# Regulation of Glucose Uptake by Muscle

## 7. EFFECTS OF FATTY ACIDS, KETONE BODIES AND PYRUVATE, AND OF ALLOXAN-DIABETES, STARVATION, HYPOPHYSECTOMY AND ADRENALECTOMY, ON THE CONCENTRATIONS OF HEXOSE PHOSPHATES, NUCLEOTIDES AND INORGANIC PHOSPHATE IN PERFUSED RAT HEART*

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Newsholme & Randle (1961) found that the concentrations of glucose 6-phosphate and fructose 6-phosphate were decreased and that of fructose 1,6-diphosphate was increased by anoxia in perfused isolated rat heart. Starvation of the rat, on the other hand, increased the concentration of hexose monophosphates and diminished that of the diphosphate. Regen, Davis & Morgan (1961) have observed in perfused hearts from alloxan-diabetic rats changes in hexose phosphate concentrations similar to those that we have seen in starvation. The conclusion was drawn from these changes in the concentrations of hexose phosphates that anoxia increased, and starvation and diabetes decreased, the rate of phosphorylation of fructose 6-phosphate to the diphosphate catalysed by phosphofructokinase (Newsholme & Randle, 1961, 1962; Newsholme, Randle & Manchester, 1962). The cause of these changes in the rate of the phosphofructokinase reaction was not known. In the studies described below the effects of fatty acids and ketone bodies *in vitro* on the concentrations of hexose phosphates in the perfused rat heart have been investigated and compared with those seen in diabetes and starvation. They were prompted by the idea that the changes seen in diabetes and starvation might be due to an increased availability of fatty acids and ketone bodies for respiration in these conditions. It has now been shown that these substrates can induce changes in hexose phosphate concentrations in hearts from normal fed rats that are similar to those seen in hearts from diabetic or starved rats.

Because anoxia and inhibitors of respiration appear to accelerate the phosphofructokinase reaction and because the provision of substrates for respiration appears to slow it, it seemed possible that this enzymic step might be controlled by changes in the intracellular concentrations of nucleotides and inorganic phosphate. We have therefore studied the effect of these factors on the intracellular concentration of these substances. While this work was in progress Mansour, Clague & Bearnink (1962) found that the activity of guinea-pig heart phosphofructokinase was diminished by ATP and increased by AMP and inorganic phosphate; similar results were obtained with rabbit skeletal-muscle phosphofructokinase by Passonneau & Lowry (1962).

A preliminary account of some of these findings has been published (Newsholme *et al.* 1962).

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## MATERIALS

*Rats.* Hearts were obtained from male albino Wistar rats (200–300 g.) fed on a stock laboratory diet (Short & Parkes, 1949) and with free access to food and water at all times, except for those described as starved, which were deprived of food for 39–42 hr. before removal of the heart. Alloxan-diabetes was induced by the intravenous administration of alloxan (60 mg./kg.) under ether anaesthesia. The animals were used 48 hr. later without starvation and all had blood glucose concentrations in excess of 300 mg./100 ml. Hypophysectomy under ether anaesthesia by the parapharyngeal route was carried out by Miss M. Thomas and animals were used after 14 days or later. Bilateral adrenalectomy was carried out through a single midline dorsal incision under ether anaesthesia and the animals were used 48 hr. later. Alloxan-diabetes was induced in adrenalectomized rats at the time of adrenalectomy.

*Chemicals.* Hexose phosphates, ATP, ADP, AMP, phosphoenolpyruvate (tricyclohexylammonium salt), 1,3-diphosphoglycerate (tricyclohexylammonium salt), NAD, $NADH_2$, NADP, tris (Sigma 121), sodium pyruvate and triethanolamine hydrochloride were obtained either from Sigma Chemical Co. (through George T. Gurr Ltd., London, S.W. 6) or from C. F. Boehringer und Soehne G.m.b.H. (through Courtin and Warner Ltd., Lewes, Sussex). Alloxan was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Heparin (given by Evans Medical Ltd.) was dissolved in 0·9% NaCl to 2500 units/ml. and diluted with an equal volume of veterinary Nembutal solution (Abbott Laboratories Ltd.) (60 mg./ml.). Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd., and used without further purification. Sodium acetoacetate was prepared by Mr B. R. Slater by

saponification of ethyl acetoacetate by the method of Davies (1943) and assayed by hypoiodite oxidation. *n*-Octanoic acid and β-methylbutyric acid were obtained from Kodak Ltd., London; they were dissolved in perfusion medium by gentle shaking and 1 equiv. of $NaHCO_3$ was added. Bovine plasma albumin (fraction V) was obtained from the Armour Pharmaceutical Co. Ltd. and freed of fatty acids, insulin and insulin antagonist by the method of Garland, Newsholme & Randle (1964). Palmitic acid (A grade) was obtained from California Corp. for Biochemical Research, Los Angeles, U.S.A. (through V. A. Howe and Co. Ltd., London, W. 11), and used without further purification. It was attached to serum albumin and solutions were prepared for perfusion as described by Garland *et al.* (1964). Crystalline insulin (given by Boots Pure Drug Co. Ltd., Burroughs Wellcome, or Novo Terapeutisk Laboratories, Copenhagen) was dissolved in 3·3 mN-HCl to yield a stock solution of 20 units/ml., which was diluted to 0·1 unit/ml. with perfusion medium. Growth hormone was prepared in this Laboratory by Mr B. R. Slater from ox anterior-pituitary lobes by the method of Wilhelmi, Fishman & Russell (1948). Solutions for injection were prepared as described by Manchester, Randle & Young (1959). Cortisol (given by Boots Pure Drug Co. Ltd.) was supplied as a solution of the free alcohol in 50% (v/v) ethanol. All other chemicals were of the purest grade commercially available.

*Enzymes.* Glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, lactate dehydrogenase and myokinase were obtained from C. F. Boehringer und Soehne G.m.b.H.

*Media.* Hearts were perfused with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing glucose (1 mg./ml.) gassed with $O_2+CO_2$ (95:5) or $N_2+CO_2$ (95:5).

## PROCEDURE

*Hearts.* These were perfused for 15 min. through the coronary circulation at 37°. When albumin was present, about 15 ml. of medium was recirculated by using the apparatus of Morgan, Henderson, Regen & Park (1961) after washing out the heart initially with 10–15 ml. of medium. In all other experiments perfusion was made with medium that flowed continuously by gravity from a reservoir 60–70 cm. above the heart. At the end of perfusion the hearts were cut from the cannula and frozen immediately in acetone–solid $CO_2$, weighed and kept frozen until extracted. Because Wollenberger, Ristau & Schoffa (1960) have obtained evidence for delay in the cooling of muscle frozen in this way, a number of the experiments were repeated and hearts frozen on the cannula by compression between two blocks of aluminium (7·5 cm. diam. × 3 cm. thick) previously cooled in liquid $N_2$. This technique is reported by Wollenberger *et al.* (1960) to freeze muscle within 0·1 sec. Rats were injected intraperitoneally with heparin (625 units) and Nembutal (15 mg.) about 5 min. before hearts were removed for perfusion.

In experiments in which measurements were made on hearts without perfusion rats were anaesthetized with Nembutal (6 mg./100 g.) and adrenalectomized (to prevent adrenaline release during removal of the heart). After a 30 min. rest period (during which they were kept warm with the heat from an electric light bulb) the chest was opened and the heart rapidly removed and frozen in acetone–solid $CO_2$.

## METHODS

*Muscle extracts.* For assay of hexose phosphates and nucleotides extracts were made as described by Newsholme & Randle (1961), except that in experiments in which nucleotides and inorganic phosphate were assayed 2·5 ml. of 10% (w/v) trichloroacetic acid was used and the extract was filtered immediately through a sintered-glass filter (porosity 1).

*Hexose phosphates.* These were assayed by the methods described by Newsholme & Randle (1961), except that glutathione (50 μM) and $HgCl_2$ (50 μM) were added to the assay medium for glucose 6-phosphate and fructose 6-phosphate to prevent reoxidation of $NADPH_2$ by glutathione reductase (Narahara & Ozand, 1960).

*ATP, ADP and AMP.* These were assayed by methods based on those described in leaflets provided by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Details are as follows. ATP was assayed by the change in extinction at 340 mμ in a Beckman spectrophotometer on the addition of a mixture of 160 μg. of glyceraldehyde 3-phosphate dehydrogenase and 40 μg. of phosphoglycerate kinase to a mixture of 0·1 ml. of muscle extract and 2·9 ml. of assay medium [triethanolamine hydrochloride (0·1 M), $MgSO_4$ (4 mM) and 3-phosphoglycerate (6 mM), pH 7·5, and containing sufficient $NADH_2$ to give an extinction of 0·6–0·7 in a 1 cm. light-path when read against a water blank]. ADP was assayed by the change in extinction at 340 mμ on adding 200 μg. of pyruvate kinase to a mixture of 0·2 ml. of muscle extract, 2·7 ml. of assay medium [triethanolamine hydrochloride (0·1 M), phosphoenolpyruvate (0·7 mM), KCl (90 mM) and $MgSO_4$ (28 mM), pH 7·5, and $NADH_2$ as above], 0·1 ml. of ATP solution (400 μg./ml. with no detectable ADP or AMP) and 100 μg. of lactate dehydrogenase. AMP was assayed by the further change in extinction on the addition to the mixture, in which the ADP assay has been completed, of 20 μg. of myokinase; it was found that this change needs to be corrected for a blank value that is the change in extinction when water is substituted for muscle extract. The reason for the need for this correction was not ascertained, but one possibility is contamination of $NADH_2$ with AMP. The correction needs to be determined for each batch of assays and has varied from zero to 0·150, but it was constant for any particular batch of assays. The recovery of AMP added to muscle extracts was 100·8±0·7% for eight determinations. No attempt was made to correct these values for the contribution of nucleotides not of the adenine series.

*Inorganic phosphate.* Inorganic phosphate was estimated photometrically by reference to 0·2 ml. of standard $KH_2PO_4$ (1 μmole/ml.) in 0·2 ml. of perfusion medium or muscle extract by the method of Berenblum & Chain as modified by Martin & Doty (1949). The trichloroacetic acid extract of frozen muscle powder was rapidly filtered (see above), and 0·2 ml. of the filtrate was taken at once and shaken with acid molybdate and isobutanol–benzene mixture and the organic phase separated immediately. Analysis of phosphate in the latter was then completed at leisure.

Table 1. *Effects of alloxan-diabetes, hypophysectomy, adrenalectomy and treatment of the rat with growth hormone and cortisol on the concentrations of hexose phosphates in perfused rat heart*

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). In rats treated with insulin, 12 units of insulin were given subcutaneously 24 and 15 hr. and 16 units 3 hr. before removal of the heart. In rats treated with growth hormone and cortisol, 0·1 mg. of growth hormone/100 g. was given at 24, 12 and 4 hr. and 2·5 mg. of cortisol/100 g. was given at 24 hr. and 1·25 mg./100 g. at 12 and 4 hr. before removal of the heart. Hearts were frozen in acetone–solid $CO_2$. Other details are given in the text. The results are given as means ±S.E.M.

| Source of heart | No. of observations | Concn. (μmoles/100 g. wet wt. of heart) Glucose 6-phosphate | Fructose 6-phosphate | Fructose 1,6-diphosphate |
|---|---|---|---|---|
| Normal fed rat (control) | 20 | 29±0·8 | 6·3±0·5 | 5·3±0·40 |
| Starved (40 hr.) rat | 6 | 38±1·5* | 8·3±0·8‡ | 2·8±0·25* |
| Alloxan-diabetic rat | 6 | 46±5·6* | 10·1±1·2* | 3·4±0·39* |
| Alloxan-diabetic rat treated with insulin | 6 | 24±2·1* | 7·0±1·1 | 4·4±1·3 |
| Hypophysectomized rat (control) | 6 | 23±0·7* | — | 0·8±0·20* |
| Hypophysectomized starved (40 hr.) rat | 6 | 25±3·1 | — | 1·1±0·21 |
| Hypophysectomized alloxan-diabetic rat | 6 | 23±1·4 | 4·4±0·4 | 0·7±0·21 |
| Hypophysectomized alloxan-diabetic rat treated with growth hormone + cortisol | 4 | 41±3·5* | 6·8±1·6 | 0·7±0·32 |
| Adrenalectomized rat | 6 | 31±1·4 | 6·6±0·4 | 3·3±0·52* |
| Adrenalectomized alloxan-diabetic rat | 6 | 34±2·1 | 7·2±1·0 | 3·1±0·68 |
| Alloxan-diabetic rat treated with growth hormone + cortisol | 6 | 76±7·0* | 19·0±3·1* | 2·5±0·70 |
| Alloxan-diabetic rat, with DL-β-hydroxybutyrate (70 mg./100 ml.) in perfusion medium | 5 | 54±6·0 | 8·4±1·1 | 3·6±0·40 |

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

*Calculation and expression of results.* Concentrations of hexose phosphates and nucleotides are calculated and expressed either as μmoles/100 g. wet wt. of heart or as intracellular concentrations (mM) by the methods given by Newsholme & Randle (1961). As explained by Newsholme & Randle (1961) this method of calculation refers concentrations to heart muscle containing (per 100 g.) 79 ml. of water, 36 ml. of which is extracellular and 43 ml. intracellular (the water contents of cardiac muscle after aerobic perfusion and blotting to remove perfusion medium in the chambers of the heart). The calculation corrects for additional water retained in the chambers of the heart by rapid freezing and for the additional water retained in the extracellular compartment by perfusion under anaerobic conditions or with salicylate (these agents do not affect the intracellular volume). The other agents used in the present study did not significantly alter the volumes of total water, extracellular water and intracellular water in the heart. The intracellular concentration of inorganic phosphate was calculated as follows. From the photometric data the total inorganic phosphate/g. wet wt. of heart was calculated. The wet and dry weights and total water content of the frozen muscle powder were also estimated by the method of Randle & Smith (1958). The intracellular water/g. dry wt. of heart under the various conditions used was about 2·1 ml. (obtained from measurements of total water and sorbitol spaces). Thus, from the total water and dry wt./g. wet wt. of heart, the intracellular water and (hence by difference from total water) the extracellular water could be calculated. The extracellular inorganic phosphate was then calculated, assuming that the concentration of inorganic phosphate in extracellular water is the same as that in perfusion medium. The intracellular inorganic phosphate was then obtained by difference, and division by the volume of intracellular water gave the intracellular concentration.

## RESULTS

*Effects of alloxan-diabetes, starvation, hypophysectomy, adrenalectomy and treatment of the rat with growth hormone and cortisol on the concentrations of hexose phosphates*

*Effects of alloxan-diabetes and starvation.* In hearts from normal fed rats perfused aerobically for 15 min. with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate (in μmoles/100 g. wet wt.) were 29, 6·3 and 5·3 respectively. In confirmation of findings by Newsholme & Randle (1961) starvation for 40 hr. increased the concentrations of the hexose monophosphates by about 50 % and diminished that of the diphosphate by about 50 % also. Similar changes were seen in hearts from diabetic rats, in confirmation of findings by Regen *et al.* (1961). However, when alloxan-diabetic rats had been injected with insulin the hexose phosphate concentrations in the perfused heart were similar to those of hearts from normal rats. On the other hand, injection of alloxan-diabetic rats with growth hormone and cortisol led to a further increase in the concentrations of glucose 6-phosphate and fructose 6-phosphate (Table 1).

*Effects of hypophysectomy.* After hypophysectomy of normal rats the concentration of glucose 6-phosphate in the perfused heart was decreased by about 25 %, whereas that of fructose 1,6-diphos-

phate was markedly decreased (by about 85 %). In hypophysectomized rats (unlike normal rats) neither starvation nor alloxan-diabetes increased the concentration of glucose 6-phosphate. On the other hand, the glucose 6-phosphate concentration was elevated in hypophysectomized alloxan-diabetic rats that had been treated with growth hormone and cortisol (Table 1).

*Effects of adrenalectomy.* In hearts from adrenalectomized rats the concentration of the hexose monophosphates was substantially normal whereas that of fructose 1,6-diphosphate was moderately lowered. Induction of alloxan-diabetes in adrenalectomized rats did not (as it did in normal rats) alter the concentrations of the hexose phosphates (Table 1).

*Effects of perfusions in the absence of insulin.* When perfusions were made with medium containing glucose (1 mg./ml.) but lacking insulin, the concentrations of glucose 6-phosphate and fructose 6-phosphate (compared with values obtained with medium containing insulin) were unchanged with hearts from fed rats; but they were diminished when normal rats were starved for 12 hr. (to decrease the tissue concentration of endogenous insulin). In hearts from alloxan-diabetic or starved rats the characteristic increase in the concentrations of glucose 6-phosphate and fructose 6-phosphate was seen with medium containing no insulin (Table 2).

*Method of freezing the tissue.* The results given above were obtained with hearts frozen in acetone–solid carbon dioxide. The effects of starvation, alloxan-diabetes and anoxia on concentrations of hexose phosphates were reinvestigated in hearts frozen with the tissue clamp of Wollenberger *et al.* (1960) (which leads to more rapid freezing of the tissue; see the Procedure section). Essentially similar results were obtained with this method of freezing though the absolute concentrations of fructose 1,6-diphosphate (but not of glucose 6-phosphate) were lower (Table 3).

*Concentrations of hexose phosphates in hearts* in vivo. It seemed desirable to attempt to measure the concentrations of the hexose phosphates in the heart *in vivo* and to compare them with those found

Table 2. *Effects of insulin, and of alloxan-diabetes, starvation, ketone bodies and palmitate in the absence of insulin, on the concentrations of hexose phosphates in perfused rat heart*

The perfusion medium contained glucose at a concentration of 1 mg./ml. (in Expts. 1, 2, 3 and 5) or 4 mg./ml. (in Expt. 4). The insulin added in Expt. 1 was glucagon-free (Novo Terapeutisk). Rats were starved for 12 hr. in Expts. 1, 3 and 4 to diminish endogenous insulin in the heart. Hearts were frozen in acetone–solid $CO_2$. Other details are given in the text. The results are given as means ±S.E.M.

| Expt. no. | Source of heart | Addition(s) to perfusion medium | No. of observations | Concn. (μmoles/100 mg. wet wt. of heart) Glucose 6-phosphate | Fructose 6-phosphate | Fructose 1,6-diphosphate |
|---|---|---|---|---|---|---|
| 1 | Normal rat starved for 12 hr. (control) | None | 6 | 15±1·6 | 4·1±0·27 | 2·7±0·19 |
| | Normal rat starved for 12 hr. | Insulin (0·1 unit/ml.) | 6 | 32±1·3* | 8·6±0·63* | 4·0±0·17* |
| 2 | Normal fed rat (control) | None | 12 | 33±2·0 | 6·8±0·51 | — |
| | Alloxan-diabetic rat | None | 6 | 46±2·7* | 8·8±0·4* | — |
| | Starved (40 hr.) rat | None | 6 | 41±3·7 | 0·9±1·4† | — |
| 3 | Normal rat starved for 12 hr. (control) | None | 6 | 19±2·1 | 3·9±0·42 | 2·7±0·23 |
| | Normal rat starved for 12 hr. | DL-β-Hydroxybutyrate (70 mg./100 ml.) | 6 | 41±4·3* | 7·1±0·63* | 1·6±0·21* |
| 4 | Normal rat starved for 12 hr. (control) | None | 12 | 26±3·4 | 6·8±1·0 | 2·8±0·16 |
| | Normal rat starved for 12 hr. | DL-β-Hydroxybutyrate (70 mg./100 ml.) | 12 | 49±2·2* | 12·2±1·2* | 2·1±0·28‡ |
| 5 | Normal fed rat (control) | Albumin (2%) | 6 | 23±2·5 | 4·4±0·35 | 1·9±0·21 |
| | Normal fed rat | Albumin (2%) + palmitate (19·5 mg./100 ml.) | 6 | 30±2·5 | 5·7±0·56 | 1·2±0·16 |

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

after 15 min. of perfusion *in vitro*. This was done by removing hearts from anaesthetized and acutely adrenalectomized rats (see the Methods section) and immediately freezing them in acetone–solid carbon dioxide. The results are given in Table 4. The concentrations of the hexose phosphates in hearts from normal fed, starved, alloxan-diabetic or adrenalectomized rats were very similar to those found after perfusion (cf. Tables 1 and 2). Particularly high concentrations of glucose 6-phosphate and fructose 6-phosphate were found in hearts from adrenalectomized alloxan-diabetic rats treated with cortisol (Table 4).

*Effects of ketone bodies, fatty acids, pyruvate and other substrates on the concentrations of hexose phosphates in perfused rat heart*

In hearts from normal fed rats perfused aerobically for 15 min. with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) the concentrations of glucose 6-phosphate and fructose 6-phosphate were increased and that of fructose 1,6-diphosphate was decreased (in hearts frozen in acetone–solid carbon dioxide) by the presence in the medium of acetoacetate, DL-β-hydroxybutyrate, butyrate, *n*-octanoate, pyruvate or lactate at the concentrations given in Table 5 (effects similar to those observed with the racemic compound were found with a sample of D-β-hydroxybutyrate kindly provided by Dr G. D. Greville). The effects of acetoacetate and of β-hydroxybutyrate increased as their concentrations in the perfusion medium were increased from 1·25 to 25 and 10 to 70 mg./100 ml. respectively. Only small changes in the hexose phosphate concentrations were observed when the perfusion medium contained isovalerate (γ-methylbutyrate) or β-methylbutyrate; other substances such as L-alanine, L-glutamate, succinate and propionate had no detectable effect. These results are given in Table 5. When hearts were perfused with medium containing no glucose (and half the concentration of $Ca^{2+}$ to decrease the force of contraction) the concentrations of glucose 6-phosphate and fructose 1,6-diphosphate were substantially lowered ($5 \pm 0{\cdot}9$ μmoles/100 g. wet wt. and none detected respectively). Perfusion with

Table 3. *Effects of alloxan-diabetes and starvation and of fatty acids, ketone bodies and pyruvate on the concentrations of hexose phosphates in perfused rat hearts frozen with the tissue clamp of Wollenberger* et al. (1960)

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Other details are given in the text. The results are given as means ±S.E.M.

| Source of heart | Addition(s) to perfusion medium | No. of observations | Concn. (μmoles/100 g. wet wt. of heart) Glucose 6-phosphate | Fructose 1,6-diphosphate |
|---|---|---|---|---|
| Normal fed rat (control) | None (aerobic) | 20 | 31±0·7 | 3·7±0·41 |
| Normal fed rat | None (anaerobic) | 2 | 21±1·0* | 8·2±0·25* |
| Starved (40 hr.) rat | None | 6 | 46±3·2* | 1·6±0·44† |
| Alloxan-diabetic rat | None | 6 | 51±6·6* | 2·2±0·60 |
| Normal fed rat | Pyruvate (60 mg./100 ml.) | 7 | 50±4·4* | 2·5±0·50 |
| Normal fed rat | *n*-Octanoate (40 mg./100 ml.) | 8 | 48±2·1* | — |
| Normal fed rat | DL-β-Hydroxybutyrate (70 mg./100 ml.) | 5 | 55±2·2* | 1·8±0·31* |
| Normal fed rat (control) | Albumin (2%) | 6 | 25±1·3 | — |
| Normal fed rat | Albumin (2%) + palmitate (19·5 mg./100 ml.) | 6 | 37±1·6* | — |

* $P < 0{\cdot}01$; † $P < 0{\cdot}02$; for other differences from appropriate control $P > 0{\cdot}05$.

Table 4. *Effects of alloxan-diabetes, adrenalectomy, starvation and treatment with cortisol on the concentrations of hexose phosphates in rat heart* in vivo

Hearts were excised and frozen immediately in acetone–solid $CO_2$ (for full details see the Experimental section). Cortisol was given by subcutaneous injection (2·5 mg./100 g. at 24 hr. and 1·25 mg./100 g. at 12 and 4 hr. before removal of the heart). Other details are given in the text. The results are given as means ±S.E.M.

| Source of heart | No. of observations | Concn. (μmoles/100 g. wet wt. of heart) Glucose 6-phosphate | Fructose 6-phosphate | Fructose 1,6-diphosphate |
|---|---|---|---|---|
| Normal fed rat | 14 | 26±2·0 | 4·6±0·36 | 6·0±0·80 |
| Alloxan-diabetic rat | 6 | 39±4·4† | 6·6±0·76‡ | 0·6±0·90* |
| Starved (40 hr.) rat | 6 | 35±4·3 | 5·4±0·15 | 2·6±0·65* |
| Adrenalectomized (48 hr.) rat | 5 | 25±1·5 | 5·0±0·37 | 2·8±0·34* |
| Adrenalectomized alloxan-diabetic rat treated with cortisol | 6 | 102±7·8* | 23·4±3·9* | 1·0±0·34 |

* $P < 0{\cdot}01$; † $P < 0{\cdot}02$; ‡ $P < 0{\cdot}05$; for other differences from appropriate control $P > 0{\cdot}05$.

β-hydroxybutyrate (70 mg./100 ml.) increased the concentration of glucose 6-phosphate to 12·5 ± 1·7 μ-moles/100 g. wet wt., whereas the concentration of fructose 1,6-diphosphate was too low to permit its detection.

To investigate the effect of palmitate on the hexose phosphate concentrations in perfused heart, it was necessary to add bovine plasma albumin as a carrier for the fatty acid (see Goodman, 1958). Hearts were therefore perfused with medium containing glucose and insulin with 2 % (w/v) bovine plasma albumin (control) or with albumin–palmitate complex. With 2 % bovine plasma albumin but no palmitate the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were 30, 5·7 and 2·1 μ-moles/100 g. wet wt. of heart respectively. Albumin thus diminished the concentration of the diphosphate without, however, affecting that of the monophosphates. The addition of palmitate–albumin complex increased the concentration of the monophosphates and diminished that of the diphosphate; thus palmitate had an effect similar to that of the short-chain fatty acids (Table 5).

The effect of octanoate, pyruvate, β-hydroxybutyrate and palmitate in hearts frozen with the tissue clamp of Wollenberger *et al.* (1960) are shown in Table 3. Essentially similar changes in the concentrations of the hexose monophosphates were seen under these conditions, and β-hydroxybutyrate and pyruvate decreased the concentration of the diphosphate (this was not assayed with other substrates). The effects of β-hydroxybutyrate and palmitate on hexose phosphate concentrations in hearts perfused with medium lacking insulin are shown in Table 2. These substrates again increased the concentration of the monophosphates and diminished that of the diphosphate. β-Hydroxybutyrate had qualitatively similar effects on the hexose phosphate concentrations at two medium concentrations of glucose, namely 1 mg./ml. and 4 mg./ml.

The changes in the hexose phosphate concentrations in hearts from normal rats induced by the presence in the medium of ketone bodies, fatty acids and pyruvate are thus qualitatively similar to those seen in the absence of these substrates in hearts from alloxan-diabetic rats or from normal rats starved for 40 hr. The changes induced by palmitate were quantitatively similar to those induced by diabetes and starvation, but other substrates exerted greater effects on the concentrations of the monophosphates (Tables 1 and 5). The glucose 6-phosphate and fructose-6-phosphate con-

Table 5. *Effects of ketone bodies, fatty acids and pyruvate and other substrates on the concentrations of hexose phosphates in perfused rat heart*

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Hearts were frozen in acetone–solid $CO_2$. Other details are given in the text. The results are given as means ± S.E.M.

| Addition(s) to perfusion medium | No. of observations | Concn. (μmoles/100 g. wet wt. of heart) Glucose 6-phosphate | Fructose 6-phosphate | Fructose 1,6-diphosphate |
|---|---|---|---|---|
| None (aerobic control) | 20 | 29 ± 0·8 | 6·3 ± 0·5 | 5·3 ± 0·40 |
| Acetoacetate (25 mg./100 ml.) | 11 | 53 ± 3·7* | 8·4 ± 0·7* | 2·0 ± 0·13* |
| Acetoacetate (5 mg./100 ml.) | 6 | 41 ± 0·9* | 7·8 ± 0·2* | — |
| Acetoacetate (1·25 mg./100 ml.) | 6 | 39 ± 1·6* | 7·2 ± 0·4 | 2·2 ± 0·40* |
| Acetoacetate (0·3 mg./100 ml.) | 6 | 29 ± 2·7 | — | 4·4 ± 0·44 |
| DL-β-Hydroxybutyrate (70 mg./100 ml.) | 11 | 56 ± 1·4* | 8·7 ± 1·0* | 2·2 ± 1·0* |
| DL-β-Hydroxybutyrate (25 mg./100 ml.) | 4 | 38 ± 2·6* | — | — |
| DL-β-Hydroxybutyrate (10 mg./100 ml.) | 5 | 37 ± 1·7* | — | 4·0 ± 0·47‡ |
| DL-β-Hydroxybutyrate (5 mg./100 ml.) | 4 | 34 ± 2·2‡ | — | — |
| DL-β-Hydroxybutyrate (1·5 mg./100 ml.) | 4 | 29 ± 1·7 | — | — |
| DL-β-Hydroxybutyrate (0·5 mg./100 ml.) | 6 | 27 ± 1·0 | — | 5·2 ± 0·60 |
| Butyrate (22·4 mg./100 ml.) | 9 | 47 ± 2·7* | 9·1 ± 0·9† | 1·8 ± 1·40‡ |
| *n*-Octanoate (40 mg./100 ml.) | 9 | 53 ± 4·0* | 9·9 ± 1·1* | 2·5 ± 0·31* |
| Pyruvate (31 mg./100 ml.) | 6 | 45 ± 4·0* | 10·2 ± 1·3* | 1·2 ± 0·30* |
| Lactate (26 mg./100 ml.) | 6 | 41 ± 1·4* | 7·5 ± 0·5 | 1·9 ± 0·18* |
| β-Methylbutyrate (30 mg./100 ml.) | 10 | 36 ± 1·9* | 9·0 ± 0·9* | 4·4 ± 0·40 |
| Isovalerate (30 mg./100 ml.) | 10 | 35 ± 2·2† | 8·5 ± 1·8 | 4·6 ± 0·37 |
| L-Alanine (26 mg./100 ml.) | 6 | 31 ± 1·2 | 6·0 ± 1·1 | 5·5 ± 0·60 |
| Succinate (33 mg./100 ml.) | 6 | 30 ± 1·3 | 6·0 ± 0·8 | 4·6 ± 0·48 |
| L-Glutamate (73 mg./100 ml.) | 4 | 24 ± 3·1 | — | 5·7 ± 0·80 |
| Propionate (36 mg./100 ml.) | 8 | 25 ± 1·3 | 5·6 ± 1·0 | 4·9 ± 0·39 |
| Albumin (2 %) (control) | 17 | 30 ± 0·8 | 5·7 ± 0·4 | 2·1 ± 0·10 |
| Albumin (2 %) + palmitate (19·5 mg./100 ml.) | 17 | 39 ± 1·1* | 7·0 ± 0·4 | 1·6 ± 0·11* |
| Albumin (2 %) + β-hydroxybutyrate (70 mg./100 ml.) | 5 | 34 ± 3·3 | 5·4 ± 0·2 | — |

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

centrations in hearts from diabetic rats perfused with medium containing DL-β-hydroxybutyrate were similar to those seen in hearts from normal rats perfused with β-hydroxybutyrate (Tables 1 and 5).

*Effects of anoxia, salicylate and 2,4-dinitrophenol on the concentrations of hexose phosphates in perfused rat heart*

In confirmation of findings by Newsholme & Randle (1961), anoxia diminished the concentrations of glucose 6-phosphate and fructose 6-phosphate and increased that of fructose 1,6-diphosphate in hearts perfused with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Similar changes were induced by salicylate (5 mM) and 2,4-dinitrophenol (50 μM) under aerobic conditions (Table 6). The effects of fatty acids and ketone bodies on the hexose phosphate concentrations were investigated in the heart perfused under anaerobic conditions. Palmitate, acetoacetate and DL-β-hydroxybutyrate failed to increase the concentrations of the hexose monophosphates, and failed to diminish that of the diphosphate. Moreover, under aerobic conditions but in the presence of salicylate (5 mM), the effects of starvation and diabetes, and of DL-β-hydroxybutyrate and palmitate, were absent; and DL-β-hydroxybutyrate was without effect in the presence of 2,4-dinitrophenol (50 μM).

*Effects of alloxan-diabetes and starvation, and of anoxia, fatty acids, ketone bodies and pyruvate, on the intracellular concentrations of ATP, ADP, AMP and inorganic phosphate in perfused rat heart*

When hearts were perfused aerobically for 15 min. with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) the intracellular concentrations of ATP, ADP, AMP and inorganic phosphate in eight separate experiments were in the ranges 9·0–11·5, 2·0–2·6, 0·20–0·44 and 6·3–10·5 mM respectively. Anoxia diminished the concentration of ATP markedly, increased that of ADP slightly and substantially increased those of AMP and inorganic phosphate. In a further experiment it was found that 1 min. of anoxia after 5 min. of aerobic perfusion was sufficient to lower the concentration of ATP from 11·6 to 10·4 mM, to increase that of ADP from 2·5 to 2·7 mM, that of AMP from 0·9 to 1·5 mM and that of inorganic phosphate from 4·5 to 6·7 mM, and to lower that of glucose 6-phosphate from 0·7 to 0·5 mM. When hearts were perfused aerobically with medium containing, in addition to the above, 2 % of bovine

Table 6. *Effects of anoxia, salicylate and 2,4-dinitrophenol on the concentrations of hexose phosphates in perfused rat heart*

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Hearts were frozen in acetone–solid $CO_2$. Other details are given in the text. The results are given as means ±S.E.M.

| Source of heart | Addition(s) to perfusion medium | No. of observations | Concn. (μmoles/100 g. wet wt. of heart) Glucose 6-phosphate | Fructose 6-phosphate | Fructose 1,6-diphosphate |
|---|---|---|---|---|---|
| Normal fed rat (control) | None (aerobic) | 20 | 29±0·8 | 6·3±0·3 | 5·3±0·40 |
| Normal fed rat | None (anaerobic) | 5 | 13±0·8* | 2·4±0·3* | 8·4±0·84* |
| Normal fed rat | Salicylate (5 mM) | 6 | 20±0·7* | 4·6±0·4* | 7·4±0·72† |
| Normal fed rat | 2,4-Dinitrophenol (50 μM) | 6 | 18±1·2* | 2·0±0·4* | 9·2±0·83* |
| Normal fed rat | Acetoacetate (25 mg./100 ml.) (anaerobic) | 5 | 15±1·9 | 2·3±0·4 | 8·0±0·86 |
| Normal fed rat | DL-β-Hydroxybutyrate (70 mg./100 ml.) (anaerobic) | 5 | 13±1·0 | 2·9±0·3 | 10·1±0·12 |
| Alloxan-diabetic rat | Salicylate (5 mM) | 6 | 20±2·0 | 3·7±0·3 | 7·1±0·84 |
| Starved (40 hr.) rat | Salicylate (5 mM) | 6 | 22±0·7 | 4·0±0·4 | 6·7±0·53 |
| Normal fed rat | Salicylate (5 mM) + DL-β-hydroxybutyrate (70 mg./100 ml.) | 6 | 20±0·9 | 4·2±0·5 | 8·1±0·33 |
| Normal fed rat | 2,4-Dinitrophenol (50 μM) + β-hydroxybutyrate (70 mg./100 ml.) | 6 | 17±1·5 | 3·5±0·4 | 9·8±1·31 |
| Normal fed rat (control) | Albumin (2%) (aerobic) | 17 | 30±0·8 | 5·7±0·4 | 2·1±0·10 |
| Normal fed rat | Albumin (2%) (anaerobic) | 4 | 24±1·4* | 4·2±0·3† | 4·3±0·50* |
| Normal fed rat | Albumin (2%) + palmitate (19·5 mg./100 ml.) (anaerobic) | 4 | 21±1·3 | 3·3±0·2 | 5·8±0·22 |
| Normal fed rat | Albumin (2%) + salicylate (5 mM) | 6 | 23±0·3* | 4·2±0·5 | 5·3±0·41* |
| Normal fed rat | Albumin (2%) + palmitate (19·5 mg./100 ml.) + salicylate (5 mM) | 6 | 21±2·9 | 4·4±0·7 | 5·8±0·82 |

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

plasma albumin, the concentrations of ATP, ADP and AMP were essentially unchanged (though the variation between individual values was diminished in each case), whereas that of inorganic phosphate was in general lower. The addition of palmitate–albumin complex did not influence the concentrations of ATP, ADP, AMP and inorganic phosphate, though that of glucose 6-phosphate was increased. In hearts from alloxan-diabetic rats or normal rats starved for 40 hr. there was no significant change in the intracellular concentrations of ATP, ADP, AMP and inorganic phosphate, though the concentration of the last was somewhat lower in each instance. When hearts from normal fed rats were perfused with medium containing acetoacetate (25 mg./100 ml.), DL-β-hydroxybutyrate (70 mg./100 ml.) or pyruvate (60 mg./100 ml.), the intracellular concentrations of ATP, ADP and AMP were unchanged. The concentration of inorganic phosphate was, however, decreased in each instance, though the change was statistically significant only in the experiments with β-hydroxybutyrate and pyruvate. When hearts were perfused with medium containing octanoate (40 mg./100 ml.) or butyrate (40 mg./100 ml.) the concentration of ATP was lowered (though the change was not statistically significant) and that of AMP increased (statistically significant in each case). The concentration of inorganic phosphate was unchanged with butyrate but increased with octa-

Table 7. *Effects of alloxan-diabetes and starvation, and of anoxia, fatty acids, ketone bodies and pyruvate, on the intracellular concentrations of ATP, ADP, AMP, inorganic phosphate and glucose 6-phosphate in perfused rat heart*

The perfusion medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Hearts were frozen with the tissue clamp of Wollenberger *et al.* (1960). Other details are given in the text. The glucose 6-phosphate concentrations are calculated from data given in Tables 2, 5 and 6. The results are given as means ±S.E.M.

| Source of heart | Addition(s) to perfusion medium | No. of observations | Intracellular concn. (mM) | | | | |
|---|---|---|---|---|---|---|---|
| | | | ATP | ADP | AMP | Inorganic phosphate | Glucose 6-phosphate |
| Normal fed rat (control) | None (aerobic) | 5 | 10·0±0·26 | 2·1±0·07 | 0·39±0·03 | 8·5±0·33 | 0·67 |
| Normal fed rat | None (anaerobic) | 6 | 6·7±0·14* | 2·3±0·03† | 0·80±0·04* | 14·4±0·60* | 0·49 |
| Normal fed rat (control) | Albumin (2%) | 6 | 10·5±0·12 | 2·0±0·04 | 0·26±0·01 | 6·3±0·80 | 0·60 |
| Normal fed rat | Albumin (2%) + palmitate (19·5 mg./100 ml.) | 5 | 10·5±0·21 | 2·0±0·08 | 0·26±0·01 | 6·4±0·80 | 0·89 |
| Normal fed rat (control) | None | 6 | 9·8±0·39 | 2·0±0·12 | 0·23±0·08 | 7·9±1·50 | 0·67 |
| Starved (40 hr.) rat | None | 6 | 10·3±0·37 | 2·0±0·12 | 0·19±0·07 | 6·8±1·51 | 1·00 |
| Normal fed rat (control) | None | 6 | 9·5±0·56 | 2·6±0·19 | 0·32±0·04 | 10·5±1·01 | 0·67 |
| Alloxan-diabetic rat | None | 6 | 8·5±0·65 | 2·2±0·06 | 0·30±0·02 | 9·0±1·30 | 1·11 |
| Normal fed rat (control) | None | 6 | 9·0±0·22 | 1·9±0·05 | 0·44±0·03 | 8·5±0·48 | 0·67 |
| Normal fed rat | DL-β-Hydroxybutyrate (70 mg./100 ml.) | 6 | 9·1±0·22 | 2·0±0·11 | 0·48±0·02 | 5·5±0·46* | 1·22 |
| Normal fed rat (control) | None | 6 | 10·7±0·20 | 2·1±0·17 | 0·20±0·02 | 7·0±0·79 | 0·67 |
| Normal fed rat | Acetoacetate (25 mg./100 ml.) | 6 | 10·3±0·30 | 2·0±0·11 | 0·21±0·02 | 5·8±0·52 | 1·15 |
| Normal fed rat (control) | None | 9 | 10·0±0·31 | 2·1±0·09 | 0·28±0·015 | 8·6±0·89 | 0·67 |
| Normal fed rat | Pyruvate (60 mg./100 ml.) | 9 | 10·1±0·39 | 2·0±0·07 | 0·26±0·02 | 6·5±0·35‡ | 1·09 |
| Normal fed rat (control) | None | 10 | 9·6±0·50 | 2·0±0·13 | 0·28±0·04 | 7·5±1·0 | 0·67 |
| Normal fed rat | Octanoate (40 mg./100 ml.) | 10 | 8·6±0·30 | 2·2±0·15 | 0·54±0·05* | 9·1±1·1 | 1·04 |
| Normal fed rat (control) | None | 6 | 11·5±0·44 | 2·0±0·06 | 0·27±0·02 | 6·6±0·7 | 0·67 |
| Normal fed rat | Butyrate (40 mg./100 ml.) | 6 | 10·3±0·31 | 2·1±0·10 | 0·61±0·02* | 6·9±0·5 | 1·02 |

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

noate (though the difference was not statistically significant). Diabetes and starvation, and each of these substrates for respiration, increased the intracellular concentration of glucose 6-phosphate under the conditions of these experiments. These results are given in Table 7.

*Concentrations of hexose phosphates, ATP, AMP and inorganic phosphate in rat heart at different times of perfusion.* The changes in the concentrations of these metabolites in rat heart that have been described above were measured in hearts perfused for 15 min. It seemed important to define how rapidly these changes occurred. In hearts preperfused for 5 min. under aerobic conditions with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) 1 min. of perfusion under anaerobic conditions led to significant falls in the concentrations of glucose 6-phosphate, fructose 6-phosphate and ATP, and to significant increases in the concentrations of fructose 1,6-diphosphate, AMP and inorganic phosphate. In experiments with β-hydroxybutyrate (70 mg./100 ml.) a significant increase in the concentrations of glucose 6-phosphate and fructose 6-phosphate and a significant diminution in that of fructose 1,6-diphosphate were detected within 3 min. of switching to perfusion with medium containing the ketone body.

## DISCUSSION

*Concentrations of hexose phosphates and the activity of phosphofructokinase.* Newsholme & Randle (1961, 1962) pointed out that a fall in the concentrations of hexose monophosphates under conditions where the rate of flow between glucose 6-phosphate and triose phosphates is increased may be attributed specifically to an increase in the rate of the phosphofructokinase reaction. Conversely, a rise in the concentrations of the hexose monophosphates associated with a diminished rate of flow could be attributed specifically to a decrease in the rate of the phosphofructokinase reaction. Emphasis would be given to these conclusions if the concentration of fructose 1,6-diphosphate increases when those of the hexose monophosphates fall and vice versa. Changes in the rate of flow between glucose 6-phosphate and triose phosphates induced by diabetes and starvation, and by anoxia, salicylate, 2,4-dinitrophenol, fatty acids and ketone bodies, were calculated (as given by Randle, Newsholme & Garland, 1964) as the sum of the difference (from the aerobic control) of uptake of glucose and net breakdown of glycogen. These results are given in Table 8 and appear to show that the rate of flow is increased by anoxia, salicylate and 2,4-dinitrophenol, and decreased by diabetes, starvation, and by fatty acids, ketone bodies and pyruvate. Taken in conjunction with the changes in hexose phosphate concentrations that these agents have been shown to induce, these results lead us to conclude that the rate of the phosphofructokinase reaction is increased in rat heart by anoxia (in confirmation of the findings by Newsholme & Randle, 1961, 1962), salicylate and 2,4-dinitrophenol, and is diminished by starvation (in confirmation of the findings by Newsholme & Randle, 1961, 1962), and diabetes, and by fatty acids, ketone bodies and pyruvate.

*Control of phosphofructokinase in perfused rat heart.* Mansour *et al.* (1962) and Mansour (1963), in

Table 8. *Effects of alloxan-diabetes and starvation, and of anoxia, salicylate, 2,4-dinitrophenol, ketone bodies, fatty acids and pyruvate, on the rate of conversion of glucose 6-phosphate into triose phosphates and beyond in perfused rat heart*

Hearts were perfused for 15 min. with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Other details are given in the text. The change in flow is calculated as the sum of the difference (from the aerobic control) of uptake of glucose and net breakdown of glycogen, from the data of Randle *et al.* (1964).

| Source of heart | Addition to perfusion medium | Change in flow of glucose 6-phosphate to triose phosphates and beyond (mg. of glucose/g. wet wt. of heart/15 min.) (difference from aerobic control) |
|---|---|---|
| Normal fed rat (control) | None (aerobic) | (2·7–3·8) |
| Normal fed rat | None (anaerobic) | +4·5 |
| Normal fed rat | Salicylate (5 mM) | +4·2 |
| Normal fed rat | 2,4-Dinitrophenol (50 μM) | +2·5 |
| Alloxan-diabetic rat | None | −1·0 |
| Starved (40 hr.) rat | None | −0·7 |
| Normal fed rat | DL-β-Hydroxybutyrate (70 mg./100 ml.) | −2·4 |
| Normal fed rat | Acetoacetate (25 mg./100 ml.) | −2·1 |
| Normal fed rat | Pyruvate (60 mg./100 ml.) | −3·1 |
| Normal fed rat | *n*-Octanoate (40 mg./100 ml.) | −1·3 |
| Normal fed rat | Butyrate (40 mg./100 ml.) | −1·9 |
| Normal fed rat | Palmitate (19·5 mg./100 ml.) | −1·2 |

experiments with guinea-pig heart phosphofructokinase, and Passonneau & Lowry (1962), with rabbit skeletal-muscle phosphofructokinase, found that the activity of the enzyme is diminished by increasing concentrations of ATP and increased by AMP and inorganic phosphate. Similar influences of these substances on the activity of phosphofructokinase in extracts of rat heart have been described by Garland, Randle & Newsholme (1963). The present studies show that the concentration of ATP is diminished and that those of AMP and inorganic phosphate are increased in rat heart by anoxia. These findings are consistent with the suggestion by Passonneau & Lowry (1962) that anoxia may accelerate the phosphofructokinase reaction by altering the concentrations of ATP, AMP and inorganic phosphate. On the other hand, no consistent changes in the concentrations of ATP, AMP and inorganic phosphate were seen in hearts from diabetic or starved rats or in hearts perfused with medium containing fatty acids, ketone bodies or pyruvate. Evidence has been presented above that these agents diminish the rate of the phosphofructokinase reaction. It has been shown, however, that citrate can inhibit phosphofructokinase in extracts of rat heart, and that each of these agents that we believe diminish the rate of the phosphofructokinase reaction in the perfused rat heart increases the intracellular concentration of citrate (Garland *et al.* 1963; Randle *et al.* 1964; Garland & Randle, 1964). These findings suggest that diabetes and starvation, and fatty acids, ketone bodies and pyruvate, may inhibit phosphofructokinase by increasing the intracellular concentration of citrate and not by altering the intracellular concentrations of adenine nucleotides and inorganic phosphate.

*Fatty acids and ketone bodies and the control of phosphofructokinase in rat heart in alloxan-diabetes and starvation.* The present studies were undertaken to ascertain, in particular, whether perfusion of hearts from normal rats with fatty acids and ketone bodies would induce changes in hexose phosphate concentrations (interpreted as showing inhibition of phosphofructokinase) similar to those seen in hearts from starved or alloxan-diabetic rats. They were prompted by the idea that the changes seen in starvation or diabetes might be due to increased availability of fatty acids derived from muscle glycerides (and perhaps also of fatty acids and ketone bodies carried over in the tissue). The present studies have shown that the effects of fatty acids and ketone bodies on hexose phosphate concentrations are similar to those of diabetes and starvation, and that the effects of each of these factors on hexose phosphate concentrations are abolished by anoxia or salicylate or 2,4-dinitrophenol. Moreover, measurements of intracellular concentrations of fatty acids and of fatty acyl-CoA and of the rate of lipolysis in rat heart have shown that there is greater availability of fatty acids formed by the accelerated breakdown of muscle glycerides in diabetes and starvation (Garland & Randle, 1964). It has, moreover, been shown that the effects of diabetes on hexose phosphate concentrations are abolished by hypophysectomy or by treatment with insulin, and intensified by treatment with growth hormone and cortisol, and that these effects are correlated with changes in the availability of fatty acids for respiration (Randle *et al.* 1964; Garland & Randle, 1964). This evidence is compatible with the idea that the effects of diabetes and starvation on the concentrations of hexose phosphates and the activity of phosphofructokinase may be due to an increased availability of fatty acids for respiration. With the demonstration that the inhibitory effects of these agents on phosphofructokinase in perfused rat heart may be mediated by an increase in the intracellular concentration of citrate this idea is capable of more exact description. As Garland & Randle (1964) have pointed out, a rise in the intracellular concentration of citrate could result either from increases in the intracellular concentration of acetyl-CoA or oxaloacetate or from a fall in the intracellular concentration of CoA. They have shown that fatty acids (and ketone bodies) increase the concentration of acetyl-CoA, but not that of oxaloacetate, and lower that of CoA. In diabetes the concentrations of acetyl-CoA and oxaloacetate are increased and that of CoA unchanged, whereas in starvation the only change was an increase in the concentration of oxaloacetate. This leads us to suggest that in diabetes the rise in citrate concentration and inhibition of phosphofructokinase are due largely to an accelerated respiration of fatty acids to acetyl-CoA. In starvation, on the other hand, the small rise in citrate concentration is most likely to be caused by the rise in oxaloacetate concentration, which may not be directly attributable to accelerated respiration of fatty acids.

The present studies have been largely concerned with the control of phosphofructokinase in rat heart *in vitro*, but it seems likely that similar control mechanisms are exerted *in vivo*. Thus it has been shown that diabetes and starvation lead to changes in hexose phosphate concentrations *in vivo* that are similar to those seen *in vitro*, and it has, moreover, been shown that the concentration of citrate in rat heart *in vivo* is increased by diabetes (Garland & Randle, 1964).

## SUMMARY

1. The concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were determined in extracts of perfused rat heart,

and the effects of alloxan-diabetes and starvation, and of anoxia, salicylate, 2,4-dinitrophenol, fatty acids and ketone bodies, were investigated.

2. Anoxia, salicylate and 2,4-dinitrophenol decreased the concentrations of glucose 6-phosphate and fructose 6-phosphate, but increased that of fructose 1,6-diphosphate. These changes have been interpreted as indicating specifically acceleration of the phosphofructokinase reaction.

3. In hearts from alloxan-diabetic or starved rats the concentrations of the hexose monophosphates were increased, whereas that of fructose 1,6-diphosphate was decreased; similar changes in the concentrations of hexose phosphates were observed when hearts from normal rats were perfused with medium containing fatty acids or ketone bodies. These changes have been attributed specifically to inhibition of the phosphofructokinase reaction by these factors.

4. In hypophysectomized or adrenalectomized rats the induction of alloxan-diabetes or starvation did not (as in the normal rat) change the concentrations of hexose phosphates. In alloxan-diabetic hypophysectomized rats treatment with growth hormone and cortisol led to changes in hexose phosphates similar to those seen in diabetic rats.

5. The effects of alloxan-diabetes and starvation, and of fatty acids and ketone bodies, on the concentrations of hexose phosphates were not observed in hearts perfused anaerobically or (under aerobic conditions) with medium containing salicylate or 2,4-dinitrophenol.

6. Evidence is presented for similar effects of alloxan-diabetes and starvation on the concentrations of hexose phosphates in rat heart *in vivo*.

7. The concentration of ATP in rat heart was decreased by anaerobic perfusion, whereas those of AMP and inorganic phosphate were increased. Alloxan-diabetes and starvation, and fatty acids and ketone bodies, had no consistent effects on the concentrations of ATP, AMP and inorganic phosphate. It is suggested that anoxia may activate phosphofructokinase by lowering the intracellular concentration of ATP (an inhibitor of the enzyme) and increasing that of AMP and inorganic phosphate (activators of the enzyme). It is suggested that alloxan-diabetes and starvation, and fatty acids and ketone bodies, decrease the rate of the phosphofructokinase reaction by raising the intracellular concentration of citrate (an inhibitor of the enzyme).

8. The possibility that the inhibitory effects of alloxan-diabetes and starvation on the phosphofructokinase reaction are due to an increased rate of respiration of fatty acids derived from muscle glycerides is discussed.

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