Regulation of acetyl-CoA carboxylase in rat mammary gland

Effects of starvation and of insulin and prolactin deficiency on the fraction of the enzyme in the active form
in vivo

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(Received 9 November 1981/Accepted 15 January 1982)

The 'initial' (I), endogenous phosphatase-activated (A) and citrate-activated (C) activities of acetyl-CoA carboxylase were measured in mammary-gland extracts of pregnant and lactating rats. There was a 10-fold increase in the A and C enzyme activities in the transition from early to peak lactation [cf. data of Mackall & Lane (1977) *Biochem. J.* **162**, 635–642], but there was no significant increase in the ratio of the initial activity to the A and C activities of the enzyme. Starvation (24 h) or short-term (3 h) streptozotocin-induced diabetes both resulted in a 40% decrease in I/A and I/C activity ratios. In starvation this was accompanied by a decrease in the absolute values of the A and C activities such that the initial activity in mammary glands of starved animals was 45% that in glands from fed animals. Insulin treatment of starved or diabetic animals 60 min before killing increased the I activity without affecting the A or C enzyme activities. Removal of the pups for 24 h from animals in peak lactation (weaning) resulted in a marked but similar decrease in all three activities such that, although the initial activity was only 10% of that in suckled animals, the I/A and I/C activity ratios remained high and unaltered. Inhibition of prolactin secretion by injection of 2-bromo- α -ergocryptine gave qualitatively similar results to those during weaning. Simultaneous administration of ovine prolactin completely prevented the effects of bromoergocryptine. It is suggested that the initial activity of acetyl-CoA carboxylase in rat mammary gland is regulated by at least two parallel mechanisms: (i) an acute regulation of the proportion of the enzyme in the active state and (ii) a longer-term modulation of enzyme concentration in the gland. Insulin appeared to mediate its acute effects through mechanism (i), whereas prolactin had longer-term effects on enzyme concentration in the gland. A comparison of initial enzyme activities (I) obtained in the present study with rates of lipogenesis measured *in vivo* [Agius & Williamson (1980) *Biochem. J.* **192**, 361–364; Munday & Williamson (1981) *Biochem. J.* **196**, 831–837] gave good agreement between the two sets of data for all conditions studied except for 24 h-starved and streptozotocin-diabetic animals. It is suggested that acetyl-CoA carboxylase activity is rate-limiting for lipogenesis in the mammary gland in normal, fed, suckled or weaned animals but that in starved and short-term diabetic animals changes in the activity of the enzyme by covalent modification alone may not be sufficient to maintain the enzyme in its rate-limiting role.

Acetyl-CoA carboxylase (EC 6.4.1.2) is the first enzyme committed to fatty acid synthesis in the lipogenic pathway. It has long been recognized as a potentially rate-limiting enzyme of this pathway because of its low activity in various tissues compared with other enzymes involved in lipogenesis (see, e.g., Korchak & Masoro, 1962). Until relatively recently its activity was considered to be regulated primarily by the direct effects of small

molecules on the enzyme. The effects of tricarboxylic acids, notably citrate, in increasing its activity concomitantly with the aggregation of the protomeric enzyme into polymeric filaments, and the inhibitory effects of long-chain acyl-CoA esters were thought to account for most of the regulatory mechanisms that would be required for the acute regulation of the activity of the enzyme in response to changes in the lipogenic rate (see, e.g., Halestrap

& Denton, 1974; Lane *et al.*, 1979). Although these mechanisms are still thought to be important in the regulation of acetyl-CoA carboxylase activity (see, e.g., McGarry & Foster, 1980) and the protomeric to polymeric transition has been shown to occur in intact isolated cells *in vitro* (Meredith & Lane, 1978; Ashcraft *et al.*, 1980), it is now widely accepted that a major form of acute regulation of the activity of the enzyme occurs through a phosphorylation-dephosphorylation mechanism (see Kim, 1979). The purified enzyme from a variety of tissues, including rat and rabbit mammary gland, has been shown to undergo reversible phosphorylation *in vitro* with consequent changes in activity (activation by dephosphorylation) (Lee & Kim, 1977; Hardie & Cohen, 1979; Hardie & Guy, 1980).

Previous work from our laboratory demonstrated that in extracts of lactating rat mammary gland the enzyme could be phosphorylated and dephosphorylated by endogenous kinase and phosphatase activities (McNeillie & Zammit, 1981; McNeillie *et al.*, 1981). The kinase activity was not stimulated by cyclic AMP nor was it inhibited by cyclic AMP-dependent protein kinase inhibitor (McNeillie *et al.*, 1981). These results suggested that phosphorylation of the enzyme in the mammary gland could occur in response to hormonal stimuli other than those mediated via the cyclic nucleotide, e.g., by insulin. In the same studies we found that the endogenous phosphatase was itself activated in the presence of Mg^{2+} and physiological (micromolar) concentrations of Ca^{2+} (McNeillie & Zammit, 1981).

The mammary gland is a major lipogenic organ in the lactating animal. The lipogenic rate in the gland as measured *in vivo* by monitoring incorporation of ^3H into lipids after injection of ^3H_2O (Agius, 1980; Agius & Williamson, 1980) increases markedly after parturition and reaches a peak at 10–14 days of lactation. The activity of acetyl-CoA carboxylase increases in parallel with the increased lipogenic activity of the gland during the transition from early to peak lactation (Mackall & Lane, 1977; Martyn & Hansen, 1981) and this increase is accompanied by a parallel increase in immunotitratable enzyme protein (Mackall & Lane, 1977). However, in these studies, the enzyme was maximally activated by pre-incubation with high concentrations of citrate at 30°C before assay. Therefore, in view of the current knowledge about regulation of the enzyme by covalent modification, these maximal activities obtained *in vitro* may have overestimated the enzyme activity *in vivo*. In addition, the previous data gave no information about the role of acute as opposed to long-term changes in the activity of the enzyme in response to changes in nutritional and/or hormonal status of the animal.

We have used the previously reported techniques

(McNeillie & Zammit, 1981; McNeillie *et al.*, 1981) to obtain initial and endogenous phosphatase-activated enzyme activity to determine the effect of reproductive state, starvation and prolactin or insulin deficiency on the fraction of acetyl-CoA carboxylase in the active state *in vivo* as well as on the maximal activity of the enzyme after activation by citrate.

Materials and methods

Animals

Female Wistar rats weighing 160–180 g were purchased from A. Tuck and Son, Battlesbridge, Essex, U.K., and mated 3 weeks later. At parturition the number of pups was adjusted to eight and the mothers were kept with their pups at all times except in the case of prematurely weaned animals when the pups were removed from the mother 24 h before killing. The animals were fed Oxoid breeding diet (Oxoid, Basingstoke, Hants., U.K.) *ad libitum*. Starved animals had their food removed 24 h before killing. All animals had continuous access to water. Animals were kept on a 12 h-light/12 h-dark cycle, the light cycle extending from 08:00 h to 20:00 h and they were killed between 09:00 h and 10:00 h, except in experiments which involved short-term insulin deprivation, when they were killed 3 h later, as were saline-injected control animals.

Experimental treatment of animals

Insulin or prolactin deficiency in animals was induced by injection of streptozotocin or 2-bromo- α -ergocryptine respectively, as described previously (Baxter & Coore, 1978). Prolactin supplementation in bromocryptine-treated animals was achieved by injection of ovine prolactin (15 mg/kg body wt.) simultaneously with 2-bromo- α -ergocryptine (10 mg/kg body wt.). Effects of insulin treatment of starved animals were studied after injection of insulin (10 units) and 2 ml of 3 M-glucose 60 min before killing as described previously (Field & Coore, 1976; Baxter & Coore, 1978). Only animals that had blood glucose > 15 mM were considered to be diabetic. No alterations in the feeding of diabetic rats were observed compared with that of saline-injected controls killed at 12:00 h. In every instance, feeding by the mothers during the first 4 h of the light phase was minimal.

Animals were anaesthetized by using a halothane/O₂ mixture (Field & Coore, 1976). The abdominal mammary gland on one side was freed of skin and abdominal-wall musculature, care being taken to maintain the circulation intact and the gland was then clamped between tongs cooled in liquid N₂. The duration of anaesthesia was standardized and

approx. 15 min elapsed between induction of anaesthesia and freezing the tissue. By using this technique 1–2 g samples could be obtained from each animal.

Extraction of tissue and assay of acetyl-CoA carboxylase activity

The tissue was pulverized and extracted as described previously (McNeillie & Zammit, 1981) followed by gel filtration on a Sephadex G-25 column (McNeillie *et al.*, 1981). Samples of the whole extract were stored frozen at -20°C for assay of DNA content. The extent of dilution of the extract by Sephadex G-25 chromatography was assessed by measurement of lactate dehydrogenase (Zammit & Newsholme, 1976) and acetyl-CoA carboxylase activities in the unchromatographed extract and in the eluate. The dilution for both enzymes was found routinely to be approx. 10-fold.

The activity of acetyl-CoA carboxylase was measured over the first 30 s at 30°C as described previously (McNeillie & Zammit, 1981; McNeillie *et al.*, 1981) in column eluate either immediately or after one of the following pre-incubation procedures: (i) 20 and 40 min in the presence of 20 mM-potassium citrate; (ii) 40 and 60 min in the presence of 5 mM-magnesium acetate and a calculated free Ca^{2+} concentration of $20\text{ }\mu\text{M}$ (see McNeillie *et al.*, 1981); (iii) preincubation as in (ii) followed by further incubation for 20 and 40 min in the presence of 20 mM-magnesium citrate. The values obtained at the two time points chosen for each condition were similar and were used to ascertain that maximal activation had been achieved for the conditions used.

DNA assay

DNA was assayed in whole homogenates (diluted 100-fold) by the method of Labarca & Paigen (1980) by using a Baird Atomic Fluoripoint fluorimeter. DNA standards (calf thymus) were made up fresh daily.

Materials

Sources of chemicals were as given by Zammit (1981) except for the following: ovine prolactin, soluble insulin and DNA were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K.; compound Hoechst 33258 (bis-benzinoid) was purchased from Fluka A.G., Buchs, Switzerland; streptozotocin was a gift from Dr. W. E. Dulin of Upjohn Pharmaceutical Co., Kalamazoo, MI, U.S.A., and 2-bromo- α -ergocryptine was a gift from Dr. E. Flukiger and Dr. H. Friedli, Sandoz, Basle, Switzerland.

Results

Initial, endogenous phosphatase- and citrate-activated activities of acetyl-CoA carboxylase

In the present study tissue extracts were prepared under conditions that were found previously to prevent changes in activity of mammary gland acetyl-CoA carboxylase due to endogenous protein kinase or phosphatase activities (McNeillie & Zammit, 1981; McNeillie *et al.*, 1981). The 'initial' (I) activity of the enzyme was obtained by performing the assays immediately after preparation of the Sephadex G-25 column eluate. The phosphatase-activated enzyme activity (A) was measured after pre-incubation of extracts in the presence of Ca^{2+} and Mg^{2+} to activate the endogenous phosphatase, and the citrate-activated (C) values were obtained after pre-incubation with citrate either before or after activation in the presence of Ca^{2+} and Mg^{2+} . Routinely, we found that the activities attained after citrate activation were the same irrespective of whether the enzyme had been activated previously by endogenous phosphatase (cf. McNeillie *et al.*, 1981; results not shown) and therefore only one citrate-activated enzyme activity (C) will be referred to below.

The ratios of the initial activity to the phosphatase-activated enzyme activity (the I/A ratio) and to the citrate-activated enzyme activity (I/C ratio) varied over a 2-fold range depending on the reproductive, nutritional and hormonal state of the animals (Tables 1–3; see below). However, the A/C activity ratio was always more or less constant (at about 55%), suggesting that endogenous phosphatase-mediated activation of the enzyme resulted in a reproducible alteration in enzyme activity in relation to the fully activated (polymerized) state attained after incubation with citrate, irrespective of the total amount of enzyme present in the gland and of the initial activity.

Changes in enzyme activity in the transition from late-pregnancy to mid-lactation

The citrate-activated acetyl-CoA carboxylase activity increased more than 10-fold in mammary gland in the transition from late-pregnancy to mid-lactation, both when the results were expressed per unit wet weight or per unit DNA content. The activity had intermediate values during the early stages of lactation (2 and 4 days) such that on day 4 of lactation the activity was about 70% that observed at mid-lactation (10–14 days). The absolute activities (expressed per mg of DNA) as well as the pattern of changes observed with increased duration of lactation were very similar to those observed by Mackall & Lane (1977). However, they were lower than those reported by Martyn &

Table 1. *Activities of acetyl-CoA carboxylase ($\mu\text{mol/min per g wet wt. or } \mu\text{mol/min per mg of DNA}$) at 30°C in the extracts of freeze-clamped mammary glands from fed or starved animals in the transition from late pregnancy to peak lactation*

Activities were measured immediately after preparation of homogenates (I), after activation with citrate (C) or after activation by endogenous phosphatase (A) (see the Materials and methods section for details). Values given are means $\pm$ s.d. Statistical significance at $P < 0.01$ (Student's t test) of the difference in activities and their ratios between fed and starved animals are indicated by * and that between values for fed pregnant or early-lactating animals and fed 10–14 day-lactating rats by †. The number of separate rats used is shown in parentheses.

	Animal	DNA (mg/g wet wt.)	Activity of acetyl-CoA carboxylase at 30°C										Activity ratios (%)	
			(μmol/min per g wet wt.)			(μmol/min per mg of DNA)								
			I	A	C	I	A	C	I/A	I/C				
19-20 day pregnant	Fed (3)	3.12 ± 0.46	0.07† ± 0.03	0.16† ± 0.08	0.25† ± 0.13	0.02† ± 0.01	0.05† ± 0.03	0.08† ± 0.04	41.7† ± 3.8	26.0† ± 1.7				
	Starved (3)	3.15 ± 0.30	0.09 ± 0.02	0.14 ± 0.04	0.22 ± 0.11	0.03 ± 0.01	0.05 ± 0.02	0.07 ± 0.04	72.3 ± 11.4	41.0 ± 5.6				
2 day lactating	Fed (4)	3.50 ± 0.36	0.69† ± 0.15	1.08† ± 0.41	1.89† ± 0.47	0.18† ± 0.05	0.27† ± 0.12	0.48† ± 0.14	63.0 ± 10.0	37.2 ± 3.6				
	Starved (4)	3.61 ± 0.24	0.36* ± 0.03	0.71 ± 0.11	1.15 ± 0.17	0.09 ± 0.01	0.18* ± 0.02	0.29 ± 0.03	52.3 ± 13.4	32.3 ± 7.9				
4 day lactating	Fed (4)	3.40 ± 0.20	0.95 ± 0.38	1.50 ± 0.45	2.37† ± 0.54	0.28† ± 0.09	0.42 ± 0.12	0.68† ± 0.12	65.5 ± 4.7	40.3 ± 5.2				
	Starved (4)	4.35* ± 0.31	0.32* ± 0.06	0.98 ± 0.45	1.36 ± 0.25	0.06* ± 0.02	0.18* ± 0.09	0.24* ± 0.06	38.6* ± 0.75	23.0* ± 1.2				
10-14 day lactating	Fed (6)	3.25 ± 0.35	1.68 ± 0.38	2.26 ± 0.56	4.20 ± 0.84	0.54 ± 0.13	0.73 ± 0.23	1.33 ± 0.29	75.0 ± 9.0	39.8 ± 3.3				
	Starved (8)	4.25* ± 0.30	0.76* ± 0.20	1.56 ± 0.39	2.96 ± 0.65	0.13* ± 0.04	0.27* ± 0.09	0.50* ± 0.27	47.3* ± 7.1	24.5* ± 2.4				

Table 2. *Effects of starvation, insulin and streptozotocin treatments on I, A and C activities of acetyl-CoA carboxylase in extracts of freeze-clamped mammary glands from 10-14 day-lactating rats*

For details of doses and modes of administration of insulin, streptozotocin and glucose see the Materials and methods section. Values are means $\pm$ s.d. Statistical significance of $P < 0.01$ of the difference between values for starved and fed animals is indicated by *, that between insulin treated animals and the controls by ‡, and that between streptozotocin-treated animals and fed rats by †. The numbers of separate rats used are shown in parentheses.

State of animal	DNA (mg/g wet wt.)	Activity of acetyl-CoA carboxylase						Activity ratios (%)	
		(μ mol/min per g wet wt.)			(μ mol/min per mg of DNA)				
		I	A	C	I	A	C	I/A	I/C
Fed									
24h starved	(6)	1.68 $\pm$ 0.38	2.26 $\pm$ 0.56	4.20 $\pm$ 0.84	0.54 $\pm$ 0.13	0.73 $\pm$ 0.23	1.33 $\pm$ 0.29	75.0 $\pm$ 9.0	39.8 $\pm$ 3.3
24h starved	(8)	0.76* $\pm$ 0.20	1.56 $\pm$ 0.39	2.96 $\pm$ 0.65	0.13* $\pm$ 0.04	0.27* $\pm$ 0.09	0.50* $\pm$ 0.27	47.3* $\pm$ 7.1	24.5* $\pm$ 2.4
24h starved	(5)	1.33§ $\pm$ 0.20	1.94 $\pm$ 0.27	2.91 $\pm$ 0.30	0.26§ $\pm$ 0.05	0.36 $\pm$ 0.04	0.56 $\pm$ 0.10	70.6§ $\pm$ 9.0	45.4§ $\pm$ 5.9
+ insulin									
+ glucose									
Streptozotocin- (60min)	(5)	1.03† $\pm$ 0.13	2.10 $\pm$ 0.23	3.49 $\pm$ 0.49	0.28† $\pm$ 0.05	0.58 $\pm$ 0.11	0.96 $\pm$ 0.21	47.8† $\pm$ 2.6	29.6† $\pm$ 2.2
Saline-injected (3h)	(5)	1.27 $\pm$ 0.31	2.20 $\pm$ 0.39	3.57 $\pm$ 0.43	0.40 $\pm$ 0.11	0.66 $\pm$ 0.12	1.07 $\pm$ 0.15	61.0 $\pm$ 12.4	37.0 $\pm$ 7.0

Table 3. *Effects of premature removal of pups (weaning) for 24h and treatment with 2-bromo-α-ergocryptine and prolactin on I, A and C activities of acetyl-CoA carboxylase in extracts of freeze-clamped mammary glands from 10-14 day-lactating rats*

For details of doses and modes of administration of bromoergocryptine and prolactin see the Materials and methods section. Values are means $\pm$ s.d. Statistical significance (at $P < 0.01$) of the difference between values for lactating and 24h-weaned or bromoergocryptine-treated animals is denoted by * and that between values for animals treated with prolactin plus bromoergocryptine and bromoergocryptine alone is denoted by †. The numbers of separate rats used are shown in parentheses.

State of animal	DNA (mg/g wet wt.)	Activity of acetyl-CoA carboxylase at 30°C						Activity ratios (%)		
		(μmol/min per g wet wt.)			(μmol/min per mg of DNA)					
		I	A	C	I	A	C	I/A	I/C	
Lactating (10-14 days)	(6)	3.25 ± 0.35	1.68 ± 0.38	2.26 ± 0.56	4.20 ± 0.84	0.54 ± 0.13	0.73 ± 0.23	1.33 ± 0.29	75.0 ± 9.0	39.8 ± 3.3
Weaned (24h)	(6)	2.39* ± 0.41	0.15* ± 0.07	0.26* ± 0.07	0.49* ± 0.11	0.09* ± 0.04	0.17* ± 0.05	0.29* ± 0.08	55.2 ± 19.0	30.3 ± 12.1
Bromoergocryptine-treated (24h)	(7)	3.89* ± 0.21	0.75* ± 0.32	1.18* ± 0.48	1.83* ± 0.54	0.20* ± 0.09	0.30* ± 0.12	0.47* ± 0.16	66.6 ± 17.4	42.0 ± 7.3
Bromoergocryptine + prolactin (24h)	(3)	3.31 ± 0.31	1.71† ± 0.43	2.59† ± 0.46	3.81† ± 0.68	0.52† ± 0.08	0.79† ± 0.07	1.16† ± 0.11	65.3 ± 5.0	44.7 ± 4.6

Hansen (1981), who also found that the activity had already reached a peak after 5 days of lactation.

Both the initial and phosphatase-activated enzyme activities also increased with lactation (Table 1) and, although there was a tendency for the I/A and I/C ratios to increase in mid-lactation, the ratios were not significantly higher than those observed for glands from early-lactating animals (Table 1).

Effects of starvation and insulin-deficiency

Starvation of lactating animals resulted in a marked decrease in initial activity of carboxylase (Table 2). This occurred as a result of the combined effects of a fall in the phosphatase- and citrate-activated enzyme activities and, more importantly, a sharp decrease in the I/A and I/C activity ratios. Thus the values for these ratios decreased from 76% to 47% and from 40% to 25% respectively in glands from rats in mid-lactation after starvation for 24 h (Table 2).

Injection of insulin into 24 h-starved mid-lactating animals 60 min before killing resulted in restoration of the I/A and I/C ratios to levels observed in fed animals (i.e. an increase of 70%) without significant changes in the A and C activities.

When mid-lactating rats were rendered insulin deficient by injection of streptozotocin (100 mg/kg body wt.) 3 h before killing (short-term insulin deficiency) there was a significant decrease in initial activity and the I/A and I/C ratios became similar to those observed after 24 h starvation (Table 2), but there was no change in the A or C values. In glands from saline-injected control animals also killed at 12:30 h there was a much smaller decrease in the initial activity, which was not significantly different from that of untreated animals killed at 09:30 h.

Effects of premature weaning and prolactin deficiency

When mid-lactating rats had their pups removed prematurely (weaned) 24 h before killing, the phosphatase- and citrate-activated carboxylase activities were decreased by about 90% (Table 3). However, the I/A and I/C activity ratios remained high and were not significantly different from those for the enzyme from glands of suckled animals. Consequently the entire fall in initial activity of the enzyme could be ascribed to a decrease in enzyme content in the gland after weaning. Inhibition of prolactin secretion by treatment of mid-lactating rats with 2-bromo- α -ergocryptine gave qualitatively similar results to those obtained after weaning but the decrease in activity was smaller. When bromo-ergocryptine-treated animals were given simultaneous injections of ovine prolactin (15 mg/kg body wt.) the effect of bromocryptine was entirely abolished and there were no changes in any of the three activities from those obtained for glands from normal suckled animals. [Injection of a smaller dose (5 mg/kg body wt.) of prolactin achieved only a partial prevention of the bromoergocryptine-induced decrease in activity (results not shown).]

Discussion

Previous studies have reported activities for acetyl-CoA carboxylase in lactating mammary gland that were measured after polymerization and maximal activation of the enzyme *in vitro* by incubation with citrate (Mackall & Lane, 1977; Martyn & Hansen, 1981) and therefore they could not be used as quantitative indices of the flux through the reaction catalysed by carboxylase *in vivo*. We have previously shown that the enzyme in extracts of mammary gland could be activated *in vitro*, in the absence of citrate, by an endogenous

Table 4. A comparison of initial activity of acetyl-CoA carboxylase ($\mu\text{mol/min per g wet wt. at } 30^\circ\text{C}$) with the flux (C_2 units/min per g wet wt. at body temperature, about 35.5°C ; see the text) through the lipogenic pathway *in vivo* calculated from data of Robinson et al. (1978), Agius (1980), Agius & Williamson (1980) and Munday & Williamson (1981) of rates of incorporation of $^3\text{H}_2\text{O}$ into lipid in mammary gland *in vivo*

Stage of lactation	Treatment	Acetyl-CoA carboxylase activity	Calculated C_2 -flux through lipogenic pathway
Early-lactating	—	0.95 ± 0.38	1.28 ± 0.57
Mid-lactating	—	1.68 ± 0.38	1.92 ± 0.58
Mid-lactating	Weaned (24 h)	0.15 ± 0.07	0.06 ± 0.05
Mid-lactating	+Bromoergocryptine (24 h)	0.75 ± 0.32	1.05 ± 0.68
Mid-lactating	+Bromoergocryptine + prolactin*	1.71 ± 0.43	1.39 ± 0.20
Mid-lactating	Starved (24 h)	0.76 ± 0.20	0.04 ± 0.01
Mid-lactating	+Streptozotocin (3 h)	1.03 ± 0.13	0.28 ± 0.18

* Dose of prolactin used in the present study was 2.5-fold higher.

phosphatase that was sensitive to Ca^{2+} and Mg^{2+} but that was completely inactive in the presence of chelators of bivalent metal cations (McNeillie & Zammit, 1981; McNeillie *et al.*, 1981). In the present study we have made use of these properties of the endogenous phosphatase to obtain initial and phosphatase-activated enzyme activities. We could then compare these activities with those obtained after maximal citrate activation. If the enzyme activities obtained *in vitro* can be used as quantitative indices of flux through acetyl-CoA carboxylase *in vivo*, it is the I activity that would be expected to correspond most closely to the catalytic activity of the enzyme *in vivo* at the time the mammary gland is freeze-clamped. A comparison of the initial activities (expressed per g wet wt.) obtained in the present study with the calculated fluxes through the lipogenic pathway obtained from the data of Williamson and his co-workers (who used $^3\text{H}_2\text{O}$ incorporation into lipid to measure the rate of lipogenesis in mammary gland *in vivo*) is given in Table 4. Although a direct comparison of the data is not possible because our results relate to activities at 30°C , whereas the temperature of the mammary gland *in vivo* is about 35.5°C (as measured by implanting a thermocouple between the gland and the abdominal musculature; E. M. McNeillie & V. A. Zammit, unpublished work), it is evident that there is good agreement between the two sets of data relating to normal fed animals and to weaned or prolactin-deficient rats. Although extrapolation from enzyme activity measurements *in vitro* to enzyme activities *in vivo* have to be made with caution, this agreement suggests that the technique used to obtain quantitative measurements of initial activity is valid and confirms the suggestion (Munday & Williamson, 1981) that under certain physiological and hormonal conditions acetyl-CoA carboxylase activity *in vitro* is rate-limiting for lipogenesis in the mammary gland.

However, in mammary glands of starved and short-term diabetic animals the initial activity of acetyl-CoA carboxylase, although significantly lower than that in normal fed animals, was several-fold higher than the calculated flux through the carboxylase reaction *in vivo* (Table 4). This observation suggested that regulation of initial activity of carboxylase solely through covalent modification of the enzyme may not be sufficient to maintain the rate-limiting role of the enzyme in the glands of starved or short-term diabetic animals. We therefore compared these results with previously reported responses of the initial activity of pyruvate dehydrogenase (another enzyme that is potentially rate-limiting for lipogenesis in the gland and that is also regulated, in part, by covalent modification) to starvation and short-term diabetes. The initial activity of pyruvate dehydrogenase also showed a

relatively small (65%) decrease in initial activity in mammary gland after starvation for 24 h (Baxter & Coore, 1978). However, initial pyruvate dehydrogenase activity, contrary to initial carboxylase activity, did decrease by $>90\%$ in glands of diabetic animals (Field & Coore, 1976). Interestingly, the converse would appear to hold in the prolactin-deficient animal. Thus, whereas initial (and total) pyruvate dehydrogenase activity in the gland only fell by 45% after 24 h-weaning (Coore & Field, 1974), the initial activity of acetyl-CoA carboxylase decreased to the same extent as the decrease in lipogenesis (see Table 4). It is suggested that the reactions catalysed by acetyl-CoA carboxylase and pyruvate dehydrogenase may alternate in being rate-limiting steps in lipogenesis from pyruvate under different hormonal conditions. However, it is to be stressed that initial activities obtained *in vitro* can only indicate changes in activity of the enzymes brought about by covalent modification and cannot predict changes that occur *in vivo* through the effects of alterations in the concentration of effector molecules (e.g., long-chain acyl-CoA, citrate) and changes in the affinity of the enzymes for their substrates and/or effectors.

Our data show that at least two parallel and complementary regulatory mechanisms control acetyl-CoA carboxylase activity *in vivo*. Initial enzyme activity was altered either by changes in the fraction of the enzyme in the active form (changes in the I/A activity ratio, e.g., after streptozotocin treatment) or by changes in the amount of enzyme present in the gland (e.g., after weaning) or by a combination of both (e.g., after starvation). The effect of insulin on the enzyme *in vivo* is likely to be due to the alteration of the activation state of the enzyme since short-term insulin deficiency resulted in a fall in the I/A activity ratio and insulin treatment of either starved or diabetic animals restored this ratio to that of normal animals without affecting maximal carboxylase activity. A similar role for insulin in the regulation of pyruvate dehydrogenase (by alteration of the fraction of the enzyme in the active form) was suggested by Baxter & Coore (1978) and a role for insulin in the acute regulation of mammary gland lipogenesis was suggested by Robinson *et al.* (1978). However, the effect of insulin on the initial activity of acetyl-CoA carboxylase cannot explain the entire decrease in initial activity observed after starvation because the maximal activity of the enzyme was also affected under these conditions. The observations that the I/A ratio was lower (though not significantly so) in saline-treated animals killed 3 h further into the light period than in normal animals suggests that the fraction of the enzyme in the active form may undergo diurnal changes in response to changes in circulating insulin concentrations that would be

expected to occur in response to feeding, which in the rat occurs primarily during the dark period. Carrick & Kuhn (1978) have shown that insulin levels may reach a minimum towards the mid-light time point.

The effects of prolactin deficiency appeared to be exerted through changes in the maximal activity of acetyl-CoA carboxylase in the mammary gland, rather than on the fraction of the enzyme in the active form. Although the data on the I/A and I/C ratios in weaned and bromoergocryptine-treated rats have to be interpreted with caution because of the very low activities of carboxylase found in the glands of these animals, the results suggest that prolactin deficiency did not result in a decrease in the I/A and I/C ratios. However, weaning and bromoergocryptine treatment are known to result in an increase in circulating insulin concentration (Agius *et al.*, 1979) and it is possible that the lack of change in the I/A and I/C ratios in glands from prolactin-deficient animals may have resulted from a compensatory increase in initial activity due to an increase in plasma insulin concentration in these animals.

General conclusions

Acute changes in initial activity of acetyl-CoA carboxylase in response to different hormonal treatment of tissues *in vitro* and *in vivo* have been reported previously (see, e.g., McCormack & Denton, 1977; Brownsey *et al.*, 1979; Geelen *et al.*, 1978; Witters *et al.*, 1979; Assimacopoulos-Jeannet *et al.*, 1981). In all instances where the effects of insulin or insulin-deprivation were studied, the hormone was found to increase the initial activity of acetyl-CoA carboxylase. In the present study this has been demonstrated for the enzyme from mammary gland and, in addition, it has been shown that this increase in activity occurred without an increase in the activity of the dephosphorylated enzyme. As in other tissues there appears to be interaction between the effects of insulin and the effects of at least one other hormone, prolactin, in the regulation of acetyl-CoA carboxylase activity in mammary gland. From the results obtained it may be suggested that regulation of acetyl-CoA carboxylase activity in the mammary gland is mediated by a combination of short-term changes in the activation state of the enzyme by insulin and by longer-term changes in the enzyme content of the gland in response to changes in prolactin levels in the circulation.

We thank Dr. R. A. Clegg for many helpful suggestions and Professor M. Peaker and Dr. W. W. Christie for interest and encouragement. E. M. McN. was a recipient of an A.R.C. post-graduate studentship.

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