Studies on acetyl-CoA carboxylase and fatty acid synthase from rat mammary gland and mammary tumours

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The activities of two lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthase, were determined in two transplantable mammary adenocarcinomas (13762 and R3230AC) carried by non-pregnant, pregnant and lactating rats, and in mammary tissue of control animals (non-tumour-carrying) of comparable physiological states. During mammary-gland differentiation of control or tumour-carrying animals, the activities of acetyl-CoA carboxylase and fatty acid synthase in the lactating gland increased by about 40–50-fold over the values found in non-pregnant animals. On the other hand, in tumours carried by lactating dams there were only modest increases (1.5–2-fold) in acetyl-CoA carboxylase and fatty acid synthase compared with the neoplasms carried by non-pregnant animals. On the basis of the K_m values for different substrates and immunodiffusion and immunotitration data, the fatty acid synthase of neoplastic tissues appeared to be indistinguishable from the control mammary-gland enzyme. However, a comparison of the immunotitration and immunodiffusion experiments indicated that the mammary-gland acetyl-CoA carboxylase might differ from the enzyme present in mammary neoplasms.

In mammalian tissues the biosynthesis of long-chain fatty acids is catalysed by the sequential actions of acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthase. Acetyl-CoA carboxylase is a biotinyl-enzyme and catalyses the formation of malonyl-CoA (reaction i). Fatty acid synthase contains 4'-phosphopantetheine, which acts as the acyl carrier during fatty acid biosynthesis. This enzyme complex catalyses reaction (ii).

mammary-gland carboxylase into the enzymically active polymer (Lane *et al.*, 1974; Ahmad *et al.*, 1978). Further results suggest that the carboxylase is also regulated by a phosphorylation–dephosphorylation cycle. Phosphorylation inactivates, whereas dephosphorylation activates, the inactive carboxylase (Carlson & Kim, 1974; Lee & Kim, 1977; Brownsey *et al.*, 1977; Hardie & Guy, 1980).

The carboxylase and synthase have been purified to apparent homogeneity from rat lactating mammary gland (Smith & Abraham, 1975; Ahmad *et al.*, 1978; Ahmad & Ahmad, 1981; Ahmad *et al.*, 1982). The properties of rat mammary-gland enzymes are similar to those of their counterparts purified from other vertebrate sources (Inoue & Lowenstein, 1972; Lane *et al.*, 1974; Maitra & Kumar, 1974; Smith & Abraham, 1975; Tanabe *et al.*, 1975).

Acetyl-CoA carboxylase catalyses the first committed and rate-limiting step of fatty acid biosynthesis. Like other vertebrate carboxylases, citrate converts the essentially inactive protomer of rat

In addition, other mechanisms, such as the ratio of the apo- to the holo-enzyme and the rates of enzyme synthesis and degradation, are important factors that might influence the flux of acetyl units into long-chain fatty acids.

Earlier studies by Sabine *et al.* (1966), Majerus *et al.* (1968) and Elwood & Morris (1968) found aberrant regulation of fatty acid biosynthesis in a number of hepatomas, since dietary manipulations did not influence fatty acid biosynthesis in liver neoplasms in a manner characteristic of liver. Neither acetyl-CoA carboxylase nor fatty acid

synthase of hepatomas was subject to changes in enzyme concentration normally observed in host liver after alteration of the diet (Majerus *et al.*, 1968). Although this information has been available for some time, the precise mechanism of regulation of fatty acid synthesis by dietary manipulations even in the normal liver is obscure at present. Nevertheless the kinetic (affinities for substrates) and heat-inactivation (etc.) properties of a single hepatoma acetyl-CoA carboxylase were investigated and found to be similar to those of the enzyme from host liver (Majerus *et al.*, 1968). However, it is not clear from these studies whether the hepatoma and host liver carboxylases are identical immunologically.

In lactating mammary gland, the conversion of acetate and glucose into fatty acids proceeds at a much greater rate than in mouse adenocarcinomas (Bartley *et al.*, 1971; Lin *et al.*, 1975). The conversion of acetyl-CoA into long-chain fatty acids proceeds via the reactions catalysed by acetyl-CoA carboxylase and fatty acid synthase. Since both the carboxylase and the synthase (especially the carboxylase) are subject to regulation by a number of mechanisms, the decreased capacity of mammary adenocarcinomas to synthesize fatty acids from acetyl-CoA could result from (a) lower amounts of these enzymes and/or (b) the presence of modified form(s) of the enzymes that are catalytically less active. The present paper addresses some of these questions. The results presented here show that, whereas fatty acid synthases of neoplastic and normal mammary tissue appear to be identical, acetyl-CoA carboxylase of mammary neoplasms may be immunologically distinct from the normal tissue enzyme. Some of these data have appeared in preliminary reports (Ahmad *et al.*, 1976; Ahmad & Ahmad, 1979).

Materials and methods

Materials

ATP, acetyl-CoA, malonyl-CoA, CoA and NADPH were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Sigma Chemical Co. (St. Louis, MO, U.S.A.) was the source for bovine serum albumin (essentially fatty acid-free), dithiothreitol, phosphocreatine, creatine kinase (EC 2.7.3.2) and $(\text{NH}_4)_2\text{SO}_4$ (enzyme grade). Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, MI, U.S.A.). $\text{Ba}^{14}\text{CO}_3$ (58 Ci/mol) was from ICN (Irvine, CA, U.S.A.) and Omnifluor from New England Nuclear (Boston, MA, U.S.A.). DEAE-cellulose (DE-52) was obtained from Whatman (Clifton, NJ, U.S.A.). All other chemicals were of the highest purity commercially available. Acetyl-CoA was also prepared by the method of Simon & Shemin (1953). Its concentration was determined enzymically (Tubbs & Garland, 1969).

Animals

Female rats of the Fischer strain were fed on Purina Laboratory Chow and water *ad libitum* and kept in quarters that provided for a 12h-light/12h-dark day. Pregnant rats were housed in separate cages. After delivery, litters were adjusted to contain eight pups. All animals were killed between 09:00 and 10:00h.

Mammary neoplasms

Mammary adenocarcinomas R3230AC (originally obtained from Dr. Russell Hilf, University of Rochester, Rochester, NY, U.S.A.) and 13762 (from the Mason Research Institute Tumor Bank, Worcester, MA, U.S.A.) were maintained by subcutaneous implant of 1mm³ pieces into the upper fore- and hind-quarters of female Fischer rats (approx. 200g). When the tumours reached 1.5–2.0cm in diameter animals were killed by cervical dislocation. Tissue was harvested and processed as described below. Time required to attain desired tumour growth was 19–21 days for adenocarcinoma R3230AC and 10 days for adenocarcinoma 13762.

Purification of rat lactating mammary-gland acetyl-CoA carboxylase and fatty acid synthase

This was done by the method of Ahmad & Ahmad (1981) and Ahmad *et al.* (1982). The highly purified acetyl-CoA carboxylase had a specific activity of approx. 15 units/mg of protein and M_r 260000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The specific activity of the fatty acid synthase was approx. 2.0–2.5 units/mg of protein when assayed at 37°C. Both these enzymes appear homogeneous by a number of criteria (Ahmad *et al.*, 1978; Ahmad & Ahmad, 1981; Ahmad *et al.*, 1982). These preparations were used for the production of antibodies.

Preparation of tissue and partial purification of enzymes

Tumour-bearing (host) and non-tumour-bearing (control) rats were obtained in each of three physiological states: non-pregnant, pregnant (16–20 days) and lactating (13–15 days). Tumours R3230AC and 13762 were harvested 20 and 10 days after transplantation respectively. Animals were killed by cervical dislocation, and mammary and/or tumour tissue was excised and then washed extensively in ice-cold 0.25M-sucrose. Each tissue was then suspended in 2vol. of 50mM-imidazole/HCl buffer, pH 6.5, containing 1mM-EDTA, 7mM-2-mercaptoethanol and 20% (v/v) glycerol, and homogenized at 4°C in a VirTis homogenizer at 22500 rev./min for three periods of 45s each interspersed with 1min rest periods. The homogenate was centrifuged at 2000g for 10min at 4°C.

After removal of the fat layer with a spatula, the supernatant solution was filtered through a loose plug of cotton covered with four layers of cheesecloth. The 2000 g pellet was resuspended in 1 vol. of the above buffer, homogenized, and treated as above. The combined supernatants were centrifuged at 100000 g for 1 h. The supernatant solution from this step could be used for direct enzyme assays, for further purification (Ahmad & Ahmad, 1981) or for precipitation with solid $(\text{NH}_4)_2\text{SO}_4$ (to 50% saturation) and subsequent determination of acetyl-CoA carboxylase and fatty acid synthase activities on the concentrated protein. Enzymes obtained from the last step were used for all immunochemical analyses, for K_m determinations and for studying the effect of temperature on catalytic activity.

Tissues were also homogenized and the enzymes partially purified by using buffers containing a mixture of known inhibitors of proteolysis [EDTA, leupeptin, antipain, pepstatin A, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK') and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') and in addition phenylmethanesulphonyl fluoride (which inhibits fatty acid synthase) for acetyl-CoA carboxylase only]. Supplementation of buffers with the inhibitors used had no marked effect on the activities of acetyl-CoA carboxylase and fatty acid synthase.

Before being assayed, fractions were dialysed [to remove $(\text{NH}_4)_2\text{SO}_4$] against 50 mM-imidazole/HCl buffer, pH 7.5, containing 1.0 mM-EDTA and 20% glycerol for 1.5 h at 4°C.

Assay of acetyl-CoA carboxylase

The reaction mixture in a final volume of 0.1 ml contained 60 mM-imidazole/HCl buffer, pH 7.5, 24 mM-magnesium acetate, 0.4 mM-EDTA, 0.10 mM-EGTA, 30 mM-sodium citrate, bovine serum albumin (0.5 mg/ml), 17.5 mM-phosphocreatine, 5.6 units of creatine kinase (in 50 mM-imidazole/HCl buffer, pH 7.5, containing 10 mg of bovine serum albumin/ml), 0.75 mM-acetyl-CoA, 25 mM- $\text{NaH}^{14}\text{CO}_3$ (800 d.p.m./nmol) and 7.2 mM-ATP. The reaction was carried out at 37°C for 5 min and terminated by the addition of 0.025 ml of 4 M-HCl. Samples (0.05 ml) were transferred to scintillation vials and evaporated to dryness in a forced air oven at 85°C. The residue was dissolved in 0.5 ml of water, 5 ml of scintillation fluid (Fricke, 1975) was added and the radioactivity was determined. One unit of enzyme activity represents 1 μmol of malonyl-CoA formed/min at 37°C.

Assay of fatty acid synthase

Fatty acid synthase was assayed spectrophotometrically as described by Smith & Abraham (1975), except that incubation temperature was 37°C. All values were recorded for NADPH oxidation occurring in the presence of malonyl-CoA.

One unit of enzyme activity represents 1 μmol of NADPH oxidized/min at 37°C.

For both the carboxylase and synthase assays, conditions were selected giving linear rates of catalysis with respect to protein concentration and time of incubation.

Immunological procedures

(a) *Antibody production.* Antibody against homogeneous fatty acid synthase was prepared in rabbits as described by Ahmad *et al.* (1979). Anti-(acetyl-CoA carboxylase) and anti-biotinyl antibodies were also raised in rabbits as described previously (Ahmad *et al.*, 1978, 1981).

The molecular weights of the polypeptides constituting acetyl-CoA carboxylase and fatty acid synthase are almost identical (Ahmad *et al.*, 1978). Since examination by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the immunoprecipitates formed on the addition of anti-carboxylase or anti-synthase would not distinguish which of the antigens was precipitated, experiments were performed to determine the specificity of the antisera prepared against these enzymes. Each antibody removed only the corresponding antigen from solution, and no cross-reactivity could be detected during immunodiffusion studies. When the carboxylase and synthase present in the normal and neoplastic tissue extracts were precipitated by the appropriate antibodies and then examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, besides the heavy and the light chains of immunoglobulin G, only the protein bands of M_r 240000–260000 were observed. These experiments established the specificity of the antisera as well as the size of the polypeptides found in tissue (normal and neoplastic) homogenates and partially purified enzyme preparations.

(b) *Ouchterlony double-diffusion analyses.* Double-diffusion analyses were performed by the method of Ouchterlony & Nilsson (1978), with minor modifications as described in Ahmad *et al.* (1978). The concentration of agarose in gel layers was decreased to 0.8% to facilitate diffusion of the high-molecular-weight acetyl-CoA carboxylase.

(c) *Immunotitration procedures.* Fatty acid synthase was incubated in duplicate with various amounts of anti-(rat lactating mammary-gland fatty acid synthase) in reaction mixtures (total volume 0.05 ml) containing 0.25 M-potassium phosphate buffer, pH 6.5, 10 mM-dithiothreitol and 0.1 mg of bovine serum albumin. After incubation at 37°C for 15 min, tubes were centrifuged at 2000 g for 10 min at 27°C. Samples (0.03 ml) of the supernatant solution were removed and assayed for synthase activity.

Acetyl-CoA carboxylase was incubated in duplicate with anti-(acetyl-CoA carboxylase) (total

volume 0.05 ml) in 60 mM-imidazole/HCl buffer, pH 7.5, containing 24 mM-magnesium acetate, 0.4 mM-EDTA, 0.1 mM-EGTA, 30 mM-sodium citrate and 0.25 mg of bovine serum albumin. After incubation at 37°C for 15 min, tubes were centrifuged at 2000 *g* for 10 min at 27°C. Samples (0.03 ml) were removed and assayed for acetyl-CoA carboxylase activity.

Immunotitrations with biotin-binding antibodies were performed as described previously (Ahmad *et al.*, 1981).

The amount of antibody required to inactivate the enzyme completely was estimated by extrapolations of the linear portions of the titration curves to zero enzyme activity. These values were used to calculate the equivalence point (i.e. the amount of antibody required to inactivate 1 munit of enzyme activity).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Activities of acetyl-CoA carboxylase and fatty acid synthase in tumours carried by virgin, pregnant and lactating animals and in the corresponding normal mammary tissue

The activities of acetyl-CoA carboxylase and fatty acid synthase were measured in mammary tissue and tumours of virgin, pregnant and lactating rats. Likewise these activities were determined on mammary tissue derived from non-tumour-bearing animals of similar physiological states. Acetyl-CoA carboxylase and fatty acid synthase activities were lowest in virgin mammary gland (Tables 1 and 2). These enzyme activities reached their highest values in lactating gland, and during mid-lactation activities of the synthase and the carboxylase were approx. 40–50-fold higher than found in the virgin mammary tissue. Similar results have been reported for rat mammary-gland acetyl-CoA carboxylase and for mouse and rabbit mammary-gland fatty acid synthases (Lin *et al.*, 1975; Mackall & Lane, 1977; Short *et al.*, 1977).

The carboxylase and synthase activities of transplantable tumours carried in virgin animals were consistently higher than the activities in mammary tissue of non-pregnant animals, but markedly lower than those found in the lactating mammary gland. These results are in accord with the previous observations (Abraham & Bartley, 1974) indicating that mammary tumours synthesized long-chain fatty acids from acetate and glucose at a much lower rate than did the lactating mammary gland. The data of Tables 1 and 2 also show that the physiological

Table 1. *Acetyl-CoA carboxylase activity of mammary gland and mammary tumours*

The 100 000 *g* supernatant solutions derived from various tissues were assayed directly for acetyl-CoA carboxylase activity as described in the Materials and methods section. In the case of the tissue obtained from non-pregnant animals, before assays the carboxylase present in the extracts was concentrated by precipitation with (NH₄)₂SO₄ (50% saturation). Each determination was made in duplicate, with the numbers of determinations (animals) in parentheses. Agreement between values was $\pm 15\%$.

Physiological state of animal	Activity (munits/mg of protein)		
	Mammary gland	Tumour R2320AC	Tumour 13762
Non-pregnant	1 (3)	4.6 (3)	2.4 (16)
Pregnant	16.7 (3)	3.8 (3)	3.3 (3)*
Lactating	53 (8)	8.4 (7)	4.0 (2)

* See Table 2 for explanation.

Table 2. *Fatty acid synthase activity of mammary gland and mammary tumours*

Fatty acid synthase activity was measured in tissue extracts prepared as described in the legend to Table 1 and assayed as described in the Materials and methods section.

Physiological state of animal	Activity (munits/mg of protein)		
	Mammary gland	Tumour R2320AC	Tumour 13762
Non-pregnant	3 (3)	12 (4)	13.5 (16)
Pregnant	27 (3)	16.6 (3)	8.2 (3)*
Lactating	123 (9)	30 (7)	24 (4)

* Tumour 13762 grew more slowly and with less frequency (all animals were injected at three sites) in all pregnant animals than in non-pregnant animals.

stimuli inducing the carboxylase and the synthase in differentiating mammary gland had little effect on the same enzymes of tumours grown in pregnant or lactating animals. The activities of acetyl-CoA carboxylase and fatty acid synthase were similar in the differentiating glands of animals, whether tumour-bearing or control (results not shown). Thus the presence of a tumour did not influence the induction of the carboxylase and the synthase during host mammary-gland differentiation.

Similarities and differences between neoplastic and normal tissue acetyl-CoA carboxylase and fatty acid synthase

Determination of K_m values. In an attempt to compare acetyl-CoA carboxylase and fatty acid

synthase enzymes from mammary adenocarcinomas and mammary gland, the kinetic properties of the enzymes carried through identical purification steps were determined. The K_m values for all the substrates, determined by double-reciprocal plots, are summarized in Table 3 and are similar for adenocarcinomas and normal mammary-gland enzymes with one exception, i.e. the K_m value for NADPH of the normal tissue synthase was about 2–3-fold lower than that found with the tumour enzymes. The reason for this difference is not clear. Nevertheless, on the basis of kinetic measurements both the acetyl-CoA carboxylase and the fatty acid synthase of neoplasms appear to be similar to the enzymes present in the control tissue.

Heat inactivation. The effect of heat on the inactivation of acetyl-CoA carboxylase obtained from different sources was also investigated. When the tumour and host mammary-gland enzymes were kept at 34°C for 10 min before assay, no change in activity was observed. However, preincubation at 37°C caused activation (30–40%) of the neoplastic tissue enzyme but inactivation (10–20%) of the enzyme from normal mammary gland. Temperatures exceeding 37°C caused the same amount of inactivation for the enzyme from both neoplastic and control tissues, with exposure to 45°C causing almost complete inactivation of all enzyme preparations investigated.

Immunodiffusion studies. The immunological cross-reactivity of acetyl-CoA carboxylase and fatty acid synthase from different sources was studied by immunodiffusion (Figs. 1 and 2). For fatty acid synthase (Fig. 2), complete fusion of the precipitin lines between tumour enzyme and control enzyme was observed, with no sign of spur formation. Such a reaction is characteristic of complete identity be-

tween neoplastic fatty acid synthase and control tissue enzyme. However, different results were obtained when the immunodiffusion experiments were performed with acetyl-CoA carboxylase. The

Fig. 1. Immunodiffusion analysis of rat mammary-gland acetyl-CoA carboxylase with anti-(lactating rat mammary-gland acetyl-CoA carboxylase)

Agarose (0.8%) plates in 50 mM-imidazole/HCl buffer, pH 7.5, containing 0.15 M-NaCl contained the following (in mg of protein): centre well, anti-(lactating rat mammary-gland acetyl-CoA carboxylase) (0.4 mg); wells 1, 3 and 5, lactating mammary-gland extract (0.068 mg); well 2, tumour-R3230AC extract (0.081 mg); well 4, tumour-13762 extract (0.091 mg). Extracts were obtained as described in the Materials and methods section. These studies were performed on at least three different preparations of antigen or antiserum, with identical results.

Table 3. K_m values for acetyl-CoA carboxylase and fatty acid synthase of normal and neoplastic tissues
 K_m values were calculated from double-reciprocal plots of initial velocity versus substrate concentrations on two different occasions. The experimental variation was about 1% and the fatty acid synthase reactions were started with malonyl-CoA.

Acetyl-CoA carboxylase			K_m value		
Substrate	Enzyme source	...	Mammary gland	Tumour 13762	Tumour R3230AC
Acetyl-CoA			$6.2 \times 10^{-5} \text{ M}$	$7.0 \times 10^{-5} \text{ M}$	$6.7 \times 10^{-5} \text{ M}$
ATP			$6.6 \times 10^{-4} \text{ M}$	$8.0 \times 10^{-4} \text{ M}$	$7.4 \times 10^{-4} \text{ M}$
HCO_3^-			$10.5 \times 10^{-3} \text{ M}$	$7.7 \times 10^{-3} \text{ M}$	$6.25 \times 10^{-3} \text{ M}$
Fatty acid synthase			K_m value		
Substrate	Enzyme source	...	Mammary gland	Tumour 13762	Tumour R3230AC
Acetyl-CoA			$1.4 \times 10^{-5} \text{ M}$	$1.8 \times 10^{-5} \text{ M}$	$1.4 \times 10^{-5} \text{ M}$
Malonyl-CoA			$2.0 \times 10^{-5} \text{ M}$	$2.5 \times 10^{-5} \text{ M}$	$2.4 \times 10^{-5} \text{ M}$
NADPH			$3.8 \times 10^{-5} \text{ M}$	$9.0 \times 10^{-5} \text{ M}$	$11.7 \times 10^{-5} \text{ M}$

Fig. 2. Immunodiffusion analysis of rat mammary-gland fatty acid synthase with anti-(lactating rat mammary-gland fatty acid synthase)

Agarose plates and enzyme extracts were prepared as described in the legend to Fig. 1. The samples were placed as follows (in mg of protein): centre well, anti-(lactating rat mammary-gland fatty acid synthase) (0.14 mg); wells 1, 3 and 5, mammary-gland extract (0.068 mg); well 2, tumour-13762 extract (0.091 mg); well 6, tumour-R3230AC extract (0.081 mg). At least three different preparations of antigen or antiserum were used, with identical results.

enzymes from both neoplastic tissues formed precipitin lines of partial identity when immunodiffused against anti-(rat mammary-gland acetyl-CoA carboxylase).

Immunotitrations of acetyl-CoA carboxylase and fatty acid synthase of control mammary gland and mammary adenocarcinomas

To estimate the quantity of acetyl-CoA carboxylase and fatty acid synthase, it is essential that a stoichiometric relationship exist between antigen and the corresponding antibody. In independent experiments this requirement was satisfied for both the enzymes by using anti-(rat mammary-gland acetyl-CoA carboxylase) and anti-(rat mammary-gland fatty acid synthase) respectively. Both the carboxylase and the synthase from the control and the neoplastic tissues were subjected to identical purification procedures before initiation of immunochemical titrations. Fig. 3 represents a typical example of immunochemical titrations of both the carboxylase and the synthase derived from normal tissue and both mammary adenocarcinomas: in all

Table 4. Immunotitration of acetyl-CoA carboxylase partially purified from control and neoplastic tissues with anti-(rat mammary-gland acetyl-CoA carboxylase)

Acetyl-CoA carboxylase was obtained from different tissues and then partially purified up to the 50%-satn.-(NH₄)₂SO₄ precipitation step. Immunotitrations and assays were performed as detailed in the Materials and methods section. All titrations were done in duplicate. Values obtained from different batches of tissue agreed within 10%. In experiments where lactating-mammary-gland and tumour tissue extracts were combined, equal parts of each were added to give the units of activity specified.

Enzyme source	Activity titrated (munits)	Antiserum required (µg/munit)
Mammary gland (lactating)	1.24, 1.15, 0.99	10.3, 12.6, 11.2
Mammary gland (pregnant)	0.963, 0.805	9.25, 12.7
Tumour R3230AC	1.1, 0.98, 0.93	8.6, 8.9, 9.3
Tumour 13762	1.2, 1.2	5.4, 6.4
Mammary gland +tumour R3230AC	1.0, 0.89	9.4, 9.6
Mammary gland +tumour 13762	1.09, 0.917	7.7, 9.9

Table 5. Immunotitration of fatty acid synthase partially purified from control and neoplastic tissues with anti-(rat mammary-gland fatty acid synthase)

Fatty acid synthase was obtained from different tissue extracts, and immunotitrations and assays for fatty acid synthase activity were performed, as described in the Materials and methods section. Agreement between tissue extracts prepared from different animals (tumour-bearing and non-tumour-bearing) was within 5%. All titrations were done in duplicate.

Enzyme source	Activity titrated (munits)	Antiserum required (µg/munit)
Mammary gland (lactating)	1.03, 1.1	11.9, 12.0
Mammary gland (pregnant)	1.2, 0.978	12.25, 11.1
Tumour R3230AC	1.4, 1.3	11.3, 11.5
Tumour 13762	0.97, 1.0	11.4, 11.6
Mammary gland +tumour R3230AC	1.18, 1.25	11.6, 11.2
Mammary gland +tumour 13762	1.02, 1.10	11.9, 10.6

cases the amount of enzyme removed is proportional to the amount of antibody added. Neither acetyl-CoA carboxylase nor fatty acid synthase was removed by non-immune serum.

Fig. 3. Immunotitration curves of (a) acetyl-CoA carboxylase and (b) fatty acid synthase from lactating rat mammary gland and mammary tumours R3230AC and 13762

Extracts were prepared, and immunotitrations and assays were performed, as described in the Materials and methods section. Only points observed to have between 45 and 100% activity remaining were used to construct a line, which on extrapolation intercepted the axis representing zero enzyme activity. The equivalence points were calculated from the amount of antibody required to inactivate 1 munit of enzyme activity. Optimal enzyme amounts had to be used such that four or five points satisfied this requirement each time a titration was performed. Values between preparations as well as duplicate determinations of the same preparation showed excellent agreement.

A summary of the immunotitration data on acetyl-CoA carboxylase and fatty acid synthase of different tissues is presented in Tables 4 and 5. Despite the fact that the activities of fatty acid synthase in mammary glands of pregnant and lactating animals are much higher than those found in tumours, the amount of anti-(rat mammary-gland fatty acid synthase) needed to neutralize an equivalent amount of activity in all these instances remained constant. Immunotitration experiments were also performed on normal mammary-gland enzyme to which had been added the enzyme derived from mammary adenocarcinomas. Addition of these proteins did not displace the neutralization point (Table 5). These results indicate that the catalytic efficiency per fatty acid synthase molecule is identical irrespective of the origin of tissue used in

these studies. Thus the changes in fatty acid synthase activity are actually determined by changing quantities of enzyme protein.

Whereas the immunotitration (Table 5) and immunodiffusion data (Fig. 2) supported the identical nature of neoplastic and normal tissue synthases, some differences were found to exist between the carboxylases of neoplastic tissues and normal mammary gland. The carboxylase activity obtained from both tumours 13762 and R3230AC was neutralized by lower amounts of anti-(acetyl-CoA carboxylase) than were needed to neutralize an equivalent amount of enzyme activity derived from lactating gland (Table 4). No such difference was detected when the immunotitration experiments were performed with the enzyme derived from mammary tissue of pregnant, lactating or involuting animals

Fig. 4. Immunotitration curves of acetyl-CoA carboxylase from various tissues against anti-(biotinyl-bovine serum albumin complex) antibody

Extracts were prepared as described in the Materials and methods section. Portions were preincubated as follows: (i) in buffer (50 mM-imidazole/HCl, pH 7.5, containing 1.0 mM-EDTA and 20% glycerol) containing 0.5 M-NaCl at 27°C for 30 min to obtain the protomer (●); or (ii) in buffer (as above) containing 25 mM-potassium citrate for 10 min at 27°C to obtain the polymer (○). Samples of the protomer and polymer were immediately taken for immunotitration and assayed for acetyl-CoA carboxylase activity by the methods described in the Materials and methods section. Immunotitrations were performed on two different enzyme preparations with similar results. Each determination was run in duplicate, with agreement within 5%.

Table 6. Immunotitration of acetyl-CoA carboxylase of different tissues with anti-(biotinyl-bovine serum albumin complex) antibody

Acetyl-CoA carboxylase was obtained from tissues as described in the Materials and methods section. Immunotitrations (anti-biotinyl antibody) and assay conditions for acetyl-CoA carboxylase are also described in the Materials and methods section. All titrations were done in duplicate. Agreement between batches was within 8%. Sedimentation coefficients were determined by layering extracts on 5–20% (w/v) linear sucrose gradients (4.6 ml volume) in 50 mM-imidazole/HCl buffer, pH 7.5, containing 1.0 mM-EDTA and either 0.15 M-NaCl or 25 mM-potassium citrate (see Ahmad *et al.*, 1981). After centrifugation at 45 000 rev./min for 1.5 h at 25°C in an SW 50L rotor of a Beckman L2-65B ultracentrifuge, fractions (15 drops each) were collected manually, and samples were assayed for acetyl-CoA carboxylase activity. Sedimentation coefficients relative to thyroglobulin were calculated.

Enzyme source	Activity titrated (munits)	Antiserum required (μg/munit)	Sedimentation coefficient	
			–Citrate	+Citrate
Mammary gland (lactating)	0.51, 0.31, 0.33	2.07, 2.1, 1.95	17	42
Tumour 13762	0.2, 0.35	2.0, 1.9	16	30
Tumour R3230AC	0.33, 0.36, 0.43	3.9, 3.5, 4.0	16	24

(results not shown). Thus results presented in Fig. 1 and Table 4 indicate that acetyl-CoA carboxylase of normal mammary tissue may differ from the enzyme present in mammary adenocarcinomas.

Effect of citrate on the hydrodynamic characteristics and immunotitration of acetyl-CoA carboxylase with anti-biotinyl antibodies

In the presence of citrate, the enzymically inactive protomeric form of mammalian carboxylases is

converted into catalytically competent polymer. This change in the hydrodynamic behaviour of acetyl-CoA carboxylase is accompanied by alterations in its conformation that affect the biotinyl group of the enzyme. In a previous study the ligand-induced changes affecting the biotinyl group of rat mammary-gland acetyl-CoA carboxylase were investigated by using biotin-binding antibodies (Ahmad *et al.*, 1981). These antibodies react with biotin and give an indication of its location.

By this technique it has been found that the biotinyl group in the polymeric form of the carboxylase is inaccessible; when the protomer generated by high salt concentration is employed, however, the anti-biotinyl antibody reacts rapidly with the biotin and inactivates the enzyme.

To determine whether the conformation around the biotinyl group of neoplastic-tissue carboxylase was similar to that observed with the normal-tissue enzyme, immunotitration studies were performed with protomeric (NaCl-induced) and polymeric (citrate-induced) forms of these enzymes, and a typical neutralization profile for the carboxylase obtained from different tissues is given in Fig. 4. Only the protomeric form of the carboxylase reacted with the anti-biotinyl antibody with attendant loss of catalytic activity.

A summary of the immunotitration data and the sedimentation coefficients determined for the protomer and polymer are presented in Table 6. On the addition of 25 mM-citrate, the carboxylase isolated from tumours acquired an intermediate-size polymeric form ($s \sim 24$ –30S), whereas the enzyme from the normal tissue sedimented in the (normally found) larger polymeric form ($s \geq 40$ S).

Almost identical amounts of anti-biotinyl antibody were required to inactivate 1 munit of acetyl-CoA carboxylase activity from mammary gland as from tumour 13762. The carboxylase isolated from tumour R3230AC, however, required about 1.5–2 times the amount of anti-biotinyl antibody to inactivate 1 munit of its activity.

Discussion

The results given in the present paper show that fatty acid synthase of both the tumours examined manifests several distinctive properties identical with those of the enzyme from normal or host mammary gland. In fact, in all the properties investigated (K_m values for substrates, immunotitration and immunodiffusion data etc.) the tumour synthase is practically indistinguishable from the normal mammary-gland enzyme. The results of immunotitration studies also show that the tissue extracts prepared from mammary adenocarcinomas and mammary gland of different stages of differentiation (non-pregnant, pregnant and lactating) contain antigen whose reactivity per unit of synthase activity is constant. Therefore, with respect to fatty acid synthase at least, both the tumours and mammary tissue contain antigenically equivalent enzymes of comparable catalytic efficiency. These results therefore support the notion that in relation to lactating gland the decreased capacity of mammary adenocarcinomas to synthesize fatty acids from acetyl-CoA is due to a diminution in synthase quantity rather than the presence of an enzyme with altered

catalytic properties. Lin *et al.* (1975) arrived at similar conclusions while investigating mouse mammary tumours.

Whereas the identical nature of fatty acid synthase of tumours and normal mammary tissue could be readily established, such was not the case when comparisons between the properties of acetyl-CoA carboxylases of neoplastic and normal tissue origins were made. During immunotitration studies with anti-(acetyl-CoA carboxylase) the tumour enzyme required somewhat lower amounts of antibody for neutralization of its activity (Table 4). Examination of the immunodiffusion data (Fig. 1), heat treatment and behaviour in the presence of citrate (Table 6) suggests that these tumours contain a modified form of acetyl-CoA carboxylase. A number of mechanisms are known that can alter the activity of mammalian acetyl-CoA carboxylase: proteolysis as well as dephosphorylation (Swanson *et al.*, 1967; Iritani *et al.*, 1969; Guy & Hardie, 1981). However, activation by heating at 37°C of acetyl-CoA carboxylase of neoplasms was not prevented when the experiments were performed in the presence of inhibitors of proteolysis or fluoride. The latter is known to inhibit phosphoprotein phosphatases. Therefore the nature of the difference between acetyl-CoA carboxylases of mammary gland and mammary adenocarcinomas is not yet understood.

The induction of these lipogenic enzymes occurring in differentiating mammary gland was not observed in both the transplantable tumours when they were carried by pregnant or lactating animals. Thus these investigations showed for the first time that the physiological factors that caused induction in differentiating mammary gland had little effect on acetyl-CoA carboxylase and fatty acid synthase of mammary adenocarcinomas. Identification of the biological factors inducing these enzymes in normal gland is of obvious interest. It might then be feasible to determine whether the effects of these modulators are modified or lost during tumorigenesis.

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