THE INFLUENCE OF HORMONES ON FETAL MOUSE MAMMARY GLANDS IN VITRO

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
BRENDA P. ALSTON
1972

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ABSTRACT

THE INFLUENCE OF HORMONES ON FETAL MOUSE MAMMARY GLANDS *IN VITRO*

By

Brenda P. Alston

The morphological development *in vivo* of mammary glands from late embryos and newborn mice were examined. Following this study, the role of hormones on mammary development were assessed in organ cultures of mammary glands from 18-day-old embryos.

The second and third thoracic mammary glands were taken from mice ranging in age from the 18-day fetus to 3 days of age. The glands were examined both in whole mount preparations and histological sections for growth and morphology with attention given to variability in the glands of different animals and within the same animal.

Most of the increase in growth occurred between the 18th and 19th days *in utero* and between birth and day 1 of age. Ductal lumen and adipose cell formation occurred at day 1 of age. Considerable variability was found within animals of the same age. Contralateral glands were compared within the same animal. There was variability

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between the second and third glands of one side but they were equal in area to the glands of the contralateral side.

In vitro studies were carried out with the second and third thoracic glands from the 18-day fetus. Explants were cultured for 3, 4, or 5 days in chemically-defined medium containing various combinations of insulin, prolactin, and corticosterone. The glands were examined histologically for the extent of survival and duct morphology. Whole mounts were examined for changes in ductal growth patterns.

Insulin elicited adipose cell development and enhanced the effects of other hormones. Prolactin effected the best growth response as shown by Lasfargues (1959) and Ceriani (1969). Corticosterone facilitated the organization and appearance of a ductal lumen.

The ductal growth pattern could be advanced to that of a one-day-old animal, but this required the full triad of hormones, a finding consistent with the hormonal requirements for the induction of lactogenesis in mammary explants from adult mice.

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By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1972

ACKNOWLEDGMENTS

The author would extend sincerest thanks to Dr. Evelyn M. Rivera who saw more in me than I saw in myself. Her confidence in me expressed through her constant encouragement helped me to accomplish this study.

My thanks also goes to Dr. Clifford Welsch who served on my committee both as technical advisor and friend, to Dr. John Shaver who also served on my committee and whose assistance in the writing of this thesis was invaluable, to Dr. Lynwood Clemens, and to Dr. Richard Fennell, professor emeritus.

A special thanks goes to Dr. H. Allen Tucker. His shoulder was always available for discouraging moments.

Finally, I wish to thank Mr. Gustav A. Ofosu for his friendship, encouragement, and assistance through which he taught me the quality of perserverance. His sensitivity to my moods was the mediating factor which, in many instances, kept me going. Nothing that I could say could adequately express my full gratitude. I can only thank him for being Gus.

This work was supported in part by a research grant from the National Institutes of Health (HD-02374) to Evelyn Rivera and a training grant (HD-00135) from NIH.

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INTRODUCTION

The importance of hormones for the development and function of the mammary gland during pregnancy and lactation has been well established. It is necessary to understand the interaction of hormones as related to function, to know what hormone elicits what response, and at what time during development hormonal influence is manifested. It is also of interest to investigate the hormonal requirements influencing mammary development during embryonic life.

Balinsky's classification (1950) of mouse mammary gland development is divided into three essential phases in the embryo:

(a) The formation of epithelial thickenings to form the individual anlagen in the course of which the growth rate decreases rapidly (advanced day 10-11);

(b) the phase of retarded growth of the individual anlagen and of slight differentiation when the anlagen shape changes little (day 11-15);

(c) the period of rapid growth corresponding to the lengthening of the primary sprout with the rate of growth during this time exceeding that of the embryo as a whole (day 16-19).

One of the early studies of mammary development in the mouse was done by Turner and Gomez (1933). According to these authors, development begins with the appearance of a mammary streak on either side of the mid-ventral line at about 10 days of embryonic life. At 12 days, a mammary line appears and at 13 days, buds appear. These buds represent the anlage. The anlage proliferate and sink into the underlying mesenchyme. This continues until day 17 at which time the bud increases in length and is eventually funnel-shaped. As the bud lengthens, central cells separate to form the lumen. This separation occurs from the distal to the proximal end, the end closest to the epidermal rudiment. By day 20, secondary sprouts appear at the distal end of the primary sprout in the form of buds or solid ball-like clusters of cells. No further change occurs until birth.

Fetal mammary development has been studied by giving in vivo hormone injections to the pregnant mother and noting the effects. Jean and Delost (1968) found that injections of somatotrophic hormone (STH) into the pregnant mother cause hypertrophy of the glands in both male and female offspring. This same effect is noted if the

fetus is injected directly (Jean, 1968). From these results it was concluded that STH can apparently cross the placenta to act on the developing glands and has the same effect as direct fetal injection. After administration of an estrogen compound to the mother at 14 days gestation, a high percentage of the glands disappeared in the newborn female offspring. If the animals were examined at 35 or 80 days of age the frequency of gland appearance increased. There was a high degree of atrophy in those glands remaining in the newborn. The differences varied according to the position of the gland and also according to the time of examination. These findings suggested that the action of injected estrogen on the fetal developing mammary gland was not an irreversible process (Jean, 1969).

Organ culture techniques have made it possible to study development of structure and function in response to specific changes in the culture medium. Using such techniques, information can be obtained regarding responses to particular hormones without the influence of other systems.

It has been established by several authors that insulin is an absolute requirement for the in vitro survival of adult mammary tissue (Elias, 1959; Rivera, 1964; Ichinose and Nandi, 1966). Insulin induced DNA synthesis and mitosis in culture (Stockdale and Topper, 1966; Turkington, 1968; El Darwish and Rivera, 1970). In hormone dependent differentiation (Topper, 1968; Turkington,

1968), insulin has also been found to enhance the effects of pituitary hormones and adrenal steroids to stimulate the formation of the apparatus for the synthesis of milk products in the adult gland. These results confirmed the findings of Rivera and Bern (1961), Rivera (1964), Jeurgens et al. (1965), Ichinose and Nandi (1966), and Stockdale et al. (1966). Other studies indicate that without insulin, subsequent milk protein will not be synthesized (Lockwood et al. 1967; Turkington et al. 1967; Topper, 1968).

Voytovitch and Topper (1967) have shown that immature glands will grow without insulin. DNA was synthesized and mitosis took place. However, without insulin, as in mature glands, immature glands could not be induced to synthesize milk proteins.

Hardy (1950) showed that 10-, 12-, and 13-day embryonic glands could be cultured up to 25 days in cock plasma and chick embryo extract with the formation of primary, secondary, and tertiary ducts without further supplementing the medium. These findings suggest that early development may not depend on specific hormonal stimuli but do not rule out the possible hormonal contributions of the plasma used.

Kratochwil (1969), culturing 12-, 13-, and 14-day embryonic mammary rudiments, found that the gland would be dormant for 5, 4, and 3 days, respectively, which is

a one-day difference in all cases from the in vivo outgrowth phase. After the dormancy, the primary sprout lengthens and branching takes place. When an older 16-day rudiment was used after the outgrowth of the primary sprout, degeneration and gland regression occurred within 48 hours in culture. Kratochwil (1969) suggests that development at this time is determined by intrinsic factors rather than hormonal stimulation.

Lasfargues (1962), in studying the effect of insulin on the mammary gland, suggested that there is a gradual increase in hormonal dependency as mammary cells differentiate from embryonic to adult stage and functional activity. Embryonic rudiments apparently survive in medium 199 with no additional supplement (Lasfargues and Murray, 1959), and with some differentiation. Lasfargues also demonstrated that three-month-old glands need estradiol and progesterone for maintenance and at the first stage of pregnancy the ovarian hormones plus at least one hypophyseal hormone are necessary.

Earlier, in 1959, Lasfargues studied the effects of exogenous hormones on cultured embryonic mouse mammary tissue for the purpose of determining the effects of individual hormones. The results were that estradiol produced no epithelial differentiation and that progesterone caused epithelial degeneration. When these hormones were combined, there was some restoration of the epithelium. The

hypophyseal hormones, prolactin and growth hormone, either alone or in combination, increased the degree of growth and differentiation of the mammary epithelium. Cortisol caused the lumen of the ducts to distend. Therefore, although some differentiation can apparently take place without hormones, Lesfargues claimed that embryonic tissue are sensitive to hormonal stimuli.

Ceriani (1969) studied the fetal rat mammary gland from the last five days *in utero*. Using a chemically defined medium with hormonal supplement, he proposed a scheme for hormonal control of development. According to Ceriani, insulin induces growth and the formation of the ductal lumen, prolactin enhances the growth response to insulin, and aldosterone induces ductal development and the appearance of secretion.

The purpose of this study was to examine the *in vivo* development of the mammary glands both grossly and microscopically from 18 days *in utero* to 3 days after birth in the Swiss albino mouse.

Raynaud *et al*. (1970) studied the variability among different strains of newborn mice. His parameters were differences in swelling of the teat, structure of the milk canal, symmetry, and the number of teats present.

For this study, the extent of growth, the degree of variability in development of contralateral glands of the same animals, and the variability of the glands of different animals in the same strain were examined.

The second part of this study concerns itself with the influence of exogenous hormones on 18-day embryonic mammary explants in organ culture using chemically defined medium. The influence of hormones, alone and in combination, on various aspects of morphology, grossly and microscopically, were also examined.

The hormones used were insulin (for the reasons enumerated previously), prolactin [because it has been found to have mitogenic effects (Dilley, 1970) and growth promoting effects (Lasfargues, 1959; Ceriani, 1969)], and corticosterone (because it is the major glucocorticoid in the mouse). Adrenal steroids have been shown to induce secretion in the immature gland (Voytovitch and Topper, 1967) and in the fetal cultured gland (Ceriani, 1969).

MATERIALS AND METHODS

Tissues. Tissues were taken from 18-day embryos up through 3 days of age from Swiss albino mice (Spartan Research Animals, Haslett, Mich.). Parent animals were selected at estrus and mated overnight with males. Pregnancy was dated from the day the females were removed from the male cage. At 18 and 19 days pregnancy, the mother was sacrificed by cervical dislocation and the fetuses were taken. The female fetuses were placed under a dissecting microscope and the second and third thoracic pairs of glands were removed by careful dissection from both sides of the mid-ventral line. The explants were as small as possible but included enough mesenchyme to insure that the entire glandular area had been removed.

After birth, age categories were determined in the following manner: newborns included less than 18 hours old; one-day-old animals ranged from more than 24 hours to less than 32 hours old; two days ranged from more than 48 hours to less than 60 hours old; and three days of age from more than 72 to less than 84 hours old. Dissection was the same for all ages.

For culture of the 18-day fetal glands, removal from the mother and dissection were done under sterile conditions. Ten animals were used per experiment with the glands from one side as the experimental and those from contralateral side as control.

Culture Methods and Hormones. Culture methods were based on those used in our laboratory. Waymouth's culture medium with glutamine (GIBCO) was used as basal medium including 3.5 mg/100 ml Penicillin G. Bovine insulin (activity 26 USP unit/mg) (Calbiochem) was dissolved in small amounts of 0.005 N HCl and Ovine prolactin (NIH P-S-8) dissolved in a small volume of 0.001 N NaOH. For both hormones, enough medium was added to make a stock solution of 100 µg/ml. Corticosterone (Upjohn) was dissolved in absolute alcohol to yield a stock solution of 100 µg/ml. The protein hormones were prepared fresh for each culture and sterilized by passing through millipore filters with an average porosity of 0.45 µ.

The media used were as follows: basal medium without hormones; prolactin; insulin; corticosterone; insulin + prolactin; insulin + corticosterone; insulin + prolactin + cortisterone. The hormones were added to the basal medium to a final concentration of 5 µg/ml for insulin and prolactin and 1 µg/ml for corticosterone.

Three circles of Whatman's filter papers were placed in a large disposable Falcon plastic petri dish

100 x 20 mm and saturated with 5 ml of sterile water. Small sterile disposable Falcon plastic petri dishes were placed on top of the filter paper and then filled with 3 ml aliquots of medium. At least two glands were put into each dish according to the particular experiment. The culture dishes were covered and placed in an air-tight plastic box and then into an incubator at 37°C with a 95% O_2 - 5% CO_2 gas flow. The rate of gas flow was adjusted to maintain the pH at 7.4. The cultures were maintained for 3, 4, and 5 days with the media changed every two days.

Preparation and Measurement of Whole Mounts. Whole mounts were prepared by a modified method of Turner and Gomez (1933). The tissues were put into a weak solution of hyaluronidase then removed after 2 minutes, and placed under a dissecting microscope, and the epidermal layer carefully teased off. The tissues were then flattened on lens paper and placed into Bouin's fixative for 12-16 hours. After pipetting off the fixative, the tissues were washed twice in water and removed from the lens paper. The tissues were then stained with Mayer's hemalum, differentiated in potassium alum, and destained in a 0.1% solution of HCl in 70% ethyl alcohol. After dehydration, the tissues were cleared and stored in methyl salicylate.

The area measurements were made by projecting the image on white paper and making a trace drawing. A Keuffel and Esser planimeter was used as the measuring device.

Histology. Whole mounts were removed from the methyl salicylate and embedded in paraplast. Serial sections were made at 7 μ and stained in hematoxylin-eosin. The glands were examined for extent of survival, duct distension or lumen formation, and adipose development.

Photography. All photos were taken on a Zeiss photoscope using Plus X Pan film by Kodak.

Statistics. The data on Table I and Tables III-VI were done by one-way analysis of variance and Duncan's New Multiple Range test (1955). The data on Table II was analyzed by using student T.

RESULTS

In Vivo Development. The 18-day fetal mammary gland from both the whole mount and histological studies showed more than merely primary duct formation (Figures 1, 2, and 9). In some instances the secondary ducts showed swellings at the distal ends. At 19 days ductal growth continued to show a marked growth increase. An increase was noted again from the newborn to 1 day of age. After this time the growth rate did not significantly change at least up to the third day of age. The growth pattern is indicated in Table I. Figures 3 and 10 represent ductal patterns of the 19-day fetal gland both in whole mount and in cross section respectively. Figures 5, 6, 7, and 8 indicate the similarities of branching patterns from day 1-3 in whole mount studies.

Microscopically, after day 1, adipose cells could be seen suggesting the formation of the fat pad. A defined lumen was also observed by day 1. It forms from the distal to the proximal end of the primary duct by canulation. The smaller ducts showed a basal membrane around the lumen surrounded by two rows of cells (Figure 12). Further

TABLE I. The general growth pattern of the mammary gland from 18 days *in utero* to 3 days after birth.

Age	Number of Animals	Size (mm^2)
18 days*	10	0.164 ± .055
19 days*	8	0.270 ± .098
Newborn	9	0.295 ± .107
1 day	9	0.369 ± .107
2 days	8	0.381 ± .139
3 days	8	0.385 ± .134

*In utero

lumen and adipose cell development can be noted in the 2-day-old gland (Figure 13) and in the 3-day-old gland (Figures 14 and 15).

There was much variability in the degree of branching at all ages. Comparisons can be made at the same age level among different animals (Figures 1, 2, 5, and 6) and for the same animal in comparing the second and third thoracic glands on one side (Figure 4). Once the glands are dissected, there is no way of distinguishing the second gland from the third. Therefore, in making the comparison, an intact side must be considered. In calculating the average sizes, contralateral sides were found to be almost equal, regardless of age group. These results are summarized in Table II.

3-Day Cultures. Culturing for 3 days without hormonal supplement to the basal medium caused no significant change in the overall mammary area. Histologically, some separation of the internal cells of the ducts was seen (Figure 16). This could be the beginning of lumen formation or possibly an indication of internal cellular death. There were some areas in the glandular and connective tissue containing pycnotic nuclei but the overall maintenance was good.

Insulin alone maintained the glandular tissue and increased the growth increment. There was little change in the connective tissue immediately surrounding the gland

TABLE II. Comparison of the measurements of the second and third thoracic glands of the contralateral sides. The glands were taken from 18-day *in utero* animals up to 3 days after birth. There is no significant difference between sides as analyzed by the Student T Test.

Age	Size (mm^2)			
	Side I	Average	Side II	Average
18 days*	0.232		0.224	
	0.220	0.226	0.228	0.226
19 days*	0.324		0.340	
	0.288	0.306	0.300	0.320
19 days*	0.240		0.248	
	0.208	0.224	0.216	0.232
Newborn	0.212		0.288	
	0.272	0.242	0.224	0.266
1 day	0.248		0.252	
	0.412	0.330	0.428	0.340
3 days	0.484		0.500	
	0.396	0.440	0.384	0.442
3 days	0.324		0.328	
	0.344	0.324	0.336	0.332
3 days	0.436		0.444	
	0.388	0.412	0.392	0.418

*In utero

but there is evidence of unilocular adipose development. Loose fibrous connective tissue surrounds the more dense connective tissue which was associated with the glandular tissue (Figure 17).

Explants cultured with corticosterone alone showed little histological difference as compared to those cultured without hormone. Again there was internal ductal cell separation but appearing more lumen-like than in explants cultured without hormone (Figure 18). Growth was also apparent. The combination of insulin and corticosterone resulted in the same growth pattern as did either hormone alone. The two hormones promoted a better defined lumen, adipose cell development, and good connective tissue maintenance (Figure 19).

Prolactin either alone or in combination with insulin maintained the glandular cells but not the connective tissue. Insulin seemed less influential in adipose cell development although connective tissue necrosis was more extensive with prolactin alone. The extent of growth was greater in tissues cultured with the two hormones than with prolactin alone.

The combination of all three hormones provided the highest growth increase, adipose development and glandular maintenance (Figures 20 and 21). However, some pycnotic nuclei did occur throughout the connective tissue surrounding the gland even with this medium.

Table IIIa-g gives the results of the different treatments and Table IV summarizes them.

4-Day Cultures. After 4 days of culture glandular tissue was maintained in media without hormones, but with some indication of breakdown beginning. The nuclei were irregular in shape with some pycnosis. However, even without hormones, there was some indication of growth. Insulin addition increased the area measurement but no specific change was observed in the histology.

The glands cultured with corticosterone alone caused glandular breakdown with patchy areas of necrosis in the connective tissue. However, there was a slight overall difference in growth as compared to the group without hormones. Corticosterone in combination with insulin improved growth and maintenance. However, the distinct lumen formation of the 72-hour culture was not apparent.

Connective tissue breakdown occurred in tissues cultured with prolactin alone. Glandular tissue was still maintained with some pycnotic areas. There was no increase in growth from the 72-hour culture. Insulin and prolactin did not appreciably improve the connective tissue maintenance; however, the glandular tissue was maintained but with some pycnotic nuclei. The average growth increased slightly over the 3-day culture.

TABLE III. The effect on growth of the individual hormone treatments in 3-day cultures. Glands were taken from 18-day fetal mice.

Expt. #	Glands/ expt.	Average Area (mm^2)		Difference
		Control	Expt.	
No Hormones (A) $d = 0.016 \pm 0.011$				
1	3	0.101	0.109	0.008
2	3	0.151	0.161	0.010
3	2	0.239	0.250	0.011
4	2	0.202	0.238	0.036
Insulin (b) $d = 0.115 \pm 0.059$				
1	2	0.170	0.286	0.116
2	2	0.340	0.474	0.134
3	3	0.124	0.236	0.112
4	3	0.133	0.239	0.106
Prolactin (c) $d = 0.119 \pm 0.063$				
1	3	0.100	0.228	0.128
2	3	0.184	0.301	0.118
3	2	0.088	0.210	0.122
4	2	0.120	0.248	0.128
Corticosterone (d) $d = 0.103 \pm 0.052$				
1	2	0.132	0.216	0.084
2	2	0.050	0.158	0.108
3	2	0.166	0.276	0.110
4	2	0.166	0.276	0.110
Insulin + Prolactin (e) $d = 0.151 \pm 0.075$				
1	2	0.186	0.352	0.166
2	2	0.182	0.336	0.154
3	3	0.185	0.333	0.148
4	2	0.060	0.194	0.134

TABLE III. Continued

Expt. #	Glands/ expt.	Average Area (mm^2)		Difference
		Control	Expt.	
Insulin + Corticosterone (f) d = 0.112 ± 0.005				
1	3	0.092	0.206	0.114
2	2	0.090	0.208	0.118
3	2	0.102	0.216	0.114
4	3	0.125	0.225	0.100
Insulin + Prolactin + Corticosterone (g) d = 0.157 ± 0.076				
1	2	0.086	0.236	0.150
2	2	0.106	0.230	0.124
3	3	0.117	0.343	0.226
4	2	0.120	0.246	0.126

TABLE IV. Summary of the hormonal effects in 3-day cultures.

Treatments	Number of Expts.	Average Area (mm^2)		Average Difference
		Control	Exptl.	
No hormones	4	0.173	0.190	0.016 ± .011[a]
I	4	0.192	0.309	0.115 ± .059[a]
PL	4	0.123	0.246	0.119 ± .063
B	4	0.129	0.232	0.103 ± .052
I + PL	4	0.153	0.304	0.151 ± .075[b]
I + B	4	0.102	0.214	0.112 ± .055
I + PL + B	4	0.107	0.264	0.157 ± .075[b]

Note: I = Insulin; B = Corticosterone; PL = Prolactin.

[a]No hormone treatment was found to be significantly different from I.

[b]I + B + PL or I + PL was found to be significantly different from PL, B, I + B, and no hormones.

Data were analyzed by Duncan's New Multiple Range Test at the 1% level.

The combination of all three hormones again resulted in the highest growth increment although areas of necrotic connective tissue were evident. The results of the individual treatments are given in Table Va-g and summarized in Table VI.

5-Day Culture. After 5 days in culture without hormones both the connective tissue and the glandular tissue showed extensive breakdown (Figure 22). It was, therefore, not possible to make whole mount measurements. In cases where the tissue remained intact, the density of the connective tissue made it impossible to distinguish anything.

Histologically, the best glandular maintenance occurred with the combination of three hormones. However, there was some indication of internal ductal disorganization indicative of cellular degeneration (Figures 26 and 27).

Explants cultured with insulin alone showed some glandular viability and again fibrous loose connective tissue surrounding the more dense connective tissue around the gland (Figure 23).

Explants cultured with corticosterone alone and in combination with insulin showed much less cellular arrangement and organization characteristic of the 3-day culture period (Figure 25).

TABLE V. Results of the individual hormone treatments in 4-day cultures.

Expt. #	Glands/ expt.	Average Area (mm^2)		Difference
		Control	Expt.	
No Hormones (a) $d = 0.083 \pm 0.043$				
1	2	0.170	0.232	0.062
2	2	0.238	0.340	0.102
3	3	0.108	0.212	0.104
4	2	0.090	0.154	0.064
Insulin (b) $d = 0.122 \pm 0.062$				
1	3	0.233	0.331	0.098
2	2	0.250	0.365	0.115
3	2	0.170	0.300	0.130
4	3	0.168	0.311	0.143
Prolactin (c) $d = 0.101 \pm 0.051$				
1	3	0.136	0.245	0.111
2	3	0.129	0.223	0.094
3	2	0.168	0.272	0.104
4	2	0.178	0.274	0.096
Corticosterone (d) $d = 0.056 \pm 0.029$				
1	3	0.149	0.191	0.042
2	3	0.164	0.220	0.046
3	2	0.158	0.222	0.074
4	2	0.128	0.190	0.062
Insulin + Prolactin (e) $d = 0.186 \pm 0.093$				
1	2	0.166	0.378	0.212
2	2	0.164	0.322	0.183
3	3	0.164	0.336	0.172
4	2	0.132	0.308	0.176

TABLE V. Continued

Expt. #	Glands/ expt.	Average Area (mm^2)		Difference
		Control	Expt.	
Insulin + Corticosterone (f) $d = 0.122 \pm 0.063$				
1	3	0.223	0.337	0.114
2	2	0.246	0.388	0.142
3	2	0.162	0.274	0.112
4	2	0.178	0.298	0.120
Insulin + Prolactin + Corticosterone (g) $d = 0.226 \pm 0.113$				
1	3	0.206	0.415	0.211
2	3	0.135	0.332	0.207
3	2	0.168	0.400	0.232
4	2	0.166	0.420	0.254

TABLE VI. Summary of hormonal effects in 4-day cultures.

Treatments	Number of Expts.	Average Area (mm^2)		Average Difference
		Control	Exptl.	
No hormones	4	0.153	0.235	0.083 ± 0.043[a]
I	4	0.205	0.327	0.122 ± 0.062[a]
PL	4	0.153	0.254	0.101 ± 0.051
B	4	0.150	0.206	0.056 ± 0.029
I + PL	4	0.157	0.336	0.186 ± 0.093[b]
I + B	4	0.202	0.324	0.122 ± 0.063[a]
I + PL + B	4	0.169	0.392	0.226 ± 0.113[c]

[a] I or I + B was significantly different from no hormones.

[b] I + PL was significantly different from PL, I, B, I + B, and no hormones.

[c] I + B + PL was significantly different from all other treatments.

Data were analyzed by Duncan's New Multiple Range Test at the 1% level.

Explants cultured with prolactin alone showed breakdown of both glandular and connective tissue such as occurred with the non-supplemented medium. The addition of insulin slightly improved glandular maintenance but with no improvement in the connective tissue (Figure 24).

DISCUSSION

Some discrepancy is found in the literature regarding the primary sprout outgrowth in the developing mammary gland of the fetal mouse. In this study, it is evident that by 18 days of fetal age, secondary and tertiary sprouts as well as primary sprouts have developed. In many cases these secondary and tertiary sprouts have terminal cell clusters or buds. The general histological pattern of the formed ducts is constant in that in no instance is there ever lumen formation before day 1 of age.

The variation in size and shape of the mammary glands within an age group may be due to several factors: litter size, individual animal size, and more important, the actual proliferative stage of the particular gland. It has been found that ductal growth occurs by the addition of the terminal bud cells (Bresciani, 1968), and the presence of such buds is an influential factor. Since most of the in vivo work was done on different litters, anything affecting the makeup of the litter might account for the variability. This includes the ratio of males to

females per litter. The larger litter size >13 seemed to favor males over females. If the litter was <10, the females seemed to be favored. Circulating androgens do affect female gland development (Jean and Jean, 1969; Kratochwil, 1971).

Culturing 18-day-old glands for 3 days did not increase overall growth of the gland with only slight effects in 4-day cultures. This is not to say that there was no glandular mitotic activity. It is possible that rates of mitosis were nearly equal to rates of cell degeneration since some cell separation was seen histologically in the ducts cultured for 3 days in hormone free medium. Responses in culture were variable. The variability and extent of development at time zero could have had effects on tissue response in vitro. Kratochwil (1969) found that once the primary duct sprouted, cultures totally degenerated and regressed after 48 hours in culture even in the presence of 10% horse serum and embryonic extract in the medium. If younger glands were used, both survival and branching were evident. Ceriani (1969), on the other hand, found that with his time zero 17-day-old rat fetal glands, 5 days before birth, survival without hormonal supplement was possible up to 9 days in vitro. At 18 days in utero, which is less than 36 hours before birth (in the mouse), ductal development has already begun. By 5 days, survival of the cultured glands was

low and complete tissue degeneration was apparent in many instances. From the results of these two authors and from those of this study, it does seem that the age of the fetal animal and the stage of development influence the effectiveness of culturing.

It can be argued that the level of circulating hormones in the animal may affect the response of the excised explants in culture. From work done by Kitchell and Wells (1952), it was concluded that the fetal hypophysis secretes ACTH causing maturation of the adrenal cortex. Such zonation is associated with adrenal activity (Milković and Milković, 1966). The studies by Kitchell and Wells were done on 18-20-day rat fetuses in which there is evidence that steroids are in circulation prior to birth.

Blázquez, Montoya, and Quijada (1970) found that during the last third of pregnancy an increase in plasma insulin occurs to compensate for the increased amount of glucose uptake from the mother. Previous evidence exists that the fetal adrenal glands maintained carbohydrate metabolism (Milković and Milković, 1966).

There is no evidence that ovarian hormones from a cycling ovary are present in the fetus. Therefore, ovarian steroids are not likely to influence fetal mammary development unless one considers maternal influences via the placenta which is known to store estrogens.

Kohmoto and Bern (1971) provide evidence that fetal mouse pituitaries contain prolactin at day 18 of gestation. Culture studies indicate that there can be secretion of prolactin at the late stages of pregnancy.

From these data, one might conclude that early fetal mouse mammary gland is indeed not hormone dependent in agreement with Hardy (1950), Lasfargues and Murray (1959), and Kratochwil (1968).

The influence of growth hormone, nevertheless, cannot be ruled out entirely. A high level of fetal growth hormone is present although its activities are unknown (Pecile and Müller, 1966). Somatotrophin must be considered in view of the work of Jean (1968) and Jean and Delost (1968) which demonstrates the growth effect of this hormone on the fetal gland.

High concentrations of growth hormone have been found to initiate DNA synthesis as does insulin in virgin and midpregnant glands in culture (Turkington, 1968), suggesting that growth hormone may stimulate DNA synthesis in vivo. The effect of insulin in fetal gland cultures may be to prevent cellular death or to allow new cell development to compensate for cellular degeneration. This is suggested in the 3-day cultures without hormones where cellular death might be beginning in contrast to the higher growth increment in response to insulin. Another possibility, as suggested by Lasfargues (1962),

is that insulin may facilitate penetration of growth factors to a cell equipped to utilize them. This might explain why insulin in combination with prolactin effects a greater growth response and with corticosterone, better cell alignment. Insulin in all cases stimulates some adipose cell development in 3-day cultures. A large amount of loose fibrous connective tissue around the more dense tissue surrounding the gland was also seen in response to insulin. The adipose development and the tissue immediately adjacent to the gland may have some influence on the ability of the gland to grow. There were always areas with pycnotic nuclei present in varying numbers depending upon the section examined.

A similar growth pattern was observed in explants cultured with corticosterone alone as with insulin alone. This cannot be as easily explained in the 3-day cultures other than the fact that there is some cellular separation in the ducts causing distension. This is in agreement with the findings of Lasfargues and Murray (1959), who cultured much younger embryonic glands with cortisol. It is difficult to determine whether this distension can account for the amount of overall growth difference between the control and the 3-day cultures. According to Lasfargues (1962), using adult mammary tissue, cortisol has no growth promoting effect on the epithelium. Lockwood, Stockdale, and Topper (1967) claim that hydrocortisone acts during

the proliferative phase. If this were also true of the fetal gland, corticosterone could be acting in some way on the newly synthesized DNA to make a viable cell population after mitosis. In combination with insulin, corticosterone appears to stimulate ductal lumen formation.

In all cases where prolactin was used in the 3-day cultures, glandular maintenance and growth were observed. In 4-day cultures connective tissue breakdown was apparent in glands cultured with prolactin alone which may have affected further development. Glandular breakdown was especially apparent after 5 days. In most cases, the breakdown started with the surrounding connective tissue and progressed from the periphery of the gland to the interior. Such findings are contrary to those of Lasfarges and Murray (1959), who reported that prolactin had a developmental effect on the mesenchymal tissue.

From these findings it may be concluded that growth responses occur with insulin either by a direct effect on the mammary cells or peripheral effects possibly on the undifferentiated mesenchyme. There is evidence of mesenchymal influence in the work of Propper (1968) with 11-14-day rabbit embryonic tissue and Kratochwil (1969) with the glands of 12-16-day fetal mice.

In all cases prolactin in combination with other hormones seemed to produce the greatest effect on growth, although when used alone growth is not suppressed.

Corticosterone appeared to act by putting the cells together in some organized manner, especially when insulin was also present. Its influence may be to prepare the cells for functional activity.

The results of this study suggest that the normal pattern of development can be altered to a greater or lesser degree by the use of exogenous hormones. Variability of the tissue itself must be kept in mind and as well as the possible effects of circulating hormones in the 18-day-old fetus. The ductual pattern could only be advanced in vitro to a stage equivalent to that in a one-day-old animal. This was accomplished by the full triad of hormones after 3 days of culture, which is the chronological equivalent to one day of age. Maximal growth was achieved after 4 days in culture. Further growth may be possible but seems unlikely due to the histological breakdown beginning by 5 days of culture. The data suggest that once maximal development is accomplished in vitro, degradation of the system begins.

By altering the processes of normal development in an actively developing system such as the fetal mammary gland, clearer knowledge and understanding of normal differentiation as well as abnormal systems may be attained.

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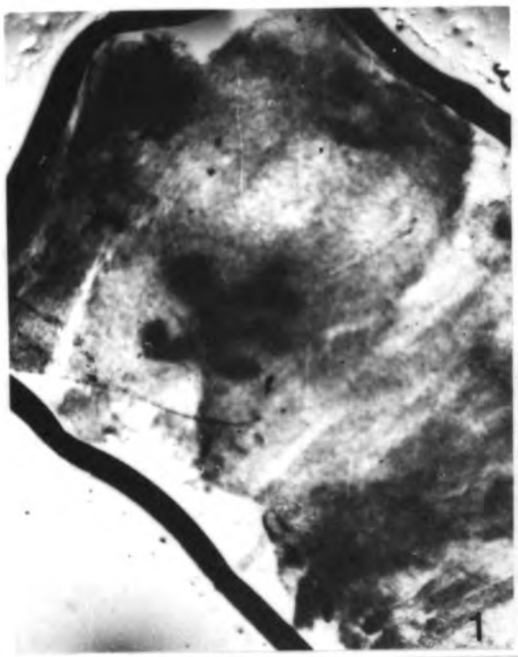
FIGURES

Figure 1. 18-day fetal gland, whole mount.

Figure 2. 18-day-old fetal gland from a different animal.

Figure 3. 19-day-old fetal gland.

Figure 4. Newborn glands, from one side showing differences in symmetry.



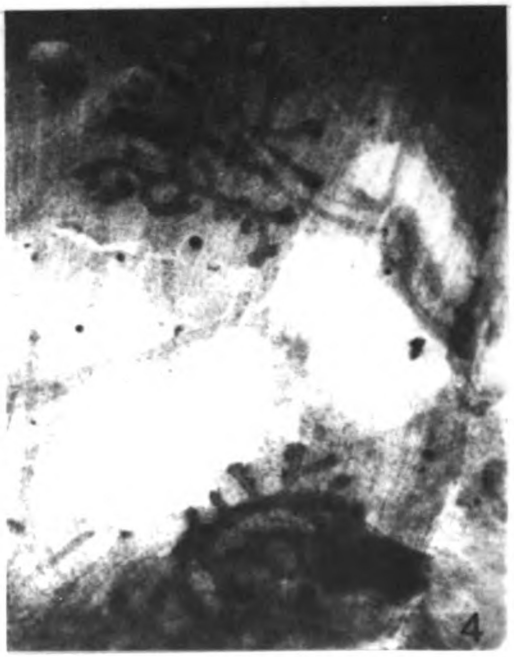


Figure 5. 1-day-old whole mount.

Figure 6. 1-day-old animal showing more complex branching from another animal.

Figure 7. 2-day-old gland.

Figure 8. 3 days old with less branching than in the 1-day animal.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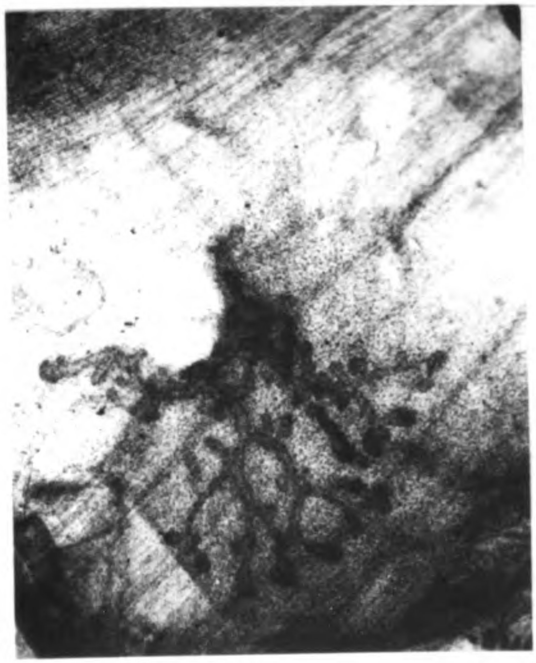


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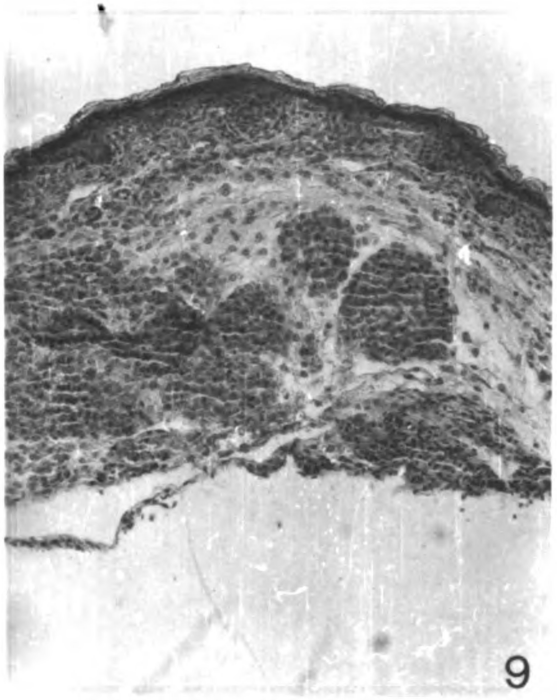
Figure 9. 18-day-old fetal duct xs.

Figure 10. 19-day duct xs.

Figure 11. Newborn duct xs.

Figure 12. 1-day bud and duct, lumen and adipose cell development.



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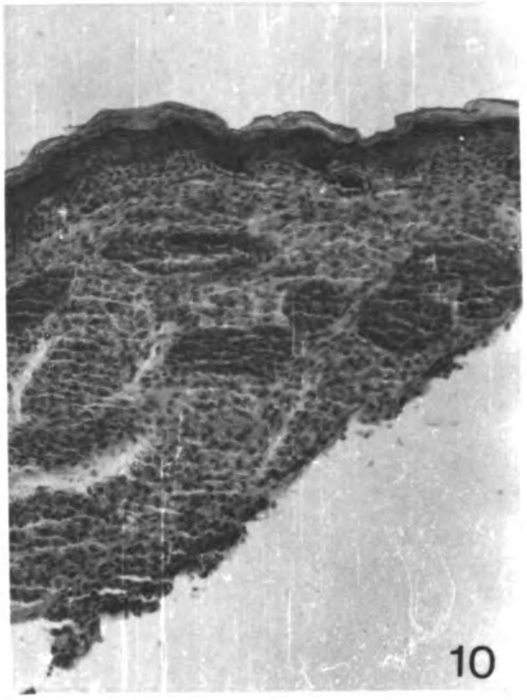

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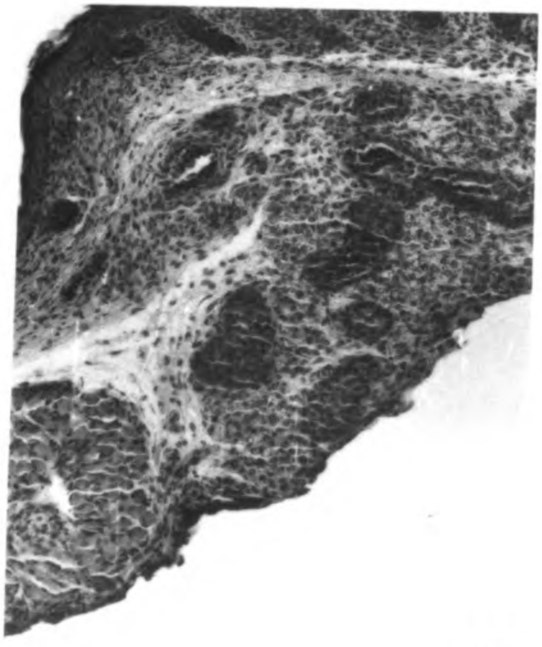

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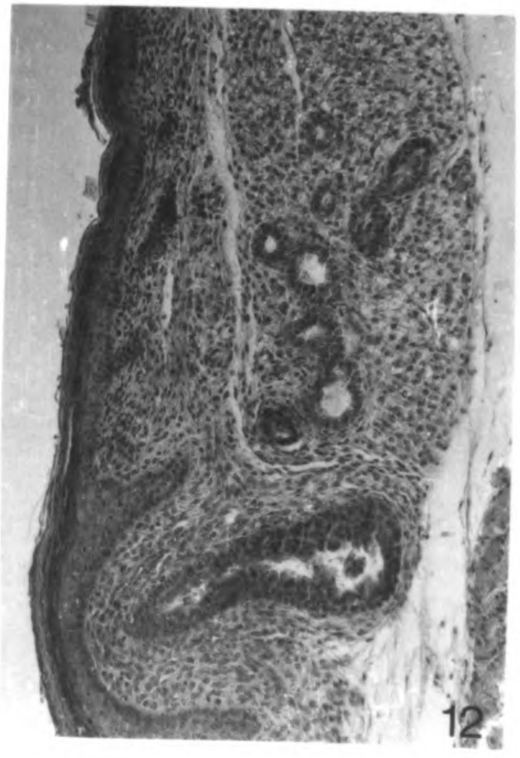

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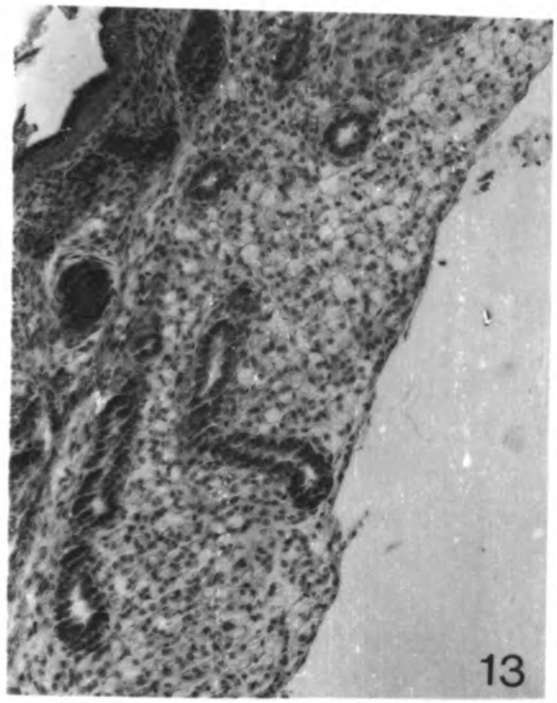
Figure 13. 2-day duct xs; note increase in adipose cells.

Figure 14. 3-day bud with lumen opening toward outside.

Figure 15. 3-day duct xs embedded in adipose cells.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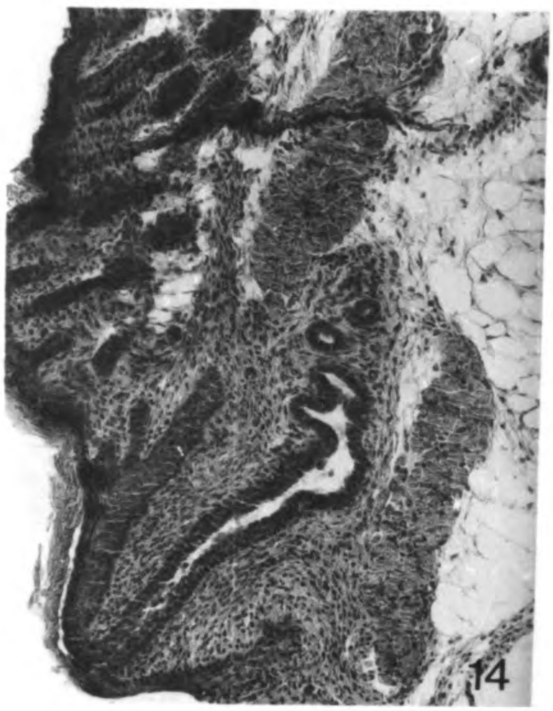
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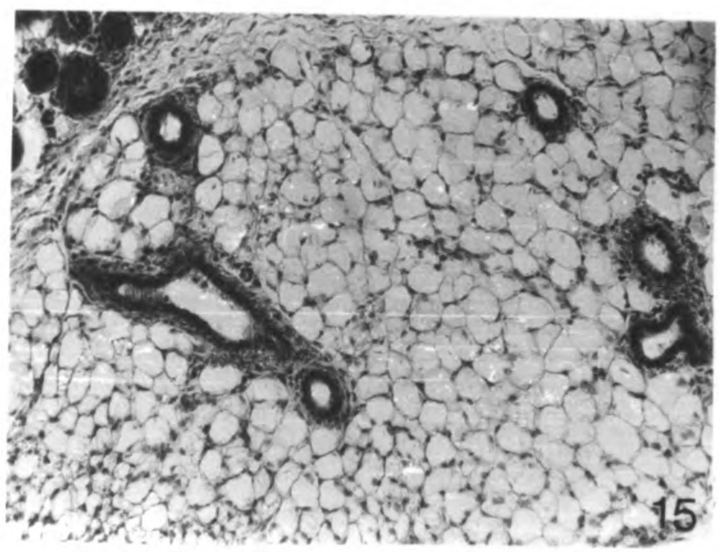
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Figure 16. Control (no hormone) after 72 hours in culture.

Figure 17. Insulin addition after 3 days in culture.

Figure 18. Corticosterone addition after 3 days in culture.

Figure 19. Combination of insulin + corticosterone after 72 hours of culture.

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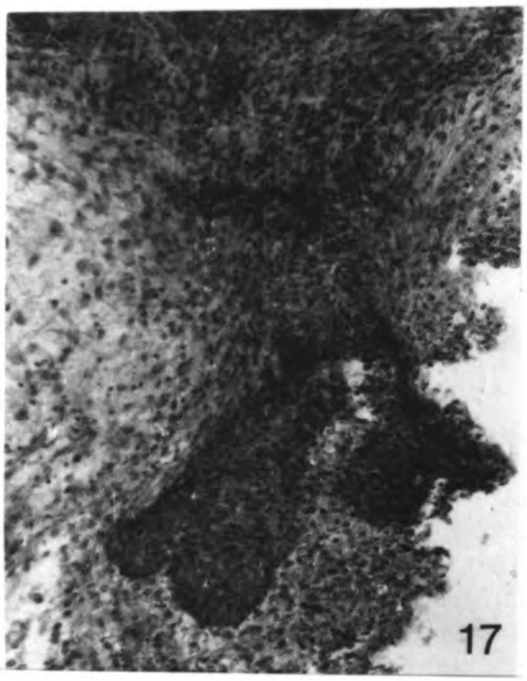

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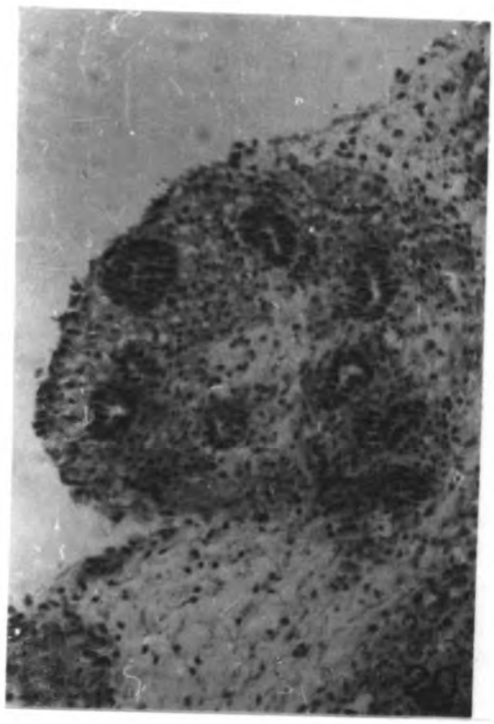
Figure 20. Insulin + corticosterone + prolactin after 72 hours; growth into adipose-like tissue with defined lumen.

Figure 21. Enlargement of above.

Figure 22. No hormones after 5 days in culture. Note massive degeneration of glandular cells.

Figure 23. Insulin addition after 5 days in culture.

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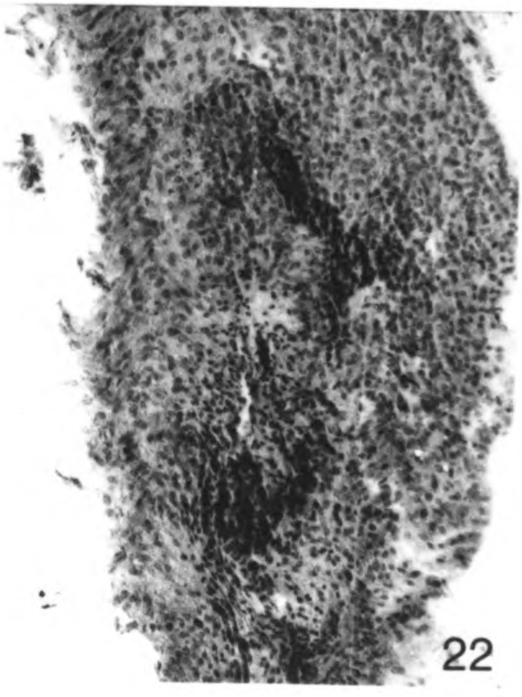
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Figure 24. Insulin + prolactin. Note peripheral degeneration after 5 days in culture.

Figure 25. Insulin + corticosterone. Note less cellular organization after 5 days in culture.

Figure 26. Insulin + corticosterone + prolactin glandular maintenance but lumen is less defined.

Figure 27. Hormone triad with duct enlargement.

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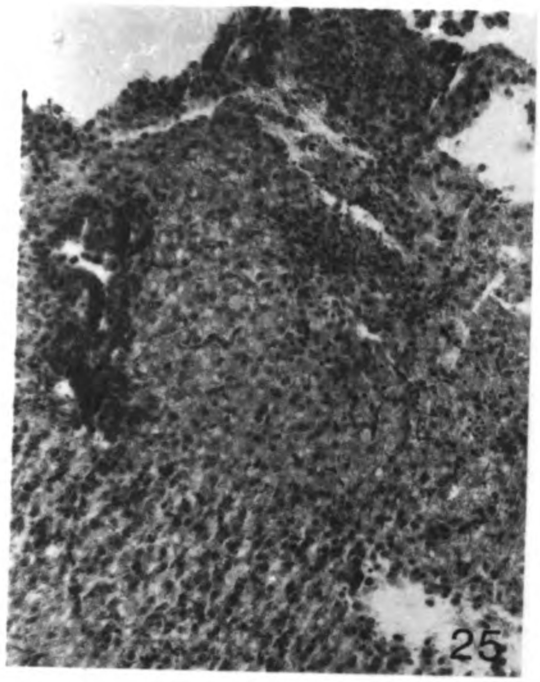

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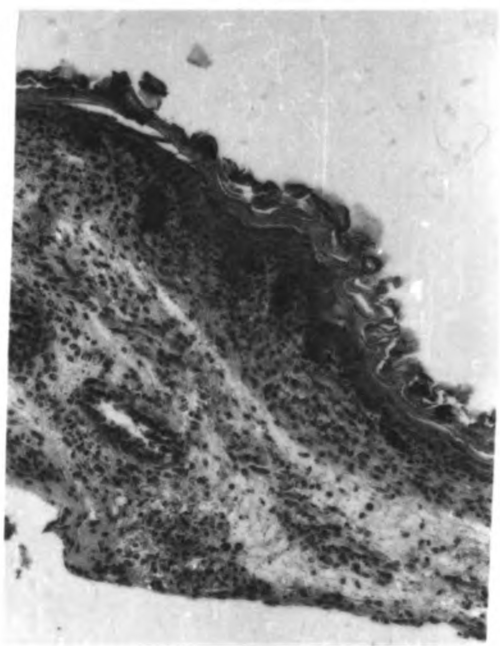

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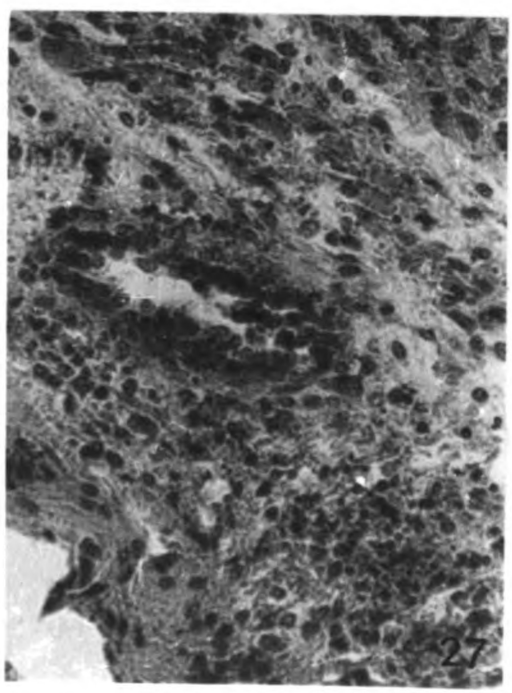

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