An ‘*in situ*’ perfusion system suitable for investigating mammary-tissue metabolism in the lactating rat

Hormonal regulation of acetyl-CoA carboxylase

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A technique is described for the non-recirculating perfusion of inguinal/abdominal mammary tissue *in situ* in anaesthetized lactating rats. Tissue viability was maintained, without resort to infusion of vasoactive chemicals which may also be effectors of cellular metabolism, for at least 90 min. Total tissue adenine nucleotides (per mg of DNA) were somewhat decreased in perfused relative to non-perfused mammary tissue. DNA content (per g wet wt. of tissue) was diminished after 90 min of perfusion to approx. 65 % of its value in control tissue. Adenylate energy-charge ratios were lower in perfused tissue in the absence of hormones than in control tissue. They were increased to control values by the presence of either insulin or isoprenaline in the perfusate. No changes occurred in flow rate of the perfusate that might account for these increases. In mammary tissue perfused without addition of hormones, acetyl-CoA carboxylase activities were similar to those measured in control tissue samples, although activity-ratio measurements implied some increase in the phosphorylation of this enzyme. Insulin or isoprenaline increased the activity of acetyl-CoA carboxylase, especially when this was measured at low concentrations of citrate. Confirming conclusions from previous experiments with mammary acini and explant preparations, insulin activated acetyl-CoA carboxylase in mammary tissue, but inhibition of its activity was not mediated by cyclic AMP.

INTRODUCTION

Aspects of the metabolism of mammary tissue in the lactating rat are subject to short-term regulation, especially in response to the dietary status of the animal. Thus, in the intact rat, both lipogenesis and lactogenesis are rapidly and markedly inhibited by starvation (Robinson *et al.*, 1978; Wilde & Kuhn, 1979; Agius *et al.*, 1980; Grigor & Warren, 1980; Bussmann *et al.*, 1984). These pathways are stimulated on re-feeding over a time scale which suggests that acute modes of regulation are involved (Munday & Williamson, 1981; Williamson *et al.*, 1983; Bussmann *et al.*, 1984; Mercer & Williamson, 1986), and several studies have focused on activity changes of acetyl-CoA carboxylase as the primary means of effecting the changed rates of lipid biosynthesis in mammary tissue associated with nutritional manipulation of the lactating rat.

Other characteristics of the control of mammary-tissue lipogenesis implicate insulin as an important determinant of the rate of this pathway *in vivo* (Robinson *et al.*, 1978; Burnol *et al.*, 1983; Page & Kuhn, 1986), perhaps in conjunction with one or more counter-regulatory hormones. However, preparations of mammary tissue *in vitro* have not been more than partially effective in illustrating these hormonal effects: thus, although the rate of lipogenesis of mammary tissue in the intact rat varies between 2.7 and 87.1 μmol of $^3\text{H}_2\text{O}$ incorporated/h per g fresh wt. of tissue in the starved and fed animal respectively (Williamson *et al.*, 1985), analogous rates in suspensions of acini prepared from mammary tissue of fed rats were 32.58 μmol of $^3\text{H}_2\text{O}$

incorporated/h per 100 mg defatted dry wt. in the absence of insulin, and 48.72 in its presence at maximally effective concentrations (Robson *et al.*, 1984). Many of the acini preparations examined (Robinson & Williamson, 1977; Agius & Williamson, 1980; Wilde & Kuhn, 1981; Williamson *et al.*, 1983) have been entirely unresponsive to insulin, as well as to its common counter-regulators, glucagon and catecholamines. In the case of glucagon, it is now known (Robson *et al.*, 1984) that mammary epithelial cells are more or less totally devoid of receptors for this hormone, and thus would not be expected to be responsive to it. For catecholamines, however, the apparent lack of mammary-cell responsiveness cannot be attributed to receptor deficiency: acinar cells of rat (Clegg & Mullaney, 1985) and ruminant mammary tissue (Clegg, 1987) have an abundance of β_2 -adrenergic cell-surface receptors roughly equal to that found on adipocytes from the same species. Furthermore, it is clear that the immediate post-receptor event (stimulation of adenylate cyclase) associated with occupancy of these receptors by adrenaline, or by the pharmacological agonist isoprenaline, proceeds normally in mammary-cell membranes (Ladha *et al.*, 1985).

Thus the most straightforward reason why mammary tissue responds *in vivo* to adrenaline with a decreased rate of lipogenesis from glucose (Bussmann *et al.*, 1984), whereas mammary cellular and slice preparations respond to the same stimulus *in vitro* either not at all or with an increase in their rate of lipogenesis (Plucinski & Baldwin, 1982; Williamson *et al.*, 1983; Robson *et al.*,

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1984; Clegg *et al.*, 1986; Munday & Williamson, 1987), may be that these particular cellular and slice preparations inadequately reflect the metabolic characteristics of the tissue in the living animal. On the other hand, it may be argued that the observed response to adrenaline in the living animal is a secondary consequence of actions of that hormone on another tissue. A mammary-tissue preparation perfused *in situ* offers some prospect of distinguishing between these two opposing views of the regulatory capacity of mammary tissue. Such a preparation has been previously described for the lactating rat (Mendelson & Scow, 1972), but its general metabolic competence was not characterized. We here describe a system for the perfusion *in situ* of mammary tissue in the lactating rat, developed from that described by Mendelson & Scow (1972), and present data that characterize the general metabolic integrity of the preparation and that define the response of acetyl-CoA carboxylase to the presence of insulin or isoprenaline in the perfusate.

EXPERIMENTAL

Animals

Female rats of the Wistar strain were fed on standard rat chow (CRM Irradiated Diet; Labsure, Cambridge, U.K.) *ad libitum* and allowed free access to water. They were housed at a constant temperature of 17 °C and under conditions of 12 h light/dark cycles, with illumination commencing at 08:00 h. Lactating animals weighing between 295 and 380 g were used on days 10–14 after the birth of their first litter, which was adjusted in number to eight pups shortly after delivery.

Surgical preparation for perfusion

Anaesthesia was induced by intraperitoneal injection of sodium pentobarbitone solution (40 mg/kg body wt.) at between 09:30 h and 10:30 h: mother and young were separated at this time. The anaesthetized rat was immobilized in the supine position, with its head and front limbs reflected to the right side to avoid respiratory difficulties.

A skin incision was made from pubis to sternum and for a further 15 mm to the left side along the posterior border of the thorax. The left inguinal–abdominal mammary tissue mass was then freed from the abdominal musculature, and ligatures were tied around the vein communicating with the pectoral mammary tissue, around the iliac branches of the ilio-lumbar artery and vein, around the external pudendal arteries and veins and around the muscular branch of the femoral artery and vein. The incision was then closed with a running suture.

A second incision was made on the medial surface of the left thigh along the lateral border of the inguinal–abdominal mammary tissue to expose the superficial epigastric artery and vein. Both these vessels were cleared of fat and connective tissue, and loose ligatures placed around them. The artery was treated locally as needed with 0.5% papaverine solution to prevent spasm. The artery was then tied and catheterized and the perfusion begun immediately with the medium described below. During this procedure, blood flow was interrupted for between 1 and 2 min. The superficial epigastric vein was catheterized within 1 min of starting perfusion. The whole procedure took up to 40 min. After cannulation,

the exposed tissue area was covered with a swab of cotton wool moistened with warm 0.9% NaCl. The body temperature of the rat was maintained throughout by the application of radiant heat from a lamp positioned above the animal.

Perfusion fluid

This was composed of 3 parts (by vol.) of medium 199 (supplied by Gibco, Paisley, Scotland, U.K.), containing 8% (w/v) bovine serum albumin and adjusted to pH 7.2 with NaOH and filtered through a 0.22 µm-pore filter (Millipore), mixed with 1 part (sedimented volume) of goat erythrocytes. The erythrocytes were prepared by sedimentation (1200 g for 10 min) from freshly drawn whole blood containing trisodium citrate (8%, w/v) as an anticoagulant. After removal by aspiration of the plasma and 'buffy coat' material, the erythrocytes were washed three times by resuspension in ice-cold 0.9% NaCl and sedimentation as above. The washed erythrocytes were stored at 4 °C as a suspension in 0.9% NaCl and used within 4 days of preparation. They were sedimented as above into a loosely packed pellet on the day of their use. This perfusion fluid was warmed to a temperature of 38 °C and maintained in gaseous equilibrium with air during use. The packed cell volume of the perfusate was $17.36 \pm 0.31\%$ (mean $\pm$ S.E.M.).

The perfusion apparatus

The perfusion apparatus was based on that used by Mendelson & Scow (1972). It consisted of a flask oxygenator/reservoir, containing up to 400 ml of perfusate and maintained at 38 °C, which was rotated at 225 rev./min and received 1.8 litres of fresh air/min to maintain O₂ saturation at 96–99%. Non-recirculating perfusion of the mammary gland was via a polyethylene input catheter (400 mm long, 1.4 mm internal diam.; Portex, Hythe, Kent, U.K.), connecting the arterial cannula to the main pumped circuit.

In the main circuit, perfusate was pumped from the reservoir by means of a flow inducer (type MHRE 100; Watson–Marlow, Falmouth, Cornwall, U.K.) at 14–30 ml/min through a blood filter and heat-exchanger coil and back to the reservoir. The heat exchanger was maintained at 38 °C and consisted of a 12 m coil of nylon tubing (internal diam. 1.7 mm). The filter material was a layer of cotton broadcloth, 0.018 m³ in area, which allowed passage of erythrocytes without any apparent damage, while filtering out clots.

Polyethylene tubing (1.65 mm internal diam.) was used throughout the main circuit, connected with Teflon variable-bore connectors (Omnifit, Cambridge, U.K.). All tubing was insulated by cladding with silicone-rubber tubing, except for 50 mm of the input catheter proximal to its junction with the arterial cannula.

The arterial cannula consisted of polyethylene tubing of 1.27 mm outer diam., 0.86 mm internal diam., heat-drawn to a tip of outer diam. 0.4 ± 0.07 mm (mean $\pm$ S.D. for the cannulae used in this work), cut to a length of 15 mm. The tip was bevelled and checked to be free from burrs. It was connected to the input catheter by a length (10 mm) of silicone-rubber tubing (2 mm outer diam., 1 mm internal diam.). The venous cannula was made similarly, except that it was heat-drawn to a tip of 0.6 ± 0.02 mm; it was connected to a 300 mm length of polyethylene tubing (1.14 mm internal diam.) which delivered venous effluent to a collecting vessel.

The perfusion circuit was arranged so that any bubbles arising were returned to the reservoir and did not pass to the animal. Mean perfusate flow rate through the mammary tissue was 2.32 ± 0.11 ml/min (mean $\pm$ S.E.M. for 24 perfused preparations); no significant differences in flow rate resulted from the inclusion of either insulin or isoprenaline in the perfusion medium. O_2 uptake was 52.5 ± 5.1 μ l/min (mean $\pm$ S.E.M. for 22 perfused preparations). A perfused-tissue weight of 4.45 ± 0.48 g (mean $\pm$ S.E.M.) was measured in eight animals. The total weight of mammary tissue in the left-hand-side abdominal/inguinal area of these same animals was 7.35 ± 0.29 g. A quite clearly defined 'core' area of tissue (weighing 2.5–3 g) adjacent to the entry point of the superficial epigastric vessels was perfused even in those preparations ranked lowest in the range of perfused-weight values. This was the area sampled by freeze-clamping for metabolite and enzyme measurements, as described below. Perfusion pressure was monitored by means of a pressure transducer (type PDCR 75; Druck, Groby, Leics., U.K.) at a point close to the arterial cannula, and could be altered by changing the pumping rate. It was controlled such that the perfusion pressure in the mammary tissue was maintained at 95–105 mmHg. Milk secretion, qualitatively assessed as visible accumulation within the tissue mass, continues in such perfused preparations (D. T. Calvert & R. A. Clegg, unpublished work) for several hours.

Collection of tissue

At 90 min after cannulation of the superior epigastric blood vessels, with perfusion still continuing, the ventral mid-line skin incision was re-opened. Abdominal skin was freed from the underlying mammary tissue by using blunt dissection as far as possible (it was necessary to cut the ducts and blood vessels running between teats and mammary tissue) and reflected anteriorly to expose the perfused tissue mass, the appearance of which was noted at this point. The tissue was then gently grasped with forceps at two points on its medial margin close to the anterior and posterior extremities of where it was free from attachment to other structures, and lifted free from the anterior abdominal wall. Flow of perfusate was not interrupted by this procedure. The 'core' area of perfused tissue (see above) was then clamped between the jaws of aluminium tongs cooled to the temperature of liquid N_2 , and cut free from the animal; the cannulae were withdrawn from the frozen tissue mass and flow of perfusion fluid was stopped. The tissue was plunged into liquid N_2 and the rat was killed.

Analytical techniques

Freeze-clamped tissue was first pulverized in a stainless-steel mortar cooled to the temperature of liquid N_2 before preparation of extracts for the measurement of metabolites. These were prepared by adding ice-cold 6% (v/v) $HClO_4$ (3.25 ml/g of tissue) to weighed portions of pulverized tissue at $-80^\circ C$ in polypropylene test tubes and immediately homogenizing the resulting 'slush' by using the PTA 10S probe of a Polytron homogenizer operated at setting 4 for 30 s. The tubes were then centrifuged at 2500 g for 10 min at $4^\circ C$. Samples of the deproteinized supernatants were neutralized and freed from perchlorate by treatment with a solution of tri-n-

octylamine in 1,1,2-trichlorotrifluoroethane as described by Khym (1975). The ATP concentration in these extracts was measured (after suitable dilution) by a luminometric technique, using both reagents and equipment supplied by LKB (Bromma, Sweden).

Nucleotide profiles were obtained by subjecting samples (100–200 μ l) of extract to reversed-phase h.p.l.c. on a C_8 column (6.2 mm $\times$ 800 mm; Du Pont Zorbax Pep/RP1) developed with a gradient of methanol in aq. 83.3 mM-triethylammonium phosphate buffer (pH 6) as described by Willis *et al.* (1986). The A_{254} of the column effluent was monitored, and an integral of the resulting trace was used to calculate the relative concentrations of ATP, ADP and AMP; components corresponding to these nucleotides were identified by peak enhancement by using standards added to samples. Internal standards, added in known quantities to samples of pulverized tissue, were used to establish the recovery of each of these three adenine nucleotides over the entire procedure; this was found to exceed 92% in all cases.

Extracts of freeze-clamped tissue were prepared and assayed for acetyl-CoA carboxylase activity as described by McNeillie & Zammit (1982), except that the gel-filtration step was omitted and the final concentration of citrate present during the assay of enzymic activity was varied. Measurements of 'expressed' activity (alternatively termed 'initial' activity by McNeillie & Zammit, 1982) were made in the absence of added citrate and in the presence of 20 mM-magnesium citrate. The 'total' citrate-activatable activity was also assayed in the presence of 20 mM-magnesium citrate, but after first pre-incubating the extract with 20 mM-magnesium citrate for 20 min at $30^\circ C$.

Other measurements

Haematocrit measurements were made on the inflowing and outflowing perfusion fluid at roughly 10 min intervals throughout perfusion, by a routine capillary-tube sedimentation method.

pO_2 , pCO_2 , % haemoglobin and % saturation of haemoglobin with O_2 were all monitored with similar frequency with a commercial blood-gas analyser (Radiometer, Copenhagen, Denmark). Flow rate of perfusate through the tissue was measured by recording the rate of outflow from the venous cannula. The rate of O_2 uptake by the perfused tissue was then calculated from the differences between the blood-gas measurements taken simultaneously on the afferent and efferent perfusate.

Measurement of the mass of tissue undergoing perfusion was made on several preparations, which, because of the nature of the procedure involved, were not used for the derivation of analytical experimental data. This was achieved by including Evans' Blue dye in the perfusate for 10 min, then discontinuing perfusion and dissecting out and weighing firstly the whole abdominal/inguinal mammary tissue mass and then secondly those regions of it that were stained deeply blue.

Chemicals

Bovine serum albumin (fraction V) and other biochemicals were from Sigma except where otherwise specified; chemical reagents were from BDH. Insulin (pig) was generously given by Boots Co. (Nottingham, U.K.).

RESULTS

Tissue oxygenation

The oxygenation status of the perfused area of mammary gland was assessed by measurement of the tissue concentration of adenine nucleotides. For comparison, these nucleotides were also measured in extracts of freeze-clamped mammary tissue from two further groups of rats. In the first of these (designated 'zero-time controls'), animals were anaesthetized and mammary tissue was exposed and prepared for freeze-clamping by using atraumatic dissection techniques: the tissue was then immediately freeze-clamped. Anaesthetized rats in the second group (designated 'non-perfused controls') were surgically prepared as if for perfusion of mammary tissue, but the superficial epigastric artery and vein were not tied off or catheterized: the skin incisions were closed with sutures, and the animal was left undisturbed (except for the administration of additional anaesthetic if signs of arousal were noted) for 90 min, the usual duration of a perfusion experiment. These measurements are recorded in Table 1, along with the DNA content (per g wet wt.) of the samples. In the perfused mammary-tissue samples, the DNA content was lower than in tissue from non-perfused controls and zero-time controls. The values for both categories of control tissue were similar to one another and also to those published for freeze-clamped mammary tissue (McNeillie & Zammit, 1982) and mammary acini (Clegg *et al.*, 1986). Thus, although there would be expected to be an accumulation of secreted milk in the tissue of animals undergoing perfusion, and of the non-perfused controls during the 90 min period when they were not suckled by their young, this cannot be the cause of the diminished content of DNA in the perfused tissue, since no diminution was seen in the non-perfused controls. When mammary tissue ATP content was expressed per mg of DNA, there was no significant difference between the two control groups, indicating that the oxygen status of the tissue in those animals that had experienced surgical intervention had not been compromised. Although the absolute value of ATP content (per mg of DNA) was lower in perfused than in non-perfused tissues ($P < 0.02$), the total adenine nucleotide content was also lower, such that the content of ATP as a percentage of the total adenine nucleotides, and the energy-charge status (Atkinson, 1968) of the tissue, were more closely similar in all three groups. Energy-charge ratios were 0.68 for perfused samples, 0.80 for non-perfused controls and 0.76 for zero-time controls. To serve a comparative function, similar data were also collected from mammary tissue whose blood supply had been deliberately interrupted by ligation of the superior epigastric blood vessels for 12 min before freeze-clamping. The absolute value of ATP content was low in tissue treated in this way ($P < 0.001$ relative to non-perfused controls and $P < 0.02$ relative to perfused tissue), and the energy-charge ratio was only 0.31. Representative profiles of adenine nucleotides in extracts from this anoxic mammary tissue and from tissue in the three other experimental groups discussed above are shown in Fig. 1.

Hormonal effects on perfused mammary tissue

The data for perfused mammary tissue reported above relate to experiments in which the perfusate was supplemented with insulin (0.1 munit/ml). As shown in

Table 1. Adenine nucleotide content of perfused and control rat mammary tissue

HClO₄ extracts of freeze-clamped mammary tissue were prepared and assayed for adenine nucleotides as described in the Experimental section. Results are mean values $\pm$ S.E.M. for the numbers of tissue samples shown in parentheses. Each tissue sample in the vertical columns was from a separate rat. Differences, compared against the values for perfused tissue, were assessed for statistical significance by Student's *t* test for unpaired samples: * $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.

Condition of tissue	DNA content (mg/g wet wt.)	Adenine nucleotide content (μ mol/mg of DNA)			Energy-charge ratio
		ATP	ADP	AMP	
Perfused	2.73 ± 0.24 (9)	0.199 ± 0.025 (9)	0.098 ± 0.23 (4)	0.038 ± 0.02 (4)	0.367 ± 0.066 (4)
Zero-time control	4.58 ± 0.11 (6)****	0.291 ± 0.025 (6)*	0.129 ± 0.012 (6)	0.047 ± 0.005 (6)	0.467 ± 0.034 (6)***
Non-perfused control	4.26 ± 0.28 (6)****	0.375 ± 0.039 (6)***	0.137 ± 0.020 (6)	0.044 ± 0.016 (6)	0.555 ± 0.042 (6)****

Fig. 1. Adenine nucleotide profiles of mammary tissue extracts

Neutralized HClO_4 extracts of mammary tissue were prepared as described in the Experimental section. Samples were as follows (energy-charge ratio shown in parentheses): (a) zero-time control (0.76), (b) non-perfused control (0.80), (c) perfused with insulin-containing medium (0.68), (d) vascular supply occluded (0.31). The A_{254} traces shown are of separations of 100 μl each of these extracts, chromatographed on a PEP RPI column. Elution conditions (1 ml/min at room temperature) were as follows: buffer A, 1% (v/v) methanol in 83.3 mM-triethylammonium phosphate buffer, pH 6.0; buffer B, 20% (v/v) methanol in the same buffer; 0–3 min, 100% A; 3–23 min, linear gradient 0–45% B; 23–30 min, linear gradient 45–100% B. Arrows show the elution positions of AMP, ADP and ATP.

Table 2, the tissue concentration of ATP and of total adenine nucleotides was lower ($P < 0.05$) when insulin was omitted from the perfusate. The energy-charge ratio fell to 0.57, whereas the DNA content was unchanged, indicating a similar degree of tissue hydration. The presence in the perfusate of 1 μM -isoprenaline (a pharmacological agent that mimics the actions of adrenaline and noradrenaline by agonistic binding to β - and, with lower affinity, to α -adrenergic receptors) restored ATP and total adenine nucleotide contents to values not significantly different from those in the presence of insulin. The energy-charge ratio was, however, greater when isoprenaline was substituted for insulin.

Activity of the enzyme acetyl-CoA carboxylase in perfused mammary tissue was affected by the presence of insulin. Expressed activity in the absence of added citrate was increased 1.6-fold relative to tissue perfused in the absence of insulin ($P < 0.05$); expressed activity in the presence of 20 mM-citrate was increased to a slightly lesser extent, by around 1.5-fold ($P < 0.05$). The 'total' activity was also higher (1.4-fold) in tissue perfused with insulin than when this agent was absent ($P < 0.05$).

Table 2. Effects of insulin and isoprenaline on adenine nucleotide content of perfused rat mammary tissue

Expression of results and evaluation of statistically significant differences (versus values from tissue perfused without additions) were as in Table 1: * $P < 0.05$; ** $P < 0.02$.

Additions to perfusion medium	DNA content (mg/g wet wt.)	Adenine nucleotide content ($\mu\text{mol}/\text{mg}$ of DNA)			Energy-charge ratio
		ATP	ADP	AMP	
None	2.87 ± 0.26 (7)	0.112 ± 0.031 (7)	0.080 ± 0.012 (4)	0.044 ± 0.01 (4)	0.57
Insulin (0.1 munit/ml)	2.73 ± 0.24 (9)	0.199 ± 0.025 (9)*	0.098 ± 0.023 (4)	0.038 ± 0.02 (4)	0.68
Isoprenaline (1 μM)	2.97 ± 0.15 (8)	0.222 ± 0.020 (8)**	0.068 ± 0.011 (8)	0.051 ± 0.012 (8)	0.75
			Total		
			0.267 ± 0.055 (4)		
			0.367 ± 0.066 (4)		
			0.342 ± 0.025 (8)		

Table 3. Acetyl-CoA carboxylase activity in rat mammary tissue: effects of perfusion with insulin or isoprenaline

Acetyl-CoA carboxylase activity was assayed in extracts of freeze-clamped mammary tissue. Tissue designated 'Non-perfused; operated' was from non-perfused control rats, and 'non-perfused; non-operated' was from zero-time control rats. Statistically significant differences (versus 'Perfused; no additions') were determined as in Table 1: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$. Activity ratios are derived from the mean values for initial and 'total' activities, both measured at 20 mM-citrate.

Tissue status	No. of preparations	Acetyl-CoA carboxylase activity ($\mu\text{mol/min per mg of DNA}$)			
		Initial activity (measured at 0 mM-citrate)	Initial activity (measured at 20 mM-citrate)	'Total' activity (measured at 20 mM-citrate)	Activity ratio
Perfused; no additions	7	0.118 ± 0.024	0.202 ± 0.039	0.439 ± 0.067	0.46
Perfused plus insulin (0.1 munits/ml)	7	$0.186 \pm 0.017^*$	$0.299 \pm 0.015^*$	$0.614 \pm 0.038^*$	0.49
Perfused plus isoprenaline (1 μM)	8	$0.191 \pm 0.028^{**}$	0.304 ± 0.038	0.561 ± 0.040	0.54
Non-perfused; operated	6	0.078 ± 0.012	0.227 ± 0.028	0.413 ± 0.028	0.55
Non-perfused; non-operated	6	0.108 ± 0.008	0.235 ± 0.015	0.467 ± 0.033	0.50
Blood vessels ligated	6	$0.038 \pm 0.005^{***}$	0.116 ± 0.015	0.432 ± 0.023	0.27

Broadly similar effects on acetyl-CoA carboxylase activity were achieved by perfusion with 1 μM -isoprenaline, but in this case only the increase in expressed activity measured in the absence of added citrate achieved statistical significance ($P < 0.02$). The activity ratio (ratio of expressed to 'total' activity, both measured at 20 mM-citrate) serves as an index of the degree of activation of acetyl-CoA carboxylase, most probably reflecting the phosphorylation state of the enzyme (McNeillie & Zammit, 1982). This was 0.46 for acetyl-CoA carboxylase in tissue perfused without insulin or isoprenaline and, on inclusion of insulin in the perfusion medium, remained virtually unchanged at 0.49. However, substitution of isoprenaline for insulin caused an activation of acetyl-CoA carboxylase, reflected by a rise in the activity ratio to 0.54. For comparison, the activity ratios in zero-time controls and non-perfused controls were 0.50 and 0.55 respectively, but only 0.27 in extracts of tissue that had been deliberately subjected to anoxia as described above. These data are summarized in Table 3.

DISCUSSION

Experiments with intact lactating rats and with rat mammary acini *in vitro* have implicated insulin as a positive (stimulatory) effector in the dietary control of lipogenesis in mammary tissue, and have further identified an involvement of acetyl-CoA carboxylase in this regulation (Williamson, 1980; McNeillie & Zammit, 1982; Munday & Williamson, 1987). The present study confirms and extends these findings by demonstrating a stimulation of acetyl-CoA carboxylase by insulin in a mammary-gland preparation of the lactating rat perfused *in situ*. It is also clear from the results presented here that the citrate-activatable activity ('total' activity) values can not be taken to represent a measure of the total quantity of acetyl-CoA carboxylase enzyme protein present in tissue extracts, since it is itself subject to acute hormonal modification on a time scale that is unlikely to be compatible with gross changes in the rates of enzyme protein synthesis or degradation.

The mechanism whereby insulin exerts its regulatory effects on mammary tissue acetyl-CoA carboxylase remains unresolved. In adipocytes, increased phosphoryl-

ation of a specific site occurs in response to insulin treatment (Brownsey & Denton, 1982; Witters *et al.*, 1983), although this modification alone is apparently without effect on the kinetic properties of the purified enzyme (Witters *et al.*, 1983; Holland & Hardie, 1985; but see also Borthwick *et al.*, 1987). It has been suggested that the activity alterations measurable in crude extracts of adipocytes after insulin treatment result from the interaction with acetyl-CoA carboxylase of a tightly bound allosteric modulator (Haystead & Hardie, 1986). In mammary tissue, however, there is evidence that insulin action is, at least under certain circumstances, mediated via a dephosphorylation of acetyl-CoA carboxylase (Munday & Hardie, 1986), which has direct consequences on its catalytic properties. Conversely, under conditions of insulin deficiency, mammary acetyl-CoA carboxylase activity declines in parallel with the incorporation of additional phosphate into the enzyme molecule (Munday & Hardie, 1986). A novel protein kinase has been described (Carling & Hardie, 1986) that may be responsible for phosphorylation of acetyl-CoA carboxylase at the distinctive serine residue implicated in these effects; activity of this kinase is stimulated by AMP (Carling *et al.*, 1987).

The search for a putative negative (inhibitory) effector serving as a counter-regulator to insulin in the control of mammary tissue metabolism has proved inconclusive. In particular, acute experiments with mammary explants and acini have yielded results suggesting that cyclic AMP does not perform this function (Plucinski & Baldwin, 1982; Williamson *et al.*, 1983; Busmann *et al.*, 1984; Robson *et al.*, 1984; Clegg *et al.*, 1986; Munday & Williamson, 1987). The present study confirms that these results were not simply a consequence of impaired function in the acini and explant preparations. As shown above, there was no inhibition of acetyl-CoA carboxylase when isoprenaline, a competent stimulator of adenylate cyclase in mammary cells, was included in the medium perfusing mammary tissue; indeed, a stimulation of expressed activity of this enzyme at low concentration of citrate was found. This is consistent with the stimulation of the rate of mammary lipogenesis reported in a number of experiments with acini and tissue explants, in which various treatments caused an elevation of the concentration of cyclic AMP in the preparation (Plucinski &

Baldwin, 1982; Clegg *et al.*, 1986; Munday & Williamson, 1987). Furthermore, it suggests that the observed decline in the rate of mammary lipogenesis when rats were injected with adrenaline (Bussmann *et al.*, 1984) was not a direct consequence of the action of that hormone on mammary epithelial cells.

The results reported here show a quite striking correlation between the activity ratio of acetyl-CoA carboxylase and the energy-charge ratio of the perfused tissue. It has been previously reported that the energy status of rat liver influences acetyl-CoA carboxylase activity in liver extracts, with AMP causing an inhibition of activity, especially at low concentrations of citrate (Yeh *et al.*, 1980). Guanine nucleotides, including GTP, have been reported to activate acetyl-CoA carboxylase (Witters *et al.*, 1981; Buechler & Gibson, 1984). More recent work by Witters *et al.* (1987) also suggests a correlation between a lowering of intracellular ATP (and GTP) and inhibition of acetyl-CoA carboxylase in rat adipocytes. Yeh *et al.* (1980) attributed the effect that they observed to an allosteric interaction of AMP with acetyl-CoA carboxylase. However, in the light of the properties of the novel acetyl-CoA carboxylase-kinase discussed above, it is at least equally probable that effects of energy-charge status on acetyl-CoA carboxylase are mediated via changes in its phosphorylation status. Further information in this regard could be gathered by affinity purification and characterization (with respect to kinetic properties and the distribution and content of alkali-labile phosphate groups within the molecule) of acetyl-CoA carboxylase from mammary tissue perfused as described in this work.

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