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Effect of Alloxan-Diabetes and Treatment with Anti-Insulin Serum on Pathways of Glucose Metabolism in Lactating Rat Mammary Gland

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1. The overall metabolic changes in lactating mammary gland in alloxan-diabetic and anti-insulin-serum-treated rats were assessed by measurement of the incorporation of ^{14}C from specifically labelled glucose, pyruvate and acetate into carbon dioxide and lipid, together with measurements of enzymes concerned with the pentose phosphate pathway and with citrate metabolism. 2. Alloxan-diabetes depressed the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose and [2-^{14}C]glucose to approx. 10% of the control rate; this was partially reversed by addition of insulin *in vitro*. The quotient

$$\frac{\text{Oxidation of [1-}^{14}\text{C]glucose}}{\text{Oxidation of [6-}^{14}\text{C]glucose}}$$

fell from a value of 17·6 in the control group to 3·9 in the diabetic group and was restored to 14·3 in the presence of insulin *in vitro*. In keeping with these results it was shown that glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were significantly decreased in alloxan-diabetic rats. 3. Alloxan-diabetes depressed the decarboxylation and the oxidation of labelled pyruvate, but not the oxidation of labelled acetate. 4. The synthesis of lipid from specifically labelled glucose was greatly decreased, that from [2-^{14}C]pyruvate was almost unchanged and that from [1-^{14}C]acetate alone was increased in alloxan-diabetic rats. However, the stimulation of lipid synthesis from acetate by glucose was small in the alloxan-diabetic rats compared with the controls. Insulin *in vitro* partially reversed all these effects. Both citrate-cleavage enzyme and acetate thiokinase activities were decreased in alloxan-diabetic rats. 5. Treatment of rats with anti-insulin serum depressed the formation of $^{14}CO_2$ from [1-^{14}C]glucose and [2-^{14}C]glucose, but increased that from [6-^{14}C]glucose. This was completely restored by the presence of insulin *in vitro*. The quotient

$$\frac{\text{Oxidation of [1-}^{14}\text{C]glucose}}{\text{Oxidation of [6-}^{14}\text{C]glucose}}$$

fell from a value of 17·6 in the control group to 3·8 in the anti-insulin-serum-treated group. There were no changes in the activity of glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase, but the hexokinase distribution changed and the content of the soluble fraction increased significantly. 6. The synthesis of lipid from specifically labelled glucose was depressed in anti-insulin-serum-treated rats; this effect was completely reversed by addition of insulin *in vitro* to the tissue slices.

There has been extensive work on the hormonal control of milk secretion (see Folley, 1956; Cowie, 1961) and it is well established that the biosynthetic processes of lactation are under hormonal control. Balmain & Folley (1951), Balmain, French & Folley (1950) and Abraham, Cady & Chaikoff (1957) have all demonstrated effects of insulin *in vitro* on the metabolism of glucose by lactating rat mammary-gland slices. Organ cultures of prelactating and lactating mouse mammary-gland tissue have also been shown to require insulin for structural maintainance and secretory response (Bern & Rivera, 1960; Rivera & Bern, 1961; Moretti & Abraham, 1966).

In the present work the overall metabolic changes in the lactating mammary gland after

inducement of diabetes by treatment with alloxan were assessed by measurement of the incorporation of ^{14}C from specifically labelled glucose, pyruvate and acetate into carbon dioxide and lipid. The changes observed in alloxan-diabetes were compared with those observed after treatment of the lactating rat with anti-insulin serum. In parallel with these studies measurements were also made of the activities of various soluble-fraction enzymes concerned with glucose catabolism and lipid synthesis.

METHODS

Animals. Primiparous albino rats of the Wistar strain were used, the litters in all cases being adjusted to eight pups. A standard diet (Rowett Research Institute formula, diet 86) and water were supplied *ad libitum*. The litter weights were taken as an index of milk secretion. Animals were killed by decapitation, and only the abdominal mammary glands were used.

Induction of diabetes by treatment with anti-insulin serum. Rats at the eleventh day of lactation were killed 1 hr. after intravenous administration of anti-insulin serum. The anti-insulin serum was kindly supplied by Dr A. Beloff-Chain (Imperial College of Science and Technology, London). This was prepared in guinea pigs against crystalline glucagon-free insulin from Burroughs Wellcome, as described by Mansford (1967). The approximate potency was such that 1 ml. of undiluted serum bound 4 milliunits of insulin. The anti-insulin serum or control injection was given directly into the sublingual vein under ether anaesthesia and the mothers were returned to their litters for the subsequent hour. Blood samples were taken from the dorsal aorta immediately before the mammary gland was excised.

Administration of anti-insulin serum (0·5 ml./100 g. body wt.) caused a rise in the blood sugar concentration from $98 \pm 6{\cdot}3$ mg./100 ml. in the controls to 237 ± 26 mg./100 ml. in the anti-insulin-serum-treated rats (Fisher's P 0·007).

Induction of diabetes by alloxan and after-care of animals. On the fourth day of lactation, diabetes was induced by subcutaneous injection of alloxan (20 mg./100 g. body wt.). The alloxan was administered in solution in 0·2 M-sodium acetate buffer, pH 5·0, made iso-osmotic with NaCl. These diabetic rats were then maintained on 2 units of protamine–zinc–insulin/day. At this stage of the experiment the average weight gain of the litters of both control and insulin-treated diabetic mothers was 13 g./day. The insulin treatment was stopped 3 days before the rats were killed on the tenth or eleventh day of lactation, i.e. 6 or 7 days after treatment with alloxan. At this stage the litter of diabetic mothers had ceased to show any gain in weight. The blood sugar concentrations of the mothers were 433 ± 90 mg. 100 ml. of blood, compared with $83 \pm 0{\cdot}5$ mg./100 ml. of the control mothers (mean values $\pm$ S.E.M. for five rats in each group).

Isotope experiments. Mammary-gland slices (250 mg.) were incubated with 4·5 ml. of Krebs–Ringer bicarbonate medium (Umbreit, Burris & Stauffer, 1949) for 1 hr. at 37° in the outer compartment of a 50 ml. conical flask fitted with a centre well of approx. 2 ml. capacity together with one of the following substrate mixtures in the presence and absence of 1 unit of insulin: (1) 100 μmoles of glucose containing 0·4 μc of [1-^{14}C]glucose, [2-^{14}C]glucose or [6-^{14}C]glucose; (2) 100 μmoles of glucose containing 0·4 μc of [1-^{14}C]glucose + phenazine methosulphate (final concn. 0·1 mM); (3) 100 μmoles of acetate containing 0·4 μc of [1-^{14}C]acetate; (4) 100 μmoles of acetate containing 0·4 μc of [1-^{14}C]acetate + 100 μmoles of glucose; (5) 100 μmoles of pyruvate containing 0·2 μc of either [1-^{14}C]pyruvate or [2-^{14}C]pyruvate. In each case the gas phase was $O_2 + CO_2$ (95:5). At the end of the 60 min. incubation, 1 ml. of 5 N-HCl was introduced into the outer compartment and 1 ml. of 5 N-NaOH into the centre well by injection through the rubber cap. A further 2 hr. was allowed before the flasks were removed from the incubation bath.

The $^{14}CO_2$ was collected as $Ba^{14}CO_3$, plated at infinite thickness on 1 cm.^{2} disks and counted in a Nuclear–Chicago gas-flow counter. The total lipid was extracted by the method of Bligh & Dyer (1959) and counted in a Nuclear–Chicago scintillation counter.

The results are expressed as μmoles of substrate utilized/hr./total gland at 37°.

Preparation of tissue extracts. The abdominal mammary glands were removed and weighed, and a 1 g. sample was taken, chopped finely in an ice-cold beaker and homogenized in 9 vol. (v/w) of homogenizing medium in a glass Potter homogenizer. The homogenizing medium contained (final concns.) 150 mM-KCl, 5 mM-$MgCl_2$, 5 mM-EDTA and 10 mM-mercaptoethanol, and was adjusted to pH 7·4 with $KHCO_3$. The homogenate was spun at 105000 g for 45 min. in a Spinco model L ultracentrifuge. The supernatant fraction was dialysed for 1 hr. against the same extracting medium and used for the determination of enzyme activities on the same day as that on which the rats were killed.

Assay of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-linked isocitrate dehydrogenase. The activities of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP oxidoreductase, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate–NADP oxidoreductase, EC 1.1.1.44) and the NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell [*threo*-D_s-isocitrate–NADP oxidoreductase (decarboxylating), EC 1.1.1.42] were measured by the methods of Glock & McLean (1953) and Ochoa (1955), as adapted by McLean & Brown (1966). One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmole of NADPH/hr. at 25°. The rate of reduction of $NADP^+$ was measured in a Unicam SP. 800 recording spectrophotometer with a constant-temperature cell housing and scale-expansion accessory.

Assay of citrate-cleavage enzyme and acetate thiokinase. The activities of citrate-cleavage enzyme [ATP–citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] and acetate thiokinase [acetate–CoA ligase (AMP), EC 6.2.1.1] were measured by the colorimetric method described by Kornacker & Lowenstein (1965). One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmole of acetyl-CoA/hr. at 37°.

Assay of ATP–glucose phosphotransferase activity. The ATP–glucose phosphotransferase activity, henceforth called hexokinase (EC 2.7.1.1) since it has a low K_m for glucose, was measured essentially by the method of Sharma, Manjeshwar & Weinhouse (1963) with modifications as described by McLean & Brown (1966); this involved the use of purified glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, giving 2 equiv. of

NADPH/mole of glucose 6-phosphate formed. The final glucose concentration used was 1mM. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose 6-phosphate/hr. at 25°. Only the hexokinase in the soluble fraction was assayed in these experiments.

Determination of nucleic acids. DNA and RNA were determined as described by Glock & McLean (1955), but with the modified diphenylamine reagent described by Burton (1956) for DNA.

Statistical analysis. The mean values are given together with their S.E.M. values. The differences are considered significant if P is no greater than 0·05. Values greater than 0·1 are quoted as not significant.

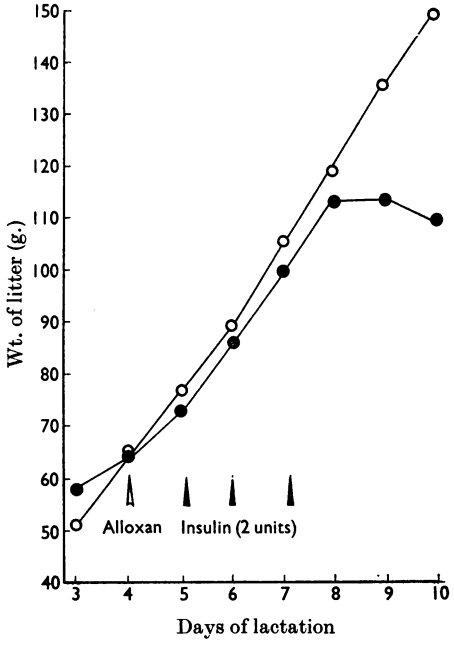

Fig. 1. Typical growth curves of litters from control rats (○) and alloxan-diabetic rats (●). Each litter contained eight pups. Alloxan (20mg./100g. body wt.) was given on the fourth day of lactation; insulin (2 units) was given on the fifth, sixth and seventh days of lactation.

RESULTS

Effect of alloxan-diabetes on metabolism in lactating rat mammary gland

Typical growth curves of litters from diabetic and control rats are shown in Fig. 1. The administration of alloxan to the mothers frequently resulted in a decreased growth rate of litters for 1 or at the most 2 days; but by the time insulin treatment was stopped the general toxicity of the alloxan had been overcome, as shown by the normal growth rate of the litters. The rats were killed relatively soon (3 days) after cessation of insulin treatment; at this stage it was clear that lactation had almost ceased, since the daily gain in weight of the litters was extremely small. In addition, it was found that the lactating mothers were not capable of surviving longer than about 4 days after cessation of insulin treatment, in contrast with diabetic male rats, which can easily be kept for 2 weeks under the same conditions. Table 1 shows that the total weight of the abdominal mammary glands was lower in the diabetic animals than in the controls. Measurement of the DNA content of the glands showed that, when it was expressed as DNA/total gland, there was no significant difference between the controls and diabetic rats. However, the RNA content had fallen from a value of 91·5mg./total gland in the controls to 55·3mg./total gland in the diabetics. For this reason it was decided to express all results as activity or units per total gland; this has the advantage of giving a value related to the total

Table 1. *Nucleic acid content of mammary glands from control and alloxan-diabetic rats*

Results are given as means ± S.E.M. The numbers in parentheses indicate the number of animals in each group. For details of nucleic acid determinations see the Methods section. Fisher's P values are given; N.S., not significant.

	Control group	Alloxan-diabetic group	Fisher's P
Body wt. (g.)	236 ± 9 (7)	203 ± 15 (7)	N.S.
Mammary-gland wt. (g.) ...	7·43 ± 0·26 (24)	3·86 ± 0·16 (24)	< 0·001
Blood sugar (mg./100ml.) ...	83 ± 0·5 (5)	433 ± 90 (5)	< 0·001
Nucleic acid content			
DNA (mg./total gland)	24·7 ± 1·7 (5)	19·2 ± 1·9 (5)	0·08
RNA (mg./total gland)	91·5 ± 6·6 (5)	55·3 ± 3·1 (5)	< 0·001

Table 2. *Effect of alloxan-diabetes on the oxidation of* [^{14}C]*glucose by lactating rat mammary-gland slices*

Mammary-gland slices (250mg.) were incubated for 1hr. at 37° in 4·5ml. of Krebs–Ringer bicarbonate solution containing 100μmoles of glucose with 0·4μc of [1-^{14}C]glucose, [2-^{14}C]glucose, [6-^{14}C]glucose or [1-^{14}C]glucose+phenazine methosulphate (PMS) (final concn. 0·1mM). Values are given as means±S.E.M. The numbers in parentheses indicate the number of animals in each group. Fisher's *P* values are given for the comparison of differences among the different groups; N.S., not significant.

	Incorporation of ^{14}C from ^{14}C-labelled substrate into CO_2 (μmoles/hr./total gland)				Fisher's *P*			
Group no.	I	II	III	IV				
Substrate	Control	Control with insulin *in vitro*	Alloxan-diabetic	Alloxan-diabetic with insulin *in vitro*	I versus III	II versus IV	I versus II	III versus IV
[1-^{14}C]Glucose	235±32 (10)	341±29 (8)	22·3±2·2 (9)	77·0±10·6 (8)	<0·001	<0·001	0·027	<0·001
[2-^{14}C]Glucose	155±14 (10)	189±18 (8)	19·1±3·3 (10)	70·0±16·9 (8)	<0·001	<0·001	N.S.	0·009
[6-^{14}C]Glucose	17·8±1·6 (10)	14·4±3·6 (8)	8·1±1·8 (10)	5·8±0·94 (8)	<0·001	0·039	N.S.	N.S.
[1-^{14}C]Glucose+PMS	456±30 (10)	479±37 (8)	177±22 (10)	200±28 (8)	<0·001	<0·001	N.S.	N.S.
$\frac{^{14}CO_2 \text{ from [1-}^{14}\text{C]glucose}}{^{14}CO_2 \text{ from [6-}^{14}\text{C]glucose}}$	17·6±2·3 (8)	28·2±4·1 (7)	3·9±0·22 (8)	14·3±1·9 (7)	<0·001	0·012	0·048	<0·001

capacity of the mammary gland for milk production and is also, since the DNA content remains constant, a value proportional to the activity or content/cell.

Oxidation of glucose. The incorporation of ^{14}C from specifically labelled glucose into carbon dioxide by mammary-gland tissue from control and alloxan-diabetic rats in the presence and absence of insulin *in vitro* is shown in Table 2. There was a highly significant decrease in incorporation of ^{14}C from all three labelled glucose substrates into carbon dioxide in the diabetic rats compared with the control. The ratio

$$\frac{^{14}CO_2 \text{ from [1-}^{14}\text{C]glucose}}{^{14}CO_2 \text{ from [6-}^{14}\text{C]glucose}}$$

fell from a value of 17·6 in the controls to 3·9 in the diabetics, emphasizing a greater decrease in $^{14}CO_2$ formation from [1-^{14}C]glucose than from [6-^{14}C]-glucose; this suggests a greater loss in activity of the pentose phosphate pathway than of the Embden–Meyerhof pathway.

Phenazine methosulphate, used as an artificial electron acceptor, doubled formation of $^{14}CO_2$ from [1-^{14}C]glucose by the controls but had an even greater effect on the diabetic tissue, causing an increase from 22·3 to 177μmoles/hr./total gland. However, despite this remarkable eightfold effect the value in the diabetic group was still well below the value observed in the control group with phenazine methosulphate.

The presence of insulin *in vitro* stimulated the incorporation of ^{14}C from [1-^{14}C]glucose into carbon dioxide by the control rats, though it had no significant effect on the incorporation of ^{14}C from glucose labelled in the C-2 and C-6 positions; indeed, the incorporation of ^{14}C from [6-^{14}C]glucose was, if anything, decreased. This differential effect is reflected in a significant increase in the ratio

$$\frac{^{14}CO_2 \text{ from [1-}^{14}\text{C]glucose}}{^{14}CO_2 \text{ from [6-}^{14}\text{C]glucose}}$$

These results are in agreement with the data reported by Abraham *et al.* (1957) and suggest a stimulation of fatty acid synthesis from C_2 units in preference to stimulation of oxidation via the tricarboxylic acid cycle.

The activities of mammary-gland hexokinase and of the two dehydrogenases of the pentose phosphate pathway are shown in Fig. 2. The hexokinase activity of the diabetic tissue was not significantly different from that of the control tissue; expressed as activity per g. of tissue there is an apparent increase in hexokinase activity in the diabetics. This increased activity is probably due to the absence of retained milk in the diabetic tissue, since the total phosphorylating capacity of the gland remained unchanged. There was a significant decrease in the activities of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the diabetics. This fall in enzyme activity is in agreement with the observed fall in glucose oxidation. The decreased glucose oxidation may

also be a result of decreased reoxidation of NADPH as a consequence of a decreased rate of lipid synthesis (Table 4). However, even in the presence of phenazine methosulphate, the rate of oxidation of [1-^{14}C]glucose was still lower in the diabetic tissue than in the controls, though the use of phenazine methosulphate did demonstrate that these dehydrogenases are capable of an enormous increase in activity when not limited by the availability of $NADP^+$.

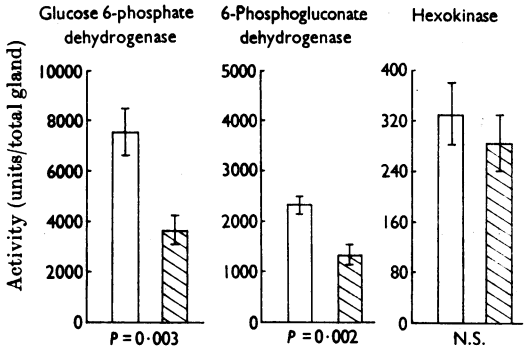

Fig. 2. Activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and hexokinase in mammary gland from control rats (□) and alloxan-diabetic rats (■). Mean values of eight rats are given; the vertical lines represent twice the S.E.M. values. For details of the assay of each enzyme see the Methods section. Fisher's *P* values for the alloxan-diabetic rats versus controls are given below the columns; N.S., not significant.

Oxidation of pyruvate and acetate. The formation of $^{14}CO_2$ from labelled pyruvate and acetate by control and alloxan-diabetic tissue in the presence and absence of insulin is shown in Table 3. There was a significant decrease in the decarboxylation of pyruvate in the diabetics, as illustrated by the use of [1-^{14}C]pyruvate. Insulin had no effect *in vitro* on this decarboxylation by either group of animals. The observed decrease in formation of $^{14}CO_2$ from [2-^{14}C]pyruvate in the diabetic mammary gland compared with the control tissue may be the result of the decreased initial decarboxylation and therefore decreased rate of entry into the tricarboxylic acid cycle; there is no evidence of a decreased rate of oxidation of C_2 units via the tricarboxylic acid cycle, since the oxidation of [1-^{14}C]acetate was the same in both control and diabetic mammary gland. In three of the four groups (I, II and IV, Table 3) the rate of oxidation of [1-^{14}C]acetate was decreased in the presence of equimolar glucose. In the alloxan-diabetic rats this effect was not observed. This is presumably a result of preferential use of acetate for lipid synthesis, as shown by the enormous increase in lipid synthesis from acetate in the presence of glucose and insulin. It is noteworthy that the fall in acetate oxidation was greatest in both control and diabetic tissue in the presence of insulin, i.e. under conditions of increased rate of glucose oxidation (Table 2) and therefore of increased availability of reduced nucleotides for fatty acid synthesis.

Table 3. *Effect of alloxan-diabetes on the oxidation of* [^{14}C]*pyruvate and* [^{14}C]*acetate by lactating rat mammary-gland slices*

Mammary-gland slices (250mg.) were incubated for 1hr. at 37° in 4·5ml. of Krebs–Ringer bicarbonate medium containing 100μmoles of pyruvate with 0·2μc of [1-^{14}C]pyruvate or [2-^{14}C]pyruvate, 100μmoles of acetate with 0·4μc of [1-^{14}C]acetate, or 100μmoles of acetate with 0·4μc of [1-^{14}C]acetate + 100μmoles of glucose. Values are given as means ± S.E.M. The numbers in parentheses indicate the number of animals in each group. Fisher's *P* values are given; N.S., not significant.

	Incorporation of ^{14}C from ^{14}C-labelled substrate into CO_2 (μmoles/hr./total gland)				Fisher's *P*			
Group no.	I	II	III	IV				
Substrate	Control	Control with insulin *in vitro*	Alloxan-diabetic	Alloxan-diabetic with insulin *in vitro*	I versus III	II versus IV	I versus II	III versus IV
[1-^{14}C]Pyruvate	1035±72 (7)	1020±74 (7)	530±55 (8)	625±34 (7)	0·002	0·002	N.S.	N.S.
[2-^{14}C]Pyruvate	162±15 (6)	192±12 (6)	67±10 (6)	92±8 (6)	0·004	<0·001	N.S.	N.S.
[1-^{14}C]Acetate	50·7±2·0 (6)	49·9±2·9 (6)	53·3±7·1 (6)	50·4±5·5 (6)	N.S.	N.S.	N.S.	N.S.
[1-^{14}C]Acetate + glucose (100μmoles)	36·9±3·4 (6)	20·2±2·3 (6)	46·5±5·5 (6)	28·1±3·3 (6)	N.S.	N.S.	0·003	0·024

Table 4. *Effect of alloxan-diabetes on the incorporation of* ^{14}C *from* [^{14}C]*glucose into total lipids by mammary-gland slices*

Mammary-gland slices (250mg.) were incubated for 1hr. at 37° in 4·5ml. of Krebs–Ringer bicarbonate solution containing 100μmoles of glucose with 0·4μc of [1-^{14}C]glucose, [2-^{14}C]glucose or [6-^{14}C]glucose. Values are given as means ± S.E.M. The numbers in parentheses indicate the number of animals in each group. Fisher's P values are given; N.S., not significant.

	Incorporation of ^{14}C from ^{14}C-labelled substrate into lipid (μmoles/hr./total gland)				Fisher's P			
Group no.	I	II	III	IV				
Substrate	Control	Control with insulin *in vitro*	Alloxan-diabetic	Alloxan-diabetic with insulin *in vitro*	I versus III	II versus IV	I versus II	III versus IV
[1-^{14}C]Glucose	194±18 (10)	281±28·5 (8)	11·5±1·7 (9)	51±9·6 (8)	<0·001	<0·001	0·025	0·001
[2-^{14}C]Glucose	361±41 (10)	548±51 (8)	15·4±2·1 (9)	84±19 (8)	<0·001	<0·001	0·013	<0·001
[6-^{14}C]Glucose	425±34 (10)	661±70 (8)	16·8±3·0 (8)	91±19 (8)	<0·001	<0·001	<0·001	0·003
$\frac{[1\text{-}^{14}C]\text{Glucose}}{[6\text{-}^{14}C]\text{Glucose}} \times 100$*	46·1±1·4	47·2±1·1	73·6±4·6	63·8±4·9	<0·001	0·006	N.S.	N.S.

* This quotient gives an approximate estimate of the percentage of ^{14}C-labelled lipid derived from glucose via the glycolytic route.

Lipid synthesis from glucose. As shown in Table 4 there was an enormous decrease in the synthesis of lipids from all three labelled glucose substrates in alloxan-diabetic rats, the values falling to about 5% of the control value. The fall in incorporation of ^{14}C from [6-^{14}C]glucose into lipid was even greater than that from [1-^{14}C]glucose (a 25-fold decrease compared with a 17-fold decrease); this differential effect is reflected in an increase in the contribution of the pentose phosphate pathway to fatty acid synthesis from 46% of the carbon atoms to 74%. The presence of insulin *in vitro* caused a large increase in the incorporation of ^{14}C from labelled glucose into lipid by the diabetic tissue (an average of fivefold stimulation). This is in contrast with the insulin stimulation observed with control mammary-gland tissue, which averaged 1·5-fold for the three labelled glucose substrates. However, despite this much greater effect on diabetic tissue, insulin caused no significant change in the percentage of carbon derived via the pentose phosphate pathway, though in the diabetics this percentage tended towards the control values, reflecting a greater stimulation of incorporation of ^{14}C from [6-^{14}C]-glucose than from [1-^{14}C]glucose.

Lipid synthesis from pyruvate and acetate. The incorporation of ^{14}C from ^{14}C-labelled pyruvate and acetate into lipid is shown in Table 5. There was some decrease in the synthesis of fatty acid from [2-^{14}C]pyruvate by diabetic tissue, though this was barely significant and in neither the diabetic nor the control tissue was there any significant stimulation by the presence of insulin in the incubation medium.

The incorporation of [1-^{14}C]acetate into fatty acids was greater in the diabetic tissue than in the controls, and was significantly increased by insulin *in vitro*, whereas that of the control tissue was not. With mammary-gland tissue from controls the presence of equimolar glucose in the incubation medium resulted in a great increase in incorporation of [1-^{14}C]acetate into labelled lipid in both the presence and absence of insulin. The stimulation by equimolar glucose of acetate incorporation in the diabetic tissue was very small compared with that in the control tissue, even in the presence of insulin.

In seeking to explain the decreased ability of diabetic tissue to synthesize fatty acids from labelled substrates it is of interest that the citrate-cleavage enzyme and acetate thiokinase were both significantly decreased in activity in the diabetic mammary gland (Fig. 3).

The NADP-linked isocitrate dehydrogenase exhibited no significant decrease in activity in the diabetic mammary-gland tissue compared with control mammary-gland tissue (Fig. 3).

Effect of treatment with anti-insulin serum on metabolism in lactating rat mammary gland

Oxidation of glucose. The incorporation of ^{14}C from specifically labelled glucose into carbon

Table 5. *Effect of alloxan-diabetes on the incorporation of* ^{14}C *from* [2-^{14}C]*pyruvate and* [1-^{14}C]*acetate into total lipids by mammary-gland slices*

Mammary-gland slices were incubated for 1 hr. at 37° in 4·5 ml. of Krebs–Ringer bicarbonate medium containing 100 μmoles of pyruvate with 0·2 μc of [2-^{14}C]pyruvate, 100 μmoles of acetate with 0·4 μc of [1-^{14}C]acetate, or 100 μmoles of acetate with 0·4 μc of [1-^{14}C]acetate + 100 μmoles of glucose. Values are given as means ± S.E.M. The numbers in parentheses indicate the number of animals in each group. Fisher's P values are given; N.S., not significant.

	Incorporation of ^{14}C from ^{14}C-labelled substrate into lipid (μmoles/hr./total gland)				Fisher's P			
Group no.	I	II	III	IV				
Substrate	Control	Control with insulin *in vitro*	Alloxan-diabetic	Alloxan-diabetic with insulin *in vitro*	I versus III	II versus IV	I versus II	III versus IV
[2-^{14}C]Pyruvate	156 ± 14·3 (5)	180 ± 15·6 (5)	116 ± 9·5 (5)	140 ± 12·0 (5)	0·055	N.S.	N.S.	N.S.
[1-^{14}C]Acetate	8·5 ± 0·6 (8)	10·2 ± 0·7 (7)	18·9 ± 4·4 (7)	32·7 ± 3·7 (7)	0·040	< 0·001	N.S.	0·040
[1-^{14}C]Acetate + glucose (100 μmoles)	225 ± 10 (6)	381 ± 37 (6)	42 ± 6·4 (6)	82 ± 14·4 (6)	< 0·001	< 0·001	0·003	0·030

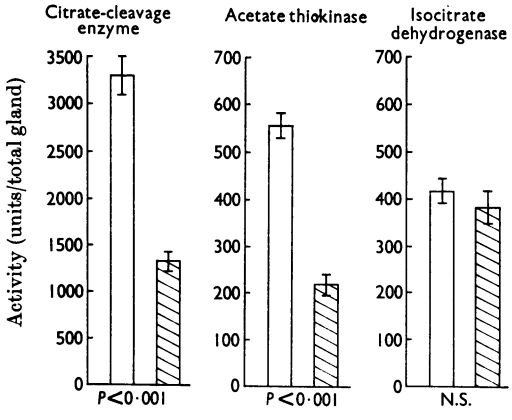

Fig. 3. Activities of citrate-cleavage enzyme, acetate thiokinase and NADP-linked isocitrate dehydrogenase in mammary-gland tissue from control rats (□) and alloxan-diabetic rats (■). Mean values of eight rats are given; the vertical lines represent twice the S.E.M. values. For details of the assay of each enzyme see the Methods section. Fisher's P values for the alloxan-diabetic rats versus controls are given below the columns; N.S., not significant.

dioxide by mammary-gland tissue from control and anti-insulin-serum-treated rats in the presence and absence of insulin *in vitro* is shown in Table 6. The formation of $^{14}CO_2$ from [1-^{14}C]glucose and [2-^{14}C]glucose was significantly decreased in the mammary-gland tissue from rats receiving anti-insulin serum, in contrast with that from [6-^{14}C]-glucose, which almost doubled. The formation of $^{14}CO_2$ from [6-^{14}C]glucose by tissue from anti-insulin-serum-treated rats was significantly decreased by the presence of insulin *in vitro* and returned to control values. These results with [6-^{14}C]glucose support the suggestion that insulin stimulated fatty acid synthesis from C_2 units in preference to stimulation of oxidation via the tricarboxylic acid cycle.

The presence of phenazine methosulphate in the incubation medium almost quadrupled the formation of $^{14}CO_2$ from [1-^{14}C]glucose by tissue from anti-insulin-serum-treated rats, increasing it to a value not significantly less than that of control tissue in the presence of phenazine methosulphate. This increased rate of oxidation by tissue both from control and anti-insulin-serum-treated rats was unaffected by the presence of insulin *in vitro*, suggesting that in the presence of an artificial electron acceptor the pentose phosphate pathway is working near its maximum potential rate, unlimited by the supply of $NADP^+$, so that the presence of insulin *in vitro* could not effect any further increase in the rate of this pathway, even by increasing the available substrate.

The formation of $^{14}CO_2$ from [1-^{14}C]glucose and [2-^{14}C]glucose by tissue from anti-insulin-serum-treated rats was significantly increased by the presence of insulin *in vitro*; this effect was similar to that of insulin *in vitro* on the oxidation of glucose by mammary-gland tissue from alloxan-diabetic rats (Table 2). However, only with mammary-gland tissue from rats treated with anti-insulin serum was the increased oxidation in the presence of insulin *in vitro* comparable with values that were obtained with control tissue with insulin *in vitro*. Thus there

Table 6. *Effect of anti-insulin serum on the oxidation of* [1-^{14}C]*glucose by lactating rat mammary-gland slices*

The rats were killed 1hr. after administration of anti-insulin serum. Mammary-gland slices (250mg.) were incubated for 1hr. at 37° in 4·5ml. of Krebs–Ringer bicarbonate solution containing 100μmoles of glucose with 0·4μc of [1-^{14}C]glucose, [2-^{14}C]glucose, [6-^{14}C]glucose or [1-^{14}C]glucose+phenazine methosulphate (PMS) (final concn. 0·1mM). Values are given as means±S.E.M. The numbers in parentheses indicate the number of animals in each group. Fisher's *P* values are given for the comparison of differences among the groups; N.S., not significant.

	Incorporation of ^{14}C from ^{14}C-labelled substrate into CO_2 (μmoles/hr./total gland)				Fisher's *P*		
Group no.	I	II	III	IV			
Substrate	Control	Control with insulin *in vitro*	Anti-insulin-serum-treated	Anti-insulin-serum-treated with insulin *in vitro*	I versus III	II versus IV	III versus IV
[1-^{14}C]Glucose	235±32 (10)	341±29 (8)	112±24 (3)	285±14 (3)	0·012	N.S.	<0·001
[2-^{14}C]Glucose	155±14 (10)	190±18 (8)	98±22 (3)	196±10 (3)	0·050	N.S.	0·015
[6-^{14}C]Glucose	17·8±1·6 (10)	14·4±3·6 (8)	32·2±3·2 (4)	18·5±1·0 (3)	0·002	N.S.	0·008
[1-^{14}C]Glucose+PMS	456±30 (10)	479±37 (8)	399±74 (3)	441±67 (3)	N.S.	N.S.	N.S.
$^{14}CO_2$ from [1-^{14}C]glucose / $^{14}CO_2$ from [6-^{14}C]glucose	17·6±2·3 (8)	28·2±4·1 (7)	3·8±0·6 (3)	16·7±0·7 (3)	<0·001	0·047	<0·001

Table 7. *Activities of hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in mammary glands from control and anti-insulin-serum-treated rats*

Values are given as means±S.E.M. Fisher's *P* values are given for the comparison of differences between the groups; N.S., not significant. Activity in the soluble fraction is given as units/total gland.

	Activity (units/total gland)		
	Control	Anti-insulin-serum-treated	Fisher's *P*
No. of animals ...	3	3	
Hexokinase	298±15	580±98	0·055
Glucose 6-phosphate dehydrogenase	9430±673	10800±261	N.S.
6-Phosphogluconate dehydrogenase	2500±79	2940±108	N.S.

was no significant difference between the oxidation of [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-glucose by tissue from control and anti-insulin-serum-treated rats in the presence of insulin (Table 6).

The activities of the enzymes hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in control tissue and tissue from anti-insulin-serum-treated rats are shown in Table 7. There was a significant increase in hexokinase activity in the treated tissue, and further study revealed this to be the result of a change in distribution of hexokinase between the particulate and soluble fractions; the hexokinase activity of the soluble fraction increased and that of the particulate fraction decreased after treatment with anti-insulin serum (Walters & McLean, 1968). There was no significant change in activity of either of the two dehydrogenases of the pentose phosphate pathway of control and treated mammary-gland tissue. This result is noteworthy with respect to the observed ability of tissue from anti-insulin-serum-treated rats to oxidize glucose at the same rate as control tissue when insulin is present *in vitro*, and suggests that the decreased oxidation apparent in the absence of added insulin was the result simply of lack of availability of substrate, due to a breakdown in the transport mechanism.

Lipid synthesis from glucose. The effect of treatment with anti-insulin serum on the incorporation of ^{14}C from labelled glucose into lipid is shown in Table 8. There was a decrease in synthesis of ^{14}C-labelled lipid from [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-glucose; however, unlike alloxan-diabetic tissue, the tissue from anti-insulin-serum-treated rats showed a decrease in synthesis of ^{14}C-labelled lipid from [1-^{14}C]glucose of the same order of magnitude

Table 8. *Effect of anti-insulin serum on the incorporation of* ^{14}C *from* [^{14}C]*glucose into total lipids by mammary-gland slices*

The rats were killed 1hr. after administration of anti-insulin serum. Mammary-gland slices (250mg.) were incubated for 1hr. at 37° in 4·5ml. of Krebs–Ringer bicarbonate solution containing 100μmoles of glucose with 0·4μc of [1-^{14}C]glucose, [2-^{14}C]glucose or [6-^{14}C]glucose. Values are given as means ± S.E.M. The numbers in parentheses indicate the number of animals in each group. Fisher's *P* values are given; N.S., not significant.

	Incorporation of ^{14}C from ^{14}C-labelled substrate into lipid (μmoles/hr./total gland)				Fisher's *P*		
Group no.	I	II	III	IV			
Substrate	Control	Control with insulin *in vitro*	Anti-insulin-serum-treated	Anti-insulin-serum-treated with insulin *in vitro*	I versus III	II versus IV	III versus IV
[1-^{14}C]Glucose	194±18 (10)	281±29 (8)	91±22 (3)	298±29 (4)	0·004	N.S.	0·003
[2-^{14}C]Glucose	361±41 (10)	548±51 (8)	207±16 (3)	500±57 (4)	0·005	N.S.	0·005
[6-^{14}C]Glucose	425±34 (10)	661±70 (8)	241±29 (4)	704±39 (4)	0·001	N.S.	<0·001
$\frac{[1\text{-}^{14}C]\text{Glucose}}{[6\text{-}^{14}C]\text{Glucose}} \times 100$*	46·1±1·4 10	47·2±1·1 (8)	36·4±5·0 (3)	38·7±1·2 (3)	N.S.	0·001	N.S.

* This quotient gives an approximate estimate of the percentage of ^{14}C-labelled lipid derived from glucose via the glycolytic route.

as the decrease in that from [6-^{14}C]glucose. This is reflected in the lack of change in the quotient

$$\frac{^{14}C\text{-labelled lipid from [1-}^{14}C\text{]glucose}}{^{14}C\text{-labelled lipid from [6-}^{14}C\text{]glucose}} \times 100$$

As with glucose oxidation, insulin *in vitro* caused a significant increase in lipid synthesis from glucose by tissue from anti-insulin-serum-treated rats. This stimulation was much larger than that observed in control tissue and there was, as a result, no difference between the values obtained with both tissues in the presence of insulin *in vitro*.

The lack of differential effect on the synthesis of ^{14}C-labelled lipids from glucose labelled in three different positions lends support to the suggestion that the decreased synthesis is the result of decreased entry of glucose into the cell, and is not the result of an effect of lack of insulin on any particular pathway of glucose metabolism.

DISCUSSION

A frequent criticism of hormonal studies on mammary gland arises from the apparently high content of adipose tissue. Wrenn, DeLauder & Bitman (1965) reported that the percentage of fat in the rat mammary gland declined from 66% in the non-pregnant gland to 15% during lactation. Weinberg, Pastan, Williams & Field (1961), studying the effect of anterior-pituitary hormones on glucose metabolism by rat mammary gland *in vitro*, found variable amounts of adipose tissue present but concluded that their results could not be accounted for by an effect only on adipose tissue. In the present work the changes observed were very large and it seemed unlikely that they could be due to a minority of the cells present.

Effect of alloxan-diabetes on the oxidation of, and fatty acid synthesis from, glucose, pyruvate and acetate. The importance of insulin in the control of carbohydrate and fat metabolism of the lactating mammary gland has been known since the studies of Folley and his collaborators, who demonstrated effects of insulin *in vitro* on the respiratory quotient, on the glucose and acetate consumption and on the lipid synthesis of lactating rat mammary gland (Balmain *et al.* 1950; Balmain & Folley, 1951; Balmain, Folley & Glascock, 1952, 1954; Folley & Greenbaum, 1960), and since the work of Abraham *et al.* (1957) on the effect of insulin on the utilization of specifically labelled glucose and the role of the two major pathways of carbohydrate metabolism in contributing glucose carbon atoms for fat synthesis in mammary-gland tissue.

There is now ample evidence for the view that there is a close gearing between the pentose phosphate pathway and reductive synthetic processes such as fatty acid synthesis. Lipid synthesis requires, in addition to the necessary enzymic machinery, a source of acetyl-CoA and of NADPH, and the present results may be examined in relation to these requirements.

(i) First, glucose entry into the mammary-gland cells may be restricted or glucose phosphorylation may be blocked in alloxan-diabetes. Measurements of hexokinase activity show that an early change is the release of particle-bound hexokinase, type I and type II, into the soluble fraction of the cell, causing a shift in the hexokinase type II/type I quotient; only 11% remained bound as opposed to 46% bound in the normal lactating mammary gland (E. Walters & P. McLean, unpublished work). Thus it is possible that glucose 6-phosphate formation may be decreased, and this could be important in the supply both of NADPH and of acetyl-CoA for lipid synthesis. In support of this, there is a marked fall in activity of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and a decrease in the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose, indicating a fall in the contribution of the pentose phosphate pathway. However, the observation that phenazine methosulphate caused an eightfold stimulation in the oxidation of C-1 of glucose, restoring this almost to the basic value (Table 2), suggested that, in fact, there was not any lack of NADPH; the pentose phosphate pathway was still rate-limited by the rate of reoxidation of NADPH. This is in keeping with the work of Abraham, Matthes & Chaikoff (1959) on liver of normal and alloxan-diabetic rats.

(ii) The second possibility is that there is a decreased rate of formation of acetyl-CoA from glucose as a precursor. Certainly there was an inhibition of about 50% in the rate of decarboxylation of [1-^{14}C]pyruvate, which would result in an impairment in the rate of formation of acetyl-CoA from pyruvate (Table 5). However, even this decreased rate of pyruvate metabolism is still greatly in excess of the rate of incorporation of ^{14}C from [1-^{14}C]glucose or [6-^{14}C]glucose into lipid (Table 4). This points to a block in metabolism between glucose 6-phosphate formation and pyruvate formation. In perfused rat heart the impairment of glycolysis by diabetes and starvation has been shown to be due to inhibition of phosphofructokinase (Randle, Garland, Hales & Newsholme, 1964), and it is possible that this same site might be involved in mammary glands from alloxan-diabetic rats.

(iii) The possibility that enzyme systems concerned in lipid synthesis are decreased in mammary gland from alloxan-diabetic rats in the same way that has been described for liver and adipose tissue (Vagelos, 1964) has yet to be investigated; however, it is noteworthy that the fatty acid-synthesizing system from diabetic rats is capable of a considerably faster rate of lipid formation than is apparent when [^{14}C]glucose is used as a substrate. Comparison of values in Tables 4 and 5 shows that, whereas only 12–17 μmoles of [^{14}C]glucose were utilized for lipid synthesis by the alloxan-diabetic animals, 116 μmoles of [2-^{14}C]pyruvate were utilized, thus revealing the far greater capacity for lipid synthesis from pyruvate; in fact, the values for alloxan-diabetic rats were only marginally lower than those for control animals. These results again point to a lesion in carbohydrate metabolism between glucose and pyruvate.

(iv) The fourth possibility involves citrate-cleavage enzyme and acetate thiokinase. The extramitochondrial pathway of citrate metabolism has been described in mammary gland, in which citrate-cleavage enzyme plays a key role (Madsen, Abraham & Chaikoff, 1964; Lowenstein, Spencer & Kornacker, 1964; Howanitz & Levy, 1965). The concurrent alteration in the rate of fatty acid synthesis and the activity of citrate-cleavage enzyme during the lactation cycle in the rat mammary gland has been demonstrated by Lowenstein *et al.* (1964) and by Howanitz & Levy (1965).

The importance of citrate-cleavage enzyme as a control point in fatty acid synthesis has been widely discussed (Srere, 1965; Kornacker & Lowenstein, 1965; Srere & Foster, 1967). It has been shown, from sequence studies of citrate-cleavage enzyme activity and fatty acid synthesis, that in rat liver during starvation the depressed lipogenesis was not due initially to decreased activity of citrate-cleavage enzyme, and it is important to distinguish between the initial changes probably mediated by enzyme modifiers rather than changes in enzyme concentration and the longer-term changes in which secondary adaptive alterations in enzyme concentration may have occurred (Srere & Foster, 1967). In the present experiments, where the period of insulin deprivation in alloxan-diabetic rats was 4 days, the longer-term changes were revealed and the marked fall in citrate-cleavage enzyme activity could be seen. The NADP-linked isocitrate dehydrogenase in the soluble fraction of the cell is also involved in the extramitochondrial pathway of citrate metabolism, but this enzyme activity remained unchanged in the mammary glands of alloxan-diabetic rats.

The behaviour of these two enzymes differs markedly from the pattern found in the mammary glands from thyroidectomized rats, a condition that also leads to a depressed rate of lipid synthesis; here citrate-cleavage enzyme activity was unchanged, whereas NADP-linked isocitrate dehydrogenase activity was significantly decreased (Walters & McLean, 1967), results that are in direct contrast with the present findings. Thus conditions of decreased lipid synthesis are not necessarily accompanied by similar modifications in enzyme pattern. A further point of contrast between these two hormonal conditions is found in acetate

thiokinase, the activity of which falls in mammary glands from alloxan-diabetic rats to less than half the control value, whereas it remains unchanged in mammary glands from thyroidectomized animals.

Effect of treatment with anti-insulin serum on glucose oxidation and fatty acid synthesis from glucose. Gregor, Martin, Williamson, Lacey & Kipnis (1963) demonstrated that, 90min. after treatment of rats with anti-insulin serum, the oxidation of [1-^{14}C]glucose in adipose tissue was depressed, whereas the direct effect of anti-insulin serum *in vitro* in decreasing the oxidation of [1-^{14}C]glucose by adipose tissue from normal rats in the absence of added exogenous insulin was demonstrated by Beloff-Chain, Catanzaro & Chain (1967).

The overall effect of treatment with anti-insulin serum on lactating rat mammary gland was a general decrease in both the oxidation of, and fatty acid synthesis from, glucose. This effect was reversed by the presence of insulin *in vitro*. The complete reversal in this condition compared with that obtained with insulin added *in vitro* to mammary-gland slices from alloxan-diabetic rats suggests that because of the relatively short period of insulin insufficiency (1hr.) no secondary changes in enzyme concentration had occurred.

The results obtained with mammary-gland tissue from diabetic rats and the effects of insulin *in vitro* could, on the whole, be accounted for if the major action of insulin on lactating mammary-gland tissue were similar to that proposed for adipose tissue, namely one of modification of glucose movement from the extra- to the intra-cellular space (Crofford & Renold, 1965*a*,*b*). One difficulty in accepting this as the complete explanation lies in the observation that the addition of phenazine methosulphate *in vitro* to tissue slices from lactating rats treated with anti-insulin serum completely restored the rate of oxidation of [1-^{14}C]glucose to $^{14}CO_2$ to the value found in the control tissue. This suggests a change in an electron-acceptor system rather than a change in the rate of glucose transport and phosphorylation.

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