis of the excretion rate per mouse per month and per 10 mgm. of creatinine in the urine. In the C3H virgin female there is no demonstrable evidence of alteration of estrogen excretion. The excretion levels in the C57 virgin females and in the C3H once-pregnant mice tend to rise with an increase in age.

There is a suggestion, from the data in Table I, and further confirmed by subsequent studies on mouse urine, that the low tumor C57 strain excrete slightly more estrogens than the high tumor C3H strain. The degree of difference is variable, but almost always present, as tested by both the vaginal smear and uterine weight technic. The C57 mice seem to excrete an average of 30 per cent more estrogen than the C3H mice. However, in view of the small quantity of excreted estrogens and the variations to be expected in the technic of bioassay this difference is not considered to be of significance. A previous pregnancy appears

to increase slightly the estrogen excretion in strain C3H.

Male and female C3H and C57 mice excrete large quantities of creatine and this appears to be related to their basic physiology, since sexual maturity in the male does not notably decrease the rate of creatine excretion as compared to the female. The creatinine-creatinine ratios were very variable within the same group, as evidenced by the large standard deviation; so only tentative conclusions can be drawn. The C3H or C57 females had similar creatine-creatinine ratios until they were 10 to 12 months old. Thereafter, creatine excretion increased in the C57 females and decreased in the C3H females. The C57 males appeared to excrete more creatine than the C3H males, and this difference remained throughout the whole period of observation (Table III).

excreted per kgm. of mouse per day, as compared to about 0.70 mgm. per kgm. of rat per day. According to Parfentjev and Perlzweig (29) adult male white mouse urine contains 0.1 per cent creatinine and 0.09 per cent creatine. Assuming that the mouse excretes 0.5 to 1.0 cc. urine per day, this would equal 0.5 to 1.0 mgm. creatinine per day, and the creatinine-creatinine ratio of 1:0.9 corresponds with our data.

Our observations indicate that the C57 female mouse excretes slightly but not significantly more estrogenic material than the C3H. Therefore these data on excretion rates of naturally occurring estrogens have produced no evidence that accounts for the difference in the incidence of mammary tumors in these mice.

Correlating these conclusions with other reports on the sex physiology of high and low tumor strains of mice must be done with caution. The steroid hor-

TABLE III: RATIO OF CREATININE : CREATINE EXCRETION AT VARIOUS AGES IN MALE AND FEMALE C57 AND C3H MICE

Age of mice, mos.	Ratio of creatinine : creatine with standard deviation per group			
	C57 Female	C3H Female	C57 Male	C3H Male
2-5	(5) *	(7)	(6)	(11)
	1:0.70 ± 0.21	1:0.80 ± 0.29	1:0.91 ± 0.34	1:0.65 ± 0.30
6-9	(10)	(9)	(8)	(4)
	1:0.74 ± 0.09	1:0.78 ± 0.08	1:0.75 ± 0.22	1:0.46 ± 0.13
10-12	(8)	(5)	(8)	(6)
	1:0.92 ± 0.33	1:0.53 ± 0.14	1:0.71 ± 0.27	1:0.51 ± 0.12
Average	(23)	(21)	(22)	(21)
	1:0.79 ± 0.23	1:0.71 ± 0.16	1:0.78 ± 0.27	1:0.57 ± 0.25

\* Figures in parentheses represent number of samples per group.

#### DISCUSSION

The presence of estrogenic and 17-ketosteroid-like substances in the urine of normal female mice is reported.<sup>1</sup> Similar observations have been recorded in rats. Four rat units (about 34 international units) per liter have been found in female rat urine, and this rose to 17 rat units per liter during pregnancy (4). Lampton and Miller (24) recovered 1.5 color units of 17-ketosteroid in the daily urine excretion of the male rat (equivalent to 0.14 mgm. androsterone) as measured by the Oesting colorimeter. These results in the rat are within the range of our findings of 30 to 150 international units of estrogen per liter of mouse urine, and 1.45 mgm. of 17-ketosteroid-like substances

extracted from the urine are the end result of a complicated metabolic cycle involving their formation, secretion, uptake by other organs, utilization, conversion into more or less biologically active substances, and, finally, destruction or excretion. The forms in which the steroids are excreted in the urine vary as to chemical structure and biologic activity. In our studies we measured the gross biologic activity of estrogens in the urine, and the quantity of a compound in the neutral fractions of benzene extracts of the urine that gave a color resembling the Zimmerman reaction in similar human urinary extracts. Only if the urinary excretion of these substances revealed great differences between the C57 and C3H mice could any inference be drawn as to the rate of formation or metabolism of these substances in each strain. Since the excretion of estrogens and 17-ketosteroid-like substances were not greatly different in the two strains, further attempts at finding significant strain differences in the urine will necessitate a qualitative and quantitative analysis of the different chemical forms of

<sup>1</sup> Since this paper was submitted for publication the presence of estrogenic material in the phenolic fraction of an extract of the urine of mice of the same order of magnitude as that published here has been reported (DORFMAN, R. I., and GARDNER, W. U. Metabolism of the Steroid Hormones. The Excretion of Estrogenic Material by Ovariectomized Mice Bearing Adrenal Tumors. *Endocrinology*, **34**:421-423. 1944).

these steroids, and the identification of the substances giving the Zimmerman reaction.

At present there is no conclusive evidence that the high mammary tumor strain mice form and metabolize estrogens differently from low tumor strains (40), and our observations are in keeping with others. It would seem fairly evident from the data available that the estrogens are essential for the growth of the mammary gland, and that they are carcinogenic only in that they bring the gland to a sufficient state of differentiation for it to evince its tumor susceptibility. Metabolic differences that have been described between strains have also failed to throw any direct light on the basis of tumor formation. That the mammary glands of low tumor mice do not respond as completely as those of high mammary tumor strains to estrogens (22), and that these differences in response are related to exposure to the milk-factor of Bittner (41) are the most significant points of difference yet encountered.

#### SUMMARY AND CONCLUSIONS

Metabolic differences between high and low tumor strains of mice and between litters of high and low tumor strain mice foster-nursed by the reciprocal mother are reviewed.

Comparative observations were made on C57 (low tumor strain) virgin and C3H (high mammary tumor strain) virgin and once-pregnant female mice. The C3H required more food than the C57 mice to maintain body weight. Both strains excreted approximately 0.5 mgm. creatinine per 20 gm. mouse per day.

The males and females of both strains excreted relatively large quantities of creatine in their urine. The creatine-creatinine ratio in the C57 and C3H females were similar until the tenth to twelfth months of age, when the creatine output rose in the C57 and fell in the C3H strain mice. The C57 male mice consistently excreted more creatine than the C3H males.

Each mouse excreted in its urine an average of 1.2 to 3.3 international units of estrogens a month. The C57 mice appeared to excrete on the average slightly more estrogenic material (30 per cent) than the C3H mice, but the difference is not considered significant. Both strains excreted 0.46 to 1.93 mgm. of a 17-ketosteroid-like substance per mouse per month with no appreciable strain differences.

Therefore, judged from urinary excretion rates, there is as yet no evidence that high mammary tumor strain mice form or metabolize estrogens or 17-ketosteroid-like material in any significantly different fashion from a low tumor strain. Estrogens are to be regarded as an essential but not a specifically carcinogenic factor in the development of spontaneous mammary tumors in mice.

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