

Metabolic Interactions of Glucose, Acetoacetate and Insulin in Mammary-Gland Slices of Lactating Rats*

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1. Utilization of 5 mM-glucose by slices of lactating mammary gland was decreased 33% on addition of acetoacetate (2 mM) to the incubation medium. This inhibition was accompanied by increases in the intracellular concentrations of citrate and glucose 6-phosphate.
2. In the presence of acetoacetate the accumulation of pyruvate in the medium approximately doubled.
3. Insulin completely reversed the inhibitory effect of acetoacetate on glucose utilization, without altering the amount of acetoacetate removed or pyruvate formed.
4. Similar results were obtained with mammary-gland slices from diabetic rats, except that insulin did not completely reverse the effects of acetoacetate.
5. Acetoacetate inhibited the formation of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$; this effect was not overcome by insulin.
6. Insulin increased the proportion of $[3\text{-}^{14}\text{C}]\text{acetoacetate}$ that was converted into lipid and decreased that oxidized to CO_2 .
7. The physiological significance of these findings is discussed.

The high activities of the enzymes of ketone-body metabolism in the lactating mammary gland of the rat suggest that this tissue is a major potential site of ketone-body utilization (Page & Williamson, 1972). The arteriovenous differences for various substrates across lactating glands of anaesthetized rats show that the gland takes up ketone bodies, but that their contribution to the total substrate uptake is less than 3% in the fed state (Hawkins & Williamson, 1972). In short-term starvation (16 h), on the assumption that no change in blood flow occurs, glucose uptake by the gland decreases by about 50%, and lactate is released. These changes are accompanied by an increase in ketone-body uptake, and this has led to the suggestion that ketone bodies may decrease glucose utilization and pyruvate oxidation by the gland (Hawkins & Williamson, 1972), as with other rat tissues: rat heart (Williamson & Krebs, 1961; Randle *et al.*, 1964), diaphragm (Randle *et al.*, 1964) and submaxillary gland (Thompson & Williamson, 1975). Alternatively, the decrease in glucose uptake may be due to the lower concentration of circulating insulin in starvation. To examine these possibilities the effects of acetoacetate and insulin on glucose utilization by rat mammary-gland slices were tested. The results indicate that in this system *in vitro* acetoacetate

decreases glucose utilization and that this effect can be reversed by insulin.

Experimental

Material

Rats. Nursing rats of the Wistar strain were used. The period of lactation varied between 6 and 13 days and the litter size varied between 5 and 13 pups. Short-term diabetes was induced in lactating rats by intravenous injection of streptozotocin (50 mg/kg body wt.). This treatment produces a rapid onset of hyperglycaemia and insulin deficiency, with the initial peak effect at 3 h (Schein *et al.*, 1971). The blood sugar of the rats at this time was 9–12 mM, but the concentrations of blood ketone bodies were not appreciably raised. Rats were killed by cervical dislocation and the sublingual mammary glands were removed as quickly as possible and placed in ice-cold Krebs & Henseleit (1932) saline.

Biochemicals. All enzymes and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Sodium acetoacetate was prepared as described by Krebs & Eggleston (1945). Insulin (glucagon-free) was obtained from Wellcome Laboratories, Beckenham, Kent, U.K. Streptozotocin was obtained from Upjohn Co., Kalamazoo, Mich., U.S.A.

Radioactive compounds. The following radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.: D-[1- ^{14}C]glucose, D-[6- ^{14}C]glucose, sodium [1- ^{14}C]pyruvate, sodium

* Dedicated to Professor Sir Hans A. Krebs, F.R.S., for his 75th birthday.

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[3-¹⁴C]pyruvate and ethyl [3-¹⁴C]acetoacetate. [3-¹⁴C]Acetoacetate was prepared from ethyl [3-¹⁴C]-acetoacetate (Krebs *et al.*, 1966).

Methods

Incubation procedure. Slices (approx. 0.2–0.3 mm thick) of lactating mammary gland were cut as detailed by Umbreit *et al.* (1972). They were placed in ice-cold Krebs & Henseleit (1932) saline, then transferred to fresh ice-cold Krebs–Henseleit saline (for 2 min), blotted and weighed on a torsion balance. The slices (about 200 mg) were then transferred into 50 ml conical flasks containing 5 ml of cold Krebs–Henseleit saline and other substrates as indicated. The flasks were gassed with O₂+CO₂ (95:5) for 1 min and quickly stoppered. When ¹⁴CO₂ was to be collected 50 ml flasks with a centre well and Suba-Seal tops (Gallenkamp and Co., London EC2P 2ER, U.K.) were used.

The slices were incubated for 1 h at 37°C in a Dubnoff-type shaker. Preliminary experiments indicated that rates of glucose and acetoacetate utilization were linear for at least 80 min.

When determination of [citrate], [ATP] and [glucose 6-phosphate] was required the slices were first removed and then 0.5 ml of 30% (w/v) HClO₄ was added to the medium. The slices were immediately homogenized for 30 s in 5 ml of ice-cold 3% HClO₄ with a Silverson model laboratory mixer–emulsifier (Silverson Machine Co. Ltd., London S.E.1, U.K.). Both the medium and the homogenized slices were centrifuged for 10 min at 3000 rev./min. The supernatant fluids were poured off and adjusted to pH 7.0 with 20% (w/v) KOH. The precipitates of KClO₄ were removed by centrifugation for 10 min at 3000 rev./min and the final supernatant fluids were decanted off and used for the determination of metabolites.

When collection of ¹⁴CO₂ was required the reaction was stopped after 1 h by injecting through the sealed top 0.5 ml of 30% HClO₄ into the outer compartment of the flask and 0.5 ml of 1 M-Hyaminate-10X hydroxide in methanol into the centre well. The flasks were incubated with shaking at room temperature for 2 h before opening. The Hyamine solution was removed with a Pasteur pipette and the centre well was washed out with 2×0.5 ml of methanol. The Hyamine plus methanol washings were made up with methanol to a volume of 2 ml and a portion was taken for measurement of radioactivity. The medium and slices were homogenized together and then treated as described above to obtain a neutral, deproteinized extract. The sediment from the first centrifugation was suspended in 5 ml of chloroform–methanol (2:1, v/v).

Lipid extraction. The method for extraction of lipid was adapted from that of Radin (1969). The chloroform–methanol extract was shaken well and then centrifuged for 10 min at 3000 rev./min. Water (1 ml) was added to the supernatant fluid, which was again

shaken and centrifuged for 10 min at 3000 rev./min. A portion of the organic layer containing the extractable lipid was taken for measurements of radioactivity.

Measurements of radioactivity. A Beckman LS-200 liquid-scintillation counter and scintillator fluid consisting of 6–8 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD; CIBA Ltd., Duxford, Cambs., U.K.), 80 g of naphthalene (Thorn Electronics, Tolworth, Surrey, U.K.), 600 ml of toluene and 400 ml of 2-methoxyethanol were used.

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); citrate (Dagley, 1963).

Calculation of flux rates. Under the experimental conditions used in this paper synthesis of lactose represented less than 10% of the glucose utilized (see also Bartley *et al.*, 1966), so that enzymically measured removal of glucose from the incubation medium was taken to represent the flux through the hexokinase reaction (phosphorylation of glucose).

The flux rate of glucose through the hexose monophosphate pathway was determined by use of [1-¹⁴C]- and [6-¹⁴C]-glucose (Katz *et al.*, 1966). The total radioactivity in CO₂ collected from [1-¹⁴C]glucose is derived from two sources: glucose carbon oxidized to CO₂ in the hexose monophosphate pathway, and glucose carbon metabolized via the glycolytic pathway and subsequently oxidized to CO₂ in the tricarboxylic acid cycle. The contribution of the latter is equivalent to the total radioactivity in CO₂ arising from [6-¹⁴C]-glucose, assuming that complete equilibration of the two C₃ units occurs at the triose phosphate isomerase step and that no randomization of label occurs before entry of pyruvate into the tricarboxylic acid cycle. The actual flux rate was calculated as follows:

$$\frac{{}^{14}\text{CO}_2 \text{ from [1-}^{14}\text{C]glucose}}{\text{Specific radioactivity of [1-}^{14}\text{C]glucose}} - \frac{{}^{14}\text{CO}_2 \text{ from [6-}^{14}\text{C]glucose}}{\text{Specific radioactivity of [6-}^{14}\text{C]glucose}}$$

It should be stressed that this gives an estimate of the flux rate, which is likely to be too low, owing to dilution of the [1-¹⁴C]glucose 6-phosphate pool with unlabelled glucose 6-phosphate recycled from the hexose monophosphate pathway.

The flux rate through the glycolytic pathway was obtained by subtraction of the flux rate through the hexose monophosphate pathway from the rate through the hexokinase reaction.

No attempt was made to differentiate the incorporation of radioactivity into the glycerol and fatty acid moieties of the lipid, so that the total radio-

activity measured is the sum of that derived from glucose carbon incorporated via the glycerophosphate dehydrogenase and acetyl-CoA carboxylase reactions.

The flux rate of glucose to lipid was calculated as follows:

$$\frac{\text{Total } ^{14}\text{C in lipid}}{\text{Specific radioactivity of [1-}^{14}\text{C]- or [6-}^{14}\text{C]-glucose}}$$

The flux rate for [6-¹⁴C]glucose gives the best estimate, because no loss of the C-6 position occurs in either the glycolytic or hexose monophosphate pathways.

The flux rates for [3-¹⁴C]acetoacetate were calculated by analogous methods to those described above.

For all these calculations, it was assumed that the concentrations of intracellular intermediates and of endogenous precursors were negligible in relation to the concentrations of the added substrates.

Results

Effects of acetoacetate and insulin on glucose metabolism

Incubation of mammary-gland slices with physiological concentrations of glucose (5mM) and acetoacetate (2mM) resulted in a decreased (about 30%) removal of glucose from the medium compared with incubation with glucose alone (Table 1). Addition of insulin resulted in no significant increase in the utilization of glucose by the slices, but when insulin, glucose and acetoacetate were included in the incubation medium the previously observed inhibition of glucose uptake by acetoacetate was abolished (Table 1). The amount of glucose utilized which can be accounted for as accumulation of lactate plus pyruvate was relatively constant (10-12%) in all the experimental conditions. The accumulation of pyruvate

approximately doubled when acetoacetate was present in the medium, and there was a concomitant decrease in the [lactate]/[pyruvate] ratio of the medium from 5.8 to 1.8; insulin did not affect these changes significantly.

In other experiments, not shown in Table 1, increase of the acetoacetate concentration in the medium to 4mM caused a 50% decrease in glucose removal; this decrease was also completely abolished by the addition of insulin. DL-3-Hydroxybutyrate (4mM) did not significantly decrease glucose removal, though there was a small inhibition in some experiments, whereas acetate (2mM) caused a slight stimulation (20%).

Intracellular concentrations of glucose 6-phosphate, ATP and citrate

In the perfused rat heart, inclusion of ketone bodies or fatty acids in the medium increases the intracellular concentrations of glucose 6-phosphate and citrate (Newsholme & Randle, 1964). These changes have been interpreted as indicating that increased [citrate] inhibits phosphofructokinase with a subsequent increase in [fructose 6-phosphate] and [glucose 6-phosphate], the latter in turn inhibiting hexokinase and decreasing glucose utilization (Parmeggiani & Bowman, 1963; Garland *et al.*, 1963). To test whether a similar situation exists in the mammary gland, [glucose 6-phosphate], [citrate] and [ATP] were measured in slices incubated under the various experimental conditions shown in Table 1. The presence of acetoacetate significantly increased [glucose 6-phosphate] and [citrate] compared with incubations with glucose alone (Table 2). Incubation with glucose plus insulin decreased [glucose 6-phosphate] and [citrate] below the values for glucose alone, and insulin abolished the increase in the two metabolites brought about by acetoacetate (Table 2). [ATP] in slices was not significantly different in any of the experimental conditions (Table 2) and the values were

Table 1. *Effects of acetoacetate and insulin on glucose metabolism by mammary-gland slices from lactating rats*

Mammary-gland slices from lactating rats were incubated as described in the Experimental section. The metabolite changes are mean values ± s.e.m., expressed as μmol/h per g wet wt. Values that are statistically different (Student's *t* test) from the incubation with glucose alone are shown by **P* < 0.01.

Substrates	No. of expts.	Concn.	Metabolite changes (μmol/h per g wet wt.)				
			Glucose removed	Lactate formed	Pyruvate formed	Acetoacetate removed	3-Hydroxybutyrate formed
Glucose	15	5mM	40.7 ± 4.1	7.6 ± 0.6	1.3 ± 0.08	—	—
Glucose + insulin	13	5mM 50munits/ml	43.8 ± 4.6	9.1 ± 0.7	1.6 ± 0.09	—	—
Glucose + acetoacetate	19	5mM 2mM	27.3 ± 3.0*	4.4 ± 1.8*	2.4 ± 0.3*	17.2 ± 1.0	2.6 ± 0.2
Glucose + acetoacetate + insulin	19	5mM 2mM 50munits/ml	45.9 ± 3.8	6.6 ± 0.6	2.4 ± 0.2*	16.9 ± 0.8	2.8 ± 0.2

Table 2. *Effects of glucose, acetoacetate and insulin on concentrations of glucose 6-phosphate, ATP and citrate in mammary-gland slices*

The results are expressed as $\mu\text{mol/g}$ fresh wt. of slice (means \pm s.e.m.). For other details see the Experimental section. Results that are statistically different (Student's *t* test) from the incubation with glucose alone are indicated by * $P < 0.01$.

Substrate	No. of expts.	Concn.	Glucose 6-phosphate	ATP	Citrate
Glucose	23	5mM	0.034 \pm 0.003	0.35 \pm 0.020	0.17 \pm 0.025
Glucose	20	5mM	0.023 \pm 0.002*	0.41 \pm 0.028	0.11 \pm 0.008*
+insulin		50munits/ml			
Glucose	22	5mM	0.061 \pm 0.003*	0.42 \pm 0.016	0.27 \pm 0.021*
+acetoacetate		2mM			
Glucose	17	5mM	0.033 \pm 0.005	0.47 \pm 0.022	0.19 \pm 0.026
+acetoacetate		2mM			
+insulin		50munits/ml			

Table 3. *Effects of acetoacetate and insulin on glucose metabolism by mammary-gland slices from diabetic rats*

Lactating rats were made diabetic as described in the Experimental section. The metabolite changes are mean values \pm s.e.m. (ten experiments) expressed as $\mu\text{mol/h}$ per g wet wt. Values that are statistically different (Student's *t* test) from the incubation with glucose alone are shown by * $P < 0.01$.

Substrates	Concn.	Metabolite changes ($\mu\text{mol/h}$ per g wet wt.)			
		Glucose removed	Lactate formed	Acetoacetate removed	3-Hydroxybutyrate formed
Glucose	5mM	21.5 \pm 1.7	10.4 \pm 1.3	—	—
Glucose	5mM	32.4 \pm 1.6*	14.0 \pm 1.3*	—	—
+insulin	50munits/ml				
Glucose	5mM	14.5 \pm 1.3*	6.2 \pm 1.1*	18.2 \pm 1.0	3.9 \pm 0.3
+acetoacetate	2mM				
Glucose	5mM	21.3 \pm 1.6	7.6 \pm 1.1	18.1 \pm 1.2	3.2 \pm 0.2
+acetoacetate	2mM				
+insulin	50munits/ml				

Table 4. *Effects of acetoacetate and insulin on the utilization of pyruvate by mammary-gland slices*

Mammary-gland slices from lactating rats were incubated as described in the Experimental section. The results are expressed as mean flux rates \pm s.e.m. ($\mu\text{mol/h}$ per g wet wt.). The numbers of experiments are given in parentheses. Values that are statistically different (Student's *t* test) from the control with pyruvate alone are indicated by * $P < 0.05$.

Substrates	Concn.	Pyruvate removed	Lactate formed	[1- ¹⁴ C]-	[3- ¹⁴ C]-	Acetoacetate removed	3-Hydroxybutyrate formed
				Pyruvate into CO ₂	Pyruvate into CO ₂		
Pyruvate	5mM	44.2 \pm 3.8	6.7 \pm 0.8	39.2 \pm 3.9	5.5 \pm 0.7	—	—
		(8)	(8)	(4)	(4)		
Pyruvate	5mM	44.9 \pm 3.0	5.4 \pm 0.3	35.7 \pm 1.6	6.3 \pm 0.4	—	—
+insulin	50munits/ml	(8)	(8)	(4)	(4)		
Pyruvate	5mM	34.0 \pm 3.2*	6.6 \pm 1.0	23.0 \pm 1.1*	4.9 \pm 0.3	14.0 \pm 1.3	2.6 \pm 0.3
+acetoacetate	2mM	(8)	(8)	(4)	(4)	(8)	(7)
Pyruvate	5mM	31.0 \pm 2.7*	5.2 \pm 0.5	24.7 \pm 2.4*	4.8 \pm 1.1	16.0 \pm 1.6	2.9 \pm 0.2
+acetoacetate	2mM	(8)	(8)	(4)	(4)	(8)	(8)
+insulin	50munits/ml						

similar to those obtained in freeze-clamped mammary gland from lactating rats (Murphy *et al.*, 1973).

Effects of short-term insulin deficiency

A number of workers have shown that insulin increases glucose utilization by slices of lactating mammary gland (Abraham *et al.*, 1957; McLean, 1960; Gumaa *et al.*, 1971). In the present experiments, insulin caused only a slight increase (not statistically significant) in glucose removal (Table 1). A possible explanation for the differences in insulin sensitivity is the amount of endogenous insulin present. To test this lactating rats were injected with streptozotocin to produce insulin deficiency (Rakietan *et al.*, 1963). The rats were killed 2h after injection, at which time the mean blood sugar had increased from 6.7 to 11.8 mm, and previous work has shown low circulating insulin at this time (Schein *et al.*, 1971).

Glucose removal was decreased by about 50% in mammary-gland slices from these short-term-diabetic rats, and in this case insulin addition to the medium caused a 50% increase in glucose utilization (Table 3). As with the slices from normal rats, acetoacetate decreased glucose utilization (about 30%), and, although insulin reversed this effect, the value obtained was similar to that with glucose alone and still well below that with glucose plus insulin (Table 3). The amount of glucose removed that could be accounted for by lactate accumulation in the medium (range 18–24%) was virtually double that found with slices from normal rats (Table 3). Insulin deficiency had no significant effect on the rate of removal of acetoacetate (compare Tables 1 and 3).

Effects of acetoacetate and insulin on pyruvate utilization

Pyruvate dehydrogenase is inhibited in rat hearts perfused with short- and long-chain fatty acids or with ketone bodies (Randle *et al.*, 1964). It was therefore decided to test whether acetoacetate or insulin affected pyruvate utilization in mammary-gland slices. Pyruvate (5 mm) utilization was decreased by about 25% by the presence of acetoacetate (2 mm). If the amount of [1-¹⁴C]pyruvate oxidized to CO₂ can be considered to represent pyruvate dehydrogenase activity the inhibition by acetoacetate was about 40% (Table 4). Acetoacetate did not significantly alter the amount of ¹⁴CO₂ derived from [3-¹⁴C]pyruvate. Insulin did not overcome the inhibition of pyruvate utilization by acetoacetate, nor did it have a significant effect on pyruvate removal in the absence of acetoacetate.

Effects of acetoacetate and insulin on the fate of glucose

Under the experimental conditions used here the glucose utilized by the lactating mammary-gland slice provides carbon for both the fatty acid and glycerol moieties of lipids; conversion of glucose into lactose is a minor pathway (Bartley *et al.*, 1966). A consider-

Table 5. Effect of acetoacetate and insulin on fate of glucose in mammary-gland slices from lactating rat

The mean flux rates \pm s.e.m. are given in μ mol/h per g wet wt. The numbers of experiments are given in parentheses. The concentrations of substrates and insulin are as for Table 1. Values that are statistically different (Student's *t* test) from the incubation with glucose alone are indicated by * *P* < 0.010.

Substrates	Glucose removed	[1- ¹⁴ C]-Glucose into CO ₂	[6- ¹⁴ C]-Glucose into CO ₂	[1- ¹⁴ C]-Glucose into lipid	[6- ¹⁴ C]-Glucose into lipid	Flux of glucose through pentose phosphate pathway	Flux of glucose through glycolytic pathway	Percentage of glucose taken up converted into lipid ([6- ¹⁴ C]glucose)	Percentage of glucose taken up converted into lipid ([1- ¹⁴ C]glucose)
Glucose	40.7 \pm 4.1 (17)	17.4 \pm 0.78 (6)	1.0 \pm 0.25 (8)	16.5 \pm 3.3 (6)	25.1 \pm 2.6 (7)	16.4	24.3	61.7	40.5
Glucose + insulin	43.8 \pm 4.6 (12)	18.9 \pm 1.5 (6)	1.0 \pm 0.27 (8)	19.4 \pm 5.2 (6)	29.6 \pm 2.9 (7)	17.9	25.9	67.6	44.3
Glucose + acetoacetate	27.3 \pm 3.0 (19)	13.5 \pm 1.2* (6)	3.8 \pm 0.53* (6)	6.4 \pm 1.2* (6)	11.3 \pm 1.7* (7)	9.6	17.7	41.4	23.4
Glucose + acetoacetate + insulin	45.9 \pm 3.8 (19)	22.4 \pm 2.5* (6)	2.7 \pm 0.31* (8)	11.6 \pm 2.9* (6)	26.9 \pm 3.3 (7)	19.7	26.2	58.6	25.3

Table 6. *Effects of glucose and insulin on the fate of acetoacetate in mammary-gland slices from lactating rat*

The mean flux rates \pm S.E.M. are given in $\mu\text{mol/h}$ per g wet wt. For their derivation see the section on calculations. The numbers of experiments are given in parentheses. Values that are statistically different (Student's *t* test) from the experiments with acetoacetate and glucose are indicated by * $P < 0.05$. Substrate concentrations are as given in Table 1.

Substrates	Acetoacetate removed	3-Hydroxybutyrate formed	[3- ¹⁴ C]Acetoacetate converted into CO ₂	[3- ¹⁴ C]Acetoacetate converted into lipid	Percentage of acetoacetate removed converted into CO ₂	Percentage of acetoacetate removed converted into lipid
Glucose+acetoacetate	17.2 \pm 1.0 (19)	2.6 \pm 0.2 (17)	6.4 \pm 0.9 (4)	6.8 \pm 0.4 (8)	37.2	39.5
Glucose+acetoacetate+insulin	16.9 \pm 0.8 (19)	2.8 \pm 0.2 (16)	3.8 \pm 0.4* (4)	9.0 \pm 1.1* (8)	22.5	53.3

able proportion of the glucose taken up by the gland is oxidized via the hexose monophosphate pathway to generate NADPH for lipogenesis; the remainder of the glucose is utilized via the glycolytic pathway. To examine the effects of acetoacetate on the proportion of glucose utilized by these two pathways the conversion of [1-¹⁴C]glucose and [6-¹⁴C]glucose into ¹⁴CO₂ and lipid was measured in the various experimental conditions listed in Table 1. From the results approximate flux rates of glucose through the two pathways were calculated.

Contrary to findings by other workers (Abraham *et al.*, 1957; Gumaa *et al.*, 1971), insulin did not significantly alter the flux rates of glucose through the hexose monophosphate and glycolytic pathways, nor did it change the rate of conversion of glucose into lipid (Table 5). In the presence of acetoacetate, the flux of glucose through the hexose monophosphate pathway was decreased by about 42%, whereas the decrease in that through the glycolytic pathway was only 26%. Insulin restored the flux rates of glucose through the two pathways to those found with glucose alone. The ratio of the flux of glucose through the two pathways remained relatively constant, except when acetoacetate was present (Table 5); this is further confirmation of the close integration between the two pathways (Gumaa *et al.*, 1971).

Acetoacetate increased the oxidation of [6-¹⁴C]-glucose to ¹⁴CO₂ about fourfold (Table 5). The mechanism for this effect may be the inhibition by acetoacetate of pyruvate oxidation via pyruvate dehydrogenase (Table 4) and the consequent greater availability of pyruvate for carboxylation to oxaloacetate. Insulin did not abolish this effect of acetoacetate, which agrees with the inability of insulin to overcome the acetoacetate inhibition of pyruvate dehydrogenase (Table 4).

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

Insulin had no effect on the removal of acetoacetate or on the formation of D-3-hydroxybutyrate (Table 1), but, because insulin lowered intracellular [citrate] when acetoacetate was present (Table 2), it was considered that it might alter the metabolic fate of acetoacetate. Incubation of [3-¹⁴C]acetoacetate plus glucose resulted in conversion of approximately equal portions of the acetoacetate removed (after correction for hydroxybutyrate formation) into CO₂ and lipid (Table 6). Kinsella (1970) has demonstrated that cultured rat mammary-gland cells can synthesize lipid from 3-hydroxybutyrate. Inclusion of insulin in the incubation medium markedly depressed the proportion of acetoacetate converted into CO₂ and increased that incorporated into lipid, without significantly altering the amount of acetoacetate utilized. The recovery of ¹⁴C in these experiments was more than 90%.

Discussion

The experiments reported here extend to another tissue of the rat the finding that acetoacetate can decrease glucose utilization, as has been demonstrated in heart (Williamson & Krebs, 1961; Randle *et al.*, 1964), diaphragm (Randle *et al.*, 1964) and submaxillary gland (Thompson & Williamson, 1975). The changes in intracellular [citrate] and [glucose 6-phosphate] suggest that the sites of inhibition of glucose utilization in lactating mammary-gland slices appear to be the same as those proposed for the other tissues examined, namely phosphofructokinase and hexokinase. However, a major difference is that in lactating mammary-gland slices two other precursors of acetyl-CoA, and hence of citrate, namely 3-hydroxybutyrate and acetate, did not inhibit glucose utilization. Acetate actually stimulated glucose removal, as has also been reported for the oxidation of uniformly labelled glucose in slices and acinar preparations of mammary gland (Greenbaum & Darby, 1964; Katz *et al.*, 1974). A possible reason for the stimulation of glucose utilization by acetate is that, unlike rat heart, 90% of the activity of acetyl-CoA synthetase is present in the cytosol of rat lactating mammary gland (B. M. Buckley & D. H. Williamson, unpublished work) and therefore acetate is less likely to give rise to increased [citrate]. A low rate of 3-hydroxybutyrate utilization (about 25% of that found with acetoacetate) may explain the failure of this compound to inhibit glucose utilization.

Unlike muscle and submaxillary gland, the lactating mammary gland can utilize appreciable amounts of glucose through the pentose phosphate pathway. At first sight it is surprising that the rise in [glucose 6-phosphate] in the presence of acetoacetate did not result in increased activity of glucose 6-phosphate dehydrogenase with relief of the inhibition of hexokinase. The reason is presumably the close 'coupling' of the flux through the glycolytic and pentose phosphate pathways in lipogenic tissues (see, e.g., Abraham *et al.*, 1957; Gumaa *et al.*, 1971; Katz & Wals, 1972). Thus primary inhibition of the glycolytic pathway at the phosphofructokinase step would be expected to result in a secondary inhibition of the pentose phosphate pathway. The mechanism of this secondary inhibition is still an open question, but one possibility is that decreased flux through the glycolytic pathway causes a decrease in lipogenesis owing to decreased availability of carbon for formation of both glycerophosphate and acetyl-CoA, and this in turn leads to an increase in the concentration of NADPH, which is an inhibitor of glucose 6-phosphate dehydrogenase from various sources (Negelein & Haas, 1935; Glock & McLean, 1953; Bonsignore & De Flora, 1972; Sapag-Hagar *et al.*, 1973; Eggleston & Krebs, 1974).

The novel finding of the present study is that insulin

completely reversed the inhibitory effects of acetoacetate on glucose utilization. This observation is analogous to the relief of the acetoacetate inhibition of glucose utilization by adrenaline described for rat submaxillary-gland slices (Thompson & Williamson, 1975). In the mammary-gland slice, the insulin effect is not mediated by a decrease in [ATP], nor does it appear to be due to increased permeability of glucose in the present experiments, because insulin did not increase glucose removal significantly and it lowered [glucose 6-phosphate] (Table 2). These facts, together with the finding that the flux of carbon (glucose and acetoacetate) to lipid was highest in the presence of insulin, suggest that the primary effect of the hormone in this system is concerned with the activation of a rate-limiting step in the lipogenic pathway. The most likely candidate is acetyl-CoA carboxylase, which has the lowest activity of all the enzymes involved in the conversion of glucose into lipid in rat mammary gland (McLean *et al.*, 1972). Activation of acetyl-CoA carboxylase in rat adipose tissue by insulin has been ascribed to the removal of long-chain acyl-CoA, which is a potent inhibitor of the enzyme (Halestrap & Denton, 1973). Alternatively some evidence exists that hepatic acetyl-CoA carboxylase activity is regulated by phosphorylation and dephosphorylation (Carlson & Kim, 1974), and it has been suggested by these authors that insulin might modify the interconversion in some tissues.

The question arises as to whether the metabolic interactions reported here have any physiological significance. Elkin & Kuhn (1975) have suggested caution in the use of mammary-gland slices for metabolic studies, because they found that lactate was produced by the slices (at rates similar to those reported here), whereas the intact gland of fed rats is known to take up lactate (Hawkins & Williamson, 1972; Elkin & Kuhn, 1975). This criticism is not necessarily valid, because virtually all tissue preparations (perfused organs, isolated cells) produce lactate when it is not initially present in the medium. At high external concentrations of lactate (5–10 mM) and in the presence of glucose, mammary-gland acinar preparations take up lactate and convert it into lipid (Katz *et al.*, 1974). Arteriovenous differences for ketone bodies across the mammary gland of lactating rats have been demonstrated (Hawkins & Williamson, 1972) and their contribution to the carbon supply of gland increased on starvation (16h) of lactating rats. If the findings reported here with slices are applicable to the situation *in vivo* then the increased availability of acetoacetate in the circulation as starvation proceeds would depress the utilization of glucose and lactate for lipid synthesis and energy supply; ketone bodies could then provide alternative substrates for both these processes. The net result in short-term starvation might be a 'sparing' of glucose for lactose synthesis, a process in which ketone bodies cannot act as substi-

tute substrates. Some evidence that insulin deficiency (and presumably hyperketonaemia) does not affect the utilization of glucose for lactose synthesis *in vivo* is the finding that induction of lactose synthesis is unimpaired in alloxan-diabetic rats (Kyriakou & Kuhn, 1973). However, long-term diabetic rats maintained on insulin show a loss of lactational performance on withdrawal of the hormone (Martin & Baldwin, 1971). Thus any metabolic interactions *in vivo* between glucose, acetoacetate and insulin would only be expected to play a role in the short-term starvation experienced by lactating rats in their natural environment.

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