Casein Turnover in Rabbit Mammary Explants in Organ Culture

By Khalidah AL-SARRAJ, John NEWBURY, David A. WHITE and R. John MAYER
*Department of Biochemistry, The Medical School, Queen's Medical Centre,
Nottingham NG7 2UH, U.K.*

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1. Explants of mammary gland from mid-pregnant rabbits were cultured in medium 199 containing insulin, prolactin and cortisol, and specific anti-casein immunoglobulin G was used to measure the amount, rate of synthesis and rate of degradation of casein in the explants in the presence of hormones and after removal of hormones from previously stimulated tissue. 2. The amount of casein in particle-free supernatants prepared from mammary explants was measured by 'rocket' immunoelectrophoresis. 3. The rate of incorporation of L-[4,5-³H]leucine into casein was measured after isolation of the casein by immunoabsorbent chromatography and polyacrylamide-gel electrophoresis in the presence of urea and sodium dodecyl sulphate. 4. Casein accumulates in mammary explants in the presence of insulin, prolactin and cortisol, but not in the absence of hormones. Removal of hormones after 24 h in culture results in a decrease in the rate of accumulation of casein in the explants. 5. Casein-synthetic rate increases in mammary explants in the presence of insulin, prolactin and cortisol, but not in the absence of hormones. Removal of hormones after 24 h in culture results in continued casein synthesis at approx. 30% of the rate in the presence of hormones. The synthetic rate does not decrease to values observed in explants cultured throughout in the absence of hormones. 6. Casein is not degraded in mammary explants during a phase of rapid casein accumulation (36-72 h) in the presence of hormones. Furthermore casein is not degraded when hormones are removed from the tissue after between 36 and 72 h in culture. 7. Casein is glycosylated in mammary explants; the extent of glycosylation parallels the rate of synthesis. The glycosylated protein is rapidly secreted from the tissue. 8. The results are consistent with the notion that after hormonal stimulation mammary explants from mid-pregnant rabbits synthesize, glycosylate and rapidly secrete casein. Removal of hormones decreases the synthetic rate of casein, but does not cause the accumulation of a pool of degradable casein in the lobulo-alveolar cells.

Lactogenesis in mammary cells is characterized by the onset of synthesis of specific milk proteins (e.g. casein and α -lactalbumin). This process is associated with two major developmental processes, cell proliferation and cytological and functional differentiation of the epithelial cells (Schmidt, 1971; Hollmann, 1974; Banerjee, 1976).

Extensive studies (*in vivo* and *in vitro*) have shown that initiation of milk synthesis is under hormonal control. Thus hormones may have stimulatory (e.g. prolactin, insulin and glucocorticoid) or inhibitory (e.g. progesterone) effects (Schmidt, 1971; Devinoy & Houdebine, 1977; Banerjee, 1976; Collier *et al.*, 1977).

One of the rate-limiting factors in the regulation of synthesis of the specific milk proteins by lactogenic hormones is the intracellular concentration of the specific mRNA species (Banerjee, 1976).

Abbreviations used: SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IgG, immunoglobulin G.

Most of the studies on casein have centred on transcription of casein mRNA and the response of this process to lactogenic hormones. Changes in concentration of casein mRNA in the mammary gland of rat, rabbit and guinea pig during pregnancy and lactation have been demonstrated by translation of casein mRNA in a reticulocyte lysate (Houdebine & Gaye, 1975; Shuster *et al.*, 1976), in a wheat-germ cell-free translation assay (Rosen *et al.*, 1975; Rosen, 1976; Craig *et al.*, 1976) and by hybridization with DNA complementary to casein mRNA (Shuster *et al.*, 1976; Rosen & Barker, 1976). The translated casein product synthesized in the cell-free systems was identified by immunoprecipitation and SDS/polyacrylamide-gel electrophoresis. Results from these studies have shown that during development a parallel enhancement of casein mRNA and casein synthesis occurs and this is under hormonal control (Devinoy *et al.*, 1978; Rosen *et al.*, 1975; Shuster *et al.*, 1976).

Similarly the hormonally stimulated increase in

the rate of casein biosynthesis in explants of mammary gland from mid-pregnant (Devinoy *et al.*, 1978) and pseudopregnant rabbits (Houdebine, 1976) is coincident with an increase in mRNA concentration.

Casein turnover has not been extensively studied during hormonally stimulated cytodifferentiation in mammary explants in organ culture, although both the rates of synthesis and degradation of casein are known to change during mammary-gland development in pregnancy and lactation *in vivo* (Hollmann, 1974).

The purpose of the present work is to study the hormonal regulation of synthesis, degradation and subsequent accumulation of casein during cytodifferentiation of mammary explants in organ culture. The effect of the removal of hormones from the culture medium has been studied as a possible model system for mammary involution *in vivo*.

Materials and Methods

Animals

The sheep used in these studies were housed at the Joint Animal Breeding Unit, Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. New Zealand White rabbits were obtained from the same source.

Materials

Agarose and barbitone were purchased from BDH Chemicals, Poole, Dorset, U.K. Glycine (AR), barbitone sodium and glass-fibre paper (Whatman GF/A) were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. L-[4,5-³H]leucine and D-[1-¹⁴C]glucosamine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Phenylmethanesulphonyl fluoride was from Sigma (London) Chemical Co., Poole, Dorset, U.K.; Sepharose 2B was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Ox insulin, sheep prolactin, cortisol 21-acetate, streptomycin sulphate and sodium benzylpenicillin were obtained from the source described by Speake *et al.* (1975). Medium 199 with and without glucose (Morgan *et al.*, 1950) was obtained from Wellcome Research Laboratories, Beckenham BR3 3BS, Kent, U.K. All other chemicals were of A.R. grade and obtained from commercial suppliers.

Preparation and culture of mammary explants

Explants of lobulo-alveolar mammary tissue were prepared from mid-pregnant rabbits as described by Forsyth & Myers (1971). Groups of 10 explants were cultured at 37°C under O₂/CO₂ (19:1) in 1 ml of medium 199 containing insulin (5 µg/ml), prolactin (1 µg/ml) and cortisol (1 µg/ml). A duplicate series of explants was also cultured in medium 199 containing

no hormones. The medium for both groups of explants also contained 21 mM-NaHCO₃, streptomycin sulphate (0.1 mg/ml), penicillin (0.12 mg/ml) and 14 mM-Hepes buffer, and was changed at 12 h intervals.

Incubation of explants

At the times indicated groups of 25 or 50 explants were removed and incubated for 15, 30 or 60 min at 37°C under O₂/CO₂ (19:1) in 0.5 ml of medium 199 (glucose-free) with or without hormones. The incubation medium also contained 5 mM-sodium pyruvate, 1.5 mM-leucine and 20 µCi of L-[4,5-³H]leucine (sp. radioactivity 50 Ci/mmol). Sometimes [1-¹⁴C]glucosamine was included in the incubations. The incubation conditions were chosen to give optimum rates of incorporation of leucine (L. E. Smith & R. J. Mayer, unpublished observations) and glucosamine (Speake & White, 1978) in this system.

Preparation of tissue extracts

After incubation each group of explants was washed (5 min each wash) at 37°C with 4 × 5 ml of medium 199 containing 10 mM-leucine and 10 mM-glucosamine, in the presence or absence of hormones. The explants were then homogenized and subsequently sonicated for 5 min at 4°C in 5 mM-EGTA, pH 7.0 (1 ml or 1.5 ml), containing phenylmethanesulphonyl fluoride (approx. final concn. 2 mM). A particle-free supernatant fraction was obtained by centrifugation of the homogenate at 15000g_{av.} for 60 min at 4°C.

Measurement of incorporation of L-[4,5-³H]leucine and D-[1-¹⁴C]glucosamine into particle-free supernatant proteins

The rate of incorporation of L-[4,5-³H]leucine and D-[1-¹⁴C]glucosamine into trichloroacetic acid-insoluble proteins of the particle-free supernatant fraction was measured essentially as described by Speake *et al.* (1975).

Duplicate samples (0.1 ml) of the particle-free supernatants were removed and bovine serum albumin (1 mg) was added. Proteins were precipitated with trichloroacetic acid (final concn. 5%, w/v) and the precipitates were collected by centrifugation for 6 min at 1400g_{av.} at room temperature. Each sediment was washed with 3 × 1 ml of 5% (w/v) trichloroacetic acid containing 10 mM-leucine and 10 mM-glucosamine, and dissolved in 0.3 ml of formic acid (90%, v/v) for measurement of radioactivity (Speake *et al.*, 1975).

Immunological procedures

Casein polypeptides were isolated and purified as previously described (Al-Sarraj *et al.*, 1978a). Antiserum to rabbit recombined casein polypeptides was

raised in a sheep by immunization for 8 weeks with rabbit recombined polypeptides coupled to Sepharose-albumin (Al-Sarraj *et al.*, 1978b). The anti-serum was processed by the method of Speake *et al.* (1975) to obtain the immunoglobulin fraction and adsorbed to give specific anti-casein antibodies by the method of Al-Sarraj *et al.* (1978b).

Immunoquantification of casein

Casein in the particle-free supernatants was quantified by means of 'rocket' immunoelectrophoresis. Immunoelectrophoretic techniques were carried out as described by Axelsen *et al.* (1973) with the Svendsen buffer system. Agarose gel (1%, w/v) containing adsorbed antiserum (76 µg of protein/ml of agarose gel) was used. Samples (2–10 µl) of the particle-free supernatant fractions were applied in the wells, and electrophoresis was carried out at 0.5 V/cm for 16 h at approx. 15°C. Purified recombined casein was used as standard.

Immunoisolation of casein

(a) *Immunoprecipitation of casein.* Samples (35–400 µl) of the particle-free supernatant fractions containing approx. 9 µg of casein were completely precipitated with the anti-casein antibodies (1 ml; 3 mg of protein/ml). The immunoglobulin fraction prepared from the serum of a non-immunized sheep was used as a control (1 ml; 3 mg of protein/ml).

The mixtures were preincubated for 30 min at room temperature (22°C) and then incubated for 48 h at 4°C. The immunoprecipitates were collected by centrifugation at 1000g_{av.} for 30 min at room temperature, and washed twice with 1 ml of 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 mM-NaCl. The final precipitates were boiled for 3–5 min in 20 µl of 0.0625 M-Tris/HCl buffer, pH 6.8, containing SDS (final concn. 2%, w/v), glycerol (final concn. 10%, w/v) and 5 M-urea. Each of the cooled preparations was mixed with recombined casein (50 µg) (as gel marker) and Bromophenol Blue (10 µl). They were then subjected to polyacrylamide-gel electrophoresis in the presence of urea and SDS as described previously (Al-Sarraj *et al.*, 1978a). The gels were stained for proteins with Coomassie Blue, and the region containing the stained casein band was cut into 10 slices, 2 mm thick, and processed for radioactivity determination (Al-Sarraj *et al.*, 1978b).

(b) *Isolation of casein by immunoadsorbent chromatography.* 'Antiserum-Sepharose' (Sepharose-anti-casein IgG) and 'control-serum-Sepharose' (Sepharose-control serum IgG) were prepared and used as described by Al-Sarraj *et al.* (1978b). The Sepharose-anti-casein IgG immunoadsorbent (0.33 mg of protein/ml) bound 7.5–10 µg of recombined casein per ml of gel. Samples of particle-free supernatant fraction were processed and casein was isolated by the method previously described for the immunoisolation of fatty acid synthetase (Paskin &

Mayer, 1978). Samples (1 ml) of the particle-free supernatant fraction were first applied to Sepharose 2B columns (3 ml) and left in contact with the columns for 4 h at room temperature. The columns were then washed with 5 mM-EGTA (10 ml) and the washings used for immunoisolation of casein. The washings contained 85% of the radioactivity and all the casein. No casein could be eluted from the Sepharose 2B by 8 M-urea containing SDS (2%, w/v) as assessed by polyacrylamide-gel electrophoresis in the presence of urea and SDS. Volumes containing approx. 18–20 µg of casein (as determined by 'rocket' immunoelectrophoresis) were then applied to antiserum-Sepharose and control-serum-Sepharose columns (3 ml). The samples were left in contact with the columns overnight at 4°C and washed with 5 mM-EGTA (12 ml). The casein was eluted with 8 M-urea, pH 7.0 (12 ml), containing SDS (2%, w/v).

The eluates were dialysed against water (with several changes) for 2 days at room temperature and freeze-dried.

Polyacrylamide-gel electrophoresis, gel slicing and processing for radioactivity determination was carried out as described above.

Radioactivity determination

Radioactivity was measured with a Packard Tri-Carb 3375 liquid-scintillation spectrometer. The dual-isotope measurements were corrected by the external-standard-ratio method. Results are expressed as d.p.m.

Fig. 1. 'Rocket' immunoelectrophoresis: standard curve for 'rocket' height and amount of casein

A linear relationship is obtained between 'rocket' height and amount of casein (0.18–0.72 µg).

Results

Techniques used to measure the amount and the rate of synthesis of casein

(i) 'Rocket' immunoelectrophoresis. The amount of casein was measured immunologically by means of 'rocket' immunoelectrophoresis, which represents a simple, quick and reproducible method for the determination of a single protein in a protein mixture, especially when a monospecific antiserum is used. The rocket-shaped precipitates are quantified by measurement of their heights (Axelsen *et al.*, 1973).

A linear relationship between 'rocket' height and amount of casein is obtained (Fig. 1). The technique

has been used here to detect as little as 10 ng of casein.

(ii) *Immunoisolation of radiolabelled casein.* The immunoabsorbent technique proved to be excellent for the isolation of casein from particle-free supernatant fractions of mammary-gland explants. Only one major radioactive peak (Fig. 2a) corresponding to the position of the casein stained band was seen with material eluted from the immunoabsorbent when analysed by polyacrylamide-gel electrophoresis in the presence of urea and SDS. The immuno-precipitation technique, however, was much less satisfactory. Several peaks of radioactivity were found (Fig. 2b) when immunoprecipitates were

Fig. 2. Polyacrylamide-gel electrophoresis of radiolabelled casein isolated by immunoabsorbent chromatography (a) or immunoprecipitation (b)

(a) Samples (0.06–0.4 ml) of particle-free supernatant fractions obtained from explants cultured for 24 h with hormones were applied to either (i) a Sepharose–anti-casein IgG column (3 ml; ●) or (ii) a Sepharose–control-serum IgG column (3 ml; ■) and left in contact with the column overnight at 4°C. Radiolabelled casein was eluted and processed for radioactivity as described in the Materials and Methods section. The position of the radioactive peak corresponds to the R_F of purified recombinant casein. (b) Samples of particle-free supernatant fractions were mixed with either (i) 1 ml of anti-casein IgG (●) or (ii) 1 ml of control-serum IgG (■), and the mixture was incubated for 48 h at 4°C. The immunoprecipitate was washed and processed for radioactivity as described in the Materials and Methods section.

analysed by the same technique. Immunoprecipitation was always accompanied by non-specific precipitation with tissue extracts prepared from explants cultured with or without hormones for various times. Clearly the incorporation into casein cannot be accurately estimated from radioactivity profiles such as shown in Fig. 2(b).

(iii) *Incorporation of L-[4,5- 3 H]leucine into casein.* The incorporation of L-[4,5- 3 H]leucine into casein was linear with time in all experiments (Fig. 3) for at least 60 min of incubation at 37°C.

Measurements of the rate of casein turnover

(a) *Effects of hormones on the amount of casein in mammary explants.* Fig. 4 shows the accumulation

Fig. 3. *Incorporation of L-[4,5- 3 H]leucine into casein* After 24 h in culture, groups of 25 explants were removed and incubated for the times indicated at 37°C under O₂/CO₂ (19:1) in 0.5 ml of medium 199 (glucose-free) containing 1.5 mM-leucine, 20 μ Ci of L-[4,5- 3 H]leucine and 5 mM-sodium pyruvate. At the end of the incubation period, each group of explants was homogenized in 5 mM-EGTA (1 ml) containing phenylmethanesulphonyl fluoride (final concn. approx. 2 mM) and a particle-free supernatant fraction obtained as described in the Materials and Methods section. Incorporation of radioactivity into casein was measured after immunoadsorbent chromatography and SDS/polyacrylamide-gel electrophoresis. Similar results were obtained with casein from explants cultured for 48 h with hormones (results not shown).

profile for casein in rabbit mammary explants in organ culture. In the absence of hormones little casein accumulates, but in the presence of hormones the protein accumulates rapidly. Removal of hormones from explants after 24 h in culture reduces the rate at which the amount of casein accumulates in the tissue. Histological and radioautographic evidence (with either [3 H]leucine or [3 H]mannosamine) show that newly synthesized casein is rapidly secreted from the lobulo-alveolar cells into the lumen. Within 6–8 h after a pulse of amino acid or amino sugar precursor, radiolabelled casein can be detected in the lumen (results not shown).

(b) *Effects of hormones on casein synthesis.* The rate of casein synthesis in explants is shown in Fig.

Fig. 4. *Effects of addition and removal of hormones on casein accumulation in mammary explants*

At the times indicated groups of 50 explants were removed from culture, incubated with radiolabelled precursors (see Fig. 5) and homogenized in 5 mM-EGTA (1.5 ml). Particle-free supernatant fractions were prepared as described in the Materials and Methods section. The amount of casein in the particle-free supernatant fraction was measured by means of 'rocket' immunoelectrophoresis. Immunodetectable casein in explants cultured with hormones (Δ), after removal of hormones (●) or in explants cultured throughout in the absence of hormones (○). The results shown for explants in the presence or absence of hormones represent means $\pm$ s.d. for the numbers of animals shown in parentheses. The results shown for explants cultured after the removal of hormones represent the mean $\pm$ half the difference for two experiments.

Fig. 5. Effects of addition and removal of hormones on the rate of casein synthesis in mammary explants. At the times indicated groups of 50 explants were

incubated for 1 h at 37°C under O₂/CO₂ (19:1) in 0.5 ml of medium 199 (glucose-free) containing 1.5 mM-leucine, 5 mM-sodium pyruvate and 20 μ Ci of L-[4,5-³H]leucine. After incubation each group of explants was homogenized and particle-free supernatants were prepared as described in the Materials and Methods section. The incorporation of radioactivity into casein was measured after immuno-adsorbent chromatography and polyacrylamide-gel electrophoresis in the presence of SDS and urea (see the Materials and Methods section). Explants were cultured in the presence of hormones (Δ), after removal of hormones at 24 h in culture ($\bullet$), or in the absence of hormones ($\circ$). The results are means $\pm$ S.D. for the numbers of animals shown in parentheses, except for values at 66 and 72 h after hormone withdrawal, which represent single observations. The maximum synthetic rate is equivalent to the 226 $\pm$ 115 d.p.m. in casein per mg fresh wt. of tissue for the four animals.

Fig. 6. Casein degradation and glycosylation

Explants (280) were cultured with hormones for 36 h and then incubated with [4,5-³H]leucine (60 μ Ci) and [1-¹⁴C]-glucosamine (42 μ Ci) at 37°C for 1 h under O₂/CO₂ (19:1) in 3 ml of medium 199 (glucose-free) containing 1.5 mM-leucine and 5 mM-sodium pyruvate. After incubation the medium was removed and the explants were washed 4 times by incubation at 37°C for 10 min with medium 199 (5 ml) containing 10 mM-L-leucine. After being washed, explants were cultured in the presence ($\bullet$) or absence ($\circ$) of hormones. At the times indicated groups of 40 explants were homogenized, particle-free supernatants prepared, casein and particle-free supernatant protein was isolated and incorporation of radioactivity measured as described in the Materials and Methods section. Incorporation of [³H]leucine into particle-free supernatant protein (a) or casein (b) was measured. Incorporation of [¹⁴C]glucosamine into particle-free supernatant protein (c) or casein (d) was also measured. Incorporation is plotted as log (d.p.m. in protein) so that any first-order decay can be observed.

5. In explants cultured throughout in the absence of hormones very low rates of casein synthesis were measured. Culture of explants with hormones results in a delayed increase in rate of casein synthesis; removal of hormones after 24 h in culture results in much lower rates of casein synthesis than in the presence of hormones. The rate of casein synthesis still increases after hormone withdrawal and does not decline to rates seen in explants cultured throughout in the absence of hormones. The increase in casein synthesis after hormone withdrawal accounts for the increase in the amount of casein in the tissue that occurs in these conditions (Fig. 4). The effects of hormonal manipulation on the synthesis of general protein in the particle-free supernatant is the same as described by Speake *et al.* (1976), i.e. a rapid increase in synthesis in the presence of hormones and rapid decrease in synthesis after hormone removal.

(c) *Putative degradation of casein.* Experiments were designed to measure putative casein degradation during a phase of rapid casein accumulation and after hormone removal. A pulse-chase technique was employed whereby explants were incubated after 36 h in culture with hormones with [^3H]leucine, carefully washed and cultured again in the presence or absence of hormones for a further 36 h. The label in casein was measured at intervals during this period. During the incubation with [^3H]leucine, explants were also cultured with [^{14}C]glucosamine. Therefore the label from this precursor in casein could also be measured. In preliminary experiments it was found that [^{14}C]glucosamine is incorporated into casein and general protein in the particle-free supernatant at rates that mimic [^3H]leucine incorporation in the presence or absence of hormones or after hormone removal (results not shown).

The results of the pulse-chase experiment, typical of three experiments performed, are shown in Fig. 6. A considerable loss of ^3H label and ^{14}C label from general particle-free supernatant protein occurs in the presence and absence of hormones (Figs. 6a and 6c) in the first 18 h after the pulse of radiolabelled precursors. Subsequently little loss of either label occurs in the presence or absence of hormones. An increase in the incorporation of both labels into casein during the first 8 h after the pulse occurs in the presence but not in the absence of hormones (Figs. 6b and 6d). Subsequently no loss of either label from casein occurs. The results indicate that some particle-free supernatant proteins (and glycoproteins) are degraded at a high rate in the presence or absence of hormones (Figs. 6a and 6c). The conditions for the precursor pulse and washing procedures were carefully designed so that radioactive isotope reutilization is minimized (Speake *et al.*, 1976; Mayer, 1979). Interestingly, increased incorporation of ^3H and ^{14}C labels (Figs. 6b and 6d) into casein occurs during the 8 h period after the pulse in the presence but not in

the absence of hormones. The data are consistent with the release of newly synthesized casein from the explants with little or no degradation intracellularly in the presence of hormones and after hormone withdrawal.

Discussion

Measurement of the amount and turnover of casein

The use of 'rocket' immunoelectrophoresis (Fig. 1) demonstrates a relatively simple and quick method for the quantitative determination of casein in the particle-free supernatant. The present work also demonstrates that the combination of immuno-adsorbent chromatography and polyacrylamide-gel electrophoresis in the presence of SDS and urea is an excellent technique for the isolation of casein from the particle-free supernatant prepared from mammary explants, since only one major radioactive peak corresponding to the position of the stained casein band is seen (Fig. 2a). Immunoprecipitation followed by polyacrylamide-gel electrophoresis in the presence of SDS and urea proved to be a much less satisfactory method for the isolation of casein, since several radioactive peaks were obtained (Fig. 2b), indicating extensive non-specific precipitation. The incorporation of radioactivity into casein cannot be accurately measured by these procedures in spite of the fact that specific anti-casein IgG was used.

Multiple peaks were also obtained when polyacrylamide-gel electrophoresis in the presence of SDS was performed on casein immunoprecipitates (Craig *et al.*, 1976). At least three of these peaks may be due to casein, since the electrophoretic system did not contain urea (Al-Sarraj *et al.*, 1978a), but others may be due to non-specific precipitation.

Casein synthesis and accumulation

Casein is synthesized at an increasing rate in the presence of insulin, prolactin and cortisol (Fig. 5) and consequently accumulates in the mammary explants (Fig. 4). Accumulation is hormone-dependent, in that hormones stimulate the increased synthesis and accumulation of casein, and hormone removal results in lower synthetic rates and therefore less accumulation of the protein in the explants. Histological and radioautographic evidence (Al-Sarraj, 1978) indicates that newly synthesized casein is rapidly released from the lobulo-alveolar cells into the extracellular lumina on hormone stimulation. It has also been demonstrated by Mancini immunodiffusion (results not shown) that casein is not secreted into the medium. Therefore casein only accumulates in the lumina of the lobulo-alveolar cells.

The hormonal enhancement of casein synthesis *in vivo* and *in vitro* is consistently accompanied by an increase in the concentration of mRNA for casein

(Houdebine & Gaye, 1975; Rosen *et al.*, 1975; Houdebine, 1976; Rosen & Barker, 1976; Terry *et al.*, 1977; Devinoy *et al.*, 1978; Houdebine *et al.*, 1978; Rosen *et al.*, 1978). Although prolactin is the primary requisite for increased casein synthesis in rabbit mammary gland *in vivo* (Houdebine *et al.*, 1978) and in mammary explants from pseudopregnant rabbits *in vitro* (Devinoy *et al.*, 1978), cortisol enhances the tissue response to the hormone. Houdebine *et al.* (1978) have shown in pseudopregnant rabbits that after prolactin stimulation cortisol was able to maintain a high rate of casein synthesis even in the presence of bromocryptine, which suppresses endogenous prolactin production. The results in Fig. 4 show that casein-synthesis rate can be partially maintained, after potentiation by insulin, prolactin and cortisol, even in the absence of hormones, at approx. 30% of that observed in the presence of hormones. This is in marked contrast with the rates of synthesis of fatty acid synthetase and general particle-free supernatant protein which decrease precipitously when hormones are withdrawn from explants (Speake *et al.*, 1975, 1976). Enhanced stability of casein mRNA relative to messengers for other proteins may explain this phenomenon (Houdebine *et al.*, 1978).

Casein degradation

Previous work on casein accumulation in mammary gland *in vivo* and *in vitro* has concentrated on aspects of transcription and translation of the mRNA for the protein. However, the accumulation profile for intracellular and secreted proteins may depend not only on quantitative changes in their rates of synthesis but also on any such changes in their rates of degradation (Paskin & Mayer, 1978; Mayer & Paskin, 1978). Work on zymogens in pancreatic rudiments in organ culture (Kemp *et al.*, 1972; Rutter *et al.*, 1973) has shown that the rates of synthesis of pancreatic secretory proteins vary as the derivative of their rates of accumulation. A decrease in synthetic rate occurs as accumulation reaches a steady-state. Zymogens appear not to be degraded intracellularly during the developmental accumulation of these secretory proteins. Other than these studies, little information is available on whether secretory proteins are degraded during cytodifferentiation.

From the histological and radioautographic studies on mammary explants in organ culture (K. Al-Sarraj, D. White & R. J. Mayer, unpublished work) it appears that casein is secreted from the lobulo-alveolar cells within approx. 9 h of synthesis. The results in Fig. 6(b) show that casein is not degraded in mammary explants when it either rapidly accumulates in the presence of hormones or more slowly accumulates in the absence of hormones. Previous work (Speake *et al.*, 1975, 1976; Mayer, 1979) has

shown that the experimental conditions used for the pulse-chase experiments shown in Fig. 6 give minimal amino acid isotope reutilization. Considerable loss of label from general particle-free supernatant protein occurs in the presence and absence of hormones (Fig. 6a). However, in the presence of hormones an initial increase in label in casein occurs, whereas subsequently there is no change in the incorporation into the secretory protein. Label released from general protein breakdown may be incorporated into casein in the presence of hormones, since the synthetic rate of casein is rapidly increasing in these conditions. The results are compatible with the synthesis and rapid release of casein from the lobulo-alveolar cells with little or no degradation either in the cells (Fig. 6b) or extracellularly (Speake *et al.*, 1976). In contrast with zymogens in pancreatic rudiments (Kemp *et al.*, 1972), little casein accumulates in epithelial cells in explants; the size of the casein intracellular pool(s) is unknown, but must be so small that no degradation of the casein can be measured by the pulse-chase technique when hormones are removed from the tissue (Fig. 6b). In these conditions a considerable increase in the rate of degradation of fatty acid synthetase occurs in the explants (Paskin & Mayer, 1978; N. Paskin & R. J. Mayer, unpublished observations). It appears therefore that casein may be rapidly synthesized and secreted in the presence and absence of hormones. This pattern of synthesis and secretion in mammary explants may indicate that hormonal stimulation produces conditions equivalent to the end of pregnancy and beginning of lactation, where secretory products are mostly extruded into the alveolar lumina and are not degraded in the cells (Hollmann, 1974).

Finally the labelling pattern of general particle-free supernatant protein and casein obtained with [^{14}C]glucosamine as precursor parallels that seen with [^3H]leucine. The results (Fig. 6c) indicate that glycoproteins are degraded in mammary explants and that concomitantly casein (Fig. 6d) is glycosylated and rapidly secreted from the tissue.

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