Regulation of Glucose Uptake by Muscle

5. EFFECTS OF ANOXIA, INSULIN, ADRENALINE AND PROLONGED STARVING ON CONCENTRATIONS OF HEXOSE PHOSPHATES IN ISOLATED RAT DIAPHRAGM AND PERFUSED ISOLATED RAT HEART*

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Membrane transport of glucose and intracellular phosphorylation of the sugar are both accelerated by anoxia in the perfused isolated rat heart (Morgan, Randle & Regen, 1959). In isolated rat diaphragm, membrane transport of glucose is accelerated by anoxia, but the effect of anoxia on glucose phosphorylation in this tissue is not known (Randle & Smith, 1958*a*, *b*). Crane & Sols (1953) have shown that muscle hexokinase is inhibited by glucose 6-phosphate and this observation suggested that anoxia might accelerate glucose phosphorylation in muscle by lowering the intracellular concentration of glucose 6-phosphate and thereby increasing the activity of hexokinase. The results of experiments recorded here appear to show that anoxia can lower the intracellular concentration of glucose 6-phosphate in heart and diaphragm, and further measurements have been made of the concentrations of fructose 6-phosphate and fructose 1:6-diphosphate in an attempt to define the enzymic step or steps which are affected by anoxia and which bring about this change in the concentration of glucose 6-phosphate.

The possibility that membrane transport of monosaccharides in muscle might be regulated by the intracellular concentration of glucose 6-phosphate has also been investigated by studying the effects of agents which alter the intracellular concentration of glucose 6-phosphate, on the membrane transport of D-xylose and D-3-*O*-methylglucose in isolated rat-diaphragm muscle. A preliminary report of these findings has been published (Newsholme & Randle, 1961).

MATERIALS

Rats. Hearts and diaphragms were obtained from male or female albino Wistar rats of 200–300 g. and 100–150 g. respectively, starved for 18–22 hr. beforehand.

Chemicals. Glucose 6-phosphate (disodium salt), fructose 6-phosphate (barium salt; converted into disodium salt by addition of an equivalent of Na_2SO_4 in solution), fructose 1:6-diphosphate (potassium salt), glucose 1-phosphate (dipotassium salt), ATP (disodium salt), AMP (sodium salt), TPN, DPN and tris were obtained from Sigma Chemical Co. (through George T. Gurr Ltd., London, S.W. 6). D-Xylose was obtained from T. Kerfoot and Co. Ltd., and D-3-*O*-methylglucose from Ayerst, McKenna and Harrison, New York. D-[^{14}C]Sorbitol was obtained from The Radiochemical Centre, Amersham, Bucks. Adrenaline was obtained from British Drug Houses Ltd. and dissolved in 20 mM-HCl to yield a stock solution of 1 mg./ml. This was prepared freshly and diluted to 20 μg./ml. with incubation medium. Heparin (gift of Evans Medical Ltd.) was dissolved in 0·9% NaCl to a concentration of 5000 units/ml. Crystalline insulin (a gift of Boots Pure Drug Co. Ltd.) was dissolved in 3·3 mM-HCl to yield a stock solution of 20 units/ml., which was diluted to 0·1 unit/ml. with perfusion or incubation medium.

Enzymes. Glucose 6-phosphate dehydrogenase, aldolase and glyceraldehyde 3-phosphate dehydrogenase were obtained from C. F. Boehringer und Soehne GmbH., through Courtin and Warner Ltd. Phosphohexoisomerase was obtained from Sigma Chemical Co.

Media. Hearts were perfused with bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing glucose (1 mg./ml.). Diaphragms were incubated in a similar medium (except that the concentration of $CaCl_2$ was decreased by one-half). The concentration of [^{14}C]sorbitol if present was 0·1 μc/ml. The concentrations of glucose, xylose or 3-methylglucose are given in the Tables. The gas phase was O_2 or N_2 containing 5% (v/v) of CO_2.

PROCEDURE

Hearts. These were perfused for 15 min. through the coronary circulation at 37° with buffer which 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 analyses were made. The rats were injected intraperitoneally with heparin (2500 units) 20 min. or more before hearts were removed for perfusion.

Diaphragms. Intact diaphragms were incubated in 10–15 ml. of medium and hemidiaphragms in 3 ml. of medium for 20, 30, 45 or 60 min. at 37° by techniques described elsewhere (Randle & Smith, 1958*a*, *b*). In all experiments control and experimental groups of diaphragms were incubated at the same time since it is established that metabolic parameters of diaphragm can vary quantitatively from day to day (Manchester, Randle & Young, 1959). At the end of incubation the diaphragm muscle was blotted gently, weighed, and frozen in acetone–solid CO_2, if analyses were

* Part 4: Battaglia & Randle (1960).

to be made for hexose phosphates. For assay of glucose, xylose, [^{14}C]sorbitol or 3-methylglucose, muscle was extracted with boiling water (Battaglia & Randle, 1960).

METHODS

Muscle extracts. For assay of hexose phosphates, frozen heart or diaphragm muscle (one heart, or six to eight hemidiaphragms, or three to four diaphragms) were powdered in a percussion mortar chilled in solid CO_2. The frozen powder was extracted with 10% trichloroacetic acid at 0° (2 ml./g. of muscle approximately), and the supernatant solution separated by centrifuge and extracted three times with a tenfold excess of ether (to remove trichloroacetic acid). The ether was then removed by suction and *in vacuo*, and the extract brought to pH 7–8 (indicator paper, number 6883, Johnsons of Hendon Ltd.) with 10% KOH (referred to as trichloroacetic acid muscle extract).

Glucose 6-phosphate. This was assayed by measuring the change in extinction at 340 mμ in a Beckman spectrophotometer on the addition of 5 μg. of glucose 6-phosphate dehydrogenase to a mixture of 0·2 ml. of trichloroacetic acid muscle extract and 2·8 ml. of assay medium (0·06 M-tris; 3 mM-EDTA; 0·1 mM-TPN; pH 8·0). Under these conditions the activity of hexokinase present as an impurity in some preparations of glucose 6-phosphate dehydrogenase was negligible (cf. Narahara & Ozand, 1960). The glucose 6-phosphate dehydrogenase preparations used were shown to be free of phosphohexoisomerase and phosphoglucomutase.

Fructose 6-phosphate. This was determined by difference, the glucose 6-phosphate content of trichloroacetic acid muscle extract being assayed (as above) in the presence or the absence of 50 μg. of phosphohexoisomerase.

Fructose 1:6-diphosphate. This was assayed by measuring the change in extinction at 340 mμ upon the addition of 200 μg. of glyceraldehyde 3-phosphate dehydrogenase to a mixture of 0·25 or 0·5 ml. of trichloroacetic acid muscle extract and 2·75 or 2·5 ml. of assay medium (0·025 M-pyrophosphate; 3·3 mM-cysteine; 7·5 mM-sodium arsenate; 0·1 mM-DPN; pH 8·5) and 200 μg. of aldolase.

Glucose, xylose and [^{14}C]sorbitol. These were assayed in muscle extracts and incubation media by methods described by Battaglia & Randle (1960).

3-Methylglucose. This was assayed photometrically in muscle extracts and incubation media by Somogyi's modification of Nelson's method (Somogyi, 1945). The values for muscle extracts were corrected for blank reduction by assays performed on extracts from diaphragms incubated in the absence of 3-methylglucose.

Total water content. This was determined by weighing muscle before and after freeze-drying.

Calculations and expression of results

Hexose phosphate concentrations in trichloroacetic acid muscle extracts were calculated by reference to standard curves constructed with glucose 6-phosphate and fructose 1:6-diphosphate. The recovery of glucose 6-phosphate or fructose 1:6-diphosphate added to trichloroacetic acid muscle extracts was 85–100% in the assay and no corrections have been applied to individual values for possible recoveries of less than 100%. For calculating the hexose phosphate contents of muscle samples the volume of extract was taken to be the sum of the volume of trichloroacetic acid and the water content of the muscle sample (determined by weighing a sample of muscle powder before and after freeze-drying).

Hexose phosphate concentrations have been calculated as μmoles/100 g. of wet muscle. Incubation medium adherent to diaphragm was removed by blotting before freezing and weighing the muscle, and the wet weight used in calculating hexose phosphate concentrations was the observed weight. Hearts, on the other hand, were frozen without opening the chambers and removing retained incubation medium, and the water content was therefore found to be abnormally high. Further, anaerobic perfusion led to the retention of additional water in the extracellular compartment (cf. Morgan *et al.* 1959). The observed wet weights were therefore corrected to a water content of 79 ml./100 g. (the water content of cardiac muscle after aerobic perfusion; Morgan *et al.* 1959). The hexose phosphate concentrations in μmoles/100 g. may be converted in each case into apparent intracellular concentrations in μmoles/100 ml. of intracellular water by dividing by the intracellular volume in ml./g. (0·44 for heart, 0·60 for diaphragm; Morgan *et al.* 1959; Randle & Smith, 1958*b*). In order to calculate the true intracellular concentration knowledge of the distribution of hexose phosphates in intracellular water is required; this is not known. In order to avoid any implication that the measured concentration represents the true intracellular concentration we have preferred to express our results in terms of μmoles/100 g. rather than as intracellular concentrations. In interpreting these results the assumption is made that changes in the measured concentration will reflect similar changes in the true intracellular concentration.

RESULTS

Effects of starvation, anoxia and insulin on hexose phosphate concentrations in heart and diaphragm

The concentrations of hexose phosphates, in hearts perfused for 15 min. or diaphragms incubated for 45 min. under aerobic conditions, were dependent upon the period for which rats had been starved beforehand (Table 1). When hearts from rats starved for 18 hr. were perfused with medium containing insulin, the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate (in μmoles/100 g.) were 22, 4·4 and 3·9 respectively. With hearts from rats starved for 30 hr. the concentrations of glucose 6- and fructose 6-phosphate were significantly increased (by 100 and 43% respectively), whereas that of fructose 1:6-diphosphate was diminished by 54%. In the absence of insulin the concentration of glucose 6-phosphate was increased 50% when the period of starvation was prolonged from 18 to 30 hr. Likewise, in intact diaphragms incubated under aerobic conditions in the presence of insulin, the concentration of glucose 6-phosphate was increased from 28 to 39 when the period of starvation was prolonged from 18 to 30 hr. In studying effects of insulin or anoxia on hexose phosphate concentrations the period of starvation was carefully controlled and was 18–22 hr.

Table 1. *Effect of insulin* (0·1 *unit/ml.*) *and anoxia on the glucose* 6*-phosphate, fructose* 6*-phosphate and fructose* 1:6*-diphosphate contents of rat-heart muscle, perfused* in vitro

Hearts were perfused for 15 min. with medium containing glucose (1 mg./ml.). Rats were starved for 18 hr. unless otherwise indicated. The number of hearts is given in parentheses.

Experiment	Insulin	Hexose phosphate content (μmoles/100 g. of wet muscle) (Mean±S.E.M.) Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1:6-diphosphate
Aerobic	–	24±1·3 (10)	4·6±0·26	3·2±0·35
Anaerobic	–	19±0·9 (10)	3·6±0·34	5·2±0·45
Difference		−5±1·6†	−1·0±0·43‡	2·0±0·57†
Aerobic	+	21±1·3 (19)	3·9±0·18	4·0±0·15
Anaerobic	+	14±0·5 (19)	2·7±0·15	5·0±0·21
Difference		−7±1·4*	−1·2±0·24*	1·0±0·26*
Aerobic	–	22±1·3 (10)	4·7±0·38	2·0±0·08
Aerobic	+	24±0·7 (10)	5·5±0·38	3·7±0·08
Difference		2±1·5	0·8±0·54	1·7±0·11*
Aerobic (starved for 18 hr.)	+	22±0·90 (29)	4·4±0·30	3·9±0·10
Aerobic (starved for 30 hr.)	+	45±1·7 (10)	6·3±2·0	1·8±0·02
Difference		23±0·8*	1·9±0·5*	−2·1±0·07*

* $P < 0·001$. † $P < 0·01 > 0·001$. ‡ $P < 0·05 > 0·01$. Other differences $P > 0·05$.

The effects of anoxia and insulin on hexose phosphate concentrations in the heart after 15 min. of perfusion are shown in Table 1. In hearts perfused aerobically without insulin the mean concentrations of hexose phosphates (in μmoles/100 g.) were glucose 6-phosphate 23, fructose 6-phosphate 4·6 and fructose 1:6-diphosphate 2·6. These correspond to intracellular concentrations of 0·51 mM-glucose 6-phosphate, 0·1 mM-fructose 6-phosphate and 0·056 mM-fructose 1:6-diphosphate (assuming uniform distribution throughout intracellular water; for volume of intracellular water see Morgan *et al.* 1959). Anoxia in the absence of insulin decreased the concentrations of glucose and fructose 6-phosphates by 21 and 22% respectively and increased that of fructose 1:6-diphosphate by 63%. Anoxia in the presence of insulin decreased the concentration of glucose 6- and fructose 6-phosphate by 33 and 30% respectively and increased that of fructose 1:6-diphosphate by 20%. Insulin (under aerobic conditions) had no significant effect on the concentrations of glucose 6- and fructose 6-phosphate but the concentration of fructose 1:6-diphosphate was increased 85% by the hormone.

The effects of anoxia on hexose phosphate concentrations in the heart (in the presence of insulin) after 5, 10 and 15 min. are compared in Fig. 1. Although the absolute concentrations of hexose phosphates varied with the period of perfusion (especially during the first 5 min.) the effects of anoxia at 5, 10 and 15 min. were essentially similar.

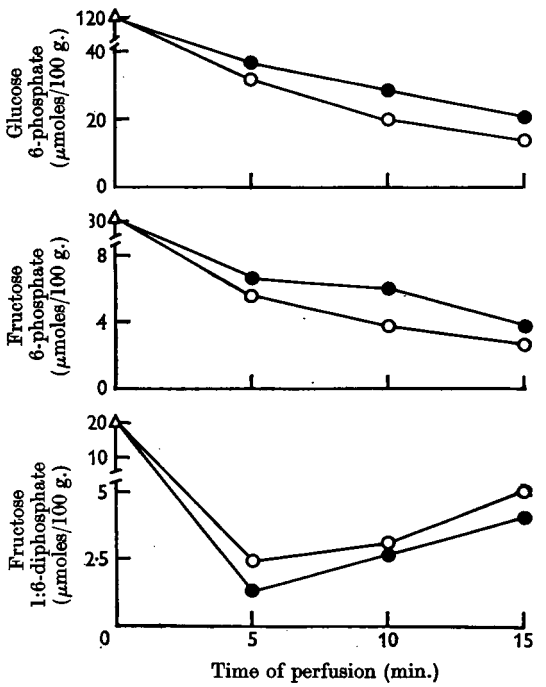

Fig. 1. Effect of anoxia on contents of hexose phosphates in hearts perfused for 5, 10 and 15 min. Hearts were perfused with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Zero-time values were obtained from hearts not subjected to perfusion. ●, Aerobic perfusion; ○, anaerobic perfusion.

The effects of anoxia, insulin and adrenaline on hexose phosphate concentrations in intact diaphragms and hemidiaphragms are shown in Table 2. In intact diaphragms incubated aerobically (but without insulin) for 20 min., the concentrations of hexose phosphates (in μmoles/100 g.) were glucose 6-phosphate 29, fructose 6-phosphate 6·7 and fructose 1:6-diphosphate 4·0. These correspond to apparent intracellular concentrations of 0·48 mM-glucose 6-phosphate, 0·11 mM-fructose 6-phosphate and 0·06 mM-fructose 1:6-diphosphate (intracellular water of 60 ml./100 g., see Randle & Smith, 1958*b*). These concentrations are very similar to those obtained in the heart (see above). Anoxia (in the absence of insulin) increased the concentrations of glucose 6-phosphate, fructose 6-phosphate, and fructose 1:6-diphosphate by 37, 16 and 33 %, whereas in the presence of the hormone anoxia increased these concentrations by 50, 86 and 65 % respectively. Insulin (under aerobic conditions) increased the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate by 50, 50 and 42 % respectively in intact diaphragms incubated for 45 min.

When hemidiaphragms were incubated aerobically, but without insulin, the mean concentrations of hexose phosphates (in μmoles/100 g.) were, glucose 6-phosphate 30, fructose 6-phosphate 7·7 and fructose 1:6-diphosphate 2·0. Anoxia in the absence of insulin was without significant effect on the concentrations of glucose 6- and fructose 6-phosphate, whereas it increased the concentration of fructose 1:6-diphosphate substantially (by 81 %). Anoxia in the presence of insulin decreased the concentrations of glucose 6- and fructose 6-phosphate by 27 and 30 % respectively and increased that of fructose 1:6-diphosphate by 33 %. Insulin under aerobic conditions increased the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate by 26, 22 and 60 % respectively.

When hemidiaphragms were incubated aerobically in the presence of insulin, adrenaline (Table 2) increased the concentrations of glucose 6- and

Table 2. *Effects of insulin* (0·1 *unit/ml.*), *adrenaline* (20 μ*g./ml.*) *and anoxia on the glucose* 6*-phosphate, fructose* 6*-phosphate and fructose* 1:6*-diphosphate contents of rat-diaphragm muscle incubated* in vitro

Intact diaphragms were incubated in medium containing glucose (2·5 mg./ml.) for 20 min. except in Expt. 3 (45 min.). Hemidiaphragms were incubated for 20 min. in medium containing glucose (5 mg./ml.). The number of muscle samples analysed is given in parentheses (see Methods).

Diaphragm preparation	Expt.	Insulin	Hexose phosphate content (μmoles/100 g. of wet muscle) (Mean ± S.E.M.) Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1:6-diphosphate
Intact	1. Aerobic	−	29 ± 1·9 (7)	6·7 ± 0·6	4·0 ± 0·24
	Anaerobic	−	40 ± 1·2 (7)	7·8 ± 0·6	5·4 ± 0·50
	Difference		11 ± 2·3*	1·1 ± 0·86	1·4 ± 0·55‡
	2. Aerobic	+	28 ± 2·4 (4)	6·2 ± 0·51	2·1 ± 0·08
	Anaerobic	+	43 ± 2·7 (4)	12·2 ± 0·85	3·4 ± 0·45
	Difference		15 ± 3·7†	6·0 ± 0·97†	1·3 ± 0·46‡
	3. Aerobic	−	17 ± 1·2 (11)	4·5 ± 0·5	1·5 ± 0·07
	Aerobic	+	26 ± 0·07 (11)	6·7 ± 0·45	2·1 ± 0·10
	Difference		9 ± 1·5*	2·2 ± 0·68†	0·6 ± 0·13*
Hemidiaphragm	4. Aerobic	−	28 ± 0·73 (6)	4·4 ± 0·55	2·6 ± 0·16
	Anaerobic	−	29 ± 0·73 (6)	6·0 ± 0·55	4·6 ± 0·09
	Difference		1 ± 0·98	1·6 ± 0·77	2·2 ± 0·16*
	5. Aerobic	+	49 ± 1·3 (8)	12·4 ± 0·41	2·9 ± 0·10
	Anaerobic	+	35 ± 0·49 (8)	8·5 ± 0·32	3·9 ± 0·24
	Difference		−14 ± 1·5*	−3·9 ± 0·52*	1·0 ± 0·26†
	6. Aerobic	−	33 ± 1·1 (4)	11·0 ± 1·1	1·5 ± 0·09
	Aerobic	+	41 ± 0·9 (4)	13·4 ± 1·7	2·3 ± 0·12
	Difference		8 ± 1·4†	2·4 ± 2·1	0·8 ± 0·15*
	7. Aerobic control	+	50 ± 1·3 (6)	11·9 ± 0·73	2·9 ± 0·13
	Adrenaline	+	68 ± 2·7 (6)	18·2 ± 1·1	2·6 ± 0·22
	Difference		18 ± 3·0*	6·3 ± 1·46†	−0·3 ± 0·35

* $P < 0·001$. † $P < 0·01 > 0·001$. ‡ $P < 0·05 > 0·01$. Other differences $P > 0·05$.

fructose 6-phosphate by 37 and 52% but did not increase that of fructose 1:6-diphosphate.

Effects of intracellular glucose 6-phosphate concentration and of nucleoside phosphates and hexose phosphates added in vitro *on the transport of* D-*xylose or* D-3-O-*methylglucose in the intact diaphragm*

When glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, fructose 1:6-diphosphate, ATP or AMP were added *in vitro* to the incubation medium, the intracellular accumulation of xylose by diaphragm muscle was slightly increased in each instance, though many of these changes were not statistically significant. Similar effects of glucose 6-phosphate and of ATP were still evident when xylose transport was accelerated by anoxia or by fluoride respectively (Table 3) (fluoride being added to retard hydrolysis of ATP in the medium by enzymes derived from the tissue preparation).

The effects of adrenaline, fluoride and anoxia on the glucose 6-phosphate concentration and the intracellular accumulations of xylose or 3-methylglucose in intact diaphragm are given in Table 4. Adrenaline increased the glucose 6-phosphate content markedly, but had no effect on the intracellular accumulation of xylose in either the presence or the absence of insulin; on the other hand, the intracellular accumulation of 3-methylglucose was moderately increased by adrenaline. Fluoride increased glucose 6-phosphate concentration and intracellular accumulation of xylose markedly, whereas anoxia produced a slight increase in glucose 6-phosphate concentration and a moderate increase in the intracellular accumulation of xylose by diaphragm.

Table 3. *Effect of hexose phosphates and nucleoside phosphates on xylose transport in rat diaphragm* in vitro

Intact diaphragms were incubated for 30 min. in medium containing D-xylose (8·6 mM); the concentration of hexose phosphates or nucleoside phosphates was 10–15 mM. There were four observations in each group.

Addition to medium	Intracellular xylose space (ml./100 g. of wet muscle) (Mean ± S.E.M.)	Difference ± S.E. of difference
None	5±1·4	
Glucose 6-phosphate	6±0·9	1±1·7
None (anaerobic)	18±1·7	
Glucose 6-phosphate	21±1·8	3±2·4
None	14±2·2	
Glucose 1-phosphate	17±0·9	3±2·4
None	8±1	
ATP	13±0·6	5±1·2*
Fluoride (10 mM)	21±1·4	
Fluoride + ATP	26±1·6	5±2·1
None	8±2·6	
Fructose 6-phosphate	14±2	6±3·3
None	11±1·4	
Fructose 1:6-diphosphate	18±1·7	7±2·2†
None	10±2·0	
AMP	15±2·7	5±2·9

* $P < 0{\cdot}01 > 0{\cdot}001$.
† $P < 0{\cdot}02 > 0{\cdot}01$. Other differences $P > 0{\cdot}05$.

DISCUSSION

The ratio of intracellular glucose 6-phosphate to fructose 6-phosphate, was not affected by anoxia, insulin or adrenaline in either heart or diaphragm (although the ratios, which averaged 4·5:1, were consistently greater than the ratio of 2·33:1 obtained at equilibrium with phosphohexoisomerase). Because the ratio was not affected by these agents, it seems unlikely either that the phosphohexoisomerase step is rate-limiting for glycolysis under any of the experimental conditions employed or that effects of anoxia or insulin on the rate of glycolysis are to be attributed to changes in the rate of the phosphohexoisomerase reaction.

In the perfused rat heart, anoxia diminished the concentrations of glucose and fructose 6-phosphates, but increased the concentration of fructose 1:6-diphosphate (in either the presence or the absence of insulin). In hemidiaphragms, anoxia diminished concentrations of glucose 6- and fructose 6-phosphate and increased that of fructose 1:6-diphosphate, when insulin was present; in the absence of insulin, anoxia raised the concentration of fructose 1:6-diphosphate without affecting those of glucose 6- and fructose 6-phosphate. In these tissues both the rate of formation of glucose 6- and fructose 6-phosphate and the rate of flow through the sequence glucose 6-phosphate → fructose 6-phosphate → fructose 1:6-diphosphate → lactate are accelerated by anoxia in either the presence or the absence of insulin. [In the absence of insulin anoxia accelerates glucose transport and thereby glucose phosphorylation in diaphragm and heart (Randle & Smith, 1958*a*; Morgan *et al.* 1959) and glycogen breakdown (unpublished observations); in the presence of insulin, transport is not affected by anoxia, which nevertheless accelerates glucose phosphorylation in the heart, but not in the diaphragm (Morgan *et al.* 1959); anoxia accelerates glycogen breakdown in both heart and diaphragm (unpublished observations). Lactate formation is accelerated by anoxia in both tissues.] Thus the overall rate of conversion of fructose 6-phosphate into the diphosphate was accelerated to a much greater extent by anoxia than either the rate of formation of glucose 6- and fructose 6-phosphate or the conversion of the diphosphate into lactate. We conclude therefore that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate is specifically increased by anoxia and that this is an

Table 4. *Effect of adrenaline* (20 μg./ml.), *fluoride* (10 mM) *and anoxia on the glucose 6-phosphate content and transport of* D-*xylose or* D-3-O-*methylglucose in isolated rat diaphragm* in vitro

Intact diaphragms were incubated for 60 min. (Expts. 1–4) or 20 min. (Expt. 5). The concentrations were: glucose, 2·5 mg./ml.; xylose, 5 mg./ml. (Expts. 1, 3 and 4) or 3·75 mg./ml. (Expt. 5); 3-methylglucose, 5 mg./ml.; insulin, 0·1 unit/ml. The number of observations is given in parentheses. In Expts. 1, 3, 4 and 5, glucose 6-phosphate was assayed in single extracts prepared from all diaphragms incubated.

Expt.	Insulin	Glucose	Non-utilizable sugar	Glucose 6-phosphate concn. (μmoles/100 g.)	Intracellular xylose or 3 methylglucose space (ml./100 g.) (Mean ± S.E.M.)	(Mean difference ± S.E. of difference)
1. Control	–	–	Xylose	35	7±1·0 (10)	
Adrenaline	–	–		176	7±0·75 (10)	0±1·3
2. Control	–	–	3-Methylglucose	—	11±1·7 (6)	
Adrenaline	–	–		—	19±1·4 (6)	8±2·2†
3. Control	+	–	Xylose	35	19±1·0 (4)	
Adrenaline	+	–		176	24±2·8 (4)	5±3·0
4. Control	–	–	Xylose	35	2±0·8 (6)	
Fluoride	–	–		94	12±0·7 (6)	10±1·1*
5. Aerobic	–	+	Xylose	24	7±2·0 (6)	
Anaerobic	–	+		33	14±1·3 (6)	7±2·4‡

* $P < 0·001$. † $P < 0·01 > 0·001$. ‡ $P < 0·02 > 0·01$. Other differences $P > 0·05$.

important rate-limiting step for glycolysis under aerobic conditions. This effect of anoxia could result either from an increase in the rate of the phosphofructokinase reaction or from a diminution in the rate of the fructose 1:6-diphosphatase reaction. These two possibilities cannot be distinguished from the results obtained in the present study.

In intact diaphragm, on the other hand, anoxia increased the concentrations of all three hexose phosphates in either the presence or the absence of insulin. In intact diaphragm therefore anoxia accelerated glucose 6- and fructose 6-phosphate formation to a much greater extent than the overall conversion of fructose 6-phosphate into the diphosphate (though the latter was clearly accelerated to a greater extent than was the conversion of the diphosphate into lactate). The increase in diphosphate concentration observed could in this instance have been brought about by the rise in fructose 6-phosphate concentration. Thus although anoxia does accelerate the overall rate of formation of the diphosphate in intact diaphragm, it is not possible to conclude from the hexose phosphate concentrations in this tissue that the overall phosphorylation of fructose 6-phosphate was rate-limiting for glycolysis under aerobic conditions, or that anoxia accelerated glycolysis in this preparation by a specific effect on this step.

In the perfused rat heart, insulin increased the concentration of fructose 1:6-diphosphate without affecting that of glucose 6- or fructose 6-phosphate. Under similar conditions the hormone has been found to accelerate the formation of glucose 6-phosphate (by accelerating transport of glucose) and to increase the rate of flow through the sequence glucose 6-phosphate → fructose 6-phosphate → fructose 1:6-diphosphate → lactate (shown by increased lactate production; unpublished observations). This would suggest that insulin may also increase the overall rate of formation of fructose 1:6-diphosphate from fructose 6-phosphate in a specific way. Since, however, glucagon can accelerate the formation of lactate from glycogen and the phosphorylation of glucose in the perfused heart (Cornblath, Morgan & Randle, 1961; M. J. Henderson, H. E. Morgan & P. J. Randle, unpublished work), and since glucagon is present as a contaminant in many insulin preparations there is the possibility that these effects of insulin may have been due to glucagon present as a contaminant in the preparations of insulin employed rather than to insulin itself.

In either hemidiaphragms or intact diaphragms, insulin increased the concentrations of all three hexose phosphates. The hormone also increased the rate of formation of glucose 6-phosphate (by accelerating glucose transport) and the rate of lactate production from glucose in diaphragm muscle under similar conditions (unpublished observations). Thus although the hormone did accelerate the overall conversion of fructose 6-phosphate into the diphosphate, this effect could have been brought about by an increase in the fructose 6-phosphate concentration rather than by a specific effect on the phosphofructokinase or fructose 1:6-diphosphatase reactions. On the other hand adrenaline, which (like insulin) increased the concentrations of glucose 6- and fructose 6-phosphate in hemidiaphragms, did not (unlike insulin) increase the diphosphate concentration.

In hearts from rats in which the period of starvation before perfusion of the heart had been prolonged to 30 hr., glucose 6- and fructose 6-phosphate concentrations were markedly increased, whereas that of fructose 1:6-diphosphate was markedly lowered. Thus it would appear that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate may be reduced in a specific way by prolonged starving. A decreased concentration of circulating insulin as a consequence of starvation could be one important factor in this respect.

As Crane & Sols (1953) have shown, the activity of muscle hexokinase is inhibited non-competitively by glucose 6-phosphate, the K_i for heart muscle hexokinase being of the order of 0·1 mM (Sols & Crane, 1954; Crane & Sols, 1955). The intracellular concentration of glucose 6-phosphate in rat hearts perfused under aerobic conditions was 0·51 mM in the present investigation (assuming that glucose 6-phosphate is uniformly distributed throughout intracellular water and that none is combined with intracellular protein). In rat heart therefore hexokinase is likely to be subject to inhibition by glucose 6-phosphate and the accelerating effect of anoxia on phosphorylation of glucose by rat heart noted by Morgan *et al.* (1959) could result from the fall in intracellular glucose 6-phosphate concentration that this agent has been shown to induce.

Other points of evidence that support the view that the intracellular glucose 6-phosphate concentration may regulate the activity of muscle hexokinase and thereby glucose phosphorylation may be summarized as follows. In the rat, prolonged starvation increased the concentration of glucose 6-phosphate in heart and diaphragm, substantially; Kipnis (1959) has already shown that phosphorylation of glucose in diaphragm muscle is impaired by starvation, and in rat heart also glucose phosphorylation appears to be moderately depressed after 30 hr. of starvation (unpublished observations). Adrenaline, in the presence of insulin, increased the intracellular concentration of glucose 6-phosphate markedly in accordance with long-standing observations concerning effects of adrenaline on the glucose 6-phosphate content of muscle (Cori & Cori, 1931–32; Hegnauer & Cori, 1934; Sutherland, 1952); in parallel experiments in which the effect of adrenaline on glucose space in the intact diaphragm was investigated, adrenaline caused intracellular accumulation of glucose (glucose space in the absence of adrenaline $-4 \pm 0{\cdot}7$ ml./100 g.; in the presence of adrenaline $7 \pm 1{\cdot}9$). It is deduced from this change that adrenaline action reduces the rate of glucose phosphorylation in diaphragm muscle in the presence of insulin (for discussion of significance of intracellular glucose accumulation see Park *et al.* 1956). Substantial accumulation of glucose in muscle cells *in vivo* follows the administration of adrenaline to rats (Kipnis, Helmreich & Cori, 1958).

Changes in the intracellular concentration of glucose 6-phosphate in heart and diaphragm can therefore be correlated with changes in the rate of glucose phosphorylation. It seems reasonable to conclude that the intracellular concentration of glucose 6-phosphate is important in the regulation of glucose phosphorylation by hexokinase in muscle, though it should be emphasized that this may not be the only factor involved. Lynen, Hartmann, Netter & Schuegraf (1959) found that anoxia, cyanide and 2:4-dinitrophenol, which increase the rate of glucose phosphorylation in yeast, reduce the intracellular concentration of glucose and fructose 6-phosphate and increase that of fructose 1:6-diphosphate in yeast. Yeast hexokinase (unlike muscle hexokinase) is not inhibited by glucose 6-phosphate and Lynen *et al.* have proposed that in yeast, intracellular phosphorylation both of glucose and of fructose 6-phosphate may be regulated by the availability of ATP to hexokinase and phosphofructokinase. The possibility that such a mechanism may also contribute to effects of anoxia on glucose phosphorylation in muscle has neither been adduced nor refuted by the present investigation.

Although intracellular glucose 6-phosphate appears to be an important regulator of glucose phosphorylation in muscle, it seems unlikely to be an important factor in the regulation of transport of monosaccharides. Glucose 6-phosphate added to the incubation medium *in vitro* accelerated xylose transport in diaphragm to a small extent but this effect was also seen with a number of other hexose phosphates and nucleoside phosphates and had previously been found with inorganic phosphate (Randle & Smith, 1958*b*). The mechanism of this effect is not known. In some instances increases in intracellular glucose 6-phosphate (e.g. in diaphragm with anoxia or fluoride) were associated with accelerated xylose transport, but adrenaline (which produced the greatest increase in intracellular glucose 6-phosphate) had no detectable effect on xylose transport. The more rapidly transported sugar 3-methylglucose did penetrate diaphragm more rapidly in the presence of adrenaline. Moreover, anoxia, which accelerates monosaccharide transport in rat heart, causes a fall in intracellular glucose 6-phosphate in this tissue. Thus it seems likely that accelerating effects of anoxia or fluoride on monosaccharide transport in muscle are related to changes other than the intracellular concentrations of glucose 6-phosphate, e.g. to the availability of high-energy phosphate compounds to the membrane transport system for monosaccharides (Randle & Smith, 1958*a*, *b*).

The results of this and earlier studies appear to show that at least two factors are responsible for the

Pasteur effect in muscle (inhibition of glycolysis by oxygen). One factor is a diminished rate of glucose transport under aerobic conditions, and this is particularly important in the absence of insulin where glycolysis is limited by glucose-transport rate. The other factor is the rate of phosphorylation of glucose by hexokinase which is inhibited under aerobic conditions; this is particularly important in heart muscle where in the presence of insulin glycolysis is limited by the rate of phosphorylation of glucose. The results of the present study suggest that the regulation of glucose phosphorylation is indirect and that it depends upon the intracellular concentration of glucose 6-phosphate, which is regulated by the rate of conversion of fructose 6-phosphate into fructose 1:6-diphosphate.

SUMMARY

1. The concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate have been assayed in extracts of perfused rat heart and isolated rat diaphragm by enzymic methods, and effects of anoxia, insulin and adrenaline on these parameters investigated.

2. In rat hearts perfused aerobically concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1:6-diphosphate (in μmoles/100 g.) were 23, 4·6 and 2·6. The corresponding concentrations in intact diaphragms were 29, 6·7 and 4·0 and in hemidiaphragms 30, 7·7 and 2·0.

3. In rat heart these concentrations were changed (as per cent of control) as follows: anoxia -21, -22 and $+63$; insulin $+9$, $+17$ and $+85$; anoxia in the presence of insulin -33, -30 and $+20$. In intact diaphragms these concentrations were changed by anoxia $+37$, $+16$ and $+33$; by insulin $+50$, $+50$ and $+42$; by anoxia in the presence of insulin $+50$, $+86$ and $+65$. In hemidiaphragms these were changed by anoxia $+4$, $+36$ and $+81$; by insulin $+26$, $+22$ and $+60$; by anoxia in the presence of insulin -27, -30 and $+33$; adrenaline in the presence of insulin $+37$, $+52$ and -12.

4. It is concluded that in heart and diaphragm the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate may limit glycolysis under aerobic conditions and that anoxia accelerates glycolysis by increasing the rate of this step in particular.

5. Evidence is presented that phosphorylation of glucose by hexokinase may be regulated in muscle by the intracellular concentration of glucose 6-phosphate (a known inhibitor of muscle hexokinase). On the other hand, monosaccharide transport in muscle does not appear to be regulated by the intracellular concentration of glucose 6-phosphate.

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