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Regulation of Glucose Uptake by Muscle

8. EFFECTS OF FATTY ACIDS, KETONE BODIES AND PYRUVATE, AND OF ALLOXAN-DIABETES AND STARVATION, ON THE UPTAKE AND METABOLIC FATE OF GLUCOSE IN RAT HEART AND DIAPHRAGM MUSCLES

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In alloxan-diabetes in the rat the uptake of glucose by heart and diaphragm muscle *in vitro* is impaired both in the absence and in the presence of insulin. The defect in glucose uptake in the absence of the hormone has been explained by the low rate of membrane transport of glucose in the insulin-deficient tissue (insulin accelerates the membrane transport of glucose); the defect in the membrane transport of glucose may be corrected by the addition of high concentrations of insulin *in vitro* but not by hypophysectomy or adrenalectomy (see Morgan, Cadenas, Regen & Park, 1961*b*; Morgan, Regen, Henderson, Sawyer & Park, 1961*c*; Kipnis, 1959). Two other defects cannot be explained in this way: first, an impaired ability of low concentrations of insulin to stimulate membrane transport of glucose; and, secondly, a decreased rate of phosphorylation of glucose. These defects are corrected by hypophysectomy or adrenalectomy or by treatment of the diabetic rat with insulin (Morgan *et al.* 1961*b*, *c*; Park *et al.* 1961; Kipnis, 1959; Riddick, Reisler & Kipnis, 1962); they can be restored in muscles of hypophysectomized diabetic rats by treatment of the animals with growth hormone and corticosteroids. The development of these defects in glucose metabolism in muscle in diabetes is thus favoured by a deficiency of circulating insulin together with a sufficiency of growth hormone and corticosteroids. The mechanism is not known. The details of the factors required for the development or for the removal of these defects are, however, the same as those required for the development of a defect in the phosphorylation of fructose 6-phosphate in muscle from the diabetic rat (Newsholme & Randle, 1964). In the latter instance the depressed phosphorylation of fructose 6-phosphate appears to be caused by an increased provision of fatty acids (and *in vivo* of ketone bodies) for respiration in tissue from the diabetic rat (Newsholme & Randle, 1964). This has prompted us to investigate the effects of fatty acids and ketone bodies on the transport, phosphorylation and metabolic fate of glucose in rat heart and diaphragm, and to compare them with those of diabetes (and also of starvation, which affects the phosphorylation of fructose 6-phosphate in the same way as diabetes). Preliminary accounts of some of these results have been published (Newsholme, Randle & Manchester, 1962; Garland & Randle, 1962; Randle, Garland, Hales & Newsholme, 1963).

Inhibitory effects of ketone bodies and acetate on glucose oxidation by muscle in rabbits *in vivo* were first shown by Drury & Wick (1953). Inhibitory effects of ketone bodies on glucose uptake and glucose oxidation in perfused rat heart *in vitro* have been described by Williamson & Krebs (1961), and similar effects of long-chain fatty acids in the form of complexes with bovine plasma albumin by Shipp, Opie & Challoner (1961). Inhibitory effects of long-chain fatty acids (as complexes with bovine plasma albumin) on the uptake and oxidation of glucose by rat hemidiaphragms have also been described in parallel studies by Bühring (1963).

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MATERIALS

Rats. Details of the rats used, diet, induction of alloxan-diabetes and hypophysectomy are given by Newsholme & Randle (1964) and Garland, Newsholme & Randle (1964).

Chemicals. Sources of sugars, enzymes, fatty acids, ketone bodies, bovine plasma albumin and other chemicals are given by Randle & Smith (1958*a*, *b*), Battaglia & Randle (1960), Newsholme & Randle (1964) and Garland *et al.* (1964). [1-^{14}C]- and [6-^{14}C]-Glucose (2 mc/m-mole) and [1-^{14}C]sorbitol (1 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. They were diluted with perfusion or incubation medium to the specific activities given in the text or Tables.

Media. Hearts were perfused with and diaphragms incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with $O_2 + CO_2$ (95:5) or $N_2 + CO_2$ (95:5).

PROCEDURE

Hearts. For measurement of glucose uptake, changes in glycogen synthesis, incorporation of [^{14}C]glucose into glycogen, intracellular glucose concentration and L-arabinose uptake, hearts were perfused for 15 or 30 min. through the coronary circulation at 37° with about 15 ml.

of medium recirculated in the apparatus of Morgan, Henderson, Regen & Park (1961*a*). The aerobic uptake of glucose with this apparatus and procedure is the same for the 0–15 min. and 15–30 min. periods of perfusion (Morgan, Randle & Regen, 1959), and oxygenation of the medium is adequate with this technique (Morgan *et al.* 1961*a*). The heart was washed out initially with 10–15 ml. of the medium used in the subsequent perfusion. For assay of intracellular glucose, L-arabinose or glycogen concentration, the heart was cut from the cannula and blotted (after opening the chambers), and frozen in acetone–solid CO_2 and kept frozen until extracted (see below).

For measurement of L-arabinose accumulation, the hearts were first perfused with medium containing glucose (at concentrations given in the text or Tables) for 5 min. to allow the tissue to recover from the period of anoxia between excision and the start of perfusion (as judged by the decline in lactate output to steady-state levels measured in pilot experiments, this required 3 min.). Perfusion was then continued for a further 10 min. with medium containing L-arabinose and glucose at concentrations given in the text or Tables. Glucose was added during the period of perfusion with pentose to restrict entry of the latter (which it does by competing with the pentose for penetration; see Morgan, Cadenas & Park, 1960). In the absence of glucose the entry of pentose was so rapid as to be nearly complete at the end of 10 min. of perfusion.

For the measurement of L-arabinose efflux, hearts were allowed to accumulate the sugar by perfusion for 10 min. under aerobic conditions with medium containing the pentose (9 mg./ml.) and from which was omitted an osmotically equivalent amount of NaCl (30 m-moles/l.). Arabinose-free medium (containing the full amount of NaCl) was then perfused by drip through for 3 min. (to remove extracellular pentose). At this point the collecting vessel was changed and perfusion continued for a further 6 min., with collection of the perfusion fluid from the heart. During this period the collecting vessel was changed each minute. Pentose was estimated in the collections obtained during the latter (6 min.) period of perfusion and in the heart at the end of perfusion. This method of estimating L-arabinose efflux is superior to that described by Morgan *et al.* (1959). In particular, the shorter period of perfusion and maintenance of iso-osmotic conditions in the medium containing L-arabinose yielded much better hearts, as judged by the force and rate of contraction. The output of $^{14}CO_2$ from hearts perfused with medium containing [1-^{14}C]- or [6-^{14}C]-glucose was measured in medium that flowed continuously through the coronary circulation from a reservoir 60–70 cm. above the heart. The heart was contained in a jacketed chamber (at 37°) of about 10 ml. capacity sealed at its upper end with a rubber bung containing the perfusion cannula and an outlet tube (to allow the chamber to be filled with medium at the beginning of perfusion); at the lower end the chamber was sealed with a rubber bung containing an outlet tube from which medium was collected. The heart was perfused initially for 5 min. with medium lacking [^{14}C]glucose and then switched to medium containing [^{14}C]glucose. A period of 3 min. of perfusion was allowed for equilibration of extracellular water with [^{14}C]glucose (shown to yield 90–95% equilibration) and perfusion fluid was then collected under heptane (15 ml.) for a further 3 min. in a graduated cylinder immersed in ice. The assay of $^{14}CO_2$ in the perfusion fluid was begun within 15 min. by a method described by Garland *et al.* (1964). Control experiments showed that there was no detectable loss of $^{14}CO_2$ from perfusion fluid kept under heptane for 2 hr. Samples of incubation media were plated and counted in a Nuclear–Chicago gas-flow counter in order that outputs of $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]-glucose could be corrected to the same specific activity of medium glucose.

Diaphragm. For measurement of glucose uptake, glycogen synthesis and lactate and pyruvate output, hemidiaphragms were incubated for 60 min. in 2 ml. of medium as described by Randle & Smith (1958*a*, *b*).

METHODS

Muscle extracts. For assay of glucose or pentose heart muscle was extracted with boiling water as described by Battaglia & Randle (1960).

Glycogen synthesis. This was assayed by the change in glycogen concentration during perfusion of rat heart or incubation of rat hemidiaphragms. The initial glycogen was measured in heart muscle on separate hearts washed through with 10–15 ml. of medium. With hemidiaphragms one member of each pair was used for assay of the initial glycogen concentration and the other incubated and used for assay of the final glycogen concentration. Glycogen was assayed in muscle as follows. Muscle was dissolved in 30% (w/v) KOH (2–4 ml./g.) and crude glycogen was precipitated and washed as described by Walaas & Walaas (1950). The crude glycogen was then hydrolysed in 2 ml. of 2N-H_2SO_4 for 3 hr. on a boiling-water bath. After cooling, the hydrolysate was diluted and brought to pH 7 by the addition of 4 ml. of M-sodium phosphate buffer, pH 7, and 2 ml. of 2N-NaOH and water to 10 ml. Glycogen glucose was then assayed on a suitable sample by the glucose-oxidase method (Huggett & Nixon, 1957).

In some experiments glycogen synthesis was measured in perfused heart with [6-^{14}C]glucose as follows. The crude glycogen was precipitated and washed as described above, and then dissolved in 5–10 ml. of water. A sample (1–2 ml.) of this solution was hydrolysed and glycogen glucose assayed to obtain the glycogen concentration as described above. The glycogen in the remainder of the solution was purified by precipitation of contaminating material at pH 4·0–4·5 (adjusted with glass electrode and 2N-H_2SO_4) followed by shaking with an equal volume of $CHCl_3$ and a drop of octan-2-ol and removal of the aqueous phase after centrifugation (contaminating material is precipitated at the interface). In control experiments the aqueous phase was shown to yield (after dialysis against distilled water and precipitation with ethanol and drying of the precipitated glycogen *in vacuo*) glycogen that on analysis contained, by wt., 96–99% of glucose. The aqueous phase was hydrolysed and analyses were made of glucose (see above) and radioactivity (after plating and assay in a Nuclear–Chicago gas-flow counter). The specific activity of medium glucose was measured in the same way. The net incorporation of medium glucose into muscle glycogen (in mg. of glucose/g. wet wt. of tissue) is given by:

$$\text{Glycogen concn. (mg./g.)} \times \frac{\text{sp. activity of purified glycogen}}{\text{sp. activity of medium glucose}}$$

Glucose. Glucose was assayed in incubation or perfusion media or muscle extracts by the glucose-oxidase method (Huggett & Nixon, 1957) after deproteinization with $Zn(OH)_2$ (Somogyi, 1945).

Pentose. L-Arabinose was estimated in muscle extract and perfusion medium deproteinized with $Zn(OH)_2$ by either the method of Roe & Rice (1948) or the method of Mejbaum (1939). The former was used for all muscle extracts and for the perfusion medium in measurements of the L-arabinose space; the latter was used for assay of the pentose in the perfusion medium in measurements of L-arabinose efflux, because of its greater sensitivity.

Fatty acid. The concentration of fatty acids in the form of complexes with albumin in perfusion or incubation media was assayed by a modification (Trout, Estes & Friedberg, 1960) of Dole's method.

Calculations. Glucose uptake, L-arabinose, glucose and sorbitol spaces and intracellular volume were calculated as described by Randle & Smith (1958*a*, *b*) and Morgan *et al.* (1959). The intracellular glucose space was calculated from:

Glucose space – sorbitol space

The rate of glycolysis was calculated from:

Glucose uptake – glycogen synthesis – intracellular glucose

The rates of L-arabinose efflux have been calculated as described by Morgan *et al.* (1959) and expressed for each minute of perfusion during the period of measurement as the percentage of intracellular arabinose lost during that minute. This method of expression is valid because the rate of efflux was linearly related to the concentration gradient (intracellular arabinose concentration–extracellular arabinose concentration) under each of the conditions used and because the concentration gradient was almost identical with the intracellular pentose concentration (the concentration of pentose in the perfusion medium was less than 0·5% of that in the heart). For purposes of comparison with the results of other studies the rate of efflux has also been calculated and expressed as the mean rate of efflux at a concentration gradient of 1 mg. of L-arabinose/ml.

Rates of glucose uptake have been calculated as mg. of glucose/g. wet wt. of muscle/hr. The water content of the heart was not changed by any of the agents used in these experiments except anoxia and salicylate. The latter treatments increase the water content of the heart by increasing the volume of extracellular water (Morgan *et al.* 1959). The weight of this additional water (calculated from the sorbitol space) was subtracted from the wet weight of hearts perfused anaerobically or with salicylate for calculation of the glucose uptake.

RESULTS

Effects of DL-β-*hydroxybutyrate and* n-*octanoate on the accumulation of* L-*arabinose and the rate of efflux of* L-*arabinose in perfused rat heart*

When hearts from normal fed rats were perfused for 10 min. with medium containing L-arabinose (2 mg./ml.) and glucose (0·5–1 mg./ml.), the intracellular arabinose space varied from 10 to 16 ml./100 g. wet wt. of muscle (the intracellular volume was 43 ml./100 g., so that the ratio of intracellular to extracellular pentose concentrations was 0·2–0·4; at equilibrium the ratio was approx. 0·8). Perfusion with β-hydroxybutyrate (70 mg./100 ml.) decreased the L-arabinose accumulation to 68, 69 and 46 % of that of the control at glucose concentrations of 0·5, 0·8 and 1·0 mg./ml. respectively (Table 1). Similarly octanoate (40 mg./100 ml.) decreased the accumulation of arabinose to 69 % of that of the control at a glucose concentration of 0·5 mg./ml. In hearts from hypophysectomized rats perfused with medium containing insulin (0·5 milliunit/ml.), glucose (0·5 mg./ml.) and L-arabinose (2 mg./ml.), β-hydroxybutyrate (70 mg./100 ml.) decreased the accumulation of pentose to 40 % of that of the control.

Table 1. *Effects of* DL-β-*hydroxybutyrate and* n-*octanoate on* L-*arabinose accumulation in perfused rat heart*

In experiments with hearts from hypophysectomized rats the perfusion medium contained insulin (0·5 milliunits/ml.). Hearts were perfused for 10 min. with medium containing glucose and arabinose at the concentrations shown after 5 min. of pre-perfusion with medium containing β-hydroxybutyrate or octanoate (or without these additions in the control experiments). Other details are given in the text. The results for the intracellular L-arabinose space are given as means ±S.E.M., with the numbers of observations in parentheses.

Source of heart	Addition to perfusion medium	Concn. in medium (mg./ml.) Glucose	Concn. in medium (mg./ml.) L-Arabinose	Intracellular L-arabinose space (mg./100 g.)
Normal fed rat (control)	None	0·5	2·0	15·7±1·6 (6)
Normal fed rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	0·5	2·0	10·7±0·4† (6)
Normal fed rat	n-Octanoate (40 mg./100 ml.)	0·5	2·0	10·9±1·4‡ (6)
Normal fed rat (control)	None	1·0	2·0	10·0±1·7 (6)
Normal fed rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	1·0	2·0	4·6±1·2‡ (6)
Normal fed rat (control)	None	0·8	2·0	16·0±1·6 (6)
Normal fed rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	0·8	2·0	11·0±1·4‡ (6)
Hypophysectomized rat (control)	None	0·5	2·0	12·0±0·4 (6)
Hypophysectomized rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	0·5	2·0	5·0±1·5* (6)

* $P < 0·01$; † $P < 0·02$; ‡ $P < 0·05$; for other differences from appropriate control $P > 0·05$.

The effects of β-hydroxybutyrate (70 mg./100 ml.) and octanoate (40 mg./100 ml.) on the rate of efflux of L-arabinose from hearts allowed to accumulate arabinose in a period of pre-perfusion with the pentose are shown in Table 2. In hearts from normal fed rats perfused with medium lacking insulin the rate of efflux averaged 21 % of the concentration of pentose in the muscle. With the addition of β-hydroxybutyrate (70 mg./100 ml.) and octanoate (40 mg./100 ml.) the rate was decreased to 60 % of that of the control. In hearts from rats starved for 18 hr. the rate of efflux was 60 % of that of hearts from fed rats, and β-hydroxybutyrate (70 mg./100 ml.) produced no diminution in the rate of efflux (the rate of efflux was slightly increased by β-hydroxybutyrate in hearts from starved rats but the difference was not statistically significant). In hearts from starved rats insulin at a concentration of 0·5 milliunit/ml. increased the rate of efflux by 230 % in the absence of β-hydroxybutyrate, but only by 160 % in the presence of the ketone body (70 mg./100 ml.). On the other hand, in hearts from fed rats perfused with a high concentration of insulin (0·1 unit/ml.) and in which transport rates are maximal (Morgan *et al.* 1959), β-hydroxybutyrate had no inhibitory effects. For comparison with the results of other studies the transport rates have also been calculated for an intracellular concentration of L-arabinose of 1 mg./ml. (see the Methods section). The rates were (in μg. of arabinose/min./g. wet wt. of heart): for heart from normal fed rat (control), 100; for heart from starved rat, 62; for heart from rat with β-hydroxybutyrate in the perfusion medium, 75; for heart from rat with octanoate in the perfusion medium, 60; for heart from rat with a high concentration of insulin (0·1 unit/ml.) in the perfusion medium, 163. The values for heart from starved rat and for that perfused with a high concentration of insulin are identical with those obtained by Morgan *et al.* (1959, 1960).

Effects of alloxan-diabetes and starvation, and of ketone bodies, fatty acids, pyruvate, anoxia and salicylate, on the intracellular glucose space and rates of glucose uptake, glycogen synthesis and glycolysis in perfused rat heart

Glucose uptake and intracellular glucose space measured in the absence of insulin. The results of these experiments are given in Table 3. When hearts from fed rats were perfused for 15 min. with medium containing glucose (1 mg./ml.), the glucose uptake in four separate experiments was in the range 8·6–10·6 mg./g. wet wt./hr. and the intracellular glucose space 0–5·5 ml./100 g. wet wt. The glucose uptake was decreased by β-hydroxybutyrate (70 mg./100 ml.) to 27 % of that of the control, by octanoate (40 mg./100 ml.) to 75 % of that of the control and by palmitate (19·2 mg./100 ml. carried by 2 % bovine plasma albumin) to 51 % of that of the control. The intracellular glucose space was not changed by β-hydroxy-

Table 2. *Effects of* DL-β-*hydroxybutyrate and* n-*octanoate on* L-*arabinose efflux in perfused rat heart*

There were 6–12 hearts perfused in each group. Details are given in the text. The values given in parentheses are the control rates of efflux. The intracellular pentose concentration was calculated as μg. of pentose/g. wet wt. of heart. The results for the average mean differences from the appropriate control are given as means ±S.E.M.

Source of heart	Addition(s) to perfusion medium	Percentage of intracellular pentose removed/min. (mean difference from appropriate control) 0–1 min.	1–2 min.	2–3 min.	3–4 min.	4–5 min.	5–6 min.	Average over 0–6 min.
Normal fed rat (control)	None	(19·9)	(20·3)	(20·7)	(21·0)	(21·2)	(22·9)	(21±0·44)
Normal fed rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	−5·5	−7·0	−8·1	−9·7	−8·8	−10·8	−8·4±0·62*
Normal fed rat	n-Octanoate (40 mg./100 ml.)	−6·0	−8·5	−9·3	−9·9	−10·5	−13·4	−8·2±0·79*
Starved (18 hr.) rat (control)	None	(14·4)	(13·6)	(13·8)	(12·8)	(10·7)	(8·3)	(12·3±1·0)
Starved (18 hr.) rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	4·0	0·9	2·2	2·2	4·3	3·7	3·0±2·1
Starved (18 hr.) rat	Insulin (0·5 milliunit/ml.)	10·3	13·6	13·0	16·5	19·3	22·9	16·1±0·89*
Starved (18 hr.) rat	Insulin (0·5 milliunit/ml.) + DL-β-hydroxybutyrate (70 mg./100 ml.)	4·8	11·5	7·3	8·8	10·6	12·0	9·2±1·12*
Normal fed rat	Insulin (0·1 unit/ml.)	10·9	10·6	10·7	8·6	10·0	7·2	9·3±3·5‡
Normal fed rat	Insulin (0·1 unit/ml.) + DL-β-hydroxybutyrate (70 mg./100 ml.)	15·3	14·9	16·2	21·3	19·0	20·2	17·8±1·8*

* $P < 0·01$; ‡ $P < 0·05$; for other differences from appropriate control $P > 0·05$.

butyrate or palmitate but was significantly increased by octanoate. In hearts from rats starved for 18 hr. and perfused for 15 min. the glucose uptake was significantly lower than that of hearts from fed rats. Nevertheless, β-hydroxybutyrate (70 mg./100 ml.) and octanoate (40 mg./100 ml.) decreased the rate of glucose uptake in hearts from rats starved for 18 hr. to 50 and 41 % of that of the control. The intracellular glucose space was not increased by β-hydroxybutyrate or octanoate. In confirmation of the findings by Morgan *et al.* (1961*b*), the glucose uptake of hearts from fed alloxan-diabetic rats was 32 % of that of the control. A similar degree of inhibition of glucose uptake was seen in hearts from rats starved for 40 hr. When glucose uptakes were measured in two successive periods of perfusion of 15 min. duration in hearts from rats starved for 18 hr., the rates of uptake were very similar, both in the presence and in the absence of β-hydroxybutyrate (70 mg./100 ml.). There was, moreover, no significant difference in the glucose spaces when these were measured after 15 min. and after 30 min. of perfusion. In further experiments (not shown) the rates of glucose uptake in hearts from fed rats measured during 15 min. and 30 min. periods of perfusion were similar.

Glucose uptake and intracellular glucose space measured in the presence of insulin. The results of these experiments are given in Table 4. When hearts from fed rats were perfused aerobically for 15 min. with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) the glucose uptake in four separate experiments was in the range 10·8–14·2 mg./g. wet wt./hr. and the intracellular glucose space 0–9 ml./100 g. wet wt. These values are substantially the same as those obtained by Morgan *et al.* (1959). In confirmation of previous findings (Morgan *et al.* 1961*b*; Newsholme & Randle, 1961), alloxan-diabetes or starvation for 40 hr. decreased the glucose uptake to 41 and 66 % of that of the control and increased the intracellular glucose (3-fold). Glucose uptake was decreased in hearts from fed normal rats to 46 % of that of the control by perfusion with β-hydroxybutyrate (70 mg./100 ml.), to 43 % with acetoacetate

Table 3. *Effects of alloxan-diabetes and starvation, and of* DL-β-*hydroxybutyrate and* n-*octanoate, on the glucose uptake, intracellular glucose space and glycogen concentration in rat hearts perfused in the absence of insulin*

The perfusion medium contained glucose (1 mg./ml.). Other details are given in the text. Intracellular glucose space was calculated from: glucose space − sorbitol space. The results are given as means ±S.E.M., with the numbers of observations in parentheses.

Source of heart	Addition(s) to perfusion medium	Time of perfusion (min.)	Glucose uptake (mg./g. wet wt. of heart/hr.)	Intracellular glucose space (ml./100 g. wet wt. of heart)	Change in glycogen concn. (mg./g. wet wt. of heart/hr.)
Normal fed rat (control)	None	0–15	9·2±1·6 (6)	5·5±1·5	—
Normal fed rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	0–15	2·6±0·7* (6)	8·0±1·9	—
Normal fed rat (control)	None	0–15	8·6±0·28 (6)	0±2·8	—
Normal fed rat	*n*-Octanoate (40 mg./100 ml.)	0–15	6·5±0·13* (6)	10±2·5‡	—
Normal fed rat (control)	Albumin (2%)	0–15	8·6±0·16 (11)	0±3	—
Normal fed rat	Albumin (2%) + palmitate (19·2 mg./100 ml.)	0–15	4·4±0·28* (11)	3±1	0±0·51
Starved (18 hr.) rat (control)	None	0–15	5·8±0·82 (12)	0±2·1	—
Starved (18 hr.) rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	0–15	2·9±0·37 (12)	4±2·1	2·8±1·1
Starved (18 hr.) rat (control)	None	15–30	5·4±0·95 (6)	0±1·7	—
Starved (18 hr.) rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	15–30	2·7±0·47† (6)	8±1·2*	0·8±0·62
Starved (18 hr.) rat (control)	None	0–15	4·1±0·5 (6)	0±1	—
Starved (18 hr.) rat	*n*-Octanoate (40 mg./100 ml.)	0–15	1·7±0·4* (4)	4±0·8†	−2·8±1·4
Normal fed rat (control)	None	0–15	10·6±0·73 (6)	—	—
Alloxan-diabetic rat	None	0–15	3·4±0·43* (6)	—	—
Starved (40 hr.) rat	None	0–15	3·2±0·50* (6)	—	—

* $P < 0·01$; † $P < 0·02$; ‡ $P < 0·05$; for other differences from appropriate control $P > 0·05$.

(25 mg./100 ml.), to 65 % with octanoate (40 mg./100 ml.), to 40 % with pyruvate (60 mg./100 ml.) and to 45 % with butyrate (35 mg./100 ml.). Each of these agents led to a marked increase in intracellular glucose (2–4-fold). In studying the effects of palmitate or oleate it was necessary to add bovine plasma albumin as a carrier for the fatty acid. Glucose uptake and intracellular glucose space were not noticeably affected by the addition of albumin (2 or 4 %) alone. Palmitate (19·2 mg./100 ml.) or oleate (32·7 mg./100 ml.) carried by 2 % bovine plasma albumin decreased the glucose uptake to 66 and 73 % of that of the control and increased intracellular glucose (2–3-fold). Similar effects of palmitate (12·8 mg./100 ml.) were observed when hearts were perfused with medium containing 4 % of albumin. The effects of different concentrations of palmitate carried by 4 % albumin on glucose uptake and intracellular glucose concentration are shown in Table 5. At concentrations of palmitate below 0·3 mM (7·7 mg./100 ml.) no change in glucose uptake or intracellular glucose could be detected. Significant inhibition of glucose uptake was observed with 0·3 mM-palmitate and maximum inhibition at 0·4 mM-palmitate. In additional experiments (not shown) the inhibitory effects of β-hydroxybutyrate on glucose uptake did not differ significantly from those described in a 15 min. perfusion when the period of perfusion was extended to 30 min. The glucose space measured after 30 min. was, moreover, very similar to that measured after 15 min. The degree of inhibition of glucose uptake induced by ketone bodies in these experiments was similar to that described by Williamson & Krebs (1961) in a much longer period of perfusion (1 hr.); similar inhibition of glucose uptake with palmitate and oleate has been described in a preliminary report by Bowman (1962).

When hearts from normal fed rats were perfused under anaerobic conditions glucose uptake was increased and the intracellular glucose concentration decreased, in confirmation of findings by Morgan *et al.* (1959). Salicylate (90 mg./100 ml.) under aerobic conditions had effects very similar to those of anoxia. Under anaerobic conditions acetoacetate (25 mg./100 ml.) and palmitate (19·2 mg./100 ml. carried by 2 % bovine plasma albumin) neither inhibited glucose uptake nor caused intracellular accumulation of glucose. Similarly, in the presence of salicylate under aerobic conditions no inhibitory effects of alloxan-diabetes or of β-hydroxybutyrate or palmitate on glucose uptake were seen, and these agents also failed to increase the intracellular glucose space. These results are in clear contradistinction to those obtained under aerobic conditions and in the absence of salicylate.

Glycogen synthesis. The results of these experiments are given in Tables 3 and 4. In hearts from normal fed rats (glycogen concentration 2·5–3·5 mg./g. wet wt.) perfusion for 15 or 30 min. with medium containing glucose (1 mg./ml.) in the presence or in the absence of insulin (0·1 unit/ml.) produced no change in the concentration of glycogen, i.e. there was no net synthesis of glycogen. In hearts from alloxan-diabetic rats or starved rats in which the glycogen concentration was initially higher (about twice the control), the concentration of glycogen fell during perfusion. In hearts from normal fed rats perfused with medium containing glucose and insulin, the addition of ketone bodies or pyruvate increased the concentration of glycogen markedly, but no significant change was seen with octanoate, butyrate or palmitate (Table 4). The concentration of glycogen in hearts perfused with medium lacking insulin was similarly increased by β-hydroxybutyrate but not by octanoate (Table 3). When glycogen concentrations were compared in hearts perfused for 15 min. and for 30 min. with medium containing β-hydroxybutyrate, the increase in glycogen concentration induced by the ketone body was much more marked in the first 15 min. of perfusion than in the second (Table 3). This suggested the possibility that much of the additional glycogen synthesized during the first 15 min. of perfusion with β-hydroxybutyrate might be derived, not from medium glucose, but from precursors of glycogen accumulated in the muscle during removal of the heart and commencement of perfusion (e.g. hexose monophosphates; see Newsholme & Randle, 1961). The rate of incorporation of medium glucose into glycogen was therefore investigated with [^{14}C]glucose (see the Methods section). The glucose concentration in the medium was 1 mg./ml. and the specific activity varied from 3 to 10 μc/ml., and insulin was present (0·1 unit/ml.); the period of perfusion was 15 min. The incorporation of medium glucose into glycogen (expressed as mg. of glucose/g. wet wt. of heart/hr.) was increased by pyruvate from $0·91 \pm 0·14$ to $1·42 \pm 0·1$ (means ± S.E.M. for six hearts; $P < 0·02$), by palmitate (19·2 mg./100 ml. carried by 2 % albumin) from $1·51 \pm 0·18$ to $1·85 \pm 0·28$ (means ± S.E.M. for six hearts; difference not significant), by octanoate (40 mg./100 ml.) from 1·2 to 1·65 (six pooled hearts in each group), and by β-hydroxybutyrate (70 mg./100 ml.) from 0·6 to 0·86 (six pooled hearts in each group). In hearts perfused without insulin the incorporation of glucose into glycogen (expressed as mg. of glucose/g. wet wt. of heart/hr.) was 0·47 in the absence and 0·53 in the presence of butyrate (20 mg./ml.) (six pooled hearts in each group). The increase in glucose incorporation into glycogen with pyruvate and β-hydroxybutyrate detected with the isotope method

is considerably smaller than the net change in glycogen concentration that they induce. This supports our suggestion that much of the glycogen deposited under the influence of pyruvate and β-hydroxybutyrate in the first 15 min. of perfusion is not formed from medium glucose. The percentage of the glucose taken up that was converted into glycogen was increased by pyruvate from 7·7 to 25·4, by palmitate from 12·8±1·52 to 24·1±3·65 ($P < 0·02$), by butyrate from 7·2 to 13·2, by β-hydroxybutyrate from 5·6 to 17, and by octanoate from 11·1 to 25.

Glycolysis rate. The changes in glycolysis rate induced by these various factors were calculated from:

Glucose uptake − intracellular glucose − change in glycogen concn.

and are shown in Table 4. The values are provisional because accurate values require a further

Table 4. *Effects of alloxan-diabetes and starvation, and of ketone bodies, fatty acids, pyruvate, anoxia and salicylate, on the glucose uptake, intracellular glucose space, glycogen concentration and glycolysis rate in rat hearts perfused with medium containing insulin* (0·1 *unit/ml.*)

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Hearts were perfused for 15 min. by recirculation (see the Methods section). Other details are given in the text. Intracellular glucose space was calculated from: glucose space − sorbitol space. Glycolysis rate was calculated from: glucose uptake − intracellular glucose − glycogen synthesis. The value in parentheses at the top of column 5 is the change in glycogen concn. in hearts from normal fed rats. The results are given as means ±S.E.M., with the numbers of observations in parentheses.

Source of heart	Addition(s) to perfusion medium	Glucose uptake (mg./g. wet wt. of heart/hr.)	Intracellular glucose space (ml./100 g. wet wt. of heart)	Change in glycogen concn. (mg./g. wet wt. of heart/hr.) (difference from control)	Change in glycolysis rate (mg. of glucose/g. wet wt. of heart/hr.) (difference from control)
Normal fed rat (control)	None (aerobic)	14·2±0·7 (14)	6±3	(0±0·8) (24)	—
Alloxan-diabetic rat	None	5·8±0·6* (13)	16±3‡	−4·4±1·8 (6)	−4·0
Starved (40 hr.) rat	None	9·4±0·4* (13)	17±2‡	−2·4±1·6 (4)	−2·8
Normal fed rat (control)	None (aerobic)	10·8±0·73 (12)	6±2·2	—	—
Normal fed rat	DL-β-Hydroxybutyrate (70 mg./100 ml.)	5·0±0·24* (12)	23±2·8*	3·4±1* (12)	−9·8
Normal fed rat	Acetoacetate (25 mg./100 ml.)	4·7±0·25* (12)	22±2·0*	2·9±1† (12)	−8·6
Normal fed rat	*n*-Octanoate (40 mg./100 ml.)	7·0±0·30* (17)	21±1·1*	0·8±1·5 (7)	−5·2
Normal fed rat (control)	None (aerobic)	14·0±1·5 (6)	9±1·7	—	—
Normal fed rat	Pyruvate (60 mg./100 ml.)	5·6±1·2* (6)	22±2·3*	4·6±1* (6)	−13·5
Normal fed rat (control)	None (aerobic)	11·8±0·7 (6)	0±0·6	—	—
Normal fed rat	Butyrate (35 mg./100 ml.)	5·3±0·4* (6)	17±1·8*	0·5±1 (6)	−7·6
Normal fed rat (control)	Albumin (2%) (aerobic)	11·8±0·8 (18)	7±2·1	—	—
Normal fed rat	Albumin (2%) + palmitate (19·2 mg./100 ml.)	7·7±0·7* (18)	23±1·7*	0·4±1 (24)	−5·0
Normal fed rat (control)	Albumin (2%) (aerobic)	10·2±0·5 (11)	6±1·9	—	—
Normal fed rat	Albumin (2%) + oleate (32·7 mg./100 ml.)	7·5±0·7* (11)	17±1*	—	—
Normal fed rat (control)	Albumin (4%) (aerobic)	11·2±0·31 (15)	9±1·5	—	—
Normal fed rat	Albumin (4%) + palmitate (12·8 mg./100 ml.)	6·6±0·62* (12)	17±1·5*	—	—

* $P < 0·01$; † $P < 0·02$; ‡ $P < 0·05$; for other differences from appropriate control $P > 0·05$.

Table 4 (*cont.*)

Source of heart	Addition(s) to perfusion medium	Glucose uptake (mg./g. wet wt. of heart/hr.)	Intracellular glucose space (ml./103 g. wet wt. of heart)	Change in glycogen concn. (mg./g. wet wt. of heart/hr.) (difference from control)	Change in glycolysis rate (mg. of glucose/g. wet wt. of heart/hr.) (difference from control)
Normal fed rat (control)	None (anaerobic)	18·7±0·63 (6)	−6±2·4	—	—
Normal fed rat	Acetoacetate (25 mg./100 ml.) (anaerobic)	17·1±0·50 (6)	−9±1·9	—	—
Normal fed rat (control)	Albumin (2%) (anaerobic)	18·0±1·6 (6)	1±2·1	−10·8±0·32	—
Normal fed rat	Albumin (2%) + palmitate (19·2 mg./100 ml.) (anaerobic)	18·4±2·1 (6)	−5±3·2	−12·0±0·36	+2
Normal fed rat (control)	Salicylate (90 mg./100 ml.) (aerobic)	17·1±1·2 (4)	4±2·1	—	—
Alloxan-diabetic rat	Salicylate (90 mg./100 ml.)	16·7±1·8 (4)	5±1·8	—	—
Normal fed rat	Salicylate (90 mg./100 ml.) + DL-β-hydroxybutyrate (70 mg./100 ml.)	16·4±1·1 (4)	4±1·3	—	—
Normal fed rat	Salicylate (90 mg./100 ml.) + albumin (2%)	14·1±0·6 (6)	—	—	—
Normal fed rat	Salicylate (90 mg./100 ml.) + albumin (2%) + palmitate (19·2 mg./100 ml.)	13·8±1·2 (6)	—	—	—

Table 5. *Effects of different concentrations of palmitate, carried by 4% bovine plasma albumin, on the glucose uptake in rat hearts perfused with medium containing insulin* (0·1 *unit/ml.*)

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.), together with albumin (4%) and palmitate as indicated. Other details are given in the text. The results are given as means ±S.E.M. with the numbers of observations in parentheses.

Initial concn. of palmitate in perfusion medium (mM)	Glucose uptake (mg./g. wet wt. of heart/hr.)	Intracellular glucose space (ml./100 g. wet wt. of heart)
0	9·9±0·5 (12)	9±1·1
0·05	10·2±1·4 (3)	—
0·20	9·4±0·5 (15)	9±1·5
0	11·2±0·3 (11)	9±1·1
0·5	6·6±0·62† (12)	17±1·5†
0	11·8±0·35 (24)	—
0·15	10·5±0·55 (4)	—
0·30	9·4±0·28† (4)	—
0·40	7·9±0·52† (4)	—
0·60	8·5±0·90† (6)	—
1·00	8·5±0·70† (6)	—
1·50	9·6±0·40† (6)	—

† $P < 0·02$; for other differences from appropriate control $P > 0·05$.

correction for glucose metabolized by the pentose phosphate pathway and this is not known (evidence for the oxidation of glucose through this pathway in rat heart is given in the next section). The results suggest that glycolysis was inhibited by alloxan-diabetes and starvation, and by β-hydroxybutyrate, acetoacetate, butyrate, octanoate, palmitate and pyruvate. The greatest degree of inhibition was seen with pyruvate, and in general fatty acids and ketone bodies exerted a greater inhibitory effect than did diabetes or starvation. Palmitate produced no inhibition of glycolysis under anaerobic conditions.

Effects of DL-β-*hydroxybutyrate on* $^{14}CO_2$ *production from* [1-^{14}C]- *and* [6-^{14}C]-*glucose*

The results of these experiments are given in Table 6. In perfused hearts from fed rats the output of $^{14}CO_2$ from [1-^{14}C]glucose was significantly greater than that from [6-^{14}C]glucose, and the C-1/C-6 ratio was 1·62. In hearts perfused with medium containing β-hydroxybutyrate the output of $^{14}CO_2$ from both [1-^{14}C]- and [6-^{14}C]-glucose was decreased, whereas the C-1/C-6 ratio was increased to 2·54. These observations suggest that some glucose is oxidized by the pentose phosphate pathway in rat heart. The rise in the C-1/C-6 ratio induced by β-hydroxybutyrate could be explained by inhibitory effects of the ketone body on the oxidation of glucose via glycolysis and pyruvate dehydrogenase (Newsholme & Randle, 1964; Garland *et al.* 1964). This might be expected to affect the oxidation of C-6 of glucose to a greater

Table 6. *Effects of* DL-*β-hydroxybutyrate* (70 *mg./100 ml.*) *on* $^{14}CO_2$ *production from* [1-^{14}C]- *and* [6-^{14}C]-*glucose in hearts perfused with medium containing insulin* (0·1 *unit/ml.*)

The perfusion medium contained [^{13}C]glucose (1 mg./ml.), insulin (0·1 unit/ml.) and either [1-^{14}C]- or [6-^{14}C]-glucose of specific activity approx. 2 μc/100 ml. Other details are given in the text. The results are given as means ±S.E.M., with the numbers of observations in parentheses.

Addition to perfusion medium	$^{14}CO_2$ production (counts per min./g. wet wt. of heart/5 min.) From [1-^{14}C]glucose	From [6-^{14}C]glucose	C-1/C-6 ratio
None (control)	2767±210 (10)	1766±110 (10)	1·62
DL-β-Hydroxybutyrate (70 mg./100 ml.)	948±75* (7)	375±31* (7)	2·54

* For difference from control $P < 0·01$.

Table 7. *Effects of palmitate and butyrate on the uptake of glucose, deposition of glycogen and glycolysis rate in isolated rat hemidiaphragms*

The incubation medium contained glucose (1·5 mg./ml.). Other details are given in the text. Glycolysis rate was calculated from: glucose uptake − glycogen synthesis. The results are given as means ±S.E.M., with the numbers of observations in parentheses.

Addition(s) to incubation medium	Glucose uptake (mg./g. wet wt. of diaphragm/hr.)	Change in glycogen concn. (mg./g. wet wt. of diaphragm/hr.) (difference from control)	Change in glycolysis rate (mg./g. wet wt. of hemidiaphragm/hr.) (difference from control)
Albumin (0·85%) (control)	3·75±0·25 (12)	—	—
Albumin (0·85%) + palmitate (0·6 mM)	3·15±0·16‡ (12)	0·05±0·20	−0·65
Insulin (1 milliunit/ml.) + albumin (0·85%) (control)	4·88±0·14 (30)	—	—
Insulin (1 milliunit/ml.) + albumin (0·85%) + palmitate (0·6 mM)	4·04±0·15* (3)	−0·05±0·31	−0·79
Insulin (0·1 unit/ml.) + albumin (0·85%)	4·95±0·21 (6)	—	—
Insulin (0·1 unit/ml.) + albumin (0·85%) + palmitate (0·6 mM)	4·85±0·20 (6)	0·3±0·36	−0·40
None (control)	2·34±0·20 (8)	—	—
Butyrate (4 mM)	1·84±0·09‡ (8)	0·15±0·25	−0·65
Insulin (1 milliunit/ml.) (control)	5·90±0·16 (8)	—	—
Insulin (1 milliunit/ml.) + butyrate (4 mM)	5·40±0·20 (8)	0·40±0·24	−0·90
Insulin (0·1 unit/ml.) (control)	6·80±0·17 (8)	—	—
Insulin (0·1 unit/ml.) + butyrate (4 mM)	6·20±0·20‡ (8)	0·25±0·22	−0·85

* $P < 0·01$; ‡ $P < 0·05$; for other differences from the appropriate control $P > 0·05$.

extent than that of C-1 of glucose if, as our results suggest, some of the latter is lost by oxidation through the pentose phosphate pathway.

Effects of palmitate and butyrate on the glucose uptake, glycogen synthesis and glycolysis rate in rat hemidiaphragms

These results are given in Table 7. Palmitate (0·6 mM) carried by 0·85 % bovine plasma albumin inhibited the uptake of glucose by rat hemidiaphragms incubated in medium containing glucose (1·5 mg./ml.) both in the absence and presence of a low concentration of insulin (1 milliunit/ml.). No inhibition of glucose uptake by palmitate was seen at a high concentration of insulin (0·1 unit/ml.). Butyrate (4 mM) inhibited glucose uptake about equally in the absence of insulin and in the presence of a low concentration (1 milliunit/ml.) and a high concentration (0·1 unit/ml.) of insulin. The rate of glycogen synthesis in hemidiaphragms was not changed by either palmitate or butyrate. The rate of glycolysis calculated from:

Glucose uptake − glycogen synthesis

was decreased by palmitate and butyrate. The rate of glycolysis calculated in this way includes glucose metabolized by the pentose phosphate pathway, but existing evidence suggests that the proportion of glucose oxidized through this pathway in the rat hemidiaphragm is small (Bloom, Stetten & Stetten, 1953). The effects of fatty acids on the disposal of glucose in rat hemidiaphragms are qualitatively similar to those in rat heart.

DISCUSSION

Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the rates of glucose phosphorylation and glucose transport in rat heart. Pertinent information obtained previously

with this preparation may be summarized as follows. In the perfused isolated rat heart either transport or intracellular phosphorylation of glucose may limit uptake of the sugar (Park *et al.* 1956; Morgan *et al.* 1959, 1961*a*, *b*). The relative importance of the two processes in the limitation of uptake has been ascertained from measurements of the intracellular glucose concentration. The intracellular glucose concentration would be expected to be low when the potential rate of phosphorylation of glucose is greater than the rate of transport of the sugar, and high when the rate of transport exceeds that of phosphorylation. Changes in transport rate may be studied in isolation with sugars such as galactose or L-arabinose, which are apparently transported by the same system as glucose but which are not phosphorylated in this tissue (Fisher & Lindsay, 1955; Morgan *et al.* 1960). Insulin accelerates the transport of glucose and other sugars but it does not directly influence the rate of glucose phosphorylation (Morgan *et al.* 1961*a*, *b*). At a glucose concentration of 1 mg./ml. (as used in the present study) the transport of glucose limits uptake under aerobic conditions and at low concentrations of insulin, whereas phosphorylation becomes a limiting factor at high concentrations of the hormone (Morgan *et al.* 1959, 1961*a*, *b*). With these considerations being borne in mind the results obtained appear to show that fatty acids and ketone bodies, like diabetes and starvation, have inhibitory effects on the phosphorylation and transport of glucose in rat heart. The evidence may be summarized as follows.

In experiments in which insulin was present and where transport rates are maximal (Morgan *et al.* 1959), diabetes, starvation, fatty acids, ketone bodies and pyruvate inhibited glucose uptake and caused marked intracellular accumulation of glucose. We conclude from these observations that these agents impaired glucose uptake by inhibiting glucose phosphorylation under these conditions. The observations in diabetes and starvation confirm observations by Morgan *et al.* (1961*b*) and Newsholme & Randle (1961). Support for the view that it is phosphorylation of glucose and not transport of the sugar that is inhibited under these conditions is provided by the observation that β-hydroxybutyrate did not decrease the rate of L-arabinose efflux in the presence of a high concentration of insulin (0·1 unit/ml.).

The results of experiments in which measurements were made of L-arabinose accumulation or L-arabinose efflux appear to show inhibitory effects of β-hydroxybutyrate and octanoate on the transport of the pentose in hearts from fed rats. In hearts from rats starved for 18 hr. the rate of transport was lower and no inhibition was seen with β-hydroxybutyrate. The higher rate of transport in hearts from fed rats presumably reflects the action of insulin carried over in the tissue from an animal with a higher blood insulin concentration (see Hales & Randle, 1963*a*). In keeping with this view transport was inhibited by β-hydroxybutyrate in hearts from starved rats when the rate was increased by the addition of insulin at a low concentration (0·5 milliunit/ml.). These observations might suggest that β-hydroxybutyrate and octanoate decreased the effect of insulin on transport at a low concentration of the hormone but not at a high concentration. Inhibitory effects of fatty acids and ketone bodies on glucose transport are more difficult to demonstrate because these agents have also been shown to inhibit glucose phosphorylation (see above). However, in experiments with hearts from fed rats perfused with medium lacking insulin, glucose uptake was markedly inhibited by β-hydroxybutyrate and less markedly by octanoate and palmitate. These effects were associated with only marginal intracellular accumulation of glucose. This could be interpreted as providing direct evidence for an inhibitory effect of these agents on glucose transport.

In studying the effects of ketone bodies on glucose uptake in the rat heart Williamson & Krebs (1961) found no inhibitory effects in the absence of insulin. We suggest that this difference may be due to the higher rates of glucose uptake achieved with hearts from fed rats in our experiments, and that this may be due to insulin carried over in the tissue and active in the shorter period of perfusion used [in the experiments of Williamson & Krebs (1961) measurements were made from 15 to 75 min. of perfusion]. This conclusion is supported by the similarity of glucose uptakes in the presence of insulin in our experiments and those of Williamson & Krebs (1961) both in the presence and in the absence of ketone bodies.

Relationship between abnormalities of lipid metabolism and disturbances of carbohydrate metabolism in muscles of diabetic rats. These studies and those described in the accompanying papers are concerned with the possibility that an increased provision of fatty acids (and ketone bodies) for respiration might be the primary event in the development of certain abnormalities of carbohydrate metabolism in the muscles of diabetic animals. The information relevant to this concept is summarized in Table 8. To establish this concept it is necessary to show: that there is increased provision of fatty acids (and ketone bodies) for respiration in the muscle of the diabetic animal; that the abnormalities of carbohydrate metabolism which are seen in the muscles of diabetic animals may be induced in normal muscle by the provision of fatty acids and ketone bodies; that factors which diminish the supply of fatty acids (and ketone bodies) for

Table 8. *Summary of effects of alloxan-diabetes on parameters of lipid and carbohydrate metabolism and of effects of fatty acids and ketone bodies on carbohydrate metabolism in rat heart and rat diaphragm muscles*

Source of tissue	Alloxan-diabetic rat	Alloxan-diabetic rat treated with insulin	Hypophysectomized alloxan-diabetic rat	Hypophysectomized alloxan-diabetic rat treated with growth hormone + cortisol	Normal fed rat	Normal fed rat	Normal fed rat
Addition to perfusion medium	None	None	None	None	Fatty acids	Ketone bodies	Pyruvate
Metabolic parameter							
Transport rate at low insulin concentrations	Inhibited*	Not inhibited*	Not inhibited*	Inhibited*	Inhibited	Inhibited	Not known
Phosphorylation of glucose	Inhibited*	Not inhibited*	Not inhibited*	Inhibited*	Inhibited	Inhibited	Inhibited
Phosphorylation of fructose 6-phosphate†	Inhibited	Not inhibited	Not inhibited	Inhibited	Inhibited	Inhibited	Inhibited
Glycolysis	Inhibited	Not inhibited	Not inhibited	Inhibited	Inhibited	Inhibited	Inhibited
Pyruvate oxidation‡	Inhibited	Not inhibited	Not known	Not known	Inhibited	Inhibited	—
Cytoplasmic $NADH_2/NAD$ ratio‡	Lowered	Not lowered	Not lowered	Not known	Elevated	Elevated	Not known
Intracellular free fatty acid concn.	Elevated	Not known	Not elevated	Not known	Elevated	Not known	Not known
Intracellular concn. of long-chain fatty acyl-CoA§	Elevated	Not elevated	Not known	Not known	Elevated	Not elevated	Not elevated
Glycerol output (lipolysis rate)§	Elevated	Not elevated	Not elevated	Elevated	—	—	—
Citrate concn.§	Elevated	Not elevated	Not known	Not known	Elevated	Elevated	Elevated
Acetyl-CoA concn.§	Elevated	Not known	Not known	Not known	Elevated	Elevated	Not known

* See Park *et al.* (1961), Kipnis (1959) and Riddick *et al.* (1962).
† See Newsholme & Randle (1964).
‡ See Garland *et al.* (1964).
§ See Garland & Randle (1964*b*).

respiration in the tissue of the diabetic animal correct the changes in carbohydrate metabolism; that agents which correct the abnormalities of carbohydrate metabolism in the tissue of the diabetic animal correct those induced by fatty acids and ketone bodies; and that the mechanism of the changes in carbohydrate metabolism is the same whether they are induced by diabetes or by fatty acids and ketone bodies.

In the alloxan-diabetic rat *in vivo* it is well established that the plasma concentrations of non-esterified fatty acids and ketone bodies are increased to levels comparable with those used in the present study (e.g. Schnatz & Williams, 1963; Steiner, Rauda & Williams, 1961). In experiments with muscle *in vitro* the tissue is removed from the influence of circulating fatty acids and ketone bodies (apart from the small amount carried over in the tissue on removal from the animal). It seems unlikely that sufficient ketone bodies are carried over to make a major contribution to the metabolism of the tissue. On the other hand, measurements made on rat heart and rat diaphragm muscles *in vitro* have shown that the intracellular concentration of free fatty acids and long-chain fatty acyl-CoA are increased in the tissue of the diabetic animal in spite of the fat that the muscle is removed from the influence of circulating fatty acids. We believe that this fatty acid is derived *in vitro* from the breakdown of muscle glyceride and that this is accelerated in the tissue of the diabetic animal. It is possible that the rate of re-esterification of fatty acid is lower in the tissue of the diabetic animal in view of the much lower concentration of glycerol phosphate (Garland & Randle, 1964*b*).

The abnormalities of carbohydrate metabolism that have been identified in the muscle of diabetic rats are compared with those that may be induced in normal muscle by fatty acids and ketone bodies *in vitro* in Table 8. One abnormality not included (high cardiac glycogen) is considered at the end of this section. The others, given in Table 8, are: impaired sensitivity of the glucose-transport system to stimulation by insulin; impaired phosphorylation of glucose by hexokinase; a diminished rate of glycolysis associated with (and we believe caused by) impaired phosphorylation of fructose 6-phosphate by phosphofructokinase; impaired oxidation of pyruvate by pyruvate dehydrogenase. Related abnormalities include a decrease in the cytoplasmic $NADH_2/NAD$ ratio and accumulations of citrate and acetyl-CoA. Each of these abnormalities in the muscle of the alloxan-diabetic rat can be induced in normal muscle by the addition of fatty acids or ketone bodies *in vitro*, except for the altered cytoplasmic $NADH_2/NAD$ ratio. This ratio, though lowered by diabetes, is increased by fatty acids and ketone bodies *in vitro*.

The increased provision of fatty acids (and ketone bodies) for respiration in the muscle of the diabetic animal may be prevented by prior hypophysectomy of the rat or by treatment of the diabetic rat with insulin (Garland & Randle, 1964*b*). These treatments correct the impaired sensitivity of glucose transport to stimulation by insulin (Park *et al.* 1961), impaired phosphorylation of glucose by hexokinase (Park *et al.* 1961) and impaired phosphorylation of fructose 6-phosphate by phosphofructokinase. Treatment of the diabetic rat with insulin also corrects impaired pyruvate oxidation, but the effects of prior hypophysectomy on this parameter have not been investigated. The injection of hypophysectomized alloxan-diabetic rats with growth hormone and cortisol increases the supply of fatty acids (and ketone bodies) for respiration, and it also restores those abnormalities of carbohydrate metabolism that are corrected by hypophysectomy (Park *et al.* 1961; Newsholme & Randle, 1964; Garland & Randle, 1964*b*).

The inhibitory effects of fatty acids and ketone bodies on the phosphorylation of glucose by hexokinase, and on the phosphorylation of fructose 6-phosphate by phosphofructokinase, are abolished in rat heart by salicylate and by anoxia. These agents also abolish the inhibitory effects of diabetes on these parameters.

Knowledge of possible mechanisms involved is limited to phosphofructokinase, hexokinase and pyruvate dehydrogenase. In each instance the inhibitory effects of fatty acids and ketone bodies and of diabetes can be explained in terms of an increased rate of formation of acetyl-CoA from fatty acids (and ketone bodies). With phosphofructokinase the inhibitory effects have been attributed to the increase in intracellular citrate concentration that these agents induce. The activity of phosphofructokinase in extracts of rat heart muscle is inhibited by citrate (Garland, Randle & Newsholme, 1963), The results of a typical experiment showing this inhibitory effect of citrate are given in Fig. 1. Inhibition of skeletal-muscle phosphofructokinase by citrate has also been described (Parmeggiani & Bowman, 1963). The rise in citrate concentration with these agents has been attributed to a rise in the concentration of acetyl-CoA with or without a fall in the concentration of CoA (Garland & Randle, 1964*b*). The mechanism of inhibition of hexokinase by these agents has yet to be established. One possibility is inhibition of hexokinase by the glucose 6-phosphate that accumulates, perhaps because of inhibition of phosphofructokinase (Crane & Sols, 1955). The inhibitory effects of these agents on the oxidation of pyruvate by pyruvate dehydrogenase have been attributed to an increase in the acetyl-CoA/CoA ratio that they induce (Garland & Randle, 1964*a*). The evidence that has been summarized appears to furnish support for the idea that increased provision of fatty acids (and ketone bodies) for respiration in muscle in alloxan-diabetes is responsible for the inhibition of hexokinase, phosphofructokinase and pyruvate dehydrogenase. The results of these studies suggest that increased provision of fatty acids (and ketone bodies) for respiration may contribute to the diminished ability of insulin to accelerate glucose transport, but in the absence of any knowledge of the mechanism involved this has yet to be established.

The concentration of glycogen in rat heart muscle *in vivo* is increased in alloxan-diabetes, and there is some evidence that this increase may be brought about by fatty acids (or ketone bodies formed from them). Thus cardiac glycogen can be increased in rats by feeding with butyrate (Lackey, Bunde &

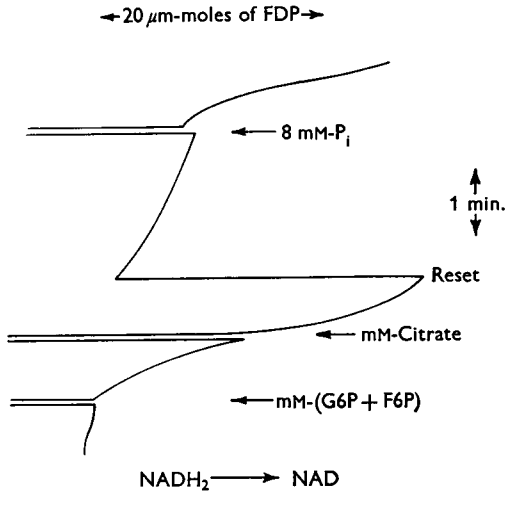

Fig. 1. Inhibition of phosphofructokinase in rat-heart homogenates by citrate. The assay medium (final vol. 3 ml.) contained: potassium acetate (150 mM); $MgCl_2$ (5 mM); imidazole buffer, pH 7·0 (20 mM); $NADH_2$ (50 μM); ATP (2·5 mM); 0·6% muscle extract made in medium of this composition (5 μl.); aldolase (20 μg./ml.); glycerol phosphate dehydrogenase (20 μg./ml.). The reaction was started by the addition of 20 μl. of 150 mM-(fructose 6-phosphate + glucose 6-phosphate) held in equilibrium with glucose phosphate isomerase. Phosphofructokinase activity was assayed by the change in fluorescence in a recording fluorimeter consequent on the oxidation of $NADH_2$ (the fructose 1,6-diphosphate formed being converted into glycerol phosphate). A 20 μl. portion of 150 mM-citrate was added at the point shown and the position of the recording stylus changed as shown. After inhibition by citrate, reactivation with 8 mM-inorganic phosphate (P_i) was demonstrable. FDP, Fructose 1,6-diphosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate.

Harris, 1946) or oleate (Bowman, 1959). The present studies show that hearts from diabetic rats, which maintain a high concentration of glycogen *in vivo*, lose glycogen *in vitro* in spite of the presence of glucose and insulin in the perfusion medium. This observation suggests that current perfusion methods may not be entirely suitable for studies of glycogen synthesis in cardiac muscle. Nevertheless, the present studies with [^{14}C]glucose suggest that fatty acids and ketone bodies may increase the proportion of glucose incorporated into glycogen (in rat heart) under conditions where glucose uptake is markedly impaired. Such an effect could be important in the development of a high concentration of cardiac glycogen in diabetes.

Quantititive aspects of effects of alloxan-diabetes, and of fatty acids, ketone bodies and pyruvate, on the reactions involved in carbohydrate metabolism in muscle. In hearts from alloxan-diabetic rats and in hearts from normal rats perfused with medium containing fatty acids or ketone bodies the degree of impairment among the various steps affected appears to be (from the greatest to the least): pyruvate oxidation, fructose 6-phosphate phosphorylation and glucose phosphorylation. This is suggested by the observations that the combined output of lactate and pyruvate is not diminished in spite of the fact that the glycolysis rate is markedly decreased, and that glucose 6-phosphate and fructose 6-phosphate accumulate in spite of the fact that glucose phosphorylation is impaired. This may be of importance in diabetes because it may lead to the maintenance of cardiac glycogen and allow for the provision of glycolytic intermediates without overall loss of glucose by oxidation. The pyruvate and lactate formed and rejected by muscle under these conditions may then be converted into glucose in the liver. If allowance is made for the latter then the degree of impairment of net glucose disposal by muscle *in vivo* under the influence of fatty acids and ketone bodies may be even greater than that detected in these experiments *in vitro*. The possible significance of these inhibitory effects of fatty acids on peripheral glucose utilization, in the development of diabetes and in the modification of insulin action on glucose disposal by growth hormone and corticosteroids has been discussed in detail by Randle *et al.* (1963) and Hales & Randle (1963*b*).

SUMMARY

1. The effects of fatty acids and ketone bodies on the uptake and metabolic fate of glucose in rat heart and rat diaphragm have been compared with those induced by alloxan-diabetes and starvation.

2. The rate of membrane transport of the pentose L-arabinose was greater in hearts from fed rats than in hearts from rats starved for 18 hr. This difference has been attributed to the higher plasma concentration of insulin (which accelerates transport of the pentose) in fed rats. DL-β-Hydroxybutyrate and *n*-octanoate inhibited L-arabinose transport in hearts from fed rats. In hearts from starved rats transport was not inhibited by these substrates unless insulin was present at a low concentration (0·5 milliunit/ml.). At high concentrations of insulin (0·1 unit/ml.) these substrates did not inhibit transport. It is suggested that these substrates may impair the sensitivity of the transport system towards stimulation by insulin. Evidence is presented for a similar inhibitory effect of these substrates on glucose transport.

3. In hearts from normal fed rats perfused with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) inhibition of glucose uptake and intracellular accumulation of glucose was induced by the addition to the perfusion medium of acetoacetate, DL-β-hydroxybutyrate, butyrate, *n*-octanoate, pyruvate, and palmitate and oleate (the last two as complexes with 2% bovine plasma albumin). It is concluded that these substrates inhibit the phosphorylation of glucose in rat heart. The effects of these substrates on glucose phosphorylation are similar to those previously demonstrated in hearts from diabetic or starved rats. The effects of these substrates on glucose phosphorylation, like those of diabetes or starvation, are abolished by salicylate and by anoxia.

4. When glucose uptake in the perfused rat heart is inhibited by fatty acids, ketone bodies or pyruvate, the rate of glycolysis is markedly decreased, whereas the net synthesis of glycogen is either unchanged or increased. Similar effects of butyrate and of palmitate (carried by 0·85% bovine plasma albumin) on the uptake and metabolism of glucose in rat hemidiaphragms have been demonstrated.

5. The output of $^{14}CO_2$ from [1-^{14}C]glucose is greater than that from [6-^{14}C]glucose in perfused rat heart, from which it is inferred that some glucose is oxidized through the pentose phosphate pathway in this tissue.

6. The evidence for the possibility that fatty acids released *in vitro* from muscle glycerides in muscle from diabetic rats are responsible for impaired rates of glucose transport, glucose phosphorylation, glycolysis, fructose 6-phosphate phosphorylation and oxidation of pyruvate by pyruvate dehydrogenase is summarized and discussed.

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Regulation of Glucose Uptake by Muscle

9. EFFECTS OF FATTY ACIDS AND KETONE BODIES, AND OF ALLOXAN-DIABETES AND STARVATION, ON PYRUVATE METABOLISM AND ON LACTATE/PYRUVATE AND L-GLYCEROL 3-PHOSPHATE/DIHYDROXYACETONE PHOSPHATE CONCENTRATION RATIOS IN RAT HEART AND RAT DIAPHRAGM MUSCLES

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In alloxan-diabetes in the rat, the uptake and oxidation of pyruvate by slices of ventricle muscle and by diaphragm muscle is impaired, and the output of lactate may be increased (Pearson, Hsieh, Du Toit & Hastings, 1949; Villee & Hastings, 1949). The cause of these abnormalities of pyruvate metabolism in diabetic muscle is not known. Newsholme & Randle (1964), Randle, Newsholme & Garland (1964) and Garland & Randle (1964*a*, *b*) have shown that two other abnormalities of carbohydrate metabolism in muscle from diabetic animals (impaired phosphorylation of fructose 6-

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