# MITOGENIC ACTIVITY OF PITUITARY HORMONES ON CELL CULTURES OF NORMAL AND CARCINOGEN-INDUCED TUMOR EPITHELIUM FROM RAT MAMMARY GLANDS

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ABSTRACT

Cell suspensions containing normal or tumor epithelium were readily obtained by enzymatically digesting rat mammary glands from perphenazine-treated (prolactin-hypersecreting) cycling, female virgin animals or hormone-responsive mammary tumors from animals treated with dimethylbenzanthracene. Cell suspensions were fractionated into predominantly epithelial and predominantly stromal cells by their differential rates of attachment to culture dishes. Both normal mammary and tumor epithelial cells were characterized by the presence of specific cell-junctional complexes, desmosome-like structures, surface microvilli, and their ability to synthesize casein. Serum-dependent protease activity was greater in cultures derived from tumors, and cells from such cultures grew in agarose whereas those from the non-neoplastic gland did not. The addition of prolactin to the culture medium stimulated DNA synthesis in primary or secondary epithelial cultures from tumors, whereas additional insulin and hydrocortisone with prolactin were required for similar levels of DNA synthesis in cultures from non-neoplastic glands. The fraction of cells synthesizing DNA was, however, smaller than that with 10% serum measured in the same time period. Both growth hormone and epidermal growth factor stimulated DNA synthesis but to a lesser extent than did prolactin. Prolactin with hydrocortisone and insulin was relatively inactive in promoting DNA synthesis of the nonepithelial cells whereas pituitary fibroblast growth factor was more active. These mitogenic effects were obtained when the hormones were added to the medium at near physiological concentrations, and paralleled the known activities of the hormones in control of mammary gland growth and development in the rat.

Growth and development of the mammary gland in mice and rats occur mainly by a process of cell multiplication rather than by hypertrophy of existing cellular units (9). The hormones which control this process in vivo have largely been identified by means of a series of endocrine gland-ablation and hormone-replacement experiments, and they include prolactin, growth hormone together with estrogens, glucocorticoids, and progesterone (18, 21, 34, 35). In organ culture, however, Topper and others have failed to show any mitogenic effect of prolactin or growth hormone although high, nonphysiological concentrations of insulin and a corticoid were shown to stimulate thymidine incorporation into DNA (19, 22, 33, 38, 40). Since pretreatment of virgin mice with prolactin

potentiated the action of insulin in mammary explant cultures, Oka and Topper (22) suggested that insulin was the primary mitogen and that prolactin sensitized the mammary tissue in vivo. Here, we reinvestigated the mitogenic role of pituitary hormones on rat mammary epithelium.

The interpretation of the effects of hormones on explant cultures, however, is complicated by the presence of several different cell types which may act in different ways. To overcome this difficulty, we have developed a system for obtaining short-term cell cultures of relatively pure epithelium from either normal or neoplastic rat mammary gland. To increase the number of epithelial cells, rats are pretreated with perphenazine (25) which causes hypersecretion of prolactin. Mammary carcinomas are induced with dimethylbenzanthracene (DMBA). The majority of these tumors require the presence of circulating prolactin, estrogens, and glucocorticoids to grow in vivo (13, 25). The mammary tissues are digested with collagenase and hyaluronidase and separated into two cell fractions, a predominantly stromal and a predominantly epithelial fraction, by their differential rates of attachment to plastic petri dishes. The degree of preservation of the isolated mammary and tumor epithelium and their various properties have been evaluated by morphological, ultrastructural, and biochemical criteria. The effects of near physiological concentrations of various hormones on DNA synthesis and cell division both in stromal and epithelial cell fractions are reported for growing primary cultures and "stationary" but not confluent, secondary cultures. The mitogenic effects of these hormones in vivo are consistent with their activity in this tissue culture system.

## MATERIALS AND METHODS

### Materials

Ovine pituitary prolactin (P-S-11), bovine growth hormone (GH-B17), luteinizing hormone (LH-B9), follicle-stimulating hormone (FSH-B1), and thyroid-stimulating hormone (TSH-B7) were kindly donated by the National Institute of Arthritis and Metabolic Disease pituitary hormone distribution program (N. I. H., Bethesda, Md.); rat prolactin, bovine fibroblast growth factor (7), and mouse submaxillary epidermal growth factor (3) were generous gifts from Dr. H. G. Kwa (The Netherlands Cancer Institute, Amsterdam), Dr. D. Gospodarowicz (Salk Institute, San Diego, Calif.), and Dr. S. Cohen (Vanderbilt University, Nashville, Tenn.), respectively. Dr. Gospodarowicz also provided the ovarian growth factor (8). Steroid hormones, insulin, collagenase (C-1030:130 U/mg), and hyaluronidase (H-2001:460 NF U/mg) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Solutions of collagenase (4 mg/ml) and hyaluronidase (2 mg/ml) in medium without serum were stored in aliquots at −20°C. Pituitary hormones were freshly dissolved in water containing a minimum of NaOH, and the steroid hormones were dissolved in 25% ethanol. All hormones were dissolved at a hundred times the final concentration in the medium. Perphenazine; 7,12-dimethylbenzanthracene and X-ray film RP/R54; goat antirabbit serum (G8-2, P4); and D-valine were obtained from Allen and Hanburys Ltd. (London, England); Eastman Kodak Co. (Rochester, N. Y.); Antibodies Incorporated (Davis, Calif.); and Koch-Light (Colnbrook, Bucks, England), respectively. Rabbit antimouse casein (5) and rat and mouse caseins were gifts from Dr. M. Feldman (Children's Hospital Medical Center, Oakland, Calif.) and Dr. S. Young (I. C. R. F., London, England), respectively. The antiserum was absorbed with mouse serum and stored with 0.01% methiolate in phosphate-buffered saline, while the caseins were prepared from milk by precipitation with renin (11, 39). Cell culture media, medium 199, Dulbecco's modified Eagle's medium (DEM), and Waymouth's medium (24) were supplied by the I. C. R. F., fetal calf serum was purchased from Gibco-Biocult (Glasgow, Scotland). Radioactive isotopes [*methyl*-$^{3}$H]thymidine (18.5 Ci/mmol) and [$^{35}$S]methionine (460 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, England).

### Animals

Virgin female Sprague-Dawley rats, aged 100–150 days, were given daily subcutaneous injections of perphenazine (1 mg/200 g body weight) for 3 days before sacrificing (25) before the removal of the normal glands. Female Sprague-Dawley rats, aged 50 days, were given a single intragastric dose of 30 mg of 9,10-dimethyl-1,2-benzanthracene (DMBA) in corn oil (13). The mammary tumors then developed during the next 6–12 wk, and growing tumors measuring 1–1.5 cm in diameter were used.

### Tissue Culture

Cells were prepared and grown at 37°C in primary and secondary cultures in two different basic media: medium 199 with a 6.0% $CO_2$ atmosphere (vol/vol) (usually primary cultures) (11), or DEM with 10% $CO_2$ (30) (usually secondary cultures). The same medium containing varying amounts of serum was used throughout the history of a culture. The established mouse line, BALB/c 3T3, was grown as described previously (30).

#### PREPARATION OF CELL SUSPENSIONS

The lower two pairs of abdominal mammary glands from two or four perphenazine-treated rats or a single tumor were cut into small pieces and then chopped

mechanically into ~200 μm cubes with a tissue sectioner (Sorvall TC2, DuPont Instruments, Sorvall Operations, Newtown, Conn.). The choppings of normal mammary gland were digested in 20 ml of medium, 10% fetal calf serum (FCS), 2 mg/ml collagenase (260 U/ml), and 1 mg/ml hyaluronidase (460 NF U/ml) at 37°C for 45 min. Released fat was separated by centrifuging at 100 *g* for 5 min and the digestion was continued for a further 3-4 h. The choppings of tumor were digested in medium containing 5% FCS, 2 mg/ml collagenase, and 1 mg/ml hyaluronidase at 37°C for up to 1½ h.

SEPARATION OF CELLS BY PRESTICKING: The cell suspensions from either the normal gland or tumor were washed three times by centrifuging through medium 199 and 5% FCS and plated into 9-cm dishes. 2 h later the medium, which contained predominantly epithelial cell clumps, was removed. This medium was used for further culture, and many intact ductal and alveolar structures were observed at this stage (slow-sticking fraction). The cells that remained adhering to the dishes were rinsed again with medium, and those remaining attached after this operation constituted the "fast-sticking" stromal cell fraction.

### Preparation of Growing Primary Cultures

Plating suspensions were prepared in medium containing 5% FCS so that sufficient epithelial clumps would produce a confluent culture in a 5-ml dish after 5 days. Epithelium grown from normal mammary glands mobilized from the clumps during 24-36 h after plating, and the colonies spread rapidly during 24-96 h and then slowly increased in size up to confluence. The maximum [$^3$H]thymidine radioactive labeling index (50-60% during a 16 h period) occurred between 48 and 76 hours. Epithelium from mammary tumors mobilized during 12-24 h to form colonies which spread rapidly during 18-96 h and then slowly up to confluence. The maximum labeling index (75-85% during a 16 h period) occurred between 48 and 76 h after plating. Hormones were added singly or in combination to the medium at the time of plating, and the medium was changed every 24 h.

To reduce further the content of fibroblastic cells, a few primary cultures of nonfractionated cell suspensions from normal glands were also plated (when indicated) in DEM in which L-valine was replaced by twice the concentration of D-valine (6) and 10% dialyzed FCS. These were then incubated for 3 days (D. Bennett and H. Durbin, unpublished results).

### Preparation of Stationary Secondary Cultures

The original digestion of the gland or tumor for preparation of primary cultures was performed in DEM. DEM was used instead of medium 199 since all the secondary cultures were maintained in DEM at 10% $CO_2$ (vol/vol). The epithelial cell clumps (slow-sticking fraction) after the presticking step were replated in 2-ml aliquots into 9-cm dishes, incubated for 16 h (to allow attachment), and then a further 8 ml of DEM with either 5% or 10% FCS, 0.5 μg/ml hydrocortisone, and 0.05 μg/ml insulin were added. The hydrocortisone and insulin maintained cell viability for longer periods of time. Similar media were added to the fast-sticking fractions. After 4-5 days the confluent cultures were washed in serum-free DEM and then digested with 0.25 μg/ml trypsin at 37°C for 2 min. After a second "presticking step" to remove contaminating stroma, the cells were replated ($2 \times 10^5$/3.5-cm dish) in the same medium described above. These constituted the secondary epithelial cultures. Secondary fibroblastic cultures were plated in exactly the same way from primary fibroblasts except that the presticking step before plating the secondary cultures was omitted. After 1 or 2 days the medium on the secondary cultures was changed and the serum concentration was reduced to 0.5%, replaced by 250 μg/ml bovine serum albumin (BSA), or the cells were washed once with DEM and the medium was replaced with DEM alone. Where indicated, 50 ng/ml insulin and 500 ng/ml hydrocortisone were also included in the medium. Incubation was continued for a further 6 days, then additional hormones or growth factors were added.

A few cultures were also grown in a "nutrient-enriched medium." These were plated and grown for 4 days as usual, and then the medium was replaced by a mixture of 1 part Waymouth and 2 parts DEM (vol/vol) (32) with 0.5% FCS. Cultures were incubated a further 2 days before the additions of the hormones. The final concentrations of the extra amino acids and vitamins over DEM alone were 0.14 mM aspartic acid, 0.3 mM glutamic acid, 0.06 mM hypoxanthine, 0.05 mM glutathione, 0.03 μM D-biotin, 0.05 μM vitamin $B_{12}$ (24).

## *Microscopy*

LIGHT MICROSCOPY: Cultures were washed twice with isotonic saline, twice with ice-cold 5% trichloroacetic acid (vol/vol), and twice with ethanol. The cultures were then allowed to dry, and were stained with Giemsa's for 10 min, washed with water, dried, and photographed with a Wild inverted microscope under phase contrast or bright field conditions as indicated on Kodak FP4 film.

ELECTRON MICROSCOPY: Primary cultures were grown on glass cover slips in petri dishes as described earlier. After 67 h the medium was removed, the cells were rinsed once with 0.1 M Sörensen's buffer (10), pH 7.3 (buffer S) and 2% glutaraldehyde at 4°C, and fixed for 3 h in the same mixture. The cover glasses were then rinsed twice with buffer S and refixed for 1 h in 1% osmium tetroxide in buffer S. The cultures on the cover glasses were dehydrated by rinsing with graded alcohols and acetone and infiltrated with Araldite which was polymerized at 70°C for 48 h. The glass was then dissolved with hydrofluoric acid and replaced by polymerized Araldite. Ultrathin sections were cut on an LKB

ultratome 1, stained with methanolic uranyl acetate and aqueous lead citrate, and examined with a Hitachi HS7S electron microscope (10).

SCANNING ELECTRON MICROSCOPY: Primary cultures on glass cover slips were obtained as described above and were fixed for up to 7 days in buffer S, 2% glutaraldehyde with 0.5% paraformaldehyde, at 4°C. The fixed cultures were dehydrated by rinsing with graded alcohols and acetone, dried at the critical point of liquid $CO_2$, and ~20 nm of gold was evaporated onto the cell surface. The cultures were then examined with a Jeol 100C electron microscope with scanning attachment (10).

## *Estimation of DNA Synthesis and Cell Division*

PRIMARY CULTURES: After a total of 67 h of incubation, 1 $\mu$Ci/ml of [$^3$H]thymidine at 1 $\mu$M was added to the cultures. 2 h later, the cultures were washed and the uptake of [$^3$H]thymidine incorporated into DNA ($^3$H-cpm/$\mu$g of DNA) was determined as before (11, 17). Results given are the mean of three separate dishes ± the standard error.

SECONDARY CULTURES: After the hormonal additions, the cells were exposed for 8 h to 3 $\mu$Ci/ml of [$^3$H]thymidine at either 1 $\mu$M or 3 $\mu$M for autoradiography or for determination of [$^3$H]thymidine incorporation into DNA, respectively. After a further 32 h the cultures were processed as previously described (30), and the total counts per minute per dish were recorded. This time period was chosen to maximize the measurable DNA synthesis without division of radioactively labeled cells in 5% or 10% serum. In the epithelial cell cultures only, percentages of radioactively labeled nuclei in the cell colonies were recorded. Cells were detached 48 h after addition of the hormones by treatment with trypsin, dispersed, and counted in a Coulter Counter (Model ZBI, Coulter Electronics Ltd., Dunstable, Bedfordshire, England) (30). Results for DNA synthesis for normal cultures are the average of two separate experiments from two pools of four mammary glands (six samples in all), while the mean results from two separate matched tumors were recorded (six samples in all) ± standard errors, except where indicated. Each experiment contained triplicate samples in both autoradiographic and scintillation counting except where indicated.

## *Measurement of Cellular Properties*

### CASEIN SYNTHESIS

Cultures of slow-sticking epithelium from normal glands or tumors were grown in 9-cm petri dishes containing 10 ml of DEM and either 10% or 5% FCS. BALB/c 3T3 mouse fibroblasts were plated at 4 × $10^5$ cells/dish and grown as described above in 10% FCS. After 3 days (~70% confluent) 100 $\mu$Ci/ml of [$^{35}$S]methionine in $^1/_{10}$ the normal methionine concentration in DEM was added and the cultures were incubated a further 4 h (11, 39). Medium was removed, the cell monolayers were washed twice with isotonic saline and stored frozen. Cells were thawed and scraped off into 1.5 ml of 0.01 M phosphate-buffered saline, pH 7.4 (PBS), frozen, and thawed again. Cell debris was removed by centrifuging at 10,000 $g$ for 20 min and the supernate was retained. Rat casein-containing proteins were precipitated with rabbit antimouse casein serum and then goat antirabbit serum according to Feldman and Ceriani (5). Mixtures contained: 0.5 ml of 0.01 M PBS, 10 $\mu$l of rabbit antimouse casein (2.5 mg/ml), and 100 $\mu$l of cell extract, and these were incubated at 37°C for 1 h. Then, 10 $\mu$l of goat antirabbit serum (15 mg/ml) was added, the mixtures were incubated at 4°C for 16 h, then diluted with 1.3 ml of cold 0.01 M PBS, mixed, and centrifuged at 1,500 $g$ for 1 h. The precipitated material was dissolved in 100 $\mu$l of buffer A (0.08 M Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol, 0.002% bromophenol blue), boiled for 2 min, and applied to a 13.5 × 9 cm rectangular gel (0.7 cm sample slot) of 10.0% polyacrylamide. The sample was electrophoresed for 4 h at 100 V in the discontinuous buffer system of Laemmli (15) until the bromophenol blue dye reached the end of the gel. The gel was removed, processed for fluorography as described by Laskey and Mills (16), dried, and photographed on blue-sensitive X-ray film. Proteins were stained with Coomassie Blue. Sufficient goat antirabbit $\gamma$-globulin was added to precipitate all rabbit globulins. In controls, additions of five and 50 times the standard amounts of both antibodies precipitated no more of the radioactive component common to rat and mouse caseins observed in extracts from mammary cells.

PROTEOLYTIC ACTIVITY: For the measurement of fibrinolytic activity the conversion of serum plasminogen to plasmin by the cell activator was measured by the serum-induced hydrolysis of casein. Slow-sticking, freshly digested cultures (epithelium) were plated at 2 × $10^5$ cells/3.5-cm dish in quadruplicate in DEM and 10% FCS. After 16 h the medium was removed, the cells were washed twice with DEM, and then 1 ml of a suspension of 0.6% agarose, 2% autoclaved powdered milk (Marvel), and 0.5% dog serum in DEM was added and allowed to solidify at room temperature. Finally, 1.5 ml of 0.6% agarose in DEM was added, allowed to solidify, and the dishes were then incubated at 37°C until the casein in parallel dishes containing the same number of SV3T3 cells had completely lysed (~50–60 h). The remaining plates were then scored on a scale of 0 to + 4 (complete hydrolysis) according to the area of white, opaque casein removed, leaving clear transparent "plaques" (28). As controls, the mouse fibroblast line BALB/c 3T3 and the simian virus-transformed line scored + 1 and + 4, respectively; there was no hydrolysis without either the dog serum or the cells.

GROWTH IN AGAROSE: The slow-sticking epithelial fractions as described above were plated at $10^5$/

3.5-cm dish in quadruplicate in DEM and 10% FCS. Cells were overlayed with a suspension of 0.33% agarose and 10% FCS in DEM for 2 wk at 37°C (28). By this time, any clumps of normal cells which were obtained immediately after plating had disintegrated. Colonies of DMBA-tumor cells could be re-isolated and grown in monolayer cultures in liquid medium again.

## RESULTS

### *Cell Properties*

Three morphologically distinct types of cell have so far been identified which attach to the dish and grow in primary cultures of mammary tissue from perphenazine-treated normal rats or DMBA-induced tumors (Fig. 1). (*1*) Cells of fibroblastic appearance most of which (90%) adhered to the plastic surface within 2 h of plating (fast-sticking) (Fig. 1*b*), approximately 6 and 3%, respectively; (*2*) cells that grew from clumps of epithelium, attached 3–24 h after plating (slow-sticking) and formed plaques or colonies of cuboidal cells (Fig. 1*c*); and (*3*) large elongated cells (also slow-sticking) that migrated from the epithelial clumps to grow between the colonies (Fig. 1*c*). This presticking step was subsequently used as a method to produce cultures enriched in mammary epithelium.

SPECIFIC EPITHELIAL CELL PROPERTIES: Colonial cells from the slow-sticking primary cultures from normal and tumor cells were examined by electron microscopy. In both cases the cells in the central area were stacked several layers thick. The surface of the cells next to the medium was covered with numerous microvilli, and junctional complexes were occasionally seen between adjacent cells (Fig. 2). The remainder of the expanding colony was composed of two or three layers of cells, and the surface exposed to the medium was also covered with numerous microvilli. Desmosome-like structures were seen between the upper two cell layers but the intermediate line (intercellular contact layer) was rarely seen in ultrathin sections cut vertical to the plane of cellular growth (Fig. 2*b*). They could be seen, however, when the plane of section was cut tangentially. The surface of the intercolonial cells was devoid of microvilli, and no desmosome-like structures were seen between overlapping cells. The large intercolonial cells were tentatively identified as myoepithelial cells. Both the presence and distribution of surface microvilli were more easily seen in the scanning electron microscope. The surface topography of the primary epithelial cultures was similar to that of the secondary epithelial cultures (Fig. 3*a* and *b*) except that in the secondary cultures the surface density of the microvilli was reduced and they became generally shorter, except where confined to regions along the intercellular junctions (Fig. 3 and unpublished results). The majority of the intercolonial fibroblastic cells from nonfractionated cell suspensions of normal glands detached after several days in medium that contained no valine or D-valine instead of L-valine, whereas most of the epithelial colonial cells remained attached (Fig. 1*d*).

Primary epithelial cultures from normal gland and tumors were also tested for their ability to synthesize casein, using a rabbit antimouse serum which conveniently precipitates only one rat casein component (5). Cultures were exposed to [$^{35}$S]methionine, and the intracellular radioactive products were precipitated with rabbit antimouse casein serum, and the precipitates were analyzed on SDS polyacrylamide gels. One radioactive component comigrated with one of the four components of purified rat casein and one of the five components of purified mouse casein (Fig. 4). This was the same component of rat casein that was precipitated by antimouse casein antiserum in agreement with Feldman and Ceriani (5). No radioactive product of this gel mobility was synthesized in mouse BALB/c 3T3 cell cultures. When normal rabbit serum instead of the anticasein serum was used to precipitate tumor cell-extracts, no specific radioactive product was observed. The specific radioactive component was also observed in secondary cultures and when [$^{14}$C]amino acids or [$^{32}$P]orthophosphate (39) were used as the radioactive precursor instead of [$^{35}$S]methionine. The four radioactive components of casein synthesized in tumor cultures were also identified after precipitation of the cell extracts with renin, an agent which precipitates many phosphoproteins (11, 39). No attempt was made to see whether radioactive casein was secreted into the medium.

TRANSFORMED CELL PROPERTIES: Primary epithelial cultures from mammary tumors had a high serum-dependent proteolytic activity as measured by the hydrolysis of casein and grew in agarose, whereas neither feature was shown by cultures from normal mammary glands (Table I). The difference in serum-dependent caseinolytic activity (probably plasminogen activator) was not so large as for simian virus-transformed and nontransformed mouse 3T3 fibroblasts (Table I) (28).

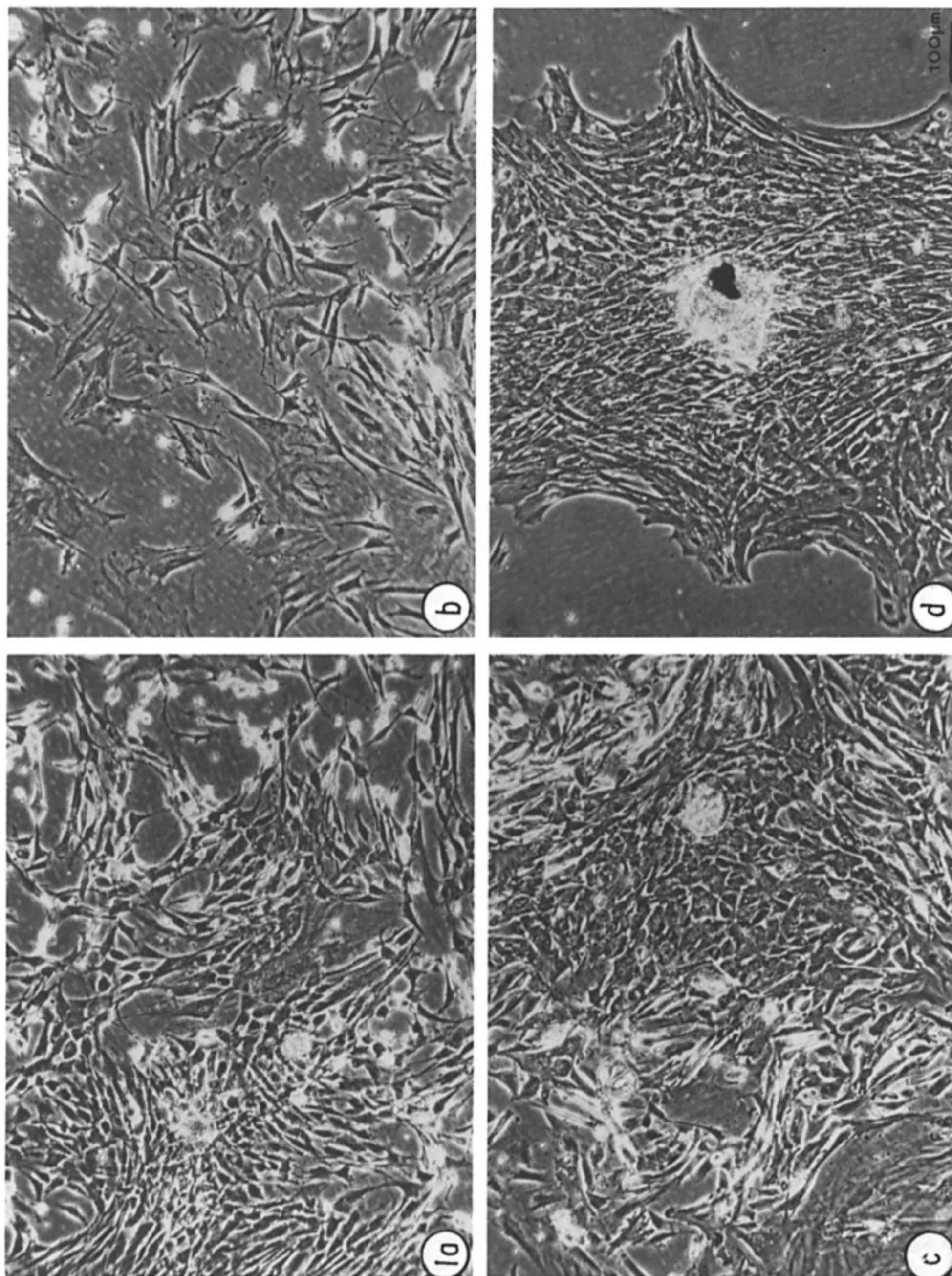



FIGURE 1 Morphology of primary cultures. Primary cultures from perphenazine-treated animals were either (*a*) plated directly or separated into (*b*) fast-sticking and (*c*) slow-sticking cell fractions and grown for 3 days. In (*d*), unseparated primary cultures were plated in medium containing twice the concentration of D-valine instead of L-valine and then incubated for 3 days. Magnification (phase contrast): × 100.

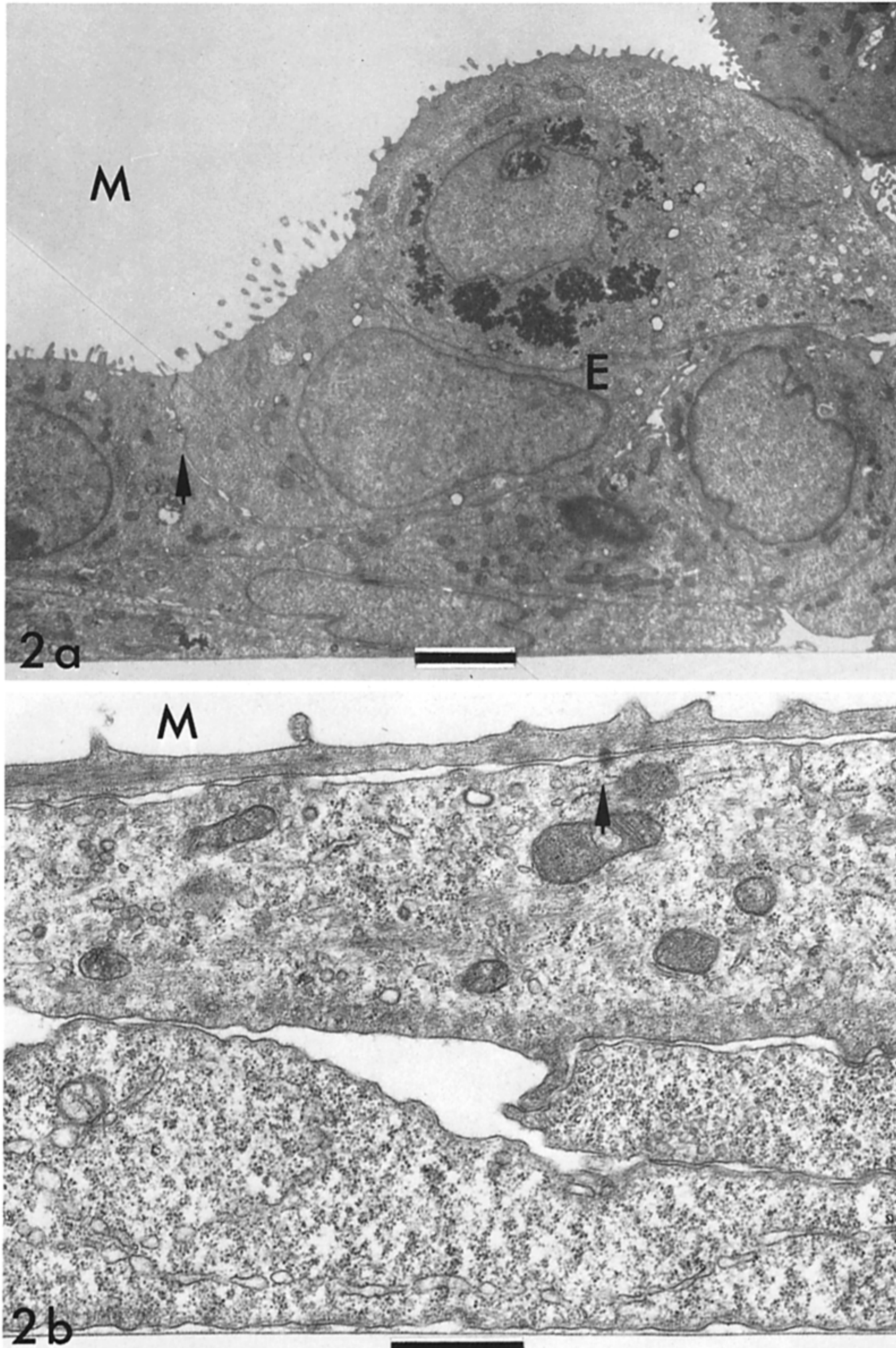
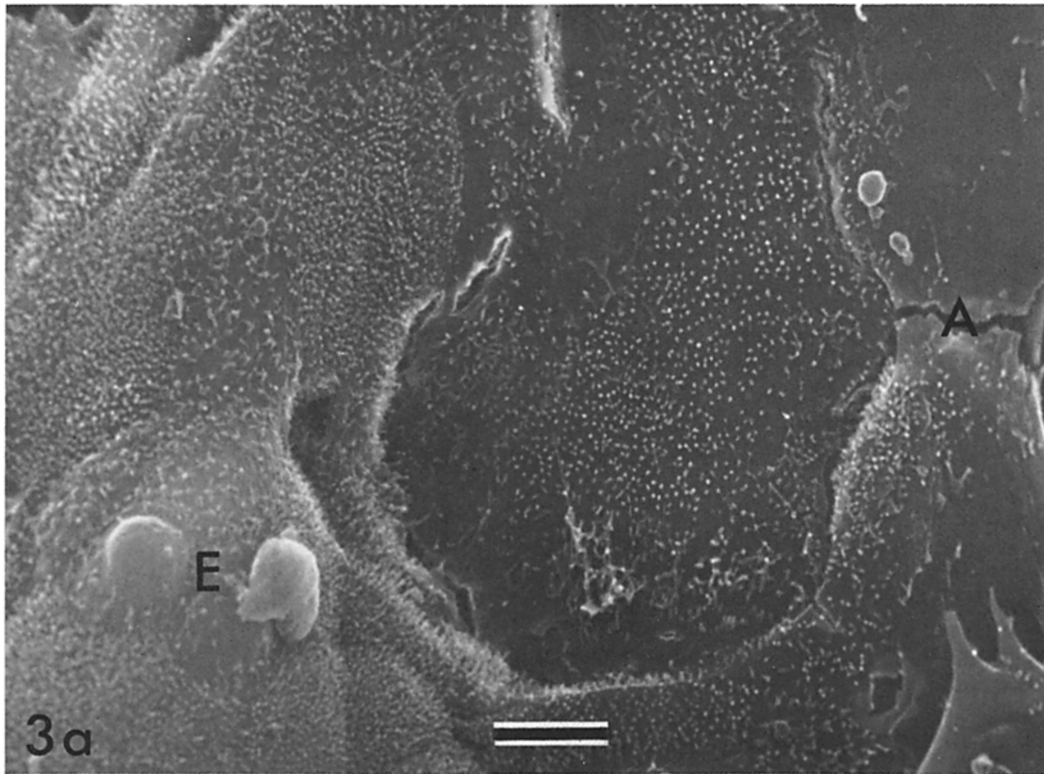



FIGURE 2 Electron micrographs of primary epithelium from tumors. Electron micrograph of a vertical section through the region of the cell contacts of epithelium from DMBA-induced tumors. Primary cultures were grown in hormone-containing medium (5 $\mu$g/ml of insulin, hydrocortisone, and prolactin) for 48 h. (*a*) The surface of the microexplant (*E*) is covered with microvilli, and a junctional complex (arrowhead) is present between the cells spreading to form the colony. M, medium. Bar, 5 $\mu$m. × 3,000. (*b*) Higher magnification showing a desmosome-like structure (arrowhead) between the two upper cells in the periphery of the colony. Bar, 1 $\mu$m. × 20,000.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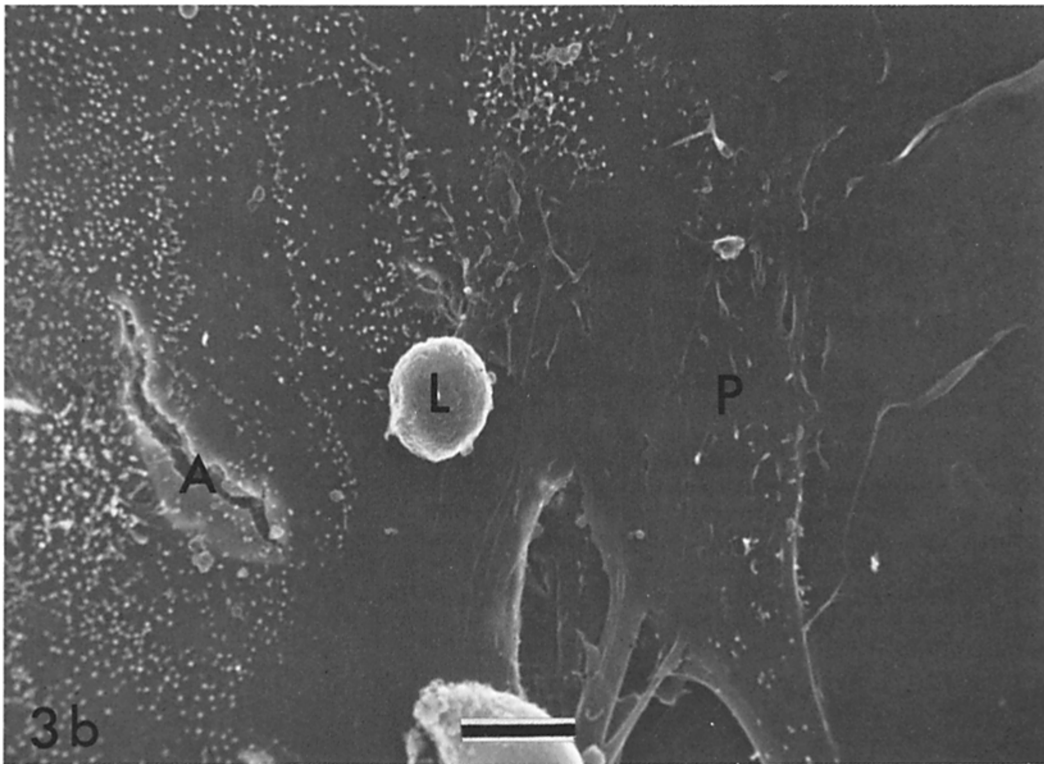
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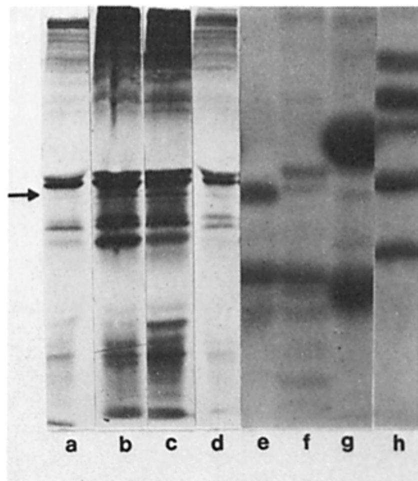


FIGURE 4 Casein Synthesis. Electrophoretogram of [$^{35}$S]methionine-containing polypeptides which were synthesized in primary rat mammary cultures and which were precipitated with rabbit antimouse casein antiserum. The low molecular weight proteins migrated to the bottom of the gel.

The [$^{35}$S]methionine-containing peptides originated from: (*a*) Balb/c 3T3 cultures; the $^{35}$S-peptides were precipitated with antimouse casein; (*b*) tumor cultures; the $^{35}$S-peptides were precipitated with antimouse casein antiserum; (*c*) normal cultures; the $^{35}$S-peptides were precipitated with antimouse casein; (*d*) tumor cultures; the $^{35}$S-peptides were precipitated with normal rabbit serum. The above samples (*a–d*) were autoradiographed (see Materials and Methods). In controls, nonradioactive polypeptides were stained and photographed (*e–g*) (see Materials and Methods). (*e*) Rat casein, (*f*) mouse casein, (*g*) rat casein preprecipitated with antimouse casein serum, and (*h*) standard proteins: phosphorylase A (100,000), BSA (68,000), catalase (58,000), alcohol dehydrogenase (41,000), and carbonic anhydrase (29,000) are shown. The two heavily stained nonradioactive polypeptides which appear solely in (*g*) arise from the antisera used. The arrow designates the electrophoretic mobility of the polypeptide common to both mouse and rat caseins. Electrophoresis was performed so that the positive electrode was at the bottom.

TABLE I

*Comparison of Caseinolytic Activity and Growth in Agarose in Primary Epithelial Cultures*

| | Caseinolytic activity (relative hydrolysis) | Growth in agarose (colonies/plate) |
|---|---|---|
| Normal gland | 2.5 | 0 |
| DMBA tumor | 4.0 | 85 ± 15 |

Slow-sticking, freshly digested epithelial cultures from perphenazine-treated rats or tumors were plated as described in Materials and Methods. The degree of removal of casein as a measurement of serum-dependent protease activity after 60 h was recorded on a scale of 0 (no hydrolysis) to + 4 (complete hydrolysis). BALB/c 3T3 and SV3T3 scored + 1 and + 4, respectively, in the same time period. The number of colonies from $10^5$ primary epithelial cells which grew in agarose is shown. $10^4$ DMBA tumor or normal cells yielded 5 ± 3 and 0 colonies, respectively.

Inoculation of cells from epithelial cultures of normal mammary gland did not give rise to tumors at the inoculation site in the rat, but mammary adenocarcinomas arose when the inoculum was that of epithelium from tumor cultures (R. C. Hallowes, unpublished results).

## *Stimulation of [$^3$H]Thymidine Uptake into DNA in Primary Cultures*

Addition of prolactin alone to tumor epithelium in medium 199 and 5% FCS caused increasing thymidine incorporation into DNA as the hormone concentration increased from 10 to 500 ng/ml; above this concentration, some inhibition occurred. In contrast, similar concentrations of prolactin had little effect in cultures from normal mammary gland (Fig. 5). In normal epithelial cultures, however, addition of prolactin with hydrocortisone and insulin caused about a twofold in-

FIGURE 3. Scanning electron micrograph of epithelium from normal glands. (*a*) Surface topography of a spreading colony from primary cultures of mammary gland from a perphenazine-treated rat. The primary cultures were grown for 48 h in medium containing hormones similar to those in Fig. 2. The microexplant (*E*) is covered with microvilli, and similar microvilli are present on the surface of the spreading colonial cells. A designates a drying artifact. Bar, 5 $\mu$m. × 3,000. (*b*) Surface topography of secondary subcultured colony derived from a primary culture of the mammary gland. The primary culture was subcultured after 48 h and the secondary culture was processed after a further 48 h in similar medium. The central colonial cells are covered with microvilli whereas the peripheral cells (*P*) have a smoother surface. A single cell, probably a lymphocyte (*L*), is also present. Bar, 5 $\mu$m. × 3,000.

crease in [$^3$H]DNA synthesis over that with hydrocortisone and insulin alone (Fig. 5). This effect was higher during 67-69 h than during 43-45 h (not shown) after cell plating.

The dose dependency curve for prolactin-induced stimulation of [$^3$H]DNA synthesis was increased by at least 10-fold when prolactin was added with hydrocortisone and insulin. Since concentrations of prolactin above 5 μg/ml were not tested, then a possible inhibition of [$^3$H]DNA in

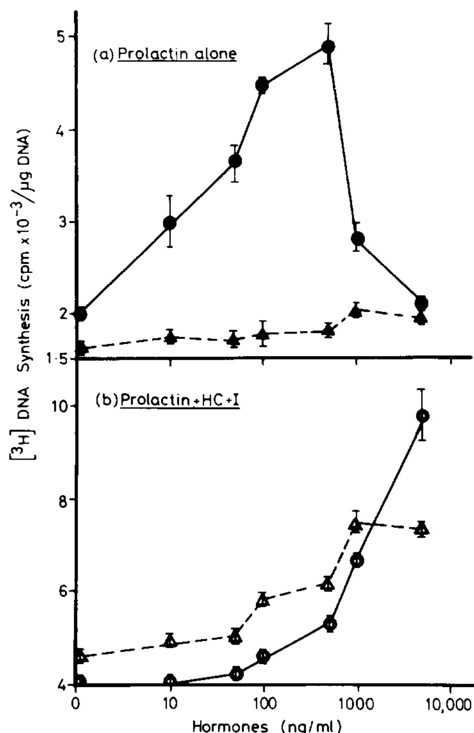


FIGURE 5 Effect of prolactin concentration on the rate of [$^3$H]DNA synthesis in primary cultures. Primary slow-sticking epithelial cultures of DMBA tumors (——) (●, ○) or normal glands from perphenazine-treated rats (- - -) (▲, △) were grown for 67 h in medium with either (*a*) different concentrations of prolactin alone (ng/ml) (●, ▲) or (*b*) different concentrations of the hormone combination, prolactin, hydrocortisone (*HC*), and insulin (*I*) (ng/ml) (○, △). The concentration of all three hormones is the same in each instance. The counts per minute of [$^3$H]thymidine incorporated per microgram of cell DNA were recorded after exposing the cultures for 2 h to [$^3$H]thymidine (see Materials and Methods). Results are the means ± standard deviations of duplicate cultures from either four pools of normal mammary glands each derived from three rats treated with perphenazine or six pools each derived from separate tumors.

the presence of hydrocortisone and insulin may not be seen as in the case when prolactin is added alone.

### *Initiation of DNA Synthesis and Cell Division in Secondary Cultures*

Secondary cultures of greater cell purity than the corresponding primary cultures were readily established from primary cultures of fibroblasts and epithelium from either mammary glands or tumors. These were grown in DEM instead of medium 199. Growth of surface cultures could be virtually stopped in 4-5 days by lowering the serum concentration to 0.5% or by replacing it by 250 μg/ml BSA. The addition of saturating concentrations of 500 ng/ml ovine prolactin (determined as described in the previous section) with 50 ng/ml insulin, 500 ng/ml hydrocortisone, and BSA induced more epithelial cells to synthesize DNA in 40 h in normal or tumor epithelium (15-20%) but failed to increase the response in fibroblasts (Table II). Cells initiating DNA synthesis in this time were located mainly within rather than between the epithelial colonies (Fig. 6). The small numbers of fibroblasts (<10%) in cultures from normal glands were not stimulated to synthesize DNA. The addition of saturating concentrations

TABLE II

*Stimulation of DNA Synthesis in Secondary Cultures from Stromal and Epithelial Fractions*

| Additions to basal medium | Radioactive nuclei: Normal cells | | Tumor cells | |
|---|---|---|---|---|
| | Stroma | Epithelium | Stroma | Epithelium |
| | % | | | |
| None | 0.4 | 1.0 | 1.6 | 0.3 |
| Prolactin | 1.1 | 2.3 | 1.3 | 10.0 |
| HC + I | 1.3 | 2.4 | 4.1 | 0.1 |
| Prolactin + HC + I | 4.4 | 21.0 | 2.1 | 15.8 |
| FGF | 19.5 | 4.2 | 8.3 | 1.6 |
| FGF + HC + I | 18.5 | 2.5 | 17.0 | 2.8 |
| Serum (10%) | 46.0 | 24.0 | 56.0 | 33.4 |

Secondary cultures from either the fast-sticking (stromal) or slow-sticking (epithelial) fractions from normal gland and tumors were grown for 2 days (Materials and Methods) and then the medium was changed to one containing 500 ng/ml hydrocortisone, 50 ng/ml insulin, and 250 μg/ml BSA (basal medium). After 6 additional days (cell density: 2 × 10$^5$) various hormones were added (ng/ml): prolactin, (500); hydrocortisone (HC), (500); insulin (I), (50); fibroblast growth factor (FGF), (50); and 10% FCS. The percentage of cells with radioactively labeled nuclei was recorded from 8 until 40 h after the additions and only epithelial cells in colonies were scored. [$^3$H] was added after 8 h. Results are the average of two separate experiments from two pools of four mammary glands or two separate matched tumors.

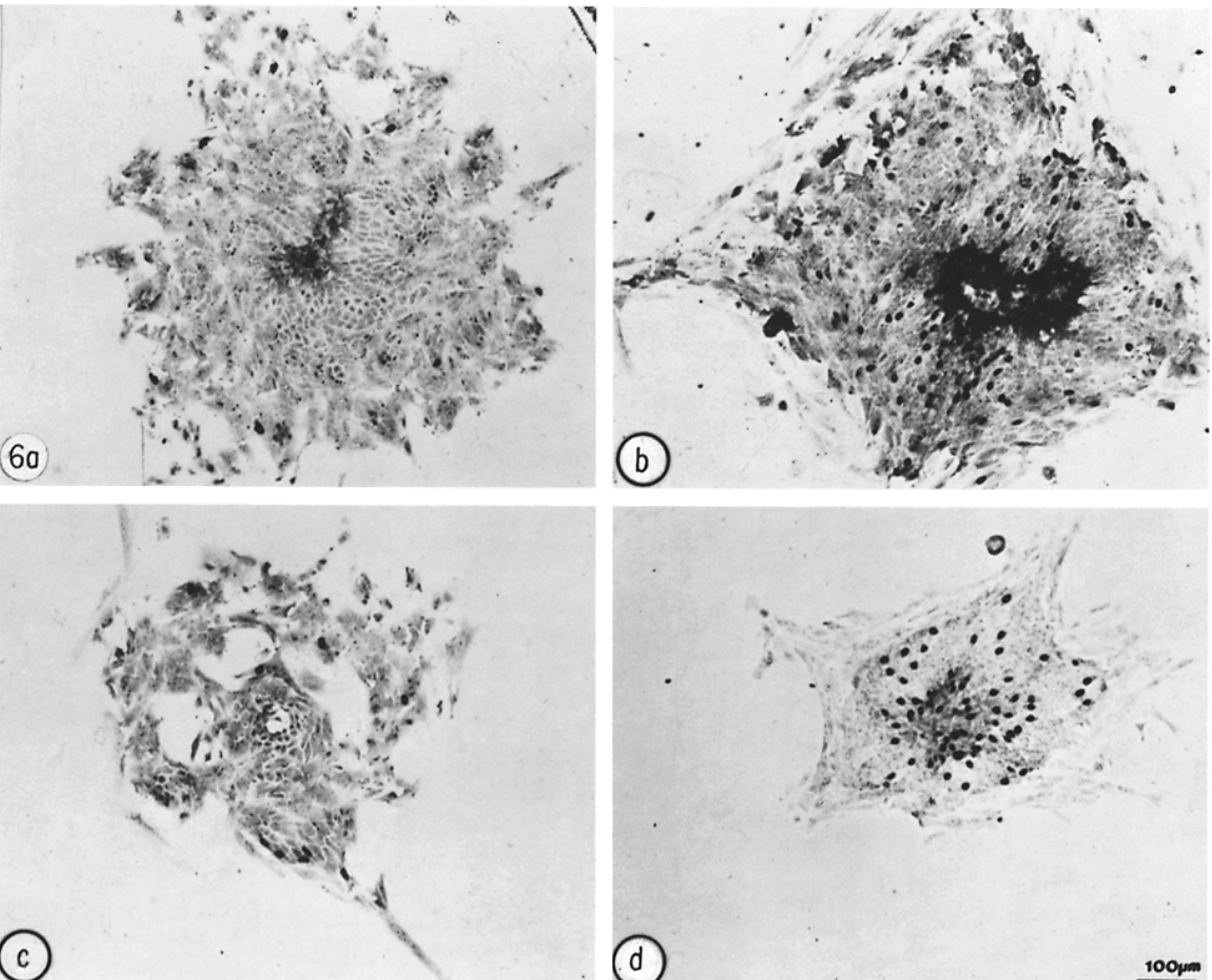


FIGURE 6 Autoradiographs of DNA synthesis in secondary cultures. Autoradiographs of (*a*) unstimulated normal secondary cultures; (*b*) prolactin, hydrocortisone and insulin-stimulated normal cultures; (*c*) unstimulated secondary tumor cultures; and (*d*) prolactin, hydrocortisone, and insulin-stimulated tumor cultures. Conditions were the same as for the epithelial fractions from Table III. Magnification (bright field): × 100.

(30) of 50 ng/ml bovine fibroblast growth factor instead of prolactin initiated more DNA synthesis in fibroblastic than in epithelial cells (Table II) in the same time period. Prolactin alone had little effect on DNA synthesis in epithelium from normal mammary glands (2–4% labeled nuclei) but was synergistic, either separately (10%) or additively (18%) with insulin and hydrocortisone. However, addition of prolactin caused a substantial increase in DNA synthesis of the epithelial cells from tumors (10%) (Table II), which was further increased by hydrocortisone and insulin (15%). Hydrocortisone and insulin had no noticeable effects. The relative effects of different hormone combinations were similar for different tumors but their absolute magnitudes varied from tumor to tumor. This amounted to a variation of 15% (Table II) to 30% (Table V) of the cells initiated to synthesize DNA by the combination of the three hormones in 40 h. A smaller difference in the means (15–20%) was observed for cultures from normal glands of single perphenazine-treated animals.

The increases in radioactively labeled nuclei over controls qualitatively paralleled both the increased incorporation of [$^3$H]thymidine into DNA in 40 h and the increased cell numbers after 48 h in the stimulated stationary cultures from both normal glands (Table III) and tumors (not shown). But quantitative differences, however, were observed in the relative increases of the three parameters after addition of different combinations of hormones. In particular, the disparity between the inhibition of [$^3$H]DNA synthesis and the increase in the labeling index upon addition of hydrocortisone to cultures containing prolactin was repeatedly observed. The same fractional increases in DNA synthesis and cell numbers were seen in cultures in which the fibroblastic cells had been reduced to 1% or less by preincubation of the cultures with medium containing D-valine. The incorporation of [$^3$H]thymidine markedly increased after 14 h, reached maximum rates during 20–28 h, and the majority of cells had divided by 48 h after the addition of the three hormones to either tumor or normal cultures. However, we have not formally shown that the cells which initiated DNA synthesis were the same cells which later divided. Serum-free controls were performed; qualitatively similar increases in the proportion of cells synthesizing DNA were observed after the addition of the three hormones to tumor cultures which had ceased growing in either a medium enriched in certain extra amino acids and vitamins compared with the standard DEM or in DEM containing no exogenously added serum or BSA (Table IV). The absolute magnitude of the response was higher in the enriched medium and lower in DEM than in DEM and 0.5% serum, although the synergistic effect of adding hydrocortisone and insulin at the same time as prolactin

TABLE III

*Stimulation of DNA Synthesis and Cell Division in Normal Epithelial Cells from Secondary Cultures*

| Additions to basal medium | DNA synthesis: [$^3$H]DNA | DNA synthesis: Labeled nuclei | Cell number ($\times 10^{-5}$) |
|---|---|---|---|
| | *cpm* | *%* | |
| None | 3,620 ± 190 | 2.0 ± 0.2 | 2.83 ± 0.06 |
| I + HC | 3,840 ± 410 | 2.1 ± 0.1 | 2.87 ± 0.08 |
| P | 3,580 ± 250 | 4.3 ± 0.1 | 2.84 ± 0.10 |
| P + I | 4,100 ± 210 | 10.3 ± 0.2 | 3.31 ± 0.05 |
| P + HC | 3,080 ± 40 | 10.0 ± 0.3 | 3.08 ± 0.11 |
| P + I + HC | 10,000 ± 450 | 17.9 ± 2.8 | 3.68 ± 0.25 |
| Serum (20%) | 31,700 ± 1,600 | 42.2 ± 2.5 | 4.21 ± 0.13 |

Secondary epithelial cultures from normal glands of perphenazine-treated rats were plated in DEM plus 10% serum and then 1 day later the medium was changed to 0.5% serum. The hormones were added after 6 days at the following concentrations: ovine prolactin (P), 500 ng/ml; hydrocortisone (HC) 500 ng/ml; insulin (I), 100 ng/ml. The counts per minute incorporated into DNA per culture, the number of radioactively labeled nuclei, and cell numbers were recorded (Materials and Methods) after 40 and 48 h, respectively. Results are the means ± standard errors of two separate experiments from two pools of four mammary glands; each experiment contained triplicate samples (six samples in all). The disparity between the counts per minute of [$^3$H]DNA synthesis and the fraction of cells with labeled nuclei observed after addition of P + HC compared with P alone was repeatedly observed.

TABLE IV

*Stimulation of DNA Synthesis in Secondary Epithelial Cultures from Tumors in Different Media*

| Additions to basal medium | DNA synthesis (% labeled nuclei): DEM | DEM + BSA | DEM + 0.5% serum | Enriched DEM + 0.5% serum |
|---|---|---|---|---|
| None | 1.2 | 0.6 | 4.1 | 8.6 |
| P | 3.0 | 10 | 16 | 33 |
| P + HC + I | 8.7 | 16 | 20 | 29 |
| Serum (10%) | 11.5 | 29 | 23 | 23 |

A single tumor was processed and the slow-sticking epithelium from secondary cultures was grown for 6 days in basal medium which contained one of the four following combinations: Dulbecco's modified Eagle's medium (column 1, DEM); DEM with 250 μg/ml bovine serum albumin (column 2, DEM + BSA); DEM with 0.5% serum (column 3); or DEM plus 0.5% serum enriched for the last two days with extra nutrients and vitamins not present in DEM (column 4, Enriched DEM + 0.5% serum). All the basal media (DEM) contained 500 ng/ml hydrocortisone, 50 ng/ml insulin added at the start of the incubation. After 6 days, prolactin (P), hydrocortisone (HC), insulin (I), or 10% fetal calf serum (serum) were added at the concentrations noted in Table III, and the percentage of cells with radioactively labeled nuclei is shown after 40 h. Samples were processed in triplicate (Materials and Methods).

was lost in the enriched medium. Finally, the growth of normal or tumor cultures stimulated with prolactin, hydrocortisone, insulin, and 0.5% serum was enhanced over that with 0.5% serum alone. The final cell densities of the cultures were ~50% (normal) and 100% higher (tumor) than without the three hormones, although these values were still only 35% (normal) or 50% (tumor) of those achieved with 10% FCS (not shown).

### *Hormonal Specificity for the Initiation of DNA Synthesis*

Various hormones and factors from the pituitary and submaxillary glands were tested for their ability to stimulate DNA synthesis in secondary cultures from tumors when added with 50 ng/ml insulin and 500 ng/ml hydrocortisone. 50 ng/ml bovine growth hormone, 10 ng/ml mouse epidermal growth factor (3), and 50 ng/ml of preparations of ovarian growth factor (8) all significantly increased the numbers of radioactively labeled nuclei in 40 h (Table V). Higher concentrations (50 ng/ml) of epidermal growth factor were inhibitory. Both ovine and rat prolactin at 50 ng/ml induced about 10% of the epithelial cells to synthesize DNA, but this was increased to a maximum of about 30% in the same time period when the concentration was 500 ng/ml. Higher concentrations were inhibitory. Fibroblast growth factor, luteinizing hormone, follicle-stimulating hormone, and thyrotropin had little effect at 50 ng/ml. Similar results were obtained for secondary cultures from normal glands (not shown).

Different steroid hormones at 5 ng/ml were also tested for their ability to synergize with prolactin and insulin in stimulating [$^3$H]DNA synthesis (Table VI). Hydrocortisone, deoxycorticosterone, progesterone, and prednisone all stimulated whereas testosterone inhibited [$^3$H]DNA synthesis. The maximum stimulations induced by hydrocortisone were activated at concentrations of 5–50 ng/ml in secondary cultures from tumors (Table VI) or normal glands (not shown). Corticosterone, the natural glucocorticoid in rats, failed to stimulate [$^3$H]DNA synthesis at 5 ng/ml but did so at 50 ng/ml; this may reflect higher affinities of hydrocortisone for cellular receptors or enhanced

TABLE V

*Pituitary Hormone Specificity for DNA Synthesis in Secondary Cultures from Tumors*

| Additions to basal medium containing hydrocortisone and insulin (ng/ml) | DNA synthesis (% labeled nuclei) |
|---|---|
| None | 4.3 ± 0.1 |
| Epidermal growth factor (10) | 13.1 ± 0.7 |
| Fibroblast growth factor (50) | 6.4 ± 1.0 |
| Growth hormone (50) | 13.9 ± 0.2 |
| Luteinizing hormone (50) | 7.9 ± 0.9 |
| Follicle-stimulating hormone (50) | 4.5 ± 0.8 |
| Thyroid-stimulating hormone (50) | 6.6 ± 1.1 |
| Ovarian growth factor (50) | 10.5 ± 1.5 |
| Prolactin (ovine) | |
| (50) | 12.4 ± 1.4 |
| (500) | 33.0 ± 1.0 |
| Prolactin (rat) | |
| (50) | 15.1 ± 2.8 |
| (500) | 29.5 ± 2.5 |
| Serum (20%) | 46.0 ± 3.0 |

Triplicate secondary epithelial cultures from two matched and pooled DMBA tumors remained for 6 days in DEM and 0.5% serum, then 500 ng/ml hydrocortisone, 50 ng/ml insulin were added (basal medium) together with various polypeptide hormones. The percentage of cells with radioactively labeled nuclei in 40 h ± SEM is recorded.

TABLE VI

*Steroid Hormone Specificity for [$^3$H]DNA Synthesis in Secondary Cultures from Tumors*

| Additions to basal medium containing prolactin and insulin | [$^3$H]DNA |
|---|---|
| | *cpm/dish* |
| None | 11,700 ± 540 |
| Estradiol | 12,100 ± 650 |
| Estradiol* | 8,340 ± 570 |
| Estrone | 8,890 ± 1,300 |
| Estriol | 9,020 ± 410 |
| Cortisone | 9,250 ± 160 |
| Aldosterone | 8,890 ± 280 |
| Corticosterone | 12,100 ± 1,300 |
| Deoxycorticosterone | 13,500 ± 800 |
| Hydrocortisone | 26,800 ± 3,700 |
| Progesterone | 15,400 ± 880 |
| Androstanolone | 10,500 ± 370 |
| Prednisone | 15,000 ± 200 |
| Testosterone | 6,120 ± 940 |
| Serum (20%) | 50,600 ± 550 |

Secondary cultures from two matched and pooled DMBA tumors were set up as described (Materials and Methods) in DEM and 0.5% serum (without further additions: 4,870 cpm/dish). Then 500 ng/ml prolactin and 50 ng/ml insulin were added (basal medium) together with different steroids at 5 ng/ml, except estradiol* at 500 ng/ml. The counts per minute of [$^3$H]thymidine incorporated into DNA per culture dish were recorded from 8 until 40 h after the additions. Corticosterone at 50 ng/ml yielded 25,870 ± 840 cpm.

protection against breakdown. Estradiol at 1–5 ng/ml caused little increase in [$^3$H]DNA synthesis (<5%) in secondary cultures from normal glands (not shown) or tumors (Table VI). At higher concentrations, 500 ng/ml estradiol inhibited [$^3$H]DNA synthesis in cultures from tumors (Table VI).

## DISCUSSION

Prolactin, growth hormone, estrogen, and progesterone have been implicated in the maintenance of the cellular architecture of the mammary gland in mice and rats (18, 21). The relative importance of pituitary hormones in vivo, particularly prolactin, has been suggested by experiments in which prolactin or prolactin plus growth hormone caused lobulo-alveolar development of the normal mammary gland or growth of DMBA-induced mammary tumors after removal of the pituitary, adrenals, and ovaries of the rat (25, 34, 35). Although Oka and Topper showed that injections of prolactin into virgin mice rendered their mammary glands additionally sensitive to the mitogenic effects of insulin and serum in organ cultures (22), prolactin per se, however, was found not to be mitogenic under similar conditions in vitro (19, 22, 33, 38, 40). Oka and Topper then suggested that prolactin acts indirectly as a mitogen rendering the mammary cells susceptible to the primary mitogens, insulin, and serum. Since then, prolactin has, however, been shown to stimulate the incorporation of thymidine into DNA in short-term organ cultures of mammary glands from immature (4), mature, midpregnant rats (11) and mice (20), and of DMBA-induced rat mammary tumors (17, 41).

In the previous studies, high nonphysiological concentrations of hormones (1–5 $\mu$g/ml) were used in mammary gland explant cultures. These cultures, however, contained several different cell types including the secretory mammary epithelial cell. Here, we establish a method to prepare surface mammary epithelial cell cultures. After the presticking step, secondary cultures from mammary glands of perphenazine-treated rats and DMBA tumors contain, respectively, <10% (about $10^4$ cells per dish) and only 1–2% contamination by cells of fibroblastic appearance before the addition of the hormones. Fibroblastic cells can be virtually eliminated from these two cultures by incubating them in a medium which contains no valine. The epithelial derivation of colonial cells is confirmed by the presence of specific junctional complexes, desmosome-like structures, and microvilli, which are all found in both mammary gland and mammary tumors (10, 42), and the colonial cells' survival in medium that contains D-valine instead of L-valine. This latter property is considered to be relatively specific for epithelial cells (6), although in our system, medium containing no valine is just as efficient as that containing D-valine in allowing selective survival of epithelial cells (D. Bennett and H. Durbin, unpublished results). The origin of the epithelial cell cultures is further substantiated by their synthesis of a phosphopolypeptide in vitro (39) which is immunoprecipitable with antiserum against mouse casein and which comigrates on polyacrylamide gels with the one common component of rat and mouse caseins (5).

Certain changes in cellular properties often accompany the transformation of fibroblasts in tissue culture (12, 23, 26–31). These include alterations in cellular architecture, increased proteolysis, the ability to grow in agar or agarose, an enhanced ability to form tumors in immune-deficient animals, a failure to reduce growth rates and accumulate in a specific region of the cell cycle ($G_1$), and an altered requirement for growth-controling hormones. So far, we have detected no microscopic differences between normal and DMBA tumor epithelial cells in culture. Epithelial cultures from DMBA tumors show an increase in serum-dependent proteolysis (probably plasminogen activator) compared with cultures from normal glands, although this difference is not so large as for simian virus-transformed and nontransformed mouse fibroblasts (28). Likewise, tumor epithelial primary cultures can grow in agarose and form new tumors in animals whereas cultures from normal glands do not, as found with established rat epithelial cell lines (2). However, a reduction in the concentration of serum to 0.5% in the medium of both growing DMBA tumors and normal epithelium causes a reduction in cellular growth such that <4% of the cells are synthesizing DNA in 32 h. This corresponds to a constant number of cells attached to the petri dishes. It does not, however, exclude a slow but continuous process of cell division matched by an equal rate of loss of attached cells to the medium. Growth recommences after the addition of 10% serum, with a wave of DNA synthesis followed by cell division (H. Durbin, unpublished results). This suggests that a large proportion of the tumor cells are in the $G_1$ phase of the cell cycle at the lower growth rate,

unlike virally transformed fibroblasts (12, 23, 29), although the exact distribution in the cell cycle is unknown.

Both growing mammary epithelium and DMBA tumor epithelium show increased rates of incorporation of thymidine into DNA with prolactin, hydrocortisone, and insulin. These hormones can also initiate an increased fraction of cells to synthesize DNA (about 20%) and divide in the specified time period in quiescent cultures maintained in low concentrations of serum. Since the cultures contain low levels of contaminating fibroblastic cells, these experiments were also repeated with D-valine-treated cultures virtually free of fibroblastic cells (Materials and Methods), with the same results. However, prolactin alone stimulates DNA synthesis in tumor but not in normal epithelium. Prolactin concentrations as low as 10 ng/ml cause a significant stimulation of thymidine incorporation into DNA. However, maximum stimulation (corresponding to 20–30% of the epithelial cells synthesizing DNA in this time) is only achieved at 200–500 ng/ml, concentrations which are slightly higher than the 10 ng/ml to 100 ng/ml range encountered in the female Sprague-Dawley rat (1). It is unlikely that this high level is due to differences in the specificity of different animal prolactins for binding to our cultured cells, since ovine, rat (Table V), and bovine (not shown) prolactins give the same results. Thus, either a subfraction of the preparation of prolactin from different sources is responsible for these growth-promoting effects (8) or additional hormones are required to sensitize the mammary epithelium to lower concentrations of prolactin. Bovine growth hormone also induces a significant fraction of the cells to synthesize DNA in 40 h, and this is probably not due to contamination by prolactin, since 50 ng/ml of growth hormone is sufficient to elicit a significant response, in accordance with its mammotrophic role in vivo (18, 21, 34). Epidermal growth factor at 10 ng/ml has also been shown to stimulate the incorporation of [$^3$H]thymidine into DNA in mouse mammary explant cultures (37), although its physiological role is unknown.

The major group of steroid hormones required to increase the growth-promoting effect of prolactin plus insulin were the glucocorticoids and, in particular, hydrocortisone at near physiological concentrations (5 ng/ml). Surprisingly, very little effect of the estrogens was observed with prolactin in cultures in low serum, although at higher concentrations (500 ng/ml) they inhibited the incorporation of thymidine into DNA in DMBA tumor epithelium (41), similar to their pharmacological effect in causing regression of DMBA tumors in rats (13, 25). From visual inspection of primary cultures from DMBA tumors, most of the epithelium arose from the lobulo-alveolar region and not the ductal-cell system. Hence, the relative lack of sensitivity to the estrogens and the increased sensitivity to progesterone (Table VI) may be a reflection of the differential sensitivity of the mature alveolar cell to these hormones (18, 21). The physiological role of insulin in mammary growth and development is unclear (22). But its requirement in tissue culture at near physiological concentrations with a pituitary hormone and hydrocortisone for maximum increases in DNA synthesis may reflect a requirement for maintenance of cell homeostasis through a supply of various nutrients (14).

The pattern of increases in [$^3$H]DNA synthesis induced by various hormones in primary and secondary cultures is similar. However, hydrocortisone can stimulate [$^3$H]DNA synthesis in growing primary cultures in 5 or 10% serum (unpublished results), but failed to stimulate DNA synthesis in quiescent secondary cultures maintained in 0.5% serum. This is similar to the effect of the glucocorticoid in promoting the initiation of DNA synthesis in mouse fibroblasts in 10% serum (36) but not in 0.5% serum or less (30) in the same time period. In secondary cultures in 0.5% serum, the fractional increase in [$^3$H]DNA synthesis and the fraction of cells with [$^3$H]thymidine-labeled nuclei in 40 h are changed in the same direction after additions of hormones, but may differ in a quantitative fashion. This possibly reflects the different methods used for their determination and standardization (Materials and Methods). The disparity that the addition of hydrocortisone to cultures containing prolactin inhibited [$^3$H]DNA synthesis but stimulated the labeling index may be explained by the reduction in the rate of [$^3$H]thymidine uptake although more cells were actually synthesizing DNA. Addition of prolactin, hydrocortisone, and insulin, however, initiates DNA synthesis in fewer cells than rat (not shown) or FCS under the same conditions, fails to support unrestricted epithelial cell growth, and yields final epithelial cell densities 50% or less than that with 10% serum. These facts suggest that additional growth-promoting agents are present in serum. The fact that primary cultures of normal cells were obtained by enzymatic digestion of prolactin-stim-

ulated glands from perphenazine-treated rats may reduce the general significance of our results in vitro. However, similar hormonally induced changes were produced in cultures from nonstimulated glands (unpublished results), and although protease treatment may temporarily damage the primary cells, their integrity is sufficiently maintained to reproduce some aspects of their physiological response.

In conclusion, we have established a method to culture relatively pure epithelial cells from normal mammary glands of perphenazine-treated rats or from DMBA-induced mammary tumors and characterized them with respect to their mammary epithelial and transformation-associated properties. The pattern of hormones that can stimulate DNA synthesis in tumor and normal mammary epithelium closely resembles that of the hormones thought to control their growth and development in the rat. The minimum requirements for pituitary prolactin, hydrocortisone, and insulin are similar to the requirements for the hormones that can stimulate DNA synthesis in some established lines of mouse fibroblasts, the bovine pituitary fibroblast growth factor replacing prolactin (7, 30). Perhaps the synergism between a corticoid, insulin, and a target-specific pituitary polypeptide may represent the basis of a general endocrine pattern in the control of cell proliferation in different tissues.

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