Cyclic AMP as a negative regulator of hormonally induced lactogenesis in mouse mammary gland organ culture

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ABSTRACT In organ cultures of mammary glands from mice in midpregnancy, addition of both insulin and prolactin induces a marked accumulation of α-lactalbumin, whereas the augmentation of casein synthesis requires the presence of insulin, prolactin, and cortisol. Addition of 0.5 mM dibutyryl cyclic AMP resulted in complete inhibition of α-lactalbumin accumulation and partial inhibition of casein synthesis. Furthermore, either cholera toxin at 0.1–0.4 μg/ml (a stimulator of adenylyl cyclase) or 3-isobutyl-1-methylxanthine (an inhibitor of phosphodiesterase) in combination with 2 mM cyclic AMP, produced a similar pattern of inhibition of α-lactalbumin and casein synthesis in cultured tissue. During culture of mammary explants in medium containing no hormone, or insulin alone, or insulin, prolactin, and cortisol, the tissue content of cyclic AMP decreased rapidly, reaching half the initial level in 24–48 hr. These results indicate that cyclic AMP plays “negative” regulatory function in hormonal induction of milk protein synthesis during the development of the mammary gland.

Terminal development of the mammary gland involves two major events: extensive proliferation of the epithelial cells during pregnancy, and the subsequent synthesis and secretion of specific milk components during the period of lactation. The transition from the growth to the lactational state at parturition is a complex process controlled by multiple interactions of various steroid and peptide hormones. Previous studies (for reviews, see refs. 1–4) have shown that synergistic actions of insulin, cortisol, and prolactin can induce precocious formation of the milk proteins casein and a-lactalbumin in nonlactating mouse mammary gland in culture, indicating that these hormones act as positive controlling factors in the initiation of lactogenesis. These results, however, do not rule out the possibility that the “switch-on” mechanisms for lactogenesis also involve disappearance of negative controlling factors during the normal transition period in vivo or in cultured tissue as a result of the translocation to an organ culture system.

Numerous studies (for review, see refs. 5–7) have documented that cyclic AMP plays a central role in hormonal regulation of cell growth and function. It has been reported (8–11) that the content of cyclic AMP in the mammary gland increases progressively during the growth stage of pregnancy and then, near parturition, decreases rapidly to the low levels seen during the lactational period. This raises the possibility that cyclic AMP acts as a negative controlling factor for lactogenesis. In studies with rat mammary gland explants in culture, cyclic AMP has been shown to inhibit the increase of enzymes associated with lipogenesis and to depress the synthesis of DNA, RNA, and fatty acids but not of the milk protein casein (12). Majumder and Turckington (13) also did not observe any effect of cyclic AMP on hormonal induction of milk protein synthesis in cultured mammary gland from mice in midpregnancy. Thus, the role of cyclic AMP in hormonal induction of milk protein synthesis remained unclear.

In recent studies (14, 15) of the dose–response relationship between cortisol and the formation of casein and α-lactalbumin in cultured mouse mammary gland, it was found that cortisol can exert dose-dependent stimulatory and inhibitory effects on the synthesis of the two milk proteins. Earlier studies (12, 13) of the action of cyclic AMP on the synthesis of milk proteins in organ culture used an inhibitory concentration (5 μg/ml) of cortisol. In the present study, we reexamined the involvement of cyclic AMP in the induction of casein and α-lactalbumin in this system. By the use of different hormone combinations and a decreased concentration of cortisol, compared to previous studies (12, 13), we have been able to identify cyclic AMP as a negative controlling factor for the induction of milk protein synthesis.

MATERIALS AND METHODS

Materials were obtained from the following sources: [14C]UDP galactose (290 mCi/mmol; 1 Ci = 3.7 × 10^10 becquerels), [3,4,5-3H]leucine (170 Ci/ml), 3H-labeled L-amino acid mixture (1 mCi/ml), and Protosol (tissue solubilizer) from New England Nuclear; medium 199 (Hanks’ salts) and Dulbecco’s phosphate-buffered saline (Ca2+ and Mg2+ free) from GIBCO; bovine galactosyl transferase (3–5 units/mg) and 3-isobutyl-methylxanthine (IBMX) from Sigma; UDP galactose, dibutyryl cyclic AMP, cyclic AMP, 3′-AMP, 5′-AMP, ADP, ATP, cyclic GMP, and a cyclic AMP assay kit from Calbiochem; GTP from Boehringer Mannheim; AG 1-X2 ion exchange resin from Bio-Rad; Hydrofluor, a liquid scintillation cocktail, from Research Products International Corporation; anti-rabbit IgG (goat) from Miles Yeda Limited; cortisol from ICN; crystalline pig insulin from Lilly; and bovine prolactin (NIH B4) from the Hormone Distribution Program, National Institute of Arthritis, Metabolism, and Digestive Diseases. Cholera toxin was a generous gift from M. Vaughan (National Heart, Lung, and Blood Institute). CSH/HeN mice in the 12th–14th day of their first pregnancy were obtained from the Animal Breeding Facility of the National Institutes of Health.

Mammary gland explants were prepared from abdominal glands and cultured as described (16). The concentrations of hormones used in the culture medium were: insulin, 5 μg/ml; cortisol, 3 μM; and bovine prolactin [free of contaminating vasopressin and oxytocin (15)], 5 μg/ml.

The rate of casein synthesis was determined by allowing cultured explants to incorporate 3H-labeled amino acid mixture (40 μCi/ml) for 4 hr at the indicated times of cultures. Explants (8–10 pieces, each weighing about 1 mg) were then harvested.

Abbreviation: IBMX, 3-isobutyl-1-methylxanthine.

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weighed, and homogenized with 50 vol (wt/vol) of phosphate-buffered saline containing 2% (wt/vol) Triton X-100. The homogenate was centrifuged at 105,000 × g for 1 hr and the resultant supernatant was used for subsequent immunoprecipitation of casein. Indirect immunoprecipitation of 3H-labeled casein was performed as described (15) using anti-mouse casein antiserum raised in rabbit against purified mouse milk casein (17) and goat anti-rabbit IgG antiserum. The washed immunoprecipitates were dissolved in 0.8 ml of Protosol and assayed for radioactivity with a toluene-based scintillation cocktail.

The rate of α-lactalbumin synthesis was determined by direct immunoprecipitation with rabbit antiserum against purified mouse α-lactalbumin, as described (18). The amount of α-lactalbumin in cultured explants was determined by a modification of the method of Fitzgerald et al. (19) with exogenous bovine galactosyl transferase and pure mouse α-lactalbumin as a standard (18). The amount of cyclic AMP in cultured explants was determined with a cyclic AMP radioimmunoassay kit, as described (20).

RESULTS

To determine the effects of cyclic AMP on milk protein synthesis in mammary cells, tissue explants prepared from mice in midpregnancy were cultured for 48 hr on media containing several combinations of insulin, prolactin, cortisol, and the cyclic nucleotides. The amount of α-lactalbumin in cultured explants was increased to a maximum by the addition of insulin and prolactin, whereas in cultures with insulin alone or insulin, prolactin, and cortisol, the level of α-lactalbumin was low (Table 1). The addition of 0.5 mM dibutyryl cyclic AMP together with insulin and prolactin completely inhibited the hormonally induced increase in α-lactalbumin. This inhibition appeared to be specific to the cyclic nucleotide: cyclic AMP also caused a partial inhibition, but sodium butyrate, 3′-AMP, 5′-AMP, ATP, ADP, and cyclic GMP, each at 2 mM (data not shown), exerted no inhibitory effect. In contrast to α-lactalbumin, casein synthesis in cultured explants was stimulated maximally by the addition of insulin, prolactin, and cortisol. Moreover, the addition of dibutyryl cyclic AMP or cyclic AMP caused only a 56% inhibition of the hormonal stimulation of casein synthesis. 3′-AMP, 5′-AMP, and cyclic GMP, each at 2 mM (data not shown), and sodium butyrate were not inhibitory. The extent of casein synthesis in cultured tissue varied among different cultures, probably reflecting the difference in the developmental stage of tissue used for organ culture.

### Table 1. Effect of dibutyryl cyclic AMP on hormonal stimulation of α-lactalbumin accumulation and casein biosynthesis in mammary gland explants in culture

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>α-Lactalbumin, ng/mg tissue</th>
<th>Casein, cpm/mg tissue/4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>5.2</td>
<td>2,230</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>15.2</td>
<td>10,800</td>
</tr>
<tr>
<td>Insulin + prolactin + cortisol</td>
<td>3.0</td>
<td>12,500</td>
</tr>
<tr>
<td>Insulin + prolactin + dibutyryl cyclic AMP</td>
<td>4.2</td>
<td>4,800</td>
</tr>
<tr>
<td>Insulin + prolactin + cyclic AMP</td>
<td>10.6</td>
<td>8,200</td>
</tr>
<tr>
<td>Insulin + prolactin + butyrate</td>
<td>16.5</td>
<td>11,000</td>
</tr>
<tr>
<td>Insulin + prolactin + cortisol</td>
<td>7.5</td>
<td>9,800</td>
</tr>
</tbody>
</table>

Mammary explants from mice in midpregnancy were cultured for 48 hr in medium containing the indicated combination of insulin, cortisol, prolactin, dibutyryl cyclic AMP (0.5 mM), cyclic AMP (2 mM), and sodium butyrate (0.5 mM). Each value represents the mean of duplicate determinations varying less than 7%.

The effect of various concentrations of dibutyryl cyclic AMP on the hormonal stimulation of the accumulation of α-lactalbumin and of casein synthesis in mammary explants is shown in Fig. 1. The inhibitory effect of the cyclic nucleotide with respect to the production of both α-lactalbumin and casein was maximal at 0.5 mM; the levels of these products were decreased by about 90% and 35%, respectively, compared to cultures without dibutyryl cyclic AMP.

To extend the above observations made after a single 48-hr period of incubation, a more detailed time-course study of the effect of dibutyryl cyclic AMP on the accumulation of α-lactalbumin and casein synthesis in cultured explants was carried out. The tissue content of α-lactalbumin remained at a low level throughout the 3-day culture with insulin alone; with insulin, prolactin, and dibutyryl cyclic AMP, or with insulin, prolactin, and cortisol plus dibutyryl cyclic AMP (Fig. 2A). The accumulation of α-lactalbumin increased continuously in explants cultured with either insulin and prolactin or insulin, prolactin, and cortisol, although the increase in the latter culture system was always less during the 3-day period but varied somewhat among different cultures. Delayed addition of dibutyryl cyclic AMP to the culture with insulin and prolactin (at 24 or 48 hr) prevented further increases in α-lactalbumin (data not shown).

The rate of casein synthesis in mammary explants cultured with insulin alone decreased rapidly to a low level during the 3-day period (Fig. 2B). In contrast, in the presence of insulin and prolactin, or insulin, prolactin, and cortisol, the rate of casein synthesis after 2 days was increased by approximately 2.5-fold and 3.5-fold, respectively, over the initial level and began to decline by day 3. The addition of dibutyryl cyclic AMP with insulin and prolactin prevented any increase in casein synthesis which remained at the initial level for 2 days and then decreased on the 3rd day. In explants cultured with insulin, prolactin, and cortisol, the addition of dibutyryl cyclic AMP inhibited the increase in casein synthesis by about 30-35% in

![FIG. 1. Effect of various concentrations of dibutyryl cyclic AMP on hormonal stimulation of α-lactalbumin accumulation and casein biosynthesis in mammary gland explants in culture. Tissue explants from mice in midpregnancy were cultured for 48 hr in medium containing the following combinations of insulin, cortisol, prolactin, and dibutyryl cyclic AMP: insulin alone (○); insulin, prolactin, and the indicated concentrations of cyclic nucleotide (●) in experiments for α-lactalbumin accumulation; insulin plus cortisol (△) or insulin, cortisol, prolactin, and the indicated concentrations of the cyclic nucleotide (▲) in experiments for casein biosynthesis. Each point is the mean of two or three separate determinations which varied less than 7% for each point. The data represent one of several similar experiments that gave essentially the same results.](image-url)
the first 2 days of culture, but on day 3 the inhibitory effect of the cyclic nucleotide was less apparent. The degree of inhibition of casein synthesis by the cyclic nucleotide in culture with insulin, prolactin, and cortisol could not be altered by frequent replenishment of culture medium during incubation.

IBMX, a known inhibitor of phosphodiesterase (21, 22), augmented the inhibitory effect of 2 mM cyclic AMP on the production of α-lactalbumin and casein in mammary explants cultured in the presence of hormones (Table 2). In tissue cultured for 48 hr with insulin and prolactin, the addition of cyclic AMP or 0.01 mM IBMX alone inhibited the accumulation of α-lactalbumin by 45 and 35%, respectively, whereas the addition of the two agents together almost completely prevented the increase in α-lactalbumin. The synergistic inhibitory effect of cyclic AMP and IBMX was not apparent when 0.5 mM IBMX was used because this concentration of IBMX alone caused total inhibition of the hormonally stimulated α-lactalbumin accumulation.

The hormonal induction of casein synthesis in mammary explants was also inhibited by the addition of 0.5 mM IBMX to cultures with insulin and prolactin or with insulin, prolactin, and cortisol. Here, too, the inhibitory effect of IBMX was synergistic with that of cyclic AMP, resulting in nearly 50% inhibition of the increase in casein synthesis that occurred in the absence of the drug. The optimal inhibitory concentration of IBMX was higher in the case of casein synthesis (i.e., 0.5 mM) than in the case of α-lactalbumin (i.e., 0.1 mM) (data not shown).

Cholera toxin is widely recognized as a stimulus for adenylate cyclase (23). The addition of increasing concentrations of cholera toxin to culture medium containing both insulin and prolactin caused progressive decreases in the amount of α-lactalbumin (Fig. 3). The inhibitory effect of the toxin was apparent at a concentration as low as 0.001 μg/ml and was maximal at a concentration above 0.1 μg/ml. In similar experiments not presented here, cholera toxin at 0.1–1.0 μg/ml also produced about 35% inhibition of casein synthesis in mammary explants cultured with insulin, prolactin, and cortisol.

It was previously shown (24, 25) that, in explants cultured in media containing insulin, a single round of mammary epithelial cell proliferation occurs prior to the increase in milk protein synthesis and that the addition of prolactin can stimulate casein synthesis in the postmitotic daughter cells that formed in explants cultured with insulin and cortisol. To determine whether the inhibitory action of cyclic AMP on milk protein synthesis involves the cell cycle traversal, the effect of cholera toxin on casein synthesis in such postmitotic cells was examined. The addition of increasing concentrations of cholera toxin caused progressive inhibition of the prolactin-dependent increase in casein synthesis (Fig. 4). At 0.1–1.0 μg/ml, the increase in casein synthesis was inhibited by 40%. As in the studies with IBMX, casein synthesis was somewhat more refractory to the inhibitory action of cholera toxin than was α-lactalbumin.

The results presented above indicate that the hormonal

![Image](https://example.com/image.png)

**Fig. 2.** Time course of the accumulation of α-lactalbumin (A) and casein biosynthesis (B) in mammary explants in culture. Tissue explants from mice in midpregnancy were cultured in medium containing insulin alone (○), insulin and prolactin (●), insulin, prolactin, and 0.5 mM dibutyryl cyclic AMP (■), insulin, prolactin, and cortisol (▲), or insulin, prolactin, cortisol, and 0.5 mM dibutyryl cyclic AMP (△). Each point represents an average of duplicate determinations which varied less than 10%.

**Table 2.** Effect of IBMX on the accumulation of α-lactalbumin and casein biosynthesis in mammary gland explants in culture

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>α-Lactalbumin, ng/mg tissue</th>
<th>Casein, cpm/mg tissue/4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>4.0</td>
<td>3,700</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>20.0</td>
<td>15,000</td>
</tr>
<tr>
<td>Insulin + prolactin + 0.01 mM IBMX</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>Insulin + prolactin + cyclic AMP</td>
<td>13.0</td>
<td>11,300</td>
</tr>
<tr>
<td>Insulin + prolactin + cyclic AMP + 0.01 mM IBMX</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Insulin + prolactin + 0.5 mM IBMX</td>
<td>4.0</td>
<td>11,000</td>
</tr>
<tr>
<td>Insulin + prolactin + 0.5 mM IBMX + cyclic AMP</td>
<td>3.0</td>
<td>8,200</td>
</tr>
<tr>
<td>Insulin + prolactin + cortisol</td>
<td>23,000</td>
<td></td>
</tr>
<tr>
<td>Insulin + prolactin + cortisol + 0.5 mM IBMX</td>
<td>13,000</td>
<td></td>
</tr>
<tr>
<td>Insulin + prolactin + cortisol + cyclic AMP</td>
<td>20,000</td>
<td></td>
</tr>
<tr>
<td>Insulin + prolactin + cortisol + cyclic AMP + 0.5 mM IBMX</td>
<td>8,300</td>
<td></td>
</tr>
</tbody>
</table>

Mammary explants from mice in midpregnancy were cultured for 48 hr in medium containing the indicated combinations. Cyclic AMP was at 2 mM; other details were as described in the legend to Table 1. Each value represents the mean of duplicate determinations varying less than 5%.

**Fig. 3.** Effect of various concentrations of cholera toxin on hormonal stimulation of α-lactalbumin accumulation in mammary gland explants in culture. The amount of α-lactalbumin in mammary tissue from mice in midpregnancy was determined after 48 hr of culture in medium containing insulin (○) or insulin, prolactin, and the indicated concentrations of cholera toxin (●). Each point is the mean of two or three separate determinations which varied less than 7% for each point.
stimulation of α-lactalbumin accumulation is more sensitive to the inhibitory effect of the cyclic nucleotide than is that of casein synthesis. Because the amount of α-lactalbumin was measured by an enzymatic assay, whereas casein synthesis was assessed by an immunological method, it is conceivable that the observed difference in the action of cyclic AMP might be due to the difference in the method of measurement of the two proteins. The effect of dibutyryl cyclic AMP on the rate of synthesis of α-lactalbumin in cultured explants was therefore examined immunologically. On the second day of culture in the presence of insulin and prolactin, the rate of α-lactalbumin synthesis was increased more than 30-fold over that in culture with insulin alone. In the presence of the cyclic nucleotide, the hormonally induced synthesis of α-lactalbumin was totally inhibited (Table 3).

To pursue further the possibility that cyclic AMP may be involved, as a negative controlling factor in the regulation of milk protein synthesis in mammary cells, it is of interest to determine the rate of cyclic AMP in explants cultured in hormone-free medium decreased rapidly.

Table 3. Effect of dibutyryl cyclic AMP on the rate of synthesis of α-lactalbumin in mammary gland explants in culture

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Rate of α-lactalbumin synthesis, cpm/mg tissue/2 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Insulin + prolactin</td>
<td>300</td>
</tr>
<tr>
<td>Insulin + prolactin+ dibutyryl cyclic AMP</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Mammary explants from mice in midpregnancy were cultured for 48 hr in medium containing the indicated combination of insulin, prolactin, and dibutyryl cyclic AMP. The rate of α-lactalbumin synthesis was determined immunologically.

within 6 hr, reaching 40% of the initial level (Table 4). Furthermore, the addition of insulin or insulin, prolactin, and cortisol accelerated the decline in the tissue level of cyclic AMP which was decreased to 50% and 35% of the initial level, respectively, at 6 hr and thereafter remained low.

**DISCUSSION**

The experimental evidence presented in this paper provides strong support for the view that cyclic AMP acts as a negative factor for the hormone-dependent differentiation of the mammary gland in vitro. It was shown that cyclic AMP, as well as its dibutyryl derivative, could inhibit the hormonally induced synthesis of casein and α-lactalbumin in cultured mammary explants. The production of the two milk proteins was also inhibited by both cholera toxin [a stimulator of adenylate cyclase (23)] and IBMX [an inhibitor of phosphodiesterase (21, 23)]. It was also shown that the tissue content of cyclic AMP decreased rapidly during culture of mammary explants in hormone-free medium and that the addition of insulin, cortisol, and prolactin accelerated the decline in cyclic AMP level. Sapag-Hagar and Greenbaum (8) showed that the tissue content of cyclic AMP in the rat mammary glands follows a biphasic pattern—i.e., a continuous increase throughout pregnancy and a rapid decline during lactation. Furthermore, there is a corresponding change in the activities of adenylyl cyclase and phosphodiesterase (8).

The transition between the increase and decrease of cyclic AMP in mammary glands coincides in time with the change in the metabolic activity of the tissue from growth to lactation (8–11). In cultured guinea pig mammary glands, Loiizzi et al. (26, 27) observed that dibutyryl cyclic AMP and IBMX impair the rate of production of lactose. In addition, Speake et al. (28) have shown that the cyclic nucleotide decreases fatty acid synthetase activity in rabbit mammary cultures. These observations, together with our present data, strongly suggest that the initiation of the lactogenic process may involve an obligatory decline in cyclic AMP level to reverse a negative controlling factor.

The present studies of the inhibitory action of cyclic AMP on the synthesis of casein and α-lactalbumin indicate that the regulatory mechanisms involved in the production of the two proteins may be different. Casein synthesis appears to be more refractory to the inhibitory action of cyclic AMP, exhibiting only partial inhibition, whereas α-lactalbumin accumulation was almost completely prevented by dibutyryl cyclic AMP, cholera toxin, and IBMX. Different patterns of response of the two milk proteins were also observed in previous studies of the action of cortisol (14, 15), progesterone (29), and thyroid hormone (30) on the development of mammary gland in organ culture. Studies in vivo (29, 31) also demonstrated that there are asynchronous increases in both the synthesis and the mRNA

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Cyclic AMP, pmol/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 0 hr</td>
</tr>
<tr>
<td>Uncultured control</td>
<td>1.13</td>
</tr>
<tr>
<td>No hormone</td>
<td>0.68</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.57</td>
</tr>
<tr>
<td>Insulin + cortisol</td>
<td>0.40</td>
</tr>
<tr>
<td>+ prolactin</td>
<td></td>
</tr>
</tbody>
</table>

Mammary explants from mice in midpregnancy were cultured under the indicated conditions, and the amount of cyclic AMP in tissue explants was determined. Each value represents the mean of duplicate determinations varying less than 7%.
level of casein and α-lactalbumin during pregnancy. At present, the reasons for such differences in the regulatory pattern of the two proteins are not known.

Another question arising from the present studies relates to the mechanism of the inhibitory action of cyclic AMP on milk protein synthesis. From the data presented, it is clear that the inhibitory effect is not exerted via cell-cycle traversal. One possibility is that it acts via cyclic AMP-dependent protein kinase, as suggested in other systems (30–34). It has been reported (35) that mouse mammary glands contain two protein kinase isozymes, one of which requires cyclic AMP for maximal activity and which increases markedly with a parallel change in the activity of cyclic AMP binding protein during pregnancy. Thus, it would be of interest to examine the possible involvement of protein kinase(s) in the action of cyclic AMP in the synthesis of milk protein in mammary gland organ culture. In addition, there is considerable evidence available that cyclic AMP acts as a modulator of various enzyme activities (5–7). For example, it was shown previously (12, 28) that cyclic AMP inhibited several enzymes of lipogenesis in mammary cells. Therefore, it is conceivable that the observed inhibition of milk protein synthesis by cyclic AMP can be attributed to its inhibitory effect on some key enzymes.

The past failure to reveal an inhibitory effect of cyclic AMP on milk protein synthesis in vitro is probably due to the difference in the culture conditions used. In the present study, we used optimal culture conditions recently established (14, 15) for the induction of casein and α-lactalbumin; in contrast, previous studies used a high concentration of cortisol (5 μg/ml) which now is known to be inhibitory for milk protein synthesis (14, 15). In addition, the present study used more precise immunochromatographic and enzymatic methods to assay casein and α-lactalbumin (14, 15, 18).

The present study has also revealed that the tissue content of cyclic AMP decreases rapidly in mammary explants cultured in hormone-free medium. There are several other instances in which translocation of mammary tissues to an organ culture system has been shown to elicit some critical changes in mammary epithelium (25, 36–39). In the case of tissue content of cyclic AMP, “culturing” may serve to free mammary tissue from an in vivo environment that effectively maintains the nucleotide at a high concentration. It was shown (8) that both progesterone and estrogen, which markedly increase in the serum during pregnancy (40), cause a considerable stimulation of the adenylate cyclase activity of the mammary gland. Thus, fortuitously, the “organ culture” system itself may play an important role in the hormone-dependent development of the mammary gland in vitro. In addition, the accelerated decline of cyclic AMP content in explants cultured in insulin-containing media may reflect the observed effect of insulin on inhibition of adenylate cyclase activity and stimulation of phosphodiesterase activity in the mammary gland (8).