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Anti-estrus Drugs on Subestrus of Ovariectomized C_3H Mice.* (26982)

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Gonadectomy in certain strains of mice leads, in time, to development of tumors of the adrenal cortex which secrete estrogen and/or androgen(1,2 and others). There is considerable evidence that these tumors develop as a result of the increased secretion of gonadotropin following castration. Nodular hyperplasia of the adrenal cortex of an intact mouse may be produced by parabiosis with a spayed partner(3). Estrogen, as well as certain other steroids(4), and hypophysectomy(5) will prevent their occurrence. Cortisone, in amounts sufficient to cause adrenal atrophy, does not prevent their development(6).

That formation of adrenal tumors and secretion of sex hormones from them may be dependent upon different mechanisms is shown by the findings that caloric restriction (7), thiourea administration(8) and unilateral adrenalectomy(9) prevent the appearance of subestrus but not the evolution of adrenal tumors. Yet secretion of estrogen by adrenal tumors may also be controlled by gonadotropin as evidenced by the observation that when an estrogen-secreting tumorous mouse is parabiosed with an intact mouse diestrus develops in the tumorous mouse, supposedly due to preferential utilization of gonadotropin by the ovaries of the intact mouse, but when a tumorous mouse is parabiosed with an ovariectomized mouse increased cornification in the vaginal smear of the tumorous mouse occurs due, supposedly, to the increased gonadotropin from the recently ovariectomized mouse acting on the adrenal tumor of its partner(10). On the other hand, administration of gonadotropin does not induce subestrus in tumorous mice on caloric restriction(11).

Inasmuch as reserpine, chlorpromazine, meprobamate(12), nidroxyzone(13), perphenazine and Enheptin† (2-amino-5-nitrothiazole) have been found to inhibit estrus in intact mice, a study of their effects on subestrus of ovariectomized mice was deemed worthwhile.

Methods and materials. C_3H female mice of the author's colony, originally obtained from Dr. J. J. Bittner, were ovariectomized and maintained on Purina Laboratory Chow. Drugs were administered in the diet after grinding with mortar and pestle and mixing with ground food. Control and drug-diets were fed *ad libitum*, except when otherwise noted. Vaginal smears were obtained by the lavage method. Mice were housed, usually 4 to 5 in a cage, in an air-conditioned, darkened-window room with automatic light cycles of 14 hours a day.

Mice were divided into 2 groups. Group I consisted of 60 animals ovariectomized between 8½ and 9½ weeks of age and placed on various drug-diets after subestrus was well established. Group II was composed of 88 mice ovariectomized between 9 and 14 weeks of age and put on drug-diets one week after ovariectomy.

A mouse was considered to be in subestrus

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† Unpublished data by the author.

TABLE I. Effect of Drugs on Subestrus Smears

Treatment	% Conc. in diet	No. mice	1st control period[a] Time[c]	No. days	% estrus[d]	Treatm. per. No. days	% estrus	2nd cont. per[b] No. days	% estrus	Toxicity No. died	% △ in body wt.
Reserpine	.0005	9	21	21	75.1	43	86.5	19	86.9	1[e]	+2.4
"	.00075[f]	7	26–30	14	85.7	28	70.4	27	50.8	0	−2.5
Chlorpromazine	.05[g]	6	24	17	85.7	39	69.2	27	34.6	0	+0.4
Perphenazine	.01–.005[h]	5	38	19	84.4	34	74.7	16	50.0	0[h]	−8.9
Meprobamate	2.0[i]	8	17–20	21	69.0	42	87.1	14	85.7	1[j]	−8.0
Nidroxyzone	.3	8	20	21	62.5	18–31[k]	61.6	—	—	8	−21.5
Enheptin	.4	8	20	21	60.1	62	58.5[l]	23	40.2	0	−15.4

[a] Immediately prior to treatment.
[b] " after "
[c] Weeks after ovariectomy.
[d] % estrus means % of total mouse days in which subestrus was noted.
[e] Died at 11 days. In addition, 2 died at 25 days due to lack of water.
[f] These mice had previously been on 2.0% meprobamate.
[g] These mice were on .1% chlorpromazine for 6 days 3 weeks previously. This concentration was toxic and 2 mice died.
[h] On .01% for 5 days, then on .005% for 29 days. One mouse died at 5 days, which is not included. These mice had previously been on reserpine (.0005%).
[i] 4.0% meprobamate to 8 other mice for 2 days proved toxic.
[j] Died at 24 days.
[k] All mice died, 18–31 days.
[l] During the last 42 days 5 mice had 68.6% estrus and 3 mice had only 18.3%.

when cornified cells were present in the smear. Leucocytes and nucleated epithelial cells were also always present, the latter appearing as single cells or clumped together. A diestrus smear consisted only of leucocytes plus or minus some epithelial cells.

Vaginal smears, taken periodically for 5 consecutive days during the first several weeks after gonadectomy in Group I, showed that subestrus had developed in an occasional mouse by 6 weeks and in most mice by 15 weeks post-ovariectomy. A general tendency toward cycling was noted but this was of a very irregular nature. Daily smearing (6 days a week) starting 17 to 23 weeks after ovariectomy was continued throughout the lifetime of mice in Group I.

Results. 1. *Anti-estrus drugs on established subestrus.* Group I mice were placed on drug therapy at 20 to 27 weeks after castration, preceded by 3 weeks of daily smearing while on control diet. Pertinent data are shown in Table I. It is apparent that none of the drugs used had any appreciable effect on estrus activity.

2. *Anti-estrus drugs on development of subestrus.* Group II mice were divided into 6 groups, one on ground control diet and 5 on drug-diets. Therapy, started one week after ovariectomy, was continued for 38 weeks, at which time surviving mice were autopsied. The data obtained from vaginal smears taken at various times are tabulated in Table II. The drugs used had no significant effect on vaginal smears at these times. The time of first subestrus noted varied from 26 to 117 days, with an average of 63 days for the entire group of mice, with no appreciable differences between the 6 sub-groups: control range, 39 to 74 days, avg. 61.5; reserpine, 41 to 74 days, avg. 63.8; chlorpromazine, 54 to 70 days, avg. 64.9; meprobamate, 42 to 95 days, avg. 63.0; nidroxyzone, 26 to 74 days, avg. 58.2, plus one at 117 days and one never showing subestrus; Enheptin, 33 to 74, avg. 60.5.

Body weights recorded weekly are summarized in Table III. During the first several weeks there were considerable variations in weights of treated mice due to changes in drug concentrations which were required in an effort to administer as high a concentration of drug as possible without excessively interfering with body growth. During the

TABLE II. Effect of Drugs on Development and Progression of Subestrus.[1]

Time after ovariectomy and effects	Control ground food	Reserpine .00025%	Chlorpro-mazine .05%	Mepro-bamate 2.0%	Nidroxy-zone .15%	Enheptin .2%
5 wk: No. estrus mice	2/15	1/13	0/15	2/15	3/15	3/15
No. mouse days	75	65	75	75	75	75
% estrus	2.7	1.5	0	2.7	5.3	5.3
9 " No. estrus mice	15/15	12/12	15/15	14/15	12/14	15/15
No. mouse days	75	60	75	75	70	75
% estrus	60.0	66.6	56.0	74.7	70.0	67.3
13 " No. estrus mice	12/15	11/12	9/13	15/15	9/13	14/15
No. mouse days	180	144	135	171	156	180
% estrus	27.8	31.9	34.8	49.7	32.7	46.7
20 " No. estrus mice	10/15	10/12	9/11	7/14	10/13	13/15
No. mouse days	75	60	55	70	65	75
% estrus	28.0	36.6	32.7	38.6	49.2	52.0
27 " No. estrus mice	11/12	9/12	9/11	8/11	10/11	12/14
No. mouse days	300	300	263	275	232	350
% estrus	38.0	13.7	19.0	15.3	18.9	22.9
36 " No. estrus mice	9/12	7/12	3/8	7/10	3/5	7/11
No. mouse days	156	156	104	127	67	143
% estrus	21.2	15.4	19.2	31.5	29.9	23.1

[1] Vaginal smears were taken for 5 days at 5, 9 and 20 weeks, for 12 days at 13 weeks, for 25 days at 27 weeks and for 13 days at 36 weeks. No. estrus mice refers to the no. of mice showing subestrus at any time during the smear period, the numerator indicating number of mice showing subestrus and the denominator total number of mice surviving in the group. % estrus means % of total mouse days in which subestrus was noted.

first 10 weeks all treated groups weighed less than the controls, the chlorpromazine and Enheptin mice only slightly less, but the reserpine, meprobamate and nidroxyzone groups markedly so. In spite of this decrease in body weight of treated mice during the period of development of subestrus, no significant change in either onset or degree of estrus activity was noted.

3. *Waning of subestrus.* The data in Table II show that there was a tendency for subestrus to decrease after a period of time. Frequency in the different groups was 56 to 75% at 9 weeks, 28 to 50% at 13 weeks, 15 to 38% at 27 weeks, and 15 to 31% at 36 weeks. A similar decrease in subestrus of ovariectomized mice with time has been noted by Dr. Carlos Martinez‡ and is also apparent from the data in Table I.

4. *Effect of PMS in diestrus mice.* A group of 12 mice from Group I that had been in continuous diestrus for 7 to 80 days (avg. 28) while on control diet was given 2 I. U. of PMS subcutaneously per mouse without effect. A dose of 20 I. U. of PMS given 7 days later induced subestrus in 3 of the 12. These mice had been ovariectomized for 230 to 298 days.

5. *Effect of restricted food intake.* A group of 9 mice from Group I in frequent subestrus, also ovariectomized 230 to 298

TABLE III. Body Weights of Mice in Table II.

Group	Avg. body weights in g				
	Begin	10 wk	Range during 10-20 wk	20-30 wk	30-38 wk
Control	19.7	25.7	24.2-26.0	25.7-26.6	25.8-26.7
Reserpine	20.1	23.7	23.7-25.3	24.9-25.8	23.9-25.0
Chlorpromazine	20.6	24.9	24.2-26.4	24.9-26.6	23.8-25.2
Meprobamate	19.9	23.0	22.5-25.4	23.9-25.0	24.0-25.0
Nidroxyzone	20.1	22.7	21.0-23.2	22.5-24.3	21.9-23.4
Enheptin	20.0	24.6	23.1-24.6	23.0-24.4	22.0-24.1

‡ Personal communication of unpublished data.

days previously, was given 2 g of control food per day per mouse for 35 to 72 days. The frequency of subestrus for the preceding 25 days of *ad libitum* control food (225 mouse days) was 53.3%, and for the entire restricted period (530 mouse days) was 48.7%. During the first 35 days of restricted intake frequency was 64.1% (9 mice), but during the last 20 days was only 21.0% (5 mice). Three mice died between 35 and 45 days and one was lost at 47 days. Of the 3 that died subestrus was present the day before death in 2, and 3 days before death in the third. Average body weight lost was 36.6% at 35 days and 40.8% at 72 days. Thus, restriction of over-all food intake had little effect on subestrus.

6. *Autopsy findings.* A total of 114 mice were autopsied. Three did not show gross evidence of adrenal tumors but had shown a high frequency of subestrus smears. In the remaining 111 mice white patches were grossly apparent in the adrenals. Microscopic examination of several adrenal glands by Dr. Carlos Martinez showed them to contain adenomas.

Discussion. In intact mice estrus cycles have been found to be decreased in ZBC mice by 74 and 84% with 0.00075 and 0.001% reserpine, by 47 and 70% with 0.2 and 0.3% chlorpromazine, by 61 and 89% with 2.0 and 3.0% meprobamate(12); in C_3H mice by 80 to 96% with 0.2% nidroxyzone(13) and by 73 to 95% with 0.1 to 0.2% Enheptin;† in ZA mice by 67 to 87% with 0.005 and 0.01% perphenazine.† These doses had only minor effects on body weight. In the present communication the concentrations of drugs used are sometimes less than the above but were approximately the maximally tolerated doses in ovariectomized C_3H mice. C_3H mice weigh less and generally tolerate less drug than the other strains used above. That these drugs inhibit estrus in intact mice but do not affect subestrus in ovariectomized mice suggests a difference in the mechanism for control of secretion of estrogen from ovaries of intact mice and of estrogen secretion from adrenals of ovariectomized mice, or, possibly, a difference in the type of estrogen secreted.

Administration of PMS had only a slight effect on the diestrus that developed in some mice many months after castration. This is in accord with the inability of gonadotropin to induce estrus reported by Martinez, *et al.* in ovariectomized, unilaterally adrenalectomized mice(9) and by King, *et al.* in caloric-restricted, gonadectomized mice(11).

The lack of effect of restricted food intake on frequency of subestrus was somewhat surprising for Casas *et al.*(7), and Ferguson *et al.*(5), found that food restriction prevented secretion of estrogen from adrenal tumors in C_3H castrated mice. These workers instituted food restriction at the time of gonadectomy whereas, in our work, mice had been castrated approximately 8 to 10 months before restriction was started. It may be that food restriction prevents the ability of adrenal tumors to start secreting estrogen but does not interfere with its secretion after initiation, due possibly to some effect on the functional, though not histological, development of cells intended to become estrogen-secreting.

Summary. Certain drugs, namely reserpine, chlorpromazine, perphenazine, meprobamate, nidroxyzone and Enheptin, previously found to have anti-estrus effect in intact mice, did not alter the frequency of subestrus nor development of subestrus in ovariectomized C_3H mice. Other findings in ovariectomized mice were: (1) frequency of subestrus tended to decrease with time and (2) restriction of food intake in mice with established subestrus did not alter frequency of subestrus.

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Study of Copper and Zinc Metabolism During Pregnancy. (26983)

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Previous studies have demonstrated that the total serum copper level increases with administration of certain hormones such as testosterone and estrogen, during pregnancy, and in different pathological states(1,2,3,4). Few studies have been reported on the effect of sex hormones on serum zinc level. The studies in this laboratory indicated that total serum zinc level declined with parenteral administration of testosterone propionate and estradiol benzoate. The mechanism of the mobilization and utilization of copper and zinc with the endogenous release or administration of sex hormones is unknown. The purpose of this investigation was to determine if total serum zinc level is changed during pregnancy.

It is known that the zinc content in serum is greater than the copper content. We found that the normal serum copper and zinc ranged from 99-113 μg/100 ml and 158-178 μg/100 ml, respectively, on 200 geriatric patients(4), as compared to 84-137 μg/100 ml and 158-285 μg/100 ml reported by others on middle aged subjects(5,6,7).

Method. Thirty female subjects were selected for this investigation. Their ages ranged from 21-40. All subjects were ambulatory with no acute or debilitating disease, and with no known disorder of copper or zinc metabolism. Twenty subjects were pregnant from 12-40 weeks. Ten non-pregnant subjects were used as controls. Venous blood was secured with the necessary precaution to avoid contamination. Duplicate determination of total serum copper and zinc was made during pregnancy and 8 weeks post partum. Colorimetric methods were employed for trace mineral determination(8,9) with modification by the author. Serum oxidase activity was determined by the method of Ravin(12), modified by Houchin(13).

Results: Table 1 gives the code, ages, weeks of pregnancy, total serum level of zinc, copper, and oxidase activity during the indicated period of pregnancy and 8 weeks after parturition. While the serum zinc level was found to be lower during pregnancy in all subjects, the serum copper level was increased in all subjects. The lowest zinc value and highest copper value were 45 and 289 μg/100 ml, respectively, for a subject 29 weeks pregnant. Eight weeks after parturition we found that the copper value decreased and the zinc value increased, approaching the normal reported range. The copper oxidase activity value ranged from 0.26-0.37 OD unit during pregnancy and declined after delivery to a range of 0.13-0.27 OD unit.

Discussion. Several investigators have observed an increase in total serum copper values in pregnancy and estrogen administration(1,5,6,4) and one author(10) has reported a decline in zinc during pregnancy. The results of investigation on copper and zinc excretion(1,5,11) during pregnancy and sex hormone administration indicated such changes are not significant. A high degree of correlation was observed between total serum copper and oxidase activity(14) in