Comparison of the Effects of Hormones on DNA Synthesis in Cell Cultures of Nonneoplastic and Neoplastic Mammary Epithelium from Rats

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SUMMARY

Mammary glands from virgin and perphenazine [1,2-hydroxyethyl-4,3-(2-chloro-10-phenothiazinyl)piperazine]-treated rats, hormone-dependent mammary tumors induced by dimethylbenzanthracene, and hormone-independent transplantable mammary tumors were digested with enzymes. Cells from the resulting epithelial microexplants and stroma were grown on plastic dishes in Medium 199 with fetal calf serum either with or without mammatrophic hormones. To determine the effects of mammatrophic hormones on the growth of the cultured cells, tritiated thymidine was added during the growth phase and its uptake into DNA was measured.

\[ {\text{[\text{H}]DNA synthesis of the stromal cells was not altered by the addition of mammatrophic hormones. \[ {\text{[\text{H}]DNA synthesis of the epithelial cultures from the transplantable tumors was insensitive to mammatrophic hormones, whereas that from nonneoplastic mammary glands and from induced tumors was stimulated by the addition of prolactin, glucocorticoids, insulin, and progesterone. Epithelial cultures from virgin and perphenazine-treated rats required both corticosterone and insulin for prolactin to initiate significant increases in \[ {\text{[\text{H}]DNA synthesis, whereas prolactin alone stimulated \[ {\text{[\text{H}]DNA synthesis in epithelial cultures from dimethylbenzanthracene-induced tumors. The effects of hormones on DNA synthesis in cell cultures from the different mammary tissues were similar to the influence of these hormones on the growth of mammary tissues in vivo.}}\]

INTRODUCTION

It is often difficult to both define and interpret the effects of a single hormone on its target cell in the breast either because of interrelated changes induced in the experimental animals by the techniques being used or because of the heterogeneous cellular composition of the target organ. Organ culture (26), in which small pieces of intact mammary gland are maintained for up to 7 days in chemically defined medium, has been used by several investigators (13, 27) to demonstrate either the effects of single hormones or the interrelationships between a number of hormones and control of mammary DNA, RNA, and milk-specific protein and carbohydrate synthesis. Most of these studies have been carried out on highly differentiated mammary gland such as those obtained from midpregnant or lactating animals. Although some reparative DNA synthesis does occur in such organ cultures (22), this technique is not entirely suitable for studies on cell proliferation in neoplastic tissues, since only a small fraction of the cells replicate during the time of culture and the cell population within the explants consists of both epithelial and nonepithelial cells. A more suitable technique would be cell culture in which numbers of cells from a defined cellular population can be studied under controlled and isolated conditions (18). There may be difficulties, however, in correlating results obtained with cell culture with those from intact organs due to possible changes caused by disrupting normal cell-cell relationships, removal of cell surface receptors, and nonphysiological growing conditions. Mammary tissue is an ideal tissue in which to evaluate the possible use of cell culture techniques to study the hormonal control of epithelium, as this tissue has been extensively studied both in the whole animal and in organ culture. This paper describes studies of the effects of hormones on primary cultures of rat normal mammary gland, rat mammary gland made hyperplastic after 2 injections of perphenazine,1 mammary adenocarcinomas induced by DMBA, and transplantable mammary adenocarcinomas. A preliminary report of part of this study has been published (17).

MATERIALS AND METHODS

Materials

Medium TC 199 was purchased from Wellcome Reagents Limited, Beckenham, Kent, England; 2 batches of FCS, supplied by Difco Laboratories, West Molesey, Surrey, England, were used for all these experiments and were not absorbed with dextran-coated charcoal to avoid the non-

1 The abbreviations and trivial names used are: perphenazine, 1,2-hydroxyethyl-4,3-(2-chloro-10-phenothiazinyl)piperazine; DMBA, dimethylbenzanthracene; FCS, fetal calf serum; BSA, bovine serum albumin; corticosterone, 11\(\beta\)-21-dihydroxy-4-pregnen-3,20-dione; cortisol, 11\(\beta\),17\(\alpha\)-21-trihydroxy-4-pregnen-3,20-dione; 17\(\alpha\)-estradiol, 1,3,5(10)-estratrien-3,17\(\alpha\)-diol; 17\(\beta\)-estradiol 1,3,5(10)-estratrien-3; testosterone, 17\(\beta\)-hydroxy-4-androstan-3-one; progesterone, 4-pregnen-3,20-dione; DEM, Dubecco's modified Eagle's medium; FGF, fibroblast growth factor; OGF, ovarian growth factor; EGF, epidermal growth factor; i.g., intragastric.
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specific absorption of unknown growth-promoting factors. Plastic dishes were from NUNC Plastics, Stone, Staffordshire, England; and BSA was from Armour Pharmaceutical Co., Ltd., Eastbourne, Sussex, England. PECAP monofilament cloth was supplied by Swiss Silk Bolting Cloth Manufacturing Co. Ltd., Zurich, Switzerland.

Collagenase type 1 (150 units/mg), hyaluronidase type I (460 units/mg), crystalline insulin (26.4 IU/mg), type V calf thymus DNA, and thymidine were purchased from Sigma Chemicals, Kingston-upon-Thames, Surrey, England.

Corticosterone, cortisol, 17α-estradiol, and 17β-estradiol were purchased from Steraloids, Croydon, England. Testosterone was purchased from BDH Biochemicals, Poole, Dorset, England, and was recrystallized from ethanol. Progesterone was purchased from G. D. Searle, High Wycombe, Buckinghamshire, England. NIH-P-S-11, 29.5 IU/mg (ovine prolactin), and NIH GH-B14, 1.04 IU/mg (bovine growth hormone), were generously donated by the NIH, Bethesda, Md. Bovine FGF (10) and OGF (11) were generously donated by Dr. D. Gospodarowicz, and mouse submaxillary EGF (8) was generously donated by Dr. S. Cohen.

Perphenazine was purchased from Allen and Hanburys Ltd., London, England. [methyl-3H]Thymidine (18.3 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. Gold Label 3,5-diaminobenzoic acid hydrochloride was purchased from Aldrich Chemical Corp., Gillingham, Dorset, England.

Animals. The female Sprague-Dawley rats were obtained from a closed colony of randomly bred rats maintained at the Imperial Cancer Research Fund's Mill Hill Unit. The normal and perphenazine-treated rats were 90 to 120 days old; a total of 12 and 24 rats, respectively, were used. Another 50 rats when 50 days old received 30 mg DMBA i.g. dissolved in 2 ml corn oil. Tumors subsequently developed in the mammary glands, and 40 growing tumors up to 15 mm in diameter were used in this study.

Transplantable Tumors. Two workers, I. W. J. Wallace, F.R.C.S., and D. Drewitt (Department of Clinical Surgery, Edinburgh University), induced mammary tumors in an inbred rat strain (ADRA-Sprague-Dawley; Animal Diseases Research Association, Moredin, Edinburgh, Scotland) by administration of DMBA. This rat strain has normal blood levels of ovarian hormones, a low level of plasma prolactin, and a low tumor induction rate in response to i.g. DMBA (3, 14). Two selected, induced tumors were subsequently passed in rats from the same strain. After the 1st few passages, neither line of transplantable tumor regressed after administration of perphenazine, a phenothiazine derivative. This indirectly increases lobuloalveolar development by decreasing the hypothalamic secretion of prolactin-inhibitory factor which, in turn, raises the level of prolactin in serum (2).

Initially, daily injections were given on 3 consecutive days to induce maximum glandular development. However, this regimen induced the secretion of milk in numbers of alveoli, and these alveoli floated in the culture medium and would not attach readily to the culture dishes. Therefore, rats were given a s.c. injection of 0.2 ml of perphenazine (5 mg/ml) equivalent to 1.0 mg/200 g body weight 29 and 5 hr prior to killing. The mammary gland was then processed as described for the normal gland.

DMBA-induced Mammary Tumors. Growing tumors, 10 to 15 mm in diameter, were dissected free of capsule and attached mammary fat, rinsed in Medium 199, and sliced into small pieces with opposed scalpel blades on sterile polythene discs. The pieces were cut into 200-μm cubes and resuspended in Medium 199 containing 5% FCS, collagenase (300 units/ml), and hyaluronidase (460 units/ml) at 37° and then incubated on the mixer for 1.5 to 2 hr at 37°.

Tissue Preparation

Normal Mammary Gland. Ninety- to 120-day-old rats were chosen because, by this age, the ductal and lobular alveolar development was more extensive than at 50 to 60 days (29). The rats were killed by an overdose of carbon dioxide and were placed in 70% ethanol solution for 5 min. The excess alcohol was removed by blotting, and the abdominal skin was peeled back, leaving the mammary gland attached to the abdominal wall. The lower 2 pairs of abdominal mammary glands (5th and 6th) on each side were dissected free, placed in sterile plastic dishes, and gently minced with opposing scalpels. The mince was transferred to sterile polythene discs and chopped into approximately 200-μm cubes with the use of a mechanical Sorvall TC.2 tissue sectioner. The choppings were resuspended in Medium 199 containing FCS plus collagenase (300 units/ml) and hyaluronidase (460 units/ml) at 37°. The suspension was inverted 28 times/min on a Matburn blood cell suspension mixer (Matburn Surgical Equipment Ltd., Portsmouth, England) at 37° for 45 to 60 min and then centrifuged at 100 × g for 3 min. The clear upper lipid layer and the cloudy digestion mixture were discarded, leaving an opalescent lipid layer and pelleted cellular material. These were resuspended in fresh collagenase-hyaluronidase mixture that contained insulin (5 μg/ml) and corticosterone (5 μg/ml), the mixture was equilibrated to pH 7.3, and the 3-ml air space above the liquid level was gassed with 0.22-μm line-filtered carbon dioxide prior to closure. The sterile bottle containing this mixture was replaced on the Matburn mixer and rotated at 20° overnight. Next morning (after 16 hr) the suspension and mixer were replaced at 37° for 1.5 to 2 hr. The pooled mammary glands from 3 rats gave sufficient epithelium for plating up to 30- x 5-cm or 60- x 3.5-cm dishes.

Perphenazine-treated Mammary Gland. Although it was possible to set up cultures of epithelium from mammary glands of mature rats, the yield of epithelium per rat was small. Therefore, rats of similar age were given s.c. injections of perphenazine, a phenothiazine derivative. This indirectly increases lobuloalveolar development by decreasing the hypothalamic secretion of prolactin-inhibitory factor which, in turn, raises the level of prolactin in serum (2).

Initially, daily injections were given on 3 consecutive days to induce maximum glandular development. However, this regimen induced the secretion of milk in numbers of alveoli, and these alveoli floated in the culture medium and would not attach readily to the culture dishes. Therefore, rats were given a s.c. injection of 0.2 ml of perphenazine (5 mg/ml) equivalent to 1.0 mg/200 g body weight 29 and 5 hr prior to killing. The mammary gland was then processed as described for the normal gland.

DMBA-induced Mammary Tumors. Growing tumors, 10 to 15 mm in diameter, were dissected free of capsule and attached mammary fat, rinsed in Medium 199, and sliced into small pieces with opposed scalpel blades on sterile polythene discs. The pieces were cut into 200-μm cubes and resuspended in Medium 199 containing 5% FCS, collagenase (300 units/ml), and hyaluronidase (460 units/ml) at 37° and then incubated on the mixer for 1.5 to 2 hr at 37°.

Transplantable Tumors. Eight tumors that were relatively free of hemorrhagic necrosis were sliced into small pieces with opposed scalpel blades and resuspended in Medium 199 containing 5% FCS, collagenase (150 units/ml), and hyaluronidase (230 units/ml). The mixture was equilibrated and
the air space was gassed prior to tube closure, as for the overnight digestion of normal mammary gland. The suspension was maintained at 4° and transferred overnight by rail to London. The overnight mixture was centrifuged at 80 × g for 3 min, and the pelleted tissue was resuspended in a digestion mixture similar to that used for the DMBA-induced tumors. The digestion was continued at 37° for 1 hr only. For control of possible deleterious effects of the overnight delay, both normal mammary gland and DMBA-induced tumor tissue were left at 4° overnight in similar overnight mixtures prior to digestion at 37°.

All Tissues. When the enzyme digestion was complete, the resulting mixture was centrifuged at 60 × g for 3 min, the supernatant was discarded, and the cell pellet was resuspended in 4 ml of Medium 199 containing 5% FCS. Then a further 16 ml of medium were added, and the pellet was resuspended and recentrifuged. The supernatant was discarded and the washing was repeated. The cell pellet was finally resuspended in 6 to 10 ml of Medium 199 containing 5% FCS, and the 2-ml aliquots were spread over the base of 9-cm dishes, avoiding contact with the sides. The dishes were placed in a gassed, water-saturated atmosphere (94% air, 6% carbon dioxide) in an incubator for up to 2.5 hr to allow single stromal cells to attach to the dish. The unattached epithelial microexplants (organoids) and single cells were washed off the dishes with Medium 199 containing 5% FCS, filtered through PECAP 51-μm-pore-size cloth filter (to remove large clumps and to ensure a uniform plating suspension of organoids), collected by centrifugation, and the cell pellet was resuspended in sufficient Medium 199 containing 5% FCS so that 0.15 ml of suspension could be plated into the required number of 3-cm dishes (0.25 ml into each 5-cm and 1 ml into each 9-cm dish). Microexplants were plated at a density equivalent to 1 to 2 × 10⁴ cells/3-cm dish [estimated by completely digesting an aliquot of final suspension with trypsin-EDTA solution (25) and counting the number of cells with an automatic cell counter].

Preparation of Hormone-containing Medium

The basic culture medium was TC199, containing 5% FCS, penicillin (1.250 IU/ml), and streptomycin (100 μg/ml). Freshly prepared stock solutions of hormones were: insulin, 5 mg/ml, in 0.005 N HCl; corticosterone and cortisol, 5 mg/ml in ethanol; prolactin, 1 mg/ml in 0.0005 N NaOH; growth hormone, 1 mg/ml in 0.0005 N NaOH; 17α- and 17β-estradiol, 0.01 mg/ml in ethanol; progesterone and testosterone, 0.1 mg/ml in ethanol; and growth factors, 5 to 50 μg/ml in 0.00025% BSA. These were added in μl volumes to 20 ml of basic culture medium to give the required concentration.

Culture Procedure

A volume of twice-concentrated hormone-containing medium equal to the original volume of cell suspension was added to each dish, mixed with the cell suspension; then the total suspension was spread over the base of the dish. This was then transferred to a gassed incubator (94% air, 6% carbon dioxide) maintained at 37°. Twenty-four and 48 hr later, the total medium was removed and replaced by freshly prepared medium (2, 4, or 8 ml for 3-, 5-, or 9-cm dishes, respectively). Three x 3-cm or 2 x 5-cm dishes were used for each experimental variable. At either 22, 43, or 67 hr after the initial cell plating, 20 μl (40 μl for 5-cm dishes) of a 0.2-mCi/ml solution of [3H]thymidine were added. Two hr later, the radioactive medium was removed and replaced by an equal volume of warm Medium 199 without serum. Five min later, this was replaced by a similar volume of medium, and the cultures were left for 10 min in the incubator. The medium was then removed, dishes were allowed to drain, and any further residual medium was also removed.

Biochemical Estimations

One ml of 1 N NaOH was added to each 3-cm dish (1.5 ml for 5-cm dish), and then the dishes were placed on a mechanical agitator at room temperature for 90 min. An equal volume of 1 N HCl was added, it was mixed well with the NaOH solution, and the solution was transferred to a glass tube. This was centrifuged at about 300 × g for 5 min to pellet any insoluble material, and aliquots of the supernatant were taken for the various estimations.

Determination of [3H]Thymidine (27). One-ml aliquots were transferred to stoppered centrifuge tubes that contained 0.4 ml BSA (1 mg/ml). Then 1.5 ml of 10% trichloroacetic acid (w/v) were added, and the mixture stood on ice for 20 min. The tubes were centrifuged at 1000 × g for 5 min, the supernatant was discarded, and the tubes were drained for 5 min. Then 0.1 ml perchorlic acid (Analar grade, 69 to 71%) plus 0.2 ml hydrogen peroxide (100 volumes) were added, the contents were mixed, and the tubes were stoppered and placed in a 60° waterbath for 30 min. The tubes were then removed and allowed to cool; 5 ml ethoxyethanol were added, and the solution was transferred to a counting vial containing 10 ml of 0.6% PPO in toluene (w/v). The radioactivity was determined in a liquid scintillation counter.

Determination of Total DNA (15). To 500-μl aliquots (ice cold) were added 100 μl of ice-cold 2 N perchloric acid; the solutions were mixed and left at 4° for 10 min. The mixture was centrifuged at 1000 × g for 1 min, the supernatant was removed as completely as possible, and 20 μl of a freshly prepared aqueous solution of 4% 3,5-diaminobenzoic acid dihydrochloride were added. After mixing to break up the deposit, the suspension was incubated at 60° for 30 min, then cooled and remixed. Then 200 μl of 0.2 N perchloric acid were added, and the solutions were mixed and centrifuged at 1000 × g for 1 min. The supernatant fluid was transferred to microtubes which were analyzed in an Amino Bowman spectrophotofluorometer. The excitation wavelength was 408 nm and the emission was measured at 520 nm. The DNA content was calculated from the emissions of external and internal standards of 0.2 to 10 μg DNA per ml in BSA (25 μg/ml).

Mammary tissue from 12 normal and 24 perphenazine-treated rats, 40 DMBA-induced tumors, and 8 transplantable tumors have been used in this study. Each culture experiment has been performed at least twice. The results
were calculated initially as cpm/μg DNA. Each biochemical determination was done in duplicate. The results of all common experimental points were pooled and the hormonal effect was finally expressed as a ratio of its effect compared to that in Medium 199 and FCS alone. Arithmetic mean ± S.E.’s were calculated where applicable, and the final results are shown as histograms; the number of cell pools for each study is given.

**Estimation of DNA Synthesis by Radioautography**

Mammary glands and tumors were digested as described, but with DEM, a less complete medium than Medium 199. Microexplants were plated at a cell density of 1 to 2 × 10⁸ cells/3-cm dish [estimated by completely digesting the organoids with trypsin-EDTA solution (25)] in 2.5 ml of DEM containing 2% FCS, 50 ng cortisol per ml and 50 ng insulin per ml for epithelium from normal mammary gland and 5% FCS, 500 ng cortisol per ml, and 50 μg insulin per ml for the other tissues. These media were changed to the test media 26 hr later for normal mammary gland cultures and 48 hr later for cultures from the other tissues. The cultures were exposed to 3 μCi [³H]thymidine per ml at 1 μM from 14 to 38 hr after addition of test media to normal mammary gland cultures and from 6 to 36 hr for the other cultures. All the cultures were processed for radioautography (25). The fraction of colonial cells that contained labeled nuclei were counted in 5 fields of at least 100 cells/field/dish, 2 dishes being used for each experimental point; i.e., 1000 total cells counted per experimental point. Four cell pools were derived from normal rats, 4 from perphenazine-treated rats, and 1 pool each from 6 DMBA-induced tumors; these results are expressed as percentages, mean ± S.E. One pool from each of 3 transplant tumors was also studied, and the results are expressed as percentages only.

**RESULTS**

**Morphological Observations**

**Stromal Cells.** Before reproducible and meaningful results could be obtained from the cultures, it was necessary to understand the behavior of the various cell populations in culture. The plating suspensions consisted of numerous single cells, probably originating from the stroma, and groups or clumps of epithelial cells (organoids). Many of the single cells were derived from blood; they did not attach to the plastic surface and their numbers were reduced at each medium change. They were most numerous in cultures from the transplantable tumors and least numerous in cultures from the normal and perphenazine-treated rats. In the latter, most of the single cells attached during the 1st 2 hr after plating. Each single cell spread out during 6 to 8 hr, and their number increased steadily up to confluence to form a dense layer of fibroblast-like cells. A few similar cells were seen in suspensions from the tumors, but most of the cells that attached did so within 30 min of plating but subsequently failed to multiply during 96 hr when the cultures then consisted of a mixture of rounded and spread cells. It was necessary to exclude as completely as possible these cell populations from the epithelial cultures for the following reasons: (a) they occupied culture surface that otherwise would have been available to the epithelialium, (b) the effects of hormones on DNA synthesis of the single cells might be different from those of the epithelium, and (c) their DNA could contribute a large proportion of the total DNA per dish. Although it was possible to separate completely these stromal cells from the epithelium by washing the dishes after 2.5 hr of plating, it was not possible to separate completely the epithelial cultures from some stromal cells by the present methods. Hence the effects of hormones or growth factors on DNA synthesis of stromal cultures were studied where possible.

**Epithelium.** The epithelial colonies always arose from small groups or clumps of cells (microexplants/organoids) and not from single cells. The attachment of organoids to the culture surface commenced after 2 to 3 hr for cells from the tumors and 3 to 8 hr for cells from normal or perphenazine-treated glands. The initial plating efficiency of the organoids was high, often approaching 100%, provided that the depth of the medium was reduced to restrict their movement. This movement also caused organoids to collect in the central region of the culture dishes where the colonies grew and soon became confluent. By spreading the plating suspension as a thin layer over the base of the culture dishes, it was possible to obtain an even distribution of organoids, each with plenty of potential colony-forming space. During the 1st 24 hr after plating, colony growth consisted of cell mobilization and spreading from the organoid, and some cell division. The large organoids often gave rise to extensive colonies just by this mobilization process, the remnant of the organoid still being present at the colony center. Therefore, a plating suspension was used that contained a population of small organoids all of similar size. For the biochemical estimations, no attempt was made to count the numbers of either organoids or single cells plated into each dish. The plating suspension was adjusted so that 0.15 ml contained 1 to 2 × 10⁸ cells. This plating density, when spread evenly over the surface of the dish, resulted in a cell growth that would not produce confluent areas for 5 days. Stromal cells that remained attached or reattached to the organoids, mobilized from the organoids, and then attached to the dish and divided. Hence, at the end of digestion, the mixture was routinely examined by phase-contrast microscopy to ensure that single cells of stromal origin were not still attached to the smooth-surfaced epithelial organoids.

**Mammary Gland Cultures from Virgin Rats 90 to 120 Days Old**

The glands were digested with enzymes until the ducts and alveoli were freed from attached stromal cells. This resulted in a mixture of large numbers of single cells (more than 80% of the total cell pellet), tubular structures that contained RBC, ducts, and alveoli (organoids). Most of the single cells, as well as the blood vessels, attached to the culture surface within 2 hr, after which time the unattached organoids were collected and replated. The organoids at-
tached during the next 24 hr, but few formed colonies initially. During the next 48 to 72 hr, colonies grew out from most organoids; more colonial growth occurred from alveolar than from ductal structures. Each epithelial colony consisted of a central area of isometric cells, often several layers thick, and a peripheral rim of more elongated cells usually in monolayers. Colonial growth slowed down 96 hr after plating but continued up to confluence, provided that the initial plating suspension was sufficiently free of fibroblasts. The remnant of the original organoid was often present at the center of each colony.

Effect of Hormones on [\(^3\)H]DNA Synthesis

Apart from the addition of FCS, the media and hormone concentrations used were similar to those reported in our organ culture studies (12, 13, 16, 17). The cultures were exposed for 2 hr to [\(^3\)H]thymidine from 67 to 69 hr, since this time period gave adequate uptake of radioactivity into DNA. A 24-hr pulse of radioactivity was also used, and this gave similar patterns of results during 45 to 69 hr postplating as those recorded for the original 2-hr pulse. For convenience, DNA synthesis in the 2-hr period was expressed as the ratio, rate of [\(^3\)H]DNA synthesis per \(\upmu\)g DNA in the basal medium with additional hormones: rate of [\(^3\)H]DNA synthesis per \(\upmu\)g DNA in the basal medium alone, since different cultured tissues showed different absolute incorporation rates in the same basal medium. The results were obtained from 4 pools of mammary glands from 3 rats each (12 rats total).

Effect of Insulin, Corticosterone, Prolactin, and Growth Hormone with Medium 199 and 5% FCS. When the hormones (5 \(\upmu\)g/ml) were added singly, only corticosterone stimulated [\(^3\)H]DNA synthesis (ratio, 2.25 \pm 0.18) above that in Medium 199 and 5% FCS (1.0), although insulin may have induced a marginal increase. Addition of insulin increased the rate of [\(^3\)H]DNA synthesis with corticosterone (ratio, 3.5 \pm 0.25) but had little effect on the rate with other hormones. The addition of prolactin (5 \(\upmu\)g/ml) further increased the rate in insulin plus corticosterone (ratio, 5.25 \pm 0.1), whereas growth hormone was without effect. Similar results were obtained when the hormones were added at 10 and 100 ng/ml, but the degree of stimulation was lower. Of the various radioactive pulse periods tested, the maximum degree of stimulation occurred during 67 to 69 hr postplating; little stimulation was seen during 22 to 24 hr.

Effect of Additional 17\(\beta\)-Estradiol, Progesterone, and Testosterone. The effects of adding either estradiol (1 ng/ml), progesterone (100 ng/ml), or testosterone (10 ng/ml) were examined during 67 to 69 hr postplating. Both 17\(\beta\)-estradiol and progesterone initiated a slight increase in [\(^3\)H]DNA synthesis in Medium 199 and 5% FCS, insulin plus corticosterone, insulin plus corticosterone plus prolactin, and insulin plus corticosterone plus growth hormone. Testosterone had little effect on the rate in Medium 199 but reduced that in insulin plus corticosterone and in insulin plus corticosterone plus prolactin to the rate in Medium 199 (Chart 1).

Mammary Gland Cultures from Perphenazine-treated Rats, 90 to 120 Days Old

The perphenazine treatment resulted in up to a 4-fold increase in the yield of alveoli of high plating efficiency over organoids from nontreated animals but without a corresponding increase in stromal cells. The epithelial organoids attached and started to form colonies by 24 hr (Fig. 1); these colonies continued to enlarge up to 72 hr (Fig. 2), after which time they grew more slowly. The colonies were marginally larger and contained more cells than those from the nontreated rats; otherwise, the cultures appeared identical.

Effects of Hormones on DNA Synthesis

The results were obtained from 8 pools of epithelium derived from 3 rats each (24 rats total).

Effect of Insulin, Corticosterone, Prolactin, and Growth Hormone in Medium 199 and 5% FCS. The relative effects of individual hormones or combination of hormones at 10 and 100 ng/ml and at 5 \(\upmu\)g/ml were similar (Chart 2) to those obtained from the epithelium of non-perphenazine-treated rats (Chart 1), but the magnitudes of some of the effects were greater. Insulin alone at both 100 ng/ml and 5 \(\upmu\)g/ml stimulated [\(^3\)H]DNA synthesis. Thus, both insulin and corticosterone were capable of a stimulatory effect; corticosterone yielded the higher ratio, but the combination of insulin plus corticosterone resulted in an even higher ratio. Similar results were obtained when cortisol replaced corticosterone. The addition of prolactin alone (10 ng/ml to 5 \(\upmu\)g/ml) was without effect, whereas an increase in [\(^3\)H]DNA synthesis occurred when prolactin was added to insulin plus corticosterone. Growth hormone did not show this effect.
The relative effect on [\(^3\)H]DNA synthesis of either a single addition of any one of these steroid hormones to either Medium 199 and 5% FCS, insulin plus corticosterone, or insulin plus corticosterone plus prolactin was similar to those obtained from non-perphenazine-treated rats, but the magnitudes were greater. Estradiol (1 ng/ml) (ratio, 7.3 ± 0.5) added to insulin plus corticosterone (5 \(\mu g/ml\)) (ratio, 5.9 ± 0.2) increased the rate of [\(^3\)H]DNA synthesis more than when added at 0.1 ng/ml (ratio, 6.5 ± 0.7); whereas, at 10 ng/ml, the rate was depressed below that in insulin plus corticosterone. 17\(\alpha\)-Estradiol (0.1 to 10 ng/ml) was without effect. Progesterone added at 25, 50, and 100 ng/ml (ratios, 6.5 ± 0.2, 7.2 ± 0.3, and 8.2 ± 0.8, respectively) increased the rate of [\(^3\)H]DNA synthesis above that measured in insulin plus corticosterone. Testosterone (1 and 10 ng/ml) reduced the rate to that in Medium 199 plus 5% FCS. Similar effects on [\(^3\)H]DNA synthesis were seen when these steroid hormones were added to insulin plus corticosterone plus prolactin.

**Effect of Serum Concentration.** The evidence of a hormonal effect on DNA synthesis was the finding that a hormone raised or lowered the uptake of [\(^3\)H]thymidine into DNA compared with that in Medium 199 and 5% FCS. Therefore, the effect of altering the FCS concentration was studied. Epithelial suspensions were plated in Medium 199 with additional hormones and 5% FCS to ensure attachment of the cells. Twenty-four hr later, the culture medium was removed and the cells were washed well with Medium 199 alone, and then fresh Medium 199 and 1, 2.5, or 5% FCS plus the additional hormones were added. When the effect of serum concentration was compared 48 hr later, the rate of [\(^3\)H]DNA synthesis in 1% FCS was lower (ratio, 0.25) than that in 2.5% FCS (ratio, 0.85), which was lower than that in 5% FCS. The rate in 1% FCS was so low that the effects of the additional hormones were not detected, but the relative hormonal effects in 2.5 and 5% FCS were similar (cf. with tumors). The addition of BSA to counter osmotic deficiencies due to reduced serum protein concentration in the medium did not alter the above results (data not shown).

**Effect of Hormones and Growth Factors on [\(^3\)H]DNA Synthesis of Stromal Cells.** The single cells that attached during the 1st 2 hr after plating and grew up to form confluent sheets of noncolonial fibroblastic cells were cultured and analyzed as for the epithelium ("Materials and Methods"). The rate of [\(^3\)H]DNA synthesis during 44 to 46 and 67 to 69 hr after plating was higher in Medium 199 plus 5% FCS than with additional insulin plus corticosterone or insulin plus corticosterone plus prolactin (5 \(\mu g/ml\)). FGF (50 ng/ml) increased the rate of [\(^3\)H]DNA synthesis, whereas epidermal and OGF (10 \(\mu g/ml\)) had little effect (Chart 3). Higher concentrations of growth factors were inhibitory.

**Cultures from Growing DMBA-induced Mammary Tumors 10 to 15 mm in Diameter.**

Enzymic digestion of the DMBA-induced mammary tumors produced a suspension that consisted of large numbers of small, irregularly shaped epithelial organoids and single cells including RBC. Only a relatively small number of single cells attached during the 1st 2 hr after plating, and most of these attached within 30 min and spread during the next 2 hr. The organoids commenced to attach after 3 days.
hr, most of them had attached within 12 hr of plating, and the epithelial cells had mobilized from the organoids and spread onto the plastic by 24 hr after plating (Fig. 3). The medium was changed at 24 hr to remove the unattached single cells and RBC. During the next 72 hr, the colonies spread rapidly and were composed of 2 cell types similar to those observed in cultures of normal glands. The central two-thirds of the colonies contained isometric, refractile cells usually several layers thick in some places; the outer one-third contained elongated less refractile cells usually spreading in a monolayer. The colonies maintained this appearance as they continued to grow up to confluence, so that confluent cultures consisted of large colonies of isometric cells separated by elongated cells (Fig. 4). The larger colony size, the increased cell density within each colony, and the progressive, steady growth of most colonies up to confluence distinguished cultures of cells from DMBA-induced tumors from those of normal or hyperplastic mammary glands. The cultures from DMBA-induced tumors were plated to yield a similar total cellular DNA content per dish to those from the nonneoplastic gland so that any possible effects due to changes in cell density were minimized (9).

**Effects of Insulin, Corticosterone, Prolactin, and Growth Hormone with Medium 199 and 5% FCS.** When these hormones were added singly at 10 ng/ml, only corticosterone increased the rate of [3H]DNA synthesis (ratio, 1.8 ± 0.2); little stimulatory effect could be demonstrated with insulin (ratio, 1.1 ± 0.2). The addition of insulin (5 μg/ml) increased the rate in medium that contained corticosterone (ratio, 2.1 ± 0.25) but had little effect on the rate with the other hormones. A greater degree of stimulation occurred during 67 to 69 hr than during 43 to 45 hr after cell plating (Chart 4). Similar response patterns were seen at hormone concentrations of 100 and 1000 ng/ml, but prolactin alone also appeared to stimulate [3H]DNA synthesis, and the effect was greater at 100 (ratio, 1.7 ± 0.2) than at 10 ng/ml (ratio, 1.4 ± 0.25); 5 μg/ml inhibited [3H]DNA synthesis. The optimum concentration of prolactin alone occurred between 100 and 500 ng/ml. No similar effect could be demonstrated for growth hormone. The absolute response to the same combination of hormones varied from tumor to tumor, but duplicate cellular pools from the same tumor gave closely similar results. The hormonal combination insulin plus corticosterone plus prolactin at 5 μg/ml produced a greater rate of [3H]DNA synthesis during 22 to 24, 43 to 45, 67 to 69, or 91 to 93 hr in cultures derived from each of the 40 DMBA-induced tumors than did any other combination of these hormones (Chart 4).

**Effect of Estradiol, Progesterone, and Testosterone.** The relative effect on DNA synthesis of a single addition of any one of the hormones to Medium 199 and 5% FCS with insulin, corticosterone, insulin plus corticosterone, or insulin plus corticosterone plus prolactin was similar to that obtained in cultures from perphenazine-treated rats. However, 2 differences were noted. The addition of 17β-estradiol (1 ng/ml) to insulin plus corticosterone plus prolactin (100 ng; 5 μg/ml) decreased the rate of [3H]DNA synthesis almost to the level in insulin plus corticosterone (Chart 5). The addition of progesterone (100 ng/ml) to insulin plus corticosterone plus prolactin (100 ng; 5 μg/ml) increased the rate of [3H]DNA synthesis (Chart 5). The addition of both estradiol and progesterone to insulin plus corticosterone plus prolactin resulted in a rate of synthesis usually slightly greater than in insulin plus corticosterone plus prolactin, but less than insulin plus corticosterone plus prolactin plus progesterone. When 17α-estradiol replaced the β isomer at the same concentration, no effect on [3H]DNA synthesis was detected. The addition of testosterone (10 ng/ml) had little effect on [3H]DNA synthesis in Medium 199 and 5% FCS alone but inhibited synthesis in insulin plus corticosterone and insulin plus corticosterone plus prolactin.

**Effect of Serum Concentration.** The serum concentrations tested were 0.5, 1.0, 2.5, and 5%, and these were...
added as described for cultures from perphenazine-treated rats. Although cell growth was higher in media that contained 2.5 or 5% serum than in lower serum concentrations, the patterns of hormonal responses were similar at all 4 serum concentrations tested. During the 67 to 69 hr after cell plating, the ratios of insulin plus corticosterone plus prolactin, and insulin plus corticosterone plus prolactin plus progesterone, were greater in medium containing 0.5% serum than in Medium 199 and 5% serum. This was due to the lower level of [3H]DNA synthesis occurring in Medium 199 and 0.5% serum than that in Medium 199 and 5% serum (Table 1).

Effect of Hormones FGF, EGF, and OGF on [3H]DNA Synthesis of Colonial Cells. The growth factors (1 and 10 ng/ml) were added to Medium 199 and 2.5% FCS with or without insulin plus corticosterone (100 ng/ml) for pools from 3 tumors only, because of the small amounts of growth factors available. As the growth factor solution also contained crystalline BSA (250 µg/ml), this was also added as a control, but alone it had no effect on the rate of [3H]DNA synthesis. FGF had little effect in Medium 199 and FCS with or without insulin plus corticosterone, whereas both EGF and OGF increased this rate. Although neither EGF nor OGF appreciably affected the rate of [3H]DNA synthesis when added at a concentration of 1 ng/ml to insulin plus corticosterone, at a concentration of 10 ng/ml, both EGF and OGF stimulated either a greater or equal rate of synthesis, respectively, to that observed in insulin plus corticosterone plus prolactin (Chart 3).

Effect of Intermediate Storage at 4° in Half-Strength Digestion Mixture for 18 Hr during the Tissue Dissociation Protocol. Since the transplanted tumors used in later sections were sent from Edinburgh to London (597 km), an overnight refrigerated rail journey was required, during which time the tissue was suspended in half-strength digestion mixture. However, no effect was observed either in the plating or colony-forming efficiency or in the pattern of colonial growth between organoids from 3 DMBA-induced tumors digested by either the standard or overnight protocol. Measurements of the patterns of responses to insulin plus corticosterone, insulin plus corticosterone plus prolactin with or without estradiol, and progesterone at 2 different hormone concentrations (100 ng/ml and 5 µg/ml) showed no differences between cultures from tumors digested by either protocol (data not shown).

Table 1
Comparison of Effects of Varying Serum Concentrations on Hormone-Stimulated DNA Synthesis in Epithelial Cultures from 3 DMBA-Induced Tumors

<table>
<thead>
<tr>
<th>Serum Concentration</th>
<th>0.5%</th>
<th>1%</th>
<th>2.5%</th>
<th>5%</th>
<th>0.5%</th>
<th>1%</th>
<th>2.5%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 199</td>
<td>1140 ± 20*</td>
<td>1184 ± 15</td>
<td>1225 ± 10</td>
<td>1784 ± 18</td>
<td>534 ± 16</td>
<td>1438 ± 8</td>
<td>1266 ± 30</td>
<td>1587 ± 20</td>
</tr>
<tr>
<td>Medium 199 + I + B</td>
<td>2219 ± 40</td>
<td>1869 ± 15</td>
<td>2503 ± 18</td>
<td>2350 ± 30</td>
<td>1726 ± 12</td>
<td>3486 ± 32</td>
<td>3314 ± 62</td>
<td>3101 ± 46</td>
</tr>
<tr>
<td>Medium 199 + I + B</td>
<td>3131 ± 50</td>
<td>3089 ± 31</td>
<td>3773 ± 42</td>
<td>3827 ± 95</td>
<td>2871 ± 17</td>
<td>4754 ± 46</td>
<td>4794 ± 81</td>
<td>4608 ± 120</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* I, insulin (5 µg/ml); B, corticosterone (5 µg/ml); P, prolactin (5 µg/ml); PG, progesterone (0.1 µg/ml).

Cultures from Growing, Transplanted Mammary Tumors

The suspensions that resulted from digestion of the 8 transplanted tumors differed from those of DMBA-induced tumors. Very large numbers of RBC and single cells were present, the epithelial organoids were very variable in size and shape but were usually small, and the total yield of organoids was less than 50% of that from a DMBA-induced tumor of similar size. The histological appearance (Fig. 5) of each tumor was variable although similar to that of DMBA-induced mammary tumors (29). Hemorrhagic necrosis was occasionally present, as well as cystic formation. One additional tumor (not used for this study) showed areas of marked squamous metaplasia. The final suspension from the digested tumor was washed several extra times in medium, and the organoids were collected by centrifugation at 60 x g for 3 min to reduce the large numbers of RBC present. The RBC interfered with the attachment of the organoids. Numerous single cells attached during the 1st 2 hr after plating, but these cells did not grow to form either colonies or cell monolayers.

The plating efficiency of the organoids was about one-half that expected from similar-sized DMBA-induced tumors. Each attached organoid produced a colony, but there was a marked variation between the appearance of individual colonies and their rate of growth. Some resembled those seen in cultures from DMBA-induced tumors but grew more slowly. The remainder showed different degrees of spatial asymmetry, often being "comma shaped," and these colonies grew even more slowly. The cells in such colonies were more elongated than isometric. The appearance of the cultures is shown in Fig. 6. Few intercolonial single cells were present.

Effects of Hormones and Serum Concentration on [3H]DNA Synthesis

The rate of uptake of [3H]thymidine per µg DNA in epithelial cultures grown in Medium 199 and 5% FCS was 4 to 5...
times the value found in cultures from DMBA-induced tumors which were plated to give similar amounts of DNA/dish. This rate of uptake remained almost constant in cultures from each transplanted tumor when insulin, corticosterone, prolactin, growth hormone, estradiol, and progesterone were added either singly or in combination at concentrations similar to those previously used (Chart 6). The effect of lowering the serum concentration was examined in 3 of the 5 tumors. In 1 tumor (Chart 7), the rate of \(^{3}H\)DNA synthesis in Medium 199 and 1% FCS was about one-third that in Medium 199 and 5% FCS. The addition of corticosterone (5 \(\mu g/ml\)) to Medium 199 and 1% FCS more than doubled the rate, but this was unaffected by the addition of insulin (5 \(\mu g/ml\)). No similar effect of corticosterone on \(^{3}H\)DNA synthesis could be demonstrated in Medium 199 and 5% FCS, nor could it be demonstrated in the other 2 tumors with a serum concentration of either 1% (ratios of 0.9 to 1.3) or 5% (ratios of 0.7 to 1.3). It was still possible that the overnight intermediate tissue dissociation step was somehow affecting the subsequent hormonal responses in culture. In a control experiment, therefore, a tumor was randomly chosen from those being routinely used at Edinburgh and was sent by rail to London. The effect of hormones and serum on DNA synthesis of cells cultured from this tumor were similar to those obtained from cultures of the DMBA-induced tumors used in London (data not shown).

Radioautographic Analysis of the Effects of Hormones on DNA Synthesis of Mammary Epithelium from Perphenazine-stimulated Rats, DMBA-induced Tumors, and Transplanted Tumors

The effects of various combinations of insulin, hydrocortisone, prolactin, and growth hormone on DNA synthesis in mammary epithelial cultures from different tissue sources was studied by a radioautographic technique. This technique measures directly the fraction of cells synthesizing DNA and is relatively insensitive to changes in the specific activities of the radioactive DNA precursors or precursor pool size in the cell. Dulbecco’s modified Eagle’s medium was used in place of Medium 199, as it is routinely used for similar studies on cell lines. The concentration of serum in the medium was reduced to 0.5% to produce relatively quiescent cultures (24) so that the ability of the hormones to reinitiate DNA synthesis was determined, rather than their ability to stimulate, maintain, or inhibit ongoing DNA synthesis. The epithelial cultures could be subdivided into 2 main groups as in the previous sections: (a) those where epithelial DNA synthesis was stimulated by addition of the mammotropic hormones, namely, mammary glands from normal and perphenazine-treated rats and from DMBA-induced tumors; and (b) those where DNA was relatively unaffected by hormonal additions, namely, epithelium from the transplantable tumors (Table 2). In the hormone-responsive epithelium, prolactin alone stimulated a marked degree of DNA synthesis in cultures from the DMBA-induced tumors, whereas a much smaller response was seen in both normal and hyperplastic epithelium. The total combination of insulin, cortisol, and pituitary hormones gave the maximum increase in DNA synthesis, in general agreement with the results obtained from the measurement of the rates of incorporation of \(^{3}H\)thymidine into DNA. Growth hormone appeared to have a more marked stimulatory effect on reinitiating DNA synthesis in quiescent cultures in medium containing 0.5% serum than was demonstrated in proliferating cultures grown in medium containing 5% serum.

DISCUSSION

There have been few studies on the proliferative effects of mammotrophic hormones on cell cultures from rat mammary glands. Cohen et al. (7) reported the establishment of a cell line from a normal mammary gland and from a single DMBA-induced mammary tumor from Sprague-Dawley rats. Both nonneoplastic and neoplastic cell lines retained some of their parental biological characteristics, and the growth of the tumor cell line was affected by prolactin and estradiol after 40 to 50 passages (6). Malan et al. (19) showed that in primary cultures of normal rat mammary epithelium the
estradiol, and progesterone was optimal for growth and term epithelial cultures are probably more likely to retain dissociation to obtain primary cultures, but hyaluronidase synthesis, and the cultures were set up so that the maxi
differentiation. We have used similar methods of tissue culture dishes. The cultures then consisted almost entirely from mammary tumors. The numbers of stromal cells could
culture medium Normal (6) Perphenazine-treated (4) DMBA-induced (6) Transplant

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Normal (6)</th>
<th>Perphenazine-treated (4)</th>
<th>DMBA-induced (6)</th>
<th>Transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEM + 0.5% FCS</td>
<td>0.5 ± 0.2*</td>
<td>3.1 ± 0.5</td>
<td>5.0 ± 1.0</td>
<td>G</td>
</tr>
<tr>
<td>DEM + I' + F</td>
<td>5.0 ± 1.3</td>
<td>4.9 ± 0.1</td>
<td>9.6 ± 1.2</td>
<td>43</td>
</tr>
<tr>
<td>DEM + I + F + P</td>
<td>1.5 ± 0.4</td>
<td>4.7 ± 1.4</td>
<td>11.0 ± 2.1</td>
<td>H</td>
</tr>
<tr>
<td>DEM + I + F + P+G</td>
<td>6.7 ± 1.9</td>
<td>15.1 ± 2.8</td>
<td>14.0 ± 2.4</td>
<td>J</td>
</tr>
<tr>
<td>DEM + G</td>
<td>0.3 ± 0.1</td>
<td>4.0 ± 0.5</td>
<td>10.0 ± 2.2</td>
<td></td>
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<tr>
<td>DEM + I + F + G</td>
<td>3.2 ± 0.1</td>
<td>11.0 ± 1.3</td>
<td>8.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>DEM + I + F + P + G</td>
<td>7.9 ± 1.7</td>
<td>17.0 ± 1.0</td>
<td>27 ± 48</td>
<td></td>
</tr>
<tr>
<td>DEM + 10% FCS</td>
<td>4.0 ± 1.2</td>
<td>42.0 ± 2.0</td>
<td>12.0 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

*a Numbers in parentheses, number of rats.  
*b Mean ± S.E.  
'I, insulin (50 ng/ml); F, cortisol (50 ng/ml); P, prolactin (500 ng/ml); G, growth hormone (500 ng/ml).

hormonal combination of insulin, aldosterone, prolactin, estradiol, and progesterone was optimal for growth and differentiation. We have used similar methods of tissue dissociation to obtain primary cultures, but hyaluronidase and serum were added to the dissociation mixture to increase the yield of viable epithelium. Large numbers of replicate cultures could be set up as routinely and as efficiently from normal and hyperplastic mammary glands as from mammary tumors. The numbers of stromal cells could be kept to a minimum by use of plating techniques that depend on differential rates of cellular attachment to plastic culture dishes. The cultures then consisted almost entirely of epithelium at least for the 1st 96 hr after plating. Manual killing of the stromal cells (7) was impractical due to the large number of dishes being used, and medium selectivity with d-valine was ineffective (24). Gradient separation (28) was more effective, but the gradients were easily overloaded by the numbers of organoids available. Recent studies suggest that the most efficient method for removing the occasional contaminating stromal cell is mild Pronase treatment (D. Bennett, unpublished observation). Such short-term epithelial cultures are probably more likely to retain their physiological response to hormones than either long-term cultures or established epithelial cell lines. Since the hormones may exert different effects on populations of growing cells than on those from more quiescent confluent cultures, this study was mainly performed during the growing phase of the cultured cells, it was confined to [3H]DNA synthesis, and the cultures were set up so that the maximum number of cells were capable of responding to growth-promoting stimuli. In this study, DNA synthesis was mainly measured by the incorporation (cpm) of [3H]thymidine into DNA during 2 hr. This experimental value is a reflection of both the average specific activity of the intracellular thymidine pool and the rate of DNA synthesis. In the present experiments, we did not attempt to determine either the size of soluble precursor pools or the effects of hormones on such pools for the following reasons. The actual number of cells in the initial plating suspension could be determined only indirectly, and we had no control over how many of these cells would replicate and help to produce the epithelial colonies. Also, both epithelial and myoepithelial cells are present within the basement membrane-bound epithelial structures (29), and both partake in colony formation (24). In a further study (24), we have been able to establish secondary cultures using known numbers of cells of known plating and colony-forming potential. In that study, we have shown that the patterns of increases or inhibitions induced by different hormones on [3H]thymidine uptake into DNA were similar in direction to those found autoradiographically in the fraction of cells synthesizing DNA and in changes in cell numbers, in both primary and secondary epithelial cell cultures from hyperplastic mammary glands and hormone-dependent mammary tumors. Similarly, in these experiments, the same pattern of hormonal effects on the fractions of cells with radioactively labeled cell nuclei was also observed for the corresponding thymidine incorporation studies in the normal, hyperplastic, hormone-dependent, and hormone-independent tumors. Thus the measurement of [3H]thymidine uptake into DNA in these systems may be a reflection of DNA synthesis, and ultimately of cell multiplication.  

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Epithelial cells from all tissues grew up to form confluent sheets in Medium 199 and FCS. The nonneoplastic epithelium showed a requirement for higher concentrations of serum (5 to 10%) than that from tumors for maximum rates of DNA synthesis. Although the rates of thymidine incorporation into DNA were measured in proliferating cultures in the presence of serum, it was still possible to show reproducible stimulation of the basal rates when additional hormones were added to the medium, and the general pattern of hormonal stimulation was independent of serum concentration. The enzymes used to digest the mammary tissues, collagenase and hyaluronidase, contain contaminating proteases that can stimulate DNA synthesis when added to cultured fibroblasts (4) and possibly cause temporary damage to the surface of the epithelial cells. However, the integrity of the primary cells was sufficiently maintained to observe the normal surface topography and ultrastructure of epithelial cells (24) and to reproduce faithfully some aspects of their physiological response in culture. In addition, this response was maintained for epithelial cells subcultured from the original primaries (24).

The overall pattern of hormonally induced increases in DNA synthesis was similar in similar tissues, but each experimental cell pool showed different absolute synthetic rates measured as cpm/μg DNA in a 2-hr period for the same additions. Similar variations in hormonal responses between pools of explants from different DMBA-induced mammary tumors (23) and from cell suspensions from different DMBA-induced tumors (1) have been reported recently. Therefore, the ratio of the absolute synthetic rate in hormone-containing medium to the absolute rate in medium without hormones was used for comparing hormonal effects on DNA synthesis between different cell pools and different tissues. These ratios were lower in cultures from neoplastic than from nonneoplastic tissues. This was caused by the higher background rates of DNA synthesis in Medium 199 and 5% FCS in cultures from the neoplastic glands. These rates could be lowered, at least for the epithelium from DMBA-induced tumors, by reducing the serum concentration to 1 or 0.5%.

Some differences were observed between the results obtained from our epithelial cell cultures and those from our previous studies on organ cultures from nonneoplastic and neoplastic rat mammary tissues (12, 16) maintained in Medium 199 without serum addition but with hormones at similar concentrations. Insulin alone induced little if any increased rate of DNA synthesis in cell culture of mammary tissues, whereas it markedly increased DNA synthesis in organ cultures of both normal mammary gland (12) and DMBA-induced tumors (16). The hormonal combination, insulin plus corticosterone plus prolactin, stimulated maximum rates of DNA synthesis in cell cultures from both nonneoplastic glands and from all 40 of the DMBA-induced tumors cultured, whereas this was not the case in organ cultures of either nonneoplastic glands or in 16 out of 32 DMBA-induced tumors. Estradiol stimulated small increases in the rate of DNA synthesis in insulin plus corticosterone plus prolactin in cultures from nonneoplastic glands, whereas it inhibited DNA synthesis in organ cultures from similar tissues. Cell cultures from 40 DMBA-induced tumors all gave similar patterns of effects of hormones on DNA synthesis, whereas, in organ cultures, 32 tumors could be clearly subdivided into 3 groups on the basis of their hormonal responses (16). Therefore, it may be rewarding to compare the effects of hormones on DNA synthesis of organoids that are encouraged to grow on surfaces to form colonies, as reported here, and those that are prevented from colonial growth in identical culture media.

There were slight differences between the effects of hormones on DNA synthesis in cultures from normal, hyperplastic mammary gland and DMBA-induced tumors and major differences between these hormone-responsive cultures and the nonresponsive cultures from transplantable, nonresponsive tumors. The pattern of responses in DNA synthesis seen in cell culture was similar to that of the mammaryotropic hormones on growth and development of mammary glands and tumors in rats (5, 20). It is concluded that the cell culture system described here accurately reflects the hormonal modulation of the growth of tissues in the intact animal, and it represents a suitable system in which to study various aspects of the growth regulation of mammary tissues derived from different biological states in the rat. In addition, it may provide a useful model system for the study of the growth control of normal, dysplastic, neoplastic human mammary epithelium and its stroma.

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Comparison of the Effects of Hormones on DNA Synthesis in Cell Cultures of Nonneoplastic and Neoplastic Mammary Epithelium from Rats


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