Control of Glucose Metabolism in Isolated Acini of the Lactating Mammary Gland of the Rat

THE ABILITY OF GLYCEROL TO MIMIC SOME OF THE EFFECTS OF INSULIN

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Inhibition of glucose uptake by acetoacetate and relief of this inhibition by insulin found previously in slices of rat mammary gland [Williamson, McKeown & Ilic (1975) *Biochem. J.* **150**, 145–152] was confirmed in acini, which represent a more homogeneous population of cells. Glycerol (1 mM) behaved like insulin (50 munits/ml) in its ability to relieve the inhibition of glucose (5 mM) utilization caused by acetoacetate (2 mM) in acini. Both glycerol and insulin reversed the increase in [citrate] and the decrease in [glycerol 3-phosphate] and the [lactate]/[pyruvate] ratio in the presence of acetoacetate. Lipogenesis from $^3\text{H}_2\text{O}$, $[3\text{-}^{14}\text{C}]\text{acetoacetate}$, $[1\text{-}^{14}\text{C}]\text{-}$ and $[6\text{-}^{14}\text{C}]\text{-glucose}$ was stimulated, whereas $^{14}\text{CO}_2$ formation from $[3\text{-}^{14}\text{C}]\text{acetoacetate}$ was decreased. Neither insulin nor glycerol relieved the acetoacetate inhibition of glucose uptake when lipogenesis was inhibited by 5-(tetradecyloxy)-2-furoic acid. From measurements of $[3\text{-}^{14}\text{C}]\text{acetoacetate}$ incorporation into lipid in the various situations it is suggested that a cytosolic pathway for acetoacetate utilization may exist in rat mammary gland. In the absence of acetoacetate, glycerol inhibited glucose utilization by 60% and increased both [glycerol 3-phosphate] and the [lactate]/[pyruvate] ratio. Possible ways in which glycerol may mimic the effects of insulin are discussed.

Previous work from this laboratory has shown that acetoacetate decreases glucose utilization in slices of lactating rat mammary gland and that addition of insulin reverses this inhibition (Williamson *et al.*, 1975). The interaction between the two substrates also occurs in rat heart (Williamson & Krebs, 1961; Randle *et al.*, 1964), diaphragm (Randle *et al.*, 1964), soleus muscle (Maizels *et al.*, 1977) and submaxillary gland (Thompson & Williamson, 1975). Present evidence indicates that the inhibition of glucose uptake by acetoacetate in these tissues is due to increased [citrate], which inhibits phosphofructokinase with subsequent elevation of [glucose 6-phosphate] and inhibition of hexokinase (Randle *et al.*, 1964). For the mammary gland, it has been postulated (Williamson *et al.*, 1975) that the decrease in flux through the glycolytic pathway causes an inhibition of lipogenesis because of the decreased availability of both glycerol 3-phosphate and acetyl-CoA, which in turn increases the concentration of NADPH, an inhibitor of glucose 6-phosphate dehydrogenase from various sources (Negelein & Haas, 1935; Glock & McLean, 1953; Bonsignore & De Flora, 1972; Eggleston & Krebs, 1974).

How insulin acts to reverse the inhibition of glucose utilization in lactating mammary-gland slices

is still an open question. Insulin reverses the inhibition of glucose uptake caused by acetoacetate in lactating mammary gland, but not in rat heart or diaphragm, which have comparatively low rates of lipogenesis compared with the gland. This suggests that the primary site of insulin action in lactating mammary gland may be in the lipogenic pathway. The use of slices has disadvantages in the study of mammary-gland metabolism, since slices contain adipocytes, whose metabolic properties, in particular their response to insulin, may differ from those of the acinar cells. Studies have indicated that active acini can be isolated from lactating mammary gland (Katz *et al.*, 1974) and that the metabolism of glucose by these acini reflects that of the gland *in vivo* in a number of different conditions (Robinson & Williamson, 1977a).

The aim of the present work was to see whether the effects of acetoacetate and insulin on glucose metabolism could also be demonstrated in acini and to test whether glycerol could mimic the effect of insulin. Glycerol was chosen because it is a potential physiological substrate of the gland, and lactating mammary gland contains an active glycerol kinase (Baldwin & Milligan, 1966), so that metabolism of glycerol can bypass the phospho-

fructokinase step in glycolysis and provide glycerol 3-phosphate. Increased provision of the latter could, via esterification, decrease the concentration of long-chain acyl-CoA, a potent inhibitor of acetyl-CoA carboxylase (Halestrap & Denton, 1973). In addition, the effects of an inhibitor of transmitochondrial membrane transport of citrate, 5-(tetradecyloxy)-2-furoic acid (Ribereau-Gayon, 1976) on the interactions between glucose, acetoacetate and insulin was studied.

Experimental

Materials

Rats. Rats of the Wistar strain (250–300 g) with between 6 and 14 pups were used after a lactation period of between 12 and 16 days. Rats were anaesthetized with Nembutal (50 mg/kg body wt.; solution in 0.9% NaCl) and the pups were allowed to continue to suckle before removal of the mammary glands.

Biochemicals. All enzymes, including collagenase (grade II), and coenzymes, were obtained from Boehringer Corp. (London), London W.5, U.K. Crystalline insulin free of glucagon was a gift of Dr. N. Z. Lazarus (Wellcome Research Laboratories, Beckenham, Kent, U.K.). 5-(Tetradecyloxy)-2-furoic acid was obtained from Dr. A. Richardson (Merrell National Laboratories, Lockland Station, Cincinnati, OH, U.S.A.).

Radioactive compounds. D-[1-¹⁴C]Glucose, D-[6-¹⁴C]glucose, ethyl [3-¹⁴C]acetoacetate and ³H₂O were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [3-¹⁴C]Acetoacetate was prepared from ethyl [3-¹⁴C]acetoacetate (Krebs *et al.*, 1966).

Methods

Preparation of acini. Acini were prepared by a modification of the procedure of Katz *et al.* (1974). For full details, see Robinson & Williamson (1977a).

Incubation procedure. The acini (about 100 mg wet wt.) were incubated in a total volume of 4 ml of Krebs–Henseleit (1932) saline containing glucose (5 mM) and other additions where indicated. The following concentrations of other compounds were routinely used: acetoacetate (2 mM); glycerol (1 mM); 5-(tetradecyloxy)-2-furoic acid (0.4 mM); insulin (50 munits/ml). For further details, see Robinson & Williamson (1977a).

Measurement of fatty acid synthesis with ³H₂O. Acini (about 100 mg wet wt.) were incubated with shaking at 37°C for 60 min in silicone-treated 25 ml conical flasks containing 0.5 mCi of ³H₂O, glucose (5 mM) and other substrates as indicated in a total volume of 4 ml of Krebs–Henseleit (1932) saline. The gas phase was O₂/CO₂ (19:1). Incubations were stopped with 0.4 ml of 60% (w/v) HClO₄ and the fatty

acids were extracted as described by Harris (1975). Since shorter-chain fatty acids formed by mammary gland are not completely extracted by pure heptane (Katz *et al.*, 1974), light petroleum (b.p. 40–60°C)/diethyl ether (9:1 v/v) was used instead of light petroleum for extraction of fatty acids after saponification of the lipid fraction.

Determination of metabolites. The following metabolites were determined by enzymic methods: glucose (Slein, 1963); L-lactate and pyruvate (Hohorst *et al.*, 1959); acetoacetate and D-3-hydroxybutyrate (Williamson *et al.*, 1962); glucose 6-phosphate and ATP (Lamprecht & Trautschold, 1963); L-glycerol 3-phosphate (Hohorst, 1963); citrate (Dagley, 1974).

Measurements of radioactivity. These were carried out as described by Williamson *et al.* (1975).

Expression and interpretation of results. These are described in full by Robinson & Williamson (1977a).

Results and Discussion

Effects of acetoacetate, insulin and glycerol on glucose metabolism

Glucose uptake by the isolated acini (Table 1) was similar to that found previously for acini (Katz *et al.*, 1974) and approx. 2-fold greater than that of mammary-gland slices (Williamson *et al.*, 1975). Acetoacetate (2 mM) decreased the utilization of glucose by the acini to 25% of the control rate (Table 1), which is an appreciably greater inhibition than that reported to occur with slices (Williamson *et al.*, 1975). Addition of insulin resulted in a reversal of the inhibition (to 72% of the control rate), but in the absence of acetoacetate, insulin did not stimulate glucose uptake (Table 1; Robinson & Williamson, 1977a). These results confirm the interactions between glucose and acetoacetate and insulin that occur in slices, and indicate that acini are still responsive to insulin after collagenase treatment. Other hormones, including sheep prolactin (0.1 μM), oxytocin (4 μM) and adrenaline (2 μM), were unable to reverse the inhibition of glucose uptake caused by acetoacetate (results not shown).

Glycerol (1 mM) behaved like insulin and increased glucose uptake in the presence of acetoacetate, to 85% of the rate found for incubations with glucose alone. However, when acetoacetate was absent glycerol decreased the uptake of glucose by 60% (Table 1). Glycerol removal was 0.33 ± 0.06 (11) μmol/min per 100 mg dry wt. in incubations containing glucose, glycerol and acetoacetate, and 0.18 ± 0.06 (9) when glucose and glycerol were present. Dihydroxyacetone, which, like glycerol, enters the glycolytic pathway at the triose phosphate stage, did not inhibit glucose uptake nor reverse the acetoacetate inhibition of glucose utilization. Likewise, acetate, which can generate acetyl-CoA, had no significant

Table 1. *Effects of acetoacetate, insulin and glycerol on glucose metabolism by mammary-gland acini from fed lactating rats*
For details of incubation procedure see the Experimental section. Where indicated glucose (5mM), acetoacetate (2mM), glycerol (1mM) or insulin (50munits/ml) were added to the incubation medium. The results, expressed as $\mu\text{mol}/\text{min}$ per 100mg defatted dry wt., are mean values $\pm$ s.d. for the numbers of observations shown in parentheses. Values that are statistically different (Student's *t* test) from incubations with glucose alone are shown:

**P < 0.0025.

Substrates added	Metabolite changes ($\mu\text{mol}/\text{min}$ per 100mg dry wt.)			[Lactate]/ [pyruvate]
	Glucose removed	Lactate formed	Pyruvate formed	
Glucose (39)	1.31 $\pm$ 0.21	0.37 $\pm$ 0.17	0.084 $\pm$ 0.032	4.4
Glucose+insulin (14)	1.29 $\pm$ 0.32	0.45 $\pm$ 0.20	0.081 $\pm$ 0.031	5.6
Glucose+acetoacetate (22)	0.33 $\pm$ 0.13**	0.12 $\pm$ 0.06**	0.087 $\pm$ 0.033	1.4
Glucose+acetoacetate+insulin (16)	0.94 $\pm$ 0.42**	0.32 $\pm$ 0.15	0.17 $\pm$ 0.05**	1.9
Glucose+acetoacetate+glycerol (14)	1.11 $\pm$ 0.19**	0.35 $\pm$ 0.16	0.079 $\pm$ 0.024	4.4
Glucose+glycerol (18)	0.54 $\pm$ 0.20**	0.32 $\pm$ 0.11	0.021 $\pm$ 0.020**	15.2

Table 2. *Metabolite concentrations in mammary-gland acini incubated under various conditions*

For details of the concentrations of substrates and insulin in the incubation see the legend to Table 1. The metabolite concentrations are mean values $\pm$ s.d., expressed as $\mu\text{mol}/\text{g}$ defatted dry wt., with the numbers of observations shown in parentheses, and were measured in the incubation medium plus acini after 60min incubation. Values that are statistically different (Student's *t* test) from incubations with glucose alone are shown: *P < 0.05; **P < 0.0025.

Substrates added	Metabolite concentrations ($\mu\text{mol}/\text{g}$ dry wt.)			
	Citrate	Glucose 6-phosphate	ATP	Glycerol 3-phosphate
Glucose	1.21 $\pm$ 0.64 (19)	0.94 $\pm$ 0.45 (15)	9.54 $\pm$ 1.96 (15)	2.81 $\pm$ 1.23 (21)
Glucose+insulin	1.13 $\pm$ 0.52 (7)	0.83 $\pm$ 0.47 (6)	8.23 $\pm$ 0.88 (6)	4.83 $\pm$ 1.21 (8)**
Glucose+acetoacetate	6.41 $\pm$ 2.36 (15)**	1.53 $\pm$ 0.38 (9)**	8.68 $\pm$ 0.90 (9)	2.09 $\pm$ 0.84 (13)*
Glucose+acetoacetate+insulin	3.69 $\pm$ 1.74 (10)**	1.44 $\pm$ 1.00 (6)	8.84 $\pm$ 2.01 (6)	2.94 $\pm$ 1.41 (12)
Glucose+acetoacetate+glycerol	2.03 $\pm$ 1.37 (7)*	1.02 $\pm$ 0.35 (3)	8.08 $\pm$ 0.98 (4)	4.71 $\pm$ 2.72 (8)*
Glucose+glycerol	1.53 $\pm$ 0.69 (7)	1.24 $\pm$ 0.55 (8)	7.75 $\pm$ 1.46 (8)*	25.2 $\pm$ 10.5 (12)**

effect on glucose uptake. D-3-Hydroxybutyrate, which was taken up by the acini to a lesser extent than acetoacetate, gave results which were qualitatively similar to those with acetoacetate (results not shown).

Lactate production by the acini did not alter significantly with the different substrate combinations, except for glucose plus acetoacetate, where, as might be expected, lactate formation decreased by 68% (Table 1). However, in contrast, pyruvate accumulation did not decrease in the presence of glucose and acetoacetate, increased 2-fold when insulin was included, and decreased by about 75% in the incubations with glucose and glycerol alone (Table 1). Assuming that the [lactate] and [pyruvate] found in the medium reflect those in the acini and that lactate dehydrogenase was in equilibrium in this system, then acetoacetate increased the free $[\text{NAD}^+]/[\text{NADH}]$ in the cytosol, whereas glycerol markedly decreased it (Table 1).

Concentrations of citrate, glucose 6-phosphate, ATP and glycerol 3-phosphate

[Citrate] increased 6-fold and [glucose 6-phosphate] increased by 60% in incubations of acini with glucose and acetoacetate (Table 2). Insulin and glycerol both

decreased [citrate] and [glucose 6-phosphate] in the presence of acetoacetate, but [citrate] was still higher than the concentration found for glucose alone (Table 2). The presence of either insulin or glycerol in incubations of acini with glucose had no effect on [citrate] and [glucose 6-phosphate]. [ATP] did not differ significantly from that with glucose alone with any combination of substrates except for glucose plus glycerol, where [ATP] decreased by 19% (Table 2). The concentrations in Table 2 are values from flasks incubated for 60min, and generally concentrations were relatively constant throughout the incubation time (results for 20 and 40min not shown). The direction of these changes in [citrate] and [glucose 6-phosphate] in the present experiments is consistent with the previous studies on slices of rat mammary gland (Williamson *et al.*, 1975), and with the postulated mechanism for the effects of acetoacetate on glucose metabolism.

The proposal being tested in the present work is that glycerol addition may be similar to a possible action of insulin to increase glycerol 3-phosphate availability and thus promote lipogenesis. In support of this proposal, insulin and glycerol increased [glycerol 3-phosphate] by 70% and 9-fold respectively in

incubations of acini with glucose (Table 2). Acetoacetate decreased [glycerol 3-phosphate] by 64% at 20 and 40 min (results not shown) and by 26% at 60 min (Table 2).

Effect of acetoacetate, insulin and glycerol on the fate of glucose

Acini were incubated with [1-¹⁴C]- and [6-¹⁴C]-glucose to measure the flux of glucose through the pentose phosphate and lipogenic pathways. The production of ¹⁴CO₂ from [6-¹⁴C]glucose, which results mainly from tricarboxylic acid-cycle oxidation, was very low, but increased with addition of acetoacetate to the incubation medium whether or not insulin or glycerol was also added (Table 3). In contrast, ¹⁴CO₂ production from [1-¹⁴C]glucose, which largely occurs in the pentose phosphate pathway, was about ten times greater than ¹⁴CO₂ production from [6-¹⁴C]glucose, and was decreased by 77% in the presence of acetoacetate (Table 3). Both insulin and glycerol increased ¹⁴CO₂ production from [1-¹⁴C]glucose in the presence of acetoacetate.

The contribution of the pentose phosphate pathway to glucose utilization can be estimated from the equation

$$PC = (G1 - G6) / [3 - (2G1 - G6)]$$

where G1 and G6 represent the percentage of the glucose uptake appearing as ¹⁴CO₂ from [1-¹⁴C]glucose and [6-¹⁴C]glucose respectively (Rognstad & Katz, 1969). From the above equation and the values in Table 3, the pentose phosphate pathway represented 10, 18, 22 and 30% of the glucose uptake for glucose plus acetoacetate, glucose, glucose plus insulin and glucose plus acetoacetate plus insulin respectively. This agrees with the findings from slices of rat mammary gland (Williamson *et al.*, 1975) and is in contrast with the observation that the ratio of the flux of glucose through the pentose phosphate pathway and the glycolytic pathway remains constant

in many situations (Bartley & Abraham, 1976; Gumaa *et al.*, 1971). The above equation is not valid for calculating the contribution of the pentose phosphate pathway in incubations with glycerol, since complete equilibration of the triose phosphate pool is assumed and no allowance is made for entry of unlabelled triose phosphate from exogenous sources (Rognstad & Katz, 1969).

The incorporation of radioactivity into lipid from [6-¹⁴C]glucose represented 56% of the glucose uptake, whereas incorporation from [1-¹⁴C]glucose accounted for a lower proportion (27%) of the glucose uptake (Table 3), owing to loss of ¹⁴CO₂ in the pentose phosphate pathway. Incubations of acini with acetoacetate decreased the incorporation of [1-¹⁴C]glucose and [6-¹⁴C]glucose into lipid by more than 90% (Table 3). This inhibition was relieved by both insulin and glycerol, which greatly increased the incorporation into lipid (Table 3). Insulin also tended to increase the incorporation into lipid with glucose alone, whereas glycerol decreased the incorporation by over 70% (Table 3), as would be expected from the decreased utilization of glucose in this situation (Table 1).

Absolute rates of lipogenesis in acini

Measurement of [¹⁴C]glucose incorporation into lipid does not give an absolute measure of the flux through the lipogenic pathway, since (a) labelled glycerol 3-phosphate may be formed from glucose, (b) variable losses of radioactive carbon as ¹⁴CO₂ may occur, particularly in incubations with [1-¹⁴C]glucose, and (c) substrates other than glucose may provide C₂ units for lipogenesis. Therefore incorporation of ³H₂O into fatty acids by acini was used to measure the absolute rate of fatty acid synthesis (Jungas, 1968). Acetoacetate decreased fatty acid synthesis by 60%, whereas both insulin and glycerol increased fatty acid synthesis in the presence of acetoacetate to equal the rate found with glucose plus insulin, which was 130% of that for glucose alone

Table 3. *Effects of acetoacetate, insulin and glycerol on the fate of glucose in mammary-gland acini*

For full details see the Experimental section and the legend to Table 1. The results are mean values $\pm$ S.D., expressed as μ mol/min per 100mg defatted dry wt., with the numbers of observations shown in parentheses. Values that are statistically different (Student's *t* test) from incubations with glucose alone are shown by: *P < 0.05; **P < 0.0025.

Substrates added	Flux rates (μ mol/min per 100mg dry wt.)			
	[1- ¹⁴ C]Glucose into CO ₂	[1- ¹⁴ C]Glucose into lipid	[6- ¹⁴ C]Glucose into CO ₂	[6- ¹⁴ C]Glucose into lipid
Glucose	0.56 $\pm$ 0.11 (20)	0.36 $\pm$ 0.08 (19)	0.038 $\pm$ 0.005 (14)	0.74 $\pm$ 0.15 (15)
Glucose+insulin	0.62 $\pm$ 0.13 (5)	0.43 $\pm$ 0.09 (5)	0.032 $\pm$ 0.000 (8)**	0.85 $\pm$ 0.21 (7)
Glucose+acetoacetate	0.13 $\pm$ 0.03 (8)**	0.01 $\pm$ 0.01 (8)**	0.052 $\pm$ 0.011 (8)**	0.08 $\pm$ 0.06 (8)**
Glucose+acetoacetate+insulin	0.59 $\pm$ 0.23 (5)	0.16 $\pm$ 0.08 (5)**	0.076 $\pm$ 0.011 (6)**	0.56 $\pm$ 0.35 (6)
Glucose+acetoacetate+glycerol	0.74 $\pm$ 0.14 (4)*	0.18 $\pm$ 0.07 (4)**	0.051 $\pm$ 0.016 (4)*	0.57 $\pm$ 0.10 (4)*
Glucose+glycerol	0.23 $\pm$ 0.08 (12)**	0.10 $\pm$ 0.05 (11)**	0.057 $\pm$ 0.042 (4)	0.11 $\pm$ 0.08 (4)**

(Table 4). The increase in fatty acid synthesis with insulin supports the trend of increased lipid incorporation from [1-¹⁴C]- or [6-¹⁴C]glucose (Table 3; see also Robinson & Williamson, 1977a). The ratio of the incorporation of [6-¹⁴C]glucose into lipid (Table 3) to fatty acid synthesis (Table 4) was decreased in the presence of acetoacetate, confirming that acetoacetate contributes carbon for fatty acid synthesis. With glycerol alone the rate of fatty acid synthesis was inhibited by 90% (Table 4).

These results are consistent with the findings in rat mammary-gland slices that ³H₂O incorporation into fatty acids was decreased in incubations with acetoacetate (10mM) and glucose (10mM) compared with glucose alone (Bartley, 1976). However, the decrease in fatty acid synthesis was not as great as that found in the present studies, and the uptake of glucose was unaffected by acetoacetate in the studies of Bartley (1976).

The rates in Table 4 are a direct measure of the incorporation of acetyl units into fatty acids, since the ratio between the incorporation of μ mol of ³H

from ³H₂O and the incorporation of μ mol of acetyl units from ¹⁴C-labelled substrates into fatty acids is very close to unity for mammary-gland preparations (Katz & Wals, 1972; Katz *et al.*, 1974; Bartley & Abraham, 1976). Thus if fatty acids with an average chain length of 11 carbon atoms are synthesized by the acini (Katz *et al.*, 1974), the pentose phosphate pathway may provide about 60% of the NADPH required for fatty acid synthesis with glucose, glucose plus insulin and glucose plus acetoacetate plus insulin, whereas with glucose plus acetoacetate it may provide only 20% of the required reducing equivalents. Presumably in this situation a greatly increased proportion of the NADPH is provided by the reaction catalysed by 'malic' enzyme in the cytoplasm (Bauman & Davis, 1974; Katz *et al.*, 1974). The present findings are similar to other estimates which suggest that the pentose phosphate pathway accounts for 20–30% of the glucose utilized and can provide 80–100% of the NADPH required for fatty acid synthesis in rat mammary-gland slices and acini (Katz & Wals, 1972; Katz *et al.*, 1974).

Effects of insulin and glycerol on the fate of [3-¹⁴C]-acetoacetate

Incorporation of [3-¹⁴C]acetoacetate into lipid and ¹⁴CO₂ accounted for 25% and 53% respectively of the acetoacetate taken up in incubations of acini with glucose and [3-¹⁴C]acetoacetate (Table 5), with formation of 3-hydroxybutyrate accounting for the remaining 22%. Both insulin and glycerol increased [3-¹⁴C]acetoacetate incorporation into lipid 2-fold and decreased oxidation of [3-¹⁴C]-acetoacetate, but glycerol had a greater effect than insulin and decreased ¹⁴CO₂ formation by 87% (Table 5). Acetoacetate uptake and 3-hydroxybutyrate formation were not affected by insulin, but were decreased by over 30% in the presence of glycerol (Table 5).

The virtual abolition of ¹⁴CO₂ production from [3-¹⁴C]acetoacetate, concomitant with a doubling of incorporation into lipid, cannot be explained by dilution of a single [¹⁴C]acetyl-CoA pool by non-

Table 4. *Rates of fatty acid synthesis in mammary-gland acini*

For full details see the Experimental section and the legend to Table 1. The results are mean values $\pm$ S.D., expressed as μ mol of ³H incorporated into fatty acid/min per 100mg defatted dry wt., with the numbers of observations shown in parentheses. Values that are statistically different (Student's *t* test) from incubations with glucose alone are shown: *P < 0.05; **P < 0.0025.

Substrates added	Fatty acid synthesis (μ mol of ³ H/min per 100mg dry wt.)
Glucose (13)	0.47 $\pm$ 0.10
Glucose+insulin (12)	0.64 $\pm$ 0.12**
Glucose+acetoacetate (9)	0.19 $\pm$ 0.10**
Glucose+acetoacetate+insulin (10)	0.62 $\pm$ 0.24*
Glucose+acetoacetate+glycerol (7)	0.62 $\pm$ 0.11*
Glucose+glycerol (11)	0.05 $\pm$ 0.04**

Table 5. *Effects of glycerol and insulin on the fate of acetoacetate in mammary-gland acini*

For full details see the Experimental section and the legend to Table 1. The results are mean values $\pm$ S.D., expressed as μ mol/min per 100mg defatted dry wt., with the number of observations in parentheses. Values that are statistically different (Student's *t* test) from incubations with glucose plus acetoacetate are shown: †*P* < 0.01; **P < 0.0025.

Substrates	Metabolite changes (μ mol/min per 100mg dry wt.)			
	Acetoacetate removed	3-Hydroxybutyrate formed	[3- ¹⁴ C]Acetoacetate converted into CO ₂	[3- ¹⁴ C]Acetoacetate converted into lipid
Glucose+acetoacetate	0.55 $\pm$ 0.12 (22)	0.11 $\pm$ 0.05 (22)	0.29 $\pm$ 0.04 (7)	0.14 $\pm$ 0.04 (7)
Glucose+acetoacetate+insulin	0.54 $\pm$ 0.08 (15)	0.09 $\pm$ 0.04 (16)	0.20 $\pm$ 0.05 (5)†	0.29 $\pm$ 0.04 (5)**
Glucose+acetoacetate+glycerol	0.37 $\pm$ 0.06 (12)**	0.05 $\pm$ 0.02 (13)**	0.04 $\pm$ 0.02 (5)**	0.31 $\pm$ 0.08 (5)**

radioactive carbon arising from glucose or glycerol. Possible explanations are that [^{14}C]acetyl-CoA formed within the mitochondria from [$3\text{-}^{14}\text{C}$]acetoacetate no longer mixes with the pool of acetyl-CoA that enters the tricarboxylic acid cycle yet still is converted into lipid, or alternatively that acetoacetate is used directly in the cytosol for lipogenesis. In support of this latter suggestion it is known that acetoacetyl-CoA synthetase and acetoacetyl-CoA thiolase, which can convert acetoacetate into acetyl-CoA, are present in the cytoplasm of rat mammary gland (Buckley & Williamson, 1975), and such a cytosolic pathway has been postulated to be of importance in the synthesis of lipids in developing rat brain (Williamson & Buckley, 1973). Some experimental evidence for this pathway in neonatal rat brain has been provided by Patel & Owen (1976). In addition, acetoacetyl-CoA generated in the cytoplasm can be converted into butyryl-CoA, which is a better primer than acetyl-CoA for fatty acid synthesis in rat mammary gland (see Scheme 1; Lin & Kumar, 1972). The existence of this cytosolic pathway in rat mammary gland bypasses the requirement for citrate efflux from the mitochondria to provide cytosolic acetyl-CoA for fatty acid synthesis. It was therefore decided to test the effects of 5-(tetradecyloxy)-2-furoic acid, which inhibits translocation of mitochondrial citrate (Ribereau-Gayon, 1976), on the observed interactions between glucose, acetoacetate and insulin.

Effect of 5-(tetradecyloxy)-2-furoic acid on glucose and acetoacetate metabolism of acini

5-(Tetradecyloxy)-2-furoic acid decreased the glucose uptake by acini by 57% and decreased incorporation of [$6\text{-}^{14}\text{C}$]glucose into lipid by 81%, whereas $^{14}\text{CO}_2$ production increased 2-fold (Table 6). Accumulation of lactate did not change, whereas that of pyruvate decreased, resulting in an increase in the [lactate]/[pyruvate] ratio in the presence of 5-(tetradecyloxy)-2-furoic acid (results not shown). In the presence of acetoacetate, 5-(tetradecyloxy)-2-furoic acid slightly decreased glucose uptake and prevented the increase in glucose uptake caused by insulin or glycerol (Table 6). $^{14}\text{CO}_2$ production from [$3\text{-}^{14}\text{C}$]acetoacetate was hardly affected by 5-(tetradecyloxy)-2-furoic acid in the presence or absence of insulin, but incorporation of acetoacetate into lipid was decreased to 25% of the maximum observed rate, which suggests that the major pathway for lipogenesis from acetoacetate under these conditions involves citrate translocation. In contrast, 5-(tetradecyloxy)-2-furoic acid increased $^{14}\text{CO}_2$ production from [$3\text{-}^{14}\text{C}$]acetoacetate in the presence of glycerol, and although there was a decrease in the rate of lipid synthesis from [$3\text{-}^{14}\text{C}$]acetoacetate, it was 2-fold higher than for glucose plus acetoacetate. This suggests that a cytosolic pathway may be involved in lipogenesis from acetoacetate in the presence of glycerol.

In incubations with 5-(tetradecyloxy)-2-furoic acid

Table 6. *Effects of inhibition of lipogenesis on glucose and acetoacetate metabolism in mammary-gland acini*
For full details see the Experimental section and the legend to Table 1. The concentration of 5-(tetradecyloxy)-2-furoic acid in the incubation mixture was 0.4 mM. Glucose removal and ^{14}C incorporation into CO_2 and lipid are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry wt. and citrate concentrations in $\mu\text{mol}/\text{g}$ defatted dry wt. The results are mean values $\pm$ S.D., with the numbers of experiments in parentheses. Values that are significantly different (Student's *t* test) in the presence of the inhibitor compared with its absence are shown: * $P < 0.05$; ** $P < 0.0025$.

Substrates added	Metabolite changes ($\mu\text{mol}/\text{min}$ per 100 mg dry wt.)			Citrate concn. ($\mu\text{mol}/\text{g}$ dry wt.)
	Glucose removed	$^{14}\text{CO}_2$ formed	[^{14}C]Lipid formed	
[$6\text{-}^{14}\text{C}$]Glucose	1.48 ± 0.10 (6)	0.038 ± 0.008 (5)	0.77 ± 0.12 (5)	0.90 ± 0.66 (5)
[$6\text{-}^{14}\text{C}$]Glucose + 5-(tetradecyloxy)-2-furoic acid	0.73 ± 0.07 (6)*	0.070 ± 0.013 (5)**	0.15 ± 0.05 (5)**	1.86 ± 0.44 (5)*
Glucose + [$3\text{-}^{14}\text{C}$]acetoacetate	0.43 ± 0.12 (5)	0.30 ± 0.05 (4)	0.15 ± 0.04 (4)	6.17 ± 0.88 (5)
Glucose + [$3\text{-}^{14}\text{C}$]acetoacetate + 5-(tetradecyloxy)-2-furoic acid	0.34 ± 0.10 (5)*	0.26 ± 0.06 (4)*	0.07 ± 0.02 (4)*	8.27 ± 0.83 (5)*
Glucose + [$3\text{-}^{14}\text{C}$]acetoacetate + insulin	1.03 ± 0.32 (4)	0.16 ± 0.08 (4)	0.29 ± 0.02 (3)	4.06 ± 0.85 (4)
Glucose + [$3\text{-}^{14}\text{C}$]acetoacetate + insulin + 5-(tetradecyloxy)-2-furoic acid	0.38 ± 0.14 (4)*	0.20 ± 0.09 (3)	0.07 ± 0.02 (3)**	9.08 ± 1.40 (4)**
Glucose + [$3\text{-}^{14}\text{C}$]acetoacetate + glycerol	1.27 ± 0.16 (3)	0.03 ± 0.02 (3)	0.31 ± 0.13 (3)	3.19 ± 1.32 (3)
Glucose + [$3\text{-}^{14}\text{C}$]acetoacetate + glycerol + 5-(tetradecyloxy)-2-furoic acid	0.42 ± 0.18 (3)**	0.15 ± 0.08 (3)*	0.16 ± 0.06 (3)*	5.54 ± 0.35 (3)*

there was a significant increase in [citrate] in the incubation medium (Table 6). If 5-(tetradecyloxy)-2-furoic acid inhibited lipogenesis by specific inhibition of citrate translocation out of the mitochondria (Ribereau-Gayon, 1976), then [citrate] might be expected to increase in the mitochondria, but if 5-(tetradecyloxy)-2-furoic acid inhibited lipogenesis at sites other than citrate translocation this might result in increased [citrate] in the cytoplasm and inhibition of glycolysis.

Effects of insulin and glycerol in rat mammary gland

These studies pose the question of how insulin and glycerol act to relieve the inhibition of glucose uptake caused by acetoacetate. In the presence of acetoacetate, [citrate] and the cytoplasmic $[NAD^+]/[NADH]$ ratio were increased, and it has been postulated that phosphofructokinase and hexokinase are inhibited by citrate and glucose 6-phosphate respectively, and that inhibition of lipogenesis by lack of glycerol 3-phosphate and acetyl-CoA results in increased $[NADPH]$ and inhibition of the pentose phosphate pathway (Williamson *et al.*, 1975). Although their precise site(s) of action have not been identified in the present studies, both insulin and glycerol reversed these changes in [citrate] and the cytoplasmic redox state and increased the rate of lipogenesis, which suggests that the mechanism involved may be similar for both.

Insulin has effects on glucose transport in adipose tissue (Crofford & Renold, 1965) and skeletal muscle (Randle *et al.*, 1964), and it cannot be excluded that insulin has a similar effect in mammary gland (McLean, 1960; Gumaa *et al.*, 1971). However, insulin did not increase the glucose uptake of acini incubated with glucose (5mM) in the present studies (see also Robinson & Williamson, 1977a), and an increased glucose concentration did not abolish the stimulatory effect of insulin on CO_2 production and lipid synthesis in isolated rat mammary cells (Martin & Baldwin, 1971b) or acini (results not shown). Furthermore, it is unlikely that an effect of insulin on glucose transport was responsible for the relief of the inhibition of glucose uptake caused by acetoacetate, since an increased glucose concentration of 10 or 20mM did not abolish the inhibition of glucose uptake by acetoacetate (2mM) (results not shown).

Pyruvate dehydrogenase may have been inhibited by acetoacetate in incubations of the acini, since an increased proportion of the glucose utilized appeared as lactate and pyruvate (Table 1). However, inhibition of pyruvate dehydrogenase did not appear to be responsible for the decreased glucose uptake, since glucose uptake was not affected when dichloroacetate (2.5mM), which activates pyruvate dehydrogenase (Denton *et al.*, 1975), was present in incubations of acini with acetoacetate (results not shown). Thus any effect of insulin to activate pyruvate

dehydrogenase in rat mammary gland, as occurs in adipose tissue (Coore *et al.*, 1971; Denton *et al.*, 1975), is unlikely to affect glucose uptake in acini under the present conditions, although effects of insulin on pyruvate dehydrogenase may be important in the control of mammary-gland metabolism in other situations (Robinson & Williamson, 1977a).

Another possible site for insulin action is acetyl-CoA carboxylase, which has low activity in rat mammary gland (Baldwin & Yang, 1974) and may be rate-limiting for lipogenesis. Acetyl-CoA carboxylase isolated from rat mammary gland shows similar properties to the enzyme from adipose tissue (Miller & Levy, 1969; Miller *et al.*, 1970) and its activation by insulin may likewise be mediated by a decreased concentration of fatty acyl-CoA, which inhibits the adipocyte enzyme (Halestrap & Denton, 1973). Insulin may decrease [fatty acyl-CoA] in mammary gland by increasing its esterification to form triacylglycerol as a result of increased availability of glycerol 3-phosphate (Howard & Lowenstein, 1965), or by activation of glycerol phosphate acyltransferase, as has been proposed for adipose tissue (Sooranna & Saggerson, 1975, 1976). Thus in the presence of acetoacetate, if the postulated decreased availability of glycerol 3-phosphate allows fatty acyl-CoA to accumulate and inhibit acetyl-CoA carboxylase activity, then an action of insulin to increase the activity of this enzyme may promote fatty acid synthesis and decrease cytosolic [citrate]. A further possibility is that the activity of acetyl-CoA carboxylase in rat mammary gland may be regulated by a phosphorylation-dephosphorylation cycle, as has been reported for the enzyme from rat liver (Carlson & Kim, 1974; Lee & Kim, 1977), and insulin may promote formation of the active form.

The finding in the present studies that glycerol behaves similarly to insulin in its ability to relieve the acetoacetate inhibition of glucose uptake suggests that increased availability of glycerol 3-phosphate may be more important than any direct effect of insulin on glycerol phosphate acyltransferase activity. Furthermore, it is possible that the action of insulin may involve changes in the redox state (Martin & Baldwin, 1971a,b; Yang & Baldwin, 1975), since insulin caused slight increases in the [lactate]/[pyruvate] ratio in incubations of acini with glucose alone or with acetoacetate present (Table 1). Thus insulin may behave like glycerol in its ability to increase the availability of glycerol 3-phosphate and decrease the cytoplasmic $[NAD^+]/[NADH]$ ratio. In the absence of acetoacetate the inhibition of glucose uptake caused by glycerol is likely to be related to these changes in [glycerol 3-phosphate] and the $[NAD^+]/[NADH]$ ratio. Direct inhibition of a rate-limiting enzyme of glucose utilization (e.g. hexokinase or phosphofructokinase) by glycerol 3-phosphate and/or limitation of the glyceraldehyde 3-phosphate

Scheme 1. Pathways of carbon flow showing possible sites of regulation of lipogenesis in rat mammary gland

dehydrogenase reaction of glycolysis by the decreased $[NAD^+]/[NADH]$ ratio are possible reasons for the decreased glucose utilization. These suggestions are reinforced by the finding that dihydroxyacetone had no effect on glucose uptake, $[glycerol\ 3-phosphate]$ or the $[lactate]/[pyruvate]$ ratio.

It has been suggested that $[glycerol\ 3-phosphate]$ will be largely determined by $[dihydroxyacetone\ phosphate]$ and the $[NAD^+]/[NADH]$ ratio in the cytoplasm in mammary gland, as is found in adipose tissue (Kuhn, 1967; Saggerson & Greenbaum, 1970), since glycerol 3-phosphate dehydrogenase has a high activity in rat mammary gland (Baldwin & Yang, 1974). In support of this proposal the changes in $[glycerol\ 3-phosphate]$ in the present experiments (Table 2) do indeed follow the changes in the $[lactate]/[pyruvate]$ ratio, and a plot of 180 individual values of $[glycerol\ 3-phosphate]$ with the corresponding value for the $[lactate]/[pyruvate]$ ratio for all substrate combinations gave a regression line with a correlation coefficient of 0.82 (results not shown). Thus changes in $[glycerol\ 3-phosphate]$ and the $[NAD^+]/[NADH]$ ratio are closely related, and either one or both of these factors may be directly responsible for both the inhibition of glucose uptake by glycerol and the relief of the acetoacetate inhibition of glucose uptake by glycerol and insulin.

Increased availability of NADH in the cytoplasm due to either glycerol or insulin might be expected to promote lipogenesis in the presence of acetoacetate (Scheme 1). Firstly $[glycerol\ 3-phosphate]$ would be increased, which would promote triacylglycerol formation (Howard & Lowenstein, 1965) and decrease $[fatty\ acyl-CoA]$. For glycerol, the 2-fold increase in glycerol uptake and the decreased $[glycerol\ 3-phosphate]$ caused by acetoacetate were probably related to the increased $[NAD^+]/[NADH]$ ratio in the cytoplasm, which would favour triose phosphate formation from glycerol 3-phosphate. Secondly, the conversion of acetoacetyl-CoA into 3-hydroxybutyryl-CoA in the pathway forming butyryl-CoA, an effective primer for fatty acid synthesis in mammary gland (Nandedkar & Kumar, 1969; Lin & Kumar, 1972; Scheme 1), would be promoted. Thirdly, oxaloacetate reduction in the cytoplasm could occur, and this would remove product from the citrate lyase reaction in addition to providing malate, which could either generate NADPH in the reaction catalysed by 'malic' enzyme or enter the mitochondria to provide more oxaloacetate for the citrate synthase reaction (Scheme 1). However, it is uncertain whether the transfer of reducing equivalents into the mitochondria via malate occurs to a significant extent in rat mammary gland, or whether pyruvate carboxylase in the cytoplasm (Gul & Dils, 1969) has a significant role in generating oxaloacetate.

These proposals to explain the interactions of glucose and acetoacetate with insulin and glycerol in rat mammary-gland acini are only speculative. It has not yet been established that phosphofructokinase in rat mammary gland is subject to inhibition by citrate. Similarly it is not known how $[fatty\ acyl-CoA]$ changes in the acini in the presence of acetoacetate, insulin and glycerol, nor whether availability of glycerol 3-phosphate is in fact limiting triacylglycerol synthesis and thus whether the observed increase in its concentration is a prerequisite for increased lipogenesis. A further complication is that acetoacetate may be metabolized in both the cytoplasm and the mitochondria, and insulin and glycerol may affect the proportions metabolized in these two compartments.

The present studies show that acini have similar metabolic characteristics to slices of rat mammary gland (Williamson *et al.*, 1975), and the novel finding is that glycerol behaves like insulin in its ability to relieve the acetoacetate inhibition of glucose uptake in incubations of rat mammary-gland acini. Evidence that the ability of acetoacetate to inhibit glucose uptake may occur *in vivo* is the finding that intravenous injection of acetoacetate decreased the arteriovenous difference for glucose across the mammary gland of fed lactating rats (Robinson & Williamson, 1977b). If the interactions *in vitro* observed in the present experiments operate *in vivo*, insulin and glycerol could both modulate the inhibition of glucose utilization brought about by acetoacetate and direct a greater proportion of this substrate to lipogenesis. Such effects are likely to be important in starvation, where ketone-body concentrations are increased and changes in blood insulin and glycerol concentrations occur. In addition it is possible that increased provision of glycerol to the gland via the action of lipoprotein lipase may 'signal' increased availability of triacylglycerols and decrease glucose utilization for lipogenesis. Finally, these experiments underline the importance of studying the effects of combinations of substrates as well as hormones on the metabolism of tissues.

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