termined both by ovarian ascorbic acid depletion following i.v. injection of fractions into immature females pretreated with gonadotrophins and by the increment in plasma LH activity which followed i.v. injection of extract into ovariectomized, estrogen progesterone-blocked rats. The peak of LH-releasing activity was found to overlap that of pressor activity but was displaced slightly so that some fractions with LH-releasing activity were devoid of pressor activity. Fractions with LH-releasing activity were devoid of ACTH-releasing activity even when the dose was increased to five times the MED for LH release. It was concluded that the LH-RF is a small polypeptide dissimilar from vasopressin and CRF.

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Autoradiographic Studies of the Epithelium of Mammary Gland as Influenced by Ovarian Hormones.* (29120)

HAROLD H. TRAURIG† AND CHARLES F. MORGAN**
University of Minnesota Medical School, Minneapolis

Many investigators have measured mammary gland growth from studies of whole mounts and histological preparations. While these methods have yielded valuable information, they did not objectively measure the contribution of cellular proliferation, secretion or cellular hypertrophy to the observed growth.

Recently, normal and experimental growth of the mammary gland has been assayed by total DNA determinations(1). This technique, however, indiscriminately measured the contribution of all the cellular components of the mammary gland. The DNA derived from connective tissue and vascular elements could be significant especially in the early phases of mammary growth.

Determination of the effects of hormonal treatment on the epithelial cells of the mammary gland alone has been made possible by the use of tritiated thymidine (T-H^3) and high resolution autoradiography (ARG). It has thus been possible to localize the tracer, having tritium as part of its molecular structure, to within one micron of its incorporation site in tissue(2). Thymidine has been shown to be a specific precursor for DNA synthesis by cells entering mitosis and is either incorporated within 30 minutes after systemic injection or degraded. Tritiated hydrogen bound to thymidine will not exchange with hydrogen in the surrounding medium, nor will the thymine moiety, to which it is attached, be replaced to a significant degree once it has been incorporated

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† Present address: Dept. of Anatomy, Univ. of Kentucky Med. School, Lexington.

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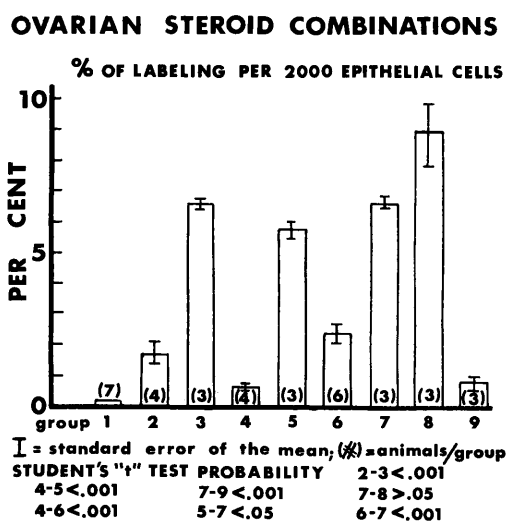

FIG. 1. Percentage of 2,000 epithelial cells labeled by T-H^3 in mammary gland of castrated, female mice following various treatments with ovarian hormones. Group (1) vegetable oil only, 10 days (d). (2) .5 μg E/d, 10 d. (3) 1.0 μg E/d, 10 d. (4) 1.0 mg P/d, 10 d. (5) 3.0 mg P/d, 10 d. (6) .5 μg E/d, 5 d + 1.0 mg P/d, 5 d. (7) 1.0 μg E/d, 10 d + 3.0 mg P/d, 5 d. (8) 1.0 μg E/d, 15 d + 3.0 mg P/d, 10 d. (9) as (7), + 5 days of no hormonal treatment before sacrifice.

into DNA. Thereafter, the only dilution of the tracer in the tissue occurs as a result of cell division(3 & 4). Hence, by the use of an incorporation period of a few hours, the percentage of cell nuclei in a population of cells demonstrating an incorporation of T-H^3 will provide an index of proliferation(3).

This investigation utilized T-H^3 and ARG to measure the growth response (cell proliferation) of the mammary gland epithelium under the influence of hormonal treatment.

Materials and methods. Thirty-six virgin female BALB/c mice, aged 9 weeks, were oophorectomized under ether anesthesia. Vaginal smears were checked to determine the success of castration. The animals were assigned to groups and treated as outlined in Fig. 1. Crystalline estradiol-17β‡ (E) and progesterone (P), in vegetable oil, were adjusted to yield the proper concentration in 0.05 ml injection volumes. At midday during the experimental period, hormones were injected subcutaneously in the dorsal thoracic region. Twenty-four hours after the final hormonal treatment, T-H^3 (0.7 μc/g/body weight; S.A. = 5.21 c/mM) was injected into the tail vein. The mice were sacrificed 6 hours later and the tissue surrounding the nipple region of the inguinal pair of mammary glands was removed and fixed in Bouin's fluid. After dehydration and clearing, the tissue pieces were embedded in piccolyte (6-8%) -paraffin and sectioned at 2 μ. Autoradiographs (ARG's) were prepared by the dipping§ and stripping‖ techniques. After an exposure period of 17 days at 4°C, the ARG's were developed in D-17 or D-19 Kodak developer and fixed in Kodafix. Processed ARG's were stained with hematoxylin, dehydrated and mounted.

After correction for background, an ARG section was selected at random and scanned under oil immersion until a mammary epithelial structure moved into the field. The number of cells composing the epithelial structure was determined as well as the number demonstrating an uptake of T-H^3 in the nucleus. The scanning procedure was continued, including all epithelial structures as they were encountered, until a total population of 2,000 epithelial cells had been counted for each animal. The percentage of labeled epithelial cells in the 2,000 cell population was determined and the mean calculated for each group. The standard error of the mean was calculated for each group and all data were tested for significance by use of the Student's *"t"* test.

Results. All groups treated with hormones (Fig. 1) showed a significantly greater percentage of labeled epithelial cells than the untreated group ($P < .001$). The 1 μg E dosage promoted a significantly greater percentage of labeled epithelial cells than the 0.5 μg E treatment (Fig. 1; $P < .001$). E treatment caused the growth of interlobular ducts and end buds (Fig. 2 & 3), however, no lobule-alveolar (L-A) differentiation was noted.

‡ Estradiol-17β and progesterone were furnished through the generosity of Dr. Edward Henderson, Schering Co.

§ NTB-3 nuclear track liquid emulsion.

‖ Kodak AR-10 fine grain autoradiographic stripping film.

Administration of 3 mg P caused a significantly greater percentage of labeled epithelial cells as compared to 1 mg P treatment (Fig. 1; $P < .001$). The epithelial structures in the mammary glands of the 1 mg P treated animals consisted of thin, poorly differentiated ducts whereas the 3 mg P treatment promoted the growth and differentiation of some L-A structures (Fig. 4).

Combinations of E + P promoted L-A

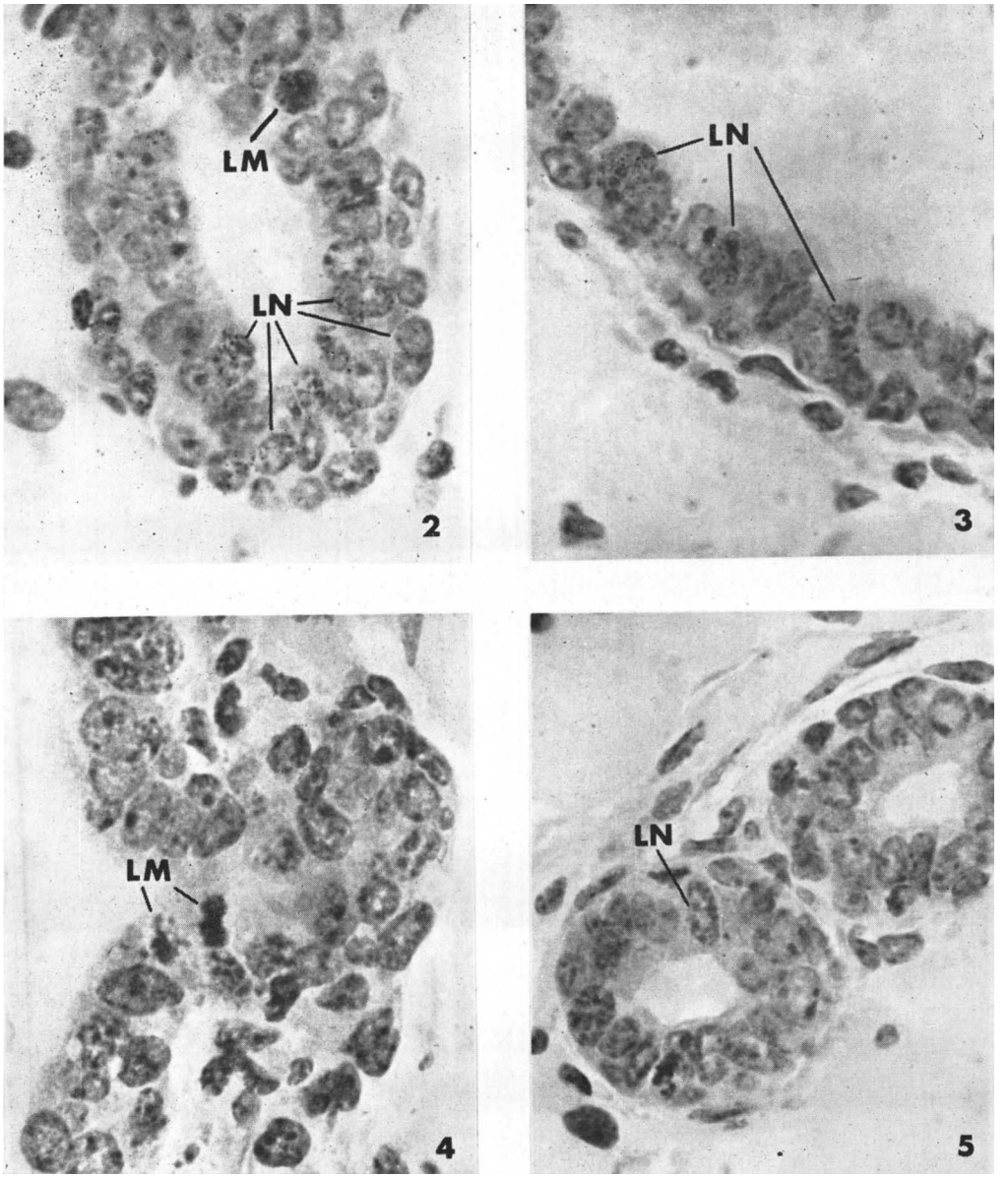

FIG. 2-5. ARG of mammary gland epithelium of castrated, hormonally treated female mice. 1260×. LN = Labeled nucleus, LM = Labeled mitotic figure.

FIG. 2. Interlobular duct. 1.0 μg E/day for 10 days.

FIG. 3. Interlobular duct. .5 μg E/day for 10 days.

FIG. 4. Interlobular duct with a developing intralobular branch. Grains over labeled mitotic figures obscured by the densely stained chromatin. 3.0 mg P/day for 10 days.

FIG. 5. Intralobular ducts. .5 μg E/day plus 1.0 mg P/day for 10 days.

growth and differentiation of the mammary gland similar to that of the earlier stages of pregnancy (Fig. 5). The 0.5 μg E + 1 mg P combination promoted a significantly greater percentage of epithelial cell labeling than 1 mg P alone (Fig. 1; $P < .001$). Combinations of 1 μg E + 3 mg P for 10 days caused a significant increase in percentage of labeled cells as compared to the value for the 0.5 μg E + 1 mg P treatment ($P < .001$). The former value was also significantly greater ($P < .05$) than that of the 3 mg P treatment. Administration of 1 μg E + 3 mg P for 15 days produced no significant increase in percentage of labeled epithelial cells as compared to the 10 day treatment (Fig. 1; $P > .05$). However, the most extensive L-A differentiation was observed following the 15 day treatment.

Administration of 1 μg E + 3 mg P for 10 days, followed by 5 days of no hormonal treatment before T-H^3 injection and sacrifice, caused a great depression in percentage of labeled epithelial cells. This depression was significant ($P < .001$) when compared to similarly treated animals sacrificed 24 hours after the last hormonal treatment (Fig. 1, Groups 7 & 9). However, no histological differences could be observed between the groups sacrificed 24 hours or 5 days after the last hormonal treatment.

Discussion. Observations have shown that the parenchyma of the mammary gland was heterogeneously dispersed in fat and connective tissue. This was replaced by new epithelium during growth and differentiation of the mammary gland. Therefore, organ weights or total DNA determinations cannot yield an accurate measurement of mammary growth until the epithelium forms the greater part of the mammary gland. Techniques that express mammary growth in terms of per cent of parenchyma/mammary gland(5) may be misinterpreted because of duct distention due to secretion or to an increase in cell size. T-H^3 and ARG offer an opportunity to selectively measure the growth response (cell proliferation) of the mammary epithelium under hormonal influence. In this investigation, the growth response of the mammary gland was expressed in terms of the per cent of T-H^3 labeled cells in a 2000 epithelial cell population. In addition, concurrent whole mount and histological studies permitted an appraisal of the degree of epithelial differentiation.

In this study of the mammary gland, an attempt has been made to separate the effect of hormonal treatment on epithelial proliferation from differentiation and secretion. A more advanced degree of epithelial differentiation was not necessarily associated with a high rate of epithelial proliferation, nor was the converse true. Mice treated with 0.5 μg E or 0.5 μg E + 1 mg P showed no significant difference in percentage of labeled epithelial cells, but whole mount preparations from the latter clearly demonstrated lobular differentiation absent in the former. The difference in percentage of labeled cells in the 1 μg E and 3 mg P treated groups was not significant, however, the 3 mg P treatment promoted considerable L-A differentiation while 1 μg E induced the growth of interlobular ducts and end buds only. Also, whole mount preparations of 1 μg E treated mice showed no more or less duct differentiation than the group treated with 0.5 μg E, but the rate of new cell formation was significantly greater in the former (Fig. 1). Therefore, epithelial cell proliferation and differentiation in the mammary gland of the mouse requires different hormonal stimuli. E promoted cellular proliferation but very little L-A differentiation. On the other hand, the most striking result of P treatment was the initiation of L-A differentiation. Whether this conclusion will stand for a wide range of dosages or lengths of treatment remains to be determined.

Summary. 1. The technique of autoradiography with tritiated thymidine has been used to measure the proliferative response of mouse mammary epithelium to hormonal treatment. 2. Estradiol-17β administered alone promoted proliferation of the mammary epithelial components but little differentiation was observed. 3. Progesterone administered alone promoted lobule-alveolar differentiation and epithelial proliferation. 4. Combinations of estradiol-17β and progesterone had, in general, an additive effect on cell

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Immunofluorescent Localization of LH and FSH in the Human Adenohypophysis.* (29121)

DAVID KOFFLER AND MARVIN FOGEL (Introduced by Hans Popper)

Departments of Pathology and Obstetrics and Gynecology, Mount Sinai Hospital, New York City

Antisera to human chorionic gonadotropin (HCG) and human menopausal gonadotropin (HMG) have previously been prepared(1,2). Anti-HCG serum contains contaminating antibodies to FSH and anti-HMG serum contains contaminating antibodies to LH. Purified antisera of strong potency may be obtained by absorption with appropriate antigen resulting in immunoelectrophoretically specific antisera to HCG and HMG(3). Anti-HCG serum inhibits biological LH activity and anti-HMG serum inhibits biological FSH activity(2).

We attempted to utilize purified antisera to localize LH and FSH in cells of the human anterior pituitary gland by immunocytochemistry. The sites of production of growth hormone(4) and ACTH(5) have been clarified by similar techniques.

Materials and methods. Preparation of antisera. Rabbits were immunized intramuscularly with 10.0 mg of HCG† and 3.5 mg of HMG‡ 4 times at weekly intervals. Aliquots of these anti-sera were absorbed with lyophilized human serum proteins. Anti-HCG serum was absorbed with HMG and anti-HMG serum was absorbed with HCG. Microimmunoelectrophoresis revealed that the antisera reacted with homologous antigen only, after allowing the plates to develop for 5 days. Globulin fractions were precipitated and aliquots of each antiserum fluoresceinated. Chromatographically purified 7s rabbit gamma globulin was injected into goats 10 mg at weekly intervals for 4 weeks. The globulin fraction was precipitated and fluoresceinated.

Human tissues. Eight pituitary glands and sections of liver, kidney and thyroid were obtained within 24 hours of autopsy and frozen in a mixture of dry ice and isopentane at −70°C.

Fluorescent antibody technique. A Leitz ortholux fluorescence microscope with HB-200 light source, 2 BG-12 exciter filters and an OG-4 barrier filter were used for observation. Cryostat sections were made at 6 μ and serum was incubated with a section for 30 minutes at room temperature. Pituitary sections were stained by the following procedures: (1) Normal rabbit serum (NRS) + fluoresceinated goat anti-rabbit gamma globulin (FGARGG); (2) Purified anti-HCG (RALH) + FGARGG; (3) Purified anti-HMG (RAFSH) + FGARGG; (4) FGARGG; (5) Fluoresceinated RALH (FRALH); (6) Fluoresceinated RAFSH (FRAFSH); (7) RALH + FRALH; (8) RAFSH + FRAFSH; (9) RALH absorbed with HCG + FGARGG; (10) RAFSH absorbed with HMG + FGARGG. Sections of

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† APL—brand of human chorionic gonadotropin generously supplied by Ayerst Laboratories.

‡ Pergonal (PR2015)—brand of human menopausal gonadotropin generously supplied by Cutter Laboratories.