Regulation of Enzyme Turnover during Tissue Differentiation

INTERACTIONS OF INSULIN, PROLACTIN AND CORTISOL IN CONTROLLING 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 in Medium 199 containing combinations of insulin, prolactin and cortisol. With hormone combinations which included prolactin, a sustained increase in the apparent rate of synthesis and in the amount of fatty acid synthetase was measurable immunologically. Maximum increase was produced with insulin, prolactin and cortisol present together. 2. With prolactin present alone, synthetase activity in the explants decreased to undetectable values after 1 day in culture, whereas the incorporation of L-[U-¹⁴C]leucine into immunodetectable material increased. Prolactin may therefore direct the synthesis of immunologically cross-reactive precursors of fatty acid synthetase which are enzymically inactive. 3. Culture with dibutyryl cyclic AMP plus theophylline in the presence of insulin, prolactin and cortisol delayed the increase in the rate of synthesis and accumulation of the synthetase. These compounds may also prevent the apparent decrease in the rate of degradation of the synthetase which occurs on day 2 of culture. 4. A large decrease in the apparent rate of degradation of the synthetase on day 2 of culture occurs during culture with hormone combinations which include prolactin. The protein obtained by centrifugation of explant homogenates for 6 min at 14000g_{av.} is degraded continuously throughout the culture period. 5. This decrease in the apparent rate of degradation of the synthetase was measured by radio-immunological precipitation. It is probably part of a regulated programme of enzyme degradation and not a reflexion of the reutilization of radioactive amino acids for the following reasons. (a) The calculated increase in the amount of the synthetase in explants on day 2 of culture with insulin, prolactin and cortisol was approximately equal to the measured increase of the enzyme complex which accumulates in the explants. This suggests little or no enzyme degradation has occurred. (b) Explants were cultured for 24 h with insulin, prolactin and cortisol. They were then incubated with L-[U-¹⁴C]leucine, washed and incubated again for up to 4½ h. L-[U-¹⁴C]leucine rapidly equilibrated with the intracellular amino acid pool. Within 10 min of incubation after washing explants to remove endogenous L-[U-¹⁴C]leucine the previously linear incorporation of L-[U-¹⁴C]leucine into total explant protein ceased. This suggests that protein is synthesized from an amino acid pool which rapidly equilibrates with amino acids in the culture medium. (c) Explants were cultured for 24 h as described in (b) but after washing they were cultured with insulin, prolactin and cortisol for 24 h. Approx. 90% of the radioactivity lost from the 'free' intracellular amino acid pool and from amino acids derived from the degradation of explant protein in this period was detected in the culture medium. This suggests that the 'free' intracellular amino acids and amino acids derived from protein degradation can equilibrate with amino acids in the medium. A residual 'free' radioactive amino acid pool was present in the tissue. (d) Casein represents approx. 20% of the protein synthesized after 1 day in culture with insulin, prolactin and cortisol. Histological evidence suggests that on day 2 of culture, casein is unlikely to be degraded in the tissue. No increase in the radioactivity incorporated into casein can be measured in the 23 h after incubation of explants with L-[U-¹⁴C]leucine as described in (b). This suggests that the incorporation of radioactivity into proteins during culture after incubation with L-[U-¹⁴C]leucine is minimal. (e) Inhibition of protein synthesis in explants by cycloheximide after incubation with L-[U-¹⁴C]leucine does not reveal a latent continuous degradation of fatty acid synthetase on day 2 of culture which might have been masked by the high rates of protein

synthesis and therefore the accumulation of the enzyme. 6. The conclusion is discussed that there is a real decrease (or even cessation) in the rate of degradation of fatty acid synthetase during the period when the enzyme accumulates in explants cultured with hormone combinations which contain prolactin.

The turnover (i.e. the synthesis and degradation) of enzymes during cytodifferentiation has not been extensively studied and is therefore not clearly understood. However, the degradation of some enzymes may be drastically decreased or even completely abolished during the period when the enzymes accumulate in cell differentiation (Philippidis *et al.*, 1972; Speake *et al.*, 1975; Every & Ashworth, 1975). Further, the rate of protein catabolism may be significantly decreased during the stimulation of growth which occurs in muscle hypertrophy (Goldberg, 1971), liver regeneration (Scornik, 1972; Swick & Ip, 1974), compensatory renal hypertrophy (Hill & Malamud, 1974) and during the stimulation of salivary gland by isoproterenol (Hill & Malamud, 1974).

The developing mammary gland is an excellent system to study enzyme turnover during differentiation. When explants of mammary gland from mid-pregnant rabbits are cultured with insulin, prolactin and cortisol cytodifferentiation occurs, as measured by the increased rate of synthesis of medium-chain fatty acids ($C_{8:0}$ and $C_{10:0}$), which are characteristic of rabbit milk. This is accompanied by an approximately sixfold increase in the apparent rate of synthesis of fatty acid synthetase in the explants. The amount of the enzyme complex in the explants increases to a maximum value after about 40 h in culture. At some stage during this accumulation of the enzyme its apparent rate of degradation decreases significantly or may fall to zero (Speake *et al.*, 1975).

The purpose of the present study is to determine the precise hormonal regulation of the rates of synthesis, accumulation and degradation of fatty acid synthetase in this mammary-explant system during differentiation. The apparent rate of degradation of the enzyme complex has been measured by immunological procedures (Speake *et al.*, 1975) and we have investigated the extent to which the apparent changes in the rate are due to radioisotope reutilization.

Materials and Methods

Animals and materials

The animals and the materials used have been described (Speake *et al.*, 1975). Dibutyl cyclic AMP (6-*N*,2'-*O*-dibutyladenosine 3':5'-cyclic monophosphate; grade II), theophylline, rennin, bovine casein, NADP⁺ and cycloheximide were obtained from Sigma (London) Chemical Co., London S.W.6, U.K.

Preparation, culture and incubation of mammary explants

These procedures were carried out as described by Speake *et al.* (1975). Explants of lobulo-alveolar mammary tissue were obtained from 16–17-day pregnant rabbits. The explants were cultured at 37°C under O₂+CO₂ (95:5) in Medium 199 (Morgan *et al.*, 1950) containing combinations of insulin (5 µg/ml), prolactin (1 µg/ml) and cortisol (1 µg/ml) as described in the Results section. To assay fatty acid synthetase activity, groups of 20 explants were removed and homogenized. A supernatant fraction was then prepared as described below. In 'pulse' experiments, groups of 10 explants were removed and were incubated with 1.0 µCi of L-[U-¹⁴C]leucine (348 mCi/mmol) for 1 h at 37°C under O₂+CO₂ (95:5) in 1 ml of Medium 199 containing insulin, prolactin and cortisol.

Preparation of tissue extracts

After incubation with L-[U-¹⁴C]leucine, the explants were washed with 4×5 ml of ice-cold Medium 199 containing insulin, prolactin and cortisol. They were then homogenized at 4°C in 0.25 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 1 mM-dithiothreitol. A supernatant and a sediment were prepared by centrifugation of the homogenate in a micro-centrifuge for 6 min at 14000g_{av.}.

Immunoprecipitation and assay of fatty acid synthetase

The immunoprecipitation of fatty acid synthetase from the explant supernatant fraction and the assay of its activity were carried out as described by Speake *et al.* (1975).

Measurement of incorporation of L-[U-¹⁴C]leucine into explant proteins

The rates of incorporation of L-[U-¹⁴C]leucine into immunodetectable fatty acid synthetase and into trichloroacetic acid-insoluble protein in the tissue extracts were measured essentially by the method of Speake *et al.* (1975). After the incubation with L-[U-¹⁴C]leucine, the explants were washed as described above. Each group of explants was homogenized in 1 ml of water. Bovine serum albumin (1 mg) was added to the homogenate, and protein was precipitated with trichloroacetic acid (final concn. 5%, w/v). The suspension was centrifuged for 6 min at 14000g_{av.}. Each sediment was washed with 3×1 ml of water, dissolved in 0.3 ml of formic acid (90%, v/v) and the radioactivity was measured.

Measurement of radioactivity of amino acids in explants

(a) *Distribution of radioactivity in amino acids in extracts of explants.* Explants were cultured with insulin, prolactin and cortisol in Medium 199 for 24 h at 37°C and were then incubated with 1.0 μ Ci of L-[U-¹⁴C]leucine (348 mCi/mmol) for 1 h at 37°C under O₂+CO₂ (95:5) in 1 ml of Medium 199 containing insulin, prolactin and cortisol. After the incubation, the explants were washed with 4 $\times$ 5 ml of ice-cold Medium 199 containing insulin, prolactin and cortisol and were then returned to culture. At the times indicated, groups of 20 explants were removed, washed thoroughly with ice-cold Medium 199 containing the three hormones, and the amino acids were extracted as described by Speake *et al.* (1975). Each sample was evaporated to dryness by rotary evaporation and then dissolved in 0.5 ml of 0.2 M-sodium citrate buffer, pH 2.2, which contained a standard amino acid mixture (50 nmol of each amino acid in a Beckman type I amino acid calibration mixture). Amino acid analyses were carried out with an analyser system described by Beckerton *et al.* (1975). The effluent from the column was divided so that 7% was mixed with ninhydrin and passed through the reaction bath for colour development. The remainder was collected in 1 ml samples and the radioactivity determined by using Triton-xylene scintillator (Speake *et al.*, 1975).

(b) *Measurement of specific radioactivity of leucine in explants.* Groups of 100 explants were cultured and were then incubated with L-[U-¹⁴C]leucine as described above in (a). Amino acids were extracted from the explant and were analysed as described by Speake *et al.* (1975). The specific radioactivity of leucine in the explants was calculated from the total radioactivity in each sample [corrected for the fraction of the total radioactivity in leucine as determined in (a) above] together with the leucine content as given by amino acid analysis.

(c) *Measurement of radioactivity in trichloroacetic acid-soluble material from explants.* After the trichloroacetic acid-insoluble material was processed as described above (see 'Measurement of incorporation of L-[U-¹⁴C]leucine into explant proteins'), the radioactivity in the combined washings, i.e. the trichloroacetic acid-soluble fractions, was measured.

Measurement of casein biosynthesis

This was measured by a modification of the method of Juergens *et al.* (1965). At the times indicated, groups of ten explants were removed from culture and were incubated under O₂+CO₂ (95:5) with L-[U-¹⁴C]leucine for 1 h at 37°C in Medium 199 containing insulin, prolactin and cortisol. After the incubation, the explants were washed with 4 $\times$ 5 ml of Medium 199 containing insulin, prolactin and cortisol and were then homogenized in 1 ml of water.

Bovine casein (10 mg) in 4 mM-Na₂HPO₄/10 mM-imidazole/0.15 M-KCl adjusted to pH 6.7 with dilute HCl was added to the homogenate. The homogenate was centrifuged for 60 min at 100 000 *g*_{av.}. Solid CaCl₂ (to give a final concentration of 10 mM) and rennin (250 μ g) were added to the supernatant and the mixture was incubated at 37°C for 30 min. The incubation mixture was centrifuged for 10 min at maximum speed in a bench centrifuge. The pellet was washed twice with 4 mM-Na₂HPO₄/10 mM-imidazole/0.15 M-KCl adjusted to pH 6.7 with dilute HCl. The pellet was suspended in 5 ml of trichloroacetic acid (final concn. 5%, w/v) and heated at 95°C for 15 min. The mixture was centrifuged at 4°C for 10 min at maximum speed in a bench centrifuge. The pellet was washed with 4 $\times$ 1.0 ml of ice-cold trichloroacetic acid (5%, w/v) and then with 3 $\times$ 1.0 ml of ethanol/diethyl ether (3:1, v/v) at room temperature (approx. 18°C). The pellet was dissolved in 0.3 ml of formic acid (90%, v/v) and the radioactivity measured. The radioactivity of casein in 1 ml portions of the culture medium was determined by the same method.

Protein determination and radioactivity measurement

Protein was determined and radioactivity was measured by Speake *et al.* (1975).

Results

Regulation of the rate of synthesis and accumulation of fatty acid synthetase in mammary explants

Fig. 1 shows that the maximum increase in the activity of fatty acid synthetase (and hence in the amount of the enzyme complex; Speake *et al.*, 1975) in mammary explants from mid-pregnant rabbits occurs when the tissue is cultured with insulin, prolactin and cortisol. The explants must be cultured with a hormone combination which includes prolactin in order to observe an increase in the amount of fatty acid synthetase on the second day in culture. The maximum increase in the apparent rate of synthesis of the enzyme is also achieved by culturing with insulin, prolactin and cortisol (Fig. 2). This increased rate of synthesis is only maintained on the second day in culture if hormone combinations which include prolactin are used.

When explants are cultured with prolactin alone (Fig. 1), the activity of fatty acid synthetase in the explants decreases to undetectable values after the first day in culture. When explants are cultured with insulin alone, the activity of the enzyme complex increases slightly on the first day in culture but subsequently decreases (Fig. 1). By contrast, when explants are cultured with insulin or with prolactin the rate of incorporation of L-[U-¹⁴C]leucine into immunodetectable enzyme increases on the first day of culture (Fig. 2). These results may indicate that prolactin can direct the synthesis of a small amount of

Fig. 1. Fatty acid synthetase activity in mammary explants cultured with combinations of insulin, prolactin and cortisol

Explants were cultured with the hormone combinations shown for the times indicated. Groups of explants were removed and were homogenized as described in the Materials and Methods section. Fatty acid synthetase activity was assayed in the supernatant fraction. Where indicated, the error bars are for the mean value $\pm$ half the difference between two independent experiments. Abbreviations: I, insulin; P, prolactin; C, cortisol.

immunologically detectable precursors of fatty acid synthetase which are enzymically inactive. Both insulin and prolactin are required to maintain high rates of synthesis of enzymically active enzyme; for maximum rates of synthesis over 2 days in culture, cortisol must also be present.

The rates of incorporation of L-[U- 14 C]leucine into trichloroacetic acid-precipitable protein isolated from the explant supernatant fraction is shown in Fig. 3. The maximum increase in the apparent rate of synthesis in this fraction also occurs when explants are cultured with insulin, prolactin and cortisol.

The apparent rate of synthesis of fatty acid synthetase and the increase in the amount of the enzyme formed are strikingly decreased when explants are cultured with insulin, prolactin and cortisol in the presence of dibutyryl cyclic AMP plus theophylline (Fig. 4). The overall effect is to delay the hormonally regulated increase in the amount of enzymically active fatty acid synthetase formed. This might again be due to the synthesis of enzymically inactive precursors of the complex. As might be expected, the overall rate of fatty acid synthesis in explants cultured

for 43 h with insulin, prolactin and cortisol in the presence of dibutyryl cyclic AMP plus theophylline is decreased to 24% of the value observed in the presence of the hormones alone (results not shown).

The specific activity and the apparent rate of synthesis of fatty acid synthetase in freshly prepared explants varies somewhat between animals (cf. Figs. 1 and 2 with Fig. 4). This appears to be related to the state of development of the gland at 16–17 days of pregnancy.

Regulation of the rate of degradation of fatty acid synthetase in mammary explants

The apparent rates of degradation of fatty acid synthetase during defined periods in culture have

Fig. 2. Apparent rates of synthesis of fatty acid synthetase in mammary explants cultured with combinations of insulin, prolactin and cortisol

Explants were cultured with the hormone combinations shown for the times indicated. Groups of explants were removed and were incubated with L-[U- 14 C]leucine. The explants were homogenized and supernatant fractions prepared. The rate of incorporation of radioactivity into immunodetectable fatty acid synthetase was then measured. Details are given in the Materials and Methods section. Where indicated, the error bars are the mean values $\pm$ half the difference between two independent experiments. Abbreviations: I, insulin; P, prolactin; C, cortisol.

Fig. 3. Apparent rates of synthesis of supernatant protein obtained from mammary explants cultured with combinations of insulin, prolactin and cortisol

Explants were cultured with the hormone combinations shown for the times indicated. Groups of explants were removed and were incubated with L-[U- 14 C]leucine. The explants were homogenized and the supernatant fraction was prepared. The rate of incorporation of L-[U- 14 C]leucine into trichloroacetic acid-insoluble material was then measured. Details are given in the Materials and Methods section. Where indicated, the error bars are for mean values $\pm$ half the difference between two independent experiments. Abbreviations: I, insulin; P, prolactin; C, cortisol.

been obtained by measuring the disappearance of radioactivity from immunodetectable fatty acid synthetase and from trichloroacetic acid-precipitable protein isolated from the explant supernatant fraction. The apparent rate of degradation of fatty acid synthetase in the explants declines drastically on the second day (18–38 h) of culture in the presence of insulin, prolactin and cortisol (Table 1), which confirms our previous observations (Speake *et al.*, 1975). This effect is only observed with hormone combinations which include prolactin (Table 1).

When dibutyl cyclic AMP plus theophylline are added to cultures containing insulin, prolactin and cortisol this decrease in the apparent rate of degradation of the enzyme complex is partially suppressed. The trichloroacetic acid-precipitable proteins isolated from the explant supernatant fraction are apparently degraded at a high rate throughout the culture period,

irrespective of the hormone combination in the medium (Table 1).

The results in Table 1 suggest that the apparent rate of degradation of fatty acid synthetase appears to be significantly decreased or even abolished during the accumulation of the enzyme on the second day in culture with insulin, prolactin and cortisol. The amount of fatty acid synthetase which is synthesized during this period should therefore approximately equal the amount of the enzyme which accumulates in the explants. In the results shown in Fig. 5, the amount of fatty acid synthetase in the explants increases during 24–48 h in culture but the apparent rate of synthesis of the enzyme remains approximately constant during this period. In these experiments the amount of the enzyme synthesized in this period can be calculated from the measured rate of enzyme synthesis. The result can then be compared with the measured increase in the amount of the enzyme which has accumulated in the tissue over this period.

The calculation is based on the assumption that during the incubation with L-[U- 14 C]leucine fatty acid synthetase is synthesized from an amino acid pool which has a similar specific radioactivity to that of the leucine in the culture medium (4780 d.p.m./nmol of leucine). After the explants had been washed thoroughly to remove contaminating culture medium, the specific radioactivity of leucine in the intracellular pool of 'free' amino acids was 4000 d.p.m./nmol of leucine (see Fig. 8).

The amount of leucine incorporated into fatty acid synthetase was calculated from the radioactivity incorporated into immunodetectable fatty acid synthetase/h (Fig. 5) and the specific radioactivity of the 'free' amino acid pool (Fig. 8). The result was 27 pmol of leucine incorporated/h per mg of fresh tissue. From the amino acid composition and the molecular weight of purified rabbit mammary-gland fatty acid synthetase (Carey & Dils, 1970), this corresponds to 42 ng of enzyme synthesized/h per mg of fresh tissue. [The molecular weight reported by Carey & Dils (1970) is about twice that of fatty acid synthetases isolated from a number of other tissues.] From the specific activity of the purified fatty acid synthetase (Carey & Dils, 1970) and the amount of supernatant protein recovered per mg of fresh tissue, this amount of enzyme synthesized corresponds to 0.42 unit (nmol of NADPH oxidized/min) of fatty acid synthetase activity produced/h per mg of supernatant fraction protein. The apparent rate of synthesis of fatty acid synthetase is constant during 24–48 h in culture, and therefore 10.08 units of enzyme activity are synthesized per mg of supernatant protein in this period. The results in Fig. 5 show that 12.2 ± 3.4 (mean $\pm$ half the difference between the results of two independent experiments) units of fatty acid synthetase activity accumulate in the tissue in this period per mg of explant supernatant

Fig. 4. Effects of dibutyryl cyclic AMP plus theophylline on the activity and on the apparent rate of synthesis of fatty acid synthetase in mammary explants cultured with insulin, prolactin and cortisol

Explants were cultured with insulin, prolactin and cortisol in the presence and absence of dibutyryl cyclic AMP (1 mM) plus theophylline (1 mM) for the times indicated. Groups of explants were removed and were incubated with L-[U-¹⁴C]leucine. The explants were homogenized and the supernatant fractions prepared. Fatty acid synthetase activity and the rate of incorporation of L-[U-¹⁴C]leucine into fatty acid synthetase were measured in the supernatant fraction. Details are given in the Materials and Methods section. (a) Fatty acid synthetase activity; (b) rate of incorporation of radioactivity into immunoprecipitable fatty acid synthetase. Abbreviations: I, insulin; P, prolactin; C, cortisol; Bu₂cAMP, dibutyryl cyclic AMP; T, theophylline.

Table 1. Changes in the apparent rate of degradation of fatty acid synthetase and of explant supernatant protein in mammary explants from mid-pregnant rabbits during culture with combinations of insulin, prolactin and cortisol

The apparent rates of degradation were measured during defined periods in culture by the disappearance of radioactivity from immunodetectable fatty acid synthetase and from trichloroacetic acid-precipitable protein isolated from the explant supernatant fraction. Details are given in the Materials and Methods section. The loss of radioactivity from protein was exponential with respect to time and was assumed to represent the degradation of protein. The apparent degradation coefficients (K_d) were calculated from gradients (i.e. slope $\times 2.303$) of graphs of log (d.p.m. in fatty acid synthetase or in the supernatant protein) versus the time after the administration of L-[U-¹⁴C]leucine. Half-lives were calculated from the relationship $t_{1/2} = \ln 2 / K_d$. Dibutyryl cyclic AMP (1 mM) plus theophylline (1 mM) were present in the culture medium in some experiments.

Period in culture when degradation was measured (h)	Culture conditions	Apparent half-lives ($t_{1/2}$, h)	
		Explant supernatant protein	Fatty acid synthetase
0-17	No hormones	58	15
15-36	Insulin	15	18
41-61	Insulin	15	19
15-36	Cortisol	20	46
41-61	Cortisol	18	38
15-36	Insulin+cortisol	18	19
41-61	Insulin+cortisol	19	20
18-38	Insulin+prolactin	44	∞
43-66	Insulin+prolactin	30	22
17-40	Prolactin+cortisol	33	216
42-65	Prolactin+cortisol	58	11
18-38	Insulin+prolactin+cortisol	28	172
43-66	Insulin+prolactin+cortisol	32	38
19-43	Insulin+prolactin+cortisol+dibutyryl cyclic AMP+theophylline	41	37
45-65	Insulin+prolactin+cortisol+dibutyryl cyclic AMP+theophylline	25	51

Fig. 5. Changes in the activity of fatty acid synthetase and in the apparent rate of synthesis of fatty acid synthetase in mammary explants cultured with insulin, prolactin and cortisol

Explants were cultured with insulin, prolactin and cortisol for the times indicated. Groups of explants were removed and were incubated with L-[U- 14 C]leucine. The explants were homogenized and the supernatant fractions prepared. Fatty acid synthetase activity and the rate of incorporation of L-[U- 14 C]leucine into fatty acid synthetase were measured in the supernatant fraction. Details are given in the Materials and Methods section. The results are mean values $\pm$ half the difference between two independent experiments. Activity of fatty acid synthetase (●); apparent rate of synthesis of the synthetase (○).

protein. The values obtained by using this calculation indicate that in the experiments shown in Fig. 5 no degradation of fatty acid synthetase occurs in the explants during the period 24–48 h in culture with insulin, prolactin and cortisol.

Radioisotope reutilization and the measurement of rates of degradation of fatty acid synthetase

The previous results and calculations suggest that a regulated programme of degradation exists for fatty acid synthetase during hormonally controlled differentiation in mammary explants. However, we must exclude the possibility that the apparent decrease in the rate of degradation of the enzyme (Table 1) is due to radioisotope reutilization during the period when the enzyme accumulates in the differentiating tissue.

The reutilization problem is inherent in the use of the 'pulse-chase' method of following the disappearance of radioactivity from immunodetectable enzymes to measure the rate of enzyme degradation. This is

because antiserum will precipitate the enzyme irrespective of whether it has been radiolabelled in the 'pulse' or in the 'chase' period. Radioactivity can be incorporated from residual amino acids into new protein which is being synthesized in the 'chase' period. This can occur at significant rates in any situation where proteins accumulate in a tissue (e.g. during growth or differentiation). The problem may be exacerbated if intracellular radioactive amino acids derived from protein degradation do not exchange with extracellular amino acids. This could occur in conditions where large quantities of amino acids are required for the synthesis of proteins which are accumulating in the tissue.

It is impossible to estimate directly the extent of radioisotope reutilization when measuring the rate of degradation of fatty acid synthetase in the experiments described so far. Nevertheless, several indirect estimations of the rate of radioisotopic equilibration with the culture medium and of the rate of radioisotope reutilization have been made for the period in culture (24–48 h) when the apparent rate of degradation of fatty acid synthetase in the explants is significantly decreased.

Previous work (Speake *et al.*, 1975) has shown that at the end of a 1 h 'pulse' with L-[U- 14 C]leucine, more than 95% of the radioactivity extracted from the explants by boiling was still associated with leucine. Preliminary results have shown that about 90% of the radioactivity extracted by boiling explants which had been cultured for 5, 18 and 25 h with insulin, prolactin and cortisol after being 'pulsed' with L-[U- 14 C]leucine was recovered in amino acids. About 40% of this radioactivity was associated with leucine.

The rapid uptake of L-[U- 14 C]leucine and the rapid loss of radioactivity from explants which had been previously cultured for 24 h in Medium 199 containing insulin, prolactin and cortisol is shown in Fig. 6. The linear rate of incorporation of L-[U- 14 C]leucine into trichloroacetic acid-insoluble material (Fig. 6a) ceases within 10 min of thoroughly washing the explants with ice-cold Medium 199 (Fig. 6b). This trichloroacetic acid-insoluble material does not contain measurable quantities of L-[U- 14 C]leucyl-tRNA, as determined in preliminary experiments by incubating samples (0.05 ml) of the homogenate with 0.2 ml of 1 M-NaOH for 1 h at 37°C (Hizi & Yagil, 1974), and it is presumed to be protein. The decrease in the radioactivity of trichloroacetic acid-soluble material in the explants (Fig. 6b) is concurrent with the appearance of a corresponding amount of radioactivity in the culture medium (which contains over 97% of trichloroacetic acid-soluble components).

The results in Fig. 7 show the radioactivity in the total explant protein (isolated as trichloroacetic acid-insoluble material in the supernatant and pellet obtained by centrifugation of the explant homo-

Fig. 6. Changes in the radioactivity of components of intracellular and extracellular compartments during and after incubation of mammary explants with L-[U-¹⁴C]leucine

(a) Explants were cultured with insulin, prolactin and cortisol for 24 h. Groups of explants were then incubated with L-[U-¹⁴C]leucine for the times indicated. The explants were homogenized and the homogenate treated with trichloroacetic acid. The radioactivity in the trichloroacetic acid-precipitable and in the trichloroacetic acid-soluble fractions was then measured. (b) Groups of explants were cultured and were then incubated with L-[U-¹⁴C]leucine as described in (a). Each group of explants was washed and then incubated again with insulin, prolactin and cortisol for the times indicated. The radioactivity in the trichloroacetic acid-insoluble and in the trichloroacetic acid-soluble fractions, as well as the radioactivity in the culture medium (97% of the radioactivity in the culture medium was soluble in trichloroacetic acid), were measured. Details are given in the Materials and Methods section. Radioactivity in the 'free' intracellular amino acid pool (○), in the total explant protein (●) and in the culture medium (□). The results are mean values $\pm$ half the difference between two independent experiments.

genate), in the explant supernatant fraction (isolated as trichloroacetic acid-insoluble material), in the 'free' intracellular amino acids (isolated as trichloroacetic acid-soluble material in the supernatant and pellet), in fatty acid synthetase (in the explant supernatant fraction), and in the culture medium (97% of the radioactivity in the medium is soluble in trichloroacetic acid). These values were obtained from an experiment in which explants were cultured for 24 h with insulin, prolactin and cortisol and then 'pulsed' with L-[U-¹⁴C]leucine. The loss of radioactivity was measured over the subsequent 25 h period in culture.

The radioactivity lost during this period from the 'free' intracellular pool of amino acids plus the total explant protein (2800 d.p.m.) is almost all recovered in the culture medium (2500 d.p.m.). Except for a small residual fraction of amino acids, the 'free' radioactive amino acids (including those derived from protein breakdown) can therefore exchange with the amino acids present in the culture medium. This

exchange must significantly decrease the extent of radioisotope reutilization in these experiments.

The results in Fig. 8 show that the specific radioactivity of the pool of 'free' intracellular leucine declines precipitously (cf. Figs. 6 and 7) to about 250 d.p.m./nmol of leucine after explants have been cultured for 18 or 25 h with insulin, prolactin and cortisol after a 'pulse' of L-[U-¹⁴C]leucine. The subcellular distribution of the 'free' intracellular pool of amino acids (including leucine) which are extracted from explants by boiling is unknown. If all (or part) of this pool of radioactive amino acids were available for protein synthesis, then a considerable rate of incorporation of radioactivity into protein would occur. It is not possible to measure directly the incorporation of radioactivity into fatty acid synthetase from these pools of possible precursors, but it should be possible to measure the incorporation of radioactivity from these pools into a protein which is not degraded in the explants.

Casein is degraded in pregnant, but not in lactating,

Fig. 7. Changes in the radioactivity of components of intracellular and extracellular compartments which occur during culture of mammary explants which have been previously incubated with L-[U- 14 C]leucine

Explants were cultured with insulin, prolactin and cortisol for 24 h. Groups of explants were incubated with L-[U- 14 C]leucine. The explants were washed at room temperature (approx. 18°C) and were then returned to culture with insulin, prolactin and cortisol. At the times indicated, groups of explants were washed and homogenized and the supernatant fraction was prepared. The incorporation of L-[U- 14 C]leucine into immunoprecipitable fatty acid synthetase as well as into trichloroacetic acid-insoluble and into trichloroacetic acid-soluble fractions of the supernatant was measured. The sediment obtained by centrifugation of the explant homogenate was suspended in water and then precipitated with trichloroacetic acid. The precipitate was washed and the radioactivity in the final precipitate and in the combined washings was measured. Details are given in the Materials and Methods section. Radioactivity in the total explant protein (○), in the supernatant-fraction protein (●), in the intracellular amino acid pool (△), in fatty acid synthetase (▲), and in the culture medium (□).

mouse mammary gland (Hollman, 1974). Casein is produced when explants of mid-pregnant rabbit mammary gland are cultured with insulin, prolactin and cortisol and it is then secreted into the lumina of the lobulo-alveolar tissue (histological evidence which is not shown). It is therefore very unlikely that the casein is degraded in the hormonally stimulated tissue. [This view is supported by the very low proportion of degradation products of β -casein which are found in bulk samples of bovine milk (D. T. Davies & D. G. Dalgleish, unpublished work).] We have found that the apparent rate of synthesis of casein increases when explants are cultured for 43 h with insulin, prolactin and cortisol. By contrast, the rate of synthesis decreases when explants are cultured in the absence of hormones (Fig. 9). When explants were cultured for 20 h with hormones, then 'pulsed' with L-[U- 14 C]leucine for 1 h, thoroughly washed and cultured for a further 23 h with hormones, there was no measurable

change in the radioactivity of casein in the explants plus the culture medium. This indicates that there is no incorporation of radioactivity from the 'free' pool of intracellular amino acids or from radioactive amino acids derived from protein degradation into casein during the 23 h period in culture after the 'pulse' with L-[U- 14 C]leucine. Casein represents about 20% of the protein synthesized by the explants after 1 day in culture in the presence of insulin, prolactin and cortisol. It therefore seems reasonable to conclude that little radioisotope reutilization occurs during the period in culture where we have studied the rate of degradation of fatty acid synthetase.

Preliminary results have been obtained in support of this conclusion by using another experimental approach. This depends on the fact that radioisotope reutilization would be blocked by the inhibition of protein synthesis. Groups of explants were cultured in the presence and absence of hormones and were then 'pulsed' with L-[U- 14 C]leucine. The rate of degradation of fatty acid synthetase was then measured over defined periods of time during which the explants were cultured in the presence or absence of cycloheximide, which was added at a concentration which inhibits protein synthesis in the explants. The results (Table 2) indicate that in the presence of the inhibitor, fatty acid synthetase was not continuously

Fig. 8. Changes in the specific radioactivity of intracellular 'free' leucine which occurs during culture of mammary explants which have been previously incubated with L-[U- 14 C]leucine

Explants were cultured with insulin, prolactin and cortisol for 24 h. Groups of explants were incubated with L-[U- 14 C]leucine. The explants were washed and were then returned to culture with insulin, prolactin and cortisol. At the times indicated, groups of explants were removed from culture. The explants were washed and amino acids were extracted from the explants. The specific radioactivity of leucine in the explants was then determined. Details are given in the Materials and Methods section. The results are mean values $\pm$ half the difference between two independent experiments.

Fig. 9. Radioactivity incorporated into casein in mammary explants during culture with insulin, prolactin and cortisol

Explants were cultured with insulin, prolactin and cortisol for the times indicated. The radioactivity incorporated into casein in the explant homogenates and in the culture medium was determined as described in the Materials and Methods section. Radioactivity incorporated into casein when explants were cultured throughout in the presence of hormones (○) and in the absence of hormones (■). Radioactivity incorporated into casein when explants were incubated with L-[U-¹⁴C]leucine and were then cultured with insulin, prolactin and cortisol for a further 23 h (Δ). The results are mean values ± half the difference between two independent experiments. The values in parentheses represent the radioactivity in casein in the culture medium.

destroyed at the high rate which characterizes explants cultured in the absence of hormones or in the first 23 h of culture in the presence of insulin, prolactin and cortisol.

The cumulative evidence from the experiments described above seems to preclude a continuous high rate of degradation of fatty acid synthetase which might have been masked by the effects of radioisotope reutilization and might therefore not have been detected by the immunological procedures used to measure enzyme degradation.

Discussion

Regulation of synthesis and accumulation of fatty acid synthetase

Hormone combinations which include prolactin are needed to sustain high rates of synthesis and accumulation of fatty acid synthetase in mammary explants (Figs. 1 and 2). Maximum effects are produced by insulin, prolactin and cortisol. This is the hormone combination required to produce correspondingly large increases in the overall rate of fatty acid synthesis and in the proportion of medium-chain fatty acids synthesized by these explants (Speake *et al.*, 1975). These changes are characteristic of differentiation in rabbit mammary gland.

The experiments where explants were cultured

Table 2. Effect of cycloheximide on the apparent rate of degradation of fatty acid synthetase in mammary explants cultured with insulin, prolactin and cortisol

In this preliminary experiment, explants were cultured throughout in the absence of hormones or with insulin, prolactin and cortisol. At the beginning of the period when degradation was measured, groups of 40 explants were removed and were incubated with L-[U-¹⁴C]leucine. The explants were washed and were then cultured with insulin, prolactin and cortisol. Groups of 10 explants were removed at intervals during each period when degradation was measured (0, 5, 18 and 23 h in culture). The explants were homogenized and the supernatant fraction was prepared. The radioactivity of immunoprecipitable fatty acid synthetase was measured. The apparent half-lives of the synthetase during the periods studied were calculated as described in the legend to Table 1. Where indicated, cycloheximide (100 μg/ml of culture medium) was added to the medium after explants had been incubated with L-[U-¹⁴C]leucine and then washed. Groups of explants were cultured throughout (i.e. for 46 h) with insulin, prolactin, cortisol and cycloheximide (100 μg/ml). No incorporation of L-[U-¹⁴C]leucine into trichloroacetic acid-insoluble material could be detected in explants after 23 h or 46 h in culture.

Period in culture when degradation was measured (h)	Culture conditions	Apparent half-life of fatty acid synthetase ($t_{1/2}$, h)
0-23	No hormones	19
0-23	+Hormones	63
23-46	+Hormones	203
23-46	+Hormones and cycloheximide	150

with only prolactin (Fig. 3) indicate that this hormone can direct the synthesis of enzymically inactive precursors of fatty acid synthetase. The accumulation of enzymically inactive precursors of fatty acid synthetase also occurs in the first 3 h after starved rats are fed on a fat-free diet (Yu & Burton, 1974). When explants are cultured with insulin, prolactin and cortisol in the presence of dibutyryl cyclic AMP plus theophylline (Fig. 4), there is a delay in the formation of enzymically active fatty acid synthetase. This might again be due to the synthesis of inactive precursors of the enzyme complex. These results confirm and extend the observations of Sapag-Hagar *et al.* (1974) that cyclic AMP inhibits the increase in the activity of enzymes (particularly of enzymes of lipogenesis) associated with the development of mammary gland which is seen when explants are cultured with insulin, prolactin and cortisol. Sapag-Hagar & Greenbaum (1974) have suggested that differentiation in mammary gland during pregnancy, the response of the tissue to hormones, and the specific programmes for the synthesis of characteristic components of milk may all be mediated by the

intracellular concentration ratio of cyclic AMP to cyclic GMP. The results presented here give some insight into the mechanism by which this control might operate.

Regulation of degradation of fatty acid synthetase

Fatty acid synthetase in mammary explants is rapidly degraded unless prolactin is included in the hormone combinations used (Table 1). When prolactin is present with other hormones, the apparent rate of degradation of the enzyme decreases dramatically on the second day in culture. This is the period when the enzyme accumulates in the tissue. Cyclic AMP may be involved in controlling this process, since dibutyl cyclic AMP plus theophylline increase the apparent rate of degradation of fatty acid synthetase in the presence of insulin, prolactin and cortisol (Table 1). By contrast with fatty acid synthetase, the protein isolated from the explant supernatant is continuously degraded in culture irrespective of the hormone combination used in the culture medium. This indicates that overall degradation of non-specific proteins occurs continuously in the explants and is not sensitive to control by hormones.

A number of lines of evidence have been presented in the Results section to indicate that the decrease in the apparent rate of degradation of fatty acid synthetase measured on the second day in culture with insulin, prolactin and cortisol by radioimmunological techniques does not reflect radioactive amino acid reutilization. All the evidence discussed below is circumstantial, but taken together it seems to justify the conclusion that a regulated programme of degradation of fatty acid synthetase exists in explants during hormonally stimulated differentiation in organ culture.

In experiments (Fig. 5) where the apparent rate of synthesis of fatty acid synthetase was constant from 24 to 48 h in culture in the presence of insulin, prolactin and cortisol, the calculated increase in the amount of fatty acid synthetase is approximately the same as the measured increase in amount. This indicates that little or no degradation of fatty acid synthetase occurs during this period in culture.

Several measurements have been made with explants which were previously cultured for 24 h in the presence of insulin, prolactin and cortisol of the equilibration of intracellular amino acids with amino acids in the culture medium.

The results in Fig. 6 show that L-[U-¹⁴C]leucine rapidly equilibrates with the intracellular amino acid pool. The rapid decrease in the intracellular L-[U-¹⁴C]leucine on incubation of thoroughly washed explants (Fig. 6b) is matched by a concurrent appearance of radioactivity (97% of which is trichloroacetic acid-soluble) in the culture medium. The apparent rate of incorporation of L-[U-¹⁴C]leucine (Fig. 6a) appears to be approximately linear. Within

10 min of washing the explants, the incorporation of L-[U-¹⁴C]leucine into total explant protein ceases. A loss of radioactivity from total explant protein is then seen within the succeeding 4½ h. The rapid cessation of incorporation of radioactivity into total explant protein suggests that protein is synthesized from an amino acid pool which rapidly equilibrates with amino acids in the culture medium.

The results in Fig. 7 show that the accumulation of radioactivity in the culture medium (97% of which is trichloroacetic acid-soluble) in the 25 h period after incubation of explants with L-[U-¹⁴C]leucine is approximately equal to the loss of radioactivity from explants in this period. The results indicate that most (90%) of the 'free' intracellular radioactive amino acids and radioactive amino acids released from the breakdown of protein can exchange readily with amino acids in the culture medium. The reutilization of radioactive amino acids from a residual amino acid pool or from those derived from protein degradation in the tissue must therefore be minimal.

The nature of the intracellular precursor amino acid pool for protein synthesis and the nature of the 'free' intracellular amino acid pool are unknown. The conflicting evidence with respect to the nature and function of amino acid pools in various cell types has been excellently reviewed by Rannels *et al.* (1975). Whatever the nature of the precursor amino acid pool for protein synthesis in lobulo-alveolar cells, it is apparent from the results in Figs. 6, 7 and 8 that the precursor pool can rapidly equilibrate with amino acids in the culture medium and that the precursor pool cannot be specifically charged from the residual radioactive amino acids in the tissue or from radioactive amino acids derived from protein degradation which occurs continuously in the tissue.

If all or part of the 'free' residual radioactive amino acids or radioactive amino acids derived from protein degradation were available for protein synthesis then a considerable incorporation of radioactivity into protein could occur. Explants were cultured for 20 h with insulin, prolactin and cortisol, incubated with L-[U-¹⁴C]leucine for 1 h, washed and then cultured for a further 23 h in the presence of these hormones. No increase in radioactivity incorporated into casein could be measured in the 23 h period after incubation with L-[U-¹⁴C]leucine. Casein represents about 20% of total protein synthesized by explants cultured for 1 day with insulin, prolactin and cortisol. Histological evidence (not shown) indicates that casein is secreted into the lumina of the lobulo-alveolar tissue in hormonally stimulated explants in culture and is therefore unlikely to be degraded in the lobulo-alveolar cells. The results indicate that the incorporation of radioactivity into protein from radioactive amino acids in the period (20–43 h) in culture after incubation with L-[U-¹⁴C]leucine (when the apparent rate of degradation of

fatty acid synthetase is drastically decreased) must be minimal. Since leucine is the second most abundant amino acid in rabbit milk caseins (R. J. Mayer, unpublished work), the secretion of casein from the lobulo-alveolar cells also precludes the utilization of L-[U-¹⁴C]leucine for protein synthesis.

The results in Table 2 suggest that when mammary explants are incubated with cycloheximide at a concentration which completely inhibits protein synthesis (Aisbitt & Barry, 1973) the degradation of fatty acid synthetase in explants apparently continues at a low rate from 23 to 46 h in culture. Experiments with cycloheximide are difficult to interpret. Nevertheless, the results (Table 2) suggest that inhibition of protein synthesis in explants does not reveal a latent continuous degradation of fatty acid synthetase which is masked by radioisotopic reutilization which could accompany the high rates of protein synthesis and enzyme accumulation which occur in explants on the second day in culture in the presence of insulin, prolactin and cortisol.

The results seem to justify the conclusion that there is a real decrease (or even a cessation in some experiments) in the rate of degradation of fatty acid synthetase during the period where the enzyme accumulates in explants cultured with hormone combinations which include prolactin. In this work several lines of evidence suggest that radioisotopic reutilization contributes minimally to the measurement of the degradation of fatty acid synthetase by radio-immunological techniques.

The role of protein degradation in the hormonally stimulated differentiation of mammary explants is obviously complex. We propose that proteins may be classed into at least three groups with respect to degradation mechanism. The first group are those enzymes (e.g. fatty acid synthetase) concerned with the biosynthesis of cell-specific products (i.e. milk components). These enzymes must accumulate rapidly in the tissue in order that milk components can be synthesized as efficiently as possible when the tissue is hormonally stimulated. To achieve this, their degradation is drastically decreased or abolished. The second group are proteins which are cell-specific products (e.g. casein). These are not degraded in the cells but are secreted into the lumina of the lobulo-alveolar cells. Such a mechanism would be similar to that proposed for the protein secretions of pancreas (Kemp *et al.*, 1972; Rutter *et al.*, 1973). The third group are non-specific enzymes and

proteins in the cell which are continuously degraded in mammary explants during differentiation. Their degradation would facilitate any enzymological and morphological remodelling which may occur in differentiation (Swick & Ip, 1974).

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