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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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phosphate and glucose) may be caused by an increased availability of fatty acids and ketone bodies for respiration in diabetes. In view of this it seemed important to test the possibility that the latter might also be responsible for abnormalities of pyruvate metabolism in muscle from diabetic animals. We have therefore compared the effect of alloxan-diabetes (and of starvation) on pyruvate metabolism in rat heart and diaphragm muscle with those of fatty acids and ketone bodies. Preliminary accounts of some of these findings have been published (Garland, Newsholme & Randle, 1962; Garland & Randle, 1962).

MATERIALS

Rats. Hearts were obtained from male albino Wistar rats (200–300 g.) and hemidiaphragms from female albino Wistar rats (100–150 g.). The animals had free access to food (diet as described by Short & Parkes, 1949) and water, except for those described as starved, which were deprived of food for 39–42 hr. Details of the induction of alloxan-diabetes were as given by Newsholme & Randle (1964).

Chemicals and enzymes. The sodium salts of [2-^{14}C]- and [1-^{14}C]-pyruvate (24·8 and 16·5 μc/mg. respectively) were obtained from The Radiochemical Centre, Amersham, Bucks. Sources of other chemicals and of enzymes were as given by Newsholme & Randle (1964).

Plasma albumin. In experiments with palmitate it was necessary to add bovine plasma albumin as a carrier for the fatty acid (Goodman, 1958). This was purified as follows, the starting material being fraction V as supplied by the Armour Pharmaceutical Co. Ltd. The albumin (50 g.) as a 4% solution in phosphate–borate buffer, pH 8·4 (Campbell & Stone, 1956), was passed through a column (5·5 cm. × 30 cm.) of Whatman cellulose powder at approx. 4 ml./min. This removes insulin and the insulin antagonist described by Vallance-Owen, Dennes & Campbell (1958). The solution was then dialysed for 24 hr. (see below) against two changes of 10 l. of distilled water at 2° and freeze-dried. The albumin was then freed of fatty acids by two extractions with anhydrous heptane (redistilled fraction b.p. 98–104°, dried over anhydrous Na_2SO_4)–acetic acid (20:1, v/v). For the first extraction 1 l. was used and for the second 500 ml. with heptane washes of 500 ml. between extractions and after the second extraction. The albumin was then dried *in vacuo* (with a water pump and a trap cooled in acetone–solid CO_2), dissolved in approx. 1 l. of distilled water and dialysed (see below) for 3–4 days against two changes of 0·9% NaCl and four to six changes of distilled water (each of 10 l.). This prolonged dialysis is needed to remove acetate (shown by studies with [^{14}C]acetate). The aqueous solution of albumin was then freeze-dried. The resulting product (as a 4% solution) was shown to be free of insulin by diaphragm bioassay, epididymal-fat-pad bioassay and immunoassay (Randle, 1954; Shepp *et al.* 1960; Hales & Randle, 1963), to be free of insulin antagonist in the rat-diaphragm bioassay of Vallance-Owen *et al.* (1958), and to contain less than 0·8 μequiv. of fatty acid/g. when titrated by a modification (Trout, Estes & Friedberg, 1960) of Dole's method. In some preparations, the initial treatment with cellulose powder was omitted; the product was apparently satisfactory by the criteria given above. The purified albumin was stored in polythene bags over silica gel at 2° until required. Albumin–palmitate complexes were obtained by injecting into a solution of albumin in medium (without $CaCl_2$, which was added afterwards) a solution of potassium palmitate in the minimum volume of hot distilled water.

Dialysis. Dialyses were made with Visking seamless cellulose tubing (Hudes Merchandising Corp. Ltd., London) purified as follows. The tubing was boiled for about 12 hr. in six changes of distilled water; during this treatment the tubing loses a brownish tinge and becomes a translucent pale blue, and the wash fluid, initially yellow and foul-smelling, becomes clear and odourless. The tubing was then allowed to stand overnight at 2° in mM-EDTA, washed with mM-HCl and distilled water, and stored in distilled water at 2°.

Media. Hearts were perfused with and hemidiaphragms incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with $O_2 + CO_2$ (95:5) or $N_2 + CO_2$ (95:5) and with glucose or pyruvate at concentrations given in the text or Tables. In experiments with bovine plasma albumin, media containing the latter were dialysed overnight against a 20-fold excess of albumin-free medium to equilibrate ions. This is especially necessary in experiments with perfused hearts because otherwise albumin removes Ca^{2+} from the medium and thereby inhibits cardiac contraction.

PROCEDURE

Muscles. Hearts were perfused for 10 or 15 min. through the coronary circulation at 37° by recirculation of 10–15 ml. of medium with the apparatus of Morgan, Henderson, Regen & Park (1961) after washing out the heart initially with 10–17 ml. of medium. When $^{14}CO_2$ production from [2-^{14}C]pyruvate was assayed the apparatus of Randle *et al.* (1964) was used. Rats were injected with heparin (625 units) and Nembutal (15 mg.) about 5 min. before hearts were removed for perfusion. Hemidiaphragms were incubated for 60 min. in 2 ml. of medium. For assay of substances in muscle, hearts or hemidiaphragms were frozen in acetone–solid CO_2 or with the tissue clamp of Wollenberger, Ristau & Schoffa (1960) cooled in liquid N_2. For the assay of substances in heart muscle *in vivo* the conditions were as described by Newsholme & Randle (1964).

METHODS

Muscle extracts. For the assay of lactate, pyruvate, glycerol phosphate and dihydroxyacetone phosphate, frozen muscle was powdered in a percussion mortar and the frozen powder extracted with 6% (w/v) perchloric acid (approx. 2 ml./g. of powder). The extract, separated by centrifugation, was neutralized (and potassium perchlorate precipitated) with an excess of 5 M-K_2HPO_4, pH 7·0, at 0°.

Media. For the assay of lactate and pyruvate, incubation and perfusion media were deproteinized by the addition of an equal volume of 6% perchloric acid (see above).

Lactate, glycerol phosphate, pyruvate and dihydroxyacetone phosphate. Lactate was assayed in 0·2 ml. of medium or muscle extracts, and glycerol phosphate in 0·2 ml. of muscle extracts, by the method of Hohorst, Kreutz & Bücher (1959). Pyruvate and dihydroxyacetone phosphate

were assayed by the change in extinction at 340 mμ in a Beckman spectrophotometer on adding 40 μg. of lactate dehydrogenase or 100 μg. of glycerol phosphate dehydrogenase to a mixture of 0·5 ml. of medium or muscle extracts and 1·5 or 2·5 ml. of assay medium [triethanolamine (0·1 M) and EDTA (1 mM), pH 7·6, and sufficient $NADH_2$ to give an extinction of approx. 0·2 in a 1 cm. light-path when read against a water blank].

$^{14}CO_2$ from [^{14}C]pyruvate. Heart perfusion medium (3 ml.) was pipetted into the outer annulus of conical flasks with a centre well into which was inserted a tube (1·2 cm. × 5 cm.) containing 0·2 ml. of M-hyamine in methanol. The flasks were sealed with skirted rubber caps, 0·7 ml. of 8 N-H_2SO_4 was injected into the outer annulus with needle and syringe, and the flasks were shaken for 3 hr. at room temperature. In experiments with hemidiaphragms, incubations were made in such flasks and hyamine and H_2SO_4 injected into the appropriate chambers at the end of incubation. After the liberation of CO_2 and absorption by hyamine, 3·6 ml. of 0·4% 2,5-diphenyloxazole and 0·05% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene was added to the hyamine (Synder, 1961) and radioactivity was assayed in a Tri-Carb liquid-scintillation spectrometer.

Glutamate, α-oxoglutarate, malate and oxaloacetate. These were assayed by the methods given by Garland & Randle (1964*b*).

Thiamine pyrophosphate. Thiamine pyrophosphate was assayed on 0·2 ml. of muscle extract in a Beckman DK2 recording spectrophotometer with the apoenzyme of yeast carboxylase as described by Holzer, Söling, Goedde & Holzer (1963). Carboxylase was prepared from dried brewer's yeast by the method of Green, Herbert & Subrahmanyan (1941), but with the omission of the final subfractionation with $(NH_4)_2SO_4$. The apocarboxylase was prepared as described by Holzer *et al.* (1963). For construction of the standard curve, 1, 0·7 and 0·3 μg. of thiamine pyrophosphate (L. Light and Co. Ltd.) were used. The recovery of thiamine pyrophosphate added to muscle extract was in the range 85–100% (0·8 μg. of thiamine pyrophosphate added to 0·2 ml. of muscle extract). Thiamine added to muscle extracts did not react in this assay.

Glycogen concentration and glucose uptake. These were estimated as described by Randle *et al.* (1964). In measuring changes in glycogen concentration during perfusion the initial glycogen was assayed in hearts washed through with 10–15 ml. of medium, and the final glycogen was assayed in further hearts perfused for the times given in the text or Tables (with correction of weights for alterations in the volume of extracellular water as described by Randle *et al.* 1964). In experiments with hemidiaphragms the initial glycogen was measured on one hemidiaphragm before incubation and the final glycogen on the other member of the pair after incubation.

RESULTS

Effects of fatty acids and ketone bodies, and of alloxan-diabetes and starvation, on the concentration of glycogen, uptake of pyruvate and output of lactate in perfused rat heart and isolated rat diaphragm

Glycogen concentration. Because the breakdown of glycogen may contribute pyruvate for metabolism it was important to investigate the effect of these agents on glycogen breakdown. When hearts from normal fed rats were perfused for 10 min. with mM-pyruvate or for 15 min. with 4 mM-pyruvate there was a fall in the concentration of glycogen in the heart of 1·8 ± 0·8 mg./g. wet wt./hr. (mean ± S.E.M. for difference between initial glycogen concentration measured in nine hearts and final glycogen concentration measured in another nine hearts after perfusion). With hearts from alloxan-diabetic rats perfused for 10 min. with mM-pyruvate no disappearance of glycogen was detected (mean change ± S.E.M. + 0·6 ± 0·57 mg./g. wet wt./hr. for ten hearts in each group). On the other hand, in hearts from rats starved for 40 hr. the glycogen concentration fell at an accelerated rate (mean change ± S.E.M. − 4·2 ± 0·63 mg./g. wet wt./hr. for nine hearts in each group). The effects of pyruvate (4 mM), of DL-β-hydroxybutyrate (5·5 mM) plus pyruvate (4 mM) and of palmitate (0·75 mM) with bovine plasma albumin (2 %) plus pyruvate (1 mM) on the concentration of glycogen after perfusion are shown in Table 1. The inclusion of these agents in the perfusion medium did not influence the concentration of glycogen measured in the heart at the end of perfusion, from which it is inferred that they did not change the rate of breakdown of glycogen.

In hemidiaphragms from normal fed rats incubated for 60 min. in medium without substrate glycogen was lost at the rate of 0·3 ± 0·1 mg./g. wet wt./hr. Similarly, in isolated rat hemidiaphragms the concentration of glycogen measured in the muscle was not altered by the inclusion in the incubation medium of pyruvate (4 mM), of butyrate (4 mM) plus pyruvate (4 mM) or of palmitate (0·6 mM) with (0·85 %) albumin plus pyruvate (1 mM) (Table 1). In hemidiaphragms from diabetic or starved rats incubated in 4 mM-pyruvate, glycogen was lost at the rate of 0·4 ± 0·28 and 0·3 ± 0·26 mg./g. wet wt./hr.

Pyruvate uptake and lactate output. In hearts from normal fed rats perfused with 4 mM-pyruvate, the uptake of pyruvate was 158–181 μmoles/g. wet wt./hr. and the output of lactate 61–94 μmoles/g. wet wt./hr. The uptake of pyruvate was decreased by 5·5 mM-β-hydroxybutyrate (31 %), by 5·5 mM-acetoacetate (51 %) and by *n*-octanoate (53 %). The output of lactate was not significantly changed by these agents. In hearts from normal fed rats perfused with medium containing pyruvate (1 mM) the uptake of pyruvate was 78 μmoles/g. wet wt./hr. and the output of lactate 62 μmoles/g. wet wt./hr. The uptake of pyruvate was diminished in hearts from alloxan-diabetic rats (45 %) and in rats starved for 40 hr. (38 %), whereas the output of lactate was unchanged. In hearts from fed rats perfused with medium containing pyruvate (1 mM) and bovine plasma albumin (2 %) the uptake of

pyruvate was 79 μmoles/g. wet wt./hr. and the output of lactate 49 μmoles/g. wet wt./hr. Palmitate (0·75 mM) carried by bovine plasma albumin (2 %) diminished the uptake of pyruvate (28 %) and increased the output of lactate (35 %). These results are given in Table 2.

When hemidiaphragms from normal fed rats were incubated in medium containing pyruvate (4 mM) the uptake of pyruvate was 29·2 μmoles/g. wet wt./hr. and the output of lactate 17·3 μmoles/g. wet wt./hr. Butyrate (4 mM) diminished the uptake of pyruvate and increased that of lactate, whereas alloxan-diabetes and starvation (for 40 hr.) diminished the uptake of pyruvate without altering the output of lactate. Butyrate (4 mM) neither diminished the uptake of pyruvate nor increased

Table 1. *Effects of pyruvate, β-hydroxybutyrate and fatty acids on the glycogen concentration in perfused hearts and isolated hemidiaphragms from normal fed rats*

Hearts were perfused for 10 min. (with mM-pyruvate) or for 15 min. (with 4 mM-pyruvate). Hemidiaphragms were incubated for 60 min. Other details are given in the text. The results are given as means ±S.E.M., with the numbers of observations in parentheses. (For all differences from appropriate control $P > 0·05$.)

Tissue	Addition(s) to perfusion or incubation medium	Glycogen concn. after perfusion or incubation (μmoles of glycogen glucose/g. wet wt. of tissue)
Heart	None	10±1·4 (6)
Heart	Pyruvate (4 mM)	11±1·2 (6)
Heart	Pyruvate (4 mM)	11±1·1 (6)
Heart	Pyruvate (4 mM) + DL-β-hydroxybutyrate (5·5 mM)	11±1·6 (5)
Heart	Pyruvate (1 mM) + albumin (2%)	14±1·1 (6)
Heart	Pyruvate (1 mM) + albumin (2%) + palmitate (0·75 mM)	15±1·7 (6)
Diaphragm	None	7±0·7 (8)
Diaphragm	Pyruvate (4 mM)	9±0·9 (8)
Diaphragm	Pyruvate (4 mM)	16±1·3 (16)
Diaphragm	Pyruvate (4 mM) + butyrate (4 mM)	16±1·3 (16)
Diaphragm	Pyruvate (1 mM) + albumin (0·85%)	13±0·8 (8)
Diaphragm	Pyruvate (1 mM) + albumin (0·85%) + palmitate (0·6 mM)	13±0·9 (8)

Table 2. *Effects of fatty acids and ketone bodies, and of starvation and alloxan-diabetes, on the metabolism of pyruvate in perfused rat heart*

Hearts were perfused for 10 min. (with mM-pyruvate) or for 15 min. (with 4 mM-pyruvate). Other details are given in the text. The results are given as means ±S.E.M., with the numbers of hearts in parentheses.

Source of heart	Addition(s) to perfusion medium	Pyruvate uptake (μmoles/g. wet wt. of heart/hr.)	Lactate output (μmoles/g. wet wt. of heart/hr.)	Pyruvate uptake not accounted for as lactate (μmoles/g. wet wt. of heart/hr.)
Normal fed rat	Pyruvate (4 mM)	162±1 (14)	94±5·2	68±8
Normal fed rat	Pyruvate (4 mM) + DL-β-hydroxybutyrate (5·5 mM)	111±7·6* (14)	108±4·4	3±5·7*
Normal fed rat	Pyruvate (4 mM)	158±17·6 (6)	61±3	97±18
Normal fed rat	Pyruvate (4 mM) + acetoacetate (5·5 mM)	78±12·4* (6)	68±2·7	10±12*
Normal fed rat	Pyruvate (4 mM)	181±14 (6)	81±4·2	100±17
Normal fed rat	Pyruvate (4 mM) + *n*-octanoate (2·8 mM)	84±9·2* (6)	78±6·1	6±6*
Normal fed rat	Pyruvate (1 mM) + albumin (2%)	79±2·5 (11)	49±1·8	30±2·6
Normal fed rat	Pyruvate (1 mM) + albumin (2%) + palmitate (0·7 mM)	57±3·4* (11)	66±6·4†	−9±5*
Normal fed rat	Pyruvate (1 mM)	78±6·4 (6)	62±12	16±13
Starved (40 hr.) rat	Pyruvate (1 mM)	43±2·7* (6)	68±7·7	−25±8*
Alloxan-diabetic fed rat	Pyruvate (1 mM)	48±4·8* (6)	71±5·1	−23±7*

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

the output of lactate in hemidiaphragms from alloxan-diabetic rats. Palmitate (0·6 mM) carried by bovine plasma albumin (0·85 %) increased the output of lactate by hemidiaphragms from normal fed rats without altering the uptake of pyruvate. These results are given in Table 3.

Effects of ketone bodies and fatty acids, and of alloxan-diabetes and starvation, on $^{14}CO_2$ production from [2-^{14}C]- and [1-^{14}C]-pyruvate by rat heart and rat diaphragm

The results of these experiments are given in Table 4. In perfused rat heart the output of $^{14}CO_2$ from [1-^{14}C]pyruvate was diminished 78 % by butyrate (4 mM), 73 % by starvation (for 40 hr.) of the rat and 86 % by alloxan-diabetes. The output of $^{14}CO_2$ from [2-^{14}C]pyruvate in perfused rat heart was diminished 66 % by β-hydroxybutyrate (5·5 mM), 78 % by starvation and 77 % by alloxan-diabetes. In rat hemidiaphragms the output of $^{14}CO_2$ from [1-^{14}C]pyruvate was similarly diminished by butyrate and by starvation and diabetes, and that from [2-^{14}C]pyruvate by butyrate and palmitate and by diabetes and starvation.

Effects of ketone bodies and fatty acids, and of alloxan-diabetes and starvation, on the metabolism of pyruvate formed from glucose or glycogen in perfused rat heart and isolated rat diaphragm

Metabolism of pyruvate formed from glucose. In hearts from normal fed rats perfused for 15 min. with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) the rate of glycolysis (calculated from:

Glucose uptake − glycogen synthesis

see Randle *et al.* 1964) was 77 μmoles of glucose/g. wet wt./hr. and the combined output of lactate and pyruvate was 25 μmoles of glucose equiv./g. wet wt./hr. (calculated as one-half of the output of lactate and pyruvate). The calculated rate of glycolysis was slightly decreased (8 %) by starvation (for 40 hr.), whereas the output of lactate and pyruvate was unchanged. The calculated rate of glycolysis was more markedly decreased (29–48 %) by alloxan-diabetes or by perfusion with butyrate, octanoate or palmitate, whereas the combined output of lactate and pyruvate was either unchanged or increased. These results are given in Table 5. In rat hemidiaphragms the calculated rate of glycolysis was decreased by butyrate and palmitate and by alloxan-diabetes and starvation, whereas the combined output of lactate and pyruvate was either unchanged or increased (see Table 6).

Metabolism of pyruvate formed from glycogen. When hearts were perfused for 10 min. with medium containing bovine plasma albumin (2 %) the rate of glycogen disappearance was 27 ± 2·1 μmoles of glycogen glucose/g. wet wt./hr. and the combined output of lactate and pyruvate was 5·8 ± 1·1 μmoles of glucose equiv./g. wet wt./hr. With the addition of palmitate (0·75 mM) (carried by 2 % albumin) the rate of glycogen disappearance was unchanged (31 ± 2·7 μmoles/g. wet wt./hr.), whereas the combined output of lactate and pyruvate was increased (to 25 ± 4·4 μmoles/g. wet wt./hr.; $P < 0·01$).

Disposal of pyruvate by pathways other than conversion into lactate in perfused rat heart. These results are given in Table 7. The disposal of pyruvate by pathways other than conversion into

Table 3. *Effects of fatty acids and of alloxan-diabetes on the metabolism of pyruvate by isolated rat hemidiaphragm*

Details are given in the text. The results are given as means ±S.E.M., with the numbers of observations in parentheses.

Source of diaphragm	Addition(s) to incubation medium	Pyruvate uptake (μmoles/g. wet wt. of diaphragm/hr.)	Lactate output (μmoles/g. wet wt. of diaphragm/hr.)	Pyruvate uptake not accounted for as lactate (μmoles/g. wet wt. of diaphragm/hr.)
Normal fed rat	Pyruvate (4 mM)	29·2±1 (16)	17·3±0·56	11·9±1·14
Normal fed rat	Pyruvate (4 mM) + butyrate (4 mM)	23·5±0·47* (16)	20·3±0·39*	3·5±0·61*
Normal fed rat	Pyruvate (4 mM) + albumin (0·85 %)	13·1±0·35 (8)	11·4±0·2	1·7±0·40
Normal fed rat	Pyruvate (4 mM) + albumin (0·85 %) + palmitate (0·6 mM)	12·9±0·42 (8)	14·1±0·6*	−1·2±0·73*
Normal fed rat	Pyruvate (4 mM)	29·2±1 (16)	17·3±0·56	11·9±1·14
Alloxan-diabetic rat	Pyruvate (4 mM)	18·5±1* (8)	17·7±0·50	0·8±1·10*
Alloxan-diabetic rat	Pyruvate (4 mM) + butyrate (4 mM)	16·1±0·4 (8)	17·5±0·85	−1·4±0·86
Starved (40 hr.) rat	Pyruvate (4 mM)	20·9±2·3‡ (8)	22·8±1·00*	−1·9±2·51*

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

lactate has been calculated in perfusions with pyruvate from:

Glycogen disappearance + pyruvate uptake − lactate output

in perfusions with glucose and insulin from:

Glycogen disappearanace + glucose uptake − (pyruvate + lactate) output

and in perfusions without these substrates from:

Glycogen disappearance − (pyruvate + lactate) output

In hearts perfused without substrate or with mM-pyruvate the rates of disappearance of pyruvate by pathways other than conversion into lactate were similar (36 and 42 μmoles of pyruvate/g. wet wt./

Table 4. *Effects of ketone bodies and fatty acids, and of alloxan-diabetes and starvation, on $^{14}CO_2$ production from [2-^{14}C]- and [1-^{14}C]-pyruvate by perfused rat heart and isolated rat diaphragm*

The specific activity of medium pyruvate varied, in different experiments, from 2 to 20 μc/m-mole. In experiments with perfused hearts $^{14}CO_2$ was collected for 2 min. after 5 min. of pre-perfusion with unlabelled pyruvate and 3 min. of pre-perfusion with [^{14}C]pyruvate. In experiments with hemidiaphragms $^{14}CO_2$ was collected for 60 min. Other details are given in the text. Control rates of $^{14}CO_2$ output are given in parentheses. The results are given as means ± S.E.M., with the numbers of observations in parentheses.

Tissue	Source of tissue	Addition(s) to perfusion or incubation medium	$^{14}CO_2$ production (counts per min./g. wet wt. of tissue/min.) (difference from control) From [2-^{14}C]pyruvate	From [1-^{14}C]pyruvate
Heart	Normal fed rat	Pyruvate (4 mM)	(7431)	—
Heart	Normal fed rat	Pyruvate (4 mM) + DL-β-hydroxybutyrate (5·5 mM)	−4923 ± 1200* (4)	—
Heart	Normal fed rat	Pyruvate (1 mM)	—	(6257)
Heart	Normal fed rat	Pyruvate (1 mM) + butyrate (4 mM)	—	−4887 ± 350* (4)
Heart	Normal fed rat	Pyruvate (1 mM)	(817)	(6257)
Heart	Starved (40 hr.) rat	Pyruvate (1 mM)	−639 ± 110* (4)	−4620 ± 500* (4)
Heart	Alloxan-diabetic rat	Pyruvate (1 mM)	−626 ± 130* (4)	−5437 ± 500* (4)
Diaphragm	Normal fed rat	Pyruvate (4 or 1 mM)	(242)	(1800)
Diaphragm	Normal fed rat	Pyruvate (4 or 1 mM) + butyrate (4 mM)	−93 ± 17* (6)	−750 ± 150* (4)
Diaphragm	Normal fed rat	Albumin (0·85%) + pyruvate (1 mM)	(108)	—
Diaphragm	Normal fed rat	Albumin (0·85%) + pyruvate (1mM) + palmitate (0·6 mM)	−8 ± 2* (6)	—
Diaphragm	Normal fed rat	Pyruvate (4 or 1 mM)	(39)	(1800)
Diaphragm	Alloxan-diabetic rat	Pyruvate (4 or 1 mM)	−24 ± 5* (4)	−1270 ± 160* (4)
Diaphragm	Starved (40 hr.) rat	Pyruvate (4 or 1 mM)	−29 ± 5* (4)	−1460 ± 150* (4)

* $P < 0.01$ for difference from appropriate control.

Table 5. *Effects of fatty acids and of alloxan-diabetes and starvation on the metabolism of pyruvate formed from glucose in rat hearts perfused with medium containing glucose* (5·5 mM) *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. Glycolysis rates were calculated as described by Randle *et al.* (1964) from: glucose uptake − glycogen synthesis. Control rates are given in parentheses. The results are given as means ± S.E.M.

Source of heart	Addition(s) to perfusion medium	No. of observations	Glycolysis rate (μmoles of glucose/g. wet wt. of heart/hr.) (difference from control)	Output of lactate + pyruvate (μmoles of glucose equiv./g. wet wt. of heart/hr.) (difference from control)
Normal fed rat	None	14	(77 ± 1·7)	(25 ± 3·5)
Starved (40 hr.) rat	None	13	−6 ± 2·4*	−3 ± 6
Alloxan-diabetic rat	None	13	−22 ± 2·4*	−1 ± 7
Normal fed rat	*n*-Octanoate (2·8 mM)	6	−25 ± 2·1*	12 ± 6
Normal fed rat	Butyrate (4 mM)	6	−37 ± 4·6*	1 ± 5
Normal fed rat	Albumin (2%)	18	(66 ± 1·7)	(20 ± 2·2)
Normal fed rat	Albumin (2%) + palmitate (0·75 mM)	18	−28 ± 2·4*	5 ± 2·2‡

* $P < 0.01$; ‡ $P < 0.05$; for other differences from appropriate control $P > 0.05$.

hr.). A significantly higher rate was seen in hearts perfused with 4 mM-pyruvate (109 μmoles/g. wet wt./hr.) or with glucose and insulin (104 μmoles of pyruvate/g. wet wt./hr.). In hearts perfused with glucose and insulin the disappearance of pyruvate by pathways other than conversion into lactate was diminished by octanoate, butyrate and palmitate (carried by 2 % bovine plasma albumin) and by alloxan-diabetes but not by starvation. In hearts perfused with pyruvate, metabolism of the latter (other than conversion into lactate) was diminished by β-hydroxybutyrate and palmitate and by alloxan-diabetes but not by starvation. In hearts perfused without glucose or pyruvate, palmitate (carried by 2 % albumin) diminished the difference:

Glycogen breakdown − lactate output

In hemidiaphragms from normal fed rats incubated in medium containing pyruvate (4 mM) disappearance of the latter (other than by conversion into

Table 6. *Effects of fatty acids and of alloxan-diabetes and starvation on the metabolism of pyruvate formed from glucose in hemidiaphragms incubated in medium containing glucose* (8·3 mM)

The change in glycolysis rate was calculated from difference for paired hemidiaphragms of: change in glucose uptake (experimental − control) + difference in glycogen concentration at end of incubation (control − experimental). The results are given as means ±S.E.M.

Source of diaphragm	Addition(s) to incubation medium	No. of observations	Glycolysis rate (μmoles of glucose/g. wet wt. of diaphragm/hr.) (difference from control)	Output of lactate + pyruvate (μmoles of glucose equiv./g. wet wt. of diaphragm/hr.) (difference from control)
Normal fed rat	Butyrate (4 mM)	6	−3·6±1·22†	−0·22±0·44
Normal fed rat	Butyrate (4 mM) + insulin (1 milliunit/ml.)	6	−5·0±1·44*	0·22±0·55
Normal fed rat	Butyrate (4 mM) + insulin (0·1 unit/ml.)	6	−4·7±1·44†	−1·28±0·44†
Normal fed rat	Albumin (0·85 %) + palmitate (0·6 mM)	8	−3·6±1·67	−0·28±0·44
Normal fed rat	Albumin (0·85 %) + palmitate (0·6 mM) + insulin (1 milliunit/ml.)	8	−3·9±1·28†	−0·28±0·44
Alloxan-diabetic rat	Insulin (1 milliunit/ml.)	12	−3·9±2·5	0·50±0·44
Starved (40 hr.) rat	Insulin (1 milliunit/ml.)	12	−8·3±3·8‡	0·61±0·56

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

Table 7. *Effects of fatty acids and* DL-β-*hydroxybutyrate, and of starvation and alloxan-diabetes, on the metabolism of pyruvate by pathways other than conversion into lactate in perfused rat heart*

Hearts were perfused for 15 min. (with 4 mM-pyruvate or with glucose + insulin) or for 10 min. (in other experiments). Other details are given in the text. Pyruvate not recovered as lactate was calculated in perfusions with pyruvate from: glycogen loss + pyruvate uptake − lactate output; in perfusions with glucose and insulin from: glycogen loss + glucose uptake − (pyruvate + lactate) output; and in perfusions without pyruvate or glucose from: glycogen loss − (pyruvate + lactate) output. The results are given as means ±S.E.M.

Source of heart	Addition(s) to perfusion medium	No. of observations	Pyruvate not recovered as lactate (μmoles/g. wet wt. of heart/hr.)
Normal fed rat	Albumin (2 %)	6	42±4·1
Normal fed rat	Albumin (2 %) + palmitate (0·75 mM)	6	12±3·2*
Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	14	104±4·1
Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + *n*-octanoate (2·8 mM)	6	30±7·2*
Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + butyrate (4 mM)	6	28±6·8
Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + albumin (2 %)	18	92±2·8
Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + palmitate (0·75 mM)	18	26±3·4*
Starved (40 hr.) rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	98±6·5
Alloxan-diabetic rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	62±7·3‡
Normal fed rat	Pyruvate (4 mM)	6	109±9·2
Normal fed rat	Pyruvate (4 mM) + DL-β-hydroxybutyrate (5·5 mM)	6	23±6·7*
Normal fed rat	Pyruvate (1 mM)	6	36±9·1
Starved (40 hr.) rat	Pyruvate (1 mM)	9	44±8·2
Alloxan-diabetic rat	Pyruvate (1 mM)	12	−8±10·8
Normal fed rat	Pyruvate (1 mM) + albumin (2 %)	6	50±6·1
Normal fed rat	Pyruvate (1 mM) + albumin (2 %) + palmitate (0·75 mM)	6	11±8·2*

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

lactate) was 15·3 μmoles/g. wet wt./hr. This was diminished by 4 mM-butyrate to 6·9, by alloxan-diabetes to 5·8 and by starvation (for 40 hr.) to 0·3 μmoles/g. wet wt./hr.

Effects of glucose plus insulin, of alloxan-diabetes and starvation, and of albumin, fatty acids and ketone bodies, on the individual outputs of pyruvate and lactate and on the lactate/pyruvate concentration ratios in the medium in perfused rat heart and isolated rat diaphragm

The results of these experiments are given in Table 8. When hearts from normal fed rats were perfused with plain medium (i.e. no glucose or other substrate added) the output of pyruvate was 1·6 ± 0·3 μmoles/g. wet wt./hr. and that of lactate was 8·5 ± 1·8 μmoles/g. wet wt./hr., and the lactate/pyruvate ratio in the perfusion medium was 5·3 ± 1·1. The addition of bovine plasma albumin (2 %) to the perfusion medium doubled the output of pyruvate without changing that of lactate, and halved the lactate/pyruvate ratio. The addition of glucose and insulin to the perfusion medium trebled the output of pyruvate, increased that of lactate 5-fold and doubled the lactate/pyruvate ratio. In hearts perfused with medium lacking glucose or insulin, β-hydroxybutyrate (5·5 mM) doubled the output of lactate without changing that of pyruvate, and doubled the lactate/pyruvate ratio. Likewise palmitate (0·75 mM) (carried by 2 % bovine plasma albumin) increased the output of lactate (5-fold) and the lactate/pyruvate ratio (4-fold) without changing the output of pyruvate significantly.

When hearts from normal fed rats were perfused with medium containing glucose (5·5 mM) and insulin (0·1 unit/ml.) the output of pyruvate was 4·8 ± 0·27 μmoles/g. wet wt./hr. and that of lactate 46 ± 7 μmoles/g. wet wt./hr., and the lactate/pyruvate ratio was 9·6 ± 1·8. The addition of bovine plasma albumin (2 %) to the perfusion medium increased the output of pyruvate (about 1·5-fold), decreased that of lactate (by 25 %) and halved the lactate/pyruvate ratio in the perfusion medium. Hearts from alloxan-diabetic rats or from normal rats starved for 40 hr. put out about twice as much pyruvate as did hearts from normal fed rats but no more lactate. The lactate/pyruvate ratio in the perfusion medium was approximately halved by diabetes and by starvation. Octanoate (2·8 mM) increased the output of lactate by hearts from normal fed rats (1·5-fold), halved the output of pyruvate and increased the lactate/pyruvate ratio (3-fold). Likewise palmitate (0·75 mM) (carried by 2 % bovine plasma albumin) increased lactate output (1·5-fold), decreased pyruvate output (by 25 %) and doubled the lactate/pyruvate ratio.

When hemidiaphragms from normal fed rats were incubated in medium containing glucose (8·3 mM) and insulin (0·1 unit/ml.) the output of pyruvate was 0·96 ± 0·08 μmole/g. wet wt./hr., the output of lactate 15·4 ± 0·55 μmoles/g. wet wt./hr. and the lactate/pyruvate ratio 16·7 ± 1·25. Diabetes and starvation approximately doubled the output of pyruvate, increased the output of lactate (about 4-fold) and decreased the lactate/pyruvate ratio by about 25 %. The addition of butyrate (4 mM) to the incubation medium in experiments with hemidiaphragms from normal fed rats approximately halved the output of pyruvate, decreased the output of lactate (by 10 %) and increased the lactate/pyruvate ratio (1·8-fold).

Lactate/pyruvate concentration ratios in the medium and lactate/pyruvate and glycerol 3-phosphate/dihydroxyacetone phosphate concentration ratios in intracellular water in perfused rat heart and rat diaphragm muscles

The results of experiments outlined in the preceding section revealed effects of ketone bodies and fatty acids, and of diabetes and starvation, on the lactate/pyruvate ratios in the perfusion medium and formed from glucose or glycogen. This ratio was decreased by diabetes and starvation but increased by fatty acids and ketone bodies. In view of these findings it seemed important to compare the lactate/pyruvate ratio in medium and muscle water and (because of the possibility that alterations in the lactate/pyruvate ratio might reflect changes in the cytoplasmic $NADH_2/NAD$ ratio) to determine the glycerol phosphate/dihydroxyacetone phosphate ratio (metabolites held in equilibrium by another NAD-linked dehydrogenase) (for discussion see Hohorst *et al.* 1959). These results are given in Table 9.

In hearts from normal fed rats perfused under aerobic conditions with medium containing glucose (5·5 mM) and insulin (0·1 unit/ml.) the lactate/pyruvate ratio in the medium was 7·9 ± 0·6 and that in the muscle was 24·5 ± 1·05. The ratio in muscle water was thus higher than that of the medium. To investigate the possibility that this difference might be due to stimulation of glycolysis during freezing of the muscle measurements were made in hearts perfused under anaerobic conditions, or with salicylate (to induce high rates of glycolysis). Anoxia increased the ratio in the medium 11-fold and salicylate increased it 6-fold. Nevertheless, the ratio remained substantially greater in muscle water. These observations suggest that the difference may be real and not due to technical difficulties in freezing muscle. Further, they suggest either that pyruvate is lost from muscle cells more rapidly than is lactate, or that lactate and pyruvate are brought into a new equilibrium in

Table 8. *Effects of alloxan-diabetes and starvation, and of fatty acids and ketone bodies, on the lactate and pyruvate output and the lactate/pyruvate concentration ratio in perfused rat heart and isolated rat diaphragm*

Details are given in the text. The results are given as means ±S.E.M.

Tissue	Source of tissue	Addition(s) to perfusion or incubation medium	No. of observations	Pyruvate output (μmoles/g. wet wt. of heart/hr.)	Lactate output (μmoles/g. wet wt. of heart/hr.)	Lactate/pyruvate ratio in medium
Heart	Normal fed rat	None	6	1·6±0·30	8·5±1·8	5·3±1·10
Heart	Normal fed rat	DL-β-Hydroxybutyrate (5·5 mM)	6	1·8±0·15	20·3±3·4*	11·3±1·08*
Heart	Normal fed rat	Albumin (2%)	6	3·1±0·72	8·6±2·3	2·8±0·84
Heart	Normal fed rat	Albumin (2%) + palmitate (0·75 mM)	6	3·8±0·69	45±8·9*	11·8±1·60*
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	14	4·8±0·27	46±7	9·6±1·80
Heart	Alloxan-diabetic rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	9·0±1·0	47±11	5·2±0·52‡
Heart	Starved (40 hr.) rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	11·0±1·1*	42±9	3·8±0·40†
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + albumin (2%)	18	7·7±0·62	33±2·4	4·2±0·43
Heart	Normal fed rat	Palmitate (0·75 mM)	18	5·9±0·47†	46±3·7*	8·0±0·90*
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	14	4·8±0·27	46±7	9·6±1·8
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + *n*-octanoate (2·8 mM)	14	2·1±0·26*	72+9‡	29·1±1·4*
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + butyrate (4 mM)	6	3·8±0·26†	48±6	16·1±1·0†
Diaphragm	Normal fed rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	0·96±0·08	15·4±0·55	16·7±1·25
Diaphragm	Alloxan-diabetic rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	1·71±0·09*	21±0·97*	12·3±0·80*
Diaphragm	Starved (40 hr.) rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	1·81±0·21*	20±0·97*	11·6±0·87*
Diaphragm	Normal fed rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	1·79±0·07	18·5±0·61	10·4±0·42
Diaphragm	Normal fed rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.) + butyrate (4 mM)	8	0·93±0·05*	16·7±0·61†	18·0±0·87*

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

Table 9. *Lactate/pyruvate concentration ratio in the medium and lactate/pyruvate and glycerol 3-phosphate dihydroxyacetone phosphate concentration ratios in muscle in perfused rat heart and isolated rat diaphragm*

In the experiments *in vitro* hearts were frozen with the tissue clamp of Wollenberger *et al.* (1960). In the experiments *in vivo* hearts were frozen in acetone–solid CO_2 mixture. Other details are given in the text. The results are given as means ±S.E.M.

Tissue	Source of tissue	Addition(s) to perfusion or incubation medium	No. of observations	Lactate/pyruvate ratio in medium	Lactate/pyruvate ratio in tissue	Glycerol phosphate/dihydroxyacetone phosphate ratio in tissue
Experiments *in vitro*						
Heart	Normal fed rat (control)	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	7·9±0·6	24·5±1·05	11·8±1·3
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) (anaerobic)	6	85±20*	129±10*	20·5±0·84*
Heart	Normal fed rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.) + salicylate (5 mM)	6	51±6·4*	124±24*	—
Heart	Normal fed rat (control)	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	14	9·6±1·8	—	10·3±1·3
Heart	Alloxan-diabetic rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	5·2±0·52*	—	5·2±0·66*
Heart	Starved (40 hr.) rat	Glucose (5·5 mM) + insulin (0·1 unit/ml.)	13	3·8±0·40*	—	3·1±0·73*
Diaphragm	Normal fed rat (control)	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	16·7±1·25	—	7·5±0·5
Diaphragm	Alloxan-diabetic rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	12·3±0·80*	—	4·3±1·0†
Diaphragm	Starved (40 hr.) rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	11·6±0·87*	—	5·0±0·8†
Diaphragm	Normal fed rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.)	8	10·4±0·42	—	7·0±1·5
Diaphragm	Normal fed rat	Glucose (8·3 mM) + insulin (0·1 unit/ml.) + butyrate (4 mM)	8	18·0±0·87	—	14·0±2·9‡
Experiments *in vivo*						
Heart	Normal fed rat (control)	—	6	—	—	2·4±0·31
Heart	Alloxan-diabetic rat	—	6	—	—	0·35±0·09*
Heart	Starved (48 hr.) rat	—	6	—	—	0·70±0·18

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

extracellular water (e.g. by lactate dehydrogenase at the muscle-cell surface or in cells in the tissue other than muscle cells).

The glycerol phosphate/dihydroxyacetone phosphate ratio in heart muscle perfused under aerobic conditions was $11{\cdot}8 \pm 1{\cdot}3$. This ratio (like the lactate/pyruvate ratio in the perfusion medium) was decreased by diabetes (by 50 %) and by starvation (by 70 %). In rat diaphragm incubated in medium containing glucose (8·3 mM) and insulin (0·1 unit/ml.) the glycerol phosphate/dihydroxyacetone phosphate ratio in muscle water was $7{\cdot}5 \pm 0{\cdot}5$. This ratio (like the lactate/pyruvate ratio in the medium) was decreased by diabetes and starvation and increased by butyrate. In rat heart *in vivo* the glycerol phosphate/dihydroxyacetone phosphate ratio in muscle water ($2{\cdot}4 \pm 0{\cdot}31$) was lower than in hearts perfused *in vitro*. Nevertheless, as in hearts perfused *in vitro*, the ratio *in vivo* was substantially lower in diabetes and starvation. Because of these findings the glutamate/α-oxoglutarate and malate/oxaloacetate ratios in rat hearts perfused with glucose and insulin have been determined for normal fed, starved and alloxan-diabetic rats (the interconversions of these pairs of metabolites are also catalysed by NAD-linked dehydrogenases). The malate/oxaloacetate ratio was decreased by starvation (for 40 hr.) from 21 to 12. The glutamate/α-oxoglutarate ratio was decreased by starvation from 61 to 29 and by alloxan-diabetes from 61 to 30.

DISCUSSION

The present study was undertaken with the object of comparing the effects of alloxan-diabetes and starvation and of fatty acids and ketone bodies on the metabolism of pyruvate in rat heart and diaphragm muscles. It has also provided further information about the reversibility of glycolysis in these muscles and about the lactate/pyruvate and glycerol phosphate/dihydroxyacetone phosphate ratios in muscle under different conditions.

Reversibility of glycolysis in heart and diaphragm muscles. The present studies have shown that, in rat heart or rat diaphragms perfused or incubated in the absence of glucose, the addition of pyruvate does not impede the breakdown of glycogen (i.e. that there is little if any net synthesis of glycogen from pyruvate, and little, if any, inhibitory effect of pyruvate on glycogen breakdown). These observations provide further support for the view (Newsholme & Randle, 1962) that glycolysis in these muscles is irreversible. In similar experiments Villee & Hastings (1949) observed slight and variable increases in glycogen concentrations in rat hemidiaphragms incubated in the presence of pyruvate. The slight increases in glycogen concentration observed occasionally by Villee & Hastings (1949) could well have been due to known inhibitory effects of pyruvate on glycolysis (Newsholme & Randle, 1964) and not to a small synthesis of glycogen from pyruvate. On the other hand, when rat hemidiaphragms are incubated in the presence of [2-^{14}C]pyruvate some 6 % of the label taken up may appear in glycogen glucose (Villee & Hastings, 1949; Hiatt, Goldstein, Lareau & Horecker, 1958). The label is found in C-2 and C-5 of the glucose residues in the glycogen molecule, with an excess in C-5. We suggest that such an incorporation of label may reflect isotope-exchange reactions in the pathway between glycogen and pyruvate and not synthesis of glycogen from pyruvate. The excess of label in C-5 of glycogen glucose may then be explained, as Hiatt *et al.* (1958) suggested, by incomplete equilibration of triose phosphates; or, as an alternative, we suggest that it might be due to an additional exchange reaction between glyceraldehyde 3-phosphate and fructose 6-phosphate catalysed by transaldolase (which would introduce further label from [2-^{14}C]-pyruvate into C-5 of glucose). Further support for the view that glycolysis is not reversible in muscle is provided by the very low or absent hexose-diphosphatase activity (Newsholme & Randle, 1962) and the apparent absence of the dicarboxylate shuttle that in other tissues facilitates the formation of phosphoenolpyruvate from pyruvate (e.g. see Hiatt *et al.* 1958). Factors in the poor reversibility of glycolysis in these muscles may thus be low or absent hexose-diphosphatase activity and limited formation of phosphoenolpyruvate from pyruvate (because of the unfavourable equilibrium of the pyruvate-kinase reaction).

Lactate/pyruvate and glycerol 3-phosphate/dihydroxyacetone phosphate concentration ratios. The changes in the lactate/pyruvate ratio that we have observed might suggest that the $NADH_2$/NAD ratio in cell sap is increased by glucose, by anoxia and salicylate (which can uncouple respiratory-chain phosphorylation), and by fatty acids and ketone bodies, and diminished by albumin and by diabetes and starvation. Support for this view is provided by the observation that diabetes and starvation diminish and butyrate increases the glycerol phosphate/dihydroxyacetone phosphate ratio. These findings may also be consistent with the observation that the total $NADH_2$/NAD ratio is decreased in diaphragm muscle in alloxan-diabetic rats (Glock & McLean, 1955). The increase in the ratio induced by glucose and salicylate may be due to the increased rate of glycolysis. The rise in the ratio with anoxia may be attributed to the lack of oxidation of $NADH_2$ by molecular oxygen and to the increased rate of glycolysis. The cause of the change in the ratio induced by fatty acids and

ketone bodies and by diabetes and starvation is not known. From the physiological point of view, the rise in the ratio induced by fatty acids and ketone bodies may facilitate esterification of fatty acids by increasing the concentration of glycerol phosphate. Similarly, the fall in the ratio in diabetes and starvation may, by lowering the glycerol phosphate concentration, favour the oxidation of fatty acids. It may also facilitate deamination of amino acids through transamination by shifting the equilibrium of the L-glutamate-dehydrogenase reaction towards α-oxoglutarate (see the Results section). The change in the ratio is under hormonal control by insulin and by pituitary and adrenocortical hormones. Thus in alloxan-diabetes the ratio may be returned to normal by treatment of the rat with insulin or by hypophysectomy or adrenalectomy.

Effects of fatty acids and ketone bodies, and of alloxan-diabetes and starvation, on the metabolism of pyruvate in rat heart and diaphragm muscles. The results summarized in Tables 2–7 appear to show that the metabolism of pyruvate in rat heart by pathways other than conversion into lactate is decreased by alloxan-diabetes and by fatty acids and ketone bodies, irrespective of whether the pyruvate is formed from glucose or glycogen, or is derived from pyruvate in the medium. In rat hemidiaphragms (but not in rat heart) an additional inhibitory effect of starvation was detected. In calculating the rate of pyruvate disposal it has been assumed that glucose and glycogen were broken down quantitatively to pyruvate. It is appreciated that the rate of pyruvate formation from glucose or glycogen may be less than the calculated rate because some glucose may be oxidized through the pentose phosphate pathway (see Randle *et al.* 1964). This might suggest as an alternative explanation for our findings that diabetes, fatty acids and ketone bodies inhibit the oxidation of glucose through the pentose phosphate pathway (and thereby increase the rate of pyruvate formation). We reject this explanation for the following reasons. In rat heart perfused with mM- or 4 mM-pyruvate the possible contribution of glycogen (20 μmoles of pyruvate/g. wet wt./hr.) was too small to account for the inhibitory effects of these agents (39–86 μmoles of pyruvate/g. wet wt./hr.). In hearts perfused with glucose and insulin this explanation could only be sustained if 75 % of the glucose taken up were oxidized through the pentose phosphate pathway and if this oxidation of glucose were totally suppressed by these agents. This would scarcely be compatible with the observations (Randle *et al.* 1964) that the ratio of $^{14}CO_2$ production from [1-^{14}C]glucose to that from [6-^{14}C]glucose (C-1/C-6 ratio) is only 1·6 and that this ratio was increased rather than decreased by β-hydroxybutyrate. In diaphragm the existing evidence suggests that little glucose 6-phosphate is oxidized through this pathway (Bloom, Stetten & Stetten, 1953). We conclude that diabetes, fatty acids and ketone bodies do impair the metabolism of pyruvate by pathways other than conversion into lactate in rat heart and diaphragm muscles.

The routes of metabolism of pyruvate that might be involved are oxidation to acetyl-CoA by pyruvate dehydrogenase, conversion into malate and conversion into alanine. Though not precluding the possibility that these agents may have some effects on the rates of malate and alanine formation, we suggest that the evidence is sufficient to establish that they inhibit pyruvate oxidation. Thus the isotope experiments showed that the formation of $^{14}CO_2$ from [1-^{14}C]- and [2-^{14}C]-pyruvate was impaired by fatty acids, ketone bodies and diabetes, and the degree of impairment observed is consistent with that calculated from the balance findings. The only exception is starvation, which in the heart impaired $^{14}CO_2$ production without affecting overall disposal of pyruvate by pathways other than lactate production. This may reasonably be explained by the accelerated breakdown of glycogen observed only in hearts from starved rats. This could decrease the formation of $^{14}CO_2$ from labelled pyruvate in the medium by furnishing unlabelled pyruvate for oxidation.

Mechanism of control of pyruvate oxidation and of the cytoplasmic $NADH_2/NAD$ ratio. Pearson *et al.* (1949) and Villee & Hastings (1949) first showed that the oxidation of pyruvate by rat heart and diaphragm muscles is impaired in alloxan-diabetes. The present study has confirmed these findings and shown further that fatty acids and ketone bodies can inhibit the oxidation of pyruvate to acetyl-CoA in these muscles. In view of the increased concentrations of fatty acids (in heart and diaphragm) and of fatty acyl-CoA (in heart) in alloxan-diabetes shown by Garland & Randle (1964*b*), it seems reasonable to suggest that the release of fatty acids from muscle glycerides at an enhanced rate is responsible for the impaired oxidation of pyruvate in diabetes. This view is supported by the identity of mechanism proposed (see below). Bing (1954–55) has suggested that a deficiency of thiamine pyrophosphate may be responsible for impaired pyruvate oxidation in diabetes. This mechanism does not appear to operate in hearts of alloxan-diabetic rats. The concentration of thiamine pyrophosphate in hearts from normal fed rats perfused for 8 min. with medium containing pyruvate (1 mM) was $12{\cdot}3 \pm 0{\cdot}50$ μg./g. wet wt. (mean ± S.E.M. for nine hearts). This was slightly decreased by alloxan-diabetes to $10{\cdot}2 \pm 0{\cdot}62$ μg./g. wet wt. ($P < 0{\cdot}02$) but starvation (which did not inhibit pyruvate oxidation) led to a similar diminution (to $10{\cdot}6 \pm 0{\cdot}46$ μg./g. wet wt.; $P < 0{\cdot}05$). Further support

for the view that concentrations of fatty acids and ketone bodies may exert an important controlling influence on the rate of pyruvate oxidation is provided by a number of experiments *in vivo* showing that pyruvate utilization is impaired in conditions associated with raised plasma concentrations of non-esterified fatty acids and of ketone bodies. These include dogs treated with growth hormone, where the suggestion was made that impaired pyruvate tolerance was due to elevated plasma concentrations of non-esterified fatty acids (Weil, Altszuler & Kessler, 1961), and also human diabetic patients (Bing, 1954–55; Fry & Butterfield, 1962), and normal people treated with corticosteroids and patients with Cushing's syndrome (Fajans, 1961; Henneman & Bunker, 1957).

In experiments involving measurements of uptake of pyruvate from the medium, the inhibitory effects of fatty acids and ketone bodies and of diabetes and starvation could be due either to changes in the rate of oxidation of pyruvate by pyruvate dehydrogenase or to changes in the rate of penetration of pyruvate into muscle cells. The latter possibility exists because the concentration of pyruvate in heart-muscle water is much lower than that in the perfusion medium (measured in hearts perfused for 10 min. with mM- or 4 mM-pyruvate and frozen with the tissue clamp). Thus with mM-pyruvate in the medium the concentration in muscle water was only 0·32 mM, and with 4 mM-pyruvate the concentration in muscle water was only 2·37 mM. Since the volume of extracellular water (measured as the sorbitol space) was 64 % of that of muscle water under these conditions, the intracellular pyruvate concentration may have been very low and the rate of penetration of pyruvate could have been a factor limiting its oxidation. On the other hand, in experiments in which the metabolism of pyruvate formed from glucose or glycogen was investigated, penetration was unlikely to have been a factor because pyruvate was presumably formed within the cell. It seems reasonable to conclude that the site of inhibition, in these instances at any rate, was the pyruvate-dehydrogenase reaction.

The mechanism of inhibition of pyruvate dehydrogenase by fatty acids and ketone bodies and by diabetes is explicable at the present time by the increased acetyl-CoA/CoA ratio that they have been shown to induce in rat heart (see Garland & Randle, 1964*a*, *b*). It has been shown with pig-heart pyruvate dehydrogenase that acetyl-CoA is a competitive inhibitor (with respect to CoA) of the oxidation of pyruvate to acetyl-CoA by the enzyme (Garland & Randle, 1964*b*). Other possible contributing factors that have yet to be investigated are the concentration of succinyl-CoA and the $NADH_2$/NAD ratio in the mitochondrion; a rise in either may inhibit pig-heart pyruvate dehydrogenase (P. B. Garland & P. J. Randle, unpublished work).

The present studies have shown that the cytoplasmic $NADH_2$/NAD ratio may be increased by fatty acids and ketone bodies in rat heart and rat diaphragm in spite of the fact that the rate of glycolysis is decreased. The increase in the ratio could be explained by reduction of cytoplasmic NAD by mitochondrial $NADH_2$ at an accelerated rate by one of the systems that have been proposed. In diabetes or starvation, on the other hand, where the oxidation of fatty acids is accelerated in heart and diaphragm muscles, the cytoplasmic $NADH_2$/NAD ratio is decreased. The reason for this change is not known, but it could be explained by the diminished rate of glycolysis and possibly by a diminished rate of reduction of cytoplasmic NAD by mitochondrial $NADH_2$.

SUMMARY

1. The rate of oxidation of pyruvate in rat hearts or diaphragms perfused or incubated *in vitro* with buffer alone or buffer containing either pyruvate or glucose and insulin has been measured, and the effects of alloxan-diabetes and starvation and of the addition of fatty acids or ketone bodies *in vitro* investigated.

2. In rat heart perfused with medium containing pyruvate the oxidation of unlabelled or of [^{14}C]-pyruvate was inhibited (by approx. 70 %) by *n*-octanoate, butyrate, palmitate, acetoacetate and DL-β-hydroxybutyrate and by alloxan-diabetes. The oxidation of pyruvate formed in the muscle from breakdown of glycogen was inhibited by β-hydroxybutyrate and palmitate. In hearts perfused with medium containing glucose and insulin the oxidation of pyruvate formed from glucose was inhibited by octanoate, butyrate and palmitate and by alloxan-diabetes.

3. In rat hemidiaphragms incubated in medium containing pyruvate the oxidation of unlabelled or [^{14}C]pyruvate was inhibited by butyrate and palmitate and by diabetes and starvation. These agents also inhibited the oxidation of pyruvate formed from glucose.

4. In rat hearts or rat hemidiaphragms perfused or incubated *in vitro* with medium lacking glucose, the addition of pyruvate to the perfusion medium did not significantly impede the breakdown of glycogen.

5. In rat heart and rat hemidiaphragms the lactate/pyruvate ratio in the perfusion or incubation medium was increased by glucose, anoxia, salicylate, fatty acids and ketone bodies, and decreased by albumin, alloxan-diabetes and starvation (for 48 hr.). The glycerol phosphate/

dihydroxyacetone phosphate ratio in the muscles was increased by butyrate and decreased by diabetes and starvation.

6. It is concluded: that the oxidation of pyruvate in rat heart and rat diaphragm muscles may be inhibited in diabetes because of the greater availability of fatty acids and ketone bodies for respiration in this condition; that glycolysis in these muscles is not reversible to any significant extent; that the $NADH_2/NAD$ ratio in cell sap is increased by glucose, anoxia, salicylate, fatty acids and ketone bodies, and diminished in alloxan-diabetes and starvation.

7. The possibility that the inhibitory effects of diabetes, fatty acids and ketone bodies on pyruvate oxidation are due to inhibition of pyruvate dehydrogenase by a rise in the acetyl-CoA/CoA ratio is discussed.

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Regulation of Glucose Uptake by Muscle

10. EFFECTS OF ALLOXAN-DIABETES, STARVATION, HYPOPHYSECTOMY AND ADRENALECTOMY, AND OF FATTY ACIDS, KETONE BODIES AND PYRUVATE, ON THE GLYCEROL OUTPUT AND CONCENTRATIONS OF FREE FATTY ACIDS, LONG-CHAIN FATTY ACYL-COENZYME A, GLYCEROL PHOSPHATE AND CITRATE-CYCLE INTERMEDIATES IN RAT HEART AND DIAPHRAGM MUSCLES

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The experimental observations summarized in the preceding papers have shown that certain abnormalities of carbohydrate metabolism in heart and diaphragm muscles of alloxan-diabetic or starved rats could be explained if the provision of fatty acids and ketone bodies for respiration is increased (Newsholme & Randle, 1964; Randle, Newsholme & Garland, 1964; Garland, Newsholme

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