Regulation of Enzyme Turnover during Tissue Differentiation

STUDIES ON THE EFFECTS OF HORMONES ON THE TURNOVER OF FATTY ACID SYNTHETASE IN RABBIT MAMMARY GLAND IN ORGAN CULTURE

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1. Explants of mammary gland from mid-pregnant rabbits were cultured with insulin, prolactin and cortisol. 2. Antibodies raised to fatty acid synthetase were used to measure the amount as well as the rate of synthesis and the rate of degradation of the enzyme in the explants over defined periods in organ culture. These measurements were also made after the hormones had been removed from the culture medium. The changes which occur in the activity of fatty acid synthetase are due to changes in the amount of the enzyme present. They are not due to activation or inactivation of the enzyme. 3. The rate of lipogenesis (measured from $[1-^{14}\text{C}]$ acetate) in the explants during culture varies independently of the amount of fatty acid synthetase both in the presence and after removal of the hormones. Hence the amount of fatty acid synthetase does not limit lipogenesis. The proportion of medium-chain fatty acids $\text{C}_{8:0}$ and $\text{C}_{10:0}$ (which are characteristic of rabbit milk) synthesized by the explants in the presence of hormones increases at about the same rate as the amount of fatty acid synthetase present. However, when hormones are removed from the medium the proportion of these acids synthesized declines as rapidly as the rate of lipogenesis and not as the amount of fatty acid synthetase present. 4. The rates of synthesis of fatty acid synthetase and of the total particulate-free supernatant protein in the explants were compared by measuring the incorporation of $\text{L-[U-}^{14}\text{C]leucine}$ into the protein of the explants. These rates increase by 5-fold and 3.6-fold respectively when explants are cultured with hormones, and they then reach approximately constant rates. When the hormones are removed there is a rapid fall in the rate of synthesis of fatty acid synthetase and of the total particulate-free supernatant protein to values which are similar to those obtained with freshly prepared explanted tissue. 5. In unstimulated explants fatty acid synthetase appears to be degraded with a half-life of 15–21 h. During the hormonally stimulated differentiation of the tissue the rate of degradation of the enzyme is considerably decreased or is switched off completely. After the amount of fatty acid synthetase has increased to a maximum the enzyme complex is again degraded with a half-life of 23–29 h. The removal of hormones after the explants have been hormonally stimulated for different times results in an increase in the rate of degradation of fatty acid synthetase. However, this increase only occurs if degradation was previously proceeding at a considerably decreased rate. The degradation of the total particulate-free supernatant protein continues throughout the period of differentiation of the explant tissue in culture. It appears to be somewhat decreased during the period of rapid maturation of the tissue during culture.

Previous work has shown the usefulness of mammary-gland explants maintained in organ culture in studying the hormonal regulation of the synthesis and accumulation of milk proteins and lactose which occurs during pregnancy [see Forsyth (1971) for review]. The technique has also been used to study the hormonal stimulation of milk-fat synthesis in mammary explants from pregnant animals (Moretti & Abraham, 1966; Mayne & Barry, 1970; Strong *et al.*, 1972; Forsyth *et al.*, 1972).

Our understanding of cytodifferentiation in developing mammary gland has been furthered by studies on mammary explants in organ culture (Owens *et al.*, 1973). This system also provides a convenient experimental model to study the regulation of enzyme turnover and of general protein turnover in the gland. This enables enzyme biosynthesis and degradation to be investigated during hormonally controlled cytodifferentiation and tissue development. The effects on enzyme turnover of

removing hormones from the culture medium can also be studied. This could mimic the situation of the mammary gland during involution.

When mammary explants from mid-pregnant mice are cultured with insulin, prolactin and cortisol there is a striking accumulation both of the enzymes involved in the synthesis of secretory products and of the secretory products themselves. These changes are obviously parameters of cytodifferentiation in the tissue [see Turkington (1972) for review]. Cytodifferentiation occurs in the epithelial cells of the rabbit mammary gland between day 18 and day 22 of pregnancy (Bousquet *et al.*, 1969). This is the period when lactogenesis is initiated and there is a striking increase in the rate of fatty acid synthesis (Strong & Dils, 1972). This increased rate of lipogenesis can be simulated *in vitro* by culturing mammary explants from 16-day pregnant rabbits with insulin, prolactin and corticosterone (Forsyth *et al.*, 1972). The present report describes the turnover of fatty acid synthetase which occurs in these explants during culture with these hormones and the effects on this turnover of removing the hormones from the culture medium. Preliminary reports of part of this work have been given (Mayer *et al.*, 1974; Dils *et al.*, 1974).

Materials and Methods

Animals

New Zealand White virgin female rabbits (albinos) were obtained from the Joint Animal Breeding Unit, School of Agriculture, Sutton Bonington, Leics., U.K. The animals were at least 6 months old at the time of mating. The experiments described used explants from mammary glands obtained from mid-pregnant (16–17-day) rabbits.

Materials

Medium 199 (Morgan *et al.*, 1950) was obtained from Wellcome Research Laboratories, Beckenham BR3 3BS, Kent, U.K. Ox insulin was a gift from Mr. V. J. Birkinshaw, Boots Pure Drug Co., Nottingham, U.K. Ovine prolactin [NIH-P-SII (26.4 i.u./mg)] was a gift from the Pituitary Hormone Distribution Program, National Institute of Arthritic and Metabolic Disease, Bethesda, Md., U.S.A. Sodium [1-¹⁴C]acetate and L-[U-¹⁴C]leucine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cortisol-21-acetate, streptomycin sulphate, sodium benzyl penicillin, fatty acids, BF₃-methanol reagent, NADPH, CoA, malonyl-CoA and bovine serum albumin were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Calcium phosphate gel and agarose were obtained from BDH Chemicals, Poole, Dorset, U.K.

DEAE-cellulose (DE-52) was purchased from Whatman Ltd., London E.C.4, U.K. Freund's complete adjuvant was obtained from Difco, Detroit 1, Mich., U.S.A. All other chemicals were of A.R. grade. The Quickfit microcentrifuge was obtained from Quickfit Instruments, Stone, Staffs., U.K.

Methods

Preparation and culture of mammary explants. Explants of lobuloalveolar mammary tissue were prepared as described by Forsyth & Myers (1971). The explants were cultured under the conditions described by Strong *et al.* (1972), i.e. in Medium 199 containing insulin (5 µg/ml), prolactin (1 µg/ml) and cortisol (1 µg/ml). The Medium 199 included 21 mM-NaHCO₃ and streptomycin sulphate (0.1 mg/ml) and was changed every day. Up to ten explants were cultured with 1 ml of the medium.

Incubation of explants. (a) Incorporation of sodium [1-¹⁴C]acetate into fatty acids. After the period in culture, duplicate groups of five explants were removed and incubated for 1 h at 37°C under O₂+CO₂ (95:5) in 1 ml of Krebs-Henseleit buffer (pH 7.4) (Krebs & Henseleit, 1932) containing 1 mM-glucose and 0.1 mM-sodium [1-¹⁴C]acetate (2 µCi).

(b) Incorporation of L-[U-¹⁴C]leucine into fatty acid synthetase and into total protein. After the culture period, groups of ten explants were removed and incubated for various times at 37°C under O₂+CO₂ (95:5) in 1 ml of Medium 199 containing 0.46 mM-L-[U-¹⁴C]leucine (1 µCi). The explants were then washed four times with Medium 199 before being processed further.

Preparation of the particulate-free supernatant fraction. Explants were homogenized at 4°C in 0.25 M-sodium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol. The particulate-free supernatant fraction was then prepared by centrifugation of the homogenate for 6 × 10⁶ g_{av.}-min.

Preparation of acetyl-CoA. Acetyl-CoA was prepared by the method of Smith *et al.* (1966).

Saponification of lipids, extraction of fatty acids and analysis of ¹⁴C-labelled fatty acids by radio-g.l.c. The procedures used have been described by Strong *et al.* (1972). A portion of the extract containing the fatty acids was dissolved in 5 ml of xylene scintillator and the radioactivity measured.

Purification and assay of fatty acid synthetase. The purification of fatty acid synthetase was carried out by the method of Carey & Dils (1970). The activity of the enzyme was measured by a modification of the assay described by Wakil *et al.* (1958) and by Lynen *et al.* (1964). The assay system contained 0.2 M-potassium phosphate buffer, pH 6.6, 1 mM-dithiothreitol, 1 mM-EDTA, 0.15 mM-NADPH, 40–50 µM-malonyl-CoA and 30 µM-acetyl-CoA (final

concentrations). Enzyme protein was added to produce an absorbance change of 0.05–0.15 unit/min at 37°C in a final volume of 1.0 ml.

Immunological procedures. Purified fatty acid synthetase (2 mg in 0.5 ml of potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol) was emulsified with an equal volume of Freund's adjuvant. Equal volumes of the emulsion were injected into four intramuscular and four subcutaneous sites on a sheep. The injections were repeated a further three times at 2-week intervals and 1 litre of blood was collected by jugular cannulation 2 weeks after the final injection. The blood was allowed to clot by being left overnight at room temperature. The serum was collected after centrifugation for 40000 g_{av.}-min and the immunoglobulin fraction was precipitated at 50% (NH₄)₂SO₄ saturation at 4°C. The precipitate was redissolved in a volume of 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl which was equal to half the original serum volume. The solution was then dialysed at 4°C overnight against the same buffer. The reconstituted immunoglobulin fraction was stored in small portions at –20°C. Non-immune serum (100 ml) was collected from the sheep before the immunization schedule. It was fractionated exactly as described for the serum which contained anti-(fatty acid synthetase). Ouchterlony double-diffusion analyses were carried essentially as described by Ouchterlony (1949) with 1% (w/v) agarose gels prepared in 0.25 M-potassium phosphate buffer, pH 7.4.

Immunotitration of fatty acid synthetase activity. Samples were prepared of purified fatty acid synthetase, of the particulate-free supernatant fraction prepared from homogenates of lactating-rabbit mammary gland (both samples were stored at –20°C before use) and of the particulate-free supernatant fraction prepared from homogenates of the explants. These were titrated with the immunoglobulin fraction (49 mg of protein/ml) which contained anti-(fatty acid synthetase) antibodies. Each incubation mixture was brought to a final volume of 0.15 ml with 20 mM-sodium phosphate buffer, pH 7.0, which contained 0.15 M-NaCl. They were then preincubated for 10 min (except for mixtures containing the purified enzyme which were incubated for 30 min) at 37°C and then incubated for 24 h at 4°C. The immunoprecipitates were removed by centrifugation in a Quickfit microcentrifuge at maximum speed for 6 min. The supernatants were assayed for fatty acid synthetase activity. The immunoglobulin fraction which had been prepared from the serum of non-immunized sheep was used as a control. No immunoprecipitates formed, nor was there inactivation of purified fatty acid synthetase, or inactivation of fatty acid synthetase in the particulate-free supernatant fractions prepared from

explants or from homogenates of lactating-rabbit mammary gland.

Measurement of the incorporation of L-[U-¹⁴C]-leucine into the particulate-free supernatant protein prepared from explant homogenates. This was measured after a sample (0.05–0.2 ml) of the particulate-free supernatants prepared from the homogenate had been precipitated with trichloroacetic acid (final concn. 5%, w/v). Bovine serum albumin (1 mg) was added to ensure a suitably sized precipitate. The precipitate was washed three times by resuspension in 1 ml of 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl and 1 mM-L-leucine, followed by reprecipitation with trichloroacetic acid (final concn. 5%, w/v). The washed precipitate was dissolved in 0.3 ml of 90% (v/v) formic acid. Triton-xylene scintillator (10 ml) was added and the radioactivity measured.

Measurement of the incorporation of L-[U-¹⁴C]-leucine into immunodetectable fatty acid synthetase. Purified fatty acid synthetase (50 µg) was added as a carrier to 0.2 ml samples of the particulate-free supernatant protein prepared from explant homogenates. A twofold excess of immunoglobulin fraction which contained anti-(fatty acid synthetase) was then added. The samples were incubated for 30 min at 37°C and then for 24 h at 4°C. The immunoprecipitates were washed three times at 4°C with 1 ml portions of 20 mM-sodium phosphate buffer, pH 7.0, which contained 0.15 M-NaCl and 1 mM-L-leucine. They were then suspended in 1.0 ml of this buffer, precipitated with trichloroacetic acid (final concn. 5%, w/v) and dissolved in 0.3 ml of 90% (v/v) formic acid. Triton-xylene scintillator (10 ml) was added and the radioactivity measured. There was negligible co-precipitation when each sample was immunoprecipitated for a second time after the addition of 50 µg of purified fatty acid synthetase. Non-specific precipitation was measured by adding the non-immune immunoglobulin fraction to another portion of each particulate-free supernatant fraction after the addition of 50 µg of purified fatty acid synthetase. The precipitate contained 10–20% of the radioactivity which was obtained by using the antiserum prepared against fatty acid synthetase. The radioactivity incorporated into fatty acid synthetase was taken as being equal to the radioactivity in the immunoprecipitate obtained with the antiserum prepared to fatty acid synthetase minus the non-specific precipitation of radioactivity obtained with the non-immune immunoglobulin fraction.

Protein determination. Protein was determined by the method of Hübscher *et al.* (1965). With dilute solutions the method of Lowry *et al.* (1951) was used.

Measurement of radioactivity. The xylene scintillator used to measure the radioactivity of fatty acids contained 0.4% (w/v) of 2,5-diphenyloxazole

and 0.01% (w/v) of 1,4-bis-(5-phenyloxazol-2-yl)-benzene in xylene. The Triton-xylene scintillator contained 0.5% (w/v) of 2,5-diphenyloxazole, 0.01% (w/v) of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 33% (v/v) of Triton X-100 in xylene. Radioactivity was measured in a Packard Tri-Carb 3375 liquid-scintillation spectrometer.

Measurement of the specific radioactivity of leucine in explants. (a) Incubation and extraction of amino acids. Explants were cultured for 1 h as described above with $1 \mu\text{Ci}$ of L-[U- ^{14}C]leucine and were then washed once with 0.9% (w/v) NaCl. Norleucine (25 pmol) was added to each sample as an internal standard. Each preparation was heated at 100°C for 2 min, and then homogenized. The homogenate was heated at 100°C for a further 10 min, and was then centrifuged for 15 min at maximum speed in a bench centrifuge. The supernatant was passed through a Millipore filter ($0.45 \mu\text{m}$), and the filtrate was made to a volume of 0.5 ml with the 0.2 M-sodium citrate loading buffer, pH 2.2.

(b) Amino acid analyses. In preliminary experiments amino acid analyses were carried out in a Locarte amino acid analyser attached to a Tracerlab Cor-flow continuous-flow radioactive detector. The results obtained showed that less than 5% of the radioactivity in leucine was associated with the other amino acids. Therefore subsequent samples were divided such that a small portion was retained to measure the radioactivity in the Packard Tri-Carb 3375 liquid-scintillation spectrometer. The rest of the sample was analysed in the Locarte amino acid analyser. Specific radioactivities were calculated from the total radioactivity in each sample together with the leucine content as given by amino acid analysis.

Results

Characterization of the antiserum to purified fatty acid synthetase

Purified fatty acid synthetase gives a single immunoprecipitation line with the antiserum to fatty acid synthetase (Plate 1). Immunoinhibition titrations were performed with the antiserum to fatty acid synthetase against purified fatty acid synthetase or the fatty acid synthetase present in a particulate-free supernatant fraction prepared from the homogenate of a mammary gland. The gland was obtained from a rabbit on the 10th day of lactation. (Both of these preparations were stored at -20°C before use.) The immunoequivalence point for purified fatty acid synthetase was given by $7 \mu\text{l}$ of antiserum (Fig. 1a). By contrast, the immunoequivalence point for the same number of activity units (expressed as nmol of NADPH oxidized/min) of fatty acid synthetase in the particulate-free supernatant fraction obtained from the lactating mammary gland was given by

$9 \mu\text{l}$ of antiserum (Fig. 1b). This discrepancy may possibly result from the presence of inactive or denatured fatty acid synthetase or of other co-precipitants in the particulate-free supernatant preparation. Subsequent immunoprecipitation re-

Fig. 1. Immunotitration of (a) purified fatty acid synthetase and (b) fatty acid synthetase present in a particulate-free supernatant fraction prepared from a homogenate of mammary gland

The gland was obtained from a rabbit on the 10th day of lactation. Fixed volumes of purified fatty acid synthetase (a) or of the particulate-free supernatant fraction (b) were titrated with antiserum to fatty acid synthetase. Each incubation mixture was adjusted to a final volume of 0.15 ml with 0.02 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Each sample was incubated for 30 min at 37°C and then for 24 h at 4°C . The immunoprecipitates were removed by centrifugation and each supernatant was assayed for fatty acid synthetase activity. Each precipitate was washed twice with 0.02 M-sodium phosphate buffer, pH 7.0, which contained 0.15 M-NaCl. The protein content was then measured. No immunoprecipitates formed nor was there inactivation of the purified fatty acid synthetase or of the fatty acid synthetase in the particulate-free supernatant fraction when an immunoglobulin fraction from non-immunized sheep was used. Fatty acid synthetase activity remaining after incubation with antiserum (○); protein in the immunoprecipitate (Δ).

EXPLANATION OF PLATE I

Ouchterlony double-diffusion analysis of fatty acid synthetase purified from mammary gland

The immunodiffusion analysis was carried out in an agarose gel (1%, w/v) in 0.25M-potassium phosphate buffer, pH7.4. The centre well contained purified fatty acid synthetase. This was prepared from mammary glands taken from rabbits on the 15th day of lactation. The antiserum to fatty acid synthetase was placed in the outer wells. The gel was stained with Coomassie Brilliant Blue (0.25%, w/v) in 50% (v/v) methanol containing trichloroacetic acid (5%, w/v).

Fig. 2. Effect of hormones on the specific activity and on the amount of fatty acid synthetase present in mammary explants during culture

After being cultured with insulin, prolactin and cortisol for the times indicated 60 explants were removed and were homogenized in 0.6 ml of 0.25 M-potassium phosphate buffer, pH 7.0, which contained 1 mM-dithiothreitol. Fatty acid synthetase activity was assayed in the particulate-free supernatant fraction which was prepared by centrifugation of the homogenate for $6 \times 10^6 g_{av.}$ -min. Immunological titration of fatty acid synthetase was then carried out. The antiserum to fatty acid synthetase was diluted with 0.02 M-sodium phosphate buffer, pH 7.0, which contained 0.15 M-NaCl so as to give a titre of 20 μ l of antiserum per unit (nmol of NADPH oxidized/min) of fatty acid synthetase activity. Increasing amounts of the diluted fraction were added to 0.1 ml portions of the particulate-free supernatant fractions. Each mixture was incubated at 37°C for 10 min and then at 4°C for 24 h. After centrifugation each supernatant was assayed for fatty acid synthetase activity. At the times indicated 60 explants were washed free of the hormone-containing Medium 199 with 4×10 ml of Medium 199. They were then transferred to hormone-free Medium 199. Fatty acid synthetase activity in explants cultured in the presence of hormones ($\circ$), and after the removal of hormones from the culture medium ($\bullet$). Immunologically detectable fatty acid synthetase in the explants cultured in the presence of hormones (Δ), and after removal of hormones from the culture medium ($\blacktriangle$). Fatty acid synthetase activity in explants which had been cultured throughout in the absence of hormones ($\square$).

actions were carried out with particulate-free supernatant fractions which had been freshly prepared from mammary-gland explants.

Effect of hormones on the activity and on the amount of fatty acid synthetase in explants

The results presented in Fig. 2 show the effects of culturing mammary explants from mid-pregnant rabbits with insulin, prolactin and cortisol. After 20 h in culture and at the subsequent times shown the specific activity of fatty acid synthetase in the explants correlated with the amount of fatty acid synthetase present as measured by immunoinhibition titration. The changes which occurred in the specific activity of the enzyme are therefore due to changes in the amount of fatty acid synthetase in the explants and are not due to activation or inactivation of the enzyme. Fatty acid synthetase activity could not be detected in particulate-free supernatant fractions obtained from freshly prepared explants of mammary gland from mid-pregnant rabbits. No estimate could therefore be obtained by immunoinhibition titration of the amount of fatty acid synthetase in these explants.

Explants were transferred to hormone-free medium after they had been cultured with hormones for 24 or 43 h. There was a decrease of about 30 and 40% respectively in the amount (and therefore in the specific activity) of fatty acid synthetase present. This indicates that the accumulation of fatty acid synthetase in the tissue is dependent on the continued presence of the hormones. Only very low values for fatty acid synthetase activity could be obtained by using explants which had been cultured throughout in the absence of hormones.

Effect of hormones on the rate of lipogenesis and on the proportion of medium-chain fatty acids synthesized by explants

Strong *et al.* (1972) and Forsyth *et al.* (1972) have described the effects of insulin, prolactin and cortisol on the rate of lipogenesis (measured by the incorporation of radioactive acetate into fatty acids) in explants of mammary gland obtained from mid-pregnant, lactating and pseudopregnant rabbits. The highest rate of lipogenesis in explants obtained from mid-pregnant rabbits was observed after the tissue had been cultured for 4 days with the three hormones. The results presented in Fig. 3 show that maximum rates of lipogenesis in explants were obtained after only 20 h in culture with this hormone combination. By contrast, the greatest amounts of fatty acid synthetase were detected in these explants after about 43 h in culture (see Fig. 2).

The removal of hormones from the culture medium after 20, 50 and 76 h caused a precipitous decrease to very low values in the rate of lipogenesis in the explants. By comparison, the amount of fatty acid synthetase in the explants only decreased by about 30 or 40% when the hormones were removed from the

Fig. 3. Effect of hormones on the rate of lipogenesis in mammary-gland explants during culture

After being cultured with insulin, prolactin and cortisol for the times indicated duplicate groups of five explants were removed from the medium. They were then incubated with sodium $[1-^{14}\text{C}]$ acetate in Krebs-Henseleit bicarbonate buffer which contained glucose for 1 h at 37°C . The lipids in each group of explants were extracted and saponified. The fatty acids were extracted and one-tenth of each extract was used to determine the radioactivity present (see the Materials and Methods section). The rates of fatty acid synthesis have been calculated by the method of Strong *et al.* (1972). The rate of lipogenesis in explants which had been cultured in the presence of hormones (○), after removal of the hormones from the medium (●) and in explants which had been cultured throughout in the absence of hormones (□). The results are expressed as means $\pm 2 \times \text{s.e.m.}$ The values in parentheses indicate the number of observations at each time-point.

medium after 24 or 43 h (see Fig. 2). These results show that the rate of lipogenesis during culture varied independently of the amount of fatty acid synthetase present. This occurred both when hormones were present and after the removal of hormones from the medium.

Forsyth *et al.* (1972) have shown that this increased rate of lipogenesis is accompanied by an increase in the proportion of $\text{C}_{8:0}$ and $\text{C}_{10:0}$ fatty acids synthesized. The proportion of these acids synthesized was greater in the presence of insulin and prolactin than in the presence of all three hormones. These medium-chain fatty acids are characteristic of rabbit milk and are the predominant fatty acids synthesized by lactating-rabbit mammary gland (Carey & Dils, 1972).

The results presented in Fig. 4 show that the proportion of the total radioactivity which was present in these medium-chain fatty acids can reach about 50% of the total after the explants had been cultured for about 48 h with insulin, prolactin and cortisol.

Fig. 4. Effect of hormones on the chain length of the fatty acids synthesized by mammary-gland explants in culture

Portions of the fatty acid samples (which were obtained as described in the legend to Fig. 3) were analysed by radio-g.l.c. To obtain sufficient radioactivity for radio-g.l.c. equal portions of the pentane-diethyl ether extracts prepared from duplicate explant preparations were combined (i.e. 90% of the pentane-diethyl ether extract prepared from each sample was combined). The percentage of the total radioactivity present in medium-chain fatty acids when explants had been cultured in the presence of hormones (○), after removal of the hormones from the medium (●) and when explants had been cultured throughout in the absence of hormones (□). The results are expressed as the means $\pm 2 \times \text{s.e.m.}$ The values in parentheses indicate the number of observations at each time-point.

This is about twice the proportion previously reported (Forsyth *et al.*, 1972).

In contrast with the increase in the rate of lipogenesis this increase in the proportion of medium-chain fatty acids synthesized correlated with the increase in the amount of fatty acid synthetase present in explants which have been cultured in the presence of the three hormones (cf. Figs. 2, 3 and 4). However, when these explants are subsequently cultured in the absence of hormones the proportion of medium-chain fatty acids synthesized decreases precipitously (Fig. 4), as does the rate of lipogenesis (Fig. 3). These changes contrast with the much slower decrease in the amount of fatty acid synthetase in the explants under these conditions (Fig. 2).

Effect of hormones on the rates of synthesis of fatty acid synthetase and of total particulate-free supernatant protein

The rate of synthesis of fatty acid synthetase increases 5-fold and the rate of synthesis of the total particulate-free supernatant protein increases 3.6-fold after explants are cultured for 40 h with insulin,

Fig. 5. Effect of hormones on the rates of synthesis of fatty acid synthetase and of total particulate-free supernatant protein

After being cultured with insulin, prolactin and cortisol for the times indicated groups of ten explants were removed and incubated for 1 h at 37°C under O₂+CO₂ (95:5) in 1 ml of Medium 199 containing 1.0 μ Ci of L-[U-¹⁴C]leucine. At the end of the incubation period each group of explants was homogenized and the particulate-free supernatant fraction was prepared. The rate of incorporation of radioactivity into the particulate-free supernatant protein and into fatty acid synthetase was measured after precipitation with trichloroacetic acid or immunoprecipitation respectively. The rate of incorporation of radioactivity into fatty acid synthetase (a) and into total particulate-free supernatant protein (b) by explants which had been cultured in the presence of hormones (○), or after the removal of hormones (●) or by explants which had been cultured throughout in the absence of hormones (□). The results are expressed as means $\pm$ 2 $\times$ s.e.m. The values in parentheses indicate the number of observations at each time-point.

prolactin and cortisol (Fig. 5). When explants are cultured in the absence of hormones the rates of synthesis of fatty acid synthetase and of the total particulate-free supernatant protein remain approximately constant. These rates are not significantly different from those shown by freshly prepared mammary explants. The removal of hormones after 20 or 40h causes a rapid decrease in the rate of synthesis both of fatty acid synthetase (Fig. 5a) and the total particulate-free supernatant protein (Fig. 5b). Within 20h of the hormones being removed the respective rates of synthesis are not significantly different from the values obtained by using freshly prepared explants. These results demonstrate the precise regulation by the hormones of the rates of synthesis of fatty acid synthetase and of the total soluble protein in these mammary explants.

Leucine concentration and the specific radioactivity of total leucine after incubation of explants with L-[U-¹⁴C]leucine

The concentration of leucine and the specific radioactivity of the total leucine pool were measured

in explants after incubation for 1 h with L-[U-¹⁴C] leucine at various times in culture (Table 1).

The concentration of leucine in the explants increases approx. 2.5-fold and the rate of uptake of L-[U-¹⁴C]leucine increases approx. 3.1-fold after the explants are cultured with hormones for 40h, and then both decline after the hormones are removed. The specific radio activity of the total leucine pool is 1.2-fold greater in explants which have been cultured for 40h with hormones than in freshly prepared explants. When explants were cultured for 19h after hormones had been removed from the medium, the specific radioactivity of the total leucine pool was 0.92 of that measured in freshly prepared explants.

These changes in the concentration of leucine are paralleled by changes in the radioactivity which is incorporated into the explants when they are incubated for 1 h with L-[U-¹⁴C]leucine. The magnitude of the changes which are measured in the specific radioactivity of the total leucine pool is therefore relatively small. Hence the rates of incorporation of L-[U-¹⁴C]leucine into fatty acid synthetase and into the total particulate-free supernatant protein are only apparent and not absolute values.

Table 1. *Effect of hormones on the leucine concentration and on the specific radioactivity of total leucine in mammary explants from mid-pregnant rabbits in organ culture*

Explants were cultured for the times indicated in the presence or after the removal of hormones from the medium. After being cultured with hormones, groups of 120 explants were transferred to 1 ml of Medium 199 which contained 1 μ Ci of L-[U- 14 C]leucine. They were then incubated for 1 h at 37°C under O₂+CO₂ (95:5). Amino acids were subsequently extracted and the amino acid analyses were carried out as described in the Materials and Methods section.

Total time in culture (h)	Initial time in culture with hormones (h)	Leucine concentration (nmol/mg fresh wt. of tissue)	Radioactivity incorporated (d.p.m./h per mg fresh wt. of tissue)	Specific radioactivity (d.p.m./nmol of leucine)
0	0	0.06	170	2880
40	40	0.16	540	3490
59	40	0.11	290	2650

Table 2. *Degradation of fatty acid synthetase and of the total particulate-free supernatant protein in mammary explants in culture*

For each measurement period four groups of 10–15 explants were removed from the culture medium. They were then incubated for 2 h at 37°C with 1 μ Ci of L-[U- 14 C]leucine in Medium 199 under O₂+CO₂ (95:5). The explants were thoroughly washed four times with fresh Medium 199 and then returned to culture. Where indicated explants were cultured with insulin, prolactin and cortisol. At intervals during each period where degradation was measured (zero time, 5, 16 and 20 or 25 h) groups of 10–15 explants were removed from culture, homogenized and a particulate-free supernatant was prepared from each group. Fatty acid synthetase or the total particulate-free supernatant protein was precipitated, washed and the radioactivity measured (see the Materials and Methods section). The apparent degradation coefficients (K_d) were calculated from the gradients of graphs of log d.p.m. in the fatty acid synthetase or in the total particulate-free supernatant protein versus the time after the administration of L-[U- 14 C]leucine (i.e. slope $\times$ 2.303). Therefore K_d is the apparent first-order coefficient for the disappearance of radiolabelled fatty acid synthetase or of radiolabelled total particulate-free supernatant protein in the explants. Half-lives are calculated from the relationship $t_{1/2} = \ln 2/K_d$.

Period in culture when degradation was measured (h)	Culture conditions	Apparent half-lives ($t_{1/2}$, h)									
		Total particulate-free supernatant protein in experiment					Fatty acid synthetase in experiment				
		1	2	3	4	5	1	2	3	4	5
0–24	No hormones	30	15	26	56	32	20	16	16	21	15
0–24	+Hormones				50					63	
24–48	+Hormones	∞	32	48	62	39	∞	28	∞	∞	172
48–72	+Hormones	63	110	31	42	28	46	120	∞	254	∞
72–96	+Hormones	18		22			23		29		
24–48	Hormones removed after 24 h		32			46		30			62
48–72	Hormones removed after 48 h		75			46		32			100

Rates of degradation of fatty acid synthetase and of the total particulate-free supernatant protein in explants in culture

The rates of degradation of fatty acid synthetase and of the total particulate-free supernatant protein are measured over defined periods during which the explants were maintained in organ culture. The disappearance of radioactivity from immunodetectable fatty acid synthetase and from the trichloroacetic acid-precipitable particulate-free supernatant protein was found to be exponential with respect to time. This was assumed to represent the degradation of protein, and first-order degradation coefficients were calculated for defined periods in culture.

Isotopic reutilization, which leads to underestimated values for the degradation coefficients, was minimized by extensively washing the explants after incubation with L-[U- 14 C]leucine. The explants were then cultured in Medium 199 which contained 0.46 mM-leucine. The results of five independent experiments are shown in Table 2. Fatty acid synthetase is degraded with a half-life of 15–21 h in explants which had been cultured in the absence of hormones for 24 h. In these experiments drastically decreased or no degradation of fatty acid synthetase could be detected in explants in periods of culture between 24 and 72 h in the presence of hormones. This period includes the time when there is rapid

accumulation of fatty acid synthetase in the explants (Fig. 2). After 72h in culture in the presence of hormones the rate of degradation of fatty acid synthetase in these explants had increased considerably ($t_{\frac{1}{2}} = 23, 29$ h). The rate of degradation of fatty acid synthetase in explants which had been maintained from 0 to 24h in culture in the presence of hormones is considerably lower ($t_{\frac{1}{2}} = 63$ h; Expt. 4) than in explants which have been cultured from 0 to 24h in the absence of hormones ($t_{\frac{1}{2}} = 15-21$ h). The period during culture in the presence of hormones when the rate of degradation of fatty acid synthetase decreased and the extent of the decrease in the rate of degradation of the enzyme complex varies between experiments. These results suggest that the rate of degradation of fatty acid synthetase is decreased as one of the initial events which occur in the hormonal stimulation of explant differentiation and development.

After hormones are removed from explants in culture the rate of degradation of fatty acid synthetase does not decrease to the value obtained by using explants which have been cultured for 24h in the absence of hormones. It might have been expected that in the absence of hormones the rate of degradation would have been the highest rate observed. The results obtained for the rate of degradation of the total particulate-free supernatant protein are equivocal. However, the rate of degradation of the total particulate-free supernatant protein resembles those observed for fatty acid synthetase although the magnitude of the changes is much smaller.

Discussion

There have been several studies on the changes which occur in the specific activity of fatty acid synthetase in a number of tissues brought about by different dietary regimes, or by experimentally induced diabetes (Gibson & Hubbard, 1960; Allmann *et al.*, 1965; Burton *et al.*, 1969; Saggerson & Greenbaum, 1970; Lakshmanan *et al.*, 1972; Volpe & Vagelos, 1974). Immunochemical techniques have shown that these changes are due to alterations in the amounts of the enzyme complex rather than to activation or inactivation of the enzyme present in the tissues (Craig *et al.*, 1972; Volpe & Vagelos, 1974).

The only studies reported on the turnover of fatty acid synthetase during development are those on liver and brain and they were restricted to comparing the turnover of the enzyme in young and adult animals (Volpe *et al.*, 1973). In the work reported in this paper, the amount of fatty acid synthetase and the rates of its synthesis and degradation in mammary tissues were measured during the hormonally regulated sequence of differentiation which occurs in organ culture and during the period after the removal of hormones from the culture medium.

The results presented in Fig. 2 show that the changes which occur in the specific activity of fatty acid synthetase are caused by changes in the amount of the enzyme complex. A similar observation has been reported for changes in the specific activity of fatty acid synthetase in developing liver and brain (Volpe *et al.*, 1973).

The rate of lipogenesis in mammary explants during organ culture changed independently of the amount of fatty acid synthetase present. This was observed when explants were cultured with hormones and after their removal (cf. Figs. 2 and 3). It is clear that the mechanism which regulates lipogenesis in this system is not related to changes in the amount of fatty acid synthetase which is present in the explants. The difference in time required to achieve the maximum rate of lipogenesis and the maximum accumulation of fatty acid synthetase in the tissue (cf. Figs. 2 and 3) shows that the amount of fatty acid synthetase does not limit lipogenesis.

Strong *et al.* (1972) have suggested that the ability of rabbit mammary tissue to synthesize medium-chain fatty acids (which are characteristic of rabbit milk) can be used as a criterion to measure the degree of differentiation in the epithelial tissue. The proportion of these fatty acids which is synthesized increases in a manner which is parallel to the increase which occurs in the amount of fatty acid synthetase when explants are cultured in the presence of hormones (cf. Figs. 2 and 4). However, the proportion of medium-chain fatty acids synthesized decreases rapidly when the hormones are removed from the medium. The synthesis of medium-chain fatty acids by rabbit mammary gland is regulated by a soluble factor which has a high molecular weight (Strong *et al.*, 1973). The time-course of the synthesis of these acids by mammary explants cultured with hormones (Fig. 4) may therefore reflect the accumulation of this factor during culture. This in turn may parallel the time-course for the synthesis of fatty acid synthetase (Fig. 2). The removal of hormones from the medium may result in the rapid loss of this factor.

The measurement of the apparent rates of synthesis of fatty acid synthetase and of the total particulate-free supernatant protein in explants will be affected by the specific radioactivity of the pool of amino acids which are the precursors for protein biosynthesis. The nature of the pool of amino acids which are the precursors for protein synthesis is controversial (Airhart *et al.*, 1974), and its size and composition cannot be accurately measured. However, it is possible to measure the concentration of leucine in the explants and the rate of uptake of L-[U- 14 C]-leucine. It is therefore possible to measure the specific radioactivity attained by the total leucine pool of the explants (Table 1). The explants had a greater concentration of leucine after being cultured for 40h

with hormones than did freshly prepared explants. This may be due to the increased rate of uptake of L-[U- 14 C]leucine by explants which had been in culture for 40 h. That hormones increase the rate of amino acid uptake by developing tissues has been shown with explants of foetal human liver (Schwartz, 1974) as well as with immature rat uterus *in vitro* (Mohri *et al.*, 1974). With mammary explants the removal of hormones from the medium results in a decrease in the concentration of leucine in the tissue and a decrease in the rate of uptake of L-[U- 14 C]leucine by the explants (Table 1).

The traditional method of measurement of the exponential loss of radioactivity from immunoprecipitated fatty acid synthetase or from the precipitated total particulate-free supernatant protein after the incorporation of L-[U- 14 C]leucine into the proteins was used to calculate the apparent degradation coefficients (K_d) for these proteins during defined periods in culture (Table 2). The apparent degradation coefficient is normally measured in steady-state conditions. It then contains two components, one of which is dependent on the absolute rate of protein degradation whereas the other is dependent on the rate of reutilization of isotope. The latter raises particular problems in the interpretation of the rates of protein degradation (Poole, 1971). Every attempt is usually made to decrease the value of the latter component. This is done by administering non-radioactive leucine to dilute the radioactive amino acid pool (Philippidis *et al.*, 1972). In the experiments reported here the period of incorporation of L-[U- 14 C]leucine into explant proteins was followed by extensively washing the explants. They were then cultured in Medium 199 which contained non-radioactive leucine. These procedures should minimize the underestimation of the absolute rates of protein degradation.

The apparent degradation coefficients reported here contain another variable. This is due to the change in the rate of degradation of proteins which occurs during the differentiation of the epithelial cells of the mammary-gland explants during culture. This variable cannot be calculated at present. It will obviously make the greatest contribution to the apparent degradation coefficients when the degradation of fatty acid synthetase is being switched off or switched on again during the accumulation of fatty acid synthetase in the explants (Table 2). This variable in the rate of degradation of fatty acid synthetase does not cause any essential deviation from linearity when $\log d.p.m.$ in the immunoprecipitated fatty acid synthetase is plotted against the time in culture. Similar results have been obtained by Philippidis *et al.* (1972) in studies on the degradation of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) in developing liver. Further,

this method of evaluating the rate of degradation of fatty acid synthetase has enabled a clear distinction to be made between different phases of degradation of fatty acid synthetase during the hormonally regulated accumulation of the enzyme complex in the explants.

The distinctions which can be made (see Table 2) are (a) that fatty acid synthetase is degraded in explants which have been cultured for 24 h in the absence of hormones, (b) that it is degraded at a much lower rate when explants are cultured in the presence of hormones for a similar time period, and (c) that there is drastically decreased or no degradation of fatty acid synthetase during the period 24–72 h in culture, and (d) after more prolonged periods of culture (72–96 h) with hormones the degradation of fatty acid synthetase occurs again at a relatively high rate. These results offer a clear demonstration that the degradation of fatty acid synthetase in explants which are cultured in the presence of insulin, prolactin and cortisol is decreased considerably or is switched off during the period when the enzyme accumulates. Degradation is switched on again when the accumulation is complete. Previous investigators have not measured the degradation of specific enzymes both before and during a sequence of cytodifferentiation. However, Philippidis *et al.* (1972) have shown that phosphoenolpyruvate carboxykinase is not degraded in neonatal liver during the first 24 h after birth. This is the period when the accumulation of enzyme is very rapid but the enzyme is subsequently degraded at a high rate. Though these authors did not measure the degradation of phosphoenolpyruvate carboxykinase in foetal liver, they deduced from the results of other experiments (Philippidis & Ballard, 1969) that the enzyme is appreciably degraded in foetal liver. The marked increase in the amount of 'malic' enzyme [malate dehydrogenase (decarboxylating) (NADP $^{+}$), EC 1.1.1.40] in the liver of the weanling rat as a result of thyroxine treatment may possibly be accompanied by a concurrent decrease in the rate of enzyme degradation (Murphy & Walker, 1974). Silpanata & Goodridge (1971) found very little degradation of the 'malic' enzyme in neonatal chick liver. However, they did not record the rate of enzyme degradation again until the amount of enzyme had increased markedly in the liver after the chicks had been feeding for 8 days. Volpe *et al.* (1973) have suggested that there is no change in the rate of degradation of fatty acid synthetase during the dramatic increase in the amount of the enzyme complex which occurs in liver after weaning. However, the rate of degradation of fatty acid synthetase during the period of enzyme accumulation was not measured.

There is a paucity of data about the degradation of specific enzymes during differentiation. Nevertheless, there are several reports of decreased rates

of protein catabolism during the stimulation of growth which occurs during muscle hypertrophy (Goldberg, 1971), liver regeneration (Scornik, 1972), compensatory renal hypertrophy and during the stimulation of salivary gland by isoproterenol (Hill & Malamud, 1974).

Cytodifferentiation has been studied in several tissues [see Rutter *et al.* (1973) for review] and in particular detail during the secondary developmental transition of the embryonic rat pancreas *in vitro* (Kemp *et al.*, 1972) when the accumulation of exocrine products (e.g. amylase, chymotrypsinogen, insulin and ribonuclease) occurs. These authors point out that where the synthesis of cell-specific proteins have been investigated their rates of synthesis vary as the derivative of the rate of accumulation of the protein. The decrease in the rate of synthesis which occurs as the accumulation reaches a steady state agrees with the absence of turnover or of secretion during the accumulation phase.

It seems reasonable to expect that proteins which are restricted to the zymogen granule and which are destined to be secreted by the cell may be treated differently from intracellular proteins which are involved in the biosynthesis of secretory products. An example of the latter is the fatty acid synthetase in mammary explants which is involved in the synthesis of milk fat for secretion. The accumulation of this lipogenic enzyme in the explants is accompanied by a stimulation of its rate of synthesis. The rate of synthesis then seems to be maintained at a high level and there is a vast decrease in the rate of degradation of the enzyme. After the accumulation of the enzyme is complete, the degradation of the enzyme complex returns to a high rate.

The mechanism of enzyme degradation, the regulation of this degradation and the integration of enzyme degradation with enzyme synthesis are not understood (Goldberg & Dice, 1974). It should now be possible with the mammary-explant system to delineate the regulatory mechanisms involved in enzyme degradation in this differentiating tissue.

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