Regulation of Glucose Uptake by Muscle

1. THE EFFECTS OF INSULIN, ANAEROBIOSIS AND CELL POISONS ON THE UPTAKE OF GLUCOSE AND RELEASE OF POTASSIUM BY ISOLATED RAT DIAPHRAGM

BY P. J. RANDLE AND G. HOWARD SMITH

Department of Biochemistry, University of Cambridge

(Received 28 March 1958)

Gemmill & Hamman (1941) showed that the uptake of glucose by isolated rat diaphragm is enhanced by the presence of insulin *in vitro*. The factors which regulate the uptake of glucose by this tissue and the mechanism of this action of insulin are largely unknown, though it is thought that insulin may increase glucose uptake by accelerating the entry of glucose into the muscle cell (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Park & Johnson, 1955). It is known that the uptake of glucose by many cells and tissues is greater under conditions where oxidative phosphorylation is impaired, e.g. under anaerobic conditions. It was of interest therefore to investigate the effects of anaerobiosis and of substances which inhibit oxidative phosphorylation on the uptake of glucose by diaphragm and to compare their effects with those of insulin.

In preliminary communications (Randle, 1956; Randle & Smith, 1957) we have noted that the uptake of glucose by isolated diaphragm in a medium buffered with bicarbonate is increased by oxygen lack and by 2:4-dinitrophenol, sodium arsenite or sodium cyanide. On the other hand, when diaphragm is incubated in a medium buffered with phosphate uptake of glucose is apparently not increased by oxygen lack (Walaas & Walaas, 1952; Demis & Rothstein, 1954) or by 2:4-dinitrophenol (Villev, Deane & Hastings, 1949; Pierce & Field, 1949). Consequently, it was important to define more clearly the conditions under which oxygen lack and substances which inhibit oxidative

phosphorylation will stimulate uptake of glucose by diaphragm.

Calkins, Taylor & Hastings (1954) and Creese (1954) have shown that potassium is lost from the muscle when isolated rat diaphragm is incubated in a bicarbonate medium under anaerobic conditions. Because of the possibility that loss of potassium from isolated diaphragm might be in some way associated with glucose uptake, we have compared the uptake of glucose with the loss of potassium under anaerobic conditions, in the presence of insulin and in the presence of substances which inhibit oxidative phosphorylation.

METHODS AND PROCEDURE

Incubation media. The composition of the basal media used is given in Table 1. A.R.-grade chemicals were used if commercially available.

Insulin. Crystalline insulin (Boots Pure Drug Co. Ltd. or Novo Terapeutisk Laboratories) was dissolved in N/300-HCl at a concentration of 20 units/ml. This stock solution was diluted with medium to the required concentration (usually 0.1 unit/ml.) just before each experiment.

Animals. Diaphragm muscle was obtained from male albino Wistar rats of 100–150 g. wt., fed with a stock laboratory diet (Bruce & Parkes, 1949). Food was withheld from intact rats for 18–20 hr. before the experiment; hypophysectomized or adrenalectomized rats, which were used 8–10 days after operation, were not starved. The animals had free access to water (or 1% NaCl for adrenalectomized rats) at all times.

Procedure. Rats were killed by decapitation and exsanguinated. Diaphragms were then rapidly removed, and

Table 1. *Composition of basal media used for incubation of isolated diaphragm*

Media containing bicarbonate were gassed with O₂ + CO₂ or N₂ + CO₂ (95:5); pH 7.4 at 37°. Tris medium and phosphate medium were gassed with O₂ or N₂; pH 7.4 at 37°. D-Glucose was present in all media (2.5 mg./ml.); this is included in the calculated osmolarity (the units m-osmoles have been suggested and defined by Gamble, 1950).

Medium	Composition (m-equiv./l.)									Calc. osmolarity (m-osmoles/l.)
	Na	K	Ca	Mg	Tris*	Cl	HCO ₃	PO ₄ †	SO ₄	
Bicarbonate‡	124.8	5.2	2.7	2.6	—	105.7	27.2	1.8	0.6	281
High phosphate-bicarbonate	133.0	5.2	2.7	2.6	—	77.9	27.2	37.8	0.6	281
Tris*	108.8	5.2	2.7	2.6	16.0	132.9	—	1.8	0.6	281
Phosphate	143.2	5.1	2.7	2.6	—	132.8	—	18.2	0.6	309

* 2-Amino-2-hydroxymethylpropane-1:3-diol.

† Assuming valency of 1.8 at pH 7.4.

‡ Gey & Gey (1936).

spread on filter paper (Whatman no. 40), and the posterior segment was removed and the remainder divided along the central tendon to yield two hemidiaphragms. These were then soaked for 5 min. at room temperature in the appropriate basal medium (previously saturated with the appropriate gas or gas mixture). They were then lightly blotted and transferred to flasks for incubation. In most experiments each hemidiaphragm was incubated in 1 ml. of medium in a 20 ml. conical flask, though in certain experiments three to eight hemidiaphragms were incubated together in 3–12 ml. of medium in a 50 ml. conical flask. The flasks were then gassed with the appropriate gas or gas mixture, and sealed with rubber bungs and incubated with shaking in a water bath at 37°. After incubation, which was usually for 1 hr., the flasks were cooled, and samples of medium taken for analysis, and the hemidiaphragms were removed, blotted with filter paper and weighed on a torsion balance. In experiments where the course of glucose uptake or potassium release was followed five to eight hemidiaphragms were incubated together in 5–12 ml. of medium in a 50 ml. conical flask fitted with a side arm and stopcock. The gas phase was then renewed or altered when samples of medium were taken during incubation.

Analytical methods

pH. This was determined with the glass electrode.

Glucose uptake. This was determined by measuring the disappearance of glucose from the medium. The glucose content of the medium was determined before and after each incubation by one or more of three methods: (i) colorimetrically by the Somogyi (1945) modification of Nelson's method, (ii) manometrically with glucose oxidase (Sigma Chemical Co., obtained from George T. Gurr Ltd.) after the method of Keilin & Hartree (1948) and (iii) colorimetrically with glucose oxidase (Sigma Chemical Co.), peroxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and *o*-dianisidine after the method of Huggett & Nixon (1957).

In some experiments the disappearance of glucose from the medium was followed with [^{14}C]glucose. [$^{14}\text{C}_6$]Glucose (15 mc/m-mole; the Radiochemical Centre, Amersham, Bucks) was added to the medium at 0.6 $\mu\text{C}/\text{ml}$. After incubation, samples of medium were desalted by addition of ethanol to 70% (v/v) and the resulting supernatant was dried *in vacuo*. The residues were taken up in a 1% (w/v) solution of unlabelled glucose and samples (5 μl .) were chromatographed on Whatman no. 1 paper with the pyridine-ethyl acetate-water (1:2:2) system of Jermyn & Isherwood (1949). The spots were located by markers, and cut out, and the glucose was eluted on to aluminium planchets, and dried under an infrared lamp and counted beneath a thin end-window Geiger-Müller tube. Individual spots gave from 700 to 1200 counts/min.; at least 1000 counts were recorded for each sample.

Potassium loss. Loss of potassium from the diaphragm was calculated from the initial and final potassium contents of the incubation medium. Potassium was estimated with an EEL flame photometer, 1:50 dilutions of medium being compared with standard solutions containing 0.1 or 0.2 m-equiv. of potassium/l.

Statistical analysis. The significance of differences between means has been established by calculating *t*; *P*, the probability of differences being due to chance, was obtained from tables for *t* (Fisher & Yates, 1943).

Table 2. *Uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions in media buffered with bicarbonate, phosphate or tris*
Composition of media is given in Table 1. The number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Rats	Medium	Gas phase	Initial pH	Glucose uptake, Mean $\pm$ s.e.m. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference $\pm$ s.e. of difference	Significance of difference (<i>P</i>)
				Aerobic	Anaerobic		
Intact	Bicarbonate	Aerobic $\text{O}_2 + \text{CO}_2$	7.4	2.6 $\pm$ 0.07 (53)	5.6 $\pm$ 0.09 (53)	3.0 $\pm$ 0.12	<0.001
	Phosphate	Anaerobic N_2	7.4	2.9 $\pm$ 0.12 (36)	3.2 $\pm$ 0.10 (36)	0.3 $\pm$ 0.15	<0.05 > 0.02
	Phosphate	Aerobic $\text{O}_2 + \text{CO}_2$	6.9	2.5 $\pm$ 0.18 (12)	3.5 $\pm$ 0.11 (12)	1.0 $\pm$ 0.21	<0.001
	Phosphate	Anaerobic N_2	6.9	2.2 $\pm$ 0.20 (24)	3.4 $\pm$ 0.13 (24)	1.2 $\pm$ 0.24	<0.001
	Tris	Aerobic O_2	7.4	2.4 $\pm$ 0.24 (6)	2.6 $\pm$ 0.22 (6)	0.2 $\pm$ 0.33	<0.6 > 0.5
Hypophysectomized	Bicarbonate	Aerobic $\text{O}_2 + \text{CO}_2$	7.4	3.6 $\pm$ 0.06 (15)	5.5 $\pm$ 0.13 (15)	1.9 $\pm$ 0.14	<0.001
Adrenalectomized	Bicarbonate	Aerobic $\text{O}_2 + \text{CO}_2$	7.4	3.0 $\pm$ 0.27 (5)	4.5 $\pm$ 0.36 (5)	1.5 $\pm$ 0.45	<0.02 > 0.01

RESULTS

Glucose uptake under anaerobic conditions

In a medium buffered with bicarbonate the uptake of glucose by isolated rat diaphragm is greater under anaerobic than under aerobic conditions (Tables 2 and 3). This has been demonstrated with methods of glucose analysis based on copper reduction (Somogyi, 1945), glucose oxidase (Keilin & Hartree, 1948) or [^{14}C]glucose (Tables 2 and 3). This effect of anaerobiosis on glucose uptake is seen with diaphragms from intact, hypophysectomized or adrenalectomized rats (Table 2). On the other hand, if diaphragm is incubated in a medium buffered with phosphate (initial pH 7.4) the anaerobic-aerobic difference in glucose uptake is not significant (Table 2; Walaas & Walaas, 1952; Demis & Rothstein, 1954).

Further experiments have been carried out in an attempt to explain this difference in response with the two media. The composition of phosphate-buffered medium differs from that of bicarbonate-buffered medium in the following respects: (i) its calculated osmotic pressure is slightly higher; (ii) it does not contain bicarbonate; (iii) it contains considerably more phosphate (Table 1). The in-

fluence of each of these differences in composition has been studied separately. The calculated osmotic pressure of phosphate medium was first adjusted to that of bicarbonate medium by omission of the appropriate amount of sodium chloride. This change did not affect the lack of response to anaerobiosis in phosphate medium (Table 4). To determine the effect of phosphate concentration, the phosphate content of bicarbonate medium was increased to more than twice that of phosphate medium, the calculated osmolarity being kept constant by appropriate reduction of the sodium chloride content (high phosphate-bicarbonate medium, Table 1). The uptakes of glucose under aerobic or anaerobic conditions and the anaerobic-aerobic difference were all enhanced in high phosphate-bicarbonate medium (Table 5). Furthermore, when diaphragm was incubated in a medium buffered with 2-amino-2-hydroxymethylpropane-1,3-diol(tris) which, though lacking bicarbonate, contained only the same amount of phosphate as bicarbonate-buffered medium (Table 1), glucose uptake was not augmented under anaerobic conditions (Table 2). In an attempt to determine whether the bicarbonate ion had a specific effect (as opposed to its buffering activity) phosphate-buffered medium was gassed with $\text{O}_2 + \text{CO}_2$ or $\text{N}_2 + \text{CO}_2$ (95:5) instead of with O_2 or N_2 . Under these conditions uptake of glucose by the diaphragm was enhanced in phosphate medium under anaerobic conditions (Table 2); but it was noted that the initial pH of the medium fell from 7.4 to 6.9 when gassed with mixtures containing 5% of CO_2 . When phosphate medium, of initial pH 6.9 and gassed with pure N_2 or O_2 , was used, a similar anaerobic-aerobic difference was observed (Table 2). It seems likely, therefore, that the stimulating effect of anaerobiosis on glucose uptake in phosphate medium gassed with mixtures containing 5% of CO_2 is related to the fall in the initial pH of the medium rather than to the bicarbonate which is thereby introduced.

The response to anaerobiosis of the glucose uptake of isolated diaphragm appears to be related to the change of pH which occurs in the medium

Table 3. *Uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions in bicarbonate medium*

Each figure is the mean of two values, obtained from the incubation of five hemidiaphragms in 3 ml. of bicarbonate medium. Glucose analyses were by the glucose oxidase method (Keilin & Hartree, 1948).

Expt. no.	Glucose uptake (mg. of glucose/g. of wet diaphragm/hr. of incubation)	
	Aerobic	Anaerobic
1	3.5	5.1
2	3.0	6.1
Uptake of [$^{14}\text{C}_6$]glucose (counts/min. in glucose/g. of wet diaphragm/hr. of incubation)		
3	101 000	394 000
4	120 000	588 000

Table 4. *Uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions in phosphate media of differing osmolarity (initial pH 7.4)*

Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Calc. osmolarity (m-osmoles/l.)	Glucose uptake. Mean $\pm$ s.e.m. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference $\pm$ s.e. of difference
	Aerobic	Anaerobic	
281*	2.2 $\pm$ 0.18 (6)	2.7 $\pm$ 0.10 (6)	0.5 $\pm$ 0.21
309†	2.4 $\pm$ 0.23 (6)	2.8 $\pm$ 0.36 (6)	0.4 $\pm$ 0.43

* Osmolarity was reduced by omission of 14 m-equiv. of NaCl/l.

† Phosphate medium as in Table 1.

Table 5. *Influence of phosphate content of bicarbonate medium on uptake of glucose by isolated rat diaphragm under aerobic or anaerobic conditions*

Composition of media is given in Table 1. Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Medium	Glucose uptake. Mean $\pm$ s.e.m. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference $\pm$ s.e. of difference (anaerobic - aerobic)	Significance of difference (<i>P</i>)
	Aerobic	Anaerobic		
Bicarbonate	2.2 $\pm$ 0.12 (12)	4.5 $\pm$ 0.12 (12)	2.3 $\pm$ 0.18	<0.001
High phosphate-bicarbonate	2.8 $\pm$ 0.14 (12)	7.0 $\pm$ 0.28 (12)	4.2 $\pm$ 0.29	<0.001
Difference $\pm$ s.e. of difference (high phosphate-bicarbonate - bicarbonate)	0.6 $\pm$ 0.18	2.5 $\pm$ 0.28	1.9 $\pm$ 0.34	—
Significance of difference (<i>P</i>)	<0.02 <0.01	<0.001	<0.001	—

Table 6. *Fall in pH of medium during incubation of isolated diaphragm under aerobic or anaerobic conditions*

Composition of media is given in Table 1. Number of observations is given in parentheses.

pH fall during incubation. Mean $\pm$ s.e.m.
(pH units/hr.)

Medium	Initial pH		
		Aerobic	Anaerobic
Bicarbonate	7.4	0.11 $\pm$ 0.15 (4)	0.11 $\pm$ 0.15 (4)
Phosphate	7.4	0.66 $\pm$ 0.08 (4)	0.60 $\pm$ 0.13 (4)
Phosphate	6.9	0.07 $\pm$ 0.14 (4)	0.35 $\pm$ 0.16 (4)
Tris	7.4	0.55 (1)	0.80 (1)

Fig. 1. Effect of anaerobiosis on uptake of glucose by diaphragm. Incubations were made in bicarbonate medium under the following conditions: ●, anaerobic for 60 min.; ○, aerobic for 60 min.; ▲, anaerobic for 20 min. then aerobic for 40 min.; △, aerobic for 20 min. then anaerobic for 40 min.

and glucose uptake is increased under anaerobic conditions. In media buffered with phosphate or tris (of initial pH 7.4) the pH falls substantially during incubation for 1 hr. and there is no anaerobic-aerobic difference either in glucose uptake or in the fall of pH. On the other hand, in phosphate medium of initial pH 6.9 glucose uptake is enhanced under anaerobic conditions and the pH change after incubation for 1 hr. is much less (Tables 2 and 6). A similar relation between initial pH and fall of pH during aerobic incubation of diaphragm in phosphate medium was observed by Stadie & Zapp (1947).

The course of glucose uptake by diaphragm in bicarbonate medium under aerobic or anaerobic conditions is shown in Fig. 1. An enhanced rate of glucose uptake under anaerobic conditions is evident at all times during incubation for 1 hr. and is detectable within 5 min. of commencing incubation. If aerobic conditions are established after a period of anaerobiosis the rate of glucose uptake falls; conversely, if anaerobic conditions are established after an initial aerobic incubation the rate of glucose uptake increases (Fig. 1). To demonstrate the statistical significance of these observations a number of hemidiaphragms were incubated (i) for 1 hr. under aerobic conditions; (ii) for 1 hr. under anaerobic conditions; (iii) for 20 min. under aerobic conditions followed by 40 min. under anaerobic conditions; (iv) for 20 min. under anaerobic conditions followed by 40 min. under aerobic conditions. The uptake of glucose was measured after a total incubation of 1 hr. in each

during incubation. With bicarbonate medium little change of pH is evident after incubation for 1 hr. under either aerobic or anaerobic conditions

Table 7. *Uptake of glucose and release of potassium by isolated diaphragm under aerobic or anaerobic conditions in bicarbonate medium*

Number of observations is given in parenthesis. Glucose analyses were by the colorimetric method of Somogyi (1945). *P* is not greater than 0.01 for differences between means, except for (iii) - (ii) where *P* is > 0.05 for both glucose uptake and potassium release.

Conditions during incubation for 1 hr.	Glucose uptake. Mean $\pm$ S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)	Potassium release. Mean $\pm$ S.E.M. (μ -equiv. of potassium/g. of wet diaphragm/hr. of incubation)
(i) Anaerobic	5.2 $\pm$ 0.24 (18)	37 $\pm$ 1.6 (12)
(ii) Anaerobic for 20 min. then aerobic for 40 min.	4.2 $\pm$ 0.16 (18)	21 $\pm$ 0.7 (12)
(iii) Aerobic for 20 min. then anaerobic for 40 min.	3.9 $\pm$ 0.11 (18)	18 $\pm$ 1.8 (12)
(iv) Aerobic	2.3 $\pm$ 0.10 (18)	6 $\pm$ 1.3 (12)

Table 8. *Glucose utilization in medium during incubation*

First incubation: 12 hemidiaphragms in 12 ml. of bicarbonate medium were incubated for 30 min. Second incubation: 1 ml. samples of medium from first incubation were incubated alone for a further 60 min. under the same conditions.

Conditions	Change in glucose content of medium (mg./ml./hr.)	
	First incubation	Second incubation
Aerobic	-0.40	+0.07
Anaerobic	-0.92	-0.04
Aerobic without tissue	0.00	-0.02

case. It was found that conditions which were partly aerobic and partly anaerobic (ii, iii) were associated with a significantly greater uptake of glucose than conditions which were wholly aerobic (iv), and with a significantly smaller uptake than conditions which were wholly anaerobic (i) (Table 7). These experiments show that the changes which occur in isolated diaphragm under anaerobic conditions and which lead to an increased uptake of glucose can be reversed if aerobic conditions are restored.

Beloff-Chain *et al.* (1953) found that phosphoglucomutase readily diffuses from diaphragm and Zierler, Levy & Andres (1953) claim that enzymes capable of dissimilating glucose 1-phosphate or fructose 1:6-diphosphate to lactic acid may leak from diaphragm during incubation in phosphate medium. On the other hand, Shaw & Stadie (1957) believe that aldolase is the only glycolytic enzyme to do so. Nevertheless, it was conceivable that some of the glucose which disappeared from the medium during incubation of diaphragm under our conditions was being utilized as a result of the action of enzymes which had leaked into the medium. In order to examine this possibility,

medium in which diaphragms had been incubated for 30 min., either under aerobic or under anaerobic conditions, was incubated by itself for a further period of 1 hr. In neither case did any more glucose disappear from the medium after the tissue had been removed (Table 8).

Goldstein, Mullick, Huddleston & Levine (1953) believe that a humoral substance may be released from muscle, during muscular contraction *in vivo*, which is capable of facilitating the entry of some sugars into the cells of other resting muscles. The possibility that a substance might be released from diaphragm during incubation under anaerobic conditions which could promote glucose uptake was therefore tested experimentally. Eight hemidiaphragms were incubated in 6 ml. of bicarbonate medium for 1 hr. under either (a) aerobic or (b) anaerobic conditions. Glucose uptake (by the method of Somogyi, 1945) was (a) 2.16 and (b) 3.93 mg./g. of wet diaphragm/hr. With each resultant medium the glucose content was re-adjusted to 2.5 mg./ml. and an equal volume of fresh bicarbonate medium added. Fresh hemidiaphragms were then incubated singly for 1 hr. under aerobic conditions in six 1 ml. portions of each of these two new media: glucose uptake in the second incubation was: (a) 2.4 $\pm$ 0.23 and (b) 2.0 $\pm$ 0.14 mg./g. of wet diaphragm/hr. (mean $\pm$ S.E.M.). No stimulation of glucose uptake had therefore occurred with the medium from anaerobic incubation.

Influence of some cell poisons on uptake of glucose

The uptake of glucose by isolated diaphragm incubated under aerobic conditions in bicarbonate medium is increased in the presence of 2:4-dinitrophenol (50 μ M or 0.25 mM), sodium arsenite (mM), sodium arsenate (10 mM), sodium cyanide (mM) or sodium salicylate (5 mM) (Table 9). Sodium azide (mM or 10 mM) was without effect on glucose uptake. Sodium *p*-hydroxybenzoate (5 mM),

Table 9. *Influence of cell poisons on aerobic uptake of glucose by isolated rat diaphragm*

Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Expt. no.	Medium	Addition	Glucose uptake.	Difference $\pm$ s.e. of difference	Significance of difference (P)
			Mean $\pm$ s.e.m. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		
1	Bicarbonate	None (control)	2.0 $\pm$ 0.15 (12)	—	—
		2:4-Dinitrophenol (50 μ M)	3.1 $\pm$ 0.15 (6)	1.1 $\pm$ 0.21	<0.001
		(0.25 mM)	5.0 $\pm$ 0.32 (6)	3.0 $\pm$ 0.35	<0.001
		Sodium arsenite (mM)	6.8 $\pm$ 0.33 (12)	4.8 $\pm$ 0.37	<0.001
2	Bicarbonate	Sodium cyanide (mM)	8.4 $\pm$ 0.36 (12)	6.4 $\pm$ 0.39	<0.001
		None (control)	2.5 $\pm$ 0.20 (12)	—	—
		Sodium salicylate (5 mM)	4.0 $\pm$ 0.28 (12)	1.5 $\pm$ 0.35	<0.01
		Sodium <i>p</i> -hydroxybenzoate (5 mM)	2.0 $\pm$ 0.21 (6)	-0.5 $\pm$ 0.29	<0.2>0.1
3	Bicarbonate	None (control)	2.6 $\pm$ 0.24 (6)	—	—
		Sodium arsenate (mM)	3.2 $\pm$ 0.18 (6)	0.6 $\pm$ 0.30	<0.1>0.05
		Sodium azide (mM)	3.0 $\pm$ 0.14 (6)	0.4 $\pm$ 0.28	<0.2>0.1
4	Bicarbonate	None (control)	3.2 $\pm$ 0.51 (6)	—	—
		Sodium arsenate (10 mM)	6.1 $\pm$ 0.51 (6)	2.9 $\pm$ 0.72	<0.01>0.001
		Sodium azide (10 mM)	2.1 $\pm$ 0.35 (6)	-1.1 $\pm$ 0.62	<0.2>0.1
5	Phosphate (pH 7.4)	None (control)	3.2 $\pm$ 0.28 (6)	—	—
		2:4-Dinitrophenol (0.25 mM)	2.6 $\pm$ 0.35 (6)	-0.6 $\pm$ 0.44	<0.3>0.2
		Sodium salicylate (5 mM)	2.8 $\pm$ 0.13 (6)	-0.4 $\pm$ 0.37	<0.3>0.2
		Sodium cyanide (mM)	3.6 $\pm$ 0.39 (6)	0.4 $\pm$ 0.41	<0.4>0.3

Table 10. *Influence of anaerobiosis on the response to insulin of isolated diaphragm in bicarbonate medium*

Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945).

Expt. no.	Concn. of insulin (unit/ml.)	Glucose uptake. Mean $\pm$ s.e.m. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		Difference $\pm$ s.e. of difference	Significance of difference (P)
		Aerobic	Anaerobic		
1	0	3.7 $\pm$ 0.31 (6)	6.4 $\pm$ 0.44 (6)	2.7 $\pm$ 0.54	<0.001
	10 ⁻³	8.0 $\pm$ 0.62 (6)	6.9 $\pm$ 0.24 (6)	-1.1 $\pm$ 0.66	<0.2>0.1
	Difference $\pm$ s.e. of difference	4.3 $\pm$ 0.7	0.5 $\pm$ 0.5	—	—
	Significance of difference (P)	<0.001	<0.4>0.3	—	—
2	0	2.3 $\pm$ 0.17 (6)	6.6 $\pm$ 0.31 (6)	4.3 $\pm$ 0.35	<0.001
	0.1	9.1 $\pm$ 0.24 (6)	8.7 $\pm$ 0.24 (6)	-0.4 $\pm$ 0.34	<0.4>0.3
	Difference $\pm$ s.e. of difference	6.8 $\pm$ 0.29	2.1 $\pm$ 0.39	—	—
	Significance of difference (P)	<0.001	<0.001	—	—

Table 11. *Influence of cell poisons on the response to insulin of isolated diaphragm in bicarbonate medium*

Number of observations is given in parentheses. Glucose analyses were by the method of Huggett & Nixon (1957).

Cell poison	Glucose uptake. Mean $\pm$ s.e.m. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		
	Insulin (0.1 unit/ml.) alone (A)	Cell poison alone (B)	Insulin (0.1 units/ml.) + cell poison (C)
2:4-Dinitrophenol (0.25 mM)	11.6 $\pm$ 0.44 (6)	5.0 $\pm$ 0.34 (6)	6.0 $\pm$ 0.42 (6)
	P; for (B - A) <0.001; for (C - A) <0.001; for (C - B) <0.2>0.1		
2:4-Dinitrophenol (0.25 mM)	—	3.3 $\pm$ 0.17 (14)	4.3 $\pm$ 0.26 (14)
	P; for (C - B) <0.01>0.001		
Sodium arsenite (mM)	11.8 $\pm$ 0.45 (10)	8.4 $\pm$ 0.36 (10)	9.5 $\pm$ 0.25 (10)
	P; for (B - A) <0.001; for (C - A) <0.001; for (C - B) <0.05>0.01		
Sodium cyanide (mM)	10.8 $\pm$ 0.42 (10)	8.8 $\pm$ 0.56 (10)	12.8 $\pm$ 0.44 (10)
	P; for (B - A) <0.02>0.05; for (C - A) <0.01; for (C - B) <0.001		
Sodium salicylate (5 mM)	11.3 $\pm$ 0.36 (6)	5.4 $\pm$ 0.31 (6)	7.8 $\pm$ 0.48 (6)
	P; for (B - A) <0.001; for (C - A) <0.001; for (C - B) <0.01>0.001		

Table 12. *Comparison of the effect of anaerobiosis, cell poisons or insulin on uptake of glucose and loss of potassium by isolated rat diaphragm*

Where *P* is not given it is not greater than 0.01. Number of observations is given in parentheses. Glucose analyses were by the colorimetric method of Somogyi (1945). Figures for glucose uptake were calculated from data given in Table 11.

Expt. no.	Medium	Addition	Potassium loss.	Glucose uptake.		Difference $\pm$ s.e. of difference	
			Mean $\pm$ s.e.m. (μ -equiv. of potassium/g. of wet diaphragm/hr. of incubation)	Mean $\pm$ s.e.m. (μ -equiv. of glucose/g. of wet diaphragm/hr. of incubation)		Potassium	Glucose
1	Bicarbonate	None (aerobic control) Insulin (0.1 unit/ml.)	8 $\pm$ 0.9 (6) 5 $\pm$ 0.9 (6)	13 $\pm$ 1.0 (6) 51 $\pm$ 1.3 (6)	—	—	—
2	Bicarbonate	None (aerobic control) None (anaerobic)	7 $\pm$ 1.3 (12) 37 $\pm$ 1.6 (12)	13 $\pm$ 0.8 (12) 28 $\pm$ 1.7 (12)	—	—	—
3	Bicarbonate	None (aerobic control) 2,4-Dinitrophenol (50 μ M) (0.25 mM)	14 $\pm$ 1.3 (12) 44 $\pm$ 1.8 (6) 47 $\pm$ 1.5 (6)	11 $\pm$ 0.8 (12) 17 $\pm$ 0.8 (6) 28 $\pm$ 1.8 (6)	—	—	—
		Sodium arsenite (mM) Sodium cyanide (mM)	37 $\pm$ 2.7 (12) 33 $\pm$ 2.1 (12)	38 $\pm$ 1.8 (12) 47 $\pm$ 2.0 (12)	30 $\pm$ 2.2 33 $\pm$ 2.0 23 $\pm$ 2.7 19 $\pm$ 2.4	6 $\pm$ 1.1 17 $\pm$ 2.0 27 $\pm$ 2.0 36 $\pm$ 2.2	—
4	Bicarbonate	None (aerobic control) Sodium salicylate (5 mM) Sodium <i>p</i> -hydroxybenzoate (5 mM)	8 $\pm$ 1.2 (14) 31 $\pm$ 1.5 (14) 8 $\pm$ 0.9 (6)	14 $\pm$ 1.1 (14) 22 $\pm$ 1.3 (14) 11 $\pm$ 1.2 (6)	—	—	—
5	Bicarbonate	None (aerobic control) Sodium arsenate (10 mM) Sodium azide (10 mM)	12 $\pm$ 2.3 (6) 28 $\pm$ 4.1 (6) 48 $\pm$ 2.4 (6)	16 $\pm$ 3.4 (6) 34 $\pm$ 3.4 (6) 12 $\pm$ 1.4 (6)	16 $\pm$ 4.7 36 $\pm$ 3.3	—	—
6	Phosphate (pH 7.4)	None (aerobic control) None (anaerobic)	13 $\pm$ 2.1 (6) 44 $\pm$ 1.8 (6)	15 $\pm$ 1.9 (6) 21 $\pm$ 1.1 (6)	—	—	—
7	Phosphate (pH 7.4)	None (aerobic control) 2,4-Dinitrophenol (0.25 mM) Sodium cyanide (mM) Sodium salicylate (5 mM)	17 $\pm$ 0.6 (6) 57 $\pm$ 2.3 (6) 28 $\pm$ 1.7 (6) 42 $\pm$ 0.9 (6)	18 $\pm$ 1.6 (6) 14 $\pm$ 1.8 (6) 20 $\pm$ 2.2 (6) 16 $\pm$ 0.7 (6)	—	—	—
					40 $\pm$ 2.3 11 $\pm$ 2.3 25 $\pm$ 1.1	—	—

* *P* > 0.05.† *P* < 0.05 > 0.01.

unlike sodium salicylate (*o*-hydroxybenzoate), did not stimulate glucose uptake (Table 9).

In a medium buffered with phosphate (initial pH 7.4) the uptake of glucose by isolated diaphragm is not increased by 2:4-dinitrophenol, sodium salicylate or sodium cyanide (Table 9), results which are in general agreement with those of Villee *et al.* (1949) and Pierce & Field (1949) for 2:4-dinitrophenol and of Smith & Jeffrey (1956) for sodium salicylate.

Uptake of glucose in the presence of insulin

As was shown by Gemmill & Hamman (1941), the uptake of glucose by isolated diaphragm is very greatly increased by insulin *in vitro* (Table 10). The maximum effect of insulin on glucose uptake is seen with the concentration used (0.1 unit/ml.) and it is much greater than that produced either by anaerobiosis or by cell poisons at the concentrations used. When diaphragm is incubated with insulin (0.1 unit/ml.) under anaerobic conditions the level of glucose uptake does not differ significantly from that attained when insulin is present under aerobic conditions (Table 10). Furthermore, insulin still stimulates glucose uptake in the presence of sodium arsenite, sodium salicylate or 2:4-dinitrophenol, though under these conditions the uptake is less than that elicited by insulin alone (Table 11). When insulin and cyanide are present together the uptake of glucose is greater than that produced by insulin or cyanide separately. Insulin at a much lower concentration (10^{-3} unit/ml.) has no significant effect on glucose uptake under anaerobic conditions although it is still able to stimulate glucose uptake under aerobic conditions (Table 10).

Potassium loss from isolated diaphragm

When isolated diaphragm is incubated under aerobic conditions in media buffered with either bicarbonate or phosphate a small but significant amount of potassium is lost from the muscle (Table 12). This loss of potassium takes place during the initial 20 min. of incubation; thereafter no further loss occurs (Fig. 2). Kamminga, Willebrands, Groen & Blickman (1950) have claimed that hemidiaphragms incubated aerobically in bicarbonate medium take up some potassium from the medium and also that insulin enhances this transfer. Despite the fact that our experiments were carried out under conditions apparently very similar to those used by Kamminga *et al.* a loss of potassium from the tissue always occurred, although this loss was perhaps smaller in the presence of insulin (Table 12).

Much more potassium is lost if diaphragm is incubated under anaerobic conditions (Table 12): the loss is apparently linear with time (Fig. 2). The marked loss of potassium induced by exclusion

of oxygen confirms the results of Creese (1954) and Calkins *et al.* (1954) and is in agreement with the known effects of anaerobiosis on other tissues. Diaphragm incubated aerobically but in the presence of 2:4-dinitrophenol (50 μ M or 0.25 mM), sodium arsenite (mM), sodium arsenate (10 mM), sodium azide (10 mM), sodium cyanide (mM) or sodium salicylate (5 mM) also loses considerably more potassium. The influence of these agents on potassium release, unlike their effect on glucose uptake, is evident whether phosphate medium or bicarbonate medium is used for the incubation (Table 12). Sodium *p*-hydroxybenzoate (5 mM), unlike sodium salicylate, does not promote loss of potassium. There does not appear to be any relation between the changes in glucose uptake and potassium loss which these agents effect (Table 12).

DISCUSSION

The present investigations have shown that oxygen lack, 2:4-dinitrophenol, sodium arsenite, sodium arsenate, sodium cyanide and sodium salicylate increase the uptake of glucose and loss of potassium by isolated rat diaphragm incubated in a medium buffered with bicarbonate. Although these factors may differ in many respects in their effects on metabolism they nevertheless have in common the property of inhibiting oxidative phosphorylation. It seems reasonable to relate their effects on glucose uptake and potassium loss to this property. The greatly increased loss of potassium from diaphragm under conditions where oxidative phosphorylation

Fig. 2. Effect of anaerobiosis on loss of potassium from diaphragm. Incubations were made in bicarbonate medium under the following conditions: ●, anaerobic for 60 min.; ○, aerobic for 60 min.; ▲, anaerobic for 20 min. then aerobic for 40 min.; △, aerobic for 20 min. then anaerobic for 40 min.

is impaired is in keeping with the view that the maintenance of a high intracellular-potassium concentration is dependent upon the selective extrusion of sodium by an active process (see for example, Hodgkin, 1958). The lack of correlation between levels of glucose uptake and potassium release under anaerobic conditions or where cell poisons are present makes it unlikely that changes in glucose uptake occur as a consequence of loss of potassium.

The uptake of glucose by very many cells and tissue preparations *in vitro* is known to be greater under anaerobic conditions, as was first recognized by Pasteur. Previous investigations had suggested that isolated diaphragm was exceptional in that it did not appear to exhibit an increased uptake of glucose under anaerobic conditions. Failure to demonstrate this manifestation of the Pasteur effect in diaphragm has now been shown to be due to the use of media buffered with phosphate. The stimulating effect of 2:4-dinitrophenol on glucose uptake is also lacking in a phosphate-buffered medium. This difference in the response of isolated diaphragm to anaerobiosis or cell poisons such as 2:4-dinitrophenol in media buffered with phosphate or bicarbonate appears to be related to the poor buffering power of phosphate medium. The unsuitability of phosphate buffers for the incubation of tissues which exhibit a high rate of glycolysis was emphasized by Dixon (1934); it is also evident, for example, in the experiments of Stadie & Zapp (1947) and of Light & Simpson (1956).

A number of factors may have to be taken into account in explanation of the increased rates of glucose utilization and glycolysis by tissues under anaerobic conditions. These include: depression of glycolysis under aerobic conditions by lack of inorganic phosphate (Johnson, 1941; Lynen, 1941) or of adenosine diphosphate (Krebs, 1954); inactivation of phosphohexokinase under aerobic conditions (Engelhardt & Sakov, 1943); shortage of coenzyme I for aerobic glycolysis (Reiner, 1947); and the lack of availability of mitochondrial adenosine triphosphate for phosphorylation of glucose under aerobic conditions (Wu & Racker, 1957). Which of these factors is of importance to the increased uptake of glucose by diaphragm (or, indeed, by other tissues) under anaerobic conditions is not known. Since inorganic phosphate can stimulate the uptake of glucose by diaphragm under aerobic conditions, availability of inorganic phosphate might appear to be an important factor. However, inorganic phosphate has been shown to inhibit oxidative phosphorylation by rat-liver mitochondria (Hunter & Ford, 1955) and its effect on glucose uptake by diaphragm under aerobic conditions may be related to this action rather than to its participation in glycolysis.

The factors listed above can account for the enhanced utilization of glucose under anaerobic conditions only if the access of glucose to hexokinase is not impeded by any kind of permeability barrier. This may be so for some cells, such as the ascites-tumour cell, which shows augmented glucose uptake under anaerobic conditions (Wu & Racker, 1957) and in which glucose utilization is apparently not limited by the rate of entry of glucose into the cell (Crane, Field & Cori, 1957). Park *et al.* (1955) believe that glucose utilization in the isolated diaphragm, however, is limited by the rate of entry of glucose into the muscle cell. Thus the factors listed above may not account for the augmented uptake of glucose by isolated diaphragm under anaerobic conditions. The factors which regulate glucose uptake in muscle, which responds to insulin with increased uptake, obviously differ in some respect from those involved in cells such as the ascites-tumour cell which do not respond to insulin. Thus the mechanism of the Pasteur effect in a tissue such as muscle in which glucose utilization is affected by insulin may differ from that of a tissue or cell which is not affected by insulin.

Levine & Goldstein (1955) and Park *et al.* (1955) believe that insulin increases the uptake of glucose by muscle (including isolated diaphragm) by accelerating the entry of glucose into the cell. In an accompanying paper (Randle & Smith, 1958) we present evidence which suggests that anaerobiosis and substances which inhibit oxidative phosphorylation also accelerate the entry of glucose into the cells of isolated diaphragm. We have therefore concluded that the entry of glucose into the cells of this preparation is restrained under basal conditions by a process dependent upon a supply of energy-rich phosphate. On the other hand, it is well known that insulin does not inhibit oxidative phosphorylation (Stadie, 1954). The mechanism of its accelerating effect on the entry of glucose into the muscle cell may therefore differ from that of anaerobiosis and that of substances which do inhibit oxidative phosphorylation. One possibility is that insulin restricts the access of energy-rich phosphate to the process concerned with the regulation of glucose entry (Randle & Smith, 1957).

The entry of glucose into the cells of isolated diaphragm appears to be accelerated by anaerobiosis and cell poisons in both bicarbonate and phosphate media (Randle & Smith, 1958). This could account for the augmented glucose utilization which these factors promote in bicarbonate medium, but it fails to explain why glucose utilization is not increased in phosphate medium. This discrepancy could be explained if the intracellular utilization of glucose was rate-limiting in phosphate medium, but this is unlikely because free

glucose does not accumulate in the muscle under these conditions (Randle & Smith, 1958). Another explanation which may prove more fruitful is that some utilization of glucose may occur at the surface of the cells of isolated diaphragm (i.e. without penetration of the cell by glucose). Thus accelerated entry of glucose would not be associated with an increased rate of glucose utilization if at the same time there was a proportionate reduction in the rate of utilization of glucose at the cell surface. Although it is thought that the enzymes responsible for glycolysis in muscle occur in the sarcoplasm (see, for example, Bailey, 1954; Perry, 1956), Shaw & Stadie (1957) have recently suggested that the formation of lactic acid from glucose by isolated diaphragm does not depend on penetration of the cell by glucose but may occur at the cell surface. If this is so then the accelerated entry of glucose which anaerobiosis and cell poisons effect may be incidental and of little significance in relation to the augmented glucose utilization induced by these factors in bicarbonate medium. Clearly, further information about the localization in the muscle cell of processes concerned with glucose utilization is needed before the mechanism of the Pasteur effect in diaphragm can be elucidated.

At a concentration of 0.1 unit/ml., insulin can still enhance the uptake of glucose by isolated diaphragm in a medium buffered with bicarbonate when incubated under anaerobic conditions or in the presence of substances which inhibit oxidative phosphorylation. Ottaway (1955) has claimed that insulin (0.2 unit/ml.) fails to stimulate glucose uptake when diaphragm is incubated in a bicarbonate medium under anaerobic conditions. This apparent discrepancy between our results and those of Ottaway may result from the much smaller increase in glucose uptake which insulin seems to effect under aerobic conditions in Ottaway's experiments (Ottaway, 1951). This explanation is supported by our finding that insulin at a much lower concentration (10^{-3} unit/ml.), which under aerobic conditions induces a much smaller increase in glucose uptake, no longer stimulates glucose uptake under anaerobic conditions.

The present investigations have been concerned solely with the influence of certain factors on glucose utilization by muscle *in vitro*. Some indication that they may have a similar effect *in vivo* is provided by the observations that sodium salicylate (*o*-hydroxybenzoate) or acetylsalicylic acid raise the metabolic rate in normal people (Cochran, 1952) and diabetic patients (Reid, Macdougall & Andrews, 1957), and lower the blood-sugar level and reduce glycosuria in diabetic patients (Reid *et al.* 1957) and in several forms of experimental diabetes (Ingle, 1950; Smith, 1952;

Smith, Meade & Bornstein, 1952); sodium *p*-hydroxybenzoate is without effect on the metabolic rate in human beings (Cochran, 1954). The present observation that salicylate, but not *p*-hydroxybenzoate, is capable of stimulating the uptake of glucose by diaphragm has led us to suggest elsewhere that these effects of salicylate on metabolic rate, blood-sugar level and glycosuria may result from an increased utilization of glucose by muscle (Manchester, Randle & Smith, 1958).

SUMMARY

1. Anaerobiosis stimulates the uptake of glucose by isolated rat diaphragm incubated in a medium buffered with bicarbonate. This effect is not observed in media buffered with phosphate or tris (of initial pH 7.4). The lack of response to anaerobiosis in the latter media appears to be related to the marked fall in pH which occurs during incubation.

2. Under aerobic conditions uptake of glucose by diaphragm in bicarbonate medium is increased by 2:4-dinitrophenol (50 μ M or 0.25 mM), sodium arsenite (mM), sodium arsenate (10 mM), sodium cyanide (mM) and sodium salicylate (5 mM). Sodium azide (mM or 10 mM) and sodium *p*-hydroxybenzoate (5 mM) do not augment glucose uptake. In a medium buffered with phosphate, 2:4-dinitrophenol (0.25 mM), sodium salicylate (5 mM) and sodium cyanide (mM) fail to stimulate glucose uptake.

3. In bicarbonate medium the uptake of glucose by diaphragm in the presence of 2:4-dinitrophenol, sodium arsenite or sodium salicylate is increased still further by the addition of insulin (0.1 unit/ml.), though the uptake of glucose achieved under these conditions is less than that with insulin alone. The levels of uptake with insulin under either aerobic or anaerobic conditions were of the same order. In the presence of both insulin and cyanide the uptake of glucose is greater than when either is present by itself.

4. There is a small loss of potassium from diaphragm incubated under aerobic conditions either in bicarbonate or in phosphate medium. The loss of potassium in either medium is very much greater under anaerobic conditions or in the presence of 2:4-dinitrophenol, sodium arsenite, sodium arsenate, sodium cyanide, sodium salicylate or sodium azide at the above concentrations. Insulin effected a slight reduction in the loss of potassium under aerobic conditions. There was no obvious quantitative relation between the loss of potassium from the muscle and the change in glucose uptake.

5. The significance of these results in relation to the mode of uptake of glucose by diaphragm, to the

Pasteur effect in diaphragm and to the mechanism of action of insulin on diaphragm is discussed.

We wish to thank Professor F. G. Young, F.R.S., for much helpful discussion and encouragement. We are indebted to Dr G. D. Greville for valuable discussions and for a sample of highly purified 2:4-dinitrophenol. Miss G. M. Bixley is thanked for skilled technical assistance and Miss M. Thomas for carrying out hypophysectomies. We are grateful to Boots Pure Drug Co. Ltd. and to Dr K. Hallas Møller of the Novo Terapeutisk Laboratories, Copenhagen, for generous gifts of crystalline insulin. The cost of this work was largely defrayed by a grant for research expenses from the Medical Research Council to one of us (P.J.R.).

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