The activities and intracellular distributions of enzymes of carbohydrate, lipid and ketone-body metabolism in lactating mammary glands from ruminants and non-ruminants

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1. The activities of several enzymes of carbohydrate, lipid, acetate and ketone-body metabolism were measured in lactating mammary glands from rats, mice, rabbits, guinea pigs, sows, sheep, cows and goats. The intracellular distributions of many of the enzymes were measured by fractional extraction. 2. Acetyl-CoA synthetase was predominantly cytoplasmic in rats and guinea pigs, but was more mitochondrial in the other species. The different location of this enzyme in rats and mice is discussed in relation to the disposal of reducing equivalents. 3. 3-Oxo acid CoA-transferase and acetoacetyl-CoA thiolase assayed at $600\mu\text{M}$ -CoA were predominantly mitochondrial in all species investigated. Acetoacetyl-CoA thiolase assayed at $8\mu\text{M}$ -CoA was predominantly cytoplasmic, except in rabbits and guinea pigs. Ruminants appeared to possess little, if any, of the cytoplasmic enzyme. 4. The activities and distributions of NADP-isocitrate dehydrogenase were consistent with a role in supplying cytoplasmic NADPH in ruminant tissue, and indicated that this system may also occur in guinea pigs.

Lactating mammary tissue uses such fuels as glucose, acetate and ketone bodies for supplying carbon, ATP and reducing equivalents for the biosynthesis of milk fatty acids and other components (for reviews see Bauman & Davis, 1974; Davis & Bauman, 1974; Smith & Taylor, 1977). The nature and extent of fuel utilization varies with species. For example, ruminant tissue (in contrast with that of other mammals) does not convert glucose into fatty acids, although it can oxidize glucose to CO_2 (Hardwick, 1966; Smith & Taylor, 1977). Such differences in fuel utilization are reflected in the activities of enzymes of the relevant metabolic pathways (Gumaa *et al.*, 1973; Baldwin & Yang, 1974). However, many of these investigations involved very few species, often only comparing rats with ruminants. Furthermore, there is little information on the intracellular distribution of enzymes involved in both cytoplasmic and mitochondrial pathways. This paper examines some aspects of mammary-gland enzymology and intracellular enzyme distributions, in eight species. For reasons given previously (Taylor *et al.*, 1978), an indirect method (fractional extraction) was used to study the intracellular location of enzymes.

Materials and methods

Sources of chemicals

Chemicals and coupling enzymes were obtained from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K., or from the sources given by Taylor *et al.* (1978), with the addition of the following: reduced acetylpyridine–adenine dinucleotide, L-malic acid, DL-isocitrate, ATP (dipotassium salt) and palmitoyl-L-carnitine, were obtained from Sigma (London) Chemical Co., Poole, U.K.; phosphocreatine (potassium salt) was obtained from Calbiochem, Hereford, U.K.; Sagatal was obtained from May and Baker, Dagenham, Essex, U.K.; Immobilon was obtained from Reckitt and Colman, Hull, U.K.

Sources of tissue

Pregnant Wistar rats, lactating guinea pigs, mice and New Zealand white rabbits were obtained through the University of Leeds Medical School and were housed with their litters in purpose-built cages and fed on standard laboratory diets. The litter sizes for guinea pigs and rabbits were two and two to four, respectively, and the average litter size for

rats and mice was eight. Sheep and goat tissue was obtained from animals housed in this Department; they received 1 kg of standard cereal ration and 0.5 kg of hay per day; the sheep suckled either one or two lambs and the goats were producing approx. 1 litre of milk per day. Sow tissue was obtained from the herd of Large Whites at the University of Leeds Pig Unit; the animals were suckling approx. ten young.

Tissue collection and homogenization

Tissue from rats and mice was obtained from 7–14-day-lactating animals, after they were killed by cervical dislocation under chloroform anaesthesia; tissue from guinea pigs and rabbits was obtained from 4–7-day and 18–21-day-lactating animals, respectively, after they were killed by exsanguination under Sagatal anaesthesia (35 mg/kg body wt); tissue from sheep (approx. 8 weeks lactating), sow (near peak lactation) and goat (peak to late lactation) was obtained by biopsy under Immobilon anaesthesia. Tissue from cows (of unknown stage of lactation) was obtained from the local abattoir, transported to the laboratory on ice and used within 2 h of death.

Tissues were rinsed in the KCl-based medium described by Taylor *et al.* (1978) and were then chopped with scissors. The chopped tissue was blotted on filter paper and a portion (30–50 mg) weighed on a torsion balance before being homogenized in 10–20 vol. of 50 mM-triethanolamine/KOH buffer, pH 7.4, containing 1 mM-EDTA, 2 mM-MgCl₂ and 2 mM-dithiothreitol. A ground-glass homogenizer was used and, after homogenization was complete (1–3 min), 10 μ l of sodium deoxycholate solution (10%, w/v) was added per ml of homogenate; the extract was rehomogenized to release latent mitochondrial enzyme activities (Taylor *et al.*, 1978). Deoxycholate was omitted when assaying ATP citrate lyase because it was found to inhibit this enzyme. Homogenates were kept on ice and assayed within 30 min of preparation.

Fractional extraction of enzymes

The procedure was modified from that described previously (Taylor *et al.*, 1978) as follows. First, the total time of extraction was cut to 90 min by omitting the extraction in phosphate buffer. Second, with several tissues it was impractical to homogenize the whole pellet; instead, a sample (approx. 50 mg) of pellet was homogenized to determine the activities retained in the tissue. These activities needed correcting because 1 g of pellet is not equivalent to 1 g of tissue, owing to loss of cytoplasm during the extraction. The correction factor was obtained by assuming 100% recovery of citrate synthase during the extraction; this is reasonable, because experiments with rat and mouse tissue,

in which the whole pellet was homogenized, gave 95–105% recoveries of the enzyme. The activity of citrate synthase in the pellet (as μ mol/min per g of tissue), activity A, was calculated as the difference between the activity in the tissue homogenate and the total activity released into the supernatant during the extraction. The correction factor was then calculated as the ratio A/B, where B is the activity of citrate synthase measured in a sample of the pellet (as μ mol/min per g of pellet); the activities of the enzymes measured in the pellet were multiplied by this factor to convert them into units of μ mol/min per g of tissue.

The third modification corrected for variations in the amount of tissue available for extraction by the medium. This was estimated as the total percentage release of lactate dehydrogenase into the medium (L) and was 60–100%. Since lactate dehydrogenase is a soluble cytoplasmic enzyme, the value of L would approach 100% if all the tissue had been available for extraction. Therefore the percentage releases for each enzyme at each time interval were multiplied by 100/L (with the value of L being estimated to the nearest 5%) to allow for the incomplete extraction.

The mean values of the total percentage activities released into the medium after 90 min are shown in Table 1. The recoveries of the enzyme activities were all greater than 85%, and preliminary experiments showed that at least 95% of the activities were released into the supernatant after thorough homogenization of the tissue in a ground-glass homogenizer. Therefore retention of these activities with the pellet reflects a non-cytoplasmic (presumably mitochondrial) location and is not the result of binding to the pellet after release from the tissue (see Taylor *et al.*, 1978).

Measurement of enzyme activities

Citrate synthase (EC 4.1.3.7), pyruvate carboxylase (EC 6.4.1.1), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and lactate dehydrogenase (EC 1.1.1.27) were assayed as described previously (Taylor *et al.*, 1978). Carnitine palmitoyltransferase (EC 2.3.1.21) was assayed in the direction of palmitoyl-CoA formation as described by Crabtree & Newsholme (1972b). Hexokinase (EC 2.7.1.1) was assayed as described by Crabtree & Newsholme (1972a), except that the rate of NADPH production was divided by 2, to allow for subsequent oxidation of 6-phosphogluconate by the 6-phosphogluconate dehydrogenase activity of the homogenates. This was shown to be necessary by comparing the rate of NADPH production in the spectrophotometric assay with the rate of glucose phosphorylation by using the direct radiochemical assay of Newsholme *et al.* (1967). The production of extra NADPH as a result of 6-phosphogluconate oxidation produced

Table 1. *Intracellular distributions of mammary-gland enzymes*

The fractional-extraction procedure, including the correction for incomplete extraction (by using lactate dehydrogenase), was as described in the Materials and methods section. The data are expressed as means $\pm$ S.E.M., with the numbers of determinations in parentheses.

Percentage of total activity released into the supernatant after 90 min extraction

Enzyme	Rat	Mouse	Rabbit	Guinea pig	Sow	Sheep	Cow
Citrate synthase	7 $\pm$ 1 (14)	3 $\pm$ 0.5 (8)	8 $\pm$ 1 (5)	7 $\pm$ 1 (6)	8 $\pm$ 2 (3)	12 $\pm$ 1 (4)	14 $\pm$ 3 (7)
Acetyl-CoA synthetase	96 $\pm$ 3 (4)	18 $\pm$ 3 (6)	52 $\pm$ 6 (5)	85 $\pm$ 4 (4)	21 $\pm$ 5 (3)	52 $\pm$ 3 (4)	24 $\pm$ 11 (4)
3-Oxo acid CoA-transferase	6 $\pm$ 3 (4)	4 $\pm$ 2 (3)	8 $\pm$ 2 (4)	9 $\pm$ 2 (4)	—	10 $\pm$ 2 (3)	7 $\pm$ 4 (3)
Thiolase (8 μ M-CoA)	91 $\pm$ 9 (4)	80 $\pm$ 4 (5)	78 $\pm$ 6 (4)	96 $\pm$ 5 (4)	80 $\pm$ 3 (3)	8 $\pm$ 4 (3)	20 $\pm$ 3 (4)
Thiolase (600 μ M-CoA)	10 $\pm$ 5 (4)	12 $\pm$ 2 (4)	53 $\pm$ 10 (4)	51 $\pm$ 5 (4)	11 $\pm$ 4 (3)	4 $\pm$ 3 (3)	10 $\pm$ 2 (4)
3-Hydroxyacyl-CoA dehydrogenase	7 $\pm$ 2 (3)	10 $\pm$ 1 (4)	14 $\pm$ 1 (4)	12 $\pm$ 2 (4)	8 $\pm$ 1 (3)	—	15 $\pm$ 2 (3)
NAD-malate dehydrogenase	72 $\pm$ 3 (4)	80 $\pm$ 4 (4)	81 $\pm$ 7 (3)	73 $\pm$ 8 (4)	—	—	63 $\pm$ 5 (3)
NADP-malate dehydrogenase	93 $\pm$ 12 (4)	97 $\pm$ 2 (5)	—	—	—	—	—
Pyruvate carboxylase	6 $\pm$ 1 (4)	5 $\pm$ 1 (4)	12 $\pm$ 4 (3)	11 $\pm$ 6 (4)	—	5 $\pm$ 4 (3)	15 $\pm$ 11 (3)
NADP-isocitrate dehydrogenase	32 $\pm$ 2 (5)	39 $\pm$ 2 (7)	51 $\pm$ 2 (4)	85 $\pm$ 4 (5)	43 $\pm$ 2 (3)	92 $\pm$ 3 (4)	97 $\pm$ 6 (5)

a lag period of 4–5 min in the spectrophotometric assay, which was not significantly improved by adding commercial 6-phosphogluconate dehydrogenase. Phosphofructokinase (EC 2.7.1.11) was assayed as described by Crabtree & Newsholme (1972a). Fructose bisphosphatase (EC 3.1.3.11) was assayed as described by Newsholme & Crabtree (1970), except that adenylate kinase (10 units/ml) was included in the assay medium and the net increase in A_{340} was divided by 2 to allow for the subsequent oxidation of 6-phosphogluconate; no significant lag period was observed in this assay. The assay for glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was based on that of Martin & Denton (1970). The assay medium contained 95 mM-triethanolamine/KOH, 0.24 mM-NADP⁺, 8 mM-MgCl₂ and 7.5 mM-glucose 6-phosphate at pH 7.4; the activity was obtained from the increase in A_{340} minus that of a control from which glucose 6-phosphate was omitted. The net increase in A_{340} was divided by 2 to allow for the subsequent oxidation of 6-phosphogluconate; this produced a lag period of 1–3 min. The assay for glutamate dehydrogenase (EC 1.4.1.3) was based on that of Martin & Denton (1970). The assay medium contained 80 mM-triethanolamine/KOH, 10 mM-2-oxoglutarate, 1.5 mM-ADP, 130 mM-ammonium acetate, 0.12 mM-NADH and 0.5 mM-EDTA at

pH 7.9; the activity was obtained from the decrease of A_{340} minus that of a control from which 2-oxoglutarate was omitted. The assay for NADP-malate dehydrogenase ('malic' enzyme, EC 1.1.1.40) was based on that of Martin & Denton (1970). The assay medium contained 80 mM-triethanolamine/KOH, 1 mM-MnCl₂, 0.24 mM-NADP⁺ and 10 mM-L-malate at pH 7.4; the activity was obtained from the increase of A_{340} minus that of a control from which malate was omitted. The assay medium for NAD-malate dehydrogenase (EC 1.1.1.37) contained 98 mM-triethanolamine/KOH, 0.12 mM-NADH and 1 mM-oxaloacetate at pH 7.4; the activity was obtained from the decrease of A_{340} minus that of a control from which oxaloacetate was omitted. The assay for NADP-isocitrate dehydrogenase (EC 1.1.1.42) was based on that of Alp *et al.* (1976). The assay medium contained 70 mM-triethanolamine/KOH, 0.24 mM-NADP⁺, 1 mM-MnCl₂, 8 mM-MgCl₂, 3.5 mM-DL-isocitrate and 10 mM-citrate at pH 7.4; the activity was obtained from the rate of increase of A_{340} minus that of a control from which isocitrate and citrate were omitted. The assay for ATP citrate lyase (EC 4.1.3.8) was based on that of Srere (1959). The assay medium contained 30 mM-triethanolamine/KOH, 1.2 mM-dithiothreitol, 0.11 mM-NADH, 7 mM-MgCl₂, 0.24 mM-CoA, 8 mM-citrate (K⁺ salt), 7 mM-ATP (K⁺ salt), 10 mM-

phosphocreatine (K^+ salt), malate dehydrogenase (12 units/ml), creatine kinase (5 units/ml) at pH 7.7; the activity was obtained from the decrease of A_{340} minus that of a control from which citrate was omitted. The assay for acetyl-CoA synthetase (EC 6.2.1.1) was based on that of Martin & Denton (1970). NAD was replaced by its 3-acetylpyridine analogue, whose reduced form has a molar absorption coefficient of 9.1×10^3 at 363 nm (Siegel *et al.*, 1959), compared with 6.3×10^3 for NADH at 340 nm; use of this analogue therefore increases the change in absorption per μmol of cofactor reduced by approx. 50%. The potential lack of stoichiometry in the assay, owing to the near-equilibrium of the indicator reaction catalysed by malate dehydrogenase (Pearson, 1965; Hardwick, 1966), was overcome by adding reduced cofactor to the assay (Guynn & Veech, 1974). The assay medium contained 105 mM-Tris/HCl, 1.1 mM-dithiothreitol, 0.38 mM-acetylpyridine-adenine dinucleotide, 0.08 mM-reduced acetylpyridine-adenine dinucleotide, 0.15 mM-L-malate (K^+ salt), 0.8 mM-CoA, 2 mM-ATP (K^+ salt), 12 mM-MgCl₂, 9.2 mM-phosphocreatine (K^+ salt), 5 mM-acetate (K^+ salt), creatine kinase (5 units/ml) citrate synthase (22 units/ml) and malate dehydrogenase (12 units/ml) at pH 8.0; the activity was obtained from the increase of A_{363} minus that of a control from which acetate was omitted. Na^+ ions were excluded from the assay because they inhibit the enzyme (see Guynn & Veech, 1974). The assay for 3-oxo acid CoA-transferase (EC 2.8.3.5) was based on that of Williamson *et al.* (1971). The assay medium contained 60 mM-Tris/HCl, 7.6 mM-MgCl₂, 0.05 mM-acetoacetyl-CoA and 60 mM-succinate at pH 8.5; the activity was obtained from the decrease of A_{313} minus that of a control from which succinate was omitted. Further controls, omitting extract, were needed to allow for a non-enzymic effect of succinate on the rate of decrease of A_{313} . The molar absorption coefficient of the acetoacetyl-CoA-Mg²⁺ complex was assumed to be 12×10^3 at 313 nm (Williamson *et al.*, 1971). It was not possible to increase the concentration of acetoacetyl-CoA to saturating values because of a non-linearity between A_{313} and high concentrations of the acetoacetyl-CoA-Mg²⁺ complex; therefore the activities of this enzyme are submaximal. Acetoacetyl-CoA thiolase (EC 2.3.1.9) was assayed, at high (600 μM) and low (approx. 8 μM) concentrations of CoA, by measuring the acetyl-CoA produced from acetoacetyl-CoA; as with the assay for acetyl-CoA synthetase, the sensitivity was increased by using the acetylpyridine analogue of NAD. The commercial acetoacetyl-CoA contained sufficient free CoA to produce a concentration of approx. 8 μM in the assay. This sufficed for the assay at low [CoA] and was removed from the control by the addition of dithiobis-(2-

nitrobenzoic acid). The assay medium contained 57 mM-Tris/HCl, 4 mM-MgCl₂, 23 mM-KCl, 0.05 mM-acetoacetyl-CoA (which also provided 8 μM -CoA), 0.45 mM-acetylpyridine-adenine dinucleotide, 0.09 mM-reduced acetylpyridine-adenine dinucleotide, 0.2 mM-L-malate, 0.6 mM-CoA (for assays at high [CoA] only), malate dehydrogenase (12 units/ml) and citrate synthase (22 units/ml) at pH 8.5. The activities at 8 μM - or 600 μM -CoA were obtained from the increase of A_{363} minus that of a control from which CoA was omitted and to which 23 μM -dithiobis-(2-nitrobenzoic acid) was added. The control rate needed correcting because CoA resulting from the enzymic plus non-enzymic hydrolysis of acetoacetyl-CoA continuously increased the concentration of 2-nitro-5-thiobenzoic acid anion, which also absorbs light at 363 nm. Preliminary experiments showed that the absorption coefficient of this anion at 363 nm was approx. 25% of that at 412 nm, at which wavelength the absorption by reduced acetylpyridine-adenine dinucleotide was insignificant. Therefore at the end of the assay, the rate of increase in absorbance of the control cuvette was measured at 412 nm. The rate of increase in A_{363} owing to acetoacetyl-CoA hydrolysis was calculated as 25% of the rate of increase in A_{412} and was then subtracted from the rate observed at 363 nm to produce the corrected control rate. Finally, the rates of increase of A_{363} owing to thiolase activity were divided by 2, since 2 mol of acetyl-CoA are produced per mol of acetoacetyl-CoA consumed.

All enzyme assays were initiated by adding samples (2–25 μl) of extract or homogenate to the assay medium, and the activities were measured at 25°C with a recording spectrophotometer (Gilford model 252 attachment to Unicam SP.500) or, for the assay of carnitine palmitoyltransferase, with a Unicam SP.600 spectrophotometer. The activities (Tables 2–5) are given as mean values $\pm$ S.E.M., with the numbers of determinations in parentheses. Preliminary experiments showed that the assay conditions were optimal for the enzymes in rat, guinea pig and cow mammary tissue, and this was assumed to be the case for the other tissues. As stated previously (Crabtree & Newsholme, 1972a), the use of these activities for precise quantitative deductions should be made with caution.

Results and discussion

Enzyme activities and metabolic fluxes

The results in Table 2 show that the activities of hexokinase and phosphofructokinase are significantly greater than the fluxes that they transmit, although in ruminant and rabbit tissue the activity of hexokinase is reasonably close to the rate of glucose phosphorylation *in vivo*. The results in

Table 3 show that, with the possible exception of ruminant tissues, the activities of acetyl-CoA synthetase are several times greater than the flux transmitted *in vivo*. Therefore, in lactating mammary tissue, the maximum activities of phosphofructokinase and (in non-ruminants) acetyl-CoA synthetase and hexokinase cannot be used to estimate maximum fluxes *in vivo*. This contrasts with muscle (Crabtree & Newsholme, 1975) and may reflect the need for mammary tissue to respond directly to changes in blood acetate and glucose concentrations (Baldwin & Smith, 1971; Kuhn, 1978). This would require an excess enzymic capacity compared with muscle, in which the major fuel-metabolizing pathways are controlled principally (and, in the case of glycolysis, almost exclusively) by feedback effects in response to changes in the demand for ATP (see Newsholme & Start, 1973; Newsholme & Crabtree, 1979).

Comparative aspects of mammary-gland enzyme activities and intracellular distribution

Enzymes of carbohydrate metabolism. The activities of these enzymes (Table 2) suggest that rat and mouse mammary tissues have the largest glycolytic potential, with that of guinea pig, sow and rabbit tissue being intermediate; lowest activities were found in ruminant tissues. These results agree with those of previous investigators (see Baldwin & Yang, 1974) and correlate reasonably well with the capacity of tissue slices to convert glucose into fatty acids (Strong & Dils, 1972) and with the activities of ATP citrate lyase (Table 3).

The presence of fructose biphosphatase in mammary tissue was first reported by Baird (1969), and the activities given in Table 2 confirm and extend his findings. All tissues investigated had low or undetectable activities of this enzyme, except those from rabbits and ruminants. In mammary gland, this enzyme is probably involved in the recombination of triose phosphates to fructose 6-phosphate to increase the amount of NADPH produced per mol of glucose 6-phosphate used in the pentose phosphate pathway. In ruminants this could be an important means of conserving glucose (see Smith, 1971), but the relatively high activity in rabbit tissue is difficult to explain. The blood glucose concentration in rabbits (approx. 6 mM; Jones & Parker, 1978) is similar to that in rats, which does not suggest that there is any restricted availability of glucose. However, the rate of fatty acid synthesis *de novo* (and hence the demand for NADPH) seems to be high in rabbit mammary tissue (see Taylor, 1979), and this may require a more efficient pentose phosphate pathway.

Enzymes of fatty acid and acetate metabolism. The activities of 3-hydroxyacyl-CoA dehydrogenase and carnitine palmitoyltransferase varied by only

Table 2. Enzyme activities and fluxes of carbohydrate metabolism

Enzyme activities are expressed as $\mu\text{mol}/\text{min}$ per g wet wt. at 25°C. Fluxes represent μmol of substrate used/min per g wet wt. at 25°C (assuming that the rate at 25°C is $0.5 \times$ that at 37°C) and are collated from the following sources: ^a Hawkins & Williamson (1972), assuming 20 g of tissue per rat and a rate of lactose synthesis of $0.22 \mu\text{mol}$ of glucose/min per g of tissue (Wilde & Kuhn, 1979); ^b Jones & Parker (1978), assuming a blood flow of $70 \text{ ml}/\text{min}$ per 100 g of tissue and a rate of lactose synthesis of $0.2 \mu\text{mol}$ of glucose/min per g; ^c Linzell <i>et al.</i> (1969), assuming a rate of lactose synthesis of $0.2 \mu\text{mol}$ of glucose/min per g; ^d Annison & Linzell (1964); ^e Bickerstaffe <i>et al.</i> (1974); ^f R. A. Smith, unpublished work; ^g Baird (1969); the fluxes glucose $\rightarrow$ glucose 6-phosphate and fructose 6-phosphate $\rightarrow$ fructose 1,6-bisphosphate were calculated as (glucose uptake $-\frac{1}{2}$ glucose released as lactose) and (glucose uptake $-\frac{1}{2}$ glucose released as lactose) respectively; the latter is overestimated because allowance was not made for the pentose phosphate pathway.								
Enzyme or flux	Mouse	Rat	Guinea pig	Rabbit	Sow	Goat	Sheep	Cow
Glucose $\rightarrow$ glucose 6-phosphate	—	0.6^a	0.45^f	0.4^b	0.1^c	0.2^d	—	0.15^e
Hexokinase	13 ± 1.4 (4)	5.2 ± 0.6 (5)	5.9 ± 1.4 (4)	0.8 ± 1.5 (4)	—	0.4 ± 0.1 (3)	0.6 ± 1 (3)	0.35 ± 0.05 (3)
Fructose 6-phosphate $\rightarrow$ fructose bisphosphate	—	0.55^a	0.4^f	0.35^b	0.05^c	0.15^d	—	0.05^e
Phosphofructokinase	9.2 ± 0.6 (4)	2.9 ± 0.3 (6)	1.5 ± 0.4 (4)	1.0 ± 0.2 (4)	1.4 ± 0.3 (3)	0.4 ± 0.1 (3)	0.6 ± 0.2 (3)	—
Fructose bisphosphatase	<0.02 (4)	0.06 ± 0.02 (4)	0.06 ± 0.02 (5)	0.35 ± 0.05 (4)	0.05 ± 0.04 (3)	0.4 ± 0.08 (3)	0.32 ± 0.05 (4)	0.52 ± 0.06^g (7)
Lactate dehydrogenase	155 ± 14 (8)	94 ± 6 (14)	38 ± 3 (7)	98 ± 11 (6)	49 ± 8 (3)	23 ± 4 (3)	28 ± 2 (4)	19 ± 2 (7)

approx. 2-fold over the range of species investigated (Table 3). If these activities reflect fluxes, these results suggest that the capacity for fatty acid oxidation [which has been shown to occur *in vivo* in sheep and goat mammary gland: Annison *et al.* (1968); Massart-Leen *et al.* (1970)] is a basic parameter which is only slightly affected by differences in the nature and amounts of fuel used for milk synthesis. It was previously observed that the activities of 2-oxoglutarate dehydrogenase in mammary tissue also varied little with species, in contrast with the capacity for converting pyruvate into acetyl-CoA (Read *et al.*, 1977).

The intracellular distribution of 3-hydroxyacyl-CoA dehydrogenase was predominantly mitochondrial in all species investigated (Table 1). This is consistent with a principal role for the enzyme in the β -oxidation of fatty acids. However, cytoplasmic fatty acid synthesis may be initiated by butyryl-CoA rather than acetyl-CoA (Nandekhar & Kumar, 1969; Lin & Kumar, 1972), and the synthesis of butyryl-CoA may require a cytoplasmic 3-hydroxyacyl-CoA dehydrogenase. Such an activity has been found in rabbit mammary gland, and, since the enzyme preferred the D-isomer of 3-hydroxybutyrate, the activity was not due to leakage of mitochondrial enzyme (Nandekhar & Kumar, 1969). The results of the present investigation (Table 1) suggest that any cytoplasmic 3-hydroxybutyrate dehydrogenase activity would not exceed approx. 10% of the total activity, and hence would be less than 0.3–0.7 $\mu\text{mol/min per g}$ (from the total activities in Table 3).

The activities of acetyl-CoA synthetase varied more with species than did the enzymes of fatty acid oxidation, with the highest activity being observed in guinea-pig mammary tissue (Table 3). Since acetate has been shown to be an important precursor of milk fatty acids in ruminants (Linzell *et al.*, 1967; Bickerstaffe *et al.*, 1974) and guinea pigs (Mepharm *et al.*, 1976), the variations of acetyl-CoA synthetase activity could reflect variations in the capacity to synthesize fatty acids from acetate. One problem with this interpretation is the relatively high activity of the enzyme in rat and mouse mammary tissue. The concentration of acetate in rat blood (approx. 0.25 mM; Buckley & Williamson, 1977) is lower than in ruminants (1–1.5 mM; Linzell, 1974), and acetate utilization by rat mammary tissue is thought to be unimportant at physiological concentrations (Kuhn, 1978). Nevertheless, the high activity of acetyl-CoA synthetase (Table 3) and its predominantly cytoplasmic location in rat mammary tissue (Table 1) suggest that acetate may sometimes serve as an important lipogenic precursor.

The intracellular location of acetyl-CoA synthetase (Table 1) showed a marked variation with species. In agreement with previous investigations

Table 3. *Acetate fluxes and enzymes of acetate and fatty acid metabolism*

Enzyme activities are expressed as $\mu\text{mol/min per g wet wt. at } 25^\circ\text{C}$. The fluxes are collated from the sources given in Table 1 plus the following: ^h Mepharm <i>et al.</i> (1976); ⁱ Spincer <i>et al.</i> (1969), assuming a blood flow of 70 ml/min per 100 g of tissue; ^k Snowswell & Linzell (1975), measured as palmitoyl carnitine formation.		Enzyme or flux		Mouse	Rat	Guinea pig	Rabbit	Sow	Goat	Sheep	Cow
Acetate $\rightarrow$ acetyl-CoA				—	—	1.05 ^h	0.15 ^b	0.05 ^j	0.35 ^d	—	0.2 ^e
Acetyl-CoA synthetase				1.3 $\pm$ 0.2 (6)	1.0 $\pm$ 0.2 (4)	3.6 $\pm$ 0.5 (4)	1.1 $\pm$ 0.3 (5)	0.6 $\pm$ 0.1 (3)	0.2 $\pm$ 0.04 (3)	0.6 $\pm$ 0.3 (4)	0.3 $\pm$ 0.05 (4)
Carnitine palmitoyltransferase				0.25 $\pm$ 0.04 (3)	0.5 $\pm$ 0.03 (4)	0.36 $\pm$ 0.03 (3)	0.28 $\pm$ 0.04 (3)	—	0.37 $\pm$ 0.06 (3)	0.75 ^(k) (4)	0.36 $\pm$ 0.07 (3)
3-Hydroxyacyl-CoA dehydrogenase				6.1 $\pm$ 0.5 (3)	3.6 $\pm$ 0.5 (4)	7.4 $\pm$ 0.8 (4)	4.5 $\pm$ 0.8 (4)	3.0 $\pm$ 0.4 (4)	—	3.9 $\pm$ 0.1 (3)	5.7 $\pm$ 0.7 (3)
ATP citrate lyase				6.1 $\pm$ 1.4 (3)	3.2 $\pm$ 0.4 (5)	0.4 $\pm$ 0.1 (7)	1.4 $\pm$ 0.6 (4)	0.5 $\pm$ 0.06 (3)	<0.02 (3)	<0.02 (2)	<0.02 (3)
Citrate synthase				69 $\pm$ 10 (8)	40 $\pm$ 4 (14)	15 $\pm$ 0.9 (6)	20 $\pm$ 3 (5)	25 $\pm$ 4 (3)	9 $\pm$ 0.7 (3)	9 $\pm$ 1.9 (4)	5.5 $\pm$ 0.7 (7)

(cited by Williamson *et al.*, 1975), the activity was predominantly cytoplasmic in the rat, and this was also the case for the guinea pig. However, in the mouse the activity was predominantly mitochondrial. This somewhat surprising difference between the rat and mouse, which were otherwise quite similar in their enzyme activities (Tables 2–5) and distributions (Table 1), could indicate that mouse mammary tissue oxidizes large amounts of acetate. However, this is unlikely, because the maximum capacity of the tricarboxylic acid cycle in this tissue is only approx. $0.2 \mu\text{mol/min per g}$ at 25°C , estimated from the activity of 2-oxoglutarate dehydrogenase (Read *et al.*, 1977). An alternative explanation is that in mouse mammary tissue acetate for fat synthesis is converted into acetyl-CoA in the mitochondria and is then transported into the cytoplasm as citrate (i.e. in the same way as the acetyl-CoA produced from pyruvate; see Bauman & Davis, 1974). This process would result in an increased rate of ATP utilization (owing to ATP citrate lyase activity plus regeneration of mitochondrial oxaloacetate by pyruvate carboxylase), compared with a direct cytoplasmic conversion of acetate into acetyl-CoA. This increased rate of ATP utilization may help to prevent the conversion of glucose into fatty acids (which may be quite high in this tissue, as evidenced by the activities in Tables 2 and 3) from being limited by an accumulation of NADH and hence ATP (see Flatt, 1970).

In sheep and rabbit mammary glands significant activities of acetyl-CoA synthetase were observed in both cytoplasm and mitochondria (Table 1). The mitochondrial activity may reflect acetate oxidation (which can account for as much as 70% of the acetate taken up by goat mammary gland; Annison & Linzell, 1964), and the cytoplasmic activity may reflect the use of acetate for fatty acid synthesis. The present results for sheep tissue differ from those of a previous investigation, which indicated a predominantly cytoplasmic location for the enzyme (Snoswell & Linzell, 1975). These workers, using a 'tissue-press' technique, obtained a cytoplasmic fraction which contained almost all of the acetyl-CoA synthetase activity, but only 2% of the citrate synthase activity of the tissue. Therefore the dif-

ference between their results and those of the present investigation is not due to leakage of enzyme from the mitochondria: it may be due to a difference in dietary status, resulting in different amounts of acetate available to the gland.

The activities of ATP citrate lyase (Table 3) were similar to those measured by previous workers (see Baldwin & Yang, 1974; Bauman & Davis, 1974) and may reflect the relative rates of conversion of pyruvate (and hence glucose) into fatty acids. Citrate synthase is also involved in this pathway, since mitochondrial acetyl units are transported into the cytoplasm as citrate. The presence of this flux through citrate synthase, in addition to that of acetyl-CoA oxidation via the tricarboxylic acid cycle, could explain the high activities of this enzyme in mouse and rat and the low activities in ruminant tissue (Table 3). In ruminants citrate synthase is involved only in acetyl-CoA oxidation, because the rate of fatty acid synthesis from pyruvate is insignificant (see Smith & Taylor, 1977). It should be noted that the activities of citrate synthase reported here (Table 3) are greater than those reported by previous workers (e.g. Gumaa *et al.*, 1973). This is most likely due to the greater degree of mitochondrial disruption obtained with deoxycholate (see the Materials and methods section).

Enzymes of ketone-body metabolism. Except for the rat (Page & Williamson, 1972), the activities of ketone-body-metabolizing enzymes do not appear to have been measured in mammary tissue. The activity of 3-oxo acid CoA-transferase, which catalyses the initial reaction of acetoacetate oxidation, was highest in rat mammary tissue, intermediate in guinea pig and mouse, and low in ruminants, sow and rabbit (Table 4). If the activity of this enzyme reflects ketone-body oxidation, it is somewhat surprising that low activities were observed in ruminant tissue, since these animals have a moderately high concentration of ketone bodies in the circulation at all times (see Linzell, 1974). However, the low activities are consistent with the results of Linzell *et al.* (1967), who found that 3-hydroxybutyrate oxidation contributed only 1–2% of the total CO_2 production of lactating goat mammary gland.

Table 4. *Enzymes of ketone-body metabolism*
Enzyme activities are expressed as $\mu\text{mol/min per g}$ of tissue at 25°C .

Enzyme	Mouse	Rat	Guinea pig	Rabbit	Sow	Sheep	Cow
3-Oxo acid CoA-transferase	13 ± 1.2 (3)	29 ± 5 (4)	8 ± 1.3 (4)	1.4 ± 0.1 (4)	2.5 ± 0.4 (3)	1.9 ± 0.4 (3)	1.9 ± 0.3 (3)
Acetoacetyl-CoA thiolase ($600 \mu\text{M-CoA}$)	5.0 ± 0.5 (4)	4.2 ± 0.3 (4)	5.8 ± 0.4 (4)	4.9 ± 1 (4)	2.2 ± 0.3 (3)	2.1 ± 0.7 (3)	0.9 ± 0.2 (3)
Acetoacetyl-CoA thiolase ($8 \mu\text{M-CoA}$)	4.2 ± 0.6 (5)	1.3 ± 0.2 (4)	12 ± 3 (4)	5.4 ± 0.9 (4)	2.0 ± 0.3 (3)	0.6 ± 0.1 (3)	0.4 ± 0.1 (3)

The retention of 3-oxo acid CoA-transferase in the tissue during fractional extraction indicates that it is predominantly, if not exclusively, mitochondrial in all species investigated (Table 1). Therefore acetoacetyl-CoA produced by this enzyme is probably unavailable to the cytoplasm (e.g. to serve as a C_4 primer for fatty acid synthesis; see above), unless there is some system for transporting acetoacetyl units across the mitochondrial membrane.

The activities of acetoacetyl-CoA thiolase were measured at high ($600\mu\text{M}$) and low (approx. $8\mu\text{M}$) concentrations of CoA. The activity measured at low [CoA] is predominantly that of an enzyme for which K_m (CoA) is less than $3\mu\text{M}$. The activity measured at high [CoA] is that of an enzyme (or enzymes) for which K_m (CoA) is approx. $20\mu\text{M}$, because at $600\mu\text{M}$ -CoA the low- K_m enzyme is almost totally inhibited (Middleton, 1973).

The fractional extraction of thiolase measured at $600\mu\text{M}$ -CoA, (Table 1) showed a predominantly mitochondrial location in all species investigated, except for rabbit and guinea pig: these results agree with those of Middleton (1973) for other tissues. This mitochondrial activity may reflect the capacity for ketone-body and fatty acid oxidation, and the results in Table 4 show that (except for the cow) it varied little with species. This is similar to the somewhat limited variation of other enzymes of fatty acid oxidation (see above). The anomalous fractionation results with thiolase assayed at $600\mu\text{M}$ -CoA in rabbit and guinea-pig tissue (Table 1) suggest that these tissues possess either a significant activity of high- K_m ('mitochondrial'-type) enzyme in the cytoplasm or a cytoplasmic enzyme which is less sensitive to inhibition by CoA.

The fractional extraction of thiolase measured at $8\mu\text{M}$ -CoA (Table 1) showed a predominantly cytoplasmic location in all species, except ruminants. The total activities varied more with species than did those measured at $600\mu\text{M}$ -CoA, being highest in guinea-pig and rabbit tissue and very low in ruminants (Table 4). The role of the cytoplasmic enzyme is unclear at present. It could be involved in converting cytoplasmic acetoacetate into acetyl-CoA for fatty acid synthesis; this would probably require a cytoplasmic conversion of acetoacetate into acetoacetyl-CoA, because 3-oxo acid CoA-transferase is predominantly mitochondrial (Table 1). A cytoplasmic enzyme which converts acetoacetate into acetoacetyl-CoA has been found in rat mammary tissue (Buckley & Williamson, 1973; 1975; Robinson & Williamson, 1978), but no investigations appear to have been carried out with other species. Alternatively, the cytoplasmic thiolase could be involved in the synthesis of acetoacetyl-CoA for use as, or conversion into, a C_4 primer for fatty acid synthesis (Buckley & Williamson, 1975).

The fractional-extraction results for ruminant

tissues suggest that they possess such low activities of the cytoplasmic thiolase that the activity measured at $8\mu\text{M}$ -CoA is predominantly that of the mitochondrial enzyme. This conclusion is consistent with the results of Smith & McCarthy (1969), who found that, with slices of cow mammary tissue, 3-hydroxybutyrate was incorporated into fatty acids predominantly as the original C_4 unit, whereas with slices of rat tissue there was a 95% cleavage and subsequent recombination of the C_4 unit before incorporation. If ketone bodies are converted into acetoacetyl-CoA in the cytoplasm (see above), then in rat tissue, which possesses a high activity of cytoplasmic thiolase (Tables 1 and 4), there would be an extensive cleavage and recombination of the acetoacetyl-CoA; in ruminant tissue the rate of cytoplasmic acetoacetyl-CoA cleavage would be so low that most of the ketone body is incorporated into fatty acids as the original C_4 unit.

Enzymes of pathways for providing cytoplasmic NADPH. NADP-malate dehydrogenase ('malic' enzyme) and pyruvate carboxylase are considered to be involved in a 'pyruvate-malate' cycle, the operation of which transfers reducing equivalents from NADH to NADP^+ and thence to fatty acids (see Martin & Denton, 1970; Bauman & Davis, 1974). In agreement with previous workers (see Baldwin & Yang, 1974; Bauman & Davis, 1974), significant activities of NADP-malate dehydrogenase (and hence the capacity for a pyruvate-malate cycle) were observed only in rat and mouse mammary tissue (Table 5), and the enzyme was predominantly cytoplasmic (Table 1). In contrast, significant activities of pyruvate carboxylase (which was predominantly mitochondrial; see also Taylor *et al.*, 1978) were observed in all species (Tables 1 and 5). This suggests that, unlike NADP-malate dehydrogenase, pyruvate carboxylase is not restricted to the pyruvate-malate cycle, but also supplies mitochondrial oxaloacetate and hence tricarboxylic acid-cycle intermediates for other purposes, possibly amino acid biosynthesis (see Clark *et al.*, 1978).

It has been suggested that ruminant mammary glands use a transhydrogenase based on NADP-isocitrate dehydrogenase (Bauman *et al.*, 1970; Gumaa *et al.*, 1973; Bauman & Davis, 1974). In this system cytoplasmic isocitrate is used to generate NADPH for fat synthesis. The resulting 2-oxoglutarate (or possibly a related metabolite such as glutamate) re-enters the mitochondria and regenerates isocitrate via reversal of the mitochondrial NADP-isocitrate dehydrogenase. The activities of NADP-isocitrate dehydrogenase (Table 5) were highest in ruminant and guinea-pig tissue, and the distribution of the enzyme showed a predominantly cytoplasmic location in these species (Table 1). These results are consistent with a role for NADP-

Table 5. *Enzymes of 'satellite' systems for producing cytoplasmic NADPH*
Enzyme activities are expressed as $\mu\text{mol}/\text{min}$ per g of tissue at 25°C

Enzyme	Mouse	Rat	Guinea pig	Rabbit	Sow	Goat	Sheep	Cow
NADP-malate dehydrogenase	12.3 ± 1.5 (5)	14.3 ± 2 (4)	<0.02 (4)	<0.02 (3)	<0.02 (3)	<0.02 (3)	<0.02 (3)	<0.02 (3)
NAD-malate dehydrogenase	301 ± 10 (4)	152 ± 28 (4)	159 ± 14 (4)	123 ± 19 (3)	—	—	71 ± 8 (3)	46 ± 4 (3)
Pyruvate carboxylase	6.0 ± 1.5 (4)	2.0 ± 0.1 (4)	0.8 ± 0.2 (4)	0.7 ± 0.1 (3)	0.3 ± 0.03 (3)	0.2 ± 0.02 (3)	0.15 ± 0.08 (3)	0.18 ± 0.03 (3)
NADP-isocitrate dehydrogenase	2.0 ± 0.2 (7)	2.4 ± 0.2 (5)	9.2 ± 0.4 (5)	3.9 ± 0.4 (4)	2.2 ± 0.3 (3)	5.0 ± 0.9 (3)	10.3 ± 0.5 (4)	10.2 ± 3 (5)
Glutamate dehydrogenase	—	3.3 ± 0.2 (4)	5.6 ± 0.5 (5)	6.9 ± 1 (4)	4.6 ± 0.8 (3)	—	4.2 ± 0.3 (4)	1.5 ± 0.3 (5)

isocitrate dehydrogenase in generating cytoplasmic NADPH in ruminant and also in guinea-pig mammary tissue, although other roles for the cytoplasmic enzyme are possible, e.g. generating 2-oxoglutarate for amino acid synthesis. In other species the distribution of NADP-isocitrate dehydrogenase was towards the mitochondria (Table 1), and the total activities were also lower (Table 5), suggesting a lesser importance for this system. Gumaa *et al.* (1973) have suggested that glutamate dehydrogenase serves as a mitochondrial transhydrogenase for the isocitrate dehydrogenase shuttle in ruminants. However, in the present investigations the activities of glutamate dehydrogenase (Table 5) showed little if any correlation with those of NADP-isocitrate dehydrogenase; in particular, the enzyme was as active in ruminant as in rat tissue. The large difference in glutamate dehydrogenase activity between rat and sheep mammary tissue reported by Gumaa *et al.* (1973) may have been due to an incomplete release of the enzyme from rat tissue, since no detergents were used to release latent mitochondrial enzymes (see Taylor *et al.* 1978).

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