Utilization of D-3-Hydroxy[3-¹⁴C]butyrate for Lipogenesis *in vivo* in Lactating Rat Mammary Gland

By ALISON M. ROBINSON and DERMOT H. WILLIAMSON
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary,
Oxford OX2 6HE, U.K.

(Received 18 August 1978)

Incorporation of D-3-hydroxy[3-¹⁴C]butyrate into lipid *in vivo* suggests that lactating mammary gland is a major site of ketone-body utilization. The incorporation decreases in short-term insulin deficiency (2h) and on starvation (24h), but increases again on re-feeding (2h). The activity of cytosolic acetoacetyl-CoA synthetase parallels the changes in nutritional state, but is not affected by short-term insulin deficiency.

Ketone bodies may regulate rat mammary-gland metabolism and provide an alternative substrate for lipogenesis and energy supply (Williamson *et al.*, 1975). Studies *in vitro* have shown that acetoacetate increases glucose utilization and hence lipogenesis, and that acetoacetate taken up by the gland is both oxidized and incorporated into lipid (Williamson *et al.*, 1975; Robinson & Williamson, 1977c). 3-Hydroxybutyrate has also been shown to be a lipid precursor in preparations of rat mammary gland *in vitro* (Kinsella, 1970; Bartley, 1976; A. M. Robinson & D. H. Williamson, unpublished work). The finding that glucose utilization by the gland was decreased when blood ketone-body concentrations in fed lactating rats were acutely increased by acetoacetate injection provided evidence *in vivo* for the proposed regulatory role of ketone bodies (Robinson & Williamson, 1977b). To investigate the possible role of ketone bodies as a lipid precursor in the gland *in vivo*, the incorporation of radioactivity into mammary-gland lipid was measured after injection of trace amounts of D-3-hydroxy[3-¹⁴C]butyrate into lactating rats in various nutritional and hormonal states. In addition, acetoacetyl-CoA synthetase, which may be involved in a cytosolic pathway for the incorporation of ketone bodies into lipid (Buckley & Williamson, 1973, 1975), was assayed in acini isolated from the gland.

Experimental

Lactating rats of the Wistar strain (250–350 g) with between 6 and 14 pups were used after a lactation period of between 10 and 18 days. Virgin rats (200–300 g) were used for comparison. The rats were fed *ad libitum* on Oxoid breeding diet (Oxoid, London S.E.1, U.K.). Starved rats (with pups) were deprived of food for 24 h and starved-refed rats were allowed

Oxoid pellets for 2 h after 24 h of starvation. Insulin deficiency was induced by streptozotocin treatment 2 h before experiments (Robinson & Williamson, 1977a).

Rats were anaesthetized with Nembutal (50 mg/kg body wt.; solution in 0.9% NaCl), and D-3-hydroxy[3-¹⁴C]butyrate (3.1 μ Ci and 0.14 μ mol in 0.5 ml of 0.9% NaCl; The Radiochemical Centre, Amersham, Bucks., U.K.; used undiluted) was injected into the tail vein. Rats were separated from their litters and kept warm on a heated stage maintained at 40°C. Blood (0.2 ml) was collected from the tip of the tail into heparinized tubes at 2, 4, 6, 8, 11 and 15 min. At 20 min after injection duplicate samples of inguinal mammary gland, parametrial adipose tissue and liver were excised and arterial blood was collected from the aorta into a heparinized tube. Weighed samples of the tissues (about 1 g in duplicate) and arterial blood (1 ml) were added to 3 ml of 30% (w/v) KOH, and lipid was saponified and extracted by the method of Stansbie *et al.* (1976). Blood samples were added to 6% (w/v) HClO₄, and radioactivity (Williamson *et al.*, 1975) and acetoacetate and D-3-hydroxybutyrate (Williamson *et al.*, 1962) were determined in the neutralized extracts.

Acini were prepared as described by Robinson & Williamson (1977a). The pellet obtained after washing with Krebs–Henseleit (1932) saline was suspended in 4 vol. (v/w) of ice-cold 0.25 M-sucrose in 10 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-2-mercaptoethanol, and homogenized (15 passes) with a motor-driven Teflon homogenizer. After centrifugation at 30 000 g for 30 min at 4°C the supernatant was used for assay of acetoacetyl-CoA synthetase (Buckley & Williamson, 1973). Assay of glutamate dehydrogenase (Schmidt, 1963) was used to confirm minimal contamination by enzymes of the mitochondrial matrix, and protein was determined by using the Folin–Ciocalteu reagent (Layne, 1957).

Results and Discussion

In fed lactating rats incorporation of D-3-hydroxy-[3-¹⁴C]butyrate into lipid in the gland was 5-fold and 100-fold greater than that of liver and adipose tissue respectively (Table 1). Incorporation of radioactivity into mammary-gland lipid was 50-fold greater in lactating rats compared with virgin rats. Starvation (24h) decreased radioactivity in mammary gland by 10-fold, and in liver by 40-fold, and these changes were reversed on refeeding (2h). Streptozotocin treatment caused a 10-fold decrease in lipid incorporation in the gland, whereas liver and adipose tissue were less markedly affected (Table 1). These large changes in lipogenesis are not likely to be due to changes in specific radioactivity of D-3-hydroxy-[3-¹⁴C]butyrate, since lipid incorporation did not show a negative correlation with blood ketone-body concentrations for the individual rats. Furthermore, injection of dimethylpyrazole, an inhibitor of lipolysis (Williamson *et al.*, 1971), into starved lactating rats decreased blood ketone-body concentrations to the fed value, whereas lipid incorporation was not increased above the rate found for starved rats (results not shown).

Incorporation of radioactivity into lipid in arterial blood was 0.0067 ± 0.0056 (4) and 0.0044 ± 0.0033 (4) % dose/ml of whole blood in fed lactating and fed virgin rats respectively. Secretion of labelled lipid into the blood from the liver is not likely to be an important source of ¹⁴C-labelled lipid in the mammary gland, since labelled triacylglycerol does not appear in the blood until 10 to 15 min after injection of labelled fatty acid (Laurell, 1959; Goransson & Olivecrona, 1964; Robinson, 1970). The total radioactivity in blood from the tail vein decreased rapidly after injection of D-3-hydroxy-[3-¹⁴C]butyrate with a half-life of between 3 and 4 min, which agrees with the value found previously

(Bates *et al.*, 1968) for male rats. The amount of D-3-hydroxybutyrate injected represented less than 5% of the total ketone-body pool of fed or starved rats and ketone-body concentrations remained constant during the course of the experiment. Although the radioactive ketone body injected was D-3-hydroxy[3-¹⁴C]butyrate, this would rapidly reach a partial isotopic equilibrium with the acetoacetate in the circulation (Bates *et al.*, 1968).

Acetoacetyl-CoA synthetase activity in acini from fed rats (Table 2) was similar to the rate of [3-¹⁴C]acetoacetate incorporation into lipid in acini of 10–20 μmol/h per g wet wt. (Robinson & Williamson, 1977c). The activity was slightly greater than that found previously in rat mammary gland (Buckley & Williamson, 1975) consistent with the loss of connective and adipose tissue in the preparation of acini. The *K_m* for acetoacetate was about 40 μM, which is similar to the value found for the enzyme from rat brain (Buckley & Williamson, 1973). In starvation, the activity of acetoacetyl-CoA synthetase decreased almost 3-fold, although on refeeding it increased to a value intermediate between that of fed and starved rats (Table 2). Streptozotocin treatment (2h) did not affect acetoacetyl-CoA synthetase activity. Thus on starvation and refeeding acetoacetyl-CoA synthetase activity shows similar changes, although of a lesser magnitude, as lipid incorporation.

In fed lactating rats, assuming that the total wet weight of gland is 24.2 g (Chatwin *et al.*, 1969), lipid incorporation in the mammary gland may account for as much as 20% of the dose of D-3-hydroxy-[3-¹⁴C]butyrate. The present study is concerned with lipid incorporation, but oxidation accounts for up to 65% of the net ketone-body utilization in rat mammary gland *in vitro* (Williamson *et al.*, 1975; Robinson & Williamson, 1977c). Hence the mammary gland may be the major site of ketone-body utiliza-

Table 1. Incorporation of D-3-hydroxy[3-¹⁴C]butyrate into lipid of mammary gland, liver and adipose tissue of lactating and virgin rats

For details of experimental procedure see the text. Incorporation of radioactivity into lipid of mammary gland, liver and adipose tissue is expressed as % dose/g wet wt. of tissue. Ketone-body concentrations (as μmol/ml of whole blood) are included for comparison. The results are mean values $\pm$ S.D., with the numbers of rats shown in parentheses. Values that are significantly different from those of the fed lactating group are shown: **P* < 0.05; ***P* < 0.0005.

State of rats	¹⁴ C incorporation into				Blood [ketone bodies]
	Mammary gland	Liver	Adipose tissue		
Lactating-fed (4)	0.82 $\pm$ 0.21	0.17 $\pm$ 0.15	0.0077 $\pm$ 0.0052		0.15 $\pm$ 0.05
Lactating-starved (24h) (4)	0.091 $\pm$ 0.080**	0.0045 $\pm$ 0.0010*	0.0032 $\pm$ 0.0015		0.65 $\pm$ 0.47
Lactating-starved (24h)+refed (2h) (4)	0.87 $\pm$ 0.26	0.13 $\pm$ 0.12	0.014 $\pm$ 0.010		0.16 $\pm$ 0.03
Lactating-fed, streptozotocin-treated (2h) (4)	0.079 $\pm$ 0.015**	0.11 $\pm$ 0.05	0.0043 $\pm$ 0.005		0.22 $\pm$ 0.06
Virgin-fed (5)	0.017, 0.014	0.12 $\pm$ 0.11	0.009 $\pm$ 0.011		0.16 $\pm$ 0.08
Virgin-starved (24h) (4)	—	0.0092 $\pm$ 0.0031	0.0022 $\pm$ 0.0004		0.97 $\pm$ 0.26

Table 2. Activity of acetoacetyl-CoA synthetase in acini from lactating rat mammary gland

For details of assay see the Experimental section. The results, expressed as $\mu\text{mol/h}$ per g wet wt. and as $\mu\text{mol/h}$ per mg of protein, are mean values $\pm$ s.d., with the numbers of rats in each group shown in parentheses. Values that are significantly different from the fed group are shown: * $P < 0.05$; ** $P < 0.0005$.

State of rats	Activity	
	($\mu\text{mol/h}$ per g wet wt.)	($\mu\text{mol/h}$ per mg of protein)
Fed (7)	12.8 ± 2.9	0.26 ± 0.05
Starved (24h) (5)	$4.6 \pm 1.6^{**}$	$0.10 \pm 0.05^{**}$
Starved (24h) + refed (2h) (6)	$9.8 \pm 2.2^*$	$0.21 \pm 0.05^*$
Streptozotocin-treated (2h) (5)	15.5 ± 3.1	0.26 ± 0.06

tion in fed lactating rats. This provides some support for the suggestion that utilization by the mammary gland in addition to the decreased ketogenic capacity of the liver may be responsible for the low blood ketone-body concentrations in lactating rats (White-law & Williamson, 1977). However, the present findings do not support the proposal that ketone bodies may be an alternative lipogenic substrate for the mammary gland in starvation (Williamson *et al.*, 1975), since lipid incorporation was decreased 10-fold compared with fed rats. This does not preclude the use of ketone bodies by the gland as a respiratory fuel in starvation.

Lipid incorporation in the present studies showed similar trends to lipogenesis measured by $^3\text{H}_2\text{O}$ incorporation (Robinson *et al.*, 1978). The decreased insulin concentration in starvation and after streptozotocin treatment and its increase on re-feeding (Robinson *et al.*, 1978) may be responsible for the changes in incorporation of D-3-hydroxy-[3- ^{14}C]butyrate into lipid in the gland. Addition of insulin increases acetoacetate incorporation into lipid in the gland *in vitro*, but the exact site of insulin action is not known (Williamson *et al.*, 1975; Robinson & Williamson, 1977c).

The changes in activity of acetoacetyl-CoA synthetase in the mammary gland on starvation and refeeding may be explained in terms of stimulation of its synthesis by insulin (or some other hormone which also decreases in starvation and increases on refeeding). Acetoacetyl-CoA synthetase activity in liver and adipose tissue of rats and mice also changes on starvation and refeeding (Stern, 1971; Buckley & Williamson, 1975; Rous, 1977; Wolczunowicz & Rous, 1977) and insulin is thought to be involved in regulation of its activity in mouse liver (Rous, 1977). After streptozotocin treatment the lack of a decrease in acetoacetyl-CoA synthetase activity is not incon-

sistent with the proposed effect of insulin to promote its synthesis, because the turnover of the enzyme may be slow. The 10-fold decrease in lipid incorporation in the streptozotocin-treated rats suggests that this enzyme is not the key regulatory step in lipogenesis from ketone bodies.

In summary, ketone bodies may have a primary role as a regulator in rat mammary gland, but they are not an alternative lipogenic substrate to glucose in starvation. High activities of the enzymes of ketone-body utilization in the gland (Page & Williamson, 1972) may therefore be required to allow ketone bodies to express their regulatory effects, and to be a supplementary lipogenic substrate for the gland when concentrations are low and not inhibitory for glucose utilization (Robinson & Williamson, 1978).

This work was supported by the Medical Research Council and the United States Public Health Service (grant no. AM-11748). A. M. R. is a Commonwealth Scholar and an honorary New Zealand Ramsay Fellow. D. H. W. is a member of the External Staff of the Medical Research Council.

References

- Bartley, J. C. (1976) *Lipids* **11**, 774-777
- Bates, M. W., Krebs, H. A. & Williamson, D. H. (1968) *Biochem. J.* **110**, 655-661
- Buckley, B. M. & Williamson, D. H. (1973) *Biochem. J.* **132**, 653-656
- Buckley, B. M. & Williamson, D. H. (1975) *FEBS Lett.* **60**, 7-10
- Chatwin, A. L., Linzell, J. L. & Setchell, B. P. (1969) *J. Endocrinol.* **44**, 247-254
- Goransson, G. & Olivecrona, T. (1964) *Acta Physiol. Scand.* **62**, 224-239
- Kinsella, J. E. (1970) *Biochim. Biophys. Acta* **210**, 28-38
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33-66
- Laurell, S. (1959) *Acta Physiol. Scand.* **47**, 218-232
- Layne, E. (1957) *Methods Enzymol.* **3**, 448-450
- Page, M. A. & Williamson, D. H. (1972) *Biochem. J.* **128**, 459-460
- Robinson, A. M. & Williamson, D. H. (1977a) *Biochem. J.* **164**, 153-159
- Robinson, A. M. & Williamson, D. H. (1977b) *Biochem. J.* **164**, 749-752
- Robinson, A. M. & Williamson, D. H. (1977c) *Biochem. J.* **168**, 465-474
- Robinson, A. M. & Williamson, D. H. (1978) in *Biochemical and Clinical Aspects of Ketone Body Metabolism* (Soling, H. D. & Seufert, C. D., eds.), pp. 107-119, Academic Press, New York and London
- Robinson, A. M., Girard, J. R. & Williamson, D. H. (1978) *Biochem. J.* **176**, 343-346
- Robinson, D. S. (1970) in *Comprehensive Biochemistry* (Florkin, M. & Stotz, E. H., eds.), vol. 18, pp. 51-116, Elsevier, Amsterdam

- Rous, S. (1977) *Arch. Biochem. Biophys.* **179**, 328–333
- Schmidt, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 752–756, Academic Press, New York and London
- Stansbie, D., Brownsey, R. W., Crettaz, M. & Denton, R. M. (1976) *Biochem. J.* **160**, 413–416
- Stern, J. B. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1001–1007
- Whitelaw, E. & Williamson, D. H. (1977) *Biochem. J.* **164**, 521–528
- Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- Williamson, D. H., Mayor, F. & Veloso, D. (1971) in *Regulation of Gluconeogenesis* (Soling, H. D. & Willms, B., eds.), pp. 92–102, Academic Press, New York and London
- Williamson, D. H., McKeown, S. R. & Ilic, V. (1975) *Biochem. J.* **150**, 145–152
- Wolczunowicz, M. & Rous, S. (1977) *Life Sci.* **20**, 1347–1352