Changes in Mammary-Gland Acetyl-Coenzyme A Carboxylase Associated with Lactogenic Differentiation

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The process leading to the rise of acetyl-CoA carboxylase activity in rat mammary tissue after the onset of lactation was investigated. The kinetics of change in enzyme activity and enzyme immunotitratable with antibody against avian liver acetyl-CoA carboxylase were determined during the course of lactogenic differentiation. The antibody inactivates and specifically precipitates acetyl-CoA carboxylase from rat mammary tissue as well as that from chicken liver cytosol. Characterization of the immunoprecipitate of the mammary tissue carboxylase by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis reveals a single biotin-containing polypeptide of about 230000 mol.wt. This molecular weight is approximately twice that reported for the avian liver enzyme. However, chicken liver cytosol prepared in the presence of trypsin inhibitor and subjected to immunoprecipitation gives rise to a biotin-containing subunit of 230000 mol.wt. as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; omission of proteinase inhibitor leads to a subunit(s) approximately one-half this size. Throughout gestation both carboxylase activity and amounts of immunotitratable enzyme remained low; however, after parturition both parameters rose concomitantly to values 30-40 times the initial values. Therefore the elevated concentration of acetyl-CoA carboxylase appears to result from an increased rate of synthesis of enzyme relative to degradation rather than to activation of a pre-existing form of the enzyme.

With the onset of lactation the mammary gland undergoes extensive change. In the rat the activities of enzymes involved in the biosynthesis of milk components rise dramatically about the time of parturition (Howanitz & Levy, 1965; Baldwin & Milligan, 1966; Kuhn & Lowenstein, 1967; Gul & Dils, 1969; Gumaa *et al.*, 1973; Baldwin & Young, 1974). In part, this increase in enzyme activity is a result of cellular changes undergone by the mammary gland during pregnancy. Mammary glands of virgin and pregnant animals are largely composed of adipose- and connective-tissue cells in which the activities of lactogenic enzymes are either low or absent. Under the influence of several hormones, mammary-gland epithelial cells proliferate and develop into secretory cells during pregnancy (Topper, 1970; Anderson, 1974). After the onset of lactation there is a rapid increase in the activities of enzymes involved in lactogenesis. This extensive rise in enzyme activity appears to be specific in that various enzyme activities increase to different extents, at different rates and with various hormonal requirements (Howanitz & Levy, 1965; Korsrud & Baldwin, 1972*a,b*; Anderson, 1974).

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the first committed step in fatty acid synthesis and is considered a likely site for regulation of lipogenesis in

animal tissues (Lane *et al.*, 1974). Consistent with a primary role for this enzyme in the initiation of lactogenesis is the finding that the activity of acetyl-CoA carboxylase in rat mammary gland rises markedly during lactation (Howanitz & Levy, 1965).

This rise in carboxylase activity is due to either an increased specific activity of the enzyme, i.e. activation, or to an increase in its concentration in the tissue. Were the specific activity of the carboxylase affected, several mechanisms for triggering this activation would be possible. For example, Carlson & Kim (1973) have reported that rat liver acetyl-CoA carboxylase is controlled by a phosphorylation-dephosphorylation mechanism. In addition, it appears that attachment of the prosthetic group is the final step in the synthesis of biotin enzymes (Moss & Lane, 1971). The rapid increase of acetyl-CoA carboxylase activity could thus reflect the timing of the addition of the prosthetic group to the apoenzyme. Alternatively, activation of acetyl-CoA carboxylase may be analogous to that of mammary tissue galactosyl transferase, whose capacity to synthesize lactose is dependent on the presence of α -lactalbumin (Brew *et al.*, 1968; Ebner & Magee, 1975). Finally, the increase in acetyl-CoA carboxylase could result from a rise in the concentration of the enzyme in

mammary tissue. Changes in nutritional and hormonal status lead to alterations in both the rates of carboxylase synthesis and degradation in liver (Majerus & Kilburn, 1969; Nakanishi & Numa, 1970; Lane *et al.*, 1974).

The results presented below indicate that the dramatic rise in carboxylase activity in mammary gland with the onset of lactation is due to an increased rate of synthesis of acetyl-CoA carboxylase relative to degradation rather than to activation of an inactive form of the enzyme.

Materials and Methods

Animals and materials

Virgin Sprague-Dawley rats, obtained from Charles River Breeding Laboratories, Wilmington, MA 01887, U.S.A., were mated with Sprague-Dawley males. Pregnant and lactating rats were housed in individual cages and fed on Purina Rat Chow *ad libitum*. All litters were decreased to eight pups 1 day after birth. For experiments involving pregnant animals, timed pregnant rats were obtained from Charles River Breeding Laboratories. Trasylol (bovine parotid trypsin inhibitor) was obtained from Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY 10022, U.S.A.

Preparation of acetyl-CoA

Acetyl-CoA was prepared by the method of Simon & Shemin (1953).

Assay of acetyl-CoA carboxylase

Acetyl-CoA carboxylase activity was determined by the [^{14}C]bicarbonate-fixation assay of Gregolin *et al.* (1968a) modified as described below. Like the acetyl-CoA carboxylases of rat liver and adipose tissue (Moss & Lane, 1971; Lane *et al.*, 1974), but unlike that of avian liver (Gregolin *et al.*, 1968a), the rat mammary-gland enzyme requires activation before assay to obtain maximum activity. Experiments (results not shown) to optimize conditions for activation showed that maximum carboxylase activity is obtained when the mammary-gland cytosol is incubated for 15 min with 10 mM-potassium citrate in assay buffer, pH 7.5 (Gregolin *et al.*, 1968a), at 37°C before initiation of the carboxylation reaction. Hence this preliminary procedure was adopted for all carboxylase assays. Long-chain fatty acids, which inhibit acetyl-CoA carboxylase, are unavoidable contaminants of mammary-gland cytosol preparations, owing to the presence of milk in the tissue. Differences in the contents of these inhibitory substances in cytosols prepared from glands at various stages of pregnancy and lactation lead to inaccuracies in the assay of carboxylase activity. The inclusion of a high concentration of bovine serum albumin (10 mg/ml) in the preincubation and assay medium to adsorb free fatty acids eliminated this course of

variation, so allowing the measurement of full carboxylase activity.

The components of the assay reaction mixture (0.5 ml total volume) were those described by Gregolin *et al.* (1968a), with the following exceptions. The concentrations of ATP, $\text{H}^{14}\text{CO}_3^-$ and acetyl-CoA were increased to 3, 20 and 4 mM respectively, since the K_m values for these substrates are higher for the rat mammary-gland carboxylase than for the chicken liver enzyme for which the original assay was designed. In addition, 10 mM-citrate replaced 20 mM-DL-isocitrate. After the 15 min preincubation with citrate, the assay was initiated by the addition of ATP, $\text{H}^{14}\text{CO}_3^-$ and acetyl-CoA; acetyl-CoA was omitted in control mixtures. Samples were removed at 1 min intervals during the 4 min assay period for the determination of incorporation of [^{14}C]bicarbonate into malonyl-CoA. [^{14}C]Bicarbonate fixation is absolutely dependent on acetyl-CoA and citrate, follows zero-order kinetics for 5 min, and is proportional to enzyme concentration up to 1 unit of carboxylase (1 unit of acetyl-CoA carboxylase catalyses the formation of 1.0 μmol of malonyl-CoA/min at 37°C).

Preparation of mammary-tissue cytosol

Mammary-tissue extracts were prepared by a modification of the procedure of Miller & Levy (1969). At appropriate times during pregnancy and lactation, rats were killed by decapitation, mammary glands were excised, rinsed repeatedly with 0.9% NaCl to remove as much milk as possible, minced with scissors and homogenized with a loose-fitting glass homogenizer with Teflon pestle (Kontes catalogue no. K886100) in 2 vol. of 50 mM-Tris/HCl buffer, pH 7.0, containing 150 mM-KCl and 5 mM-EDTA. Cell breakage by this procedure was 85–95% as a routine. All further operations were carried out at 4°C. After samples were removed for DNA determination, the crude extract was centrifuged for 10 min at 2000g and the supernatant filtered through gauze to remove fat and then centrifuged for 30 min at 40000g. The supernatant was filtered again and centrifuged for 45 min at 73000g. The final high-speed supernatant was filtered and stored at –20°C. Enzyme activity was not altered by freezing and thawing.

Preparation of chicken liver acetyl-CoA carboxylase

Acetyl-CoA carboxylase for the induction of antibody formation was purified to homogeneity from chicken liver as previously described (Gregolin *et al.*, 1968a; Moss & Lane, 1972). The specific activity of the purified enzyme when maximally activated with 10 mM-citrate was 9.0 units/mg of refractometrically determined protein, a value identical with that reported earlier (Gregolin *et al.*, 1968a) for the homogeneous carboxylase. This enzyme preparation gave

rise to a single hypersharp sedimenting boundary ($s_{20,w} = 58S$ at 1.0mg/ml in the presence of 50mm-potassium phosphate, pH 7.0) in the analytical ultracentrifuge.

Preparation of antibody against purified chicken liver acetyl-CoA carboxylase

Antibody against the chicken liver carboxylase was generated in male New Zealand White rabbits by injecting 0.19mg of the homogeneous enzyme (0.76mg per rabbit) mixed with Freund's complete adjuvant into each foot pad. At 2 and 3 weeks the procedure was repeated and after 5 weeks the antiserum was collected. The antiserum was used directly for the carboxylase immunoinactivation studies and double-diffusion analysis. For immunoprecipitation of carboxylase for SDS*/polyacrylamide-gel electrophoresis, the immunoglobulin G fraction of the antiserum was precipitated by raising the $(NH_4)_2SO_4$ concentration of the serum to 33% saturation in the presence of 6mm-sodium borate, pH 8.3, and 150mm-NaCl. After redissolving the precipitate and repeating the precipitation procedure twice, the antibody preparation was dialysed for 2 days against this borate-buffered NaCl.

Ouchterlony double-diffusion analysis

Ouchterlony double-diffusion analyses were carried out in 0.5% agarose gels containing 50mm-potassium phosphate, pH 7.5, and 150 mm-NaCl. Plates were developed for 72h at 4°C, washed exhaustively with 50mm-potassium phosphate, pH 7.5, containing 150mm-NaCl, and stained with Amido Black. Rat mammary-gland extracts for double-diffusion analysis were concentrated by raising the $(NH_4)_2SO_4$ concentration to 30% saturation with the solid salt. After centrifugation, the pellet, which contained all of the acetyl-CoA carboxylase activity, was redissolved in 50mm-potassium phosphate, pH 7.0, containing 1mm-dithiothreitol and 0.5mm-EDTA, and dialysed against the same buffer overnight at 4°C.

Quantitative immunoinactivation of rat mammary-gland cytosol and purified chicken liver acetyl-CoA carboxylase

For the quantitative immunoinactivation experiments with antiserum against the carboxylase, rat mammary-tissue cytosols were used without additional treatment, but the chicken liver carboxylase was dialysed for 24h at 4°C against 50mm-potassium phosphate, pH 7.0, containing 10mm-potassium citrate, 1mm-dithiothreitol and 0.5mm-EDTA. Samples containing 1–100munits of carboxylase were incubated in a total volume of 0.5ml with 50 μ l of antiserum for 2h at 25°C in 50mm-Tris/HCl buffer, pH 7.2, containing 150mm-NaCl and 1mm-dithiothreitol. With the chicken liver enzyme, 5mg of

bovine serum albumin/ml was also present. Samples of the incubation mixtures were then assayed for the carboxylase activity remaining.

Subunit characterization of immunoprecipitates from rat mammary-tissue and chicken liver cytosol

Immunoprecipitates of acetyl-CoA carboxylase from rat mammary-tissue and chicken liver cytosols were dissociated with 1% SDS and subjected to SDS/polyacrylamide-gel electrophoresis. Mammary tissue or liver cytosols were prepared as described above in the presence or absence of bovine parotid-gland trypsin inhibitor (Trasylol; 200 Kallikrein inactivator units/ml; Trautschold *et al.*, 1967). To 1600 μ l of cytosol containing 180munits of carboxylase was added 400 μ l of the antibody solution in 50mm-Tris/HCl buffer, pH 7.2, containing 1mm-dithiothreitol and 0.5mm-EDTA. After 30min at 37°C and overnight at 4°C, the reaction mixture was centrifuged at 2000g for 10min, and the immunoprecipitate washed twice with cold 0.9% NaCl and dissolved in 0.3ml of 1M-acetic acid (Silpananta & Goodridge, 1971). After 30min at room temperature, the solution was freeze-dried and the residue dissolved in 200 μ l of 25mm-sodium phosphate, pH 7.2, containing 1% mercaptoethanol, 1% SDS, 5% (w/v) sucrose and 800mm-urea. Samples were subjected to SDS/polyacrylamide-gel electrophoresis (5% acrylamide gels; acrylamide/methylenebisacrylamide ratio, 30:1, w/w) in 25mm-sodium phosphate buffer, pH 7.2, containing 0.1% SDS as described by Weber & Osborn (1969). After exposure to 5% (w/v) trichloroacetic acid for 15h, the gels were stained with Coomassie Blue in 5% (v/v) acetic acid.

Determination of biotin

The (+)-biotin content of the stained protein bands on SDS/polyacrylamide gels was determined by microbiological assay with *Lactobacillus plantarum* 17-5 (A.T.C.C. 8014) as described by Gyorgy (1967). Polyacrylamide-gel slices were hydrolysed with 2M- H_2SO_4 for 3h at 120°C in sealed tubes to release covalently bound biotin. Samples were neutralized, diluted and stored frozen until assayed.

Determination of protein and DNA

Protein was determined by the method of Lowry *et al.* (1951) and DNA by the modified diphenylamine method of Burton (1968). Bovine serum albumin was used as a protein standard and calf thymus DNA as a DNA standard.

Results

Changes in mammary-tissue acetyl-CoA carboxylase activity during gestation and lactation

The acetyl-CoA carboxylase activity of mammary tissue from rats was determined at different stages

* Abbreviation: SDS, sodium dodecyl sulphate.

of pregnancy and lactation. Cytosols from this tissue were incubated with citrate at 37°C to activate the enzyme maximally and then assayed for carboxylase activity by the [14 C]bicarbonate-fixation assay. As shown in Fig. 1, cytosolic carboxylase activity in this tissue is low and remains relatively constant from mid-gestation to parturition, the specific activity being about 1–2 munits/mg of cytosolic protein. With the onset of lactation, however, carboxylase activity rises abruptly for a short period, followed by a slower rise over the next 2 weeks, and reaches a peak specific activity of 50 munits/mg of cytosolic protein 13 days *post partum*. At its maximum, the acetyl-CoA carboxylase activity in mammary tissue exceeds that of other lipogenic tissues, such as liver (Gregolin *et al.*, 1968a) and adipose tissue (Moss *et al.*, 1972) by a factor of 10–15-fold. It is evident (Fig. 1) that the pattern of change in carboxylase activity is the same during gestation and lactation whether activity is expressed per unit weight of protein or DNA, although the latter best reflects activity per cell.

To determine whether the post-partum rise in carboxylase activity is due to an increased specific activity of the enzyme, i.e. activation, or to an increase in its concentration in the tissue, changes in enzyme content were determined by immunotitration. It was necessary therefore to prepare and characterize antibody against acetyl-CoA carboxylase.

Characterization of anti-(acetyl-CoA carboxylase) antibody and the immunotitration procedure

Pure chicken liver acetyl-CoA carboxylase was used as antigen owing to the impracticality of obtaining purified rat mammary-tissue acetyl-CoA carboxylase in sufficient quantity for use as an antigen. The chicken liver carboxylase preparation had a specific activity of 9 units/mg of protein, which

is equal to that established for the pure enzyme (Gregolin *et al.*, 1968a; Moss & Lane, 1972). Further, this preparation was judged to be homogeneous by analytical ultracentrifugation and by SDS/polyacrylamide-gel electrophoresis as indicated in the Materials and Methods section. Fortunately, antibody raised against the chicken liver enzyme effectively inactivates and immunoprecipitates the mammary-tissue acetyl-CoA carboxylase.

Ouchterlony double-diffusion analysis of the rabbit anti-(chicken liver acetyl-CoA carboxylase) antibody reaction with carboxylases from rat mammary gland and chicken liver reveals a single precipitin line in each case (Plate 1). However, a spur from the precipitin line of the chicken liver carboxylase runs beyond that of the rat mammary-gland enzyme. This spurring pattern indicates a cross-reaction of partial identity, i.e. the mammary-gland enzyme possesses fewer antigenic determinants than the liver enzyme when tested against the antibody to chicken liver acetyl-CoA carboxylase.

To ascertain whether acetyl-CoA carboxylase is specifically precipitated from mammary tissue cytosol by antibody against the avian liver enzyme, immunoprecipitates of both were subjected to polypeptide analysis by SDS/polyacrylamide-gel electrophoresis. As shown in Plate 2 (gel *a*), in the mammary-tissue cytosol the major stained polypeptide band was found at a position in the gel that corresponds to a mol.wt. of 230000; the two fast-moving bands of mol.wt. 50000 and 25000 correspond to immunoglobulin G subunits. Slices cut from the gels at the positions indicated (Plate 2) were assayed for biotin by microbiological assay with *Lactobacillus plantarum* after hydrolysis of the slices to release covalently bound biotin. Only the 230000-mol.wt. band (gel *a*, Plate 2) contained a significant amount of biotin

Fig. 1. Changes in acetyl-CoA carboxylase activity and immunotitratable enzyme content of rat mammary tissue during gestation and lactation

Acetyl-CoA carboxylase activity [expressed in terms of protein (○) and DNA (●)] and immunotitratable enzyme (Δ) (acetyl-CoA carboxylase) were determined on mammary-tissue cytosols as described in the Materials and Methods section. Where indicated the error bars are for the mean values $\pm$ s.d.

EXPLANATION OF PLATE I

Ouchterlony double-diffusion analysis of rat mammary-gland acetyl-CoA carboxylase

Agarose (0.5%) gels contained 20mM-potassium phosphate, pH7.4, and 150mM-NaCl. The centre well contained 10 μ l of rabbit anti-(acetyl-CoA carboxylase) serum; wells 1, 3 and 5 contained 5 μ g of purified chicken liver acetyl-CoA carboxylase, and wells 2, 4 and 6, 1.1mg of mammary-gland cytosol protein from a rat that had been lactating for 7 days.

EXPLANATION OF PLATE 2

SDS/polyacrylamide-gel electrophoresis of immunoprecipitates of rat mammary tissue and chicken liver acetyl-CoA carboxylase

Cytosols obtained from rat mammary tissue and chicken liver were precipitated with rabbit antibody against the chicken liver carboxylase as described in the Materials and Methods section. Immunoprecipitates were dissociated in a 1% SDS solution containing 8M-urea and analysed by SDS/polyacrylamide-gel electrophoresis. Gel (a), rat mammary tissue (day 13 of lactation) cytosol immunoprecipitate. Gel (b), chicken liver cytosol immunoprecipitate. Gel (c), purified chicken liver acetyl-CoA carboxylase (5 μ g). Gel (d), purified chicken liver acetyl-CoA carboxylase (50 μ g). Slices were removed from gels at the positions indicated by the numbers on the left and were analysed for (+)-biotin by microbiological assay (see Table 1 for results) as described in the Materials and Methods section. The numbers on the right indicate the positions of molecular-weight standards [myosin heavy chain, 207000; *Escherichia coli* β -galactosidase (EC 3.2.1.23), 135000; muscle phosphorylase (EC 2.4.1.1), 100000; pyruvate kinase (EC 2.7.1.40), 57000].

(Table 1), which confirms its identity as the major, if not sole, polypeptide of mammary-gland acetyl-CoA carboxylase and indicates that the immunoprecipitation procedure is specific.

Nevertheless, a small but reproducible amount of biotin was detected in a barely visible stained protein band of 125000 mol.wt. This finding is interesting and perhaps important, since the purified chicken liver acetyl-CoA carboxylase is known (Gregolin *et al.*, 1968b; Guchhait *et al.*, 1974) to be composed of subunits in the 130000-mol.wt. range. As illustrated in Plate 2 (gel c, light load, and gel d, heavy load), the chicken liver carboxylase, purified as previously described (Gregolin *et al.*, 1968a; Moss & Lane, 1972) and dissociated with SDS, produces a major band(s) at 125000 and a minor band at 230000 daltons (see gel d). Acetyl-CoA carboxylase immunoprecipitated from avian liver cytosol prepared in the presence of bovine parotid-gland trypsin inhibitor, gives rise to a major band on SDS/polyacrylamide gels (Plate 2, gel b) of 230000 mol.wt. with a minor band of 125000 mol.wt. Both bands contain quantities of bound biotin approximately in proportion to the intensity of the protein stain (Table 1). When trypsin inhibitor was omitted during enzyme extraction and immunoprecipitation (experiments not shown), the 230000-mol.wt. band was virtually absent and the biotin-containing band in the 125000-mol.wt. region became predominant. The 125000-mol.wt. polypeptide can also be generated by brief exposure of unproteolysed carboxylase to 0.01% trypsin (EC 3.4.21.4) for 5 min. It is evident that native acetyl-CoA carboxylase from both rat mammary gland and chicken liver is composed of subunits of 230000 mol.wt. Proteolytic cleavage of that native enzyme during purification produces polypeptides approximately one-half the native subunit size, particularly with the liver enzyme. Tanabe *et al.* (1975) have reported similar findings with rat liver acetyl-CoA carboxylase.

The reactions of rabbit anti-(chicken liver acetyl-CoA carboxylase) antibody with the rat mammary tissue and chicken liver acetyl-CoA carboxylases were examined quantitatively. Centrifugation of the immunoprecipitated carboxylase leads to more than an equivalent loss of soluble carboxylase activity from the supernatant solution. This excessive activity loss is indicated by a slope much lower than 1.0 beyond the equivalence point in immunotitrations in which supernatant carboxylase activity is plotted versus carboxylase activity added at a fixed amount of added antibody. At least part of the apparent loss of soluble carboxylase activity appears to result from instability of the enzyme during the long incubation period required for quantitative immunoprecipitation. It also appears that because of the tendency of the carboxylase to aggregate into a filamentous form (Gregolin *et al.*, 1966), more than an equivalent amount of carboxylase protomer may be complexed in the insoluble immunoprecipitates. A simpler and more quantitative immunoinactivation procedure was therefore developed to titrate acetyl-CoA carboxylase. Carboxylase-containing samples were incubated in the presence of a fixed amount of antibody and the enzyme activity remaining after 2 h was determined. This procedure is acceptable, since the antibody totally inactivates rat mammary-tissue acetyl-CoA carboxylase in solution. Enzyme stability was significantly increased with this procedure. In addition, titrations were linear with enzyme concentration and reproducible equivalence points were obtained. Finally, control incubations conducted with normal serum produced no significant enzyme inactivation. As shown in Fig. 2, the antibody was neutralized by a smaller amount of the original antigen (purified chicken liver acetyl-CoA carboxylase) than of the rat mammary-gland cytosolic carboxylase. Two-thirds as much chicken liver acetyl-CoA carboxylase (20 munits) was required to neutralize 50 μ l of antiserum as of the mammary-

Table 1. Biotin content of SDS/polyacrylamide-gel slices

The polyacrylamide-gels shown in Plate 2 were stained and sliced, hydrolysed with NaOH, and assayed for (+)-biotin by microbiological assay as described in the Materials and Methods section. n.d., Not determined.

Gel slice no.	Mol.wt.	(+)-Biotin content (ng/stained protein band)		
		Gel (a) (rat mammary tissue cytosol)	Gel (b) (chicken liver cytosol)	Gel (c) (purified chicken liver carboxylase, 5 μ g)
1	230000	6.2	2.5	0.0
2	125000	0.02	0.13	1.5
3	105000	0.0	0.0	n.d.
4	50000*	0.0	0.0	n.d.
5	Blank	0.0	0.0	n.d.

* Immunoglobulin G heavy chain.

tissue carboxylase, which required 30 munits. This finding is not unexpected, since chicken liver acetyl-CoA carboxylase was the antigen against which the antibody was raised and since double-diffusion analysis (Plate 1) indicated only partial antigenic identity of the two carboxylases.

Changes in mammary tissue acetyl-CoA carboxylase content during gestation and lactation

As pointed out above, the rise in carboxylase activity which begins abruptly after parturition may result from activation of enzyme synthesized previously, i.e. during gestation. The various mechanisms by which this activation could occur have been discussed above.

It was therefore decided to obtain a measure of the total concentration in mammary tissue of acetyl-CoA carboxylase protein, including enzymically inactive forms that might serve as precursor of active carboxylase. Hence the concentration of acetyl-CoA carboxylase protein in mammary tissue was assessed at various stages of pregnancy and lactation by determining the number of units of carboxylase activity required to neutralize a fixed amount of anti-carboxylase antibody. The only assumption required is that all forms of the carboxylase should react effectively with the antibody. Immunoinactivation titrations were conducted on mammary tissue cytosols in the same manner as described for Fig. 2.

However, carboxylase activities of cytosols from pregnant rats or rats during the first 24 h of lactation were too low to be titrated by this procedure. In such cases immunotitrations were conducted with highly

active extracts from rats in peak lactation. To each enzyme-antibody incubation was added a constant amount of the inactive extract. If inactive acetyl-CoA carboxylase protein was present in the 'inactive cytosol', the equivalence point of the reaction of the antibody with the active extract should be lowered, since the inactive acetyl-CoA carboxylase protein would neutralize a fraction of the antibody, thus decreasing its effective concentration. The reaction between antibody and the highly active extract was also conducted in the absence of the inactive extract. As shown in Fig. 3, the equivalence point of the reaction between antiserum and an extract from a 13-day-lactating rat is not lowered by an extract obtained from a rat immediately after parturition (2 h *post partum*).

Equivalence points for mammary-tissue cytosols obtained from rats from day 14 of pregnancy to day 19 of lactation are summarized in Table 2. The values determined for inactive extracts are not significantly lower than those for the highly active extracts. All results are, in fact, within the variance of the data. Thus during the entire course of pregnancy and lactation the amount of immunologically reactive enzyme per unit of carboxylase activity remained constant. As would be expected, therefore, post-partum changes in the immunotitratable enzyme content of mammary tissue are closely paralleled by changes in carboxylase activity (Fig. 1).

The possibility was also considered that an inhibitory cytosolic factor(s), present in the mammary tissue of rats just before the rise in carboxylase activity, might account for the low enzyme activities observed. Reciprocally, a cytosolic factor present in active

Fig. 2. Immunotitration of purified chicken liver acetyl-CoA carboxylase and rat mammary tissue cytosol with antibody against the chicken liver carboxylase

Increasing amounts of enzyme sample were added to 50 μ l of anti-(chicken liver acetyl-CoA carboxylase) serum, and enzyme activity remaining after incubation for 2 h at 25°C was determined as described in the Materials and Methods section. ○, Purified chicken liver acetyl-CoA carboxylase. ●, Mammary-tissue cytosol from a rat on day 13 of lactation.

Fig. 3. Immunotitration of rat mammary cytosols obtained from rats immediately after parturition

Increasing amounts (5–125 μ l) of cytosol from a rat lactating for 13 days were added to 50 μ l of anti-(acetyl-CoA carboxylase) serum in the presence (○) and absence (●) of 20 μ l of cytosol from a rat immediately after parturition and acetyl-CoA carboxylase activity remaining after incubation for 2 h at 25°C was determined.

Table 2. *Acetyl-CoA carboxylase activities and equivalence points of immunotitrations of mammary-tissue cytosol at different stages of gestation and lactation*

For details see the Materials and Methods section. Results are means $\pm$ s.d. for the numbers of samples in parentheses.

Stage	Acetyl-CoA carboxylase activity (munits/mg of protein)	Equivalence point (munits)
Day 14 of gestation	1.7 $\pm$ 0.4 (2)	14.8 $\pm$ 0.2 (2)*
Day 20 of gestation	1.2 $\pm$ 0.4 (2)	15.1 $\pm$ 0.2 (2)*
At parturition	1.7 $\pm$ 0.8 (2)	18.0 $\pm$ 0.4 (2)*
Day 1 of lactation	6.0 $\pm$ 1.0 (2)	17.3 $\pm$ 1.2 (2)
Day 2 of lactation	13.0 $\pm$ 5.0 (2)	14.7 $\pm$ 1.3 (2)
Day 7 of lactation	31.0 $\pm$ 6.0 (4)	17.3 $\pm$ 1.4 (4)
Day 8 of lactation	32.0 (1)	15.5 (1)
Day 13 of lactation	50.0 $\pm$ 14.0 (3)	17.4 $\pm$ 2.0 (3)
Day 19 of lactation	20.0 $\pm$ 5.0 (2)	16.4 $\pm$ 2.9 (2)

* The equivalence point of an enzymically active cytosol (from mid-lactation) was determined in the presence of cytosol from a rat at this stage of gestation.

cytosols from lactating tissue might activate latent carboxylase in cytosols from non-lactating tissue. To determine whether either extract could affect the activity of acetyl-CoA carboxylase in the other, incubation of each cytosol alone and in combination with the other were conducted in the absence of anti-serum under a variety of conditions. In one case, for example, the activity of acetyl-CoA carboxylase in the cytosol from 13-day-lactating rats was 0.53 munit/ml in both the presence and the absence of the cytosol obtained from the rat at parturition. Thus there is neither an activator of carboxylase in the extract from 13-day-lactating rats nor an inhibitor in the inactive extract. It is concluded therefore that the dramatic increase in acetyl-CoA carboxylase activity after the onset of lactation can best be explained by a rise in cellular enzyme concentration. Modification of a pre-existing inactive form of the enzyme does not appear to play even a secondary role in the activation process.

Discussion

Acetyl-CoA carboxylase is thought to play an important role in regulating the carbon flux into the lipogenic pathway of animal cells (Lane *et al.*, 1974). In mammary tissue the activity of acetyl-CoA carboxylase is quite low throughout pregnancy and then rises markedly from the second day *post partum* to late lactation. The present investigation shows that the rise in carboxylase activity associated with the

onset of lactation is due to an increase in cellular concentration of the enzyme rather than to activation of carboxylase or apocarboxylase already present in the tissue. This conclusion is based on the finding that the amount of immunotitratable enzyme parallels the increase in enzyme activity (Fig. 1 and Table 2).

The observed elevation in acetyl-CoA carboxylase content may be the result of increased rates of enzyme synthesis, decreased rates of degradation, or most likely a combination of the two factors. Studies with mammary-gland explants from mid-pregnant rabbits (Speake *et al.*, 1975, 1976) indicate that a hormonally induced elevation of the concentration of fatty acid synthase is the result of decreased rates of enzyme degradation as well as elevated rates of synthesis. The process of mammary-gland differentiation may be facilitated by decreased degradation of key enzymes such as acetyl-CoA carboxylase and fatty acid synthase.

The change in lipogenic enzyme content appears to constitute a late event in the differentiation of the secretory epithelial cells of mammary tissue in preparation for the synthesis of milk triglyceride. It is likely that this change of carboxylase content occurs primarily in epithelial cells of the mammary gland and not the other major cell types of this tissue. It has been established (Rees & Eversole, 1964) that only epithelial cells increase as a percentage of total mammary tissue mass in the transition from the latent state to lactation, whereas connective-tissue cells and adipocytes decrease. In the non-lactating mammary tissue of the rat, epithelial, connective-tissue and adipose cells comprise less than 5%, approx. 90% and 10% respectively, of the total cell mass, whereas in lactating tissue the distribution changes to approx. 70%, 30% and less than 2% respectively.

While the specificity of the immunoprecipitation of acetyl-CoA carboxylase was being assessed, it became apparent that carboxylase precipitated with antibody from both rat mammary tissue and avian liver possess subunits considerably larger than previously realized (Gregolin *et al.*, 1968b; Inoue & Lowenstein, 1972; Guchhait *et al.*, 1974). It is evident that during its extraction from tissues, particularly from chicken liver, the carboxylase is susceptible to limited proteolytic cleavage by a trypsin-like proteinase. The action of this proteinase(s) generates polypeptides of approx. 125000 mol.wt., which is about one-half that of the native subunit (230000) (Plate 2). However, cleavage by the proteinase can be prevented by the inclusion of the bovine parotid-gland trypsin inhibitor, Trasylol, in the homogenization medium. Moreover, brief exposure of the unproteolysed carboxylase to trypsin generates a polypeptide(s) of similar size, i.e. 125000 daltons, which cannot be resolved by SDS/polyacrylamide-gel electrophoresis from those pro-

duced by the endogenous tissue proteinase. Interestingly, the 'clipped' enzyme retains full catalytic activity. It appears that acetyl-CoA carboxylase may be one of a growing list of enzymes having two domains which are linked by an exposed polypeptide chain which is particularly susceptible to attack by proteinases. The existence of two distinct domains in the subunit of acetyl-CoA carboxylase would be consistent with the finding (Moss & Lane, 1971; Lane *et al.*, 1974) that the enzyme possesses two catalytic centres each of which carries out one of the half-reactions which comprise the overall catalytic process.

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