Hormone-Dependent Differentiation of Mammary Gland: Sequence of Action of Hormones in Relation to Cell Cycle

Abstract. *Differentiation of mouse mammary gland in vitro requires insulin, hydrocortisone, and prolactin. The epithelial cells must first divide in order to synthesize casein in response to these hormones. Insulin is required for the initiation of DNA synthesis and is also necessary during G_1 phase (after mitosis). Prolactin can elicit the overt differentiative responses after mitosis. Activity of hydrocortisone precedes that of prolactin, that is, after mitosis it is not capable of eliciting the differentiative response.*

Differentiation of mouse mammary gland requires insulin, hydrocortisone, and prolactin (*1–3*). Epithelial cells of mammary gland must first divide in order to synthesize casein in response to these hormones (*4, 5*). Insulin is the only hormone required for proliferation of these cells (*3–5*). Our intent in this study was to determine whether both hydrocortisone and prolactin need to be present during proliferation, or whether one of them could act after mitosis in this complex differentiative process.

Synthesis of DNA and mitosis occur extensively during the first 48 hours of culture in the epithelial cells of mid-pregnancy mammary gland explants, but both processes virtually cease by the 3rd day (Table 1). This provided us with an opportunity to establish which hormones need to act during the proliferative phases of the cell cycle. Explants were first incubated with insulin, insulin and hydrocortisone, or insulin and prolactin, and were then placed on medium containing all three hormones (second period). Insofar as all the systems contained insulin, the degree of epithelial cell proliferation was the same in each case (*3–5*). The rate of synthesis of authentic casein (*2, 6*) and the histological appearance of the tissue (*3*) were used as the criteria of differentiation. If the second period of incubation was initiated after an incubation period of only 1 day, when the cells were still engaged in DNA synthesis and mitosis, the level of casein synthesis reached at the end of the second incubation period was independent of the initial hormone composition (Fig. 1). Thus, under these conditions, one cannot detect a particular sequence in the actions of hydrocortisone and prolactin. However, if the first incubations were maintained until proliferative activity markedly declined (that is, for 3 days), then, during the subsequent 24-hour period, the rate of casein synthesis increased only in that system which contained hydrocortisone during proliferation (Fig. 2, *IF-3H*). Such effects of prolactin have also been observed after baseline synthesis of certain electrophoretic components (*6*) of the precipitate induced with rennin and Ca^{++} has declined to virtually zero during the first incubation (*7*). Thus, the actual effect of prolactin was much larger than the apparent effect shown in Fig. 2. Similarly, the histological appearance characteristic of explants cultured with all three hormones (*3*) was observed only in the tissue that had first been incubated in the presence of hydrocortisone. These results demonstrate that hydrocortisone acts during the proliferative periods of the cell cycle of mammary epithelium, but that prolactin can act after mitosis. The conclusion that prolactin can act after mitosis is supported by the results of experiments with colchicine (Fig. 3). Colchicine, an effective mitotic inhibitor in this system (*4*), does not prevent the action of prolactin after the tissue is incubated for 3 days in medium containing insulin and hydrocortisone.

If hydrocortisone is removed during the second incubation period, the augmentation of casein synthesis is still manifest. This may be due to the presence of residual hydrocortisone after the first incubation in the medium with insulin and hydrocortisone. However, if after such an incubation the explants are cultured in medium containing hydrocortisone and prolactin, then no augmentation in casein synthesis occurs. This indicates that, in addition to its action in relation to DNA synthesis, insulin is necessary after mitosis. Both

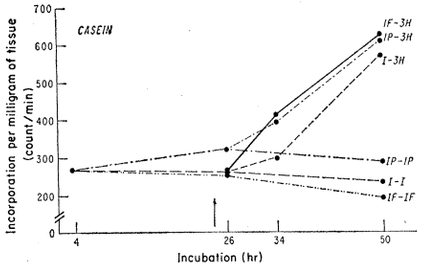

Fig. 1. Incorporation of $^{32}P_i$ (inorganic phosphate-32) into casein. Explants were prepared as described in Table 1 and cultured in the presence of the designated hormones insulin (*I*), hydrocortisone (*F*), and prolactin (*P*). Final concentration of each hormone was 5 μg/ml. At 24 hours, as indicated by the arrow, explants were transferred to a second incubation medium containing either the same hormones present during the first incubation or insulin, hydrocortisone, and prolactin (*3H*). Casein biosynthesis was measured by the incorporation of $^{32}P_i$ (15 μc/ml) during a 4-hour period. After homogenization of the tissue and centrifugation of the homogenate at 105,000*g*, isotopically labeled casein was isolated from the supernatant by precipitation with rennin and calcium ions in the presence of casein as a carrier (*2*). Casein biosynthesis is plotted at the midpoint of such 4-hour periods.

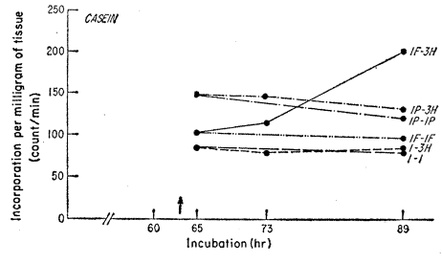

Fig. 2. Incorporation of $^{32}P_i$ into casein. Experimental conditions were the same as those described in Fig. 1, except that the second incubation was initiated after 63 hours of culture. Similar results have been obtained in five experiments.

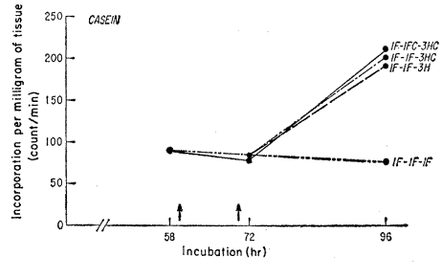

Fig. 3. Effect of colchicine on action of prolactin after mitosis. During the first incubation period (60 hours) all explants were cultured on *IF* medium (shown on the right side of the figure). At the time designated by the first arrow, explants were transferred to new media whose compositions are also shown (*C*, colchicine). Ten hours later, at the time designated by the second arrow, explants were transferred again to media whose compositions are indicated. Colchicine (0.025 μg/ml) inhibits division of mammary epithelial cells within 10 hours, as evidenced by studies of the mitotic index (*5*). Pulse labeling of casein with $^{32}P_i$ was performed as described in the legend to Fig. 1.

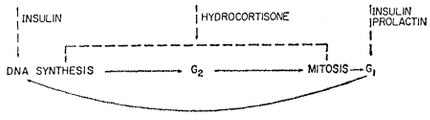

Fig. 4. The action of hormones in relation to cell cycle of mammary epithelium.

effects of insulin may be observed when fructose is substituted for glucose in the synthetic medium.

These results and those reported recently (*4, 5*) constitute a partial resolution of the multiple hormone requirements of this system. It appears that the covert and overt differentiative phases of development in mouse mammary gland in vitro are separable on both a temporal and hormone-dependency basis. Insulin is the hormone involved in the stimulation of mammary epithelial cell proliferation. However, if cell proliferation is permitted to occur in the absence of hydrocortisone, the new cells are incapable of subsequently differentiating (as judged by casein synthesis and histological development) in response to the presence of all three hormones. On the other hand, cells formed in the absence of prolactin (that is, those formed in a medium with insulin and hydrocortisone) can subsequently respond in the medium with all three hormones.

The action of the hormones in relation to the mammary epithelial cell cycle may then be depicted as in Fig. 4. Insulin is involved in the initiation of DNA synthesis (*3–5*) and is required during the phase after mitosis (G_1). Prolactin can elicit the overt differentiative responses after mitosis. Hydrocortisone activity precedes that of prolactin; it is not capable of eliciting a differentiative response after mitosis. It is not known which of the other phases of the cell cycle—DNA synthesis, G_2 (the period between the cessation of DNA synthesis and the commencement of mitosis), and mitosis—coincide with the covert action of hydrocortisone.

Table 1. Epithelial cells incorporating tritiated thymidine and undergoing mitosis during a 5-day culture period in the presence of insulin. Abdominal mammary glands of a C3H/HeN nulliparous mouse halfway through pregnancy (10 to 12 days) were removed with aseptic technique. Explants (0.5 to 1.0 mg) were prepared and cultured in sterile Medium 199 (Microbiological Associates). Insulin was added to a final concentration of 5μg/ml. Explants used for the study of DNA synthesis by autoradiography were exposed to 1 μc of tritiated thymidine (specific activity, 6.0 c/mmole) for 24 hours prior to fixation at the designated time. After fixation in Bouin's solution, explants were sectioned at 5 μ, and the slides were dipped in liquid photographic emulsion. Explants taken for histological examination were fixed, sectioned at 7 μ, and stained with Delafield's hematoxylin. Similar results were obtained when explants were cultured in the presence of all three hormones—insulin, hydrocortisone, and prolactin.

Length of incubation (hr)	Cells incorporating thymidine-H^3 (%)	Mitotic figures (No./1000 epithelial cells)
0		10
24	42.6	23
48	44.9	7
72	3.6	1
96	1.5	1
120	1.1	1

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12 January 1967

Automobile Paint Effective as an Insect Attractant

Abstract. *Two acrylic auto paints were effective attractants for sap beetles,* Stelidota geminata *(Say) and* Glischrochilus fasciatus *(Oliver). Response to the synthetic lures was sufficient to encourage additional testing of their potential in survey and control programs.*

Endemic populations of nitidulids have been present in Michigan for many years and have been reported in weekly surveys (*1*), but not until 1966 did *Stelidota geminata* suddenly reach outbreak proportions in parts of southwestern Michigan and become a serious pest of strawberries in some counties. Estimates of losses for the 1966 strawberry season exceeded $3,000,000 (*2*). During the outbreak of *S. geminata* on 16 July 1966 in East Lansing, Michigan, so many *Glischrochilus fasciatus* responded to a paint container and to the spots of new paint on an automobile that the painting of rust spots on the car had to be stopped (*3*). The paint being used was General Motors midnight blue, Dupli-Color touch-up, stock No. DP-GM 44 (*4*).

Members of the various species of nitidulids are associated with a saprophytic existence. They are attracted to many kinds of decomposing vegetation, particularly fermented fruit juices; some live on flowers, fungi, and carrion, and a few are predacious (*5–7*). Nearly all except the genus *Carpophilus* lack economic importance, though some are a considerable nuisance around public picnic areas. Other genera have been listed as pests of corn, especially sweet corn, in the North Central states (*8*), and *Glischrochilus* spp. have been associated with worm-damaged ear corn in Michigan (*1*).

Specific studies of the attraction of the Nitidulidae to synthetic lures have not been reported. However, Dorsey and Leach (*9*), who were investigating the comparative attractiveness of tree wounds to insects, found that *Stelidota geminata* (Say) and *Glischrochilus fasciatus* (Oliver) were important vectors of oak wilt. Also, Daugherty and Brett (*6*) included some attractant work in their investigations of nitidulids on sweet corn and found that when banana pulp was used as the attractant, nitidulids from five genera, including *G. fasciatus* and *Stelidota* spp., collected at their bait stations.

The results obtained with *G. fasciatus* led us to a field investigation of paints as an attractant for *S. geminata*. A series of paints was exposed in vials containing a wick saturated with 2 ml/vial and suspended on sticky board field traps in Berrien County, Michigan. These traps were yellow cards (Fig. 1) suspended from a pole 2 to 3 feet (0.6 to 0.9 m) above ground. Replicates were placed within a pear orchard, within a strawberry patch adjacent to the pear orchard, and within a strawberry patch adjacent to a vineyard. Collections were highest within the strawberry patch adjacent to the pear orchard and lowest within the strawberry patch adjacent to the vineyard. Initial response occurred near dusk, an indication of crepuscular behavior. As the season progressed, the nitidulids dispersed into the cherry and peach orchards adjacent to the