Activity Rhythm and Breast Cancer in Pituitary-Isografted C and D_8 Mice; Effect of Hypothalamus.* (25348)

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Mühlbock and Boot have shown that the estrous cycle of mice can be significantly altered by pituitary isografts(1,2).† Estrous, in turn, in the rodent, is locked in defined phase relations with 24-hour rhythms, such as that in body activity(3,4). Are the latter rhythms biologically more basic, however, and do they persist under conditions which alter or obliterate estrous(4,5)? It was of interest, then, to record gross motor activity and to find that a 24-hour periodic activity-pattern persists after pituitary isografting. In the same study, mammary carcinogenesis from pituitary isografts was analyzed and experimentally modified.

Materials and methods. Virgin female recipient and donor mice of 2 strains were used. The strains had been maintained by brother-to-sister mating for over 15 years and differed in that the C's (Bagg albino) had no demonstrable mammary tumor agent (MTA), while the D_8's had a demonstrable MTA. From weaning and throughout the study, the mice had Purina Fox Chow and tap water continuously available. They were kept under natural conditions of illumination, in wooden cages with a wire mesh top, 5 mice in an 11″ x 5¾″ x 5¼″ space. Beginning during second month of life and continuing 7, 8, 14, or 16 times (at one-week intervals, or exceptionally, at 2-week intervals), recipient mice were given subcutaneously by trocar one of the following treatments: Pools of either 5 (I) or 3 (II) pituitaries, or one (III) hypothalamus with 3 pituitaries, or one (IV) hypothalamus alone, or 5 (V) or 3 (VI) pieces of femoral muscle. The 6 C-recipient groups and 2 D_8 recipient groups (I and V) consisted of 10 mice each. The other 4 D_8 recipient groups consisted of 5 mice each. Right and left axilla were used in alternation as sites of consecutive treatments. Age of donors (of same stock as recipients) varied from 3 to 5 weeks. Transplantations were done with assembly-line procedures immediately after killing donors by cervical dislocation by day (08-16). Fig. 1 shows area removed as "hypothalamus"‡ as well as pituitary. Cancer rate and age were evaluated by inspection and palpation, the diagnosis verified on biopsy or autopsy material stained with hematoxylin-eosin and, frequently, also by tests of transplantability. Two of 40 D_8 recipients and 6 of 60 C recipients died non-cancerous before one year of age. Cancer incidence comparisons were by Fisher's exact test (6) with and without omission of deaths prior to one year of age, with similar results. During second year of life gross motor activity was measured concomitantly on 10 non-cancerous C mice from different experimental groups (including animals which developed later verified breast cancer). Activity was recorded for several weeks on each mouse, in individual electronic actometers(4,7) kept at 24°C in light daily from 06 to 18 hrs.

Results. Activity counts of individual C mice which had received different treatments, in each case, were on the average higher during daily dark periods than during light periods (*cf.* Fig. 2). Persistence of a 24-hour activity rhythm synchronized with the regimen of light and dark seems established for mice bearing pituitary isografts, in which pseudopregnancies reportedly replace normal estrous cycle(1,2). If periodic changes underlying activity rhythm, *e.g.*, in blood corticoids (8,9), also are synchronized with lighting

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† Drs. Mühlbock and Boot discussed activity-rhythm measurements in pituitary-isografted mice with one of the authors (FH) and allowed him early insight into extensive and valuable data on estrous behavior in such mice.

‡ Authors are indebted to Dr. Berry Campbell, Univ. of California at Los Angeles, for advice regarding hypothalamus removal.

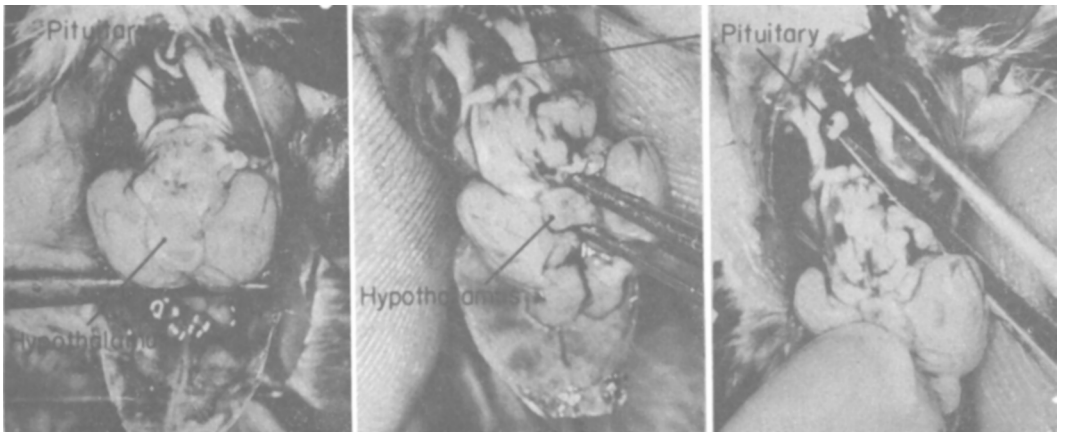


FIG. 1. Tissue removal from donors (see text).

schedule in mice bearing pituitary isografts, the data suggest persistence of the adrenal cycle(4,5,8-10) under conditions which significantly alter the estrous cycle.

In addition to changes with time, overall-activity levels differed from mouse to mouse. Similar differences existed, however, among individual mice from the same treatment-group (and were recorded earlier on untreated mice), although the actometers had been calibrated before use. Further work is needed to explore possible differences among experimental groups in 1) amplitude or level (rather than period) of light-dark synchronized activity rhythm or in 2) behavior of period of rhythm in the absence of such synchronization.

Breast cancer development in some groups

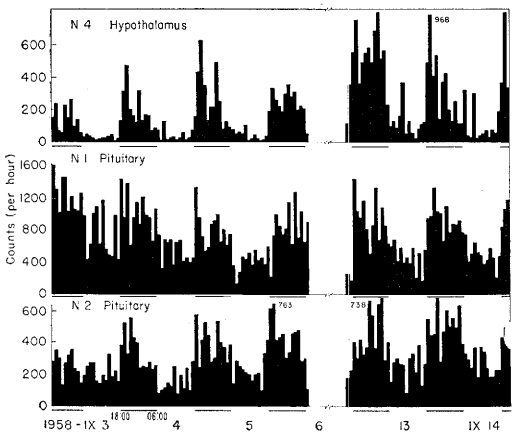
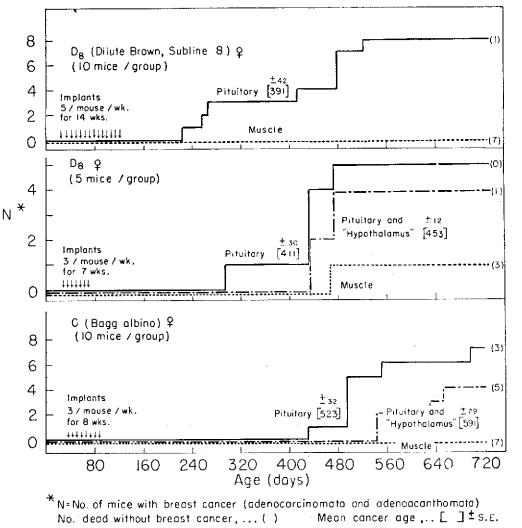


FIG. 2. Light-dark synchronized activity rhythm whether or not pituitaries were isografted. Activity in arbitrary units is higher during dark periods (above horizontal bars).

given pituitary isografts is shown in Fig. 3 (with corresponding data on mice given muscle). The upper sections show data on D_8



*N=No. of mice with breast cancer (adenocarcinomata and adenoacanthomata)
No. dead without breast cancer, ... ( ) Mean cancer age ... [ ] ± S.E.

FIG. 3. Breast cancer incidence in 65 of recipients studied—work involving also 1390 donors of corresponding inbred stock, sex, and age. Incidence in virgin D_8 mice is low, whether or not muscle treatments are given; it is significantly raised by pituitary isografts. In non-cancerous C mice pituitary isografts induce breast cancer, though at a more advanced cancer age than in D_8's. Insertion of hypothalamus at times of pituitary isografting delays mammary carcinogenesis.

mice with MTA: The number of mice with breast cancer was 1 of 15 D_8's given muscle [0 of 5 D_8's given hypothalamus (not graphed)], as compared to 13 of 15 D_8's given pituitaries (all maintained as virgins). Fig. 3 (bottom) also shows data on virgin C

TABLE I. Body Weights of Mice without Cancer, at About 16 Months of Age.*

Treatment	C: No. of inserts (No. of mice)	C: Body wt, g	D_8: No. of inserts (No. of mice)	D_8: Body wt, g
Muscle	16 × 4 (5)	32.0 ± 1.5†	14 × 5 (7)	25.4 ± .4†
"	8 × 3 (8)	29.6 ± .7	7 × 3 (5)	32.2 ±1.0
Hypothalamus	" (8)	31.4 ± .7	" (5)	32.6 ± .2
Pituitary	16 × 4 (3)	29.0 ± 2.0	14 × 5	‡
"	8 × 3 (5)	31.8 ± 1.0	7 × 3	‡
" & hypothalamus	" (7)	33.0 ± 1.1	" (2)	28.0 ± .0

* See text. † Mean ± S.E. ‡ Those alive all with breast cancer.

mice, without demonstrable MTA, revealing again the carcinogenic effect of pituitary isografts, in agreement with results on other stocks without demonstrable MTA(2), recently reviewed(1). Differences in cancer incidence between muscle and pituitary treatment-groups are significant for each stock: The P values for the 2 available comparisons on D_8 mice were $<.01$ and $<.03$ respectively, and $<.01$ for the C mice.

Breast cancers recorded were adenoacanthomata or adenocarcinomata, both types being found in C's as well as D_8's with pituitary isografts. Squamous metaplasia was seen more often, however, in C mice without MTA than in D_8 mice with MTA, in agreement with results of earlier work on other stocks(11).

The carcinogenic effect of pituitary isografts (Fig. 3) is in close agreement with earlier work(1,2,12,13); its significance is underlined by control of secular variation, since all treatment groups compared were set up concomitantly and by control of experimental procedures, since the standards of reference are data from mice given muscle rather than data from colony at large, a procedure previously used, which does not allow for control of trauma (*e.g.*, from trocar puncture or from "irritation" by inserted tissues). Control of exposure to experimental procedures was sought since trauma might affect carcinogenesis, although some data supporting this suggestion were not statistically significant(14). In this study at 16 months of age, body weight of D_8 mice given 14 muscle treatments was lower than that in mice given only 7 such treatments, but this possible effect of trauma was not apparent in comparison of C mice given 8 or 16 treatments (Table I). Herein trauma, as such, was not studied, only its possible effect was controlled. The contribution of pituitary isografts themselves to breast cancer development in the mouse stands out more significantly, whether or not a demonstrable MTA is present.

Stock and number of implants in relation to breast cancer incidence and cancer age. Although cancer developed in mice without (C) as well as with MTA (D_8), the question might be raised whether presence of agent enhanced carcinogenesis (*cf.* 1,2). Cancer incidence in C mice given totals of either 80 or 24 pituitaries was 7 of 10 in each group while for the D_8's given 70 or 21 pituitaries, the number of cancers was 8 of 10 and 5 of 5 respectively. Comparisons of these small samples of mice with different genetic background, receiving slightly different total numbers of treatments, do not reveal an effect of stock (and agent) or number of implants upon cancer incidence. Cancer age, in turn, seems to be affected by both factors, stock (agent) and number of implants. An analysis of variance (Table II) reveals these effects to be significant at about 10%. The difference in cancer age between pituitary isografted mice from the C and D_8 stocks is more clearly seen when the groups

TABLE II. Stock (Agent) and Number of Implants in Relation to Cancer Age.

Stock	MTA	No. of implants	Cancer age (No. of mice)
C	—	24	523 ± 32 (7)
		80	428 ± 40 (7)
D_8	+	21	411 ± 30 (5)
		70	391 ± 42 (8)

Analysis of variance*

Source	DF	MS	F†
No. of implants	1	.0373	3.08
Stock (agent)	1	.0364	3.01
Interaction	1	.0057	<1.00
Within	23	.0121	

* On $\log_{10}$ transformation of data.
† $F_{.90}(1, 23) = 2.94$.

receiving the smaller number of implants are compared [t computed for difference (in $\log_{10}$ transformed data) = 2.56; P = .028]. The effect of number of implants, in turn, appears more clearly in data on the C stock, not complicated by presence of MTA (t = 1.868; P = .088). In close agreement with Mühlbock and Boot(1,2), it seems that cancer age is shortened by either presence of MTA or by increase in number of implants; either effect, however, may be obscured by the other, that of the number of implants by the presence of the agent and vice versa. It also seems pertinent that in subsequent work from this laboratory, on 100 pituitary-isografted D_8 recipients, breast cancer incidence was over 20% by one year after implantation of only 2 pools, each composed of 3 pituitaries. For this stock, the number of implants is therefore not among the more critical experimental variables in mammary carcinogenesis from pituitary isografts.

Effect of hypothalamus. In some animals, a hypothalamus was inserted into the axilla (in the same trocar with the pituitary) at time of pituitary implantation. Cancer incidence in animals thus treated was slightly less than in mice given only the pituitary (Fig. 3) but the difference was not statistically significant in either stock. Noteworthy differences were found, however, in cancer age between animals given only the pituitary on one hand and the pituitary and hypothalamus on the other. At ~ 520 days post-implantation, one-half of the C mice given only pituitaries (5 out of 10) had breast cancer as compared to none of the (10) C mice given pituitary and hypothalamus (P = .05). The differences in cancer age as a function of whether or not the hypothalamus had been inserted were ultimately not significant at 10% in either stock. This lack of statistical significance in the C data, however, resulted from the fact that one animal from the group given only pituitaries developed cancer at a very late age and thus raised the mean cancer age of the pituitary group.

In the interim, additional groups each composed of 20 D_8 mice were given 2 pools of pituitary alone (I) or pituitary and muscle (II) or pituitary and cerebral cortex (III) or pituitary into one axilla and hypothalamus into the other axilla (IV), while another two groups (of 20 mice each) received the pituitary and hypothalamus in the same axilla either jointly by the same trocar (V) or successively through different trocars directed at the same site (VI). At the time of this writing, 11 months post-treatment, tumor incidence in the latter two groups (V and VI) is about one-fourth that in either of the former groups (I to IV). These latter findings confirm the impression gained from Fig. 3 and suggest further that mechanical factors cannot be held solely responsible for an apparent anti-cancer effect of the hypothalamus when it is inserted at the time of pituitary isografting. May has suggested that grafted dying tissues have chemical action while they are being replaced by immigrated elements and that nerve fibers may electively be attracted by tissues which are no longer, or which are not yet innervated(15). At any rate, the mode of action of the as yet ill-defined "hypothalamus"-insert (Fig. 1) awaits analysis with particular reference to the morphology or function of the grafted pituitary.

Summary. A light-dark synchronized activity rhythm persists in pituitary-isografted C mice. The adrenal cycle probably persists in mice with a significantly altered estrous cycle(1,2). Multiple pituitary isografts are associated with adenocarcinomata and adenoacanthomata in virgin female mice of the D_8 and C stocks. The carcinogenic effect of the pituitary isografts is statistically significant and not a result of trauma. Number of implants as well as presence or absence of mammary tumor agent also are significant variables, but neither variable is critical to carcinogenesis from pituitary isografts, under the conditions studied. Insertion of the hypothalamus at the time of pituitary implantation delays mammary carcinogenesis by mechanisms as yet unknown.

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Apparent Loss of Glycine on Acid Hydrolysis. (25349)

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Studies concerning loss of amino acids during acid hydrolysis of protein containing materials have been reported(1,2). For the most part, such losses resulted from outright destruction, racemization, or irreversible binding to human material. The present report is concerned with an apparent loss of glycine, as determined microbiologically, with increasing time of acid hydrolysis. This loss is due to the presence of glycine peptides in the short time hydrolysates which promote greater growth of the assay organism than a corresponding amount of free glycine. The relationship of these findings to the antagonistic effects of certain amino acids in the basal medium upon glycine was investigated.

Methods. Hydrolysates were prepared by autoclaving 1 g of material with 20 ml of 3 N HCl in a sealed tube at 15 lb 121°C for varying time. The hydrolysates were taken to dryness, resuspended in water and made up to pH 4.0 with KOH. After filtering through a sintered glass funnel, the resulting clear filtrate was adjusted to pH 6.8, made to desired volume and stored under toluene at 5°C. Glycine was estimated microbiologically with *Leuconostoc mesenteroides* strain P-60 (ATCC 8042) using a commercially prepared amino acid assay mixture.* After distributing 2 ml of assay medium, samples and standards were added and total tube volume was made to 3 ml. The tubes were autoclaved for 10 min. at 15 lb 121°C, cooled, and inoculated with one drop of a washed suspension of test organism. Incubation was carried out at 37°C for 72 hours and the resulting acidity titrated with 0.025 N NaOH.

Results. Typical results illustrating effect of acid hydrolysis upon glycine content of soybean oil meal are shown in Fig. 1. The apparent glycine reaches a maximum at one hour, and thereafter drops rapidly to 6 hours. Additional autoclaving to 24 hours results in a further slight loss in activity. In separate experiments, where glycine was added prior to hydrolysis, recovery values ranging from 97.1 to 100.8% showed that this apparent loss was not due to destruction of free glycine. In addition to decrease in glycine activity with increased time of hydrolysis, a downward drift with increasing aliquot of sample assayed was observed. These results are shown in Table I. In the various hydrolysates, decrease in assayed glycine value is less with larger aliquot of sample and more pronounced with smaller aliquot. However, as time of hydrolysis is lengthened these differences become less significant.

Therefore, it seemed expedient to examine

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