2. ABSTRACT

The mammogenic and lactogenic effect of various hormones on rat mammary gland has been studied in vitro using an organ culture technique. The effects have been assessed mainly by light microscopy. The study has been confined mainly to the effects and interactions of the lactogenic protein hormones, corticosteroids and progesterone. It establishes the importance of the presence of insulin and serum and of the type of serum for the hormonal effects in vitro. These effects have been studied mainly on the explants obtained from 13 days pregnant rats. In addition explants from non-pregnant rats and at various stages of pregnancy and lactation were studied.

2.1. Experiments with mammary glands of pregnant rats

Prolactin

The mammogenic effect of prolactin manifested itself as increased alveolar development, and the presence of mitotic activity and thymidine incorporation. A high concentration of virgin rat serum in the medium was necessary for the growth response. Human, rabbit, fetal calf or horse serum could not or only partly replace rat serum. The mammogenic effect was obtained with rat, human, bovine and to a lesser extent with ovine prolactin preparations.

High mitotic activity was present in the pregnant rat mammary gland explants after 24 h of culture especially if insulin was added. Prolactin was not needed for the occurrence of this wave of mitoses. The effect of prolactin on mitotic activity became distinct after 72 h. Prolactin also increased the thymidine incorporation after 24 and 72 h of culture.

Although some effect was observed at concentrations between 10 to 27 ng/ml rat prolactin in the medium, a sharp increase in response occurred in the range of 27 to 115 ng/ml medium. At higher concentrations the response obtained in a 3 days culture did not increase. The prolactin concentration in the medium remained fairly constant during the three days
of culture.

A mammogenic response was also obtained with lactating rat serum and it correlated with the prolactin level measured in the sera by radioimmunoassay.

In addition to a proliferative effect prolactin had a weak effect on secretion: it promoted the accumulation of small intracellular fat droplets and promoted some intraluminal secretion and formation of intracellular fat vacuoles locally in the explants. However, the secretory response remained incomplete, even after 6 days of culture.

Progesterone, like prolactin had a proliferative effect on pregnant rat mammary gland in vitro in a medium containing virgin rat serum and insulin. At low dose levels a synergism could be shown for the two hormones. Testosterone had a weak proliferative effect, but a synergism with prolactin was not observed. An interaction between prolactin on the one hand and oestradiol (except for an inhibition at high concentrations) or thyroxin, on the other hand, could not be demonstrated.

A high concentration of insulin was favourable for an optimal effect of prolactin, but a proliferative effect could still be demonstrated in the absence of insulin added to the medium.

Human placental lactogen

Human placental lactogen (hPL) has a proliferative and some lactogenic effect on pregnant rat mammary gland in vitro. For the proliferative effect rat serum must be added to the medium. HPL combined with virgin rat serum can simulate - with some quantitative differences - the effect of 13 days pregnant rat serum in vitro. HPL was approximately four times less active than rat prolactin in a medium containing rat serum. The activity present in one ml of 13 days pregnant rat serum was comparable to 1 to 5 µg rat prolactin added to one ml of virgin rat serum.

Rabbit anti-hPL serum had a toxic effect on the rat mammary gland explants. No conclusive evidence was found for a cross-reactivity between hPL and rat chorionic mammotrophin, present in pregnant rat serum.
Growth hormone, ACTH

Human and porcine growth hormone preparations showed mammo-
genic activity in vitro, but their activity was much less - on a weight basis - than that of rat prolactin. Evidence for an effect of adrenocorticotropic hormone was equivocal.

Corticosteroids

The corticosteroids induce a secretory response: intraluminal secretion and intracellular fat vacuoles appear. Rat serum was needed for an optimal effect. Heterologous sera could not replace rat serum. A maximum secretory response with cortisol was obtained when the culture medium contained rat serum as well as insulin and a lactogenic protein hormone. When only one of these factors was present the response was virtually absent. When two factors were present a response developed but it remained submaximal.

Prolactin potentiated the corticosteroid effect. The potentiation was dose-related. A similar effect was exerted by growth hormone preparations and human and rat lactogenic hormones of placental origin. The cortisol-potentiating effect of pregnant rat serum appeared on day 8 of pregnancy and disappeared to a large extent shortly after parturition. The cortisol-potentiating effect of lactating rat serum was less predictable.

An effect of adrenocorticotropic hormone, progesterone, testosterone or thyroxin on the cortisol-induced response could not be demonstrated. Oestradiol may slightly stimulate the secretory effect.

The mammary gland of virgin rats or of rats early in preg-
nancy appeared to be less sensitive to cortisol and aldosterone than the mammary gland of rats late in pregnancy. The secretory response to cortisol developed in 13 days pregnant rat explants only after two days of culture and reached a maximum around day 6. The presence of a lactogenic protein hormone did not accele-
rate the response. The lag period of the cortisol effect was reduced in explants of rats later in pregnancy. The reaction to the corticosteroids was dose-related. The addition of 0.17 μg cortisol per ml medium gave a response. Both corticosterone and aldosterone produced an effect similar to that of cortisol, but
higher concentrations were needed. Some effect could be obtained with deoxycorticosterone but at relatively high concentrations. With the corticosteroids added, the number of mitoses induced by the mammogenic hormones tended to decrease.

**Progesterone**

Progesterone had a mammogenic effect, which appeared to be less than that of the lactogenic protein hormones. It had no lactogenic effect. High concentrations of oestradiol suppressed the effect of progesterone. An interaction with testosterone was not found. Insulin was needed for a maximum effect.

**2.2. Experiments with mammary glands of lactating rats**

When mammary explants obtained from lactating rats were cultured in a medium to which only virgin rat serum and insulin had been added, secretion persisted only on the first day of culture. High concentrations of glucose in the medium stimulated the appearance of fat vacuoles. On the second day a wave of mitoses was observed. Thereafter a rapid involution followed.

Prolactin added to a medium containing virgin rat serum had a proliferative effect on lactating rat explants: it increased thymidine incorporation and mitotic activity on day 2 and 3 of culture. It had also some effect on secretion: it seemed to decelerate the breakdown of the accumulated secretion products. Cortisol had a strong effect on secretion: in its presence the secretory aspect of lumina filled with eosinophilic material, and fat vacuoles was maintained for 3 days. This effect was observed with mammary explants obtained at various stages of lactation. A synergism between prolactin and cortisol could not be demonstrated.

After addition of lactating rat serum instead of virgin rat serum the same results were obtained. When prolactin was replaced by the placental lactogen present in pregnant rat serum, an effect on mammary explants from lactating rats could not be demonstrated, while a synergism between the lactogen and cortisol could still be shown in explants from late pregnant rats. An effect of progesterone together with oestradiol was not seen.
either in the presence or in the absence of cortisol.

A sequence of hormonal control is proposed for the development of the mammary gland and the induction of secretion. In this sequence the lactogenic protein hormones and progesterone regulate proliferation, the lactogenic hormones (with the corticosteroids) regulate the morphological differentiation, while for a full secretory response increased corticosteroid levels are necessary. The lactogenic protein hormones potentiate the corticosteroid effect, but cannot induce secretion at high concentrations. The proposed sequence may explain some of the developments occurring in vivo.

Rat serum and insulin are essential for the in vitro condition. Arguments for the assumption that only "stem cells" are able to divide, were not found.

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3. INTRODUCTION

The response of mammary gland cells to hormones has been determined using various culture techniques (for a review, see Banerjee 1976). From results obtained with mouse mammary gland in vitro Turkington (1972) has proposed a general schedule for the sequence of hormonal actions and cellular changes. In this schedule substances such as epithelial growth factor, growth hormone, serum growth factor and especially insulin are involved in the multiplication of stem cells. It is assumed that if cell division takes place in the presence of cortisol, some daughter cells can be transformed in the next step into secretory cells by prolactin or placental lactogen. For this final transformation insulin must also be present. These conclusions were based on results obtained with mammary gland explants cultured in a chemically defined medium. A different role for prolactin has been suggested by other authors. El-Darwish & Rivera (1970) demonstrated an initial stimulation of DNA synthesis by insulin and prolactin and subsequently by prolactin and by prolactin plus cortisol. Mayne & Barry (1970) confirmed the stimulating effect of prolactin combined with corticosterone on DNA synthesis. Dilley (1971a) and Koyama et al. (1972) reported a similar prolactin effect on rat mammary gland. Besides prolactin, progesterone also showed mammogenic (= growth promoting) activity. Reviewing the evidence Forsyth & Jones (1976) concluded that rather than insulin, a complex of hormones might be involved, its essential constituents being insulin, prolactin and steroids from ovaries and adrenals.

The present study is an outcome of the earlier observation that pregnant and lactating rat serum had a mammogenic effect on rat mammary gland in vitro (Peters et al. 1976). With regard to pregnant rat serum, the activity was present from day 8 of pregnancy until shortly after parturition. The pregnancy factor is formed in the placenta (Peters et al. 1977) and was considered to be rat chorionic mammotrophin (rCM), alternatively referred to as rat placental lactogen by Robertson & Friesen (1975). Its human analogue, human placental lactogen (hPL) also possessed
in vitro mammogenic activity in a first trial (Peters 1977a). The addition of a high concentration of insulin to the culture medium was not essential for the mammogenic activity of pregnant rat serum, but it enhanced the effect strongly. In lactating rat serum mammogenic activity was erratic. Prolactin seemed to be the mammogenic factor (Peters et al. 1976). The activity of prolactin could be demonstrated in vitro (Peters 1977b).

The apparent ease with which the "lactogenic" hormones could be shown to be mammogenic when virgin rat serum was present in the medium suggested that the serum itself played an essential role. It was surprising that such a high concentration (50%) of rat serum could be added to the medium without exerting a toxic effect on the rat mammary explants. Forsyth & Parke (1973) reported a toxic effect of various types of serum when added to the medium in excess of 30%. An explanation for the absence of a toxic effect with high concentrations could conceivably be found in the nature of the serum used. The present study was set up to explore this possibility.

Its aims have been to establish the importance of serum and the type of serum for the organ culture of rat mammary explants. It shows to what extent the in vitro responses to hormones vary with the medium. The study is confined to the effects and interactions mainly of the lactogenic hormones, corticosteroids and progesterone.
4. MATERIALS AND METHODS

4.1. Serum

Serum ($S_0$) was obtained from nulliparous, adult female albino rats. Collection and preparation have been described elsewhere (Peters et al. 1976). In addition serum was collected from pregnant rats ($S_{1-21}$, number indicating day of pregnancy) and from lactating rats ($SL_{1-21}$). Sera obtained from rats treated otherwise are described for the various experiments under "Experimental conditions". Human, rabbit, fetal calf and horse serum were obtained commercially (membrane filter sterilized, Flow Laboratories, Irvine, Scotland). Anti-hPL serum was obtained both commercially (hPL-immunoassay Kit) and as a gift by Dr. H. Friesen.

4.2. Hormones

The hormones were obtained from Organon, Oss, The Netherlands, unless stated otherwise.

The following stock solutions (1 mg/ml aqua dest.) of protein hormones were used: ovine prolactin (5 IU/mg), rat prolactin (of various potency, see appropriate "Experimental conditions"), human prolactin (potency unknown), bovine prolactin (14-15 IU/mg Netherlands' Cancer Institute preparation, and 12.8 IU/mg, NIH preparation), human growth hormone (potency unknown), porcine growth hormone (0.4 U/mg, Sigma Chem. Co. Inc., St. Louis, MO, USA), human placental lactogen (potency unknown), thyroxin (L-thyroxine sodium, BDH, England), adrenocorticotrophins (Organon porcine preparation 28 U/mg; Sigma porcine preparations Grade II, 86.1 U/mg and 150 U/mg; Organon tetracosactide, pure preparation). The solutions were membrane sterilized (Millipore Filter Corp., Brussels, Belgium; 0.45 μm, Ø 25 mm), divided in small portions and stored at -22°C. The ovine prolactin preparation was glass-filter sterilized (Jenaer Ganzglas-Bakterienfilter G5, Schott, Mainz, W-Germany). The human, bovine and rat prolactin preparations, and human growth hormone were gifts
from Dr. H.G. Kwa, The Netherlands' Cancer Institute, Amsterdam, the human placental lactogen was a gift from Dr. H. Friesen, Winnipeg, Canada, while tetracosactide was a gift from Organon, Oss, The Netherlands. When insulin (I) was added to the culture medium, the final concentration was 50 μg/ml (bovine; 23.6 or 25.7 IU/mg). Trowell's T8 was obtained with insulin already added to the solutions from Flow Laboratories, Irvine, Scotland. The following stock solutions of steroids in ethanol were used: progesterone (P; 10 mg/ml), 17-β oestradiol (O; 0.5 μg/ml), testosterone propionate (T; 10 mg/ml), cortisol (F; 8 mg/ml), aldosterone (A; 8 mg/ml), corticosterone (B; 8 mg/ml) and deoxycorticosterone (D; 8 mg/ml). The steroid solutions were stored at +4°C. The final concentration of ethanol in the culture medium did not exceed 0.05%, unless stated otherwise.

4.3. Medium

The medium consisted of either 100% Trowell's T8 (Paul 1970) or a mixture of serum and t8, a simplified formula adapted from Trowell's T8, i.e. without amino acids, vitamins and phenol red. The final glucose concentration is stated under the "Experimental conditions". No antibiotics were used. In one experiment Waymouth MB 752/1 (Flow Laboratories, Irvine, Scotland) was used.

4.4. Culture

The technique has been described elsewhere (Peters et al. 1976). The number of mammary glands used and the number of explants cultured per experiment are stated under "Experimental conditions". Size of the explants obtained from rat mammary glands at various stages of development during pregnancy and lactation were generally uniform (approximately 2 x 2 x 1 mm) except for some experiments using lactating rat mammary gland (see "Experimental conditions"), when the size was reduced to 1.5 x 1.5 x 1 mm. It took approximately 30 min to prepare one gland for explantation.
4.5. Histology & Grading

Histology and grading have been described elsewhere (Peters et al. 1976). Sections were stained with haematoxylin and phloxin. The following parameters were of importance: alveolar development or size (size is used instead of development when in the experiments concerning the lactating rat mammary gland this parameter seemed to be controlled mainly by the degree of accumulation of secretion in the lumina), cytoplasmic opalescence, vacuolization and eosinophilic material in the lumina (secretion) whereby a distinction was made between secretion containing cell debris, and homogeneous secretion. Mitoses and pyknosis were noted. Necrosis was estimated as the percentage of the area of the section showing necrosis. In some experiments the material supporting the explants could be dissolved in acetone enabling an evaluation of the amount of outgrowth at the bases of the explants.

RNA was stained with methylgreen/pyronin, RNA-ase was used to control specificity (Spannhof 1967). For the detection of lipids the following methods were applied using frozen sections after formol fixation: Sudan Black B in propylene glycol (probably neutral fats) (Spannhof 1967); copperphthalocyanin method (Luxol Fast Blue) after Elftman's controlled chromation (phospholipids) (Pearse 1968). For the estimation of the total amount of lipids in the culture frozen sections were stained with Phosphine 3R, microphotographs were taken at a magnification of 200 x avoiding necrotic parts, and the amount of fluorescent material in each microphotograph was estimated with a point counting system (adapted from Weibel & Elias 1967).

4.6. Electron microscopy

For electron microscopy explants were cut into fragments of approximately 1 mm³ and fixed in 2.5% glutaraldehyde in 0.067 M phosphate buffer, pH 7.4 at +4°C, postfixed in 2% OsO₄ in the same buffer, dehydrated in graded ethanol and embedded in Epon (Luft 1961).
4.7. Labelling Index

\(^{3}\text{H}\)-thymidine (5 μCi/ml), Radiochemical Centre, Amersham, England) was added 3\(\frac{1}{2}\) h before the end of the experiment. Details are described elsewhere (Peters et al. 1976). \(^{14}\text{C}\) glucose (D-glucose-U-[\(^{14}\text{C}\)]; 16.1 μCi/mg glucose; Radiochemical Centre, Amersham, England) was dissolved in the chemically defined part of the medium, membrane-filter sterilized and added to the medium at 1 μCi/ml medium. The cultured material was treated with NCS (Nuclear Chicago, Des Plaines, Illinois) and \(\text{H}_2\text{O}_2\). Activity was measured after addition of the scintillation cocktail, with a Mark 1 liquid scintillation counter. Total lipids were isolated according to Overturf & Dryer (1969).

4.8. Radioimmunoassay

Rat prolactin levels in the medium were determined in some experiments by the method described by Kwa et al. (1972) at the Netherlands' Cancer Institute.

4.9. Glucose, lactate, amino acids and proteins

The glucose concentration in the medium was measured by the 0-toluidin method (Blutzucker Merckotest\(^R\), E. Merck, Darmstadt, W-Germany). The lactate concentration was determined enzymatically (Biochemica Test Combination, Boehringer, Mannheim, W-Germany). Amino acids were determined using fluorescamine (Fluram, Roche Diagnostica, F. Hoffmann - La Roche, Basle, Switzerland). The protein concentration was measured by the method of Lowry et al. as modified by Oyama & Eagle (1956).

4.10. Statistical analysis

Differences in grading between groups were tested for statistical significance using the distribution-free test of Wilcoxon (Wabeke & van Eeden 1970). Differences between in-
cidences were tested with the Chi-square test, differences between means with Student's t-test (Croxton 1959). A level of significance of 5% or 1% was chosen.
5. EXPERIMENTS AND RESULTS

5.1. Hormonal responses of virgin rat mammary glands and rat mammary glands at various stages of pregnancy

5.1.1. Prolactin

5.1.1.1. Importance of the presence of homologous rat serum in the medium

Experimental conditions

Media: 1) 100% T\textsubscript{8} (T\textsubscript{8}); 2) 50% S\textsubscript{0} + 50% t\textsubscript{8} (S\textsubscript{0}); glucose: 4 mg/ml; hormones: I + two rPrl preparations (a and b; 25 IU/mg) in the concentrations of 0, 0.08, 0.31, 1.25 and 5 µg/ml; the hormone combinations were added to each of the two media; 3 days culture using 4 mammary glands (MP\textsubscript{13})*.

Results

The mammary tissue collected for culture showed grade 4 to 5 of alveolar development. The acinar cytoplasm was opalescent. Traces of intraluminal secretion or vacuolization in the acini were rarely observed. Mitoses were seen infrequently. Table 1 shows that in 100% T\textsubscript{8} the rPrl preparations a and b had almost no effect. The cells were opalescent and contained a few vacuoles. This coincided with the presence of pyknosis. These effects were not dose-dependent and a difference between the two preparations was not observed. There was, however, a slight indication that under these culture conditions prolactin caused the appearance of some eosinophilic material in the acinar lumina. In this respect preparation a was more active than preparation b. With S\textsubscript{0} both preparations a and b produced an increase in the degree of alveolar development, and of mitotic activity. Traces of vacuolization, coinciding with cytoplasmic opalescence, and secretion occurred. The maximum response was, however, obtained at different concentrations for the two prolactin preparations. Table 1 shows that preparation a produced a significant but sub-

* MP\textsubscript{13}: Mammary glands of 13 days pregnant rats.
Table 1  Effect of various concentrations of two rat prolactin preparations (a and b) on mammary gland explants in vitro (MP). The medium was either 100% Trowell's (T8) or it contained virgin rat serum (S0: 50%); 3 days culture; + insulin supplementation.

<table>
<thead>
<tr>
<th>Prolactin µg/ml</th>
<th>Alveolar development (grade)</th>
<th>Mitotic activity (%)</th>
<th>Cytoplasmic opalescence (%)</th>
<th>Vacuolization (%)</th>
<th>Secretion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T8</td>
<td>S0</td>
<td>T8</td>
<td>S0</td>
<td>T8</td>
</tr>
<tr>
<td>preparation a</td>
<td>0</td>
<td>3-3</td>
<td>0</td>
<td>17</td>
<td>75</td>
</tr>
<tr>
<td>0.08</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>0.31</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>1.25</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>preparation b</td>
<td>0.08</td>
<td>2-3</td>
<td>3</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>0.31</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>42</td>
<td>67</td>
</tr>
<tr>
<td>1.25</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>83</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>2-3</td>
<td>4</td>
<td>0</td>
<td>100</td>
<td>67</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.

2: % of explants showing response indicated.

No of explants: 9-12.

maximal effect on alveolar development and mitotic activity at 0.08 µg/ml. A maximum effect on alveolar development was obtained at 0.31 µg/ml, whereas 1.25 µg/ml was required for a maximum incidence of secretion and vacuolization. Preparation b of rat prolactin produced a moderate increase of mitotic activity at a concentration of 0.31 µg/ml, while the highest concentration (5 µg/ml) produced 100% mitotic activity but otherwise a response which was still submaximal.

5.1.1.2. Efficacy of heterologous sera

Experimental conditions

Type of serum: human, rabbit, horse, fetal calf and virgin rat (S0); medium: 50% T8 + 50% serum, serially diluted with T8 to 25, 12.5, 6.3, 3.1 and 0% serum; glucose: 4 mg/ml; hormones: I + rPrl (25 IU/mg; 1.25 µg/ml); 3 days culture; 4 mammary glands (MP) were used for culture.
Table 2  Effect of various types of serum added in different concentrations on the changes in rat mammary gland (MP) induced by rat prolactin (1.25 µg/ml). 3 days culture with insulin supplementation.

<table>
<thead>
<tr>
<th>In vitro response</th>
<th>Type of serum</th>
<th>% of serum in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0  3.1  6.3  12.5  25  50</td>
</tr>
<tr>
<td>Alveolar development (grade)¹</td>
<td>rat (virgin, 9)</td>
<td>3  3  4  5  4-5  6</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>3  4-5  4  5  4</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>3  3  3  3  3</td>
</tr>
<tr>
<td></td>
<td>fetal calf</td>
<td>3  3  3  3  3</td>
</tr>
<tr>
<td></td>
<td>horse</td>
<td>3  3  3  3  3</td>
</tr>
<tr>
<td>Secretion(%)²</td>
<td>rat (virgin, 9)</td>
<td>42 82 83  42 50  0</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>67 57 50  55 25</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>50 17 25  0 0</td>
</tr>
<tr>
<td></td>
<td>fetal calf</td>
<td>25 0 8  8 0</td>
</tr>
<tr>
<td></td>
<td>horse</td>
<td>50 67 83  75 75</td>
</tr>
<tr>
<td>Vacuolization(%)²</td>
<td>rat (virgin, 9)</td>
<td>33 27 33  25 33 33</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>25 0 8  9 8</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>25 0 0  0 0</td>
</tr>
<tr>
<td></td>
<td>fetal calf</td>
<td>25 0 0  8 0</td>
</tr>
<tr>
<td></td>
<td>horse</td>
<td>33 50 42  57 17</td>
</tr>
</tbody>
</table>

¹: Median value of semiquantitative grading of response indicated.
²: % of explants showing response indicated.

No of explants per group: 11-12.

Results

Table 2 shows that when a standard concentration of prolactin (1.25 µg/ml) was added to a medium with 0 to 50% serum of various origins, the addition of rabbit, fetal calf and horse serum did not improve the alveolar development as compared with 100% T8 (Fig. 1a); only the addition of virgin rat serum did increase the degree of alveolar development (Fig. 1b) and this effect depended on the concentration of rat serum in the medium. Human serum also had an effect, the maximum effect being obtained at 6.3 to 25%.

Fig. 2 shows that a similar trend was observed for the mitotic activity. The highest mitotic activity was obtained with virgin rat serum (100% mitotic activity at 25% and 50% serum in the medium).
Fig. 1 Morphological changes in the mammary gland of a 13 days pregnant rat in organ culture. 

a Mammary gland explant after 3 days culture in a medium of 100% Trowell's T8, containing insulin (50 µg/ml and rat prolactin (1.25 µg/ml). The alveoli are poorly developed (grade 2-3). Scale: 100 µ.

b Explant from the same mammary gland as a but after 3 days culture in a medium of 50% virgin rat serum + 50% chemically defined medium (t8). The medium contained 4 mg glucose/ml, insulin (50 µg/ml) and rat prolactin (1.25 µg/ml). The alveoli are well developed (grade 5). Scale: 100 µ.

Table 2 shows that some droplets (vacuolization), and some intraluminal eosinophilic material (secretion) were seen locally in the acinar epithelium in less than 50% of the explants with prolactin and 100% T8. These changes tended to disappear with the addition of high concentrations of the different sera, there being some variations between the different types of sera.

Fig. 2 shows the incidence of pyknosis. Without serum, pyknosis was present in 100% of the explants. Pyknosis was completely suppressed by virgin rat serum at 6.3%, by human serum with a maximum effect between 12.5% and 25% and by horse serum with a maximum effect at 25%. Rabbit serum suppressed pyknosis incompletely with a maximum effect at 12.5% and fetal calf serum had no effect at all.

5.1.1.3. Effect of prolactin on $[^3]$H-thymidine incorporation

Experimental conditions

Medium: 50% $S_0 + 50$% t8; glucose: 4 mg/ml; hormone combinations:
1) I; 2) I + rPrl (25 IU/mg, 0.5 μg/ml); 3½, 24 and 72 h culture with [³H]-thymidine added to the medium 3½ h before the end of the culture period; 6 mammary glands (MP₁₃) were used, one explant of each per group.

Results

When the labelling index was determined for explants cultured in a medium with virgin rat serum and insulin, the index was after 3½, 24 and 72 h of culture 5.8 ± 0.76, 9.9 ± 1.02 and 3.3 ± 0.74% respectively (X ± SEM, n=6 for each group). When in addition prolactin was present in the medium, the corresponding values were 4.9 ± 1.16, 14.0 ± 1.09 and 9.6 ± 0.49% (n=6 for each group). The difference between the two groups was statistic-

Fig. 2 Effect of five different types of serum added in various concentrations to the medium, on the % of explants showing one or more mitoses (left) or pyknosis (right). The medium contained 1.25 μg rPrl/ml medium. 3 Days culture with insulin supplementation. Serum a = rat; b = human; c = rabbit; d = fetal calf; e = horse. Arrow: 0% of serum. No of explants per group: 11-12 (MP₁₃).
ally significant at 24 h and 72 h of culture (at the 1% level). In both groups the percentage of labelled cells was significantly (at the 1% level) less on day 3 of culture than on day 2.

5.1.1.4. Radioimmunoassay and effect in vitro

Experimental conditions

Medium: 50% S₀ + 50% t8; glucose: 4 mg/ml; hormones: I + two different prolactin preparations (rPrl a and b) in the concentrations of 0, 0.6 and 5 μg/ml; 1, 2 and 3 days culture; the medium was collected from each culture vessel separately and the prolactin concentrations determined by radioimmunoassay. These values were compared with the concentrations determined in the original solutions at the beginning of the experiment and in media stored in culture vessels for 3 days but without mammary gland explants; 4 mammary glands (MP₁₃) were used for the culture.

Results

From Table 3 it appears that the prolactin concentration remains largely constant. The slight decrease in concentration after three days was also found in culture media maintained under similar conditions, but without mammary gland explants.

When no prolactin was added (Group A), the prolactin concentration was approximately 10 ng/ml. In this group the alveolar development decreased steadily from grade 5 in the explants at the beginning of the culture to grade 3 after three days of culture. Similarly cytoplasmic opalescence decreased. However, mitotic activity increased sharply during the first day, only to decrease on the second day, reaching a low level on day 3. Some traces of secretion were seen on day 2. When the prolactin level was increased to approximately 27 ng/ml (group B), the changes in the explants during the three days of culture were similar to those of group A. However, on day 2 the decrease of alveolar development was less and the mitotic activity was still increased on this day.

When the prolactin concentration was raised to approximately 115 ng/ml (group C), the alveolar development and cytoplasmic...
Table 3 Effect of various concentrations of rat prolactin on mammary gland explants (MP), cultured for 1, 2 and 3 days (exp 5). Prolactin levels (mean) ± SEM, n=4, ng/ml were determined by radioimmunoassay at the end of the culture period for each culture vessel separately; 0 indicates level in the medium at the start of the experiment, 3* the level after 3 days under similar conditions, but with no explants present in the vessel.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of culture</th>
<th>Rat prolactin (ug/ml) (x ± SEM)</th>
<th>Alveolar development (grade)</th>
<th>Mitotic activity (%)</th>
<th>Cytoplasmic opalescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>grade 1</td>
<td>grade 2</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>11.2</td>
<td>5</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.4 ± 0.73</td>
<td>4</td>
<td>92</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.2 ± 0.53</td>
<td>4</td>
<td>42</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.3 ± 0.65</td>
<td>3</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>10.0 ± 0.54</td>
<td>3</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>31.2</td>
<td>4</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30.5 ± 4.02</td>
<td>4-5</td>
<td>83</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>27.4 ± 1.29</td>
<td>4-5</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26.6 ± 0.80</td>
<td>3</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>27.7 ± 2.24</td>
<td>3</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>131</td>
<td>4-5</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>171 ± 41.5</td>
<td>4-5</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>113 ± 3.3</td>
<td>5</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>116 ± 1.2</td>
<td>5</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>117 ± 6.2</td>
<td>5</td>
<td>100</td>
<td>58</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>226</td>
<td>4</td>
<td>83</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>232 ± 7.3</td>
<td>4</td>
<td>83</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>216 ± 6.4</td>
<td>5</td>
<td>100</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>205 ± 9.4</td>
<td>5</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>196 ± 21.4</td>
<td>5</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>1793</td>
<td>5</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2062 ± 105</td>
<td>5</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1885 ± 53</td>
<td>5</td>
<td>92</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1698 ± 58</td>
<td>4-5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3*</td>
<td>1694 ± 129</td>
<td>4-5</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

A: endogenous prolactin; B and C: + rat prolactin preparation b; D and E: + preparation a.

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated; for mitotic activity the percentage of explants showing grade 1 of activity and those showing grade 2 are recorded separately.

No of explants per group: 12
opalescence were maintained and the increased mitotic activity in the explants did not decline. Traces of secretion— but not of vacuolization— were seen on days 2 and 3. When the prolactin concentration was increased to approximately 210 ng/ml (group D) or 1700 ng/ml (group E), the effect on the explants was similar to that obtained in group C, except for the appearance of traces of vacuolization.

5.1.1.5. Prolactin in lactating rat serum

5.1.1.5.1. Radioimmunoassay and effect in vitro

Experimental conditions

Serum: 21 serum samples ($S_L$) were collected between day 5 and 32 of lactation. The rats were nursing 4-17 young; medium: 20% $S_L$ + 80% t8; glucose: 3.2 mg/ml; hormones: + I; 3 days cul-

![Graph](image)

**Fig. 3** Effect of various rat prolactin (rPrl) concentrations as measured by radioimmunoassay in a medium containing 20% lactating rat serum on alveolar development (median grade), on mitotic activity (% of explants) and on cytoplasmic opalescence (grade 1 or more; % of explants). Relationships are suggested by drawn lines. 3 Days culture with insulin supplementation per group: 9-12(MP).
ture; the medium was collected and pooled for each serum separately and the prolactin concentration measured by radio-immunoassay; 4 mammary glands (MP₁₃) were used.

Results

Figure 3 shows the relationship between the concentration of rPr₁ measured in the medium after 3 days and the response in vitro. Increase of rPr₁ concentration from < 2 to 40 ng/ml was associated with a regular and steep increase of the median grade for alveolar development and of the percentage of explants showing one or more mitoses per section, or cytoplasmic opalescence (grade 1).

5.1.1.5.2. Prolactin concentrations in lactating rat serum after L-dopa treatment

Experimental conditions

Serum: Serum samples were collected from rats nursing 8 young during 6 days and a) weaned for 8½ h, b) weaned for 8 h but nursing for ½ h after a saline injection sc, and c) weaned for 8 h but nursing for ½ h after a sc injection of 0.5 ml of an L-dopa suspension per 100 g bw; L-dopa (Merck, Mannheim, W-Germany, 4184425) was suspended (12 mg/ml) in a solution of 1 mg Na₂S₂O₅ per ml saline; each group contained 4 rats; medium: 20% serum + 80% t8; glucose 3.2 mg/ml; hormones: + I; 3 days culture; the medium was collected and pooled for each serum separately and the prolactin concentration measured by radio-immunoassay; 4 mammary glands (MP₁₃) were used.

Results

Figure 4 shows the presumed relationship between the concentration of rPr₁ measured in the medium after 3 days of culture and the response in vitro. RPr₁ levels were less than 2 ng/ml medium in the group of weaned rats and the response in vitro was low. RPr₁ levels were high in nursing rats (75 - 160 ng/ml medium containing 20% serum), except for one rat in this group. The high rPr₁ levels were associated with a high response in vitro. After L-dopa administration rPr₁ levels fell in the range of 11-78 ng/ml medium. The height of the response
alveolar development
mitotic activity
cytopl. opalesc.

Fig. 4 Relationship between the concentration of rat prolactin (rPrl) in a medium containing 20% serum of 6 days lactating rats and alveolar development (median grade), mitotic activity (% of explants) and cytoplasmic opalescence (grade 1 or more; % of explants). Rats were weaned for 8½ h (solid circles), weaned for 8 h followed by ½ h nursing (open circles) and weaned for 8 h followed by a sc injection of 6 mg L-dopa/100 g bw and nursing ½ h (solid squares). The relationships are suggested by the drawn lines. 3 Days culture with insulin supplementation. Each group consists of 4 animals; no of explants per group: 8-12.

in vitro fell generally in line with the response obtained for the other groups. For the total group of 12 sera the response showed a regular steep increase in the range of < 2-80 ng rPrl/ml medium.

5.1.1.6. Effect of bovine and human prolactin preparations

Experimental conditions

Bovine: medium: 50% S₀ + 50% t8; glucose: 2 mg/ml; hormones: I in combination with 3 prolactin preparations i.e. bovine A and B, and rPrl (25 IU/mg), in the concentrations of 0, 1.6, 3.1, 6.3, 12.5, 25, 50 and 100 µg/ml; 3 days culture using 6 mammary glands (MP₃).

Human: medium: 50% S₀ + 50% t8; glucose: 2 mg/ml; hormones:
I in combination with 2 prolactin preparations i.e. human and rat (preparation c), in the concentrations of 0, 0.4, 1.6, 6.3 and 25 µg/ml. Comparison with 50% S₁₃ + 50% t₈ + I; 3 days culture using 5 mammary glands (MP₁₃).

Results

Bovine: Table 4 shows that all three preparations produced an increase of alveolar development, secretion and vacuolization. Mitotic activity was seen in all groups cultured with prolactin; the incidence (approximately 50%) did not vary in the range of concentrations used, or with the type of prolactin preparation. Alveolar development with 1.6 µg/ml of rat prolactin (rPrl, Table 4) was significantly (at 5% level) higher than with 1.6 µg/ml bovine prolactin (A, B) but not significantly different from the effect of 3.1 µg/ml bovine prolactin. With all three preparations maximum response was obtained with 3.1 to 6.3 µg/ml.

Table 4 Effect of various concentrations of three different types of prolactin preparations on rat mammary gland explants (MP₁₃) in vitro.

<table>
<thead>
<tr>
<th>Prolactin µg/ml</th>
<th>Alveolar development (grade)¹</th>
<th>Secretion (%)²</th>
<th>Vacuolization (%)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bovine A</td>
<td>bovine B</td>
<td>rPrl</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1.6</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3.1</td>
<td>5-6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6.3</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>12.5</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

A: bovine (NIH).
B: bovine (The Netherlands' Cancer Institute).
rPrl: rat prolactin.

Medium contained virgin rat serum (50%) and was supplemented with insulin; 3 days culture.

¹: Median value of semiquantitative grading of response indicated.
²: % of explants showing response indicated.

No of explants per group: 17-18.
Secretion and vacuolization were never observed in all explants, but were only seen locally in a number of explants, even at the highest concentrations. No signs of toxicity such as pyknosis were noticed.

Human: The control medium produced a poor development (grade 4) and almost no mitotic activity, cytoplasmic opalescence, secretion or vacuolization. Addition of 0.4 µg rat or human prolactin per ml medium produced a decrease of pyknosis and an increase of both the alveolar development (grade 6) and mitotic activity (87%). The cytoplasm was opalescent and locally some vacuolization and secretion developed but in a minority of the explants. Increasing the concentration of either rat or human Prl did not produce a further increase of the response. Signs of toxicity were not noticed. S₁₃ produced changes similar to those produced by S₀ plus rat or human Prl.

5.1.1.7. Interaction of prolactin with various hormones

5.1.1.7.1. Progesterone and 17 β-oestradiol

5.1.1.7.1.1. Interaction with progesterone and 17 β-oestradiol

Experimental conditions

Medium: 50% S₀ + 50% T8; glucose: 2 mg/ml; hormones: I (50 µg/ml), Prl (ovine, 10 µg/ml), P (0.5 µg/ml) and O (0.05 µg/ml). The following combinations of hormones were used: I; I + oPrl; I + P + O; I + P + O + oPrl; the same hormone combinations were tested in a medium in which S₀ was replaced by S₁₆ from day 0-3 and S₁₉ from day 3-6 of culture; 6 days culture; 6 mammary glands (MP₁₃) were used for culture.

Results

When mammary gland explants were cultured for 6 days in a medium with virgin rat serum and I, the median grade for alveolar development was 3 with mitoses seen in 6% of the explants (n=18). When oPrl was added, the alveolar development increased to grade 4 (significant at 1% level) and the mitotic activity to 23% (n=17). When P and O were added the alveolar
development increased to grade 4 (significant at 1% level) and mitotic activity to 39% (n=18). When P and O were added to the combination of I and oPrl, the alveolar development was grade 5 (increased significantly at 5%) and the mitotic activity 75% (n=16). Secretion and vacuolization did not develop in these groups.

When pregnant rat serum was added to I instead of virgin rat serum, the mean grade for alveolar development was 6, while 72% of the explants showed mitotic activity (grade 1), 50% secretion (grade 0-1) and 78% vacuolization (grade 1, n=18). When oPrl or P plus O were added alone or in combination, similar results were obtained with none of the minor differences being statistically significant.

5.1.1.7.1.2. Dose related interactions with progesterone regarding morphological criteria

Experimental conditions

Medium: 50% S0 + 50% t8; glucose: 6 mg/ml; hormones: I (50 μg/ml), P: 0, 0.05, 0.5, 5 and 50 μg/ml; rPrl (3-5 IU/mg): 0, 0.5, 5 and 50 μg/ml; each of the concentrations of P was combined with each of the concentrations of rPrl; 3 and 6 days cultures on two different sets of 5 mammary glands (MP_j3) each.

Results

Morphological changes. Table 5 shows, that rPrl alone (P: 0 μg/ml) increased the alveolar development with increasing concentration. Mitotic activity was increased considerably at the highest dose in the 3 days culture (Fig. 5). However, in the 6 days culture this effect was slight. According to Table 6 rPrl without P produced traces of secretion and vacuolization, especially in the 6 days culture with 50 μg/ml rPrl.

Table 5 shows that P alone in the 3 days culture increased the alveolar development already at a concentration of 0.05 μg/ml. A maximum effect was obtained at 0.5 μg/ml. In the 6 days culture the effect of P alone was somewhat smaller. P increased the mitotic activity (Fig. 5) to a relatively small extent, the optimum concentrations being 0.5 μg/ml in the 3 days
Table 5 Effect of various concentrations of prolactin (rPrl) in combination with several concentrations of progesterone (P) on the degree of alveolar development (median grade) in mammary gland explants (MP) cultured for 3 and 6 days in a medium containing virgin rat serum (50%) and supplemented with insulin.

<table>
<thead>
<tr>
<th>Progesterone (μg/ml)</th>
<th>3 days rPrl (μg/ml)</th>
<th>6 days rPrl (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3 a 4 b 5 6</td>
<td>4 5 6 7</td>
</tr>
<tr>
<td>0.05</td>
<td>4 a b c d</td>
<td>4 5 6 7</td>
</tr>
<tr>
<td>0.5</td>
<td>5 5 5 6</td>
<td>5 5 5 6</td>
</tr>
<tr>
<td>5</td>
<td>5 5 5 6</td>
<td>5 5 5 6</td>
</tr>
<tr>
<td>50</td>
<td>5 5 5 6</td>
<td>5 5 5 6</td>
</tr>
</tbody>
</table>

a: Difference with 0 μg/ml P + 0 μg/ml rPrl sign. at 1% level.
b: Difference with 0 μg/ml P + 0 μg/ml rPrl sign. at 5% level.
c: Difference with groups receiving either 0.05 μg/ml P or 5 μg/ml rPrl sign. at 1% level.
d: Difference with groups receiving either 0.05 μg/ml P or 50 μg/ml rPrl at 1%.
e: Difference with 0 μg/ml P, 0 μg/ml rPrl sign. at 1% level.

No of explants per group: 14-15.

Table 6 Effect of various concentrations of rat prolactin (rPrl) on secretion and vacuolization in mammary gland explants (MP), cultured for 3 and 6 days and the interaction with various concentrations of progesterone added to a medium containing virgin rat serum (50%) and insulin. Results for 0, 0.5 and 5 μg/ml prolactin in the 3 days culture showed no response for each combination.

<table>
<thead>
<tr>
<th>Culture period</th>
<th>rPrl (μg/ml)</th>
<th>Secretion (%) 1</th>
<th>Vacuolization (%) 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone (μg/ml)</td>
<td></td>
<td>Progesterone (μg/ml)</td>
</tr>
<tr>
<td>3 days</td>
<td>50</td>
<td>47 33 7 7 0</td>
<td>7 7 7 0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>13 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20 27 0 7 0</td>
<td>7 13 0 20 7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>86 87 80 73 0</td>
<td>93 100 80 66 0</td>
</tr>
<tr>
<td>6 days</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>13 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20 27 0 7 0</td>
<td>7 13 0 20 7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>86 87 80 73 0</td>
<td>93 100 80 66 0</td>
</tr>
</tbody>
</table>

1: % of explants showing response indicated.
* semiquantitative grading of the degree of secretion significantly (at 5% level) less than in the group with 50 μg/ml rPrl alone.

No of explants: 14-15.
Fig. 5  Effect of various concentrations of prolactin (rPrl) in combination with several concentrations of progesterone (P), indicated by numbers, on mitotic activity (% of explants) in mammary gland explants (MP) cultured for 3 (left) and 6 (right) days in a medium containing virgin rat serum (50%) and supplemented with insulin. No of explants per group: 14-15.

culture and 5 μg/ml in the 6 days culture. P alone did not induce either secretion or vacuolization.

Fig. 5 and Table 5 show that when P and rPrl were combined, the lower concentrations of P and rPrl had a synergistic effect on alveolar development and mitotic activity, which was especially marked in the 3 days culture. In the 3 days culture the optimum synergistic effect on mitotic activity was already obtained at 0.05 μg/ml P. At 50 μg/ml P a suppression of the effect of rPrl was obtained at the highest concentrations of rPrl. In the 6 days culture the optimum of synergism was found with 0.5 and 5 μg/ml P. Table 6 shows that P, especially at the highest concentration, depressed the weak effect of rPrl on secretion and vacuolization.
5.1.1.7.1.3. Dose related interactions with progesterone on $[^3]H$-thymidine incorporation

Experimental conditions
Medium: 50% S_0 + 50% t8; glucose: 2 mg/ml; hormones: I (50 μg/ml), P: 0, 0.13, 0.25, 0.50 and 1.00 μg/ml, rPrl (25 IU/mg): 0, 0.08, 0.16 and 0.32 μg/ml; each concentration of P was combined with each concentration of rPrl; 3 days culture on 1 mammary gland (MP_13); $[^3]H$-thymidine (5 μCi/ml) added 3½ h before the end of the culture period at 3 days.

Results
When or rPrl were not added, the labelling index after 3 days was 2.1 ± 0.62% (X ± SEM, n=6). When rPrl was added in the concentrations of 0.08, 0.16 and 0.32 μg/ml the index increased to 4.4 ± 1.28%, 4.9 ± 1.30% and 5.1 ± 1.27% respectively (n=6 for each group). The differences with the control group were not statistically significant. When P was added in the concentrations of 0.13, 0.25 and 1.00 μg/ml, 0.13 μg/ml produced already a significant increase to 5.8 ± 0.72% (n=6; at 1% level). When this concentration was combined with rPrl, the results obtained were 4.3 ± 0.88%, 6.2 ± 1.49% and 7.6 ± 1.33% (n=6 for each group) for 0.08, 0.16 and 0.32 μg/ml rPrl. At higher concentrations of P no indication was found for a regularly increasing synergism between rPrl and P.

5.1.1.7.1.4. Dose related interactions with oestradiol

Experimental conditions
Medium: 50% S_0 + 50% t8; glucose: 6 mg/ml; hormones: I (50 μg/ml), O: 0, 0.005, 0.05, 0.5 and 5 μg/ml, rPrl (3-5 IU/mg): 0, 0.5, 5 and 50 μg/ml; each of the concentrations of O was combined with each of the concentrations of rPrl; 3 and 6 days cultures on two different sets of 5 mammary glands (MP_13). Results
No significant effect could be demonstrated for O alone in the 3 days culture except that 5 μg/ml O decreased the alveolar development (Table 7). rPrl alone increased the alveolar develop-
Table 7 Effect of various concentrations of rat prolactin (rPrl) on the in vitro response of mammary gland explants (MP13) in a 3 days culture and the interaction of prolactin with various concentrations of oestradiol added to a medium containing virgin rat serum (50%) and insulin.

<table>
<thead>
<tr>
<th>Oestradiol (ug/ml)</th>
<th>Alveolar development (grade) 1</th>
<th>Mitotic activity (%) 2</th>
<th>Secretion (%) 2</th>
<th>Vacuolization (%) 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.5</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>0</td>
<td>¼</td>
<td>½</td>
<td>¾</td>
<td>6</td>
</tr>
<tr>
<td>0.005</td>
<td>½</td>
<td>5</td>
<td>* 5</td>
<td>6</td>
</tr>
<tr>
<td>0.05</td>
<td>½</td>
<td>5</td>
<td>¾</td>
<td>6</td>
</tr>
<tr>
<td>0.5</td>
<td>¾</td>
<td>½</td>
<td>¾</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
<td>¾</td>
<td>4</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.

2: % of explants showing response indicated.

* Difference with the group receiving 0 µg/ml and 0.5 µg/ml rPrl significant at 5% level.

No of explants per group: 15.

ment at 50 µg/ml. Its effect on mitotic activity, secretion and vacuolization remained low. When rPrl and O were combined, 5 µg/ml O suppressed the effect of rPrl on the alveolar development, mitotic activity and vacuolization. Eosinophilic material in the acinar lumina (secretion) remained present. At lower concentrations O had no consistent effect on the results obtained with rPrl. No effect of O, except for a toxic reaction with 5 µg/ml, could be demonstrated in the 6 days culture.

5.1.1.7.2. Interaction with testosterone and insulin

Experimental conditions

Medium: 50% S0 + 50% t8; glucose 4 mg/ml; hormones: I (50 µg/ml) and T (1 µg/ml) in the combinations: no hormones; I; I + T. To each of these three combinations was added 0, 0.08, 0.31, 1.25 and 5 µg/ml rPrl (25 IU/mg); 3 days culture using 4 mammary glands (MP13).
Results

When the medium contained 50% virgin rat serum and no hormones were added, mitotic activity was low after 3 days of culture (Table 8). Table 8 shows that alveolar development was low and cytoplasmic opalescence absent (see also Fig. 7a). No traces of a secretory response were seen. Pyknosis was present. When various concentrations of rPrl were added, the mitotic activity increased with increasing concentrations (Fig. 6). The alveolar development intensified slightly (Fig. 7b, Table 6) and although grade 2 of cytoplasmic opalescence was not seen (Table 8), grade 1 was reached in 0, 17, 42 and 67% of the explants with concentrations of rPrl increasing from 0 to 5 μg/ml. A secretory response was not seen. Pyknosis remained present.

When I was added to the medium containing virgin rat serum, mitotic activity was present in more than half of the explants,

Table 8  Effect of various concentrations of prolactin (rPrl) on the in vitro response of mammary gland explants (MP) cultured during 3 days and interaction with insulin (I; 50 μg/ml) and testosterone (T; 1 μg/ml); -: no hormones added except rPrl. The medium contained virgin rat serum (50%).

<table>
<thead>
<tr>
<th>rPrl μg/ml</th>
<th>Alveolar development (grade)²</th>
<th>Cytoplasmic opalescence (%)²</th>
<th>Mitotic activity (grade)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>I</td>
<td>I+T</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>3</td>
<td>4³</td>
</tr>
<tr>
<td>0.08</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.31</td>
<td>3</td>
<td>5</td>
<td>4-5</td>
</tr>
<tr>
<td>0.125</td>
<td>3</td>
<td>5</td>
<td>4-5</td>
</tr>
<tr>
<td>0.5</td>
<td>3</td>
<td>5-6</td>
<td>4-5⁵</td>
</tr>
</tbody>
</table>

1: Median value.
2: % of explants showing grade 2 of cytoplasmic opalescence.
3: Differences between group - and I significant at the 5% level (or less) for each rPrl concentration.
4: Difference with I (0 μg/ml rPrl) significant at 1% level.
5: Difference with I + 5 μg/ml rPrl significant at 5% level.

No of explants per group: 12.
Fig. 6 Effect of various concentrations of prolactin (rPrl), on the mitotic activity in mammary gland explants (% of explants showing mitotic activity) cultured for 3 days; and the interaction of rPrl with insulin (I; 50 μg/ml), and testosterone (T; 1 μg/ml). The medium contained 50% virgin rat serum. The hormone combinations are indicated in the figure. Results for the groups with no insulin (-) added to the medium were median grade 0 or 1. No of explants (MP13) per group: 12.

but the grade of activity was low (grade 1, Fig. 6). Alveolar development increased slightly but cytoplasmic opalescence, secretion and vacuolization remained absent (Table 8). The addition of I decreased the pyknosis. When I and rPrl in various concentrations were added, the mitotic activity was present in all explants and the grade was high (Fig. 6). The alveolar development increased and cytoplasmic opalescence occurred (Fig. 7c). The effects increased when the prolactin concentration was increased from 0.08 to 5 μg/ml (Table 8). Vacuolization or
Fig. 7 Morphological changes in the mammary gland of a 13 days pregnant rat in organ culture;

a Mammary gland explants after a 3 days culture in a medium containing 50% virgin rat serum and 50% chemically defined medium. No insulin or prolactin added. The alveoli are poorly developed (grade 2). Scale: 100 μ.

b Similar to a but with 5 μg/ml prolactin added. Certain alveoli are poorly developed, others better and in appearance similar to c. Scale: 100 μ.

c Similar to a but with 50 μg/ml insulin and 5 μg/ml prolactin added. The alveoli are well developed (grade 5). Scale: 100 μ.

secretory material was not observed.

The addition of T to a medium containing virgin rat serum and I resulted in a significant increase in the degree of mitotic activity (Fig. 6) and alveolar development (Table 8). The effect on cytoplasmic opalescence was not clear when the percentage of explants with grade 2 opalescence was recorded as was done in Table 8. If however grade 1 opalescence was recorded, opalescence
was significantly (at the 1% level) higher than in the group without T. When in addition rPrl was added, neither a consistent synergism nor an antagonism was observed, except that T lowered the grade of mitotic activity (Fig. 6) and of alveolar development obtained with 5 µg/ml rPrl (Table 8).

5.1.1.7.3. Interaction with thyroxin

Experimental conditions

Medium 50% S₀ + 50% t8; glucose: 4 mg/ml; hormones: I (50 µg/ml), rPrl (25 IU/mg): 0, 0.08, 0.31 and 1.25 µg/mg, Th: 0, 0.031, 0.125 and 0.5 µg/ml; each of the concentrations rPrl was combined with each of the concentrations of Th; in addition the following combinations: I + Th (1 µg/ml); I + P (1 µg/ml); 50% S₁₃ + 50% t8 + I to each of these last three combinations was added 0 and 0.5 µg/ml Th; 3 days culture on 4 mammary glands (MP₁₃).

Results

Table 9 gives the results for mitotic activity and cytoplasmic opalescence. When the medium contained 50% virgin rat serum and insulin, mitotic activity was absent and cytoplasmic opalescence was present in only 17% of the explants. When prolactin was added, mitotic activity appeared and cytoplasmic opalescence developed fully. The addition of thyroxin to the medium with or without prolactin did not produce any consistent change.

When testosterone or progesterone was added to virgin rat serum and insulin, some mitotic activity appeared but cytoplasmic opalescence developed only in about 30% of the explants (Table 9). The addition of 0.5 µg/ml thyroxin had no effect. When the medium contained 50% 13 days pregnant rat serum and insulin, mitotic activity was high and cytoplasmic opalescence was noted in all sections (Table 9). The addition of 0.5 µg/ml thyroxin had no effect. The degree of alveolar development was not affected by the addition of thyroxin in any of the groups.
Table 9  Effect of various concentrations of thyroxin on the in vitro response to prolactin, of mammary gland explants (MP\textsubscript{13}) cultured during 3 days. Similarly thyroxin alone or in combination with testosterone or progesterone in a medium containing virgin rat serum (50%) and insulin (50 \textmu g/ml); similarly thyroxin combined with pregnant rat serum (13 days, 50%) and insulin.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Hormones</th>
<th>Mitotic activity (grade)\textsuperscript{1}</th>
<th>Cytoplasmic opalescence (%)\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type \mu g/ml</td>
<td>0   0.03 0.13 0.5</td>
<td>0   0.03 0.13 0.5</td>
</tr>
<tr>
<td>Virgin rat</td>
<td>Prolactin</td>
<td>0    1    1    1</td>
<td>17   25  25   8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.08  2    2    1-2 2</td>
<td>83   82  67   91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.31  3    2    2    2</td>
<td>100  83  83   83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.25  2    2-3  4    4</td>
<td>100  100 91  100</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>1    1    1    1</td>
<td>30   18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3     2    2    2</td>
<td>100  100</td>
</tr>
<tr>
<td>Pregnant rat</td>
<td>Progesterone</td>
<td>1    1    1    1</td>
<td>33   50</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of the mitotic activity.
2: % of explants showing cytoplasmic opalescence.

No of explants per group: 9-12.

5.1.2. Human placental lactogen

5.1.2.1. Importance of homologous rat serum

Experimental conditions

HPL was added to the medium in concentrations of 0, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5 and 25 \textmu g/ml. The medium was supplemented with insulin. The combination hPL + I was added to two different media: 100\% T8 and 50\% t8 and compared with 50\% S\textsubscript{13} + 50\% t8 + I; glucose: 4 mg/ml. 3 Days culture with 4 mammary glands (MP\textsubscript{13}).

Results

Table 10 shows the results obtained with the various media and hormone concentrations. When only I is added to the
media, the degree of alveolar development is low in T8 and S₀, but well maintained in S₁₃. In T8 the addition of hPL produced only a slight increase but in S₀ the increase was clear-cut.

The latter effect was constant in the range of 0.4 to 6.3 μg/ml, but at 12.5 μg/ml the alveolar development was decreased.

From the three basic media used only S₁₃ produced a strong mitotic activity. Addition of hPL did not produce an increase with T8, but the response was marked with S₀. The latter effect was present in the range of 0.4 to 6.3 μg/ml hPL. At 12.5 μg/ml mitotic activity was suppressed.

Cytoplasmic opalescence was present in almost all explants cultured in S₁₃ and absent in those cultured with T8 or S₀. When hPL was added, cytoplasmic opalescence developed but in the case of T8 it was accompanied by a degeneration of the explants with a high degree of pyknosis.

Vacuolization and secretion developed only with S₁₃ in the

Table 10 Effect of hPL on rat mammary gland explants (MP₃) in vitro. T8: medium of 100% T8; S₀: medium of 50% virgin rat serum plus 50% T8; S₁₃: medium of 50% 13 days pregnant rat serum plus 50% T8. The media were supplemented with insulin (50 μg/ml).

<table>
<thead>
<tr>
<th>hPL (μg/ml)</th>
<th>Alveolar development (grade)¹</th>
<th>Mitotic activity (%)²</th>
<th>Vacuolization (%)²</th>
<th>Secretion (%)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T8</td>
<td>S₀</td>
<td>S₁₃</td>
<td>T8</td>
</tr>
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<td>0</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>0</td>
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<td>0.4</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>1.6</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>3.1</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>6.3</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>12.5</td>
<td>4</td>
<td>4</td>
<td>4-5</td>
<td>0</td>
</tr>
</tbody>
</table>

¹: Median value of semiquantitative grading of response indicated.
²: % of explants showing response indicated.

3 Days culture.

No of explants: 10-12 per group.
acini of approximately half of the number of explants. The addition of hPL did not produce secretion in T8, but in S₀ secretion appeared in a minority of the explants. Secretion at 12.5 μg/ml.

5.1.2.2. Comparison of effects of hPL with effects of prolactin or pregnant rat serum

Experimental conditions

HPL and rPrl were added to the medium in concentrations of 0, 0.08, 0.16, 0.31, 0.63 and 1.25 μg/ml (rPrl 25 IU/mg). Medium: 50% S₀ + 50% t8; glucose 4 mg/ml; + I (50 μg/ml). The effects of hPL and rPrl were compared with the effects of three sera, collected from 13 days pregnant rats. The three sera were added to the medium in concentrations of 0, 1.6, 6.3, 12.5 and 25%. S₀ was added to obtain a final serum concentration of 50%; 3 days culture with 4 mammary glands (MP₁₃).

Results

Fig. 8 shows that an obvious dose-effect relationship was obtained with the three S₁₃-sera, hPL and rPrl for the development of a certain level of cytoplasmic opalescence (grade 2). This grade was reached by half of the explants when rPrl was added at 0.16 μg/ml, but the concentration of hPL had to be 4 to 8 times higher. A comparable response was obtained with 3.2, 3.2 - 6.3 and 6.3 - 12.5% serum of the three pregnant rats respectively. The activity of 1 ml of pregnant rat serum is therefore equivalent to the activity of approximately 2 to 5 μg rPrl in 1 ml S₀.

The mitotic activity at the chosen level of grade 2 was reached in half of the cultured explants at 0.08 - 0.16 μg/ml rPrl and hPL. The dose-effect relationship was less clear for hPL. A comparable degree of mitotic activity was reached in 50% of the explants in two samples of the pregnant rat sera at less than 1.6%, and in the third sample at 3.2 - 6.3%. Therefore, in this respect 1 ml of the latter serum is equivalent to 2 - 3 μg rPrl in 1 ml S₀.

The results obtained with the semiquantitative evaluation
Fig. 8 Effect of serum of three 13 days pregnant rats (S\textsubscript{13}, a, b and c) and hPL or rPrl on the percentage of mammary gland explants (MP\textsubscript{13}) showing grade 2 or more mitotic activity and cytoplasmic opalescence. Arrows indicate values obtained with a medium containing neither serum nor hormones. 3 Days culture; no of explants: 12 per group.

of the degree of development did show some dose-effect relationship with the S\textsubscript{13}-sera, rPrl and hPL, but the relationship was not distinct enough to permit a comparison between the activity of S\textsubscript{13}, hPL and rPrl.
5.1.2.3. Cross reactivity of hPL and rat chorionic mammotrophin

5.1.2.3.1. Interaction with rabbit anti-hPL serum I: commercial preparation

Experimental conditions

Rabbit anti-hPL serum was obtained commercially. The freeze-dried anti-serum was reconstituted with t8 and sterilized by millipore filtration (1 vial, containing 6 ml rabbit serum in a dried form, dissolved in 24 ml t8). In one series this rabbit anti-hPL serum was added to the medium in a final concentration of 25% of reconstituted anti-serum, in another series no anti-serum was added.

HPL and rPrl were added to the medium in concentrations of 0, 0.31, 1.24 and 5 μg/ml medium. The medium contained 25% S₀, 50% t8 and 25% anti-serum or t8.

S₁₃ was added to the medium in concentrations of 0, 1.6, 6.3 and 25%. S₀ was added to obtain a final serum concentration of 25%. In addition the medium contained 50% t8 and 25% anti-serum or t8. The final glucose concentration of the media was 4 mg/ml, + I (50 μg/ml). Explants of MP₁₃ were used.

Results

Table 11 shows that with 0% S₁₃ + I the degree of alveolar development and mitotic activity was low and that cytoplasmic opalescence, secretion and pyknosis were absent. Addition of pregnant rat serum produced an increased alveolar development and mitotic activity. Cytoplasmic opalescence and traces of secretory material appeared. Pyknosis was absent.

When rabbit anti-hPL serum was added to these media the effect of pregnant rat serum on alveolar development, mitotic activity and secretion disappeared. Some cytoplasmic opalescence was still present, but its aspect had changed. The cells seemed to be swollen and the cytoplasm stained very faintly. This development was accompanied by the appearance of a high degree of pyknosis. Some acini were necrotic. The connective tissue also showed pyknosis. The mammary glands showed some variations in their reaction to the rabbit anti-serum. Sometimes the
Table 11 Effect of 13 days pregnant rat serum (S₁₃), hPL and rPrl on rat mammary gland explants (MP) and the effect of the addition of rabbit anti-hPL serum (AS, 25%) to the medium.

<table>
<thead>
<tr>
<th>Supplement to the Medium</th>
<th>Alveolar development (grade)¹</th>
<th>Mitotic activity (%)²</th>
<th>Cytoplasmic opalescence (grade)¹</th>
<th>Secretion (grade)¹</th>
<th>Pyknosis (grade)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁₃ (μg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>33 0 0 0</td>
<td>0 17 0 0</td>
<td>0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>3</td>
<td>33 3 0 0</td>
<td>25 17 0 0</td>
<td>0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>3</td>
<td>33 8 0 0</td>
<td>33 58 0 0</td>
<td>8 0 0 0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>100 0 1-2 0</td>
<td>92 50 1 0-1</td>
<td>25 0 0 3</td>
<td></td>
</tr>
<tr>
<td>rPrl (μg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.31</td>
<td>4</td>
<td>92 0 1-2 0</td>
<td>83 50 1 0-1</td>
<td>17 0 0 3</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td>100 0 2 0</td>
<td>100 50 2 0-1</td>
<td>50 0 0 3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>100 0 3 0</td>
<td>100 42 3 0</td>
<td>42 0 0 3</td>
<td></td>
</tr>
<tr>
<td>hPL (μg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.31</td>
<td>3</td>
<td>75 0 1 0</td>
<td>75 8 1 0</td>
<td>8 0 0 3</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>4</td>
<td>75 0 1-2 0</td>
<td>92 42 1-2 0</td>
<td>33 0 0 3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>83 0 2 0</td>
<td>100 50 1-2 0</td>
<td>17 0 0 3</td>
<td></td>
</tr>
</tbody>
</table>

¹: Median value of semiquantitative grading of response indicated.
²: % of explants showing response indicated.

3 Days culture with the media supplemented with insulin.
No of explants: 12 per group.

appearances of an eosinophilic mass in the lumen was distinct. This material showed the presence of many nuclear remnants.

The addition of either rPrl of hPL to virgin rat serum and insulin produced changes which were similar to those obtained with pregnant rat serum (Table 11). The response obtained with 25% S₀ + 0.31 μg/ml rPrl or 1.25 μg/ml hPL was equivalent to that of 25% S₁₃.

When rabbit anti-hPL serum was added to the media containing S₀ + rPrl or hPL, the explants reacted to the anti-serum similar to those cultured in S₁₃ plus anti-serum.

5.1.2.3.2. Interaction with rabbit anti-hPL serum II:
preparation Dr Friesen

Experimental conditions
Lyophilized rabbit anti-hPL serum (preparation Dr H.G. Friesen) was reconstituted with T8 and sterilized by millipore filtration. The anti-serum was added to the medium in concentrations of 0, 6.3, 12.5 and 25%. $S_0$ and $S_{13}$ respectively were added to the medium in concentrations of 0, 6.3, 12.5 and 25%.

Fig. 9 Effect of rabbit anti-hPL serum on the mammotrophic activity of 13 days pregnant rat serum ($S_{13}$) and on the appearance of nuclear remnants in the acinar lumen of explants (MP) cultured with $S_{13}$ or $S_0$ (virgin rat serum). Mitotic activity, vacuolization and the presence of nuclear remnants are expressed as % of explants showing the response indicated. 3 Days culture with insulin supplementation; no of explants: 11-12 per group.
The medium was made up to 100% by adding T8 + I (50 µg/ml). Explants of MP₁₃ were used.

Results

Figure 9 shows that 25% anti-serum suppressed the mitotic activity produced by S₁₃, but the suppression was incomplete. The incidence of secretory activity was depressed by the anti-serum in the same way as vacuolization (Fig. 9). Depending on the concentration of anti-serum, nuclear remnants were seen in the acinar lumina with increasing frequency. This tendency was observed in the media containing rat serum as well as in 100% T8. The tendency was suppressed to some extent by high concentrations of S₁₃. High concentrations of S₀ had no such effect. Besides the intraluminal nuclear remnants, pyknosis in the explants increased also with higher concentrations of the rabbit anti-hPL serum.

When S₀ was added in concentrations of 0-25% the median grade of alveolar development remained 2 to 3. The addition of anti-serum (0-25%) had no consistent effect on the degree of alveolar development. When S₁₃ was added to the medium, the median grade increased from 3 (0% S₁₃) to 4-5 (6.3% S₁₃) and 5 (12.5 and 25% S₁₃). The addition of anti-serum had no effect except for a decrease to grade 4 when 25% anti-serum was present.

5.1.3. Growth Hormone

5.1.3.1. Human growth hormone

Experimental conditions

Medium: 50% S₀ + 50% t8; glucose 2 mg/ml; hormones: I + 0, 0.8, 3.1, 12.5 and 50 µg hGH/ml (potency unknown); compared with 50% S₁₃ + 50% t8 + I. 3 And 6 days culture (change of medium on day 3), with the same set of 4 mammary glands (MP₁₃).

Results

Figure 10 shows the results obtained in a 3 days culture. Alveolar development, mitotic activity and cytoplasmic opalescence were increased with 3.1 µg hGH/ml and more. Maximum effect was reached at 12.5 µg hGH/ml. The effect of S₀ + 3.1. µg hGH/ml was
comparable to that of $S_{13}$ except for mitotic activity, which did not reach the level of $S_{13}$. The addition of hGH tended to increase necrosis, especially at 12.5 and 50 μg/ml. Secretion or vacuolization was noticed neither with $S_0 + hGH$, nor with $S_{13}$. The results for the 6 days culture (Fig. 11) were generally similar to those of the 3 days culture. With $S_0$ or $S_{13}$ necrosis was estimated to be 15% of the section area. It was 15, 45, 65 and 70% with 0.8, 3.1, 12.5 and 50 μg hGH/ml respectively.

![Graph](image)

**Fig. 10** Effect of various concentrations of a human growth hormone preparation (hGH), added to a medium containing virgin rat serum and insulin, on alveolar development ($\bigtriangleup$—$\bigtriangleup$, AD; median grade), mitotic activity ($\bullet$—$\bullet$, Mi; % of explants) and cytoplasmic opalescence ($\blacksquare$—$\blacksquare$, CO; of explants) in mammary gland explants (MP$^{13}$) cultured for 3 days (A) and 6 days (B). The lowest concentration of hGH that produced a change in effect statistically significant from the control at the 5% level or at the 1% level is indicated by one, respectively two arrow heads. $S_{13}$: results obtained with serum of 13 days pregnant rats. No of explants per group: 11-12.
Fig. 11 Effect of various concentrations of rat prolactin (rPrl; • — •) and a procine growth hormone preparation (pGH, Δ—Δ) on alveolar development (median grade), mitotic activity (% of explants with grade 2 or more) and cytoplasmic opalescence (% of explants with grade 2 or more) in mammary gland explants (MP13) cultured in a medium with virgin rat serum and insulin for 3 days. No of explants: 11-12 per group.

5.1.3.2. Porcine growth hormone

Experimental conditions

Medium: 50% S0 + 50% t8; glucose 4 mg/ml; hormones: I + 0, 0.63, 1.25, 2.5 and 5 μg pGH/ml (porcine Somatotropin, 0.4 U/mg); compared with I + 0.02, 0.04, 0.08, 0.16, 0.31, 0.63 and 1.25 μg rPrl/ml (25 IU/mg). 3 Days culture with 4 mammary glands (MP13) Results

Figure 11 shows that pGH increased significantly all three indices at 5 μg/ml. The pGH preparation was, however, on a weight basis approximately 8 to 16 times less potent than the rPrl preparation. With pGH pyknosis was not prominent.
5.1.4. Adrenocorticotropic hormone.

5.1.4.1. Interaction of rPrl and ACTH

Experimental conditions

Medium: 50% S₀ + 50% t₈; glucose 4 mg/ml; hormones: I + 0, 0.31 and 5 µg ACTH/ml (Organon; 82 IU/mg; 1 mg/ml aqua dest.); one series assayed with rPrl (25 IU/mg; 1.25 µg/ml medium) another series without rPrl; 3 and 6 days culture (change on day 3) with 4 mammary glands (MP₁₃).

Results

Table 12 shows that in the absence of added rPrl 0.31 µg ACTH/ml medium had no effect but 5 µg ACTH/ml were needed to obtain a significant effect. The effect was noticeable on alveolar development, mitotic activity and cytoplasmic opalescence. Vacuolization was not seen and secretion only in a few explants.

Table 12

<table>
<thead>
<tr>
<th>ACTH (µg/ml)</th>
<th>3d</th>
<th>6d</th>
<th>(%)²</th>
<th>3d</th>
<th>6d</th>
<th>(%)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>3</td>
<td>58</td>
<td>17</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>(+ I) 0.31</td>
<td>3</td>
<td>3</td>
<td>58</td>
<td>44</td>
<td>42</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>1*</td>
<td>4**</td>
<td>83</td>
<td>75</td>
<td>42</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>ACTH (µg/ml)</th>
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<th>6d</th>
<th>(%)²</th>
<th>3d</th>
<th>6d</th>
<th>(%)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5-6³</td>
<td>4³</td>
<td>5³</td>
<td>1³</td>
<td>1³</td>
<td>17</td>
</tr>
<tr>
<td>(+ I + rPrl)⁵</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>27</td>
</tr>
</tbody>
</table>

¹: Median grade of response.
²: % of explants showing response indicated.
³: Difference in grade with corresponding group cultured with I significant at 1% level.
⁴: Difference in response with group 0 µg ACTH/ml significant at 5% level.
⁵: Idem at 1% level.

No of explants: 11-12.
The addition of 1.25 μg/ml rPrl resulted in a high degree of alveolar development and mitotic activity. The effect was less in the 6 days culture. Cytoplasmic opalescence was present in all explants. Some showed vacuolization and secretion. The effect was greater than with 5 μg/ml ACTH. In the presence of rPrl, ACTH did not produce a consistent effect. Some parameters were significantly increased in the 6 days culture.

5.1.4.2. Dose effect relations of ACTH and rPrl

Experimental conditions

Medium: 50% S₀ + 50% t8; glucose 4 mg/ml; hormones: I + 0, 0.63, 1.25, 2.5 and 5 μg/ml of either (α) p ACTH (Organon preparation, 28 U/mg), (β) p ACTH (Sigma, Grade II, 86.1 U/ml), (γ) p ACTH (Sigma, 150 U/mg) and (δ) tetracosactide (Organon, pure preparation); compared with 0.02 - 1.25 μg rPrl (25 IU/mg).

3 Days culture with 4 mammary glands (MP₁₃).

Results

None of the four ACTH preparations produced a statistically significant effect as compared with the control group 0 μg/ml. A dose-response relationship was not found. Pyknosis was not prominent.

5.1.5. Corticosteroids

5.1.5.1. Efficacy of heterologous sera

Experimental conditions

Type of serum: virgin rat serum (S₀), horse serum, human serum, rabbit serum and fetal calf serum, either heat-inactivated or untreated; media: 1) 50% serum + 50% t8; 2 mg glucose/ml medium + I (50 μg/ml); 2) 100% Trowell's T8; 3) 100% Waymouth MB 752/1 + I. Hormones: one group 20 μg/ml cortisol (F), another group no hormones except I; 6 days culture with change of medium after 3 days; 4 mammary glands (MP₁₃) were used for culture.

Results

In a 6 days culture with the medium S₀ + I (Table 13) the
Table 13  Effect of various types of medium on mammary gland explants (MP\textsuperscript{13}) in vitro; 6 days culture. Sera: S\textsubscript{0} = virgin rat; S\textsubscript{H} = horse; S\textsubscript{H} = human; S\textsubscript{R} = rabbit; S\textsubscript{F} = fetal calf; S\textsubscript{F} = fetal calf, heat-inactivated. Medium: either 50% serum + 50% chemically defined medium or 100% chemically defined medium (T8 = Trowell's T8; W = Waymouth's MB 752/1). The effect of insulin (I; 50 µg/ml) + cortisol (F; 20 µg/ml) was compared with I alone.

<table>
<thead>
<tr>
<th>Type of Medium</th>
<th>Alveolar development (grade)\textsuperscript{1}</th>
<th>Necrosis (%)\textsuperscript{2}</th>
<th>Pyknosis (%)\textsuperscript{2}</th>
<th>Secretory response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cytoplasmic opalescence (%)\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>I+F</td>
<td>I+F</td>
<td>I+F</td>
<td>I+F</td>
</tr>
<tr>
<td>S\textsubscript{0}</td>
<td>3 6**</td>
<td>0 33</td>
<td>0</td>
<td>0 1***</td>
</tr>
<tr>
<td>T8</td>
<td>2 3</td>
<td>25 82**</td>
<td>50</td>
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</tr>
<tr>
<td>S\textsubscript{H}</td>
<td>2 3*</td>
<td>0 75**</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>S\textsubscript{HU}</td>
<td>2 2</td>
<td>18 100**</td>
<td>70</td>
<td>2 3*</td>
</tr>
<tr>
<td>S\textsubscript{Ra}</td>
<td>2-3 3</td>
<td>30 83</td>
<td>20</td>
<td>1 3**</td>
</tr>
<tr>
<td>S\textsubscript{F}</td>
<td>2 2-3</td>
<td>0 50</td>
<td>10</td>
<td>1 2**</td>
</tr>
<tr>
<td>S\textsubscript{Fi}</td>
<td>2 1**</td>
<td>33 92*</td>
<td>20</td>
<td>2 3</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated.
* Indicating a significant difference at the 5% level.
** Idem at the 1% level.

No of explants per group: 9-12.

Alveolar development was low (median grade 3), necrosis was absent and pyknosis occurred only sporadically. Cytoplasmic opalescence, vacuolization or secretion were absent or almost absent. When F (20 µg/ml) was added to S\textsubscript{0} + I, the explants responded with a significant increase of the alveolar development, pyknosis, cytoplasmic opalescence, vacuolization and secretion as indicated in Table 13.

When serum was not added to the medium and the explants were cultured in Trowell's T8 or Waymouth (W) with insulin added, the alveolar development was low and necrosis and pyknosis were prominent (see Table 13). The addition of F increased the alveolar development in T8 and the acini showed cytoplasmic opalescence (grade 1). Vacuolization or secretion did not develop. Approximately half of the group showed some eosinophilic material in
the lumen, but nuclear remnants were present. These effects of F were absent in medium W. F increased the necrosis both in T8 and in W. In the latter group only 3 explants from the group of 12 were not totally degenerated after a 6 days culture.

When virgin rat serum (S₀) was replaced by horse, human, rabbit and fetal calf (either heat-inactivated or untreated), the response of the explants was generally poor. Table 13 shows that the alveolar development was low in the culture with I added. Necrosis and pyknosis were marked, horse serum and fetal calf serum both native and inactivated being somewhat less unfavourable than the other heterologous sera. An effect of F on alveolar development was present only with horse serum and inactivated fetal calf serum. Cytoplasmic opalescence was prominent only with fetal calf serum. Using these sera F produced also some vacuolization (Table 13). Some secretion was seen only with horse serum and inactivated fetal calf serum present. The total secretory response was best using the latter type of serum, but the response varied greatly between different mammary glands.

In all groups mitotic activity was absent.

5.1.5.2. Efficacy of homologous serum, insulin and rat prolactin

5.1.5.2.1. Efficacy of serum and hormones

Experimental conditions

Media: 1) 100% T8; 2) 50% S₀+50% t8 (8 mg glucose/ml), no insulin; 3) 50% S₀+50% t8 (8 mg glucose/ml) + I (50 μg/ml). Hormones α) no hormones; β) + F (20 μg/ml); γ) + rPrl (25 IU/mg; 2.5 μg/ml medium); δ) + F + rPrl. 3 And 6 days culture (with change of the medium after 3 days; 4 mammary glands (MP₁₃) were used, a different set for the 3 and the 6 days culture.

Results

Medium no 1: 100% chemically defined medium plus insulin, (T8; Table 14: group 1-4).

Table 14 shows that when explants are cultured in 100% T8 in the presence of I, the alveolar development is very poor (group 1; grade 2) after 3 and 6 days. Pyknosis is present
Table 14: The effect of virgin rat serum and insulin S on the secretory response of mammary explants (MP) induced by prolactin and cortisol. The 3 and 6 days cultures were performed each on a different set of mammary glands. I: insulin (50 μg/ml); rPrl: prolactin (2.5 μg/ml); F: cortisol (20 μg/ml).

<table>
<thead>
<tr>
<th>Group No</th>
<th>Hormones added</th>
<th>Alveolar development (grade)</th>
<th>Pyknosis (grade)</th>
<th>Mitotic activity (%)</th>
<th>Cytoplasmic opalescence (%)</th>
<th>Vacuolization (%)</th>
<th>Secretion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>I+F</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>8</td>
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<td>I+rPrl+F</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
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<td>1</td>
<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
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<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>7</td>
<td>rPrl</td>
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<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>rPrl+F</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative response indicated.  
2: % of explants showing response indicated.  
* Difference with the various groups (no 1, 5 and 9), receiving neither rPrl nor F, significant at the 5% level.  
**Idem at the 1% level.  
No of explants per group: 11-12.

especially after 6 days. 50% of the explants show cytoplasmic opalescence (grade 1). Mitotic activity, vacuolization or secretion are absent. When F is added (group 2), the median degree of alveolar development is increased after 3 days. The incidence of cytoplasmic opalescence is increased after 6 days. F increased the pyknosis to maximum levels after 6 days. The explants showed a degree of degeneration close to necrosis. The addition of rPrl (group 3) to T8 + I produced only an increase in cytoplasmic opalescence after 6 days (difference with group 1 significant at 5% level). Mitotic activity was absent. In comparison with group 3 (T8 + I + rPrl) the addition of F (group 4) produced a sharp increase in alveolar development after 3 and 6 days (Table 14), coinciding with an increase in cytoplasmic opalescence (grade 2) and secretion after 3 days and vacuolization.
after 3 and 6 days. F increased the degree of pyknosis, especially after 6 days. The explants were then almost necrotic.

Medium no 2: 50% virgin rat serum without insulin (Table 14: group 5-8). Table 14 shows that when explants were cultured in S0 without I the alveolar development is very poor (grade 1 and 2) after 3 and 6 days. A high incidence of cytoplasmic opalescence (grade 1) was present after 6 days. Pyknosis was not marked. The addition of F to this type of medium (group 6) caused an increase in alveolar development after 3 days, and in cytoplasmic opalescence (grade 2) and vacuolization (grade 1) after 6 days (see Table 14). Secretion was not observed. The addition of rPrl (group 7) increased, in comparison with group 5, the alveolar development after 3 days and cytoplasmic opalescence after 3 and 6 days (significant at the 5% or lower level). Some mitotic activity was present; pyknosis was absent. The addition of F to S0 + rPrl (group 8) resulted in an increase in cytoplasmic opalescence after 3 days, in alveolar development, vacuolization (grade 3) and secretion after 6 days (see Table 14), but the latter response was very poor. In comparison with group 6, the presence of rPrl in group 8 increased the alveolar development and cytoplasmic opalescence after 3 days, and the alveolar development, vacuolization and secretion after 6 days (significant at the 5% or lower level).

Medium no 3: 50% virgin rat serum plus insulin (Table 14: group 9-12). Table 14 shows that when explants are cultured in a S0 + I medium, the median development remained 3 to 3-4, that some mitotic activity and cytoplasmic opalescence were present especially after 6 days. Pyknosis, vacuolization or secretion were absent. The score for alveolar development was higher than in the absence of either serum (group 1) or of insulin (group 5; differences significant at 1% level). The addition of F to S0 + I (group 10) increased the median alveolar development, vacuolization and secretion after 6 days as shown in Table 14. F suppressed the mitotic activity after 6 days. In comparison with group 2 or 6, which lacked either serum or insulin the results for alveolar development, vacuolization and secretion were increased (differences significant at 1% level). When rPrl was added to S0 + I
(group 11, compare with group 9 of Table 14), the median alveolar development and mitotic activity (grade 3 after 3 days, grade 2 after 6 days) remained high, the cytoplasm was opalescent (grade 1) especially after 3 days and some vacuolization had appeared after 6 days. The differences with group 9 were significant at the 5% or lower level. Addition of F to $S_0 + I + rPrl$ (group 12) produced an increase in the score for alveolar development, cytoplasmic opalescence, vacuolization or secretion after 3 and/or 6 days. It suppressed mitotic activity (grade 2 after 3 days, grade 1 after 6 days). The presence of rPrl was responsible for an increase in alveolar development, mitotic activity and cytoplasmic opalescence (grade 2-3) after 3 days, and in mitotic activity also after 6 days (compare groups 10 and 12, differences significant at 1% level). However, vacuolization and secretion were not affected by the presence or absence of rPrl, the results for alveolar development, mitotic activity, vacuolization and secretion with $S_0 + I + rPrl + F$ (group 2) were superior (significant at 1% level) to those with either $T8 + I + rPrl + F$ (group 4) or $S_0 - I + rPrl + F$ (group 8).

5.1.5.2.2. Effect of various rat prolactin concentrations on cortisol induced morphological changes

Experimental conditions
Medium: 50% $S_0 + 50\% t8$; glucose 4 mg/ml medium; hormones: I (50 $\mu$g/ml) and F (20 $\mu$g/ml) in the combinations F and I + F. To each of these two combinations was added: 0, 0.08, 0.31, 1.25 and 5 $\mu$g/ml rPrl (25 IU/mg); 3 days culture using 4 mammary glands (MP$_{13}$).

Results
When F was added to a medium containing virgin rat serum ($S_0$) but no insulin, the degree of alveolar development was low (grade 3) and mitotic activity, cytoplasmic opalescence, vacuolization or secretion absent or almost absent (Table 15). When 0.08 - 5 $\mu$g/ml rPrl was added to $S_0 + F$ (Table 15), the difference with the result obtained with 0 $\mu$g/ml rPrl became significant at the 5% or lower level for the mitotic activity and cytoplasmic
Table 15 The effect of various concentrations of rat prolactin on mammary gland explants (MP,) in vitro, cultured either with cortisol (F, 20 μg) or with cortisol plus insulin (I, 50 μg/ml). 3 Days culture.

<table>
<thead>
<tr>
<th>Rat Prolactin (μg/ml) medium</th>
<th>Alveolar development (grade)</th>
<th>Mitotic activity ($)</th>
<th>Cyttoplasmic opalescence ($)</th>
<th>Vacuolization ($)</th>
<th>Secretion ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F I+F</td>
<td>F I+F</td>
<td>F I+F</td>
<td>F I+F</td>
<td>F I+F</td>
</tr>
<tr>
<td>0</td>
<td>3 4</td>
<td>8 50</td>
<td>8 50</td>
<td>17 100</td>
<td>0 67</td>
</tr>
<tr>
<td>0.08</td>
<td>3 5</td>
<td>58 100</td>
<td>67 100</td>
<td>50 92</td>
<td>0 100</td>
</tr>
<tr>
<td>0.31</td>
<td>4 6</td>
<td>67 100</td>
<td>67 100</td>
<td>67 83</td>
<td>25 75</td>
</tr>
<tr>
<td>1.25</td>
<td>3-4 5</td>
<td>67 92</td>
<td>92 100</td>
<td>67 83</td>
<td>33 92</td>
</tr>
<tr>
<td>5</td>
<td>4 5</td>
<td>42 100</td>
<td>75 100</td>
<td>75 67</td>
<td>50 83</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated.

No of explants: 12.

opalescence at 0.08 μg/ml rPrl, for the alveolar development and vacuolization at 0.31 μg/ml rPrl and for the secretion at 5 μg/ml rPrl.

When insulin (50 μg/ml) was added to S₀ + F, the degree of all five responses presented in Table 15 increased significantly. When 0.08 - 5 μg/ml rPrl was added to S₀ + F + I (Table 15), the differences with the result obtained with 0 μg/ml rPrl became significant at the 5% or lower level for the alveolar development, mitotic activity and cytoplasmic opalescence with 0.08 μg/ml rPrl. Vacuolization and secretion did not increase significantly by the addition of rPrl. The degree of the alveolar development, mitotic activity, vacuolization and secretion was increased significantly (at the 5% or lower level) by the addition of insulin to S₀ + F + rPrl (0.08 - 5 μg/ml).

5.1.5.2.3. Cortisol induced ultrastructural changes in the presence of S19.

Experimental conditions

Media: 1) 90% S19 + 10% glucose solution (20 mg/ml aqua dest.), 2) idem + 20 μg F/ml medium. 5 Days culture (with change of the medium after 3 days); 1 mammary gland of a 13 days pregnant
rat was used. 9 Fragments per group were prepared for electron microscopical examination.

Results

The tissue cultured in the medium without F and I added showed extensive degeneration and was not suitable for examination. Parts of the tissue cultured with F added but not I showed viability. Figures 12 and 13 show details of such parts. In figure 12 lipid droplets and vacuoles with proteinaceous material are present. Figure 13 shows in addition a well developed Golgi apparatus containing some granules.

5.1.5.3. Interaction of cortisol with various hormone preparations

5.1.5.3.1. Pregnant rat serum

5.1.5.3.1.1. Interaction of cortisol and serum at various stages of pregnancy

Experimental conditions

Medium: 50% serum collected each day during pregnancy and 2 days post-partum + 50% t8; glucose 2 mg/ml; I (50 µg/ml) and F (5 µg/ml); 3 and 6 days cultures, each with 5 mammary glands of 13 days pregnant rat. In the case of the 6 days culture the medium was changed after 3 days.

Results

Using rat serum collected from day 9 to 21 of pregnancy supplemented with I (50 µg/ml) and 5 µg/ml F, a high degree of alveolar development, some mitotic activity, and a distinct
Fig. 14 Morphological changes in the mammary gland of a 13 days pregnant rat in organ culture:

a. Mammary gland explant after 3 days culture in a medium containing 50% mammotrophic-inactive serum ($S_0$) with insulin and cortisol (5 µg/ml). The alveoli are well developed (grade 4). The lumina are prominent and some eosinophilic material (secretion: grade 1) is present. Vacuolization is not prominent. Scale: 100 µ.

b. Mammary gland explant after 3 days culture in a medium containing 50% mammotrophic-active serum ($S_{13}$) with insulin and cortisol (5 µg/ml). The alveoli are well developed (grade 5). In some parts the lumina are prominent and contain eosinophilic material (secretion: grade 1). Vacuolization is clearly visible in the acini (grade 2). Scale: 100 µ.

c. Similar to Fig. 14a, but after 6 days culture. Alveolar development: grade 4. Acinar lumina are in certain areas prominent and contain secretion (grade 2). Vacuolization is marked (grade 2). Arrow indicates local necrosis of the acinus. Scale: 100 µ.

d. Similar to Fig. 14b, but after 6 days culture. Alveoli are distended (development: grade 6) with secretion (grade 3) and vacuolization (grade 4). Scale: 100 µ.

e. Detail of Fig. 14d. The acini are distended with dark-staining secretion in which some nuclear remnants are visible. The cells are distended with big vacuoles, some of which seem to be extruded into the lumen. Scale: 25 µ.

f. Mammary gland explant after 3 days culture in a medium containing 25% virgin rat serum, 25% 19 days pregnant rat serum with insulin and cortisol (0.63 µg/ml). The alveolar cells are swollen and the cytoplasm is opalescent (grade 3). The lumina are small. Scale: 20 µ.
secretion and vacuolization in the acini were observed after 3 days culture (Fig. 14b, 15). Before day 9 of pregnancy the activity of the serum actually declined from day 0 to a minimum on day 6 (difference in alveolar development and secretion significant at 1% level). Compared with day 0 serum (Fig. 14a) the following parameters became significantly different (at the 5% level): using day 9 serum, degree of development and vacuolization of the acini, using day 10 serum, secretion in the acini and vacuolization in the ducts and using day 13 serum, mitotic activity. With serum collected on the day after parturition the degree of development, mitotic activity in the acini, vacuolization in acini and ducts, and secretion were significantly lower (at 5% level) than with serum collected on day 21 of pregnancy.

After a 6 days culture with I + F the effect of the various sera on the development was retained (Fig. 14d, 14e, 15). Again the activity of the serum decreased during the first week of pregnancy (alveolar development day 0 compared with day 7: significant at 1% level). After day 7 the activity of the sera increased and significant differences as compared with day 0 serum (Fig. 14e) were obtained with day 9 serum, with respect to development, vacuolization of acini and ducts, and amount of secretion (at 1% levels). Again the activity in the serum dropped significantly between day 21 of pregnancy and the day after parturition.

5.1.5.3.1.2. Influence of insulin on the effect of cortisol + S19 on mammary glands

Experimental conditions

Medium: 50% S19 + 50% t8. Hormones added: 1) F (20 µg/ml), 2) I (50 µg/ml) + F (20 µg/ml); 3 and 6 days culture, each with 4 different mammary glands of 13 days pregnant rats. Change of the medium after 3 days in the 6 days culture.

Results

When the mammary explants were cultured during three days with I and F added, cytoplasmic opalescence was present in 100%
Fig. 15 Effect of serum collected on different days during pregnancy and lactation (lact.) on rat mammary gland in vitro in a 3 days (left) and 6 days culture (right). Insulin and cortisol were added to the medium, which contained 50% rat serum. Top: degree of development (median value of semi-quantitative grading). Middle: % of explants showing one or more mitotic figures in acini (—) and ducts (—, left). Bottom, left: % of explants showing secretion (—) or vacuolization (—) of the acini; right: median value of semiquantitative grading of secretion (—) or vacuolization (—) of the acini. Number of explants: 13-15 per group.

of the explants (median grade 3), secretion in 92% (grade 1) and vacuolization in 50% (grade 0-1). The median of alveolar development was grade 5 and mitotic activity was present in 58% of the explants (median grade 1). When only F but no I was added, cytoplasmic opalescence developed in 67% of the
Fig. 16  Morphological changes in the mammary gland of a 13 days pregnant rat in organ culture:

a  Mammary gland explant after a 6 days culture with 50% 19 days pregnant rat serum with cortisol (20 μg/ml) and insulin (50 μg/ml). Both secretion and vacuolization are prominent. Scale: 20 μ.

b Similar to a, but without insulin. Vacuolization is prominent but the degree of secretion is low. Many nuclei stain darkly. Scale: 20 μ.

explants (grade 1). The cell size was small. Secretion or vacuolization were not seen. The alveolar development scored grade 2-3. Mitotic activity was absent. Pyknosis was prominent in all explants.

When the mammary explants were cultured during 6 days with I and F added, the cytoplasmic opalescence had disappeared. Secretion and vacuolization were present in all explants and highly developed (grade 4 and 5 respectively; Fig. 16a). Vacuolization was present in the ducts of 100% of the explants (grade 4). The alveolar development scored grade 6. Mitotic activity was absent. When only F but no I was added 58% of the explants were necrotic. In the remaining 5 explants cytoplasmic opalescence was absent. Two explants showed some secretion. Vacuolization was prominent in both acini (grade 4, Fig. 16b) and ducts. The alveolar development was grade 4. Mitotic activity was absent. Pyknosis was prominent.

5.1.5.3.2. Lactating rat serum
5.1.5.3.2.1. Interaction of cortisol and rat serum of late pregnancy and during lactation

Experimental conditions

Sera were collected from rats on days 19, 20 and 21 of pregnancy and on 21 days of lactation following parturition. Serum collected from 2-3 rats on a certain day of lactation was pooled and assayed; medium: 50% serum + 50% t8; glucose: 2 mg/ml; hormones: I + F (5 ug/ml); 3 days culture with 4 mammary glands (MP₁₃).

Results

As shown in Table 16 the alveolar development and vacuol-

<table>
<thead>
<tr>
<th>Serum collected on day</th>
<th>Alveolar development (grade)¹</th>
<th>Mitotic activity (%)²</th>
<th>Acini Vacuolization (grade)¹ (%)²</th>
<th>Secretion Vacuolization (grade)¹ (%)²</th>
<th>Ducts Vacuolization (%)²</th>
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</thead>
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<td>8</td>
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</tr>
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<td>3</td>
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<td>42</td>
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<td>92**</td>
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</tr>
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<td>5</td>
<td>6**</td>
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<td>92**</td>
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<td>0</td>
<td>58*</td>
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<tr>
<td>20</td>
<td>5-6</td>
<td>8</td>
<td>0</td>
<td>42*</td>
<td>1*</td>
</tr>
</tbody>
</table>

¹: Median of semiquantitative grading.
²: % of explants showing response indicated.

Statistical significance for the difference with virgin rat serum indicated by ** at the 1% level, by * at the 5% level.

No of explants: 11-12.
ization of the acini were significantly increased with serum collected during the last three days of pregnancy and during days 2 to 6 of lactation, as compared with the serum from virgin rats. Later during lactation the effect of the serum became erratic. Significantly increased vacuolization of the ductal epithelium was seen using the serum from the pregnant rats and from a few days during lactation. Significant increase of secretion was distributed erratically over the period of investigation. Mitotic activity was hardly ever significantly increased, not even during pregnancy. Cortisol seemed to suppress the mitotic activity which activity is marked when insulin alone is added (Peters et al. 1976). This suppression of mitotic activity by F was more outspoken in the case of pregnant rat serum than of lactating rat serum.

5.1.5.3.2.2. Effect of weaning

Experimental conditions

Sera were collected on 5-6 days following weaning on days 0, 10 or 21 of lactation; sera of non-pregnant rats served as control; sera from 2-4 rats were pooled and assayed; medium etc.: see 5.1.5.3.2.1.

Results

Weaning after parturition significantly suppressed, on days 0, 2, 3 and 4, the alveolar development, mitotic activity (day 2, 3, 4), vacuolization (day 2, 3, 4, 5) and secretion (day 2, 3) as shown in Table 17.

Table 17 shows that on certain days during lactation the serum had a statistically significant effect. Sera from animals weaned either on day 0 (group 3), 10 (group 4) or day 21 (group 5) after parturition suppressed significantly on days 0, 2, 3 and 4 the alveolar development, mitotic activity (day 2, 3, 4), vacuolization (day 2, 3, 4, 5) and secretion (day 2, 3) as shown in Table 17.

Table 17 shows that on certain days during lactation the serum had a statistically significant effect. Sera from animals weaned either on day 10 or 21 were on all days as active as the
Table 17  The effect of weaning on days 0 (group 3), 10 (group 4) and 21 (group 5) of lactation on the mammotrophic activity of serum collected on 5-6 consecutive days. Medium supplemented with insulin plus cortisol; 3 days culture (MP_{13}).

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum collected on day</th>
<th>Alveolar development (grade)</th>
<th>Mitotic activity (S)</th>
<th>Vacuolization (grade)</th>
<th>Secretion (grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>W</td>
<td>N</td>
<td>W</td>
</tr>
<tr>
<td>1</td>
<td>virgin pregnancy</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>5</td>
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<td>lactation</td>
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<td>4</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>42</td>
</tr>
</tbody>
</table>

1: Median of semiquantitative grading.

2: N of explants showing the response indicated.

3: Explants showing a high degree of pyknosis in acini and ducts.

Statistical significance for the difference between serum of weaned and non-weaned rats indicated by * at the 5% level, by ** at the 1% level.

N = non-weaned

W = weaned

No of explants: 11-12.

serum from non-pregnant rats. The effect of weaning was least clear as regards secretion.
5.1.5.3.2.3. Effect of L-dopa

Experimental conditions

Sera were collected from rats nursing 8 young during 6 days and a) weaned for 8½ h, b) weaned for 8 h but nursing for ½ h after a saline injection sc and c) weaned for 8 h but nursing for ½ h after a sc injection of 6 mg L-dopa/100 g bw; the experiment was part of another experiment described in 5.1.1.5.2.; medium: 20% serum + 80% t8; glucose: 3.2 mg/ml; hormones: I + 20 μg F/ml; 3 days culture (MP13); the medium was collected and pooled for each serum separately and the prolactin concentration measured by radioimmunoassay; 4 mammary glands were used.

Results

Figure 17 shows the relationship between the concentration of rPrl measured in the medium after 3 days of culture and the response in vitro. The serum of weaned rats had low rPrl concentrations (< 2 mg/ml medium) and showed a relatively low activity with respect to alveolar development, mitotic activity and cytoplasmic opalescence. Three out of four sera of rats nursing for 30 min after 8 h weaning showed the combination of high rPrl levels and high in vitro activity. The results for the sera obtained from rats nursing after weaning but treated with L-dopa fell more or less between the results for the two previous groups except for the alveolar development. In this case high scores were obtained at low rPrl levels in the medium (< 10 ng/ml).

In comparison with the groups cultured with insulin but no cortisol added to the medium, the combination of insulin plus cortisol produced at rPrl levels of less than 2 ng/ml medium, a significant (at 1% level) increase of the alveolar development and the grade of cytoplasmic opalescence (see "Lactating rat serum after L-dopa"). A synergism between cortisol and high rPrl levels was not seen for the alveolar development, but was present for the degree of cytoplasmic opalescence. An antagonism between the two factors was found for the mitotic activity.

The development of vacuolization and secretion after 3 days of culture is shown in figure 18. Vacuolization and
Fig. 17 Relationship between the concentration of rat prolactin (rPrl) as measured by radioimmunoassay in a medium containing 20% serum of 6 days lactating rats, and alveolar development (median grade), mitotic activity (% of explants) and cytoplasmic opalescence (grade 3 or more; % of explants) in mammary gland explants (MP₃) cultured for 3 days in a medium supplemented with insulin and 20 μg cortisol/ml medium; 4 rats were weaned for 8½ h (●), 4 rats were weaned for 8 h followed by ½ h nursing (○) and 4 rats were weaned for 8 h followed by a sc injection of 6 mg L-dopa/100 g bw and nursing for ½ h (■); the results are part of the experiment shown in Fig. 4; no of explants per group: 8–12.

secretion increased after 3 days of culture; they increased sharply at low rPrl levels (< 10 ng/ml medium) but decreased at higher rPrl levels. Vacuolization and secretion were not seen regularly in the groups cultured without cortisol added to the medium (see secretion 5.1.1.5.2.).

5.1.5.4. Ovine prolactin

Experimental conditions

Medium: 50% S₀ + 50% T8; glucose 2 mg/ml medium, hormones: I (50 μg/ml), Prl (ovine, 10 μg/ml), P (0.5 μg/ml), O (0.05 μg/ml)
and F (20 μg/ml). The following combinations of hormones were used: I + P + O + F and I + P + O + F + Prl; 6 days culture, change of medium after 3 days; 6 mammary glands (MP13) were used for culture.

Results

When mammary explants were cultured during 6 days in a medium containing virgin rat serum, progesterone, oestradiol and insulin either without or with ovine prolactin (10 μg/ml), the median degree of alveolar development was grade 5 and 6 respectively, of secretion grade 2 and 3, and of vacuolization grade 3 and 3. The differences between the groups with or without ovine prolactin were not statistically significant.

5.1.5.5. Human prolactin

Experimental conditions

Medium: 50% S0 + 50% t8; glucose 2 mg/ml. Hormones: I
(50 µg/ml), F (20 µg/ml) and 0, 0.39, 1.56, 6.25 and 25 µg/ml Prl, one series with a rat prolactin preparation (12.8 IU/mg), another with a human prolactin preparation (potency unknown); 3 days culture; 5 mammary glands (MP_{13}) were used.

Results

Table 18 shows the results obtained with 5 µg/ml and human or rat prolactin added in a concentration of 0.4 and 1.6 µg/ml. Results obtained with 6.3 and 25 µg/ml Prl are comparable with those of 1.6 µg/ml and have been omitted from Table 18.

When the explants were cultured in S_{0} + I, the alveolar development was low (grade 4). Mitotic activity was low and cytoplasmic opalescence, vacuolization and secretion were absent or almost absent (Table 18). Both prolactin preparations produced an increase of the alveolar development and mitotic activity

Table 18: The effect of various concentrations of a human and a rat prolactin preparation on mammary gland explants in vitro cultured with insulin (I, 50 µg/ml) or with insulin (I, 50 µg/ml) plus cortisol (F, 20 µg/ml). 3 Days culture (MP_{13}).

<table>
<thead>
<tr>
<th>Prolactin µg/ml</th>
<th>Alveolar development (grade)</th>
<th>Mitotic activity (%%)</th>
<th>Cytoplasmic opalescence (%)</th>
<th>Vacuolization (%)</th>
<th>Secretion (%%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>I+F</td>
<td>I</td>
<td>I+F</td>
<td>I</td>
</tr>
<tr>
<td>Human Prolactin</td>
<td>0</td>
<td>4</td>
<td>5**</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>6**</td>
<td>6**</td>
<td>87</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>6**</td>
<td>6**</td>
<td>80</td>
<td>36</td>
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<td>1.6</td>
<td>6**</td>
<td>6**</td>
<td>93</td>
<td>33</td>
</tr>
</tbody>
</table>

* Difference with the group 0 µg/ml prolactin significant at the 5% level.
** Idem at 1% level.
1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated.
3: Difference with the median semiquantitative response of the corresponding group receiving insulin but no cortisol, significant at the 5% level.
4: Idem at the 1% level.

No of explants per group: 14-15.
Traces of vacuolization and secretion and some opalescence were observed. A maximum effect was obtained with both human and rat prolactin at 0.4 to 1.6 µg/ml. With 25 µg/ml a decline in the incidence of secretion occurred.

When cortisol was added to the medium S₀ + I without exogenous prolactin, the alveolar development was increased, in comparison with the control containing no cortisol. Little mitotic activity and cytoplasmic opalescence were observed, but vacuolization and secretion were found in a high percentage of the explants (Table 18). Cortisol affected the response to both human and rat prolactin in the same way. The alveolar development was little affected whereas mitotic activity declined. The secretory response, which was presented in the explants only locally with prolactin alone, became more generalized by the addition of cortisol, but this was not marked in this 3 days culture. On the other hand, the synergism between the two prolactin preparations and cortisol with regard to the cytoplasmic opalescence was clear-cut (Table 18). Maximum response was obtained with 0.4 - 1.6 µg/ml of both preparations.

5.1.5.6. Human placental lactogen

Experimental conditions

HPL was added to the medium in concentrations of 0, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5 and 25 µg/ml. The medium was supplemented with insulin and cortisol (F, 1.25 µg/ml). The combination hPL + I was added to two different media: 100% T₈ and 50% S₀ + 50% t₈, and compared with 50% S₁₃ + 50% t₈ + I; glucose: 4 mg/ml.

3 Days culture with 4 mammary glands (MP₁₃).

Results

Table 19 shows that with I + F, the alveolar development was still low in T₈ (compare Fig. 19b (T₈ + I + F) with 19a (T₈ + I)) and S₀, but higher in S₁₃. The addition of hPL to T₈ + I + F (Fig. 19c) or S₁₃ produced some increase, but the effect was most marked in S₀ (Fig. 19d). However, at 12.5 µg/ml hPL the degree of alveolar development was decreased. Mitotic activity was virtually absent in T₈ and S₀. It was noted in approximately
Table 19 Effect of hPL on rat mammary gland explants in vitro. T8: medium of 100% T8; S0: medium of 50% virgin rat serum plus 50% T8; S13: medium of 50% 13 days pregnant rat serum plus 50% T8. The media were supplemented with insulin (50 μg/ml) and cortisol (1.25 μg/ml). Explants (MP13).

<table>
<thead>
<tr>
<th>hPL (μg/ml)</th>
<th>Alveolar development (grade)1</th>
<th>Mitotic activity (%)2</th>
<th>Vacuolization</th>
<th>Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T8 S0 S13 T8 S0 S13 T8 S0 S13 T8 S0 S13</td>
<td>T8 S0 S13 T8 S0 S13 T8 S0 S13</td>
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<tr>
<td>0</td>
<td>3 4 5 0 17 42 0 92 92 0 0 58 83 100 92 2 2-3</td>
<td>1-2</td>
<td>50%</td>
<td>100</td>
</tr>
<tr>
<td>0.4</td>
<td>4 5 6 0 33 42 0 92 92 0 42 75 100 100 92 2 2-3</td>
<td>1-2</td>
<td>50%</td>
<td>100</td>
</tr>
<tr>
<td>0.8</td>
<td>4 5 6 0 42 67 10 83 92 40 50 75 100 100 92 3 2</td>
<td>1-2</td>
<td>50%</td>
<td>100</td>
</tr>
<tr>
<td>1.6</td>
<td>4 6 8-6 0 57 50 8 92 75 25 33 42 100 92 100 2-3 1-2</td>
<td>2</td>
<td>50%</td>
<td>100</td>
</tr>
<tr>
<td>3.1</td>
<td>4 5 6 0 50 33 0 92 83 25 33 92 100 83 100 2-3 2</td>
<td>2</td>
<td>50%</td>
<td>100</td>
</tr>
<tr>
<td>6.3</td>
<td>3 4 6 5 0 42 67 17 92 75 17 33 50 93 92 92 3 1</td>
<td>1</td>
<td>50%</td>
<td>100</td>
</tr>
<tr>
<td>12.5</td>
<td>3 4 3-4 0 8 0 10 13 10 10 0 0 60 8 0 1 0</td>
<td>0</td>
<td>50%</td>
<td>100</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated.
3 Days culture.
No of explants: 10-12 per group.

half of the explants in S13. The addition of hPL produced no mitotic activity in T8, but in S0 the mitotic activity was increased to values of approximately 50% (Table 19). Addition of hPL to S13 produced no increase of mitotic activity. The concentration of 12.5 μg/ml hPL suppressed the activity obtained in S0 and S13.

Vacuolization of acini or ducts did not develop with I + F in T8. In S0 vacuolization developed in acini but not in the ducts, in S13 it developed in both acini and ducts. The addition of hPL to T8 produced the appearance of some vacuolization in a minority of the explants (Fig. 19c). After addition to S0 no further increase of vacuolization was observed in the acini, but some vacuolization developed in the ducts. The secretory response due to hPL developed in part of the explants (Fig. 19d). The addition of hPL to S13 produced no further increase of vacuolization in either acini or ducts, although the incidence using hPL + S13 was generally at a higher percentage level than using hPL + S0; 12.5 μg/ml hPL suppressed the vacuolization. Secretion
Fig. 19  Morphological changes in the mammary gland of a 13 days pregnant rat in organ culture:

a  Mammary gland explant after 3 days culture in a medium of 100% T8 (containing insulin). The alveoli and ductal epithelial lining are poorly developed (grade 2). The acinar lumina are small and contain no eosinophilic material. Vacuolization is not present. Scale: 50 μ.

b  Mammary gland explant after 3 days culture in 100% T8 with 1.25 µg/ml cortisol added. A secretory response is almost absent. Some lumina are slightly distended; in a few lumina some granular material is present. Scale: 50 μ.

c  Mammary gland explant after 3 days culture in 100% T8 with 1.25 µg/ml cortisol and 6.3 µg/ml human placental lactogen added. The lumina are distended and some material is present (secretion: grade 2). A few vacuoles are visible in the acinar epithelium. Scale: 50 μ.

d  Mammary gland explants after 3 days culture in a medium containing 50% virgin rat serum with 1.25 µg/ml cortisol and 6.3 µg/ml human placental lactogen added. Note the secretory inactive appearance of some groups of acini. Lumina are not distended and the secretory material is almost absent. Scale: 50 μ.
did develop with I + F in either T8, S0 or S13. However, with I + F in T8 nuclear remnants were present in the secreted material. The degree of secretion was graded to be equivalent for the three media; 12.5 μg/ml suppressed the appearance of secretory material, 6.25 μg/ml to some extent with S0 and S13. 25 μg/ml hPL caused necrosis of the explants with T8, S0 and S13.

5.1.5.7. Human growth hormone

Experimental conditions

Medium: 50% S0 + 50% t8; glucose 2 mg/ml; hormones: I + F (5 μg/ml) + 0, 0.8, 3.1, 12.5 and 50 μg hGH/ml (potency unknown); compared with 50% S13 + 50% t8 + I + F. 3 And 6 days culture (change of medium on day 3), with the same set of 4 mammary glands (MP13).

Results

Figure 20 shows the results in a 3 days culture. In the presence of F the only parameter significantly increased was vacuolization (starting at 0.8 μg hGH/ml). Maximum effect was obtained with 3.1 μg hGH/ml. The effect of S0 + hGH was comparable to that of S13 except for alveolar development and mitotic activity, which did not reach the level of S13, and for vacuolization, which was more developed than with S13. In the 6 days culture mitotic activity was not observed in any of the groups. A significant effect of hGH on alveolar development and vacuolization, (Fig. 21 expressed as median grades) was seen starting at 3.1 μg/ml. These results were comparable to those obtained with S13. Secretion in the acini was significantly increased at 12.5 μg hGH/ml. Nuclear remnants were observed in the secretion at 50 μg/ml. As regards vacuolization and secretion, results for the ducts went parallel with those for the acini. Without hGH 5-10 and 15% of the secretion surface was estimated to be necrotic with S13 and S0 respectively. Adding hGH at 0.8, 3.1, 12.5 and 50 μg/ml increased the percentage to 25, 30, 50 and 40% respectively.
Fig. 20 Effect of various concentrations of a human growth hormone preparation (hGH), added to a medium containing virgin rat serum, insulin and 5 μg cortisol/ml, on the alveolar development (Δ—Δ, AD; median grade), mitotic activity (●—●, MIT; % of explants), vacuolization (○—○, VAC; % of explants) and secretion (■—■, Secr; % of explants) in mammary explants (MP) cultured for 3 days. The lowest concentration of hGH that produced a change in effect statistically significant from the control at the 5% level or at the 1% level is indicated by one, respectively two arrow heads. S13: results obtained with serum of 13 days pregnant rats. No of explants: 11-12 per group.

Fig. 21 As Fig. 20. The results for alveolar development, vacuolization and secretion are expressed as median grades. No of explants: 11-12 per group.
5.1.5.8. Adrenocorticotrophin

Experimental conditions

Medium: 50% S₀ + 50% t8; glucose 4 mg/ml; hormones: I + F (1.3 µg/ml) + 0.31 and 5 µg ACTH (82 IU/ml); one series assayed with rPrl (25 IU/mg; 1.3 µg/ml medium), another series without rPrl 3 and 6 days culture (change on day 3), with the same set of 4 mammary glands (MP₁₃).

Results

Table 20 shows that the addition of 0.31 µg ACTH/ml to I + F had no significant effect, whereas 5 µg/ml significantly increased mitotic activity, vacuolization and secretion after

Fig. 21
Table 20 Effect of 0, 0.31 and 5 μg ACTH/ml on the in vitro response of mammary gland explants (MP) cultured during 3 and 6 days in a medium containing virgin rat serum and cortisol (1.3 μg F/ml), supplemented either with insulin (I + F) or insulin plus 1.25 μg/ml rat prolactin (I + rPrl + F).

<table>
<thead>
<tr>
<th>ACTH μg/ml</th>
<th>Alveolar development (grade)$^1$</th>
<th>Mitotic activity (%)$^2$</th>
<th>Cyttoplasmic opalescence (grade)$^1$</th>
<th>Vacuolization (grade)$^1$</th>
<th>Secretion (grade)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3d 6d</td>
<td>3d 6d</td>
<td>3d 6d</td>
<td>3d 6d</td>
<td>3d 6d</td>
</tr>
<tr>
<td>I+F</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>5 5</td>
<td>50 42</td>
<td>1 0</td>
<td>0 3-4</td>
<td>2 4</td>
</tr>
<tr>
<td>0.31</td>
<td>3-4 5-6</td>
<td>25 25</td>
<td>1 0</td>
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<td>5 6</td>
<td>83** 36</td>
<td>1-2 0</td>
<td>3** 5**</td>
<td>3-4* 6</td>
</tr>
<tr>
<td>I+F+rPrl</td>
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</tr>
<tr>
<td>0</td>
<td>6 6-7</td>
<td>83 25</td>
<td>2 0</td>
<td>3 5</td>
<td>3 5-6</td>
</tr>
<tr>
<td>0.31</td>
<td>6 6-7</td>
<td>92 58</td>
<td>2 2</td>
<td>3 5</td>
<td>3 5-6</td>
</tr>
<tr>
<td>5</td>
<td>6 7</td>
<td>73 58</td>
<td>2 0</td>
<td>0 6-7</td>
<td>2 5</td>
</tr>
</tbody>
</table>

$^1$: Median grade of response.
$^2$: % of explants showing response indicated.

* Difference in response with 0 μg ACTH/ml significant at the 5% level.
** Idem at 1% level.

No of explants: 11-12 per group.

3 days, and only vacuolization after 6 days. When the explants were cultured in I + F + rPrl, the presence of rPrl resulted in a significant increase of all 5 parameters presented in Table 20, after 3 and 6 days. The addition of either 0.31 or 5 μg ACTH/ml had no statistically significant effect.

In comparison with S₀ + I + rPrl, F suppressed the mitotic activity but increased vacuolization and secretion (compare with Table 12).

5.1.5.9. Progesterone

Experimental conditions

Medium: a) 100% T8, b) 50% S₀ + 50% t8, c) 50% S₁9 + 50% t8; glucose: 4 mg/ml; + I; F in concentrations of 0, 0.25, 0.5 and 1.0 μg/ml, with or without P (0.5 μg/ml); 3 days culture; 4 mammary glands of 19 days pregnant rats (Fig. 22a). The size of the explants was reduced by approximately 50%.

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Fig. 22 Morphological changes in the mammary gland of a 19 days pregnant rat in organ culture:

a Aspect of the gland at the beginning of the culture. The alveoli are well developed (grade 6). The cytoplasm is opalescent. Some vacuoles are noticeable. The acinar lumina are small and contain traces of secretion. Scale: 100 μ.

b Mammary gland explant after a 3 days culture in a medium containing 50% serum of 19 days pregnant rats with insulin (50 μg/ml), cortisol (1 μg/ml) and progesterone (0.5 μg/ml) added to the medium. In certain acini a secretory response has not developed, in other acini the lumina are formed and filled with secretory material. Some vacuolization has developed. Scale: 100 μ.

Results

Table 21 shows that using T8 alveolar development was not maximal and mitotic activity absent. Moreover the cytoplasm of the acinar cells stained weakly. Some vacuoles were seen scattered through the alveoli; degeneration was apparent from a generalized and high degree of pyknosis. With F added, the only marked effects were an increase in the number of explants showing vacuolization and nuclear remnants in the acini. Some eosinophilic material was present in the acinar lumen, but nuclear remnants were prominent in the "secretory product". Addition of P did not change the morphology in any respect.

When the explants were cultured in a medium with 50% S₀ without F or P, maintenance of alveolar development was still poor and mitotic activity virtually absent (Table 21), but in comparison with 100% T8, the degree of pyknosis was less. Addition of F to this medium produced some increase in alveolar development, but mitotic activity remained low (Table 21). The
Table 21: The effect of progesterone (P; 0.5 μg/ml) added to 3 different types of medium in combination with various concentrations of cortisol (F), on mammary gland explants of 19 days pregnant rats; insulin supplementation (I; 50 μg/ml); t8: simplified formula; 3 days culture.

<table>
<thead>
<tr>
<th>Medium</th>
<th>F (μg/ml)</th>
<th>Mitotic Activity (grade)</th>
<th>Vacuolization (grade)</th>
<th>Secretion (grade)</th>
<th>Nuclear Remnants (grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>I+P</td>
<td>I</td>
<td>I+P</td>
</tr>
<tr>
<td>100% t8</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50% virgin rat</td>
<td>0.25</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>serum +</td>
<td>0.5</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>50% t8</td>
<td>1.0</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50% 19 days rat</td>
<td>0.25</td>
<td>6</td>
<td>6</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>serum +</td>
<td>0.5</td>
<td>6</td>
<td>6</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>50% t8</td>
<td>1.0</td>
<td>6</td>
<td>6</td>
<td>91</td>
<td>91</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated.
3: Difference tested between grades of mitotic activity, not frequencies.

Significant differences between the result obtained and the control (no progesterone) are indicated by one asterisk (5% level) or by two asterisks (1% level). 

No of explants: 11-12.

Addition of F induced vacuolization in the acini, already at the lowest concentration of F. Vacuolization of the ductal epithelium was not prominent. Secretion became marked and was not dominated by the presence of nuclear remnants. When in addition P was added alveolar development did not increase, but the addition of P resulted in a consistent increase of mitotic activity; at 1 μg/ml F this increase became less (Table 21). The secretory response to F was slightly decreased, but this decrease was rarely significant.

When 50% S19 was used instead of S0, the explants showed, in the absence of exogenous F or P, a maintenance of the high
degree of alveolar development (Table 21). A high mitotic activity was found, and traces of vacuolization and secretion were observed. When F was added, the alveolar development increased. The suppression of the mitotic activity by F was not marked (Table 21). Vacuolization became generalized and reached a higher grade, than in the explants cultured with \( S_0 \) (with 0.5 \( \mu \)g/ml F significantly at the 1% level). The difference between the two types of serum was especially noticeable with respect to the vacuolization of the ductal epithelium. Nuclear remnants were inconspicuous in this group. Additional progesterone had little or no effect on the effect of F in the medium with \( S_19 \) (compare Fig. 22a and Fig. 22b). The effect on the secretory response was only statistically significant in one instance.

5.1.5.10. Testosterone and oestradiol

Experimental conditions

Medium: 25% \( S_{19} \) + 25% \( S_0 \) + 50% \( t8 \); + I (50 \( \mu \)g/ml); glucose 4 mg/ml; F added in concentrations of 0, 0.17, 0.31, 0.63 and 1.25 \( \mu \)g/ml; one series of concentrations of F with no other hormones added, one with 1 \( \mu \)g/ml testosterone (T) added and one with 0.05 \( \mu \)g/ml oestradiol (O) added; 3 days culture with 4 mammary glands of 13 days pregnant rats.

Results

Without F added, the \( S_{19} \)-medium containing 25% \( S_{19} \) + 25% \( S_0 \) produced grade 5 of alveolar development, a high mitotic activity (grade 2), grade 1 of cytoplasmic opalescence and no secretion or vacuolization (Table 22). When F was added in concentrations of 0.17 to 1.25 \( \mu \)g/ml, the alveolar development increased slightly but irregularly to grade 5 or 5-6. The mitotic activity decreased significantly (Table 22) to grade 1. F increased the cytoplasmic opalescence markedly: 0.17 \( \mu \)g/ml F produced grade 2 (difference with 0 \( \mu \)g/ml F sign. at 1% level). Higher concentrations gave maximum opalescence (Fig. 14f). Secretion and vacuolization developed, but only in part of the explants and the degree was low (grade 1).

When testosterone (T) was added, no effect was observed on
Table 22. Mammary gland explants obtained from 13 days pregnant rats. The effect of cortisol (F). The medium contained 25% virgin rat serum and 25% 19 days pregnant rat serum (-). In one group 1 μg/ml testosterone (T) was added, in another group 0.05 μg/ml oestriadiol (O). The medium was supplemented with insulin.

<table>
<thead>
<tr>
<th>F µg/ml</th>
<th>Mitotic activity (%)</th>
<th>Secretion (%)</th>
<th>Vacuolization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+T</td>
<td>+O</td>
</tr>
<tr>
<td>0</td>
<td>67</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>0.17</td>
<td>42</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>0.31</td>
<td>42</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>0.63</td>
<td>58</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>1.25</td>
<td>33</td>
<td>42</td>
<td>83</td>
</tr>
</tbody>
</table>

1: % of explants showing response indicated.
2: Difference with the grade of mitotic activity in group 0 µg/ml F significant at 5% level.
3: Idem at 1% level.
4: Difference with the corresponding group receiving neither oestradiol (O) nor testosterone (T) significant at 5% level.
5: Difference with the corresponding group receiving T significant at 5%.

No of explants: 12 per group.

the results obtained with either S₁₉-medium alone or in combination with F (Table 22). 0.17 µg/ml F produced a significant increase of the cytoplasmic opalescence (grade 2, significant at 1% level). When oestriadiol (O) was added, no effect was observed on the results obtained in the S₁₉-medium without the addition of F. The combination of F with O did not change the results for alveolar development or cytoplasmic opalescence. 0.17 µg/ml F produced again a significant increase of opalescence (grade 2, significant at 1% level). The combination of O + F suppressed the mitotic activity more than F alone, but the difference was not statistically significant. The combination of O with 1.25 µg/ml F increased both the percentage of explants showing secretion and vacuolization (Table 22), and the degree of the response (grade 2 versus grade 1 with 1.25 µg/ml alone). The differences in secretion and vacuolization between O + F and T + F were not significant at 1.25 µg/ml, but they were so at
5.1.5.11. Thyroxin

Experimental conditions

Medium: 50% S₁₃ + 50% T₈; glucose: 4 mg/ml; hormones: I + F (20 µg/ml) with and without 0.5 µg/ml thyroxin; 3 days culture with 4 mammary glands (MP₁₃).

Results

After 3 days the results were for alveolar development grade 5 and 5-6, for mitotic activity 50 and 67%, for cytoplasmic opalescence grade 3-4 and 4, for vacuolization 33 and 58 and for secretion 83 and 100 (grade 2) respectively without and with thyroxin. None of the differences were statistically significant.

5.1.5.12. Influence of cortisol on rat mammary glands at various stages during pregnancy.

5.1.5.12.1. Sensitivity of rat mammary glands to cortisol at various stages of pregnancy

Experimental conditions

Medium: 50% S₀ + 50% T₈; glucose 2 mg/ml medium. Hormones: I (50 µg/ml), P (0.5 µg/ml), O (0.05 µg/ml) and F (20 µg/ml). The following combinations of hormones were used: I; I + P + O; I + P + O + F; 3, 6 and 9 days culture with change of the medium after 3 and 6 days, mammary glands were used from non-pregnant rats (M₀) and 4, 7, 10, 13, 15 and 19 days pregnant rats (M₄ etc.), of each 2, except 4 days pregnant (4 glands).

Results

The mammary gland of pregnant rats.

The acini of the non-pregnant rat mammary gland are not well developed (grade 2-3). The ducts are prominent, but the epithelial lining is poorly developed. The cells of the acini are small with compact nuclei. Mitoses, secretion and vacuolization have not been observed. After 4 or 7 days pregnancy the mammary gland was slightly more developed, reaching grade 3.
On day 10 the alveolar development was grade 4, on day 13 4-5, on day 15 and 17 grade 5-6, on day 19 grade 6. The cytoplasm of the acinar cells is opalescent on day 10 (grade 1), the degree increases later during pregnancy and reaches grade 2 on day 19. Some vacuoles are then notable, but the acinar lumina remain small and intraluminal eosinophilic material is almost absent. The ducts are not prominent, but the lining is well developed.

When explants obtained at various stages of pregnancy were cultured during 3, 6 or 9 days, the mammary tissue tended to reach an identical low level of differentiation irrespective of the differences which existed at the beginning of the culture. The median alveolar development was generally grade 4 after 3 and 6 days, but decreased to grade 1-3 after 9 days. At that time no consistent difference was found between the $M_0 - M_{19}$. Some mitotic figures were observed in 25% or less of the groups of explants after 3 days. Cytoplasmic opalescence was not marked, secretion and vacuolization were absent. The degree of pyknosis was low (grade 1) in the $M_0 - M_{10}$ after 3 days, but higher (grade 3) with $M_{13} - M_{19}$. The difference in degree of pyknosis between these two groups became less marked after 6 days whereas after 9 days pyknosis was high in all groups (grade 3). Necrosis remained low (0-10% of sections) except for the $M_{19}$ after 9 days of culture (60%). Considerable outgrowth (grade 4) was found in the 6 and 9 days culture.

The presence of progesterone and oestradiol did not result in a statistically significant effect. Mitotic activity was present at a slightly higher level.

The presence of F resulted in a generally higher median grade for alveolar development. The median did not change greatly between the 3, 6 and 9 days culture, but it showed a higher level with $M_{13} - M_{19}$ in the 3, 6 (see Fig. 23) and 9 days culture. Mitotic activity was absent. After the 3 days culture vacuolization of the acini was present in 0-50% of the number of explants in the $M_0 - M_{17}$ but the $M_{19}$ proved to be more sensitive with 92% of the
Fig. 23 Response of mammary gland explants obtained from non-pregnant rats (day 0 of pregnancy) and from 4 to 19 days pregnant rats to a hormone combination of insulin (50 µg/ml), progesterone (0.5 µg/ml), oestradiol (0.05 µg/ml) and cortisol (20 µg/ml). 6 Days culture. Culture medium: 50% virgin rat serum + 50% chemically defined medium. Grade: median value of semiquantitative grading of response indicated. AD: alveolar development; V: vacuolization; S: secretion. No of explants per group: 11-12 (except day 4: 22).

number of explants showing vacuolization (median grade 2). At the time the ducts still showed no vacuolization. After the 6 days culture vacuolization was notable in every type of mammary gland (Fig. 23), but the median degree was higher in the M_{15}-M_{19}. As regards the ducts the two groups did not differ. Some vacuolization of the ducts was found in about half of the number of explants in all groups after 6 days. The degree of vacuolization
in the acini was slightly further increased after 9 days. Secretion was almost absent after 3 days culture with M₀ (present in 10% of the number of explants), but otherwise present in 50 to 100% of the number of explants of M₄-M₁₈. After 6 days 40 to 50% of the number of explants showed some secretion. The median degree of secretion was higher with M₁₀-M₁₈ (Fig. 23). This tendency was even more marked after 9 days when medians of grade 3-4 were reached for M₁₃-M₁₈ while secretion in M₀-M₇ was then almost absent. Pyknosis was present during the entire period of culture and generally at a high level (grade 3). Necrosis was still almost absent after 3 days, but it appeared after 6 days (approximately 25% of the sections) and increased to approximately 50% after 9 days of culture. F suppressed outgrowth (grade 2).

5.1.5.12.2. Dose effect relations of cortisol with mammary glands at various stages of pregnancy

Experimental conditions
Medium: 50% S₀ + 50% t8; glucose 4 mg/ml medium; hormones: I (50 μg/ml) + rPrl (25 IU/mg; 5 μg/ml) to which was added 0, 0.16, 0.31, 0.63, 1.25, 2.5 and 5 μg/ml F; 3 days culture; 4 mammary glands were used from non-pregnant, and 13 and 19 days pregnant rats, each set cultured in medium drawn from the same pool.

Results
The mammary gland of pregnant rats is described in section 5.1.5.12.1.

Figure 24 shows the effect of cortisol on mitotic activity, cytoplasmic opalescence, vacuolization and secretion in mammary explants of non-pregnant (M₀), 13 days (M₁₃) and 19 days (M₁₉) pregnant rats, cultured during 3 days in the same medium of S₀ + I + rPrl.

M₀: When no F is added, S₀ + I + rPrl produced a high mitotic activity, grade 1 of cytoplasmic opalescence which reached grade 2 only in one explant, and no secretion or vacuolization. The median alveolar development was grade 3. The addition of F
Fig. 24  Response of mammary gland explants obtained from non-pregnant rats (M₀), 13 days (M₁₃) and 19 days (M₁₉) pregnant rats. 3 Days culture. Culture medium: 50% virgin rat serum + 50% chemically defined medium; insulin (50 µg/ml), prolactin (rat, 5 µg/ml). The culture medium was supplemented with 0, 0.16, 0.31, 0.63, 1.25 and 5 µg/ml cortisol. %: percentage of explants showing response indicated. CO: cytoplasmic opalescence (grade 2 or higher); V: vacuolization (M₀ and M₁₃: % of explants with grade 1 or higher; M₁₉: grade 5); S: secretion (M₀ and M₁₃: % of explants with grade 1 or higher; M₁₉: grade 5); MA: mitotic activity (grade 2 or higher). No of explants per group: 11-12.

suppressed the mitotic activity (difference between grades with 0 and 0.16 µg/ml F significant at the 5% level). The suppression did not show a good relationship with the concentrations of F in the range from 0.16 to 5 µg/ml. Cytoplasmic opalescence (grade 2, Fig. 24) increased sharply in the range of 0.16 to 1.25 µg/ml F. The appearance of some secretion (median grade 1) in the explants ran parallel with the change
of cytoplasmic opalescence. The alveolar development remained approximately at the level of grade 3.

M$_{13}$: When no F was added to S$_0$ + I + rPrl, mitotic activity in the M$_{13}$ explants was high, reaching grade 2 (or higher) in 73% of the number of explants. Cytoplasmic opalescence of grade 2, vacuolization and secretion were absent (Fig. 24, middle). The median alveolar development was grade 5. The addition of F to S$_0$ + I + rPrl suppressed the mitotic activity. The difference between the grades with 0, and 0.16 µg/ml F was significant at the 5% level. The further decrease of mitotic activity with higher concentrations of F was not regular and the difference between 0.16 µg/ml and 5 µg/ml was not significant. The number of explants showing grade 2 of cytoplasmic opalescence increased sharply with 0.16 µg/ml (Fig. 24). One hundred percent incidence was obtained with 1.25 µg/ml F. A low degree of vacuolization (grade 1) was observed in about half of the number of explants in the range of 0.16 to 1.25 µg/ml F. At higher concentrations the vacuolization tended to increase slightly and it reached the median grade 2 with 5 µg/ml F. Grade 5 was seen in only one explant with 2.5 µg/ml F. Secretion (grade 1) appeared in 42% of the number of explants with 0.16 µg/ml F. At higher concentrations of F, the percentage increased and reached 100% with 1.25 µg/ml F. (Fig. 24). The median degree of secretion reached grade 2 with 5 µg/ml F. In none of the explants a grade 6 of secretion was observed. The median alveolar development remained close to grade 5 after the addition of F.

M$_{19}$: When no F was added to S$_0$ + I + rPrl, mitotic activity was high (grade 2 or more in 68% of the number of explants, Fig. 24). Cytoplasmic opalescence was prominent (grade 2: 91%). In all explants vacuolization was observed in the acini. The median degree was grade 2-3. The maximum response in this group was grade 3 of vacuolization. Secretion was observed in all explants but one. The median degree was grade 2-3, the maximum response being grade 3 of secretion. The median alveolar development was grade 6.

The addition of 0.16 µg/ml F to S$_0$ + I + rPrl suppressed the mitotic activity but the effect was not statistically sig-
significant. The percentage of explants showing grade 2 (or higher) of mitotic activity decreased regularly in the range of 0.16 to 1.25 μg/ml F (Fig. 24). The difference between the degree of activity with 0.16 and 5 μg/ml F was significant (at the 5% level). The median degree of cytoplasmic opalescence remained approximately grade 2, irrespective of the concentration of F. Vacuolization was observed in all explants with each concentration of F. The number of explants with grade 5 of vacuolization reached 50% with 0.16 μg/ml F and 100% with 5 μg/ml F. Secretion was also observed in all explants with each concentration of F. The number of explants that reached grade 5 of secretion was still low with 0.16 μg/ml F (25%, see Fig. 24), but was increased to 100% with 2.5 μg/ml F. The median alveolar development increased by the addition of 0.16 μg/ml F to grade 6-7. At still higher concentrations it reached grade 7. The difference in alveolar development with the group 0 μg/ml F became significant (at the 5% level) with 0.63 μg/ml F.

5.1.5.13. Time related changes in the response of mammary glands to cortisol

5.1.5.13.1. Effect of serum

Experimental conditions

Media: a 100% T8 (T8); b 50% S0 + 50% t8 (S0); c 50% S13 + 50% t8 (S13); glucose: 4 mg/ml; hormones: I (50 μg/ml) and I + F (20 μg/ml); explants were fixed at logarithmically spaced intervals: 1 1/8, 2½, 4½, 9, 18, 36 and 72 h after the onset of culture. The medium was changed after 3 days and the series of intervals repeated. Each of the three media was assayed separately on 4 mammary glands (MP13).

Results

The effects are reported separately for the various parameters.

Alveolar development.

Figure 25 shows, that with T8 + I the alveolar development declined rapidly after 18 h and reached a minimum after 72 h. With T8 + I + F results were similar for the first 36 h. There-
change of medium

Fig. 25
Fig. 25 Time-related changes of the median degree of alveolar development graded for mammary gland explants cultured for two periods of 3 days with change of the medium after the first period. The medium was either Trowell's T8 (T8) or t8 plus serum (S: serum of non-pregnant rats; S: 13 days pregnant rat serum). Each of the three media was tested on a different set of 4 mammary glands (13 days pregnant rats). To the medium was added either insulin (50 µg/ml; I; ○—○) or insulin plus cortisol (20 µg/ml; I + F; ●—●). No of explants per group: 11-12.

after the decline stopped till 72 + 36 h. Differences between T8 + I and T8 + I + F were significant (at 1% level) from 72 + 1 1/8 h till 72 + 36 h. With S + I the alveolar development gradually decreased from 36 h till 72 + 72 h. With S + I + F it remained at the same level till 72 + 4½ h and increased thereafter slightly towards a maximum at 72 + 36 h. As compared with S + I the effect of F became significant (at 1% level) after 72 h. With S + I the alveolar development at 0 h was maintained during 72 + 72 h. With S + I + F it increased after 72 + 18 h and the difference with S + I was significant (at 1% level) at 72 + 36 h.

Mitotic activity.

Figure 26 shows that with T8 + I or T8 + I + F mitotic activity remained virtually absent. With S + I it showed a peak at 18-36 h, but declined thereafter. With S + I + F the changes followed approximately the same pattern. With S + I the mitotic activity in the acini increased sharply after 4½ h. The median degree reached grade 2 or more after 36 h. After the change of medium the percentage of explants with grade 2 dropped temporarily to 50 and 25% after 72 + 1 1/8 and 72 + 2½ h, but increased thereafter to 75, 67 and 92% at 72 + 4½, 72 + 9 and 72 + 18 h. At 72 + 36 and 72 + 72 h the percentage was 50 and 0% respectively. The mitotic activity in the ducts showed an increase only after 36 h with a maximum at 72 + 18 h followed by a decrease. With S + I + F the percentage of explants showing mitoses increased during the first 36 h, but at that time the degree of activity was already significantly less (at 5% level). Thereafter activity decreased and remained less (at 1% level) than with S + I during the ramining time. Mitoses in the ducts were seen irregularly and in no more than 27% of the explants.
Fig. 26
Fig. 26 Time-related changes of the percentage of explants showing one or more mitoses in the acini per section (mitotic activity). Explants of 13 days pregnant rat mammary glands were cultured for two periods of 3 days with change of medium after the first period. The medium was either Trowell's T8 (T8) or t8 plus serum (S0; non-pregnant rat serum; S13: 13 days pregnant rat serum). Each of the three media was tested on a different set of 4 mammary glands. To the media was added either insulin (50 µg/ml; I; □ — □) or insulin plus cortisol (20 µg/ml; I + F; ● — ●). Mitotic activity in the ductal epithelium is presented separately for the medium S13 I (Δ — Δ).

No of explants per group: 11–12.

Cytoplasmic opalescence.

Figure 27 shows that with T8 + I the cytoplasm remained opalescent till 18 h. Thereafter the cytoplasm lost the appearance rapidly. With T8 + I + F the disappearance occurred less rapidly. It showed wide fluctuations. With S0 + I the cytoplasmic opalescence disappeared after 18 h at the rat found for T8 + I. With S0 + I + F the opalescence remained present, but the median degree decreased from grade 2 at 18 h to grade 1 thereafter. With S13 + I and S13 + I + F the opalescence was maintained at the level present at 0 h. With S13 + I + F the opalescence was present in only 42% at 72 + 72 h.

Vacuolization.

With T8 + I and S0 + I vacuolization did not develop, with S13 + I irregularly and only in some explants. Figure 28 shows the results for the acini with T8 + I + F, S0 + I + F and S13 + I + F. With all three media the percentage increased after 18–36 h. Thereafter vacuolization was present in almost all explants, except for T8 + I + F in which case the percentage dropped to a low level at 72 + 72 h. With the latter medium the degree of vacuolization remained a low grade 1; with the other two media the degree increased at a fairly comparable rate till 72 + 36 h. Thereafter the degree increased only for S13 + I + F. T8 + I + F did not vacuolize the ducts. With S0 + I + F vacuolization of the duct developed after 72 h in 13, 33, 67 and 100% of the explants after 72 + 1 1/8, 72 + 9, 72 + 36 and 72 + 72 h respectively. The maximum degree was grade 2. The time course was similar to that with S13 + I + F. It increased from 0% at 72 + 1 1/8 h to 50, 75, 100 and 100% at 72 + 9, 72 + 18, 72 + 36 and 72 + 72 h, while the degree increased from median grade 1 at 72 + 18 h to grade 3 and
Fig. 27 Time-related changes of the percentage of explants showing cytoplasmic opalescence in the acini. The 13 days pregnant rat mammary gland explants were cultured during two periods of 3 days with change of medium after the first period. The medium was either Trowell's T8 (T8) or t8 plus non-pregnant rat serum (S₀). The two media were tested on a different set of 4 mammary glands (13 days pregnant rats). To the media was added either insulin (50 μg/ml; I; ■—■) or insulin plus cortisol (20 μg/ml; I+F; ⧫—⧫). No of explants per group: 11-12.
Fig. 28
Fig. 28 Time-related changes of the median grade of vacuolization (upper part) and of the percentage of explants showing vacuolization in the acini (lower part). The 13 days pregnant rat mammary gland explants were cultured during two periods of 3 days with change of medium after the first period. To the medium was added 50 μg/ml insulin (I) plus 20 μg/ml cortisol (F). The medium was either Trowell’s T8 (T8; • — •) or T8 plus serum (S;Δ—Δ; non-pregnant rat serum; S13;□—□; 13 days pregnant rat serum). No of explants per group: 11-12.

4 at 72 + 36 and 72 + 72 h respectively.

Secretion.

Figure 29 shows the results for the acini with T8 + I + F, S0 + I + F and S13 + I + F. The appearance of eosinophilic material in the lumen ran parallel with the development of vacuolization. The difference at 72 + 72 h between S0 + I + F and S13 + I + F was minimal. With T8 + I + F the presence of nuclear remnants in the eosinophilic material was prominent during the entire culture period.

5.1.5.13.2. Interaction with insulin

Experimental conditions

Medium: 50% S21 + 50% t8; glucose 4 mg/ml; hormones:
a: no hormones; b: + I (50 μg/ml); c: + F (20 μg/ml); d: + I + F; explants were fixed after 1, 2 and 3 days after the onset of culture; 6 mammary glands of 19 days pregnant rats.

Results

At 0 h the alveolar development was grade 6-7 and it remained approximately 6 with either S21 + I or S21 + I + F. With both S21 or S21 + F the alveolar development decreased sharply to grade 5, 4 and 3 on days 1, 2 and 3 respectively.

Figure 30 shows that at 0 h only some fragments showed the presence of mitoses. With S21 + I mitotic activity was still low after 1 day, although in some explants already 4 to 6 mitoses per section could be observed. Mitotic activity increased sharply after 2 and 3 days. A similar pattern was seen with S21 + I + F, but the activity on day 2 and 3 was significantly less than with S21 + I. With either S21 or S21 + F no mitoses were found except for a few after 1 day.
Fig. 29 As figure 28 for secretion.
Fig. 30 Time-related changes of the percentage of 19 days pregnant rat mammary gland explants showing mitotic activity, vacuolization and secretion in the acini. The median grade of the response is indicated by numbers. The explants were cultured for a period of 3 days without change of medium. The medium contained serum of 21 days pregnant rats. To the medium was added 50 μg/ml insulin (I; ⋅—⋅) or insulin plus 20 μg/ml cortisol (I + F; □—□). Differences between I and I + F statistically significant at the 5% or at the 1% level, are indicated by one, respectively two arrow heads. The explants were obtained from 6 mammary glands (starting material: day 0; n = 6). No of explants per group: n = 18.

At 0 h the acinar cells were swollen and opalescent (grade 3). With S$_{21}$ + I the median grade was 4, 3 and 3 on days 1, 2 and 3 respectively, while with S$_{21}$ + I + F it stayed 4. The difference with S$_{21}$ + I was significant (at 1% level) on day 2 and 3. With S$_{21}$ and S$_{21}$ + F the opalescence remained grade 3. Figure 30 shows that at 0 h a low degree of vacuolization (grade 1) was observed in 4 of the 6 fragments. With S$_{21}$ + I the incidence dropped after 1 day, while with S$_{21}$ + I + F vacuolization developed strongly after 2 days. With S$_{21}$ the percentage de-
creased to 50%, 39 and 20% on days 1, 2 and 3, with $S_{21} + F$ to 56, 39 and 28% respectively.

At 0 h secretion was not seen in the acinar lumina. Small intracellular eosinophilic inclusions were noticed, but only rarely. These inclusions became more prominent during the 3 days culture with $S_{21} + I$ or $S_{21} + I + F$. With $S_{21}$ or $S_{21} + F$ they were observed after 1 day, but they had disappeared thereafter. Figure 30 shows the appearance of eosinophilic material in the lumen. With $S_{21} + I$, 8 out of 18 explants showed some secretion on day 3, but it appeared only locally in the explants and the degree was low. With $S_{21} + I + F$ the secretion increased sharply from day 1 to 3. Differences with $S_{21} + I$ were statistically significant on day 2 and 3. With either $S_{21}$ or $S_{21} + F$ secretion did not develop.

Some pyknosis (grade 1) was notable in approximately 50% of the explants after 1 day with $S_{21} + I$ or $S_{21} + I + F$. On day 2 and 3 it disappeared. However, with $S_{21}$ pyknosis was prominent (grade 3) during the entire culture. With $S_{21} + F$ pyknosis increased to grade 3, 4 and 5 on day 1, 2 and 3 respectively.

5.1.5.14. Dose-response relationships for various corticosteroids

5.1.5.14.1. Cortisol

5.1.5.14.1.1. Effect on 13 days pregnant rat mammary glands

Experimental conditions

Medium: 50% virgin rat serum ($S_0$), serum of 13 days pregnant rats ($S_{13}$) or of 19 days pregnant rats ($S_{19}$) + 50% $t_8$; + I (50 µg/ml), glucose 2 mg/ml. Cortisol was added in concentrations of 0.03, 1.25, 5, 20 and 80 µg/ml. The final concentration of ethanol was 0.01 ml/ml in all media; 3 and 6 days culture with mammary explants of 13 days pregnant rats; 6 mammary glands for the 3 days culture, 6 other glands for the 6 days culture, the medium being changed after 3 days.
Results

\( S_0 \)

When the medium contained \( S_0 \) without F added, the median for the alveolar development was grade 4 after 3 days, and grade 2-3 after 6 days of culture. Mitotic activity was found only in a few explants, while secretion or vacuolization was almost absent (Fig. 31). With various concentrations of F added to \( S_0 \) the alveolar development fluctuated between grade 4 and 5 after 3 days of culture. Similar values were obtained after 6 days. F had no effect on the mitotic activity (Fig. 31). In the 3 days culture the presence of secretory material in the lumen increased steadily by the addition of 0.31 to 5 \( \mu \)g/ml F (Fig. 29). In the 6 days culture secretion was already present in almost all the explants at the lowest cortisol concentration. The median degree was grade 2. In the 3 days culture the presence of vacuolization in the acini depended on the concentration of F added (Fig. 31). At 80 \( \mu \)g/ml F 94% of the explants showed vacuolization of the acini. After 6 days the acini of all explants showed vacuolization at 0.31 \( \mu \)g/ml F. The median was grade 2. It did not increase at higher concentrations of F. Vacuolization of the ductal epithelium still showed some dose-dependency after 6 days (Fig. 31).

\( S_{13} \) or \( S_{19} \)

When \( S_{13} \) or \( S_{19} \) was added (without F) instead of \( S_0 \), the alveolar development was grade 6 after 3 days and 6 to 7 after 6 days of culture (difference with \( S_0 \) significant at 1% level). The mitotic activity (Fig. 31) was increased in the 3 days and slightly less in the 6 days culture (sign. at 1% level). A high percentage of the explants showed some degree of secretion and vacuolization (Fig. 31). When F was added in various concentrations the median for the alveolar development remained grade 6 to 7 after 3 days, and grade 7 to 7-8 after 6 days of culture. A concentration of 0.31 \( \mu \)g/ml F did not affect mitotic activity, but with 1.3 and 5 \( \mu \)g/ml mitotic activity was suppressed in the 3 days culture (Fig. 31). This suppression was more complete in

*Results are presented only for the concentrations of 0.31, 1.3 and 5 \( \mu \)g/ml cortisol (F). Results obtained with 20 and 80 \( \mu \)g/ml were similar to those obtained with 5 \( \mu \)g/ml. Exceptions will be mentioned separately.
the 6 days culture. The effect of F added to S_{13} or S_{19} on the secretion was not very marked in the 3 days culture. Secretion became notable in all explants (Fig. 31), but did not reach more than grade 2 with 5-20 μg/ml F. However, in the 6 days culture the effect of F on secretion became very distinct. Already at a concentration of 0.31 μg/ml F the median for secretion was grade 4. The effect of F was significantly increased (at 1% level) by the presence of either S_{13} or S_{19} instead of S_{0}. Some nuclear remnants were observed in the secretory material. When F was added to the media with S_{13} or
S_{19}, vacuolization of the acini was present in all explants after 3 (Fig. 31) and 6 days of culture. The median was grade 1 to 2 after 3 days, but grade 4 to 4-5 after 6 days of culture. The response was maximal with 0.31 μg/ml F. A similar effect was observed in the ducts (Fig. 31). The effect of F on vacuolization was significantly increased (at the 5% or lower level) by the presence of S_{13} or S_{19} in the medium instead of S_{0}.

5.1.5.14.1.2. Effect on virgin rat mammary glands

Experimental conditions
Similar to section 5.1.5.14.1.1. except for the use of the mammary glands of virgin rats instead of 13 days pregnant rats.

Results (see note section 5.1.5.14.1.1.)

S_{0}
Without the addition of F (0 μg/ml, in Table 23), the median degree of alveolar development was grade 3 in the 3 days experiment and grade 2 after 6 days of culture. After 3 days of culture relatively few explants showe mitoses (Table 23). Secretion and vacuolization were absent. When F was added, the increase of the alveolar development was not consistent. Mitotic activity remained low (table 23). After 3 days of culture secretion and vacuolization were still almost absent, but in the 6 days experiment secretion was observed. 5 μg/ml F was needed for a low degree of response (grade 1). The results for vacuolization followed a similar trend (grade 2 with 5 μg/ml).

S_{13} or S_{19}
Without F added with S_{13} in the medium the median degree of alveolar development was increased to grade 4 after 3 days and to grade 5 after 6 days of culture (differences with S_{0} significant at 5% level). With S_{19} the alveolar development was not increased in comparison with S_{0} except slightly after 6 days (grade 3, not significant). With S_{19} some necrosis developed. Table 23 shows that in comparison with S_{0} after 6 days an effect of S_{13} or S_{19} on the mitotic activity was present (differences between median grades significant at 5% level). Secretion and vacuolization were noted in only a few explants. When F was added,
Table 23 Mammary gland explants obtained from virgin rats. The effect of cortisol and aldosterone on the acini. The medium contained 50% serum from virgin rats (S₀) or 13 days (S₁₃) and 19 days pregnant rats (S₁₉). The explants were cultured during 3 or 6 days, each culture period with 6 rat mammary glands. Cortisol and aldosterone were tested each on different mammary glands. Medium supplemented with insulin.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cortisol</th>
<th>Aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>S₀</td>
<td>S₁₃</td>
</tr>
<tr>
<td>Culture period</td>
<td>3 6 3 6 3 6</td>
<td>3 6 3 6 3 6</td>
</tr>
<tr>
<td>Mitotic activity (μg/ml)</td>
<td>0 33 0 44 28 33</td>
<td>6 0 40 17 25 29</td>
</tr>
<tr>
<td>Secretion (%)</td>
<td>0 0 0 0 0 0</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>Vacuolization (%)</td>
<td>0 0 0 0 0 0</td>
<td>0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

No of explants: 15-18 per group.

%: percentage of explants showing response indicated.

the alveolar development did not increase. A suppression of the mitotic activity was observed especially in the 6 days experiment, but it was not marked (Table 23). Secretion was still poorly developed after 3 days, but after 6 days with S₁₉ the effect obtained with 1.2 and 5 μg/ml F was more obvious (grade 1). The effectivity of S₁₉ was less. The effect of S₁₃ and S₁₉ stood out more clearly as regards the vacuolization. It was not prominent after 3 days of culture (Table 23), but vacuolization developed in the majority of the explants after 6 days. 0.31 μg/ml F with S₁₃ or S₁₉ produced vacuolization (grade 1 and 2 respectively) in contrast to none with a similar concentration of S₀ (Table 23). Grade 3 of vacuolization was obtained with 1.3 and 5 μg/ml F plus S₁₃ or S₁₉.
5.1.5.14.2. Aldosterone

5.1.5.14.2.1. Effect on 13 days pregnant rat mammary glands

Experimental conditions

Similar to section 5.1.5.14.1.1. except for the addition of aldosterone instead of cortisol; 5 mammary glands for the 3 days and the 6 days culture.

Results (see note section 5.1.5.14.1.1.)

$S_0$

With $S_0$ in the medium without the addition of aldosterone (A) the median grade for alveolar development was grade 4 in both the 3 and the 6 days culture. Mitotic activity was low, and secretion and vacuolization were absent (Fig. 32). When A was added the alveolar development in both cultures increased only slightly up to grade 5 at 5 μg/ml. Mitotic activity remained present, but in a low percentage of the explants (Fig. 32). Secretion and vacuolization did not develop to any great extent in the 3 days experiment. After 6 days some secretion had appeared with 1.3 μg/ml A (grade 1, Fig. 32). Vacuolization of the acini was present only with 5 μg/ml A or more (93% of the explants, grade 1). Vacuolization of the ductal epithelium remained almost absent (Fig. 32).

$S_{13}$ or $S_{19}$

Without A added, $S_{13}$ and $S_{19}$ produced - in comparison with $S_0$ - an increase of the alveolar development (grade 5 to 6) and mitotic activity (Fig. 32). In this experiment secretion was present only in a few explants after 3 and 6 days. Vacuolization was almost absent.

The addition of A to $S_{13}$ or $S_{19}$ resulted in a slight increase of the alveolar development (grade 6 to 7) without a distinct dose-dependence. Results for the 3 and 6 days culture were identical. A suppression of the mitotic activity by A was not distinct in the 3 days experiment (Fig. 32). In the 6 days culture the suppression seemed to be dose-dependent but 1.3 - 5 μg/ml A was necessary for a complete suppression. The addition of A to either $S_{13}$ or $S_{19}$ did not result in a high secretory response.
after 3 days. However, after 6 days of culture the effect was much more marked. 0.31 μg/ml A produced some secretion (grade 1) with S_{13} or S_{19}, a higher degree of secretion was present in all explants, reaching grade 3 at 1.3 μg/ml A and grade 4 at 5 μg/ml A (difference with S_{0} significant at 5% or lower level). In combination with S_{13} or S_{19}, A induced vacuolization in slightly more than half of the explants (Fig. 32) in the 3 days experiment. The response obtained with 0.31 μg/ml A was equal to that at higher concentrations. After 6 days 0.31 μg/ml A produced vacuolization of the acini in 66% of the explants (grade 1). With 1.3 and 5 μg/ml the response increased to 100% and grade 3 to 4. 5 μg/ml A was needed for a high percentage of vacuolization in the ductal epithelium (Fig. 32). Secretion and vacuolization...
tended to decrease with 20 and 80 μg/ml A.

5.1.5.14.2.2. Effect on virgin rat mammary glands

Experimental conditions
Similar to section 5.1.5.14.2.1. except for the addition of aldosterone instead of cortisol.

Results (see note section 5.1.5.14.1.1.)

\( S_0 \)
Without A the median grades for the alveolar development were similar to those of section 5.1.5.14.1.2. Mitotic activity, secretion and vacuolization were virtually absent.

The addition of A had no effect on the alveolar development or mitotic activity. Even after 6 days of culture, secretion and vacuolization had not developed to any great extent (Table 23).

\( S_{13} \) or \( S_{19} \)
Without A added, neither \( S_{13} \) nor \( S_{19} \) in this experiment did increase the alveolar development after 3 days in comparison with \( S_0 \), but grade 3 was maintained after 6 days of culture. Mitotic activity was present, but in less than 50% of the explants (Table 23). Secretion and vacuolization were absent. When A was added, the alveolar development was increased only distinctly with \( S_{13} \) after 6 days of culture. Suppression of the mitotic activity by A was not clear-cut. Secretion and vacuolization were not well developed after 3 days, but they were prominent after 6 days of culture (difference with \( S_0 \) significant at 5% level or less). 0.31 μg/ml A had not much effect. 1.3 μg/ml A or more was necessary for a maximum response (Table 23).

5.1.5.14.3. Cortisol, aldosterone, corticosterone and deoxy-corticosterone

5.1.5.14.3.1. Effects in a synthetic medium

Experimental conditions
Medium: 100% T8 (T8); glucose 4 mg/ml; hormones: I (50 μg/ml + rPrl (5 μg/ml; 25 IU/mg) + 0, 0.16, 0.31, 0.63, 1.25, 2.5 and
5 µg/ml cortisol (F), aldosterone (A), corticosterone (B) or deoxycorticosterone (D); each medium contained 0.01 ml alcohol/ml; with medium T8 6 days of culture with 4 mammary glands (MP

Results

The condition of the explants was poor after 6 days. With one gland the results were much better than with the other three.

Table 24 shows that on addition of the corticosteroids almost invariably a large area of the at random selected section was necrotic or otherwise contained mainly pyknotic nuclei. This reaction was not seen in the control group. The degree of necrosis did not correlate to the degree of secretory response; necrosis was consistently greatest with D, which produced a poor secretory response. The alveolar development was poor (grade 3) and mitotic activity was absent. Vacuolization (see Table 24) was best develop-

Table 24 Effect of various concentrations of 4 corticosteroids on necrosis, secretion and vacuolization in 13 days pregnant rat mammary gland explants cultured in 100% Trowell's T8 during 6 days (with change of medium after 3 days). The medium contained 5 µg rPrl/ml.

<table>
<thead>
<tr>
<th>Dosis µg/ml</th>
<th>Necrosis (% section area)¹</th>
<th>Secretion (%)²</th>
<th>Vacuolization (grade)³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>0.16</td>
<td>40</td>
<td>15</td>
<td>55</td>
</tr>
<tr>
<td>0.32</td>
<td>45</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>0.65</td>
<td>30</td>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>1.25</td>
<td>40</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>2.5</td>
<td>30</td>
<td>70</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>35</td>
<td>45</td>
</tr>
</tbody>
</table>

F: cortisol.
A: aldosterone.
B: corticosterone.
D: deoxycorticosterone.

¹: median %.
²: % of explants showing secretion.
³: median grade of vacuolization.

No of explants per group: 11-12.
ed with F, least with D. Compared to 0 μg/ml, each one of the 4 steroids produced a response, which was, however, poorest with D. F, A and B showed no dose-response relationships, D to some extent.

Approximately two-thirds of the central area of the explants (0 μg/ml steroid, see Table 24) showed some eosinophilic material in the lumen (grade 1). The material stained light-red and was flocculent. Nuclear remnants were not prominent. The degree of secretion with either F, A or B remained generally low (grade 1). With D the response was poor. Dose-response relationships were not obtained.

5.1.5.14.3.2. Effect of serum

Experimental conditions
Similar to 5.1.5.14.3.1., but instead of 100% T8, 50% SP₀ + 50% t8 (S₀) was used. 3 Days culture with 4 mammary glands (MP₁). Results

With S₀ + I + rPrl the alveolar development was grade 5. When the steroids had been added, the alveolar development remained 5 to 6 without a distinct dose-response relationship. Figure 33 shows that compared to 0 μg steroid/ml, the steroids decreased the mitotic activity, significantly at the 5% level with 5 μg steroid/ml. The effect of the four steroids was comparable.

The steroids increased the cytoplasmic opalescence of the cells. The effect was significant (at 5% or less) at 0.16 μg F, A or D/ml and at 0.32 μg B/ml. As regards the number of explants reaching grade 3 of opalescence (see Fig. 33), F was most potent and D the least. The response towards B, - the main naturally occurring glucocorticosteroid in the rat - increased steeply in the range of 0.32 to 1.25 μg/ml. A similar trend was observed for vacuolization and secretion, B being slightly less active than A as regards vacuolization.
Fig. 33 Effect of various concentrations of 4 corticosteroids on mitotic activity (median grade), cytoplasmic opalescence (% of explants with grade 3 or more), vacuolization and secretion (% of explants showing response) in 13 days pregnant rat mammary explants cultured in a medium containing virgin rat serum, insulin and rPrl, for 3 days. F: cortisol (■ — ■); A: aldosterone (Δ — Δ); B: corticosterone (● — ●); D: deoxycorticosterone (□ — □). No of explants per group 11-12.

5.1.5.15. Progesterone

5.1.5.15.1. Interaction with oestradiol

Experimental conditions

Medium: 50% S₀ + 50% t8; glucose: 6 mg/ml; + I; P in concentrations of 0, 0.25, 2.5 and 25 μg/ml; O: 0, 0.025, 0.25 and 2.5
µg/ml; each concentration of progesterone combined with each concentration of oestradiol. 3 And 6 days culture with change of the medium at day 3. Two different sets of 5 mammary glands of 13 days pregnant rats were used.

Results.

When P alone was added to a medium containing S₀ + I, the explants of 13 days pregnant rat mammary glands responded with a dose-dependent increase of mitotic activity both in the 3 and 6 days culture, as shown in figure 34. The degree of alveolar development was also increased.

The addition of O alone to a medium of S₀ + I had little effect. It did not influence the effect of P, except for some suppression of the mitotic activity with 2.5 µg/ml O especially in the 6 days culture (Fig. 34). No indications of a secretory activity were seen with P and/or O.

![Fig. 34](image_url) The effect of progesterone and oestradiol (indicated by numbers) added to a medium containing 50% virgin rat serum, on the mitotic activity (% of explants) of mammary gland explants (MP₃). 3 And 6 days culture (left and right respectively). Insulin supplementation (50 µg/ml). The different concentrations of oestradiol are indicated in the figure.
5.1.5.15.2. Interaction with insulin and testosterone

Experimental conditions

Medium: 50% S₀ + 50% t8; glucose: 2 mg/ml. Hormonal combinations: 1) no hormones, 2) + I (50 µg/ml), 3) + P (1 µg/ml), 4) + I + P, 5) + I + T (1 µg/ml), 6) + I + P + T. 3 Days culture; 2 mammary glands of 13 days pregnant rats.

Results

When mammary gland explants of a 13 days pregnant rat were cultured for 3 days in a medium containing 50% S₀ but no I (group 1, Table 25), the alveolar development was low and mitotic activity almost absent. When I alone was added (group 2), the alveolar development increased significantly and mitotic activity was noted in almost all explants. When P alone was added (group 3), the alveolar development was significantly higher than in group 1, but mitotic activity remained low. With I + P added (group 4), the grade of alveolar development and mitotic activity was higher than in group 1, 2 or 3.

When the medium contained 50% S₀ and I (group 2, Table 25),

| Group | Hormones added | Alveolar Development (grade) || Mitotic Activity (grade) |
|-------|----------------|-------------------------------|--------------------------|
| 1     | none           | 2**                           | 17                       | 0**                      |
| 2     | I              | 3                             | 83                       | 1                        |
| 3     | P              | 3**3                         | 25                       | 0                        |
| 4     | I+P            | 4**                          | 92                       | 2-3**                    |
| 5     | I+T            | 4**                          | 92                       | 2**                      |
| 6     | I+P+T          | 5**                          | 83                       | 2**                      |

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing response indicated.
3: Difference between group 3 and 1.

** Significant differences between the result obtained and the result of group 2 (1% level).

No of explants: 12.
the addition of either P (group 4) or T (group 5) resulted in a significant increase of the grade of the alveolar development and of the mitotic activity. When both steroids were added (group 6) no changes were produced which were statistically significant from those obtained with either hormone separately (group 4 and 5).

5.2. Hormonal responses of the lactating rat mammary gland

5.2.1. Effect at various stages

Experimental conditions

Mammary glands of 21 days pregnant (MP\(_{21}\)), 3 - 7 - 10 - 16 - and 21 days lactating (ML\(_3\), ML\(_7\), ML\(_{10}\), ML\(_{16}\), ML\(_{21}\), resp.) and 10 days non-nursing rats (after 21 days lactation) (MN). Medium: 50% S\(_0\) + 50% T8; approximately 2 mg glucose/ml. The following hormones were added: I (50 \(\mu\)g/ml), Prl (ovine; 10 \(\mu\)g/ml); P (0.5 \(\mu\)g/ml); O (0.05 \(\mu\)g/ml) and F (20 \(\mu\)g/ml) in the following combinations a) I, b) I + oPrl, c) I + P + O, d) I + F, e) I + F + oPrl and f) I + F + P + O. Duration of culture 3, 6, 9 and 12 days (the medium being changed at 3, 6 and 9 days). For each stage of the mammary gland 4 different glands were used. The results for the 3 days lactating rats are reported in detail, for the other glands only alveolar size, vacuolization and secretion are reported.

Results

MP\(_{21}\), ML and MN.

On day 21 of pregnancy the acini of the mammary gland are extended (grade 6-7). The cells are opalescent (grade 3) and some vacuolization has developed. The lumina are still rather small and contain some eosinophilic material. Mitoses are not observed. During lactation the lumina are small. In some areas they contain little material and the cells are cubic or cylindric. In other areas the lumina are large and engorged with material and the cells are flattened, while vacuolization is not prominent (Fig. 35a). Staining with Sudan Black B shows mainly multiple fine black intracellular droplets. The cytoplasm is dense, is baso-
Fig. 35 Morphological changes of lactating rat mammary glands in organ culture (I).

a Aspect of the mammary gland of a 3 days lactating rat. The alveolar lumina are distended to a variable degree. Vacuolization of the epithelium is absent. Connective tissue and adipose tissue are not prominent. Scale: 100 µ.

b Aspect of the mammary gland of 21 days lactation followed by 10 days weaning. The alveoli are poorly developed. Connective tissue and adipose tissue are prominent. Scale: 100 µ.

c Mammary gland explant from a 3 days lactating rat after 9 days culture in a medium containing 50% virgin rat serum, insulin (50 µg/ml) and cortisol (20 µg/ml). The tissue has lost the original organization (compare with a). Some alveolar lumina are distended (size grade 6) with secretory material (secretion grade 3). Vacuolization is prominent (grade 4). Some connective tissue and adipose tissue is present. Some outgrowth is present at the base of the explant (grade 1). Necrosis at the top of the explant is indicated by an arrow. Scale: 100 µ.

d Mammary gland explant from a 3 days lactating rat after 12 days culture in a medium containing 50% virgin rat serum and insulin (50 µg/ml). Alveolar development is poor (grade 2) and comparable with that of b. Secretory activity is not notable. Connective tissue and adipose tissue are prominent. Connective tissue outgrowth at the base of the explant is indicated with arrows. Scale: 100 µ.
philic with haematoxylin and is strongly positive with pyronin. Staining with Luxol Fast Blue after chromation shows a strong blue colour in the cytoplasm. After 10 days of nursing - following 21 days of lactation - the alveolar size is reduced to grade 2-3 (Fig. 35b), mitoses, vacuolization and secretion are absent.

ML₃: 3, 6, 9 and 13 days of culture.

Fig. 36 shows that with I (curve a) the size of the alveoli decreases regularly with the duration of the culture period. The
aspect after 12 days culture is shown in Fig. 35d. Some vacuolization was present after 3 days, but later it had disappeared. Eosinophilic material (i.e. secretion) containing nuclear remnants was prominent in the alveolar lumina after 3 days, but it decreased thereafter and had disappeared after 9 and 12 days (see also Fig. 35d). Approximately 50% of the total section area was estimated to be necrotic after 3 days, but necrosis decreased regularly until only a trace was left after 12 days (Fig. 36). The degree of pyknosis followed a similar pattern. Outgrowth developed predominantly at the basis of the explants (Fig. 35d, arrows). It was still absent after 3 days, but increased rapidly thereafter to reach a maximum after 6 to 9 days. When instead of I either I + P + O or I + oPrl was added to the medium, the presence of P + O or oPrl had no definite effect (see Fig. 36, curves a, b and c).

When I + F was added (curve d in Fig. 36), the alveolar size seemed not to decrease till after 9 days (see also Fig. 35c). Vacuolization already present after 3 days, developed further to a maximum at 9 days (Fig. 36). Secretion was maximal at 3 days. The secretion contained nuclear remnants. From day 3 to 6 secretion seemed to decrease but thereafter it remained constant (Fig. 36 curve d). The necrosis disappeared with I + F less completely than with I. The results for pyknosis were similar for all treatments (Fig. 36 curve d). F suppressed the outgrowth (Figs 35c and 36 curve d).

When I + F + P + O or I + F + oPrl were added instead of I + F, the presence of P + O or oPrl had no definite effect (Fig. 36, curves d, e and f).

MP, ML and MN: 3, 6, 9 and 12 days culture.

In figure 37 only the results for the combinations I and I + F and for the parameters alveolar size, vacuolization and secretion are compared. The addition of P + O or oPrl to media containing either I or I + F had no significant effect. The results for ML_{16} and ML_{21} were essentially similar to those for ML_{7}.

With I the alveolar size decreased to grade 1 or 2 after 12 days of culture irrespective of the type of mammary gland.
Fig. 37 Effect of 3, 6, 9 and 12 days culture on insulin (I, 50 μg/ml) and cortisol (F, 20 μg/ml) induced changes on mammary gland explants obtained from pregnant (21 days: MP_{21}) rats, lactating (3-7-16-21 days: ML_{3-21}) rats and from rats lactating for 10 days followed by 10 days of non-nursing (MN-S). Grade: median value of the semi-quantitative grading of the response indicated. No of explants per group: 10-12.

cultured (Fig. 37). Some vacuolization was present after 3 days in ML_{3}, ML_{7} and ML_{16}. It showed a tendency to disappear after a 6 days culture except when MP_{21} was used in which case some vacuoles remained present. Secretion - with nuclear remnants
- was observed in the explants of ML₃₋₂₁, but only after 3 days. An exception was ML₁₆, in the explants of which the necrosis did not disappear with time and eosinophilic material was left in the lumina. However, the nuclear remnants in the secretion disappeared.

With I + F the alveolar size remained at a high level (Fig. 37). Some vacuolization was present after 3 days except with MN. The difference with group I was not clear-cut in the 3 days culture, but with I + F the vacuolization became more prominent after 6 and 9 days, in contrast to the media containing only I. Except for MN secretion was present after 3 days and it was maximal at that time for ML₃, ML₇ and ML₁₆. It remained present for 12 days. Nuclear remnants were prominent after 3 days. With MN the secretion was apparent only after 6 days.

Necrosis was present after 3 days with ML₃₋₂₁. It decreased after 6 and 9 days, but incompletely in the case of ML₇ and ML₁₆. Especially with MP₂₁ and MN necrosis developed after 12 days. F diminished the outgrowth in all groups.

5.2.2. Effect of prolactin and cortisol - various parameters

Experimental conditions

Medium: 50% S₀ + 50% t8; 4 mg glucose/ml. Hormones: I (100 µg/ml), rPrl (25 IU/mg; 10 µg/ml) and F (20 µg/ml) in the following combinations: a) I, b) I + F, c) I + rPrl and d) I + rPrl + F. Duration of culture: 1, 2 and 3 days. One mammary gland of a 6 days lactating rat was used. Glucose, lactate, amino acids and free glycerol concentrations in the media were determined. Similar hormone combinations were tested in a 3 days culture on two other glands and the explants stained with either methylgreen/pyronin or Luxol Fast Blue and Sudan Black B. Similarily a number of explants were prepared in a 3 and 6 days culture for electron microscopy.

Results

With I the alveolar distension found after one day (grade 7-8, Table 26) was significantly reduced after 3 days (Fig. 38). Mitotic activity was not observed after one day. It showed a
Fig. 38 Morphological changes of lactating rat mammary gland in organ culture (II).

a Mammary gland explant obtained from a 5 days lactating rat after 3 days culture in a medium containing 50% virgin rat serum with insulin supplementation (100 μg/ml) (S₀ + I). The alveolar size is still high (size grade 6) and the lumen is filled with secretory material (secretion grade 1), which contains numerous vacuoles and some nuclear remnants. Vacuolization of the epithelium is sparse (grade 1). Scale: 100 μ.

b As a The secretory material in the lumen is vacuolized and contains nuclear remnants. The cytoplasm of the alveolar cells is slightly opalescent. A few intracellular vacuoles are present. Scale: 25 μ.

c As a but cultured in S₀ + I + rPrl (10 μg/ml). In comparison with a the alveoli are more distended (size grade 7). The intraluminal material shows different qualities: in certain areas, (especially in the upper part of the explants), the secretion contains no nuclear remnants except for some isolated small spots of light-red staining cellular debris (see also d). This secretion stains slightly violet and was found in apparently viable parts of the explants. Another type of eosinophilic material is often seen in the lower and more central parts of the explants. This intraluminal material stains light-red and nuclear remnants are abundant. Some intracellular vacuoles are present (grade 2). Scale: 100 μ.

d As c The cytoplasm of the cells is more compact than in b. Intracellular vacuoles are present but some contain globules of the same material as present in the lumen (arrows). These vacuoles may represent resorption vacuoles and not lipid droplets which have left the vacuoles after fixation and embedding. Scale: 25 μ.

e As a but cultured in S₀ + I + F (20 μg/ml). In comparison with a the alveoli are more distended (size grade 7) with mainly compact secretory material (secretion grade 3-4). Vacuolization is prominent (grade 3). Scale: 100 μ.

f As e The cytoplasm is compact and contains large vacuoles. Mitoses are indicated by arrows. Scale: 25 μ.
Table 26  Effect of rat prolactin (rPrl, 10 µg/ml) and cortisol (F, 20 µg/ml) on mammary gland explants obtained from a 6 days lactating rat, cultured during 1, 2 and 3 days.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Day of Culture</th>
<th>Alveolar development (grade)¹</th>
<th>Mitotic activity (%)²</th>
<th>Vacuolization (grade)¹</th>
<th>Secretion (grade)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>7-8</td>
<td>0 0</td>
<td>3 2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>92 2³</td>
<td>2³ 2</td>
<td>7**</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>63</td>
<td>39 0¹</td>
<td>3 1</td>
<td>1</td>
</tr>
<tr>
<td>I + rPrl</td>
<td>1</td>
<td>7</td>
<td>4 0</td>
<td>3 3</td>
<td>1**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>100 1,³</td>
<td>2** 3</td>
<td>2**</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7**</td>
<td>93 1*</td>
<td>1 2*</td>
<td>2**</td>
</tr>
<tr>
<td>I + F</td>
<td>1</td>
<td>8</td>
<td>4 0</td>
<td>3 2</td>
<td>3**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>92 2</td>
<td>2** 3</td>
<td>3**</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7**</td>
<td>78 1</td>
<td>3** 3</td>
<td>3**</td>
</tr>
<tr>
<td>I + rPrl + F</td>
<td>1</td>
<td>7</td>
<td>0 0</td>
<td>2 2</td>
<td>2**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>96 3**</td>
<td>2** 3**</td>
<td>2**</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7**</td>
<td>83 1*</td>
<td>3** 4**</td>
<td>4**</td>
</tr>
</tbody>
</table>

1: Median value of semiquantitative grading of response indicated.
2: % of explants showing one or more mitoses per section.
3: Difference with the corresponding result for group I, 1 day culture significant at the 5% level.
4: Difference with the corresponding result for group I, 2 days culture significant at the 5% level.

* Difference with the corresponding result for group I on same day of culture, significant at the 5% level.
** Idem at the 1% level.
No of explants per group: 22-24.

peak on the second day with a significant fall on day 3 (Table 26). The vacuolization of the alveolar epithelium, which was prominent on day 1 (grade 3, Table 26) was decreased on day 2 and 3 (Fig. 38). On day 3 the cytoplasm had become more opalescent (grade 3). The eosinophilic staining of the cytoplasm had become weak. The alveolar lumen was distended with eosinophilic material (secretion, Table 26). This material showed different qualities (Fig. 38b and d). The homogeneous type of secretion tended to disappear on day 2 and 3. Material containing nuclear remnants remained present, but the nuclear remnants became less prominent on day 3. This material was found in necrotic parts of the explants, but also in apparently viable
alveoli. In approximately half of the explants some necrotic area was present in the centre of the explants. In these areas the epithelium of the alveoli showed rows of pyknotic nuclei with markedly eosinophilic cytoplasm. On day 2 and 3 the pyknosis of the nuclei tended to fade. Pyknosis was also found scattered through more viable parts of the explants. It was present in 94% of the explants after 1 day and had decreased to 39% on day 3.

With I + rPrl the following differences with I alone were noted (Table 26): the alveolar size did not decrease (Fig. 36c); the mitotic activity which showed a peak on day 2, was still significantly increased on day 3; vacuolization decreased at a slower rate. The cytoplasm became less opalescent (difference significant at 5% level; compare Fig. 38d with 36b). The secretion of both types was maintained on day 2 and 3 (Fig. 38c) with a significant difference on day 3 (Table 26). Results for necrosis and pyknosis were similar.

With I + F differences with I alone were (Table 26): the alveolar size did not decrease (Fig. 38e, f) with a significant difference on day 3, vacuolization did not decrease. The cytoplasm did not turn opalescent (grade 1, difference significant at 1% level), but remained strongly basophilic. The homogeneous type of secretion tended to increase. The results for mitotic activity, necrosis and pyknosis were similar to those with I alone.

With I + rPrl + F the differences with I alone were similar to those obtained with I + F, except for the mitotic activity on day 2 which was significantly higher (at the 5% level) than in group I. Secretion was significantly increased on day 2 in comparison with group I alone (Table 26).

Staining for RNA and fat; electron microscopy. The staining of RNA was intense in the non-cultured control tissue. In cultured explants staining with pyronin showed a positive reaction of the cytoplasm in the alveoli which appeared to be viable and which contained homogeneous secretion. The cytoplasm did not stain in necrotic areas. In viable parts staining with Luxol Fast Blue produced a blue colour of the cytoplasm.
Fig. 39 Detail of a mammary gland explant obtained from a 6 days lactating rat and cultured for 6 days in 50% virgin rat serum + insulin (50 µg/ml). The lumen is small. Some cells (A) show a de-differentiated aspect: RER is sparse and not arranged in layers, and protein granules or fat droplets are absent. Other cells (B) still show the presence of an apical Golgi-apparatus (G) and protein containing vesicles (arrows) and some small fat droplets (Li); Lu: lumen. Scale: 1 µ.

In other parts masses of similar blue stained material were deposited in the lumina. Round vacuoles present in the cytoplasm were not stained. With Sudan Black B, however, they were stained black.

Electron microscopy of the cultured explants showed an extremely variable ultrastructure, but in some alveoli the cells were uniformly dedifferentiated after a 6 days culture with the medium $S_0 + I$ (Fig. 39). The lumen was small and empty. Some small secretory granules were still present in certain cells. After 3 days of culture with $I + F$ at least some
Fig. 40 Detail of mammary gland explant obtained from a 6 days lactating rat and cultured for 3 days in 50% virgin rat serum + cortisol (20 μg/ml) + insulin (50 μg/ml). The lumen (Lu) is filled with protein granules. In the cells the Golgi apparatus (G) is still well developed and is situated apically. Protein containing granules are seen (arrows). The RER is arranged in layers. The fat globule (Li) shows "degeneration". Scale: 1 μ.

cells had maintained a secretory appearance (Fig. 40) with some layered RER and secretory granules present. The fat droplets showed "degeneration" of their contents.

Changes of the chemical composition of the medium.

With I the free glycerol concentration in the medium had increased on day 1 only to some degree (Fig. 41, curve 1), but later it rose rapidly. The amino acid concentrations fell during the first day, but thereafter it increased rapidly. The glucose concentration which decreased on day 1, had remained uncharged on the second day, and was further decreased on day 3. The lactate concentration increased during the first day only to some extent, but later the lactate production was accelerated.

With I + rPrl, no significant difference with group I was found. With I + F the increase of the free glycerol was depressed
on both day 2 and 3 (Fig. 41, curve 2). Similarly the amino acid concentrations were lower on day 1, 2 and 3. The glucose concentration was not affected, but the lactate concentration was lower on day 2. With I + rPrl + F the effect was almost similar to that of I + F. Significant changes in the glucose concentration were observed on day 2 and 3.
concentration occurred on day 1 and 2, but the pattern was inconsistent (Fig. 41).

5.2.3. Effect of insulin and rat prolactin on $[^3H]$-thymidine incorporation

Experimental conditions

Medium: 50% $S_0$ + 50% t8; 4 mg glucose/ml. Hormones: a) I (100 μg/ml) and b) I (100 μg/ml) + rPrl (25 IU/mg; 5 μg/ml).

$[^3H]$- Thymidine was added after 3½, 24, 48 and 72 h of culture 3½ h before the end of the experiment. One mammary gland of a 6 days lactating rat was used.

Results

Thymidine incorporation on day 0, 1, 2 and 3 respectively. The labelling indices were determined in a culture either with I or with I + rPrl. The results were for I: 0.8 ± 0.24% ($\bar{x}$ ± SEM, n = 6), 4.9 ± 1.10 (n = 6), 6.7 ± 1.54 (n = 6) and 3.8 ± 0.46 (n = 12) for day 0, 1, 2 and 3 respectively. The corresponding results for I + rPrl were 0.8 ± 0.22 (n = 6), 5.5 ± 0.99 (n = 6), 11.2 ± 1.59 (n = 6) and 6.2 ± 0.72 (n = 12). The difference between I and I + rPrl was significant at 5% level on day 3.

5.2.4. Effect of glucose concentration

5.2.4.1. Various parameters

Experimental conditions

Medium: 50% $S_0$ + 50% t8, which contained 0, 1, 2, 4 and 8 mg glucose/ml. Hormones: I (50 μg/ml), rPrl (25 IU/mg; 10 μg/ml) and F (20 μg/ml) in the combinations a) I, b) I + rPrl and c) I + F, each of these 3 combinations tested with each of the five different glucose concentrations. 3 Days culture with one mammary gland of a 6 days lactating rat. Lactate and glucose concentrations after culture were determined for the two highest glucose concentrations.
Table 27  Effect of glucose concentration on the changes produced by insulin (I, 50 μg/ml), rat prolactin (10 μg/ml) and cortisol (F, 20 μg/ml) in mammary gland explants obtained from a 6 days lactating rat.

<table>
<thead>
<tr>
<th>Glucose (mg/ml)</th>
<th>Alveolar development (grade)¹</th>
<th>Mitotic activity (g)²</th>
<th>Vacuolization (grade)³</th>
<th>Secretion (grade)³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I+Prl</td>
<td>I+F</td>
<td>I+Prl</td>
<td>I+F</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5-6</td>
<td>50</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>6</td>
<td>6**</td>
<td>63</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>5-6</td>
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<tr>
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<td>63</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

¹: Median value of semiquantitative grading of response indicated.
²: % of explants showing one or more mitoses per section.
³: Difference with the corresponding result for group with 0 mg glucose/ml significant at the 5% level or less.
* Difference with the corresponding result of group I significant at the 5% level.
** Idem at the 1% level.
No of explants per group: 8.

Results

With I an increase of the glucose concentration from approximately 0 to 4 mg/ml medium had no effect on the alveolar size (Table 27). The mitotic activity was decreased at 4 mg/ml. Both vacuolization and secretion increased significantly. With I + rPrl the effect of glucose was in general similar to I alone, except for secretion which did not increase (Table 27). With I + F the increase of the glucose concentration, stimulated vacuolization while secretion remained essentially unaltered. A significant increase in alveolar size, vacuolization and secretion was only observed at 0.5, 1 and 2 mg/ml respectively (Table 27). Nuclear remnants in the secretion were more prominent (significant at the 1% level). Lipid material was less regularly deposited in the alveoli after 3 days of culture than in the glands of rats, 12 or 20 h after weaning (compare Fig. 42a with Fig. 42b). Lipids were distinctly present in the presumptive cellular debris accumulated in some alveolar lumina. The lipids
made up approximately 20% of the section surface. A correlation between the glucose concentration and amount of lipid material, expressed as percentage of surface, could not be demonstrated for either group I, I + rPrl or I + F. Differences between the groups were not consistent.

When the glucose and lactate concentrations were determined for the cultures with approximately 2 and 4 mg glucose/ml medium, the glucose concentration had decreased for group I, I + rPrl and I + F from 2.2 mg/ml to 1.4 ± 0.09, 1.5 ± 0.08 and 1.4 ± 0.19 mg/ml (± SEM, n = 3) respectively and only slightly more from 3.9 mg/ml to 2.9 ± 0.20, 3.0 ± 0.08 and 3.0 ± 0.15 mg/ml (n = 3) in the group with 4 mg glucose/ml. The lactate concentration increased from 0.18 mg/ml medium to 0.37 ± 0.015, 0.37 ± 0.022 and 0.30 ± 0.015 with 2.2 mg/ml glucose. Lactate concentration was increased to 0.52 ± 0.027, 0.56 ± 0.027 and 0.57 ± 0.049 when the medium contained 3.9 mg/ml glucose. A significant (at 5% level) effect of F on the lactate concentration was present only in the culture with the lower glucose level of 2 mg/ml.
5.2.4.2. [$^{14}$C] Glucose uptake

Experimental conditions

Medium: 50% S$_{13}$ + 50% t8; 4 mg glucose/ml; hormones: a) I (50 µg/ml) and b) I (50 µg/ml) + F (20 µg/ml). A culture of a 13 days pregnant rat mammary gland served as a control; 3 days culture with one mammary gland of a 6 days lactating rat. [$^{14}$C] glucose was added to the medium to a final concentration of 1 µCi/ml.

Results

With 1 µCi/ml glucose added, the activity in ML$_6$ explants after 3 days was 17.4 ± 2.85 nCi/mg wet weight (x ± SEM; n = 5) for I, 12.8 ± 1.83 (n = 5) for I + F. These results were compared with those for MP$_{13}$, which were 6.0 ± 0.45 nCi/mg (n = 5) for I, and 6.5 ± 0.82 (n = 5) for I + F.

In two samples of ML$_6$ explants cultured with I, the percentage of [$^{14}$C] incorporated into the lipid fraction was 80.5% in both samples; with I + F the corresponding values were 83% and 84%.

5.2.5. Effect of lactating and pregnant rat serum

5.2.5.1. Lactating rat serum

Experimental conditions

Medium: 50% S$_0$ or SL$_{10}$ + 50% t8; glucose either 2 or 4 mg/ml. Hormones: I (50 µg/ml), rPrl (3-5 IU/mg; 25 µg/ml) and F (20 µg/ml) in the combination a) I, b) I + F, c) I + rPrl, d) I + rPrl + F, each combination tested with two different sera and two different concentrations. 3 Days culture with 4 mammary glands of 10 days lactating rats.

Results

With S$_0$ + I and 2 mg glucose/ml, the alveolar size scored grade 5 after 3 days (Table 28). Mitotic activity was present in 50% of the explants. Vacuolization was not prominent, but the alveoli were distended with eosinophilic material. When the glucose concentration was increased to 4 mg/ml, the most marked
Table 28  Effect of virgin rat serum (S₀) and 10 days lactating rat serum (SL₁₀), at two glucose concentrations on the changes produced by insulin (I, 50 μg/ml), rat prolactin (rPrl, 25 μg/ml) and cortisol (F, 20 μg/ml) in mammary gland explants obtained from four 10 days lactating rats. Serum added to the medium in a concentration of 50%; 3 days culture.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Glucose mg/ml</th>
<th>Hormones</th>
<th>Alveolar development (grade)¹</th>
<th>Mitotic activity (%)²</th>
<th>Vacuolization (%)² (grade)¹</th>
<th>Secretion (grade)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₀</td>
<td>2</td>
<td>I</td>
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<td>50</td>
<td>0-1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I+rPrl</td>
<td>4-5</td>
<td>0</td>
<td>0-1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I+F</td>
<td>6</td>
<td>25</td>
<td>1</td>
<td>3-4*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I+rPrl+F</td>
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<td>18</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>S₀</td>
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<td>I</td>
<td>6</td>
<td>0</td>
<td>100</td>
<td>3</td>
</tr>
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<td></td>
<td></td>
<td>I+rPrl</td>
<td>6</td>
<td>10</td>
<td>91</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I+F</td>
<td>6</td>
<td>0</td>
<td>83</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I+rPrl+F</td>
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<td>33</td>
<td>100</td>
<td>2-3</td>
</tr>
<tr>
<td>SL₁₀</td>
<td>2</td>
<td>I</td>
<td>6</td>
<td>11</td>
<td>67</td>
<td>3</td>
</tr>
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<td></td>
<td></td>
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<td>6</td>
<td>10</td>
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</tr>
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<td></td>
<td></td>
<td>I+F</td>
<td>6</td>
<td>8</td>
<td>100</td>
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</tr>
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<td></td>
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<td>6</td>
<td>18</td>
<td>91</td>
<td>1</td>
</tr>
<tr>
<td>SL₁₀</td>
<td>4</td>
<td>I</td>
<td>6</td>
<td>17</td>
<td>100</td>
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</tr>
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<td></td>
<td>I+rPrl</td>
<td>5-6</td>
<td>17</td>
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</tr>
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<td></td>
<td></td>
<td>I+rPrl+F</td>
<td>6</td>
<td>10</td>
<td>90</td>
<td>3</td>
</tr>
</tbody>
</table>

¹: Median value of the semiquantitative grading of response indicated.
²: % of explants showing response indicated.

* Difference with the corresponding result for group I significant at the 5% level.

No of explants per group: 9-12.

change was an increased vacuolization (significant at the 1% level).

With SL₁₀ + I no significant differences with group S₀ + I were seen. Increasing the glucose concentration from 2 mg/ml to 4 mg/ml again resulted in a significant increase in vacuolization (Table 28).

With I + rPrl + either S₀ or SL₁₀', the presence of rPrl had no significant effect (Table 28). With I + F, F significantly increased the degree of secretion in some groups. This effect seemed to be independent of the type of serum or the glucose concentration. With I + rPrl + F the results did not differ significantly from those with I + F.

In this experiment approximately half of the explants showed necrosis. Pyknosis was prominent and intraluminal nuclear debris was abundantly present. A difference in these respects between
the various groups was not observed.

5.2.5.2. Pregnant rat serum

Experimental conditions

Medium: 50% $S_0$ or $S_{13} + 50\%$ t8; glucose 2 mg/ml. Hormones: a) I (50 µg/ml) and b) I (50 µg/ml) + F (20 µg/ml) mammary glands of 21 days pregnant rat served as control. 3 And 6 days culture (medium was changed on day 3), with 2 lactating and 2 pregnant rat glands.

Results

To control the effectiveness of the pregnant rat serum, its effect on $MP_{21}$ was determined (Table 29). In comparison with

Table 29 The effect of virgin rat serum ($S_0$) and 13 days pregnant rat serum ($S_{13}$) on the changes produced by insulin (I, 50 µg/ml) and cortisol (F, 20 µg/ml) in mammary gland explants obtained from two 21 days pregnant rats and two 10 days lactating rats. Serum added in a concentration of 50%; 3 and 6 days cultures.

<table>
<thead>
<tr>
<th>Mammary gland</th>
<th>Serum</th>
<th>Hormones</th>
<th>Days of Culture</th>
<th>No of explants</th>
<th>Alveolar development (grade)</th>
<th>Vacuolization (%) (grade)</th>
<th>Secretion (%) (grade)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant day 21</td>
<td>$S_0$</td>
<td>I</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>$S_{13}$</td>
<td>I</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>70</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>66</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>89</td>
<td>3**</td>
<td>92</td>
</tr>
<tr>
<td>$S_0$</td>
<td>I+F</td>
<td>3</td>
<td>12</td>
<td>5</td>
<td>75</td>
<td>1**</td>
<td>100</td>
</tr>
<tr>
<td>$S_{13}$</td>
<td>I+F</td>
<td>3</td>
<td>12</td>
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<td>1**</td>
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<td></td>
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<td></td>
<td>I+F</td>
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<td>89</td>
<td>3**</td>
<td>92</td>
</tr>
<tr>
<td>Lactating day 10</td>
<td>$S_0$</td>
<td>I</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>$S_{13}$</td>
<td>I</td>
<td>3</td>
<td>11</td>
<td>5</td>
<td>70</td>
<td>60</td>
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<td></td>
<td>I</td>
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<td></td>
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<tr>
<td>$S_0$</td>
<td>I+F</td>
<td>3</td>
<td>12</td>
<td>5-6</td>
<td>83</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>$S_{13}$</td>
<td>I+F</td>
<td>3</td>
<td>12</td>
<td>5</td>
<td>83</td>
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<tr>
<td></td>
<td>I+F</td>
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<td></td>
<td>I+F</td>
<td>3</td>
<td>12</td>
<td>5-6</td>
<td>83</td>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

1: Median value of the semiquantitative grading of the response indicated.

2: % of explants showing response indicated.

* Difference with the corresponding result for group cultured with $S_0$, significant at the 5% level.

** Idem at the 1% level.
$S_0 + I, S_{13} + I$ increased the alveolar size of the MP$_{21}$ explants significantly both in the 3 and 6 days culture. The effect on vacuolization and secretion was not significant except for the secretion after 6 days. The degree of secretion remained low (Table 29). With I + F, the alveolar size, vacuolization and secretion increased generally in comparison with I. This was found to be the case with both $S_0$ and $S_{13}$. The effect of $S_{13}$ was significantly synergistic with the effect of F, especially after 6 days.

In comparison with $S_0 + I, S_{13} + I$ had no significant effect on ML$_{10}$ explants (Table 29). With I + F, $S_{13}$ showed some synergism in the 6 days culture, but only the effect on alveolar size reached significance.

Mitoses were seen only sporadically in this experiment. A culture for 6 days resulted in a high percentage of necrotic explants.

5.3. Ultrastructure of epithelial cells during mitosis

Experimental conditions

In vivo material. Specimens were obtained from 9, 10 and 13 days pregnant rats and from lactating rats two days after parturition.

In vitro material. Mammary explants from non-pregnant and 13 days pregnant rats were cultured during 1, 2 and 3 days in serum-containing media. The media were supplemented with insulin and in some instances with cortisol. In one experiment explants derived from a lactating rat two days post-partum, were cultured during two days in a medium containing non-pregnant rat serum, insulin and rat prolactin (5 µg/ml). The presence of mitotic figures in thin sections was spotted by light microscopy and examined in detail by electron microscopy.

Results

The ultrastructure of a total of seven cells in mitosis was studied in the in vivo material. Three cells (10 and 13 days pregnant, 2 days lactating) showed neither lipid nor protein droplets, one cell (10 days pregnant) showed only protein drop-
Table 30  Total number of mitoses observed in 13 days pregnant rat mammary explants cultured for 1, 2 or 3 days in media not supplemented with hormones or supplemented with either rat chorionic mammotrophin (rCM, added as pregnant rat serum) or rCM and cortisol (F, 20 μg/ml), and the number of cells showing the presence of lipid droplets and/or protein granules.

<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Total no of cells during mitoses</th>
<th>no of cells showing lipid droplets</th>
<th>no of cells showing protein granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>no hormones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>+rCM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>+rCM + F</td>
<td></td>
<td></td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>13</td>
<td>7</td>
</tr>
</tbody>
</table>

droplets and the remaining three cells (9 and 10 days pregnant) showed both types of products (see Fig. 43).

The in vitro results with explants from a 13 days pregnant rat mammary gland are summarized in Table 30. The majority of the cells in mitosis showed lipid (Fig. 44) with or without droplets. The presence of various hormones seemed to have no effect on their frequency. Fig. 45 illustrates that the mitosis can occur in a cell with a well developed Golgi apparatus which contains some secretory material. Moreover, the appearance of the Golgi apparatus in the dividing cell is similar to that in adjacent cells.

Two mitoses were seen in explants derived from a virgin rat after 3 days culture with rCM and F added to the medium. Both cells contained fat globules but no protein droplets. One mitosis was seen in an explant derived from a two days lactating rat after two days culture. The dividing cell contained fat globules, protein granules and a well developed Golgi apparatus with a fine-granular product. A well developed RER, arranged in layers, was not observed.
Fig. 43 Alveolar cell of a 10 days pregnant rat mammary gland in vivo. The cell is in mitosis with the centriole (c) visible. The cell contains lipid droplets (Li) and small vacuoles (v) containing a proteinaceous core, which is comparable to the material in the larger vacuole (V) present in the adjacent cell. Scale: 1 µm.
Fig. 44 Mitosis of an alveolar cell of a 13 days pregnant rat mammary gland, cultured for 2 days in a medium containing pregnant rat serum, insulin and cortisol. The nuclear membrane has disappeared; centrally in the cell cross-sections of chromosomes are visible. The cell contains multiple fat droplets (Li) at the periphery of the cytoplasm. Lu: lumen. Scale: 1 μm.
Fig. 45 Mitosis of an alveolar cell of a 13 days pregnant rat mammary gland, cultured for 1 day in a medium containing pregnant rat serum and insulin. The nuclear membrane has partially disappeared. Cell organelles are arranged at the periphery. The cell contains multiple small fat droplets (Li) and Golgi vacuoles (G) containing fine granular material, comparable in aspect with the vacuoles (V) in the adjacent cells. Scale: 1 μm.
6. DISCUSSION

6.1. General aspects

Since the conclusions reached concerning the actions of hormones are based on certain characteristic responses of the mammary explants in vitro, the nature of these responses is considered first.

Mammary explants can respond in vitro by growth, (morphological) differentiation of the cells, secretion, regression and degeneration. Each in vitro response is defined by a combination of histological changes. The various responses are summarized in Fig. 46.

Growth is defined by a high score for both mitotic activity and alveolar development. By mitotic activity is meant the number of mitotic figures in alveoli and ducts separately, per given histological section. Counting the number per section is less informative, but also less time consuming than counting the number of mitoses per 2000 cells or more (Dilley 1971a). In our experience the counting per section provides sufficient discrimination between groups of explants derived from the same mammary gland. The (lobulo-)alveolar development is another index for growth. Generally a high mitotic activity is accompanied by a relatively high degree of alveolar development. Dilley (1971b) showed for whole-mounts of the immature rat mammary gland that the alveolar development was proportional to and dependent on mitotic activity. However, the score for alveolar development can be high in the absence of considerable mitotic activity. The size of the cells and distension of the lumen with secretory products are also contributory factors.

During culture changes in the morphology of the cells occur. Cytoplasmic opalescence is an index for one of those changes. Various factors contribute to cytoplasmic opalescence. The cells may be large, while the cytoplasm stands out prominently and stains well and homogeneously. In this case, however, the score for opalescence remains low. Progesterone and testosterone show such an effect. Another possible morphological
Fig. 46  Schematic representation of the various types of responses of 13 days pregnant rat mammary gland MP\textsubscript{13} in vitro after 3 and 6 days of culture (3 & 6 resp.). Growth is indicated by an increase of alveolar development and the presence of mitotic figures (M). The secretory response can be either incomplete or complete. The incomplete response is characterized by maintenance of the cytoplasmic opalescence present in the starting material, the presence of some dispersed fat vacuoles and the appearance of intraluminal eosinophilic material (secretion, S), while there is no obvious increase of response from day 3 to day 6 of culture. Mitoses may remain present. The complete secretory response is characterized by a maximally developed vacuolization and secretion after 6 days of culture. After 3 days cytoplasmic opalescence is present and vacuolization and secretion are still incompletely developed. When the explants respond with regression, the alveolar development decreases progressively. The cells become compact. After 3 days of culture pyknosis (p) is notable in the explants. In case of degeneration, the alveolar development decreases progressively while pyknosis is notable after 3 and 6 days. The cellular aspect is variable: some cells appear to be compact with pyknotic nuclei, other cells appear to be bloated with pale-staining nuclei. The lumen may contain cellular debris (R).
change is the accumulation of small fat globules in the cytoplasm. Since fat is dissolved during the histological treatment of the explants the globules are transformed into tiny vacuoles, giving the cytoplasm, which stains only faintly, a foamy appearance. This is referred to as "opalescence" (see Fig. 13f). A high score for cytoplasmic opalescence indicates that the hormone(s) or other factor(s) in the medium stimulate or maintain synthesis and accumulation of fat.

The secretory response which may be complete or incomplete (see Fig. 46) has various aspects: in the first place it is noted whether the response has developed only locally or has spread throughout the explants; secondly vacuolization and secretion are rated separately, for both alveoli and ducts. Vacuolization can be preceded by an increase in cytoplasmic opalescence. When the small fat globules fuse to larger globules (visible as vacuoles after preparation for the histological examination), cytoplasmic opalescence is replaced by vacuolization. The term secretion is reserved for the appearance of stainable (eosinophilic) material in the alveolar lumen (see Fig. 13e). Not all the stainable material in the lumen consists of secreted milk proteins. Some of it may be cellular debris as is indicated by the presence of nuclear remnants and can be identified by electron microscopy (Njio 1976).

When the secretory response develops, it is at first visible locally in the explants (3 days culture, Fig. 46). Then its development may stop - as is seen with prolactin and other lactogenic protein hormones - or it may spread over the entire explant - as is the case with the corticosteroids. Differences in culture conditions inside the explants may be important for the local secretory response with prolactin: the response is mostly confined to the upper-central part of the explant, whereas mitotic activity is concentrated in the periphery in agreement with the observations by Prop & Hendrix (1965). To evaluate the response properly transverse sections must be made roughly through the middle part of the explant.

A distinction must be made between regression and degeneration (Fig. 46). When the explant reacts with a decrease of al-
Alveolar development, a loss of cytoplasmic opalescence, a decrease of cell size, an absence of mitoses after 3 and 6 days, a wave of pyknosis and a prominence of connective tissue, the response is interpreted as a "regression" or an in vitro involution. Regression occurs in a culture of explants taken from well-developed mammary glands. The response indicates that the medium lacks the hormone concentrations necessary to maintain the original stage of development but not the other factors like rat serum and insulin which are necessary for an optimal maintenance of the explants. Pyknosis is prominent during the period in which the explants fall back to a lower level of development but tends to disappear when a new equilibrium is reached between the mammotrophic activity of the medium and the mammary development.

The response is interpreted as degeneration, if the pyknosis is spread through the explants, affects both the epithelium and the connective tissue, and is seen in both the 3 and 6 days culture. Alveolar development decreases and mitotic activity is absent. Sometimes the cells become swollen and the cytoplasm stains pale with an increased score for opalescence. The degree of degeneration varies. When the degree is low, functional decline may occur before visible signs of degeneration (Rivera 1974). A high degree of degeneration results in necrosis of the explants. Degeneration implies that the medium either is deficient in essential metabolic factors, such as insulin or rat serum, or contains toxic factors such as those present in heterologous sera. The degeneration which develops in (over)extended alveoli by stasis of secretory products is a special case (for comment see Rivera 1974).

With extensive degeneration the explants cannot be expected to react normally to hormonal stimulation and definite conclusions cannot be drawn. Local degeneration has less significance. Central degeneration and/or necrosis occurs when the diameter of the explant is too large to permit adequate diffusion. A thin sector of degeneration at the surface of the explants implies suboptimal conditions e.g. a position too far above the surface of the medium.
6.1.1. Various types of response obtained in vitro

6.1.1.1. Role of serum

The importance of serum for mammary culture has been reported by Frantz et al. (1972), Turkington & Majumder (1973) and Hsueh & Stockdale (1975). Similarly, the present results show that a high concentration of rat serum is an important condition for the viability of rat mammary gland in vitro. Both the concentration of the serum and its type are important. Heterologous serum seems to have a complex effect: when pyknosis is taken as an index for degeneration, the culture is seen to improve with the addition of certain types of heterologous sera in low concentrations whereas at higher concentrations a "toxic" effect starts to prevail. Although both the degree of the improvement and the optimal concentration vary with the type of serum used in the present experiments, the maximum concentrations tolerated generally correspond with those reported by Forsyth & Parke (1973) for the bioassay of prolactin. It is uncertain whether the degree of "toxicity" of the heterologous sera is inherent to the species or to a particular sample. Franz et al. (1972) have reported that some human plasma samples (at 30% in the medium) produced necrosis of mouse mammary explants, while other samples improved viability.

The "toxic" reactions of rat mammary explants limit the suitability of the organ culture as a bioassay for mammotrophic activity present in heterologous serum samples. Moreover, it complicates the use of heterologous anti-sera such as rabbit anti-rPrl serum (Peters et al. 1976) or rabbit anti-hPL serum in the present series of experiments.

Serum seems to be involved in every response of the mammary explants, but especially in the mitotic activity and the cytoplasmic opalescence/vacuolization. Secretion can develop to a certain degree without serum. Rat serum is a decisive factor for the response to the lactogenic hormones Prl, hPL and rCM (added in the form of serum fractions, Peters et al.
1977). Serum influences the response to other hormones such as insulin at 24 h, cortisol and progesterone. Since serum factor(s) are involved in every response and in every hormonal action, and since the lack of these factor(s) causes degeneration rather than regression, the effect of the serum is considered to be non-specific. It is essential for the capacity of the cells to react to hormonal stimulation. The presence of these factors in the serum - reportedly polypeptide in nature (Turkington & Majumder 1973) - is not exclusively associated with periods of mammary growth in vivo. Virgin rat serum by itself shows little proliferative or secretory activity, but it contains the factors which "permit" the hormones to act.

Prop (1976) has pointed out the dilemma in the use of serum in the medium. In fully synthetic media all substances are known, but serum supplemented media are often superior for the survival and maintenance of the cells. This superior maintenance compensates in the present experiments the introduction of unknown factors. The effects of the hormones obtained in the rat serum containing medium, seemed to agree better with the in vivo situation, whereas under other circumstances in vitro findings were difficult to reconcile with those seen in vivo, especially with respect to proliferation (Porter 1974, Banerjee 1976).

Virgin rat serum contains basal hormone concentrations. Their presence in the medium might be a prerequisite for the effect of every hormone added to the medium. This does not diminish the relevance of the results for the in vivo situation. The addition of a particular hormone to a medium supplemented with virgin rat serum imitates the situation encountered in vivo when the concentration of a particular hormone or set of hormones rises and other factors remain constant.

6.1.1.2. Role of insulin

The effect of the high insulin concentration in the medium on the explants resembles in many ways the effect of virgin rat serum. Like serum, insulin has an effect on every step of the mammary cell development, from cell division to secretion. It
interacts with other hormones tested on the mammary gland. In the absence of a high insulin concentration the explants degenerate. For these reasons the insulin effect is likewise considered to be non-specific. It seems to amplify the effect of the various hormones as initially suggested by Lasfargues (1962). The mitogenic effect of the high insulin concentration on 13 days pregnant rat mammary explants may be a separate phenomenon. A similar wave of mitoses was observed in explants from late pregnant and lactating rats. The wave was absent when the medium lacked rat serum. Some mitotic activity and thymidine incorporation occurs in the absence of high insulin concentrations when the medium contains prolactin (in the present experiments) or rCM (Peters 1977b).

In agreement with Turkington & Kadohama (1972) a high insulin concentration was found necessary for secretion. The dependence may not be total: an indication for secretory activity or at least maintenance of secretory products could be demonstrated for the fully differentiated explants from a 19 days pregnant rat, cultured for 5 days with 95% rat serum but no high insulin. Otherwise secretion is minimal in the absence of high insulin.

Unlike the need for serum factor(s), the need for high insulin is considered to be an in vitro artefact. This necessity for an abnormally high concentration of insulin in order to obtain a full hormonal response diminishes the relevance of the in vitro results for the in vivo situation. This may perhaps be less so for the type of hormonal response involved than for its degree, since high insulin may make the explants abnormally sensitive. For instance the mitotic activity in explants cultured with high insulin and prolactin surpasses the activity seen in vivo at the comparable stage of pregnancy. High insulin therefore distorts the dose-response relationships. Minimum and maximum effective concentrations in vitro may be different from those in the in vivo situation.
6.2. In vitro effect of various lactogenic protein hormones

6.2.1. Prolactin

6.2.1.1. Demonstration of mammogenic and lactogenic activities

The effect of prolactin on the morphology of pregnant rat mammary gland in vitro includes both a mammogenic effect (i.e. growth with an increase in mitotic activity, thymidine incorporation and alveolar development) and a lactogenic effect (i.e. morphological differentiation, cytoplasmic opalescence and an incomplete secretory response: locally some vacuolization and secretion, see Fig. 46).

The mammogenic effect of prolactin is highly dependent on the composition of the medium. Both the concentration and the type of serum are important factors. A growth promoting effect of prolactin could not be demonstrated when the medium contained only T8. The mammogenic effect appeared, however, and increased steadily when rat serum was added up to a concentration of 50%, while the levels of glucose, insulin and prolactin remained approximately constant. This effect of rat serum could not be due to endogenous prolactin in the serum, since a high concentration of an active prolactin preparation (1.25 μg/ml) had been added. In the presence of heterologous sera the proliferative effect of prolactin was decreased or absent. In the present experiments human serum was the most synergistic of the heterologous sera. The results suggest that the low percentage of heterologous serum added to the culture medium by Koyama et al. (1972) and Prop (1976) may have been important for the subsequent in vitro demonstration of the mammogenic effect of prolactin on rat and mouse mammary gland. Others (Mayne & Barry 1970; Dilley 1971a) have obtained evidence for such mammogenic effect of prolactin without serum added to the medium. This could not be confirmed under our culture conditions.
6.2.1.2. Dose-response relationships for various prolactin preparations

Various preparations of ovine, bovine, human and rat prolactin have been tested on the mammary gland in vitro. The morphological changes produced by these preparations when added to a medium with a high concentration of virgin rat serum and insulin, were similar. However, the concentration - on weight per volume basis - at which these changes were obtained varied considerably.

It was possible to use proliferation - i.e. alveolar development and mitotic activity - and cellular differentiation - i.e. cytoplasmic opalescence - as histological endpoints for the assessment of mammotrophic activity instead of the lactogenic response (Franz et al. 1972; Forsyth & Parke 1973).

A good correlation was not always found between the activity of a preparation expressed in units and the effective dose range in vitro. Two different rat prolactin preparations with the same activity in units showed a marked difference in activity in vitro. For unknown reasons the actual prolactin concentrations differed greatly from the expected concentrations after various procedures, such as sterilization. These facts indicate that a radioimmunoassay of the culture may be necessary in order to draw a final conclusion about the relative activity of a prolactin preparation in vitro.

When the results obtained by radioimmunoassay were correlated with the proliferative effect of prolactin in vitro, the steep slope of the dose-response relationship fell within the "physiological" range of 27 to 115 ng per ml medium. The culture was sufficiently sensitive to detect a slight difference between 10 and 27 ng. This degree of sensitivity would be in the same range as that reported by Forsyth & Parke (1973).

In a 3 days culture 115 ng rat prolactin per ml medium measured by radioimmunoassay produced a maximum response. This value may differ from the optimal concentration in vivo since the in vitro results were obtained in the presence of abnormally high levels of insulin. Moreover, in other experiments or in
the case that the culture is prolonged and the sensitivity towards prolactin of the mammary explants decreases, higher concentrations of prolactin are necessary for the maximum response. Therefore, no definite conclusion can be reached with respect to the dose-response relationship for prolactin in vivo.

When the medium contained virgin rat serum and insulin, while corticosteroids were not added, the secretory response to prolactin was not prominent. Vacuolization and secretion developed only locally in the explants of a limited number of the 13 days pregnant rat mammary glands. In agreement with earlier results (Peters 1977b), the dose-response relationship for this effect was poor. Even when the bovine prolactin concentration was increased to 100 µg/ml, the secretory response became neither general nor maximal. In other experiments high prolactin concentrations seemed even to suppress the incomplete secretory response. The synergism between corticosteroids and prolactin on lactogenesis is discussed elsewhere in relation-ship to the action of the corticosteroids.

6.2.1.3. Mammogenic activity of lactating rat serum

The mammogenic activity of lactating rat serum poses a special problem (Peters et al. 1976). The prolactin concentration increases to 300 - 350 ng/ml serum after a suckling stimulus (Chen et al. 1974). At these serum concentrations of prolactin a mammogenic effect of lactating rat serum should be demonstrable in vitro.

Previously it has been proposed that the mammogenic activ-ity in lactating rat serum could be explained by the presence of increased prolactin levels during lactation (Peters et al. 1976). An argument for such a point of view has been the single observation that rabbit anti-prolactin serum inhibits the mammogenic effect of lactating rat serum. However, the use of heterologous sera, which cannot be avoided, introduces the unpredictable factor of "toxicity". To add other arguments, the mammogenic activity of a series of lactating rat sera was
correlated with the actual prolactin level present in the sample as measured by radioimmunoassay. In this way it could be demonstrated that the serum activity correlates with the prolactin level in the lactating rat serum. Moreover, the range of prolactin concentrations in which the mammogenic response increased steeply, i.e. lower than 40 ng/ml, corresponded reasonably well with a similar range determined for rat prolactin added to the medium.

Although these results indicate that the mammogenic effect of lactating rat serum could be explained by the presence of high levels of prolactin, they cannot exclude the possibility that growth hormone contributes to the effect. Growth hormone has a mammogenic effect in the system used, even though it could not be demonstrated that the effect is present at concentrations actually measured in rat serum in vivo during lactation. In order to establish the importance of growth hormone, L-dopa has been administered to some lactating rats. Meites (1977) reports that in rats which have been weaned for 8 h, followed by nursing during 30 min the administration of L-dopa results in a low serum concentration of prolactin but a high concentration of growth hormone. If the serum of rats treated in this way still shows mammogenic activity, the importance of growth hormone is strongly suggested. For reasons unknown L-dopa failed to suppress prolactin concentrations in the serum sufficiently and the mammogenic activity still present after L-dopa could well be explained by the prolactin present in the samples. Further experiments must be performed to answer this question.

6.2.1.4. Time related effects of prolactin

Dilley (1971a) reported that the insulin plus prolactin maintained the initial mitotic activity in mammary glands from immature rats, although the sensibility of the explants seemed to be decreased after 5 days of culture. Similarly, in the present experiments, the mitotic activity obtained with a certain concentration of prolactin in pregnant rat explants
did not remain at a constant level. The explants, cultured in a medium with insulin and virgin rat serum but with no prolactin added, showed a sharp increase in the mitotic activity during the first 24 h, followed by a sharp fall to a low level after 3 days. A similar sequence was found for the thymidine incorporation. The cytoplasmic opalescence, present in the material at day 0 of culture, was maintained for 24 h. Thereafter the opalescence decreased parallel with the mitotic activity. The effect of the addition of prolactin to this system of virgin rat serum and insulin can be defined as the prevention of the decline after 24 h: the mitotic activity remains at a high or higher level and the cytoplasmic opalescence is maintained. A similar effect has been obtained by Mayne & Barry (1970) for prolactin plus corticosterone in mouse explants. In the present experiments the same kind of sequence was obtained for rCM, present in pregnant rat serum.

At a high concentration of prolactin the mitotic activity is maintained for at least 3 days, but after 6 days of culture the number of mitoses was decreased. At submaximal concentrations of prolactin the prevention of the decline after 24 h is less effective. At low concentrations a (slight) difference in mitotic activity could be observed on day 2 between approximately 10 and 27 ng of prolactin per ml medium, but on day 3 the difference had disappeared. The results show, that the decline of mitotic activity is not caused by a disappearance of prolactin from the medium. The prolactin levels in the medium were stable during the three days of culture. The reason for the decrease of response may be a decline of the functional activity of the cells in vitro as discussed by Rivera (1974). A depletion of insulin and/or serum factors in the medium may be another explanation.

6.2.1.5. Interaction of prolactin with various hormones

The mammogenic hormone of pituitary origin was thought to synergize with progesterone to stimulate lobulo-alveolar development (see review by Anderson 1974). Recently, however, progesterone has been demonstrated to antagonize the stimulating effect
of prolactin on the level of its receptors (Djiane & Durand 1977). This finding is hard to reconcile with the concept of a synergism between progesterone and prolactin on mammary growth. Such a synergism has been demonstrated previously for progesterone and rCM (Peters 1977b; Peters et al. 1977). The present results indicate the presence of a similar synergism for progesterone and prolactin, and support the results of Dilley (1971b) and Koyama et al. (1972). When the proliferative effect of prolactin in vitro is maximal, progesterone adds little to the effect, but when it is submaximal, progesterone shows a synergism. The interaction between the two hormones was obvious when histological endpoints, i.e. mitotic activity and alveolar development, were used. The results for thymidine incorporation were less consistent.

In agreement with Koyama et al. (1972) oestradiol by itself has no clear effect on the explants nor did it influence the effect of prolactin, except at very high concentrations. Dilley (1971b) reported that the addition of the combination of oestradiol and progesterone increased the thymidine incorporation and alveolar development obtained with prolactin in immature rat mammary gland. The present results support the finding of Dao & Sinha (1972), that progesterone might be the more active component in this hormone combination.

Testosterone has a weak growth promoting effect at the one concentration used under the present experimental conditions. It did not interact to a great extent with the effect of prolactin. In vitro Turkington & Topper (1967) and Palmiter (1969) obtained an inhibition of the DNA synthesis in mouse mammary gland explants by androgens, but their culture conditions were different. In vivo a direct, local growth promoting effect of testosterone on rat mammary gland has been demonstrated by Ahren & Hamberger (1962).

An antagonism in vivo between prolactin and thyroxin at the level of the rat mammary gland has been described by Mittra (1974). The present results do not provide evidence for such an antagonism in vitro. Thyroxin had no effect either on the action of other growth promoting hormones used as controls, such as
testosterone or rCM in pregnant rat serum.

6.2.2. Human placental lactogen

6.2.2.1. In vitro effect

HPL has a mammogenic effect in vitro: it stimulates mitotic activity and maintains the alveolar development present in the well developed 13 days pregnant rat mammary gland. A similar mammogenic activity has previously been demonstrated for rCM, the rat equivalent of hPL (Peters et al. 1977). The effect of hPL could be observed only when the medium contained virgin rat serum and insulin. When 100% Trowell's T8 was used as medium, mitotic activity was not apparent.

The demonstration of a proliferative effect of hPL on mammary tissue supports the suggestion that hPL provides the mammogenic stimulus required for mammary gland growth during pregnancy (Anderson 1974). Such an action would be expected to be exerted in vivo rather than a lactogenic effect as proposed by Turkington (1972) since this hormone circulates at high concentrations before lactogenesis and disappears at the time when lactogenesis is initiated.

In addition to the growth promoting effect, some lactogenic activity of hPL could be demonstrated. For this effect the type of medium used was important in the three days culture period used. Without cortisol traces of secretion developed with hPL and some vacuolization, but not to the degree of vacuolization seen with pregnant rat serum. These effects of hPL could be observed only when virgin rat serum and insulin were added to the medium at a high concentration. With a 100% chemically defined medium (T8) the incipient secretory response was not seen. The localized and poor development of secretion and vacuolization has been observed also for the rat equivalent rCM (Peters et al. 1977).

Since the effect of pregnant rat serum on the pregnant rat mammary gland in vitro is similar to that obtained with either hPL or rPrl added to virgin rat serum, the organ culture tech-
nique has been used to estimate the level of mammotrophic activity in pregnant rat serum. Assuming that the activity in pregnant rat serum is solely the result of the rCM present, the activity of rCM in one ml of pregnant rat serum would be equivalent to that of 1 - 5 μg rPrl. A comparison with hPL would be more meaningful, but the results for hPL were often less distinct. The rPrl preparation appeared on a weight basis four times more active than the hPL preparation.

6.2.2.2. Cross reactivity with rat chorionic mammotrophin

The possibility of a cross-reactivity between hPL and rCM has been explored by trying to neutralize the effect of rCM in pregnant rat serum by rabbit anti-hPL serum. An immunochemical similarity between rat and human lactogens of placental origin was suggested by Leak & Burt (1969), who showed a cross-reaction between anti-hPL serum and rat placental extract. Furthermore, a marked effect of rabbit anti-hPL serum during pregnancy of the rat in vivo has been reported by Gusdon (1972), although this effect may not have been specifically related to an inhibition of placental factors (Yamini et al. 1975).

Organ culture is not very suitable for the use of anti-sera. In one experiment a toxic reaction of the explants to the heterologous rabbit serum developed, irrespective of the presence of rPrl, hPL or pregnant rat serum. When in a second experiment both the source of anti-serum and the concentration in the medium were changed, the anti-serum caused some decrease of the mammotrophic activity of pregnant rat serum. This could be interpreted as indicating a low level of cross-reactivity between rCM and hPL. However, the decrease in activity of the pregnant rat serum could be explained better as the result of an aspecific toxic reaction, because histologically, the decrease of activity was accompanied by the appearance of nuclear remnants. Moreover, anti-hPL serum could not suppress the activity of the pregnant rat serum to the level of activity of the controls i.e. the combination of rabbit anti-serum and virgin rat serum. Therefore, the results offer little support for the existence of significant
cross-reactivity between hPL and rCM.

6.2.3. Growth hormone

Both the human (hGH) and porcine growth hormone (pGH) preparations showed in vitro mammogenic activity with, in addition, a stimulation of the cytoplasmic opalescence. The combination of hGH plus virgin rat serum produced morphological changes, which were similar to those obtained with pregnant rat serum. However, in the 3 days culture the combination of hGH and virgin rat serum was less effective than 13 days pregnant rat serum with respect to mitotic activity, but more effective as far as cytoplasmic opalescence is concerned. This trend towards more function and less growth with hGH is at variance with the conclusion reached by Banerjee (1976). This author ascribed to growth hormone an effect on alveolar morphogenesis, but not on functional differentiation of the mammary cells. The present results do not support this conclusion. Most likely growth hormone stimulates both growth and differentiation, the latter to a level just short of full lactogenesis like prolactin and the placental lactogen. The decrease of mitotic response with the hGH preparation might be explained by the toxic impurities present in the preparation as suggested by the increase of necrosis in the explants. The pGH preparation, which did not produce necrosis, induced concomitant growth and cytoplasmic opalescence.

The steep part of the concentration-effect curve for hGH fell in the range of 0.78 to 3.15 μg/ml and for pGH at concentrations higher than 0.63 μg/ml. These concentrations are too high to postulate a role for GH in vivo. Moreover, possible traces of prolactin present as a contamination of the pGH preparation could contribute to the overall effect. Although rat growth hormone may show greater activity than either hGH or pGH, the results do not provide evidence that GH is a major factor in the complex of mammogenic hormones.
6.3. Progesterone

The present results are in agreement with previous observations (Koyama et al. 1972, Peters 1977b, Peters et al. 1977) concerning a proliferative effect of progesterone in vitro. The mammary gland of rats late in pregnancy is still sensitive to this effect of progesterone. The results support the concept that progesterone may have a similar effect in vivo (Chatterton 1970). The growth response to progesterone can be detected in vitro only under certain culture conditions i.e. in the presence of virgin rat serum and of insulin. The light microscopic criteria for the proliferative activity of progesterone are a high degree of alveolar development and an increased mitotic activity. With light microscopy no signs of morphological differentiation were observed. Although the acinar cells showed an increase in size when progesterone was added to the medium, there was neither opalescence such as is obtained with prolactin, nor secretory activity. For this reason progesterone was initially considered to cause growth of the mammary epithelium without morphological differentia-
tion. This effect would then be different from that of prolactin which causes both (Peters 1977b).

The proliferative effect of progesterone in vitro requires the presence of both virgin rat serum and insulin, added in a concentration which exceeds that found in vivo. In the present study it could be demonstrated that the effect of progesterone was not totally dependent on the presence of insulin.

Finally, no specific interaction could be demonstrated between progesterone and either oestradiol (in agreement with Koyama et al. 1972) or testosterone. The latter hormone showed a slight proliferative effect on the mammary gland which re-
sembled that of progesterone.
6.4. Corticosteroids

6.4.1. Cortisol-induced changes

Cortisol initiates secretory activity in the rat mammary gland in vitro. The morphological changes in the gland are similar to those obtained in vivo (Talwalker et al. 1961) in the pregnant rat by the administration of high doses of cortisol. The histological appearance in vitro after addition of cortisol is that of "sham lactation" in vivo (Freud & Uyldert 1948). Ultrastructural changes produced by cortisol in vitro have been examined by electron microscopy by Njio & Peters (1977). The secretory response produced by the corticosteroids can be evaluated by a semiquantitative grading of secretion and vacuolization separately. In addition cytoplasmic opalescence can also be graded. An increase in cytoplasmic opalescence precedes vacuolization. The cells seem to be swollen with small fat vacuoles. This change serves as an index for the cortisol effect in the 3 days culture of the 13 days pregnant rat mammary gland. The degree of alveolar development is a less useful index. With cortisol, alveolar development is more an index for secretory activity than for growth or maintenance (see Fig. 46). The reason for this is that after 6 days of culture under optimal conditions the alveoli are distended with secretory products. The degree of "alveolar development" at this stage is not to be compared with the alveolar development seen in a 3 days culture when the lumina are still small and the alveolar development depends for its score on factors such as growth and cell size. Finally mitotic activity may be used as an index. There may be a relationship between the decrease of mitotic activity by cortisol and a high secretory activity. One possibility may be that high secretory activity and mitotic activity is incompatible. Another is that the distension of the alveoli reduces the number of alveolar cells per given section and thereby decreases the chance of encountering a mitotic figure. The number of mitoses not per section, but per actual number of cells remains to be studied.
6.4.2. Time related changes in explants due to cortisol

The histological changes occurring in the 13 days pregnant rat mammary explants during culture with cortisol are interpreted as follows:

6.4.2.1. Cytoplasmic opalescence and vacuolization

When the medium lacks cortisol, only the presence of rCM (in pregnant rat serum) can prevent the loss of the cytoplasmic opalescence present in the material at the start of the culture. The rate of disappearance in vitro with a medium containing serum is similar to the rate with 100% T8. The disappearance of the small fat globules from the cells is part of the total regression due to hormonal withdrawal. This disappearance takes place in viable tissue, as is shown by the fact that the disappearance occurs during the wave of mitoses which takes place when the medium is supplemented with serum. Loss of fat globules can be prevented by rCM - or similar hormones - but this hormone is not the only factor involved. Cortisol is another hormonal factor which promotes the accumulation of fat droplets. For this effect cortisol is not totally dependent on a high concentration of rCM or prolactin. This can be concluded from the fact that the effect is still seen with virgin rat serum, which contains only a low prolactin concentration. However, cortisol and rCM are synergistic as regards the production of fat globules. With respect to the fat content of the cells corticosteroids show one property which the lactogenic protein hormones do not possess, i.e. promotion of the fusion of the fat globules. Fusion of the small droplets to large ones (fat vacuoles) sets in after 18 to 36 h with cortisol. Cortisol is responsible for this fusion. In agreement with the observation of Collier et al. (1977) on cow mammary gland explants, it was observed that the large fat droplets tended to remain intracellular and that a massive transport towards the lumen did not occur in vitro in the rat explants, as it does in vivo around the time of parturition (see also Njio 1976). Although rCM and related
hormones may stimulate the accumulation of small fat globules, the effect never reaches the stage of a fully developed vacuolization. In addition it was noted that the presence of rCM in the medium did not seem to accelerate the process of fusion, but increased only the final degree of vacuolization. The lactogenic protein hormones potentiate the effect of cortisol in the sense that vacuolization with cortisol plus rCM (pregnant rat serum) progresses during the entire culture period of 6 days, while cortisol without rCM (virgin rat serum) reaches its maximum after 4½ days of culture and thereafter levels off.

A different situation is obtained when the medium lacks serum. The combination of cortisol plus 100% T8 induces some vacuolization, but the score remains low and the vacuolization short-lived. It starts to disappear again after 4½ days, at a time when generalized degeneration of the explants sets in. In cow mammary gland explants no vacuolization was seen at all after 3 days of culture, although fatty acid synthesis was shown to be temporarily increased (Collier et al. 1977)

6.4.2.2. Secretion

Eosinophilic material appears in the lumen of the alveoli at the same time as vacuolization in the cells of the alveoli. Its appearance is not influenced by the composition of the medium. This finding is surprising, considering the fact that the eosinophilic material which appears with cortisol plus 100% T8 consists of cellular debris (Njio 1976) and is of a quite different nature than the material which appears with cortisol plus serum either with or without rCM. The latter combinations produced material similar in morphology to that seen in vivo by electron microscopy (Njio 1976) and is considered to be true secretion. RCM and the other lactogenic protein hormones do not cause frank secretion by themselves, but they potentiate cortisol. This potentiation of secretion with cortisol is less easily distinguished by light microscopy than the potentiation of vacuolization. An argument can be advanced against the assumption of a true potentiation of secretion by rCM and similar hormones.
It is argued (Rivera 1974) that because rCM and similar hormones produce proliferation of the alveoli, the potentiating effect on secretion could merely be the consequence of more cells being involved in secretion rather than of increased secretory activity on the part of individual cells. However, in the present case electron microscopy has shown that after 6 days culture with cortisol plus rCM secretory granules are still present in the cells whereas they had largely disappeared from the cells in the absence of rCM (Njio & Peters 1977). This indicates that the interaction between cortisol and rCM occurs at the level of the cell and that the presence of high lactogenic protein hormone concentration is necessary for the continuation of the secretory process. As far as light microscopic observations are concerned this latter property is shared by rCM, prolactin and a growth hormone preparation. A similar effect caused by ACTH is less obvious.

6.4.2.3. Alveolar development and mitotic activity

The addition of cortisol to the medium causes an increase of the score for alveolar development of the explants. This effect is essentially independent of the type of medium used, but the difference in alveolar development between a medium with and without cortisol is most marked with the virgin rat serum medium. Cortisol apparently suppresses mitotic activity which would be in agreement with results reported by Koyama et al. (1972). The suppressant effect of cortisol could not be observed with 100% T8 for the simple reason that with 100% T8 there is no mitotic wave at 24 h culture. This observation suggests that at least with midpregnant mammary explants the mitotic wave is not a prerequisite for the development of a secretory response. Vacuolization and "secretion" still developed and apparently not at a different rate than in explants which first went through a wave of mitoses at 24 h or in explants which maintained a high mitotic activity after 24 h. Earlier results indicated that cortisol suppresses rCM-induced mitotic activity after 3 and 6 days of culture. Subsequent studies of
cortisol provides more details of time-related effects: cortisol does not suppress the mitotic wave which appears with virgin rat serum medium plus insulin at 24 h, and this wave subsides after 3 days. Cortisol suppresses more selectively the mitotic activity which continues after 24 h when rCM is added to the medium. These results, however, do not warrant interpretation as a specific antagonism between cortisol and rCM with respect to proliferation. Competition for essential (serum) factors, exhaustion of the medium and accumulation of toxic products may be involved. Furthermore, the number of cells per given section may decrease due to alveolar distension, resulting in an artificial decrease in the number of mitoses per given section. Further studies are necessary to answer this question.

6.4.3. Stimulation of the effect of cortisol by serum of pregnant and lactating rats

In comparison with virgin rat serum, serum collected during pregnancy or lactation can be a much greater stimulus for the secretory response towards cortisol. The explanation is that the effect of these sera is due to two factors, one being rat serum as such, the other the presence of high concentrations of either rCM or prolactin/growth hormone. During pregnancy the cortisol-stimulating effect is time-related. In comparison with serum collected on day 0 of pregnancy (= day of insemination) the serum collected at the end of the first week of pregnancy stimulated less the effect of cortisol. A decrease of the serum prolactin concentration as described by Amenomori et al. (1970) may be the explanation. Serum collected from day 8 to the end of pregnancy greatly stimulated the effect of cortisol. The stimulation appeared and disappeared synchronously with mammmogenic activity of the collected serum. Time of appearance and disappearance agree well with those for rCM (Shiu et al. 1973), although the temporary decline in the rCM concentration around day 14 of pregnancy described by these investigators did not show in the present experiments. Except for this discrepancy the effect of late-pregnant rat serum can be explained by the
effect of virgin rat serum plus rCM. This assumption is supported by the observation that virgin rat serum plus factor(s) released by placenta fragments simulate the cortisol-stimulating effect of late-pregnant rat serum.

Previously it has been demonstrated that the cortisol-potentiating effect of rCM was dose-dependent (Peters 1977b). In the present study evidence was obtained that rCM seemed to shift the dose response relationship for corticosteroids to lower concentrations. This tendency could be demonstrated for both cortisol and aldosterone on both the virgin and the pregnant rat mammary gland explants.

When cortisol-potentiation was correlated with the prolactin concentration in the serum of lactating rats treated in various ways, a steep increase of the cortisol effect was obtained in a concentration range of lower than 20 ng prolactin/ml medium. When the prolactin concentration was increased further, the cortisol effect decreased progressively. However, the possibility of an interference by growth hormone present in the samples cannot be excluded. The effect was registered in a 3 days culture. A reversal of the potentiation at high concentrations of prolactin was not seen in other experiments when the culture period was extended to 6 days.

6.4.4. Interaction of cortisol with prolactin, growth hormone and human placental lactogen: a cortisol potentiating effect

Potentiation of the effect of cortisol on the mammary explants after 3 and 6 days of culture includes various visible aspects of secretory activity: an increase of cytoplasmic opalescence (especially after 3 days), of vacuolization and of secretion (especially after 6 days). Those lactogenic protein hormone preparations which showed mammogenic activity when added to virgin rat serum plus insulin, also showed a cortisol-potentiating activity. They shared this characteristic with the factors responsible for the mammogenic activity of pregnant and lactating rat serum - as discussed previously - and with
rat placenta extracts (Peters et al. 1977). The lactogenic protein hormones exert their cortisol-potentiating and mammogenic effects simultaneously, although the first effect seems to occur at the cost of the second. The cortisol-potentiating effect of prolactin is dose-dependent. If the presence of prolactin is essential for the effect of cortisol, the minimal amounts of prolactin present in virgin rat serum (approximately 10 ng/ml medium after dilution) should be sufficient to elicit a submaximal response. A similar concentration of ovine prolactin produced approximately 50% of the maximum response in mouse mammary gland (Frantz et al. 1972). In the present study the addition of 80 ng/ml prolactin in a 3 days culture stimulated the effect of cortisol maximally but the mammogenic effect of prolactin continued to increase at concentrations higher than 80 ng/ml.

6.4.5. Interplay of cortisol, prolactin, serum and insulin

The interplay between serum, insulin and prolactin with respect to the cortisol effect has been schematized in Table 31. All three factors play a role in the secretory response of the mammary explants to cortisol. Table 31 shows that in the absence of either one of the three factors cortisol is unable to produce a secretory response in vitro. When only one of the three factors is added some vacuolization develops, but secretion is still absent or minimal and moreover of doubtful origin, (i.e. cellular debris). When any combination of two of the three factors is added, cortisol produces a definite but submaximal secretory response. The full and maximum in vitro response is obtained only in the presence of all three factors simultaneously. Table 31 shows that the demonstration of the interaction between cortisol and prolactin is very much dependent on the presence of both serum and insulin in the medium.
Table 31 Dependence of the secretory effect of cortisol on the absence (-) or presence (+) of serum (virgin rat serum containing a low prolactin concentration), insulin (I, added to the medium at the high concentration of 50 μg/ml) and prolactin (Prl, added to the medium at high but physiological concentrations). The secretory response is made up of the appearance of cytoplasmic opalescence and vacuolization (indicating intracellular accumulation of fat) and secretion (appearance of eosinophilic material in the alveolar lumen, indicating protein secretion). The secretory response is graded, varying from 1, indicating absence of response, to 4, indicating maximum response.

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6.4.6. Regulating of the final step towards a full secretory response: corticosteroid versus prolactin

Topper (1970) and Turkington (1972) have proposed prolactin as the hormone regulating the final step towards full secretory activity of mouse mammary cells. Cortisol is held to act at an earlier step at which the mammary cells differentiate after mitosis. Arguments for this sequence are the findings that insulin plus cortisol (without serum) were unable to produce secretory activity, but that the addition of prolactin to the combination of insulin and cortisol produced secretory activity. This suggests strongly that prolactin regulates the final step. This impression is strengthened by the fact that the effect of prolactin on the appearance of secretion is dose-dependent (Kleinberg & Franz 1971; Forsyth & Parke 1973). Similar observations were made for rat mammary explants in the present
study. However, a different conclusion is reached: corticosteroids regulate the final step. Prolactin and other lactogenic protein hormones regulate growth and differentiation and potentiate – not regulate – the secretory effect of the corticosteroids. The arguments which favour prolactin as the final regulator can be advanced also for cortisol. Insulin plus prolactin (without serum) were unable to produce secretory activity, but the addition of cortisol to the combination of insulin and prolactin produced some secretory activity. Moreover, the effect of cortisol on the appearance of secretion is dose-dependent. Table 31 shows the possibility of a reconciliation of these apparently contradictory results as regards the final step in the sequence of hormone action. When hormone action is determined in a culture system with only one of the three cortisol-potentiating factors present in the medium i.e. insulin, the addition of a second factor, prolactin, becomes decisive for the appearance of the cortisol effect. Under these circumstances prolactin permits the effect of cortisol. The fact that the effect of prolactin is dose-dependent, is not necessarily an argument against a permissive function of prolactin. A comparable situation is encountered when the mammogenic effect of prolactin is shown to be dependent on the concentration of serum added. In this instance, which is clearly a permissive effect, there is also dose-dependency. In the present discussion it is argued that a hormone effect can only be established when the explants are cultured under optimal conditions, e.g. when prolactin and cortisol are tested in the presence of both serum and insulin. When serum and insulin are both present, prolactin shows a dose-dependent effect on growth and differentiation while its effect on secretory activity is weak and remains weak even at higher than physiological dose-levels. On the other hand secretory activity shows a dependency on the dose of corticosteroid added to the culture medium. Prolactin can be considered as "lactogenic" only to the extent that it determines the height of the secretory response obtainable with corticosteroids in rat explants.
6.4.7. Interaction of cortisol with various hormones

6.4.7.1. Interaction with adrenocorticotropic hormone

The one ACTH-preparation tested on its cortisol-potentiating effect in these experiments showed some activity. This result may bear little relationship with the in vivo situation since the effect was weak even at high concentrations, and the activity may have been due to active impurities in the ACTH-preparation. Frantz et al. (1972) reported ACTH to be inactive in the mouse mammary gland culture.

6.4.7.2. Interaction with testosterone, oestradiol and thyroxin

An interaction between cortisol and either testosterone or thyroxin could not be demonstrated. However, interaction has not been studied for a range of concentrations, but only for single concentrations. Oestradiol potentiated the secretory effect of cortisol, but only to a minor extent. This requires further study. These in vitro results are at variance with some in vivo observations whereby oestrogens and testosterone suppress lactation and thyroxin increases milk yield (see review of Meites 1974).

6.4.7.3. Interaction with progesterone

According to Kuhn (1971) lactogenesis develops in vivo in the rat due to a fall in the progesterone level just before parturition. A high level of progesterone is thought to block lactogenesis. This notion is supported by the observations of Prop (1972) who reports the absence of casein production by explants of immature mouse mammary glands when progesterone is added to the medium even in very low concentrations. Forsythe & Myres (1971) did not detect a progesterone effect on the response to prolactin in rabbit mammary gland explants. A secretory response was still obtained by Uyldert & Villaudy (1967) in the presence of cortisol and progesterone in the
culture of mid-pregnant rat mammary gland. In previous experiments (Peters 1977b) progesterone seemed sometimes to suppress the appearance of traces of secretory activity, which developed when mid-pregnant rat serum was used. The present study does not give further evidence for such an inhibition.

The possibility that progesterone inhibits lactogenesis by antagonizing cortisol has been explored on the fully developed mammary gland of 19 days pregnant rats. Explants of these glands reacted with proliferation to both progesterone and 19 days pregnant rat serum in the presence of insulin. A concentration of exogeneous cortisol as low as 0.25 μg/ml was sufficient to induce a secretory response. No consistent antagonism by progesterone to the effect of this low concentration of cortisol could be demonstrated. Cortisol suppressed to some extent the mitogenic activity of progesterone. Since cortisol also tends to suppress the mitogenic activity of the lactogenic protein hormones rCM, prolactin and hPL, this anti-mitotic effect may be non-specific i.e. the result of an induced tendency towards a non-dividing functional state of the cells.

6.4.8. Relative potency of cortisol, aldosterone, corticosterone and deoxycorticosterone

Dose-response relationships have been determined for four corticosteroids simultaneously on explants of 13 days pregnant rat mammary glands. The choice of medium is important. Rivera (1964) and Turkington et al. (1967) both selected a chemically defined medium with insulin and prolactin added in high concentrations. In our first experiment a similar medium was used. In agreement with the results of Rivera (1964) and Turkington et al. (1967) obtained using mouse mammary explants, deoxycorticosterone was less active than cortisol, corticosterone or aldosterone while the explants were also less well maintained. Rivera (1964) reported that cortisol and corticosterone were equally active, Turkington et al. (1967) found that cortisol and aldosterone were equally active and both more active than corticosterone. The present results on rat mammary explants indicate
that cortisol is more active than either corticosterone or aldosterone, which were of approximately equal potency. Although the relative potency of the four corticosteroids could be estimated using a chemically defined medium, the dose-response relationships were poor and the differences in potency could not be quantified. When a second assay virgin rat serum was added to the medium, better dose-response relationships were obtained. It could be estimated that for a certain secretory response approximately four times less cortisol was needed than either corticosterone or aldosterone and at least sixteen times less than deoxycorticosterone. Not only were higher concentrations of deoxycorticosterone needed to obtain some secretory response, but also with deoxycorticosterone the maximum response seemed to be much less than with cortisol, corticosterone and aldosterone.

The in vitro results agree with the observations made in vivo. Nandi & Bern (1961) showed that the naturally occurring corticosteroid in mice, corticosterone, was less active than cortisol in inducing a secretory response in vivo. A similar conclusion was reached by Talwalker et al. (1961) for the rat mammary gland. Although corticosterone is less potent than cortisol, the morphological changes produced by the two corticosteroids were similar. Therefore the results obtained with cortisol in vitro may be considered relevant for the rat in vivo. The objection could be made that higher concentrations of the less potent corticosterone are needed to obtain a secretory response and that these concentrations could exceed those reported for rat serum in vivo. However, the present results for corticosterone show that a sharp increase of secretory response occurs within a range of corticosterone concentrations present in the serum in vivo.

6.4.9. Change in sensitivity of the mammary gland to cortisol during pregnancy

In agreement with the observations by Rivera & Bern (1961) and Prop (1966) on the mouse mammary gland, the present results
indicate that the stage of mammary development is important for the response to cortisol. The explants from virgin rat mammary glands are less sensitive towards corticosteroids than explants from pregnant rats, in particular after 10 days of pregnancy. Secretion is the factor primarily altered. A similar critical change in sensitivity around day 9 has been observed by Frantz et al. (1972) for the mouse mammary gland. The change may be related to the appearance of rCM in the serum (Shiu et al. 1973; Peters et al. 1976). During the same period the ultrastructure of the mammary cells becomes more differentiated (Njio 1976). Ultimately, the secretory response to cortisol of virgin rat mammary explants cultured with virgin rat serum should be compared with the secretory response to cortisol of late pregnant rat mammary explants cultured with late pregnant serum. This would provide the most relevant information on the total increase of secretory response occurring in vivo. In this study an attempt has been made to determine various contributing factors. An increased response to cortisol late in pregnancy is partly due to the presence of high levels of lactogenic protein hormones in the serum, partly to the existing high level of glandular development. Besides these two factors the sensitivity of the alveolar cells at the end of pregnancy towards cortisol may be increased. However, the demonstration of an increased sensitivity is difficult. First of all there are many difficulties in comparing the results for the virgin explants with those for late pregnancy explants. The total secretion produced by late pregnancy explants is many times that of virgin explants, but this is not necessarily due to an increased sensitivity. The production per individual cell may be the same and the difference in overall secretion is due to the largely increased number of cells in late pregnancy explants. The degree of secretion therefore cannot be taken as an index for the cellular sensitivity to the corticosteroids. An attempt has been made to solve the problem not by measuring the amount of secretion, but by establishing minimum and maximum concentrations necessary for a minimum and maximum response, i.e. a shift in the dose-response relationship.
A second difficulty concerns the choice of hormones to be added to the assay medium. Whereas in vivo the virgin mammary gland has been exposed to low basal levels of hormones, the pregnant rat mammary gland has been exposed to high concentrations of rCM and progesterone. When cortisol is tested in a medium with low hormone concentrations (except for insulin), the situation for explants from pregnant mammary is that of a hormone withdrawal. On the other hand when cortisol is tested in a medium with a high rCM plus progesterone concentration, the virgin mammary becomes exposed to more than one stimulus at once and this may change the secretory response to cortisol. Therefore, the sensitivity was established under various conditions. In a first experiment rCM was left out of the medium by adding virgin rat serum instead of pregnant rat serum while cortisol and the ovarian steroids were added to the medium in excess (the presence of a high concentration of progesterone might have affected the secretory response of e.g. the virgin mammary gland selectively). Under these conditions the degree of the secretory response became increased from approximately day 10 of pregnancy onwards.

In a following experiment the sensitivity of the virgin mammary was compared with 13 and 19 days pregnant mammary explants under optimal conditions, i.e. with insulin, virgin rat serum and prolactin added to the medium in high concentrations. However, under these conditions the explants from the various mammary glands all showed a clear response to 0.16 μg cortisol per ml medium, the lowest concentration tried, and all showed a maximum response, except for vacuolization, at approximately 1.25-2.5 μg cortisol per ml medium. Therefore the experiment failed to demonstrate an increased sensitivity to cortisol for the pregnant mammary gland.

However, a better indication of an increased sensitivity towards corticosteroids was obtained when cortisol was replaced by aldosterone, a less potent corticosteroid for the mammary gland. The effect of aldosterone was determined in a medium both with and without rCM added. In the absence of rCM the virgin explants did not respond even to 5 μg aldosterone per ml
medium, while the pregnant explants already showed some secretory response at 1.3-5 μg/ml. When rCM was present in the medium the response of the virgin rat explants increased sharply in the range from 0.31 to 1.25 μg aldosterone per ml medium. For pregnant rat explants this occurred at concentrations lower than 0.31 μg aldosterone. These results show that in vitro pregnant rat explants react to lower corticosteroid concentrations than virgin rat explants. This indicates that in vivo the mammary gland at the end of pregnancy may respond to changes in corticosteroid concentrations in the serum to which the virgin rat mammary gland is indifferent, partly because the cells are more sensitive and partly because the gland is exposed to high concentrations of rCM, which potentiates the effect of the corticosteroids. The reason for the increased sensitivity at the end of pregnancy may be the high degree of ultrastructural development in the cells (Njio 1976) under the influence of rCM and possibly progesterone.

6.5. Hormonal response of lactating rat mammary gland explants

6.5.1. Problems in culturing lactating mammary gland explants

6.5.1.1. General considerations

The culture of mammary explants obtained from lactating rats poses some special problems. As pointed out by Rivera (1974), at the time of explantation endogenous hormones may still be present in the explanted tissue which ensure, during a limited period, the continuation of the secretory processes in vitro. On the other hand the fully developed secretory apparatus may continue to function even in the absence of hormonal stimulation until involution sets in. Therefore, engorgement of the explants with secretory products shortly after explantation seems unavoidable. In vitro expulsion of the secretory products from the alveolar lumina is impossible. As a consequence stasis of the secretory products occurs and subsequently regression sets in. Therefore, this in vitro model
is not very suitable for establishing the role of various hormones in the function of the lactating rat mammary gland in vivo. In vivo the lactating gland is either drained at regular intervals and hormonally stimulated, or engorged, but in this case not stimulated. A situation comparable to the in vitro model is found only in experimental in vivo situations, such as when one set of lactating glands of a rat is allowed to be sucked while excretion of the other glands is experimentally prevented, or when the nursing rat is weaned and exogenous hormones are administered to test their efficacy in retarding involution. In the present experiments prolactin and cortisol have been tested under comparable conditions in vitro.

6.5.1.2. Technical aspects

Central necrosis develops readily in the mammary explants obtained from lactating rats. The vulnerability of the tissue may be increased late during lactation. To some extent central necrosis can be prevented by reducing the size of the explants. The presence of central necrosis does not seem to alter the hormonal response of the remaining viable tissue, but it complicates the assessment of hormone effects. It becomes difficult to distinguish between secretion and cellular debris in the lumen with routine histological staining, all the more so because the nuclear remnants, which are diagnostic for debris, are dissolved and become less and less distinct with the passage of time. Similarly it is difficult to distinguish between changes caused by involution and those caused by damage of the tissue following explantation. When not too extensive, the necrotic area becomes organized and disappears when the culture period is prolonged, but mammary explants especially from late lactation seem to be less capable of eliminating the areas of necrosis. Lack of oxygen may be one, but not the only cause of necrosis. The degree of luminal engorgement at the time of explantation may also be a factor. There may be a different degree of stasis in different parts of the same gland.
The outer zone of the explant may be drained during the cutting of the tissue and consequently have a better chance to survive. The variability of the material affects the response to various hormones. Consequently a larger number of explants per group than usual is necessary in order to demonstrate statistically significant hormone effects.

6.5.1.3. Choice of medium

The composition of the medium is an important modifier of the response to hormones. By increasing the glucose concentration, the lactate production and lipid production are apparently stimulated. The formation of fat vacuoles can be stimulated to such a degree that it becomes difficult to demonstrate hormonal effects. There is some evidence that under these circumstances mitotic activity is suppressed. A high glucose concentration seems also to stimulate secretion, but this effect is less distinct than the stimulation of vacuolization.

Insulin may disappear from the medium at a high rate during the culture of lactating rat explants. When media with 100 μg/ml insulin - twice the concentration used for the culture of pregnant rat explants - were exposed to lactating rat explants of regular size for 3 days, the media injected subcutaneously into mice did not produce a hypoglycemic effect in contrast to media collected from virgin and pregnant rat mammary cultures (unpublished results, Leeuwin 1972).

6.5.2. Time related changes in a non-stimulating medium (virgin rat serum plus insulin)

6.5.2.1. Secretory activity

On the first day of culture the lumina are filled with material which at least in part seems to represent true secretory products. Large fat vacuoles appear similar to those seen in vivo after weaning, but in vitro they are
deposited less regularly in the cells. The concentration of amino acids in the medium decreases, which is consistent with the synthesis of milk protein on that day. The glucose consumption is high as is the release of lactate. The rise of the free glycerol concentration in the medium is small, suggesting that the breakdown of the triglycerides present in the milk and in the mammary cells (Kinsella 1973) is low on the first day.

On the second day the breakdown of the secretory products sets in gradually. Glucose consumption decreases, possibly reflecting the end of fat synthesis. Glycerol levels in the medium increase, indicating an acceleration of lipolysis. Instead of big fat droplets, there appear multiple small droplets with "degeneration" of fat (recognizable as a less well preserved fixation of the fat by osmium and the appearance of plicae in the fat; unpublished electron microscopic observations). The multiple small fat droplets could explain the reappearance of cytoplasmic opalescence. The remaining large vacuoles are not all fat vacuoles. The increase of the concentration of amino acids in the medium likewise reflects increased proteolysis.

Breakdown of the tissue continues until, after 6 days of culture, the remaining tissue appears dedifferentiated and adipocytes have become prominent. After 12 days the involution seems to be complete for at that time the light microscopic appearance resembles the in vivo involuted gland or the virgin rat mammary gland with a few ducts and sparse, undeveloped alveoli.

6.5.2.2. Mitotic activity

Thymidine incorporation and mitotic activity are low on the first day; they reach maximum values on the second day. A decrease is observed on the third day. Mitotic activity and thymidine incorporation show no predilection for areas with tissue damage. The wave of mitoses on the second day is considered to be an in vitro artefact caused by the high level of
insulin. Because of the breakdown of insulin in vitro, the mitotic pattern may be partly dependent on changes in the insulin concentration during the culture.

6.5.3. Hormone effects

When the effect of various hormones on the development of the involutionary changes was studied, the results indicated that the reaction of the lactating rat mammary gland towards prolactin and cortisol was in many ways comparable to that of the pregnant rat mammary gland. No effect could be demonstrated for progesterone plus oestradiol.

6.5.3.1. Prolactin

Prolactin has a proliferative effect with a stimulation of both thymidine incorporation and mitotic activity. It acts in synergism with insulin. An effect of prolactin on secretory activity in the lactating rat explants could be demonstrated, but it was not marked and seemed to exist merely of a deceleration of the breakdown of the secretory product on the second and third day of culture. Prolactin was not capable of maintaining even a semblance of secretory activity in the lactating mammary explants.

6.5.3.2. Cortisol

Cortisol did maintain the appearance of secretory activity. The difference with the untreated control group was especially distinct when the culture period was extended from 6 to 9 days. In this respect mammary glands obtained at different stages of lactation all showed a similar reaction. Cortisol prevented the disappearance of both vacuolization and intraluminal eosinophilic material. Regression and reorganization of the remaining tissue occur since the end result of 9 days of culture with lactating rat explants was in most respects similar to that obtained with pregnant rat explants or explants from a rat...
weaned for 10 days cultured during 9 days with cortisol.

It remains to be proven that the cortisol effect demonstrated on lactating rat explants is related to galactopoiesis in vivo. An objection is that the light microscopic effect of cortisol on vacuolization did not reach its maximum during the first few days, but only after a culture period of 6 to 9 days. This lag period is difficult to reconcile with an in vivo role for corticosteroids. A possible explanation for the lag period could be that the effect of cortisol involves a selection of newly formed cells or undifferentiated cells which survived trauma and involution. In this case the in vitro effect of cortisol demonstrated is irrelevant for galactopoiesis in vivo. There are, however, indications that cortisol acts already on the first day of culture. Such an effect could not be demonstrated by light microscopy, but the decrease in concentration of amino acids in the medium was significantly greater in the presence of cortisol and this could be interpreted as the result of an increased milk protein synthesis. Some caution is needed with this interpretation. The effect on amino acid concentration may be due to other, aspecific effects of cortisol on the explants, e.g. a suppression of the outgrowth of the explant. Similarly cortisol might suppress lysosomal activity and thereby proteolysis. Such an aspecific effect could just as well explain the effect of cortisol on amino acid concentration.

While an effect of cortisol is not yet distinct on the first day of culture, an effect on vacuolization and secretion can be demonstrated by light microscopy as early as the second and third day. A better maintenance of the fat globules and an inhibition of the free glycerol release into the medium can be demonstrated too on these days. Cortisol did not suppress the wave of mitotic activity in the lactating rat explants.

So far as secretory activity is concerned prolactin and other lactogenic protein hormones like rCM acted in synergism with cortisol on virgin and pregnant rat mammary explants. A similar synergism could not be shown for the lactating rat mammary explants. When virgin rat serum was replaced by
lactating rat serum - which could conceivably contain factors essential for milk production - the effect of cortisol was identical with both sera. Replacing prolactin by another kind of lactogen, i.e. rCM (pregnant rat serum), had no effect on the lactating rat explants, while at the same time an effective synergism between rCM and cortisol could still be demonstrated on the mammary explants of the 21 days pregnant rat, selected as a control. Possibly the low endogenous prolactin concentration in the lactating rat explants themselves or in the virgin rat serum added to the medium, is sufficient for the effect of cortisol on the lactating rat explants. More likely the assay method used may be inadequate to demonstrate subtle effects.

6.6. Stem cells

Turkington & Kadohama (1972) have proposed a sequence for the proliferation of mammary alveolar cells in vitro. This sequence starts with the division of an undifferentiated "stem cell". Mills & Topper (1970) state that cell differentiation is coupled with mammary differentiation. These papers seem to imply that in vitro cell division is limited to undifferentiated cells, i.e. "stem cells".

In vivo autoradiography of lactating mammary cells has shown that DNA synthesis and milk secretion can take place simultaneously in the same cell (Traurig 1967). Traurig (1967) rejects the possibility that increased DNA turnover, endogenic DNA duplication and polyploidy may explain the labelling of the cells, since these phenomena do not occur in mammary glands (cf. Persson, 1960). However, these phenomena have been studied intensively during the last decade and much more information has become available (Kiefer & Sandritter 1976), which makes a reinvestigation of mammary alveolar cells necessary. In vitro in a lactating rat mammary gland a labelled cell probably divides only once and cells incorporating $^3$H-thymidine form such a heterogeneous population that it is improbable that all cells incorporating $^3$H-thymidine are in a stage preliminary to division (J. van Marle, unpublished results).
The history of a cell can be guessed from the presence of secretory products from a previous secretory stage (cf. Feltkamp 1972). Therefore the presence of secretory products was investigated in dividing mammary cells, both in vitro and in vivo material. It was found that protein granules as well as lipid globules were present in the cytoplasm of dividing cells. In this respect dividing cells did not appear to differ from adjacent non-dividing cells. Both types of cell possess the same organelles, the same protein granules and lipid globules to a comparable degree.

The differentiation of an alveolar cell is apparent from the presence of protein granules and lipid globules in the cytoplasm. Turkington & Kadohama (1972) assume that mid-pregnancy mouse mammary gland contains a mixture of cell types, varying from undifferentiated to fully differentiated cells. Mills & Topper (1970) described the presence of a few well developed alveoli with cells containing secretory products. At day 14 protein granules and fat droplets were more uniformly present. This was also found in the mammary gland of the 13 days pregnant rat (Njio 1976). In this case only a few cells can be described as undifferentiated, while most cells are differentiated and contain protein granules and/or fat globules. These premature secretory products develop abundantly in the cells at approximately day 9-10 of pregnancy, at a time when the mammotrophic activity of rCM appears in the maternal serum (Shiu et al. 1973, Peters et al. 1976).

Considering the presence of protein granules and lipid globules, indicating a certain degree of differentiation, in the cytoplasm and the results of $^3$H-thymidine incorporation in such a varying cell population, it becomes apparent that the stem cell concept is too rigid. It appears that a mitotic response is not restricted to undifferentiated cells (i.e. stem cells) but that (partly) differentiated cells as well can respond to a mitogenic factor in the serum.
6.7. Sequence of actions of various hormones in vitro

Conclusions reached concerning the sequence of hormonal actions are summarized in fig. 47. The sequence presented is obtained in vitro for a medium with a sufficient amount of virgin rat serum and a high insulin concentration. When in this type of medium the concentration of "lactogenic" protein hormones and/or progesterone is increased, cell division is stimulated. In agreement with Topper (1970) and Turkington (1972) an intermediate step is assumed between cell division and full secretory function. It represents the transformation of an undifferentiated cell, possibly after an obligatory division, towards a differentiated cell with an ultrastructure adapted to a future secretory function. A high concentration of "lactogenic" protein hormones causes the stage of differentiation. A concomitant high progesterone concentration does not inhibit the differentiation. Electron microscopy shows that the differentiated ultrastructure of the cells is actually maintained better in vitro by the combination of rCM with progesterone than by either one of these hormonal factors separately (Njio unpublished observation). A high "lactogenic" protein hormone concentration is not sufficient by itself to cause the transformation of a differentiated cell into an actively secretory cell. An increase in corticosteroid concentration is necessary for this final step. The "lactogenic" protein hormones potentiate the effect of the corticosteroids. In the present in vitro study progesterone did not inhibit the induction of the secretory response by the corticosteroids. The schematic representation in fig. 47 does not take into account the situation in which a high corticosteroid concentration is combined with a low lactogenic protein hormone concentration. This latter combination seems only relevant for the experimental situation in which high doses of corticosteroids are administered to the animals in vivo. The present in vitro results indicate that under these conditions high corticosteroid levels can cause the transformation of an undifferentiated cell into a fully developed, but submaximally operating secretory cell. The same
Fig. 47  Diagrammatic representation of the sequence of hormonal action on rat mammary gland in vitro simulating the development during pregnancy. The stages of in vitro development include proliferation, morphological differentiation and secretory activity. The scheme does not imply that cell division is an obligatory step towards secretory activity. The sequence shows the effects obtained in a medium containing a sufficient concentration of rat serum and insulin. The question mark besides "TARGET" indicates uncertainty about the stage of development at which cells become a target for the mitogenic hormones. The specific mitogenic hormones for the mammary cells are placental lactogens, prolactin and progesterone. The role of growth hormone is not well established. Morphological differentiation and secretory activity are indicated by the presence of fat (shaded circles) and secretory protein granules (dots). At the stage of morphological differentiation fat globules are numerous but small and protein granules rare and located intracellularly. The stage of differentiation is promoted by the placental lactogens, prolactin and possibly growth hormone. Their action is increased by corticosteroids. The corticosteroids promote secretory activity, thereby potentiated by the lactogenic hormones.
sequence of hormonal action is put forward tentatively to summarize events in lactating rat explants.

6.8. Relevance of in vitro results for the situation in vivo

In vitro results are relevant (a) when the morphological changes induced in vitro resemble those occurring in vivo, (b) when hormonal effects in vitro are obtained at concentrations which occur in the serum in vivo and (c) when changes in mammary gland morphology are associated in vivo with changes in the serum concentrations in vivo of those hormones shown to produce similar changes in mammary explants in vitro.

6.8.1. Morphology

Njio (1976) compared the in vitro ultrastructural changes with those in vivo during pregnancy and lactation in the rat. Except for quantitative differences the light and electron microscopic morphology of the cultured explants of pregnant rats after appropriate hormonal treatment for 6 days resembled the morphology of the mammary gland in vivo at the time of parturition. It was not possible to obtain in vitro the morphological changes found during lactation and weaning in vivo.

6.8.2. Effective concentrations

Rat chorionic mammotrophin (pregnant rat serum), human placental lactogen and prolactin have been shown to be active in vitro at concentrations encountered in the serum in vivo, as discussed previously. This has not been the case for growth hormone and even less so for ACTH. The minimal concentrations of insulin and testosterone active in vitro have not been determined. Of the active steroid hormones both progesterone and corticosterone exerted an effect at concentrations found to occur in vivo. In the case of aldosterone the evidence remains equivocal.
6.8.3. Correlation between mammary development and hormone serum levels in vivo

Porter (1974), Ceriani (1974) and Banerjee (1976) among others have commented on the discrepancy between the in vitro sequence of hormone action as proposed by e.g. Turkington (1972) and the in vivo situation. Our in vitro results for hormone activity can explain changes in mammary development in vivo and they can be correlated with concomitant changes in hormone serum concentrations. They suggest that the proliferation of the rat mammary gland is under control of the serum prolactin and/or placental lactogen and, in addition, progesterone. In this respect the lactogenic protein hormones are more powerful than progesterone. However, the effect of the combination of protein hormones with progesterone should be taken into account separately. There is mutual potentiation which may be of significance for certain in vivo situations, such as pregnancy, when the concentrations of both the protein hormones and progesterone are increased. The correlation between these in vitro results and the stages of proliferation which occur in vivo suggests various explanations for development of the mammary gland occurring at different times in the lifespan of the rat. In the newborn rat this could be (partly) the result of placental lactogen in the amniotic fluid (Peters 1977a) in addition to an incipient function of the pituitary gland (see review of Ceriani 1974). An increase of progesterone and prolactin serum concentrations could account for mammary growth at puberty (see review of Ceriani 1974). The same combination of hormones could be responsible for the mammary growth during the first week of pregnancy, but growth during the last two weeks of pregnancy may be the result of placental lactogen in the rat. This would support – at least for the rat – the original theory of Halban in 1904/1905 (see review of Forsyth 1974), in which the placenta is said to control the mammary growth during pregnancy. The low prolactin serum levels in the rat during pregnancy (Amenomori et al. 1970) must add little to the effect of the high placental lactogen level during
the last two weeks of pregnancy. The same cannot be said with certainty for progesterone which may act on a different receptor. Otherwise progesterone may have little importance for the mammary growth late in pregnancy since ovariectomy at that stage does not affect mammary growth (see review by Ceriani 1974) and since the serum retains its mammotrophic activity after ovariectomy (Peters & van Marle 1976). With respect to the proliferation of the lactating rat gland this gland is still sensitive to the mammogenic effect of prolactin. This hormone may be important, but the roles of growth hormone and progesterone have not been established in the present study. Organ culture of lactating rat mammary explants seems to be an inappropriate technique.

6.8.4. Lactogenesis

In vitro secretory activity is induced by a family of corticosteroids. This suggests that one of the members may play a similar role in vivo, in the lactogenesis at the time of parturition. Results obtained with cortisol are applicable for corticosterone, the naturally occurring hormone in the rat, which in vitro differs from cortisol essentially only in the possession of a lower activity. In vitro corticosteroids may exert at least a dual effect: one may be non-specific, such as improving the viability of the mammary cells; the other may be specific, such as affecting lactogenesis and the appearance of secretory products. Concerning the first effect electron microscopy has confirmed an improvement of the viability of explants cultured in 100% Trowell's T8 with cortisol (Njio 1976). This non-specific effect of cortisol is, however, not observed when the explants are cultured under more optimal conditions, i.e. in a medium containing rat serum. Concerning the second effect, i.e. secretory activity, the present results indicate that the effect of the corticosteroids on secretory activity cannot be explained solely as a "permissive" or "modulating" effect (Rivera 1974). Apart from the question whether or not there is a lactogenic corticosteroid effect in vivo it can be said at
least that this lactogenic effect is triggered by a corticosteroid in vitro. Relevance for the in vivo situation depends upon whether an increase of the corticosterone (plus aldosterone) concentration occurs at about the time of parturition. ACTH and corticosterone serum concentrations which are low in the pregnant rat increase at the time of parturition (see Review of Meites 1974).

Sensitivity of the mammary cells may become important at this stage. Corticosteroids can be shown to be similarly active in the rat in vivo since severe stress can induce lactogenesis in this species after estrogen priming (Nicoll et al. 1969). The present in vitro results support the in vivo observations. A sharp increase in in vitro response occurs in a range of concentrations demonstrated to occur in the rat in vivo. A corticosterone concentration shown to be present in vivo on the day of parturition (Voogt et al. 1969) induces secretory activity in vitro.

The increase of the corticosterone concentration at about the time of parturition has been judged by Kuhn (1971) to be insufficient to account for the onset of lactogenesis. Conclusions on this point are, however, complicated by the fact that the sensitivity of the mammary gland at the time of parturition is not taken into account. At the time of parturition the dose-response relationship for corticosterone appears to have shifted to lower concentrations. Firstly, the mammary gland shows an increased sensitivity to corticosteroids, while secondly the sensitivity is further increased by the presence of (still) high concentrations of placental and pituitary lactogenic protein hormones. The latter synergistic effect has also been observed in vivo (Meites et al. 1963). Therefore, even a relatively small increase of corticosterone (plus aldosterone) concentration may become a factor which triggers lactogenesis at about the time of parturition after the placental and pituitary hormones "have caused the readiness to lactate just short of full milk secretion" (Josimovich et al. 1974). A similar increase in corticosterone concentration would be ineffective in the mammary gland of the virgin rats, or rats.
early in pregnancy. The role of progesterone in lactogenesis has been stressed by Kuhn (1971). The indication for an inhibitory effect of progesterone on the onset of secretory activity in vivo could not be confirmed in vitro. There would be less need for a lactogenesis-inhibiting hormone during pregnancy if it is accepted that the so-called lactogenic protein hormones derived from the placenta and pituitary gland are predominantly involved in growth and differentiation and not in the induction of full secretory activity. However, a fall in progesterone concentration may be one factor in the timing of lactogenesis.

6.8.5. Lag period of cortisol effect

With respect to the importance of corticosteroids for lactogenesis in vivo, one in vitro observation needs explanation. The in vitro corticosteroid effect shows a lag period. This complication has already been discussed for the lactating rat explants. If acute changes in the corticosterone serum concentrations were of importance for lactogenesis in vivo, a rapid and fairly instantaneous response should be demonstrable in vitro. The stage of pregnancy could be a decisive factor for the reactivity of the mammary tissue. "Immaturity" could explain a delay in reaction of the two to three days in the still not fully developed mammary gland of the 13 days pregnant rat. However, even though explants from a 19 days pregnant rat show a shorter lag period, it still takes one to two days for a fully developed reaction while the appropriate serum (of 21 days pregnant rats) had been added to the medium. This serum collected on the day before parturition and at about the time of lactogenesis has still appreciable mammotrophic activity (Peters et al. 1976), while the progesterone level can be expected to be low (Wiest 1970). As regards the lag period, the speed of reaction of explants taken on the very day lactogenesis starts in vivo (21 to 22 days of pregnancy) would be the most suitable model to test the question of the lag period. However, this material is too vulnerable to the trauma of ex-
plantation to be of use (unpublished observation). Any lag period in reaction of the surviving cells in 21 - 22 days of pregnancy explants can be interpreted as due to the adaptation to in vitro conditions or recovery from the trauma of dissection (Rivera 1974).

The possibility should be considered that the lag period of the corticosteroid effect is an in vitro artefact caused by a mitotic wave. The wave of mitotic activity in the explants after the first day of culture even in the presence of corticosteroids may prevent temporarily the development of a secretory response. Assuming that this wave is an in vitro artefact caused by the high concentration of insulin, a culture of 19 or 21 days pregnant explants without the addition of insulin may be suited to establish the effect of the mitotic wave on the onset of secretory activity. However, this was technically not feasible (unpublished results). Explants from this stage of pregnancy were extremely sensitive to lack of sufficient insulin in the medium. A widespread degeneration set in before a secretory response developed.
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