The differential actions of cortisol on the synthesis and turnover of α -lactalbumin and casein and on accumulation of their mRNA in mouse mammary gland in organ culture

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Cortisol was previously shown to exert different, concentration-dependent, effects on the accumulation of casein and α -lactalbumin in mammary glands from mid-pregnant mice cultured in the presence of insulin and prolactin [Ono & Oka (1980) *Cell* **19**, 473–480]. The present study demonstrated that the addition of 30 nM-cortisol to the medium containing insulin and prolactin resulted in a marked enhancement of the rate of synthesis of both α -lactalbumin and casein in cultured tissue. The addition of 3 μ M-cortisol in combination with insulin and prolactin caused a marked decrease in the rate of α -lactalbumin synthesis, but increased casein synthesis substantially. Similar changes were also observed in the amount of translatable mRNA for α -lactalbumin and casein in mammary explants cultured with insulin, prolactin and the two concentrations of cortisol. The study of the turnover of the milk proteins in cultured explants showed that virtually all of the casein synthesized remained intact in tissue explants cultured with 3 μ M cortisol, whereas about 45% of casein disappeared in 40 h from explants cultured with 30 nM-cortisol. In contrast, the two concentrations of cortisol did not differentially affect the disappearance of α -lactalbumin, which was about 55% in 40 h. These results indicate that the concentration-dependent differential actions of cortisol on the accumulation of α -lactalbumin and casein are exerted through its effects on the rate of synthesis and turnover of the two proteins as well as on the accumulation of their mRNA species.

The regulation of the synthesis of the major milk proteins, casein and α -lactalbumin, during the development of the mammary gland is of particular interest, because these proteins can serve as specific markers for the differentiated function of mammary epithelium. Their production is controlled by the multiple interactions of various peptide and steroid hormones (Topper & Freeman, 1980). Previous studies (Topper & Freeman, 1980) have shown that the synergistic actions of insulin, cortisol and prolactin can stimulate the accumulation of the milk proteins in mouse mammary gland cultured in a serum-free chemically defined medium.

Our previous studies of the dose-response relation between cortisol and the accumulation of the two milk proteins in cultured mammary tissue have

shown (Ono & Oka, 1980a) that the optimal concentration of cortisol, 30 nM, for the accumulation of α -lactalbumin is at least 100 times lower than that for the accumulation of casein. Moreover, cortisol at concentrations above 300 nM has been shown to cause a progressive decrease in the amount of α -lactalbumin in cultured tissue (Ono & Oka, 1980a,b). In those studies, however, the amount of α -lactalbumin was measured by an enzymic assay in the lactose synthase system, whereas casein production was assayed by an immunological method. Thus it was decided to determine whether the differential effects of cortisol on the accumulation of casein and of α -lactalbumin involved alterations in either synthesis or degradation of the milk proteins, or were due merely to the difference in analytical methods. In the present study, we have used immunochemical procedures to examine the effect of cortisol on the rate of synthesis and degradation of α -lactalbumin and casein as well as on the accumulation of their mRNA molecules in cultured mammary tissue.

Abbreviations used: SDS, sodium dodecyl sulphate; poly(A)⁺ RNA, poly(A)-containing RNA.

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Experimental

The materials used for the present studies were obtained from the following sources: medium 199 (Hanks' salts) and Dulbecco's phosphate-buffered saline (Ca^{2+} - and Mg^{2+} -free) from GIBCO; cortisol, UDP-galactose, sodium deoxycholate and spermidine from Calbiochem; UDP-[U- ^{14}C]galactose (sp. radioactivity 289 Ci/mol), L-[^3H]leucine (sp. radioactivity 52 Ci/mmol and 145 Ci/mmol), Triton X-100 and Protosol (tissue solubilizer) from New England Nuclear; GTP (trisodium salt), ATP (disodium salt), phosphocreatine and creatine kinase from Boehringer Mannheim; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Tris, sucrose, cycloheximide, unlabelled amino acid kit, sodium penicillin G, bovine α -lactalbumin and bovine galactosyltransferase from Sigma; heparin from Fisher Scientific; 1,4-dithiothreitol from Schwarz/Mann; wheat germ from General Mills; reagents for polyacrylamide-gel electrophoresis, sodium dodecyl sulphate and AG 1-X2 ion-exchange resin from Bio-Rad; and goat anti-(rabbit immunoglobulin G) serum from Miles-Yeda. Crystalline pig zinc insulin was a gift from Lilly Laboratories. Bovine and sheep prolactin preparations (NIH-B4 and NIH-S 13) were obtained from the Hormone Distribution Program, NIADDK, National Institutes of Health. Each preparation of prolactin was further purified to remove contaminating vasopressin and oxytocin by ultrafiltration with ultrafiltration cell model no. 12 and PM-10 membrane (Amicon), by the method of Ono & Oka (1980b). Oligo(dT)-cellulose was obtained from Collaborative Research, and phenol (distilled before use) from Mallinckrodt. C3H/HeN mice in days 12–14 of their first pregnancy were obtained from the Animal Breeding Facility of the National Institutes of Health.

Organ-culture methods

Explants of abdominal mammary glands were prepared from C3H/HeN mice 10–12 days into their first pregnancy and were cultured as described previously (Topper *et al.*, 1975). Medium M199 with Hanks' salts (GIBCO) was used throughout these experiments. NaHCO_3 and sodium penicillin G were added to final concentrations of 350 mg/ml and 35 mg/ml respectively, and the pH was adjusted to 7.4 with 1 M-NaOH. The final concentration of insulin and prolactin was 5 $\mu\text{g}/\text{ml}$. The concentration of cortisol was specified in each experiment.

Enzymic assay of galactosyltransferase and α -lactalbumin activities

The individual activities of the two components of lactose synthase, galactosyltransferase and α -lactalbumin, were measured in the lactose synthase reaction by a modification (Nagamatsu & Oka,

1980) of the method of Fitzgerald *et al.* (1970). The explants were homogenized with 9 vol. of 20 mM-Tris/HCl buffer, pH 7.4, containing 10 mM- MgCl_2 , 100 mM-KCl and 2% Triton X-100. After centrifugation at 700 g for 20 min, 10 μl or 15 μl portions of the supernatant fluids were mixed with bovine α -lactalbumin (final concn. 0.2 mg/ml) or bovine galactosyltransferase (final concn. 0.4 unit/ml; 1 unit represents 1 $\mu\text{mol}/\text{min}$ per ml) for assay of the transferase or α -lactalbumin activity, respectively, and then total volume was adjusted to 50 μl with the above buffer without the detergent. The concentration of the detergent was kept constant in the assay mixture because it affected the lactose synthase reaction. The amount of exogenously added α -lactalbumin and the transferase exceeded the maximal endogenous amounts in assay samples by 250-fold and 25-fold respectively, which allowed linearity with respect to time and the amount of test samples. The reaction was initiated by addition of 50 μl of a solution containing 27 mM-Tris/HCl buffer, pH 7.4, 6.8 mM- MnCl_2 , 1.4 mM-UDP-galactose supplemented with approx. 60 000 c.p.m. of UDP-[^{14}C]galactose, 2 mM-GTP and 40 mM-glucose, and the reaction mixture was incubated at 37°C for 20 min. Parallel assays were also performed for the background radioactivity contributed by hydrolase, endogenous acceptor and endogenous transferase in test solutions. The bovine α -lactalbumin used in the assay did not contain any detectable lactose synthase activity. Other details are described by Nagamatsu & Oka (1980).

Measurement of protein synthesis in cultured tissue, and immunoprecipitation of casein and α -lactalbumin

At the indicated times, tissue explants were washed in 4 $\times$ 4 ml of leucine-free medium M199 supplemented with the appropriate combination of hormones, by repeated blotting and soaking, and then incubated for 2 h in 2 ml of leucine-free medium M199 containing 100 μCi of [^3H]leucine (sp. radioactivity 145 Ci/mmol)/ml. The explants were weighed, frozen and homogenized with 49 vol. (v/w) of Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) containing 2.5% Triton X-100 and 10 mM-leucine. After centrifugation at 150 000 g for 55 min, the resultant supernatant fluid was used for the subsequent analyses.

The rate of total protein synthesis was determined by measuring 5%-trichloroacetic acid-insoluble radioactivity. A 20 μl portion of the tissue extract was spotted on a 2.3 cm Whatman 3MM filter-paper disc. The paper disc was immersed in cold 10% trichloroacetic acid for 5 min, heated in 5% trichloroacetic acid at 90°C for 10 min, and then washed first in cold 5% trichloroacetic acid and then in ethanol. The radioactivity was extracted in 0.8 ml

of Protosol and counted with 10 ml of toluene-based scintillation cocktail (Nagamatsu & Oka, 1980).

The incorporation of radioactivity into α -lactalbumin was measured by direct immunoprecipitation and subsequent SDS/polyacrylamide-gel electrophoresis. As the standard procedure, the tissue extract (500 μ l) was mixed with 25 μ l of 20 mM-disodium EDTA, pH 7.4, containing 2 μ g of purified mouse α -lactalbumin (Nagamatsu & Oka, 1980) as carrier, and incubated with 400 μ l of anti-(mouse α -lactalbumin) serum at 25°C for 20 min, and the mixture was kept at 4°C overnight. The immunoprecipitate was washed with Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), pH 7.4, containing 2% Triton X-100, 1 mM-EDTA and 5 mM-leucine, by the method described previously (Nagamatsu & Oka, 1980). The washed precipitate was dissolved in a solution containing 2% SDS and 0.5% dithiothreitol at 95°C for 5 min and subjected to SDS/polyacrylamide-gel electrophoresis. The gel was fixed, stained, sectioned and treated with Protosol as described previously (Nagamatsu & Oka, 1980). Radioactivity incorporation into α -lactalbumin was expressed as c.p.m. in the α -lactalbumin peak after subtraction of the background. In parallel experiments, ^3H -labelled purified α -lactalbumin (1–4.2 μ g) was incubated with anti-(α -lactalbumin) serum to ensure quantitative recovery in the immunoprecipitation procedure.

Incorporation of radioactivity into casein was determined by indirect immunoprecipitation with anti-(mouse casein) antibody prepared in rabbits, as described previously (Ono & Oka, 1980b).

Isolation of mRNA species from mammary explants

Mammary explants were homogenized with 7 vol. of 10 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-KCl, 5 mM-magnesium acetate, 0.4 mM-cycloheximide, 0.1 mg of heparin/ml, 2% Triton X-100 and 0.5% sodium deoxycholate by eight to ten strokes in a Teflon/glass homogenizer. The homogenate was centrifuged at 5000 g for 10 min at 4°C and the resulting supernatant was used for a source of total RNA. Post-nuclear RNA was extracted with an equal volume of phenol/chloroform (1:1, v/v) as described previously (Takemoto *et al.*, 1980). Poly(A)⁺ RNA was isolated by two cycles of oligo(dT)-cellulose chromatography, by the method of Aviv & Leder (1972).

Translation of mRNA in a wheat-germ cell-free system

A 30000 g supernatant extract of wheat germ was prepared by the method of Roberts & Paterson (1973). The translation reaction mixture consisted of 24 mM-Hepes/KOH, pH 7.6, 1 mM-ATP, 50 μ M-GTP, 2 mM-dithiothreitol, 8 mM-phosphocreatine, 40 μ g of creatine kinase/ml, 30 μ M of the appro-

priate unlabelled amino acids, 74 mM-KCl, 1.5 mM-magnesium acetate, 0.4 mM-spermidine, 20–100 μ l of the wheat-germ extract, 0.4 mCi of [^3H]leucine (sp. radioactivity 52 Ci/mmol)/ml and the appropriate amount of mRNA, and was incubated at 25°C for 120 min. The reaction was stopped by dilution with Dulbecco's phosphate-buffered saline and addition of unlabelled leucine (8 mM) as described previously (Takemoto *et al.*, 1980). The amount of mRNA added was adjusted to yield linearity with respect to the extent of amino acid incorporation. The amounts of casein and α -lactalbumin in the released polypeptides were measured by immunoprecipitation of the supernatant obtained after centrifugation of the reaction mixture at 150000 g for 55 min (Takemoto *et al.*, 1980). The specificity of this procedure was determined by measuring the ability of various proteins to compete for antibody-binding sites with the nascent polypeptides of each milk protein. Product analysis of both milk proteins recognized by the specific antiserum was performed by SDS/polyacrylamide-gel electrophoresis in the buffer system of Laemmli (1970). The translational activity of mRNA for α -lactalbumin and casein in each sample was determined from the linear portions of the translation assay by use of several different inputs of RNA samples.

Results

Galactosyltransferase and α -lactalbumin are the two components of lactose synthase (Fitzgerald *et al.*, 1970). As shown in Fig. 1, the galactosyltransferase activity in tissue cultured with insulin alone or with insulin and cortisol (1 μ g/ml, 3 μ M) was similar to that found in fresh uncultured tissue. The combination of insulin and prolactin caused about a 2-fold increase in enzyme activity in 48 h culture. The addition of increasing concentrations of cortisol, up to 1 μ g/ml, with insulin and prolactin increased the activity further in a dose-dependent manner. In contrast, the activity of α -lactalbumin increased about 10-fold after culture with insulin and prolactin when compared with that in fresh tissue or in tissue cultured with insulin alone or with insulin and cortisol. The addition of cortisol, up to 0.01 μ g/ml (30 nM), increased the activity of α -lactalbumin in tissue cultured with insulin and prolactin, but at higher concentrations of cortisol the activity of α -lactalbumin was markedly decreased.

Fig. 2(a) shows the temporal changes in the rate of casein synthesis in mammary explants cultured for 48 h in the presence of insulin and prolactin or the triple hormone combination with 0.01 or 1 μ g of cortisol/ml. The initial rate was maintained in the presence of insulin and prolactin, whereas casein synthesis in the two triple-hormone systems increased almost to the same extent. The amount of

casein relative to the total extractable proteins was about 15–20% of the initial value or that in tissue explants cultured with insulin and prolactin, and

Fig. 1. Effect of different cortisol concentrations on galactosyltransferase and α -lactalbumin activities in mammary-gland explants in culture

Mammary-tissue explants from mice in mid-pregnancy were cultured for 48 h in medium containing the following combinations of hormones; insulin alone (I), insulin and cortisol (1 μ g/ml) (IF), or insulin, prolactin (IP) and the indicated concentrations of cortisol (F). T_0 denotes freshly prepared mammary explants. The activities of galactosyltransferase (●) and α -lactalbumin (○) in tissue explants were measured as described in the Experimental section.

about 45–60% in those cultured in the triple-hormone systems. The initial rate of casein synthesis declined by about 65% in explants cultured with insulin and cortisol for 48 h (results not shown). During the 2 h pulse period, measurement of the acid-soluble radioactivity of [3 H]leucine in explants revealed no difference with respect to the various hormone treatments.

In contrast with casein synthesis, a substantial increase in the rate of α -lactalbumin synthesis occurred in explants cultured with insulin and prolactin (Fig. 2b). The combination of 0.01 μ g of cortisol/ml, insulin and prolactin caused a greater and more prolonged increase in the rate of α -lactalbumin synthesis. The amount of α -lactalbumin increased from 0.02–0.04% at the beginning of culture to 0.25–0.27% of the total extractable proteins in explants cultured with the three hormones for 48 h. When the concentration of cortisol was increased from 0.01 to 1 μ g/ml at 0, 10 or 24 h, the synthesis of α -lactalbumin was markedly decreased. Under the same conditions, casein synthesis was not inhibited (Fig. 2a).

Fig. 3 shows SDS/polyacrylamide-gel electrophoresis profiles of [3 H]leucine-labelled α -lactalbumin synthesized in mammary explants cultured in the presence of insulin and prolactin and the combination of these hormones with a low and a high concentration of cortisol. In each case, there are two species of α -lactalbumin, the major A form (apparent mol.wt. 14 000) and a minor B form

Fig. 2. Time course of synthesis of casein (a) and α -lactalbumin (b) in mammary-gland explants in culture. Mammary-tissue explants from mice in mid-pregnancy were cultured in medium containing insulin and prolactin (IP: □), insulin, prolactin and cortisol (0.01 μ g/ml) (IPF_{0.01}: ○) or insulin, prolactin and cortisol (1 μ g/ml) (IPF_{1.0}: ●). Two groups of tissue explants were cultured in the IPF_{0.01} system for 10 h (▲) or 24 h (△) and then transferred to the IPF_{1.0} system for the remainder of the culture period. At the indicated time points, the rates of synthesis of casein and α -lactalbumin were determined as described in the Experimental section.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of α -lactalbumin synthesized by mammary-gland explants in culture

Mammary-tissue explants from mice in mid-pregnancy were cultured for 30h in medium containing (a) insulin, prolactin and cortisol ($0.01 \mu\text{g/ml}$) ($\text{IPF}_{0.01}$), (b) insulin and prolactin (IP), or (c) insulin, prolactin and cortisol ($1 \mu\text{g/ml}$) ($\text{IPF}_{1.0}$). At 28h of culture, tissue explants were pulse-labelled with

(apparent mol.wt. 18500). The relative amount of the two species of α -lactalbumin was not altered appreciably by these combinations of hormones. Our previous studies (Nagamatsu & Oka, 1980) indicated that the B form is a glycosylated species of mouse α -lactalbumin, whereas the A form is not. Authentic mouse α -lactalbumin competitively inhibited immunoprecipitation of material from peaks A and B (Fig. 3 and Table 1), indicating that they are not contaminants of their immunoprecipitates.

Fig. 4 shows that the amounts of the functional mRNA for both casein and α -lactalbumin in explants cultured with insulin and cortisol did not increase during 46h of incubation over those of uncultured tissue. Treatment with insulin and prolactin, however, increased the amounts of mRNA for the two proteins substantially, and the additional treatment with $0.01 \mu\text{g}$ of cortisol/ml produced greater increases. The addition of $1 \mu\text{g}$ of cortisol/ml in combination with insulin and prolactin caused a similar large increase in casein mRNA, but that of α -lactalbumin mRNA was greatly decreased.

$[^3\text{H}]$ leucine ($200 \mu\text{Ci/ml}$) for 2h, and ^3H -labelled α -lactalbumin was isolated by immunoprecipitation with mouse anti- α -lactalbumin serum. Each precipitate was processed for SDS/polyacrylamide-gel electrophoresis. About 10–11mg of tissue equivalents was applied to each gel. BPB indicates the position of the dye marker at the front; A and B indicate the positions of the A and B forms of α -lactalbumin respectively.

Fig. 4. Hormonal influence on mRNA activity for α -lactalbumin, casein and total protein in mammary-gland explants in culture

Mammary tissue explants from mice in mid-pregnancy were cultured for 46h in medium containing insulin and cortisol ($0.01 \mu\text{g/ml}$) ($\text{IF}_{0.01}$), insulin and prolactin (IP), insulin, prolactin and cortisol ($0.01 \mu\text{g/ml}$) ($\text{IPF}_{0.01}$) or insulin, prolactin and cortisol ($1.0 \mu\text{g/ml}$) ($\text{IPF}_{1.0}$). T_0 denotes freshly prepared mammary explants. Poly(A)⁺ RNA was isolated from cultured tissue and translated in a wheat-germ cell-free system as described in the Experimental section. mRNA activity for α -lactalbumin, casein and total protein was determined as described in the Experimental section and normalized for poly(A)⁺ RNA obtained from 1g of cultured tissue. □, Total protein mRNA activity; ■, casein mRNA activity; ▨, α -lactalbumin mRNA activity.

The inhibitory effect of cortisol ($1\mu\text{g/ml}$) was also found when the concentration of the steroid was increased from $0.01\mu\text{g/ml}$ at 24 hr: the amount of α -lactalbumin mRNA was about one-fifth of that in explants cultured with insulin, prolactin and cortisol ($0.01\mu\text{g/ml}$) at the end of 46 h. The changes in the amounts of total mRNA were generally in line with those of casein mRNA under various culture conditions examined, except that the increase was largest in the presence of insulin, prolactin and cortisol ($1\mu\text{g/ml}$).

The effect of the two concentrations of cortisol on degradation and/or release of the two milk proteins was examined by pulse-chase experiments shown in Fig. 5. The data indicated that in the presence of $1\mu\text{g}$ of cortisol/ml radioactivity in casein decreased only slightly during 40 h, whereas the amount of labelled casein declined by as much as 45% in explants cultured with $0.01\mu\text{g}$ of cortisol/ml. The decrease did not represent loss to the medium, since only about 1% of the total cellular casein was found in the medium during 40 h of the chase.

As shown in Figs. 5(b) and 5(c), the extent of disappearance of α -lactalbumin and total non-casein-proteins was about 50% in 40 h in culture, regardless of cortisol concentration. The maximum amount of total protein in the medium was about 3.4% of the initial value. In contrast with casein and total non-casein proteins, approx. 20% of α -lactalbumin present at the beginning of culture was found in the medium at 40 h of chase incubation in

the presence of either a high or a low concentration of cortisol. The amount of α -lactalbumin was too small to affect the amount of total non-casein proteins in the medium. The analysis of α -lactalbumin in both explants and the medium by SDS/polyacrylamide-gel electrophoresis showed the presence of the major (A) and minor (B) forms of α -lactalbumin: the relative amount of the two forms was essentially the same in terms of cortisol concentration and their presence in tissue and the medium (Table 1).

In the experiments presented in Fig. 5, addition of 5-fold excess (5 mM) of unlabelled leucine during the chase period gave essentially the same results in terms of the effect of cortisol on the extent of degradation of casein, α -lactalbumin and non-casein proteins in cultured explants (results not shown), suggesting that the labelled amino acid was effectively diluted with unlabelled amino acid so that further incorporation of the radioactivity into proteins was insignificant during the chase period.

Discussion

Previous studies (Ono & Oka, 1980*a,b*) showed that the addition of cortisol in increasing concentrations augments the accumulation of α -lactalbumin at 0.0005 – $0.01\mu\text{g/ml}$ and inhibits this process at 0.1 – $1\mu\text{g/ml}$ in mouse mammary gland cultured in the presence of insulin and prolactin. On the other hand, the accumulation of casein is

Fig. 5. Time course of disappearance of casein, α -lactalbumin and non-casein protein in mammary-gland explants in culture

Mammary-tissue explants from mice in mid-pregnancy were first cultured for 30 h in medium containing insulin, prolactin and cortisol and then allowed to incorporate [^3H]leucine ($200\mu\text{Ci/ml}$) during the final 2 h of culture, as described in the Experimental section. At the end of labelling, explants were thoroughly washed with cultured medium and transferred to radioisotope-free culture medium containing insulin, prolactin and cortisol, either $0.01\mu\text{g/ml}$ (IPF_{0.01}; $\circ$) or $1\mu\text{g/ml}$ (IPF_{1.0}; $\bullet$). At the indicated time points after transfer, the amounts of ^3H -labelled casein and α -lactalbumin remaining in cultured tissue as well as the amount of ^3H -labelled α -lactalbumin recovered in the medium were determined immunochemically as described in the Experimental section. The amount of radioactivity in non-casein proteins was estimated by subtracting the amount of ^3H -labelled casein from that of total protein.

Table 1. *Relative amounts of A and B forms of α -lactalbumin in cultured mammary explants and medium during the pulse-chase experiments*

Experimental details are given in the legend to Fig. 5. ^3H -labelled α -lactalbumin was isolated by immunoprecipitation, and each precipitate was analysed for the relative amounts of the A and B forms of α -lactalbumin by SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. The pattern of distribution of radioactivity was identical with those shown in Fig. 3. About 50 mg of tissue equivalents was applied to each gel, and the results were normalized as c.p.m./10 mg of tissue.

Chase conditions	A form		B form	
	Explants	Medium	Explants	Medium
Zero time	2510	0	190	0
24 h				
Cortisol (0.01 $\mu\text{g/ml}$)	1540	400	120	30
Cortisol (1 $\mu\text{g/ml}$)	1500	390	135	33
40 h				
Cortisol (0.01 $\mu\text{g/ml}$)	710	550	55	40
Cortisol (1 $\mu\text{g/ml}$)	695	560	55	42

enhanced by cortisol in a dose-dependent manner, reaching a maximum at 1 $\mu\text{g/ml}$. In the present study we have demonstrated that the stimulatory and inhibitory effects of cortisol on the accumulation of α -lactalbumin are due to alterations in its rate of synthesis and the amount of its mRNA rather than to altered turnover or change in the enzymic activity of this protein. The data also indicate that both a low and high concentration of cortisol equally cause the maximal increase in the rate of casein synthesis and in the amount of its mRNA, but a high concentration of cortisol is necessary for decreasing the turnover of casein in this system. These results can account for the maximal accumulation of casein in mammary explants cultured with a high concentration of cortisol (Ono & Oka, 1980*a,b*).

The present study indicates that under the optimal conditions casein synthesis increased about 3-fold, amounting to 45–60% of total protein in cultured tissue, whereas α -lactalbumin synthesis increased about 10-fold, comprising 0.25–0.27% of total protein. The difference in the relative amount of the two proteins reflects the concentration of each protein in the mouse milk (Jenness, 1974; Nagamatsu & Oka, 1980). A greater increase in α -lactalbumin synthesis in the culture system may be related to the state of the mammary gland at the time of explantation, i.e. mid-pregnancy. During the pregnancy, casein synthesis increases progressively,

whereas the amount of α -lactalbumin remains low in mammary glands of mice and rats (Turkington *et al.*, 1968; Nakhasi & Qasba, 1979). This is partly because α -lactalbumin synthesis is more sensitive to the inhibitory action of progesterone (Turkington & Hill, 1968). The differential changes in the amount of mRNA for casein and α -lactalbumin in the mammary gland during pregnancy have been observed in mice (Takemoto *et al.*, 1980) and in rats (Nardacci *et al.*, 1978). Translocation of mammary tissue from the situation *in vivo* to an organ culture may serve to free mammary cells from an inhibiting environment, and this may be more apparent with α -lactalbumin synthesis because it is more suppressed *in vivo*.

The data presented here indicate that there is generally a good correlation between the amounts of the functional mRNA for casein and α -lactalbumin and the rate of synthesis of the two milk proteins in mammary explants cultured under different conditions. However, in culture with insulin and prolactin, the rate of casein synthesis did not increase in spite of enhanced amounts of its mRNA. These results suggest that, in addition to transcription, the post-transcriptional or translational control of casein-gene expression is important, as suggested by Bathurst *et al.* (1980*a,b*) and others (Houdebine *et al.*, 1978; Guyette *et al.*, 1979). Bathurst *et al.* (1980*b*) showed the preferential accumulation of the abundant and moderately abundant mRNA sequences in the cytoplasm of the lactating guinea-pig mammary gland: the former, representing milk-protein mRNA species, predominated in the poly(A)⁺-RNA fraction, whereas the latter, which predominated in the non-poly(A)⁺-RNA fraction, probably represents mRNA sequences that are translated into various enzymes and structural proteins necessary for the synthesis and secretion of milk proteins as well as the general metabolic activities during lactogenesis. Thus the expression of milk-protein genes requires co-ordinate cellular changes, including the accumulation of milk-protein mRNA molecules and other relevant metabolic and structural alterations, which can be regulated at transcriptional and translational levels. Earlier studies (Mills & Topper, 1970) showed that the development of endoplasmic reticulum, the primary site of milk-protein synthesis, required cortisol, and mammary explants treated with insulin and prolactin lack this organelle.

Casein and α -lactalbumin, like other secretory proteins, are mainly synthesized on polyribosomes associated with endoplasmic reticulum, undergo post-translational modification, and are transported into the Golgi apparatus for secretion, as outlined in the model proposed by Siekevitz & Palade (1960). The attachment of mRNA for secretory proteins is facilitated by translation of the 5'-end of the mRNA

on free polyribosomes, giving rise to a relatively small hydrophobic 'signal' peptide which binds to a specific receptor on the membrane and thus facilitates the attachment of polyribosomes to the membrane (Milstein *et al.*, 1972; Blobel & Dobberstein, 1975a,b). The 'signal' sequence is cleaved subsequently by proteolysis. The cell-free translation of mRNA for casein and α -lactalbumin from the mammary glands of various species, in the absence of the membrane fragments, yielded precursor proteins with signal peptides (Craig *et al.*, 1976; Lingappa *et al.*, 1978; Mercier *et al.*, 1978; Craig *et al.*, 1979; Takemoto *et al.*, 1980). The present and earlier work (Ono & Oka, 1980a,b) showed that cultured mammary explants synthesize and modify casein and α -lactalbumin polypeptides, as the lactating gland does, but the ability of cultured tissue to secrete these proteins appears abnormal. The present data indicate that only 1% of casein and 20% of α -lactalbumin in mammary explants are released or secreted into the medium. At present, it is unknown whether the milk proteins in the medium are actually secreted or leaked out from the tissue. Since mammary cells derived from mid-pregnant mice can synthesize and readily secrete milk proteins into medium in a primary cell culture system (Y. Taketani & T. Oka, unpublished work), it is likely that in cultured explants the secretory-duct system is severely damaged during the preparation of tissue explants or closed off at its end during the wound-healing process. The differential extent of secretion of casein and α -lactalbumin has been also observed in previous studies of cultured guinea-pig mammary gland (Burditt *et al.*, 1981) and the secretory activity of oocytes of *Xenopus laevis* after the micro-injection of milk-protein mRNA species (Lane *et al.*, 1980). These studies suggested that the primary structure of the polypeptide is an important determinant of the rate of secretion. Our present study showed no difference in the relative amounts of the non-glycosylated and glycosylated forms of α -lactalbumin in tissue and the medium. The two forms of α -lactalbumin are immunologically indistinguishable and have the same amino acid composition (Nagamatsu & Oka, 1980). These results suggest that post-translational modification of this protein is not critical for the secretory process. At present, however, the intracellular mechanisms involved in regulation of the secretion of various peptides are largely unknown.

Studies of the effect of cortisol on the degradation of casein, α -lactalbumin and non-casein proteins in organ culture of the mammary gland revealed some interesting differences. The turnover of both α -lactalbumin and non-casein proteins is greater than that of casein, and this is not affected by the concentration of cortisol. In contrast, casein remained stable in the presence of 1 μ g of cor-

tisol/ml, whereas it was substantially degraded in the presence of 0.01 μ g of cortisol/ml. The difference in the effect of the two concentrations of cortisol is not due to a dilution effect of newly formed casein in explants, since the rate of casein synthesis is almost the same in the two culture systems. The hormone-dependency of casein turnover was also reported by Wilde *et al.* (1980) in a study of rat mammary gland in culture. In a study of the involution of the mammary gland, Ossowski *et al.* (1979) reported that cortisol inhibits this process by blocking the production of plasminogen activator. At present, the physiological significance of the stabilizing effect of cortisol on casein is conjectural, because of its high concentration. Also, the reason for the differential stability of casein and α -lactalbumin remains unsolved. On the basis of the differential effects of hormones and NH_4Cl on the turnover of casein and fatty acid synthase, Wilde *et al.* (1980) proposed two components of the degradation system in the mammary gland, one occurring within the lumen of the endoplasmic reticulum and the other in lysosomes. Although both casein and α -lactalbumin are secreted normally into milk, α -lactalbumin is present, in substantial amounts, in the Golgi apparatus to facilitate the production of a milk sugar, lactose, in combination with galactosyltransferase (Fitzgerald *et al.*, 1970). Thus it is possible that the difference in the intracellular localization of casein and α -lactalbumin produces the differential susceptibility of the two proteins to the degradative systems. Alternatively, the difference is due to the intrinsic chemical nature of the individual polypeptides, which determines the substrate-preference for proteolysis.

In conclusion, the present study provides further evidence for the view that glucocorticoids play an important role in the complex regulatory processes associated with differentiation of the mouse mammary gland (Mills & Topper, 1970; Sakai & Banerjee, 1979; Ganguly *et al.*, 1980; Ono & Oka, 1980a,b).

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